A ROLE FOR CAPPUCCINO AND CHICKADEE IN REGULATION OF VESICLE TRANSPORT DURING DROSOPHILA DEVELOPMENT

by

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Dedicated

In memory of my dad

P.N. Balasundaram
# TABLE OF CONTENTS

**LIST OF FIGURES** ........................................................................................................................................ 9

**LIST OF TABLES** ........................................................................................................................................ 11

**ABSTRACT** ................................................................................................................................................. 12

**1. INTRODUCTION** .................................................................................................................................... 14

**Overview** .................................................................................................................................................. 14

Drosophila oogenesis ................................................................................................................................. 16

Establishment of polarity ............................................................................................................................ 18

Formins: A new class of actin nucleators .................................................................................................. 19

Capu is a member of the formin family ...................................................................................................... 22

Spire ............................................................................................................................................................. 24

Chickadee ..................................................................................................................................................... 27

The actin and microtubule cytoskeleton in Drosophila ovary .................................................................. 28

*capu spir* and *chic* share phenotypes .................................................................................................. 30

**2. CAPU AND CHIC REGULATE YOLK PROTEIN BIOGENESIS DURING DROSOPHILA DEVELOPMENT** ....................................................................................................................... 42

**Background** .............................................................................................................................................. 42

The endocytic pathway ............................................................................................................................... 44

The Drosophila oocyte as an endocytic cell ............................................................................................... 45

Regulation of cytoskeleton by Capu ......................................................................................................... 48
# TABLE OF CONTENTS – Continued

**Results**

- Loss of function mutation in *capu* results in enlarged yolk granules...49
- The large yolk granule phenotype is due to *capu* loss of function...51
- The biogenesis defect is not due to internalization of large yolk granules...53
- Kinetics of early endocytosis is not affected in capu mutants...53
- Abnormal fusion and large endosomes...54
- Actin and large vesicles...56
- Loss of function mutation in *chic* result in enlarged yolk granules...57
- Interaction of Profilin with Capu is necessary for yolk granule biogenesis...58

**Discussion**

- Large endosomes and vesicle fusion...61

**3. A GENOME-WIDE DEFICIENCY SCREEN TO IDENTIFY INTERACTORS OF CAPU AND SPIR.**

**Background**

- 91

**Results**

- Screen Design...96
- A primary screen for *capu* interactors identifies the chromosomal region 49C1-50D2...97
- A secondary screen for *capu* interactor narrowed the interacting interval to chromosomal region 50A-50D2...98
TABLE OF CONTENTS – Continued

A primary screen for spir interactors identifies the chromosomal region 55A-55F ................................................................. 99

A secondary screen for spir interactors narrowed the interacting interval to 55A-55C1 .................................................................. 100

Discussion .............................................................................................................................................................................. 103

4. FUTURE PERSPECTIVES .................................................................................................................................................. 120

APPENDIX A: MATERIALS AND METHODS ...................................................................................................................... 123

REFERENCES ................................................................................................................................................................. 128
LIST OF FIGURES

Figure 1.1 Drosophila oogenesis.................................................................34
Figure 1.2 Localization of determinants during oogenesis...........................36
Figure 1.3 Domain organization of Formin and Spir proteins.......................38
Figure 1.4 Microtubule bundling seen in capu and spir mutants....................40
Figure 2.1 The formin multigene family....................................................65
Figure 2.2 Schematic of the pathway of yolk granule biogenesis in Drosophila  oocytes.................................................................67
Figure 2.3 Enlarged yolk granules in capu mutants.....................................69
Figure 2.4 The enlarged compartments in capu mutant oocytes are accessed by Lysotracker.................................................................71
Figure 2.5 The number of yolk granules is decreased and volume increased in capu mutants compared to wild type.................................73
Figure 2.6 The large yolk granule phenotype is due to capu loss of function.....75
Figure 2.7 Vesicle size increases during oogenesis in capu mutants..............77
Figure 2.8 Endocytic tracer uptake............................................................79
Figure 2.9 Exposure to cytochalasin D does not cause large vesicle defect.....81
Figure 2.10 Enlarged yolk granules in chic mutants...................................83
Figure 2.11 The number of yolk granules is decreased and volume increased in chic mutants compared to wild type.................................85
Figure 2.12 The large yolk granule phenotype is due to chic loss of function....87
LIST OF FIGURES – Continued

Figure 2.13 Interaction of Profilin with Capu is necessary for Profilin function in endosome biogenesis.................................................................89

Figure 3.1 Pole cells are absent in embryos from capu or spir/+ rhoA/+ mothers.................................................................106

Figure 3.2 Schematic of the deficiency screen design.................................108

Figure 3.3 Deficiency Df(2R)CX1 interacts with capu........................................110

Figure 3.4 Deficiency Df(2R)PC4 interacts with spir.................................112

Figure 3.5 Schematic of the P-element Excision strategy to excise mapmodulin locus.................................................................114
LIST OF TABLES

Table 3.1 List of deficiencies tested for the screen.................................................116
Table 3.2 Summary of interactions of spir with chromosomal region 55A-55F...............................................................119
Establishment of polarity is a critical process that occurs early during development. In *Drosophila melanogaster*, axis determination occurs by localization of determinants during oogenesis. Mutations in *cappuccino (capu)* lead to defects in polarity establishment of both the anterior/posterior (A/P) and dorsal/ventral (D/V) axes during oogenesis. In the oocytes laid by *capu* mutant females, determinants that define these axes are either mislocalized or are absent. Several lines of evidence suggest that the regulation of cytoskeleton by the gene product encoded by *capu* is involved in *Drosophila* oogenesis.

Capu, a member of the formin family of proteins, known to be regulators of actin dynamics, interacts both genetically and physically with *chickadee (chic)* which encodes the actin binding protein Profilin. I show here that mutations in both *capu* and *chic* lead to defects in the endocytic uptake of yolk into developing oocytes. I show that mutations in these loci lead to accumulation of abnormally large yolk granules and that this is a post internalization defect in the oocyte of *capu* and *chic* females. I also present evidence which indicates that an interaction with *capu* is necessary for *chic* regulation of yolk granule biogenesis.

This is the first evidence for a formin subfamily of formin proteins to have a role in endocytosis. While this new function identified for the actin associated
proteins Capu and Profilin indicates that regulation of actin cytoskeleton plays a role in endocytosis during oogenesis, the mechanism of this regulation and possible actin independent roles played by Capu and Profilin in this process are yet to be determined.

Like capu, mutations in spire (spir) also show defects in A/P and D/V axes during oogenesis. Spir is an actin binding protein and like capu, mutations in spir shows defects in cytoskeletal architecture and suggests that capu and spir alter microtubule distribution in the oocyte during oogenesis.

To identify molecular partners of capu and spir and their roles during oogenesis, I performed a genome-wide deficiency screen to identify regions of the genome that interact with these genes. I identified regions in the genome that showed interaction with capu and spir. While I was able to narrow down the region of interaction to a smaller cytological interval, gaps in the deficiency coverage and lack of mutants in those regions prevented me from identifying interacting loci in those regions.
CHAPTER 1

INTRODUCTION

Overview

Polarity establishment in a developing cell is a critical process and in many invertebrates and vertebrates, the axes are set up in the egg itself (Albertini and Barrett, 2004; Manseau and Schupbach, 1989; Melton et al., 1989; Wodarz, 2002). In Drosophila, anterior/posterior (A/P) and dorsal/ventral (D/V) axes are established during oogenesis by the asymmetric localization of bicoid, oskar, and gurken mRNAs to respective poles within the oocyte (Grunert and St Johnston, 1996; Manseau and Schupbach, 1989; Neuman-Silberberg and Schupbach, 1993). The localization of these transcripts depends on the polarized organization of the oocyte cytoskeleton. Both actin and microtubule cytoskeletons have been implicated in axis formation in Drosophila (Riechmann and Ephrussi, 2001). For instance, in the establishment of A/P polarity during Drosophila oogenesis, microtubules direct the transport of oskar mRNA to the posterior of the oocyte (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991). This defines the future posterior pole of the embryo. An actin-based cortical anchor is required to maintain this polarized distribution of oskar mRNA (Ephrussi et al., 1991; Gunkel et al., 1998; Rongo et al., 1995).
Studies in the Manseau lab had focused on the role of two genes, *cappuccino* (capu) and *spire* (spir), mutations in which result in disruption of oocyte polarity early in oogenesis. *capu* and *spir* are maternal effect loci that affect the organization of both the anterior-posterior and dorsal-ventral axes of the Drosophila egg and embryo (Emmons et al., 1995; Manseau and Schupbach, 1989; Wellington et al., 1999). The gene products they encode are necessary for the localization or stabilization of developmentally important molecules to specific regions within the cytoplasm of the developing egg. *capu* and *spir* mutant females produce embryos that are dorsalized. Mutant embryos lack pole cells and abdominal segments (Manseau and Schupbach, 1989). The proper development of the pole cells and abdomen is achieved by the localization of gene products to the posterior pole of the developing oocyte (Bardsley et al., 1993; Ephrussi et al., 1991; St Johnston et al., 1991). In *capu* and *spir* mutants these gene products are produced but are not localized to the posterior pole, indicating that *capu* and *spir* are required either for the transport or for localization of these molecules to the posterior pole.

Several lines of evidence suggest that the determinant localization defect in *capu* and *spir* mutants is due to misregulation of the cytoskeleton. In *capu* and *spir* mutants, the microtubules are severely misorganized, a phenotype that can be phenocopied by disruption of the actin cytoskeleton by exposure to cytochalasin D (Emmons et al., 1995; Theurkauf, 1994; Manseau et al., 1996). While Spir has
been shown to directly bind actin (James, 2001; Wellington et al., 1999), capu interacts both physically and genetically with Profilin, an actin regulating protein, encoded by the chickadee (chic) gene in Drosophila (Cooley et al., 1992; Manseau et al., 1996). Mutations in chic also show the microtubule phenotype in addition to failure to localize Staufen and Oskar mRNA to the posterior pole, similar to mutations in capu and spir (Manseau et al., 1996). Finally, Capu is a member of the formin homology family of proteins that are potent regulators of actin dynamics (Emmons et al., 1995).

Here, I briefly present an overview of Drosophila oogenesis and establishment of polarity of the oocyte and describe in detail the genes and proteins that I analyzed during this dissertation.

**Drosophila Oogenesis**

The primary purpose of oogenesis is to assemble a large cell containing plenty of resources for the construction of the embryo. Oogenesis commences with the asymmetric division of the stem cell to produce a cystoblast and another germline stem cell (Riechmann and Ephrussi, 2001). The cystoblast undergoes 4 mitotic divisions with incomplete cytokinesis to form a 16-cell germline cyst. The 16 cells are interconnected by actin rich cytoplasmic bridges called the ring canals. The number of ring canals that a cell is connected to, depends on the round of cell division from which it is derived. The one cell that is connected by four ring
canals becomes the oocyte, while the other fifteen become nurse cells that provides cytoplasmic components and nourishment to the developing oocyte (Figure 1.1) (Dobens and Raftery, 2000; Spradling, 1993; Theurkauf, 1997). Once the oocyte is determined, it is positioned at the posterior of the 16-cell cyst and this determines early formation of A/P polarity. Bidirectional signaling between the oocyte and the surrounding layer of follicle cells helps in this positioning (van Eeden and St Johnston, 1999).

The Drosophila egg chamber consists of the fifteen germline derived nurse cells and an oocyte. The oocyte-nurse cell complex is surrounded by a layer of somatically derived follicle cells. The egg chambers develop sequentially in tubular structures called the ovariole. The Drosophila ovary is composed of 16-20 such ovarioles (Figure 1.1). Each ovariole is made of a chain of progressively mature egg chambers. New egg chambers are formed at the anterior end of the ovariole, called the germarium while the more mature chambers are found toward the posterior end. Oogenesis comprises of 14 stages, according to the staging of oocyte growth (Cummings and King, 1970). The first seven are the previtellogenic stages, and stages 8 to 10 are the vitellogenic stages and stages 11 to 14 are mainly involved in the maturation of the egg and formation of egg coats. At the completion of vitellogenesis, the oocyte grows rapidly as a result of the addition of the nurse cell cytoplasm (Dobens and Raftery, 2000; Spradling, 1993; Theurkauf, 1997). As the egg chambers mature they progress through the ovariole and leave the ovary.
Establishment of polarity

As the egg is being formed, the A/P and D/V axes are established. Signaling events between the oocyte and the surrounding layer of follicle cells triggers early axis formation (van Eeden and St Johnston, 1999). In the establishment of the A/P axis of the egg, the anterior fate is the default fate (Schupbach, 1987). In the absence of posterior specification, the posterior adopts the anterior fate. *gurken* (*grk*), encoding a secreted transforming growth factor (TGF-β-like) protein polarizes the oocyte by two rounds of signaling (Neuman-Silberberg and Schupbach, 1993). The first round of Gurken signaling induces the follicle cells overlaying the oocyte to adopt a posterior fate. Gurken sends a signal from the oocyte to the posterior follicle cells which is received by the Erythrocyte Growth Factor (EGF) receptor homologue, Torpedo. In response to this, the follicle cells adopt a posterior fate (Figure 1.2) (Manseau and Schupbach, 1989; Schupbach, 1987).

After the A/P axis is formed, the D/V axis is established. In the D/V axis formation, the ventral fate is the default fate (Neuman-Silberberg and Schupbach, 1993). Movement of the oocyte nucleus to the dorsal anterior corner of the oocyte signifies the first step in the establishment of the dorsal-ventral axis formation. Gurken subsequently localizes to the dorsal anterior corner, adjacent to the oocyte nucleus (Neuman-Silberberg and Schupbach, 1993). This restricts the distribution of Gurken to the dorsal side of the oocyte, where it activates the
EGF receptor in the surrounding epithelium of follicle cells. This signaling conveys to the follicle cells that they are situated on the dorsal side of the oocyte and initiates a surge of activities in the follicle cells on the dorsal and ventral sides of the oocyte to establish the D/V axis of the developing embryo (Figure 1.2) (Saunders and Cohen, 1999).

The terminal follicle cells at the anterior and posterior ends of the egg chamber are competent to differentiate as posterior follicle cells if they receive the Gurken signal, while the remaining follicle cells can only adopt dorsal fates in response to Gurken. This differential response to Gurken signaling suggests that there are different sets of follicle cells that respond to the signal. During these signaling events, the oocyte inside the egg chamber grows in size along with the egg chamber itself, leading to altered geometry of the oocyte and the surrounding follicle cells, thus resulting in differential response to Gurken signaling (van Eeden and St Johnston, 1999).

**Formins: A new class of actin nucleators**

Formins, a family of conserved proteins from yeast to humans, have been shown to be involved in the reorganization of the actin cytoskeleton (Evangelista et al., 2003; Wallar and Alberts, 2003; Wasserman, 1998; Zigmond, 2004). The first formin to be identified was the limb deformity (ld) locus in mouse, mutations in which affect proper formation of limbs and kidneys in mice (Kleinebrecht et al., 1982; Mass et al., 1990). Formins have been shown to function in various
cytoskeleton based cellular processes such as cell polarization, cytokinesis, embryonic development and hair cell stereocilia formation (Castrillon and Wasserman, 1994; Chang et al., 1997; Emmons et al., 1995; Leader and Leder, 2000; Lynch et al., 1997).

Formins are multidomain proteins that share regions of sequence homology. Two well conserved FH (Formin Homology) domains, the FH1, and the FH2 domains (Castrillon and Wasserman, 1994) have been shown to function in actin assembly (Evangelista et al., 2002; Sagot et al., 2002a). There is a loosely defined FH3 domain (Petersen et al., 1998). Some formins have been shown to interact with Rho family members through a Rho binding domain (RBD) found at the amino terminus (Kohno et al., 1996). A fifth domain called the Diaphanous Auto regulatory Domain (DAD) is found toward the C terminal (Figure 1.3) (Alberts, 2001). Based on sequence homology formins have been subdivided into three subfamilies, the formin, the Bni1 and the diaphanous subfamilies (Zeller et al., 1999).

Though formins have been implicated in a variety of functions such as cell polarity and cytokinesis, only recently a role for formins in the nucleation of actin filaments has been identified (Pruyne et al., 2002; Sagot et al., 2002b). While Arp2/3 has been considered as a well characterized nucleator of branched actin filaments (Stradal and Scita, 2005), the source of unbranched actin filaments had
remained obscure in cells. Recently formins have been shown to be direct nucleators of straight, unbranched actin filaments involved in direct assembly of actin structures independent of Arp2/3 (Copeland and Treisman, 2002; Pruyne et al., 2002; Sagot et al., 2002b).

The well conserved FH2 domain of formins is involved in the nucleation of actin filaments and has been shown to nucleate actin \textit{in vitro} (Pruyne et al., 2002; Sagot et al., 2002b) and also the assembly of actin \textit{in vivo} (Copeland and Treisman, 2002; Evangelista et al., 2002; Sagot et al., 2002a; Sagot et al., 2002b). The mechanism of FH2-mediated actin nucleation occurs by the stabilization of an actin dimer, forming a nucleation unit that enables barbed end growth (Pring et al., 2003). A role for the actin monomer binding protein, Profilin in formin-mediated actin nucleation is also known. Profilin binds the proline rich FH1 domain of formins. The \textit{in vivo} assembly of actin filaments by budding yeast formin, Bni1p requires both the FH2 and FH1 domains to mediate filament assembly (Evangelista et al., 2002; Sagot et al., 2002a; Sagot et al., 2002b). \textit{In vitro}, Profilin has been shown to enhance the nucleation mediated by Bni1p FH1-FH2, as mutants in Profilin, unable to bind the proline rich FH1 domain have no effect in enhancing this formin mediated nucleation (Pring et al., 2003; Sagot et al., 2002b). This suggests that the FH1-Profilin interaction is essential for the increased nucleation of actin filaments by formins.
There is also evidence that formins regulate the microtubule cytoskeleton (Emmons et al., 1995; Evangelista et al., 1997; Ishizaki et al., 2001). In budding yeast the spindle orientation is indirectly controlled by the assembly of actin cables (Yin et al., 2000). Also, formin 2 is required for actin-dependent spindle positioning and polarity establishment in mouse oocytes (Leader et al., 2002). Studies on the mammalian formin, mDia have demonstrated a clear link for formins and the microtubule cytoskeleton (Ishizaki et al., 2001; Palazzo et al., 2001).

**Capu is a member of the formin family**

The Drosophila protein Capu, encoded by the *cappuccino (capu)* gene is a member of the formin family and belongs to the formin subfamily of proteins (Emmons et al., 1995). Sequence analysis of Capu indicates that the carboxy terminal half of the protein is closely related to the limb deformity locus (ld) (Emmons et al., 1995). Like capu, the ld locus functions in polarity establishment, mutants in which have abnormal limb development and kidney aplasia (Woychik et al., 1990).

Members of the formin family are considered to link RhoGTPase signaling to regulation of the actin cytoskeleton (Evangelista et al., 1997; Kohno et al., 1996).
RhoGTPases are bimodal switches that regulate actin dependent processes. Formins have been shown to interact with members of the Rho family through the Rho binding domain (RBD) at their amino terminus (Evangelista et al., 1997; Watanabe et al., 1997). Capu binds strongly with RhoA and weakly with other members of the Rho family such as CDC42 and Rac1. capu also interacts with RhoA in vivo as females doubly heterozygous for mutations in rhoA and capu lay embryos with no pole cells (James, 2001). Thus, formins may act as intermediates that link RhoGTPase signaling to the actin cytoskeleton.

The FH1 domain of formins is a highly conserved proline rich region that has been shown to interact with the actin binding protein, Profilin (Figure 1.3) (Evangelista et al., 1997). Members of the formin family are thought to regulate the actin cytoskeleton by binding to Profilin and other actin binding proteins (Chang et al., 1997; Evangelista et al., 1997; Manseau et al., 1996; Watanabe et al., 1997). Mutations in capu shares phenotypes with mutations in spir and chickadee (chic), a gene that encodes the Drosophila homolog of Profilin. capu interacts with chic genetically and physically (Emmons et al., 1995; Manseau et al., 1996; Wellington et al., 1999). Drosophila Profilin is required for actin polymerization (Cooley et al., 1992). chic also shares the microtubule phenotype with capu and spir and exhibits dorsal-ventral eggshell defects, resembling capu in its function to localize determinants to the posterior pole of the oocyte (Manseau et al., 1996).
The phenotypes seen in capu are likely due to the misregulation of the actin or the microtubule cytoskeleton (Emmons et al., 1995; Manseau et al., 1996; Theurkauf, 1994; Wellington et al., 1999). It is not clear whether they regulate the microtubule cytoskeleton via actin or vice versa or if they regulate both in parallel to achieve polarity. The microtubule phenotype seen in capu, spir and chic mutants suggests that they probably act directly to regulate the microtubule cytoskeleton (Figure 1.4). Members of the formin family have also been linked to the regulation of the microtubule cytoskeleton (Ishizaki et al., 2001). Capu has a strong association with the actin cytoskeleton, yet has only microtubule phenotypes and thus, probably serves as a connection between the actin and microtubule cytoskeleton. Like Capu, the budding yeast formin Bni1 and the mammalian formin, mDia have been shown to be required for functions that involve both the cytoskeletons (Fujiwara et al., 1999; Ishizaki et al., 2001).

**Spire**

Spir is a novel gene that encodes a protein that contains 4 Wasp Homology 2 (WH2) domains (Figure 1.3) (Bi and Zigmond, 1999; Wellington et al., 1999). WH2 domains bind G-actin in other proteins such as yeast verprolin and the Wiskott Aldrich Syndrome Protein (WASP) suggesting a function for Spir in regulating the actin cytoskeleton (Machesky and Insall, 1998; Miki et al., 1998; Symons et al., 1996). Studies from our lab have shown that Spir binds to actin in
the two hybrid system through its WH2 domains and also Spir protein fragments containing the WH2 domains have been shown to directly bind to G-actin (James, 2001; Wellington et al., 1999).

Previously only two classes of nucleating factors were known, formins and Arp2/3, to be able to nucleate actin polymerization in vivo. Recently Spir has been shown to nucleate actin filaments. The four WASP homology 2 (WH2) domains and an unique actin binding site seem to be responsible for this nucleation mediated by Spir (Baum and Kunda, 2005; Quinlan et al., 2005). The WH2 domains found in other actin regulating proteins such as N-WASP are not capable of nucleating actin filaments suggesting that this function is unique for Spir (Quinlan et al., 2005). Spir acts independently of the Arp2/3 and formins to induce new actin filaments in vitro and also has been shown to trigger actin assembly in vivo (Quinlan et al., 2005).

Spir has been shown to colocalize with Rab11 in NIH 3T3 cells (Kerkhoff et al., 2001). Rab11 GTPases are involved in the transport process from the transgolgi and in recycling processes. A dominant negative Spir blocks exocytosis of a viral protein indicating that Spir could be an effector of Rab GTPases and is involved in vesicle transport, suggesting a link between Spir and intracellular transport (Kerkhoff et al., 2001).
The mammalian homologue of the Drosophila Spir, spir-1 and the mouse formin-2 gene has been shown to have overlapping expression pattern in the mouse embryo and adult brain suggesting that Spir and formins play roles in the same pathway in mammals similar to flies (Schumacher et al., 2004).

Spir was recently identified in the yeast two-hybrid system as a protein interacting with DJNK, the Drosophila homolog of Jun N-terminal kinase (Otto et al., 2000). Spir is phosphorylated by DJNK in vitro, suggesting that multiple signaling inputs may impinge on Spir. Spir also induces actin polymerization upon transfection into mammalian cells, suggesting that Spir proteins can promote actin polymerization in vivo (Otto et al., 2000). Actin filament clustering around the nucleus was observed when Spir was ectopically expressed in mouse fibroblasts (Otto et al., 2000).

The amino terminal of Spir binds to wild type and dominant negative RhoA, Rac1 and CDC42 in two hybrid interaction (Wellington et al., 1999). A direct interaction between Spir and Rho family members has also been observed in vitro (James, 2001). Genetic interactions between spir and mutants in certain Rho GTPases, where mutant spir heterozygotes in trans with mutant rhoA heterozygotes show the pole cell absent phenotype, confirm the observed in vitro interactions (James, 2001). This suggests that Spir may link signaling from Rho family GTPases to the actin cytoskeleton.
**Chickadee**

*Drosophila chickadee* encodes the actin binding protein, Profilin (Carlsson et al., 1977; Lassing and Lindberg, 1985). Profilins are small proteins that interact with actin monomers and also interact with proteins having a type of Poly-L-proline helix (Machesky and Insall, 1998; Schluter et al., 1997). Profilin’s interaction with actin monomers suppresses growth at the pointed end, allowing growth at the uncapped barbed end (Blanchoin and Pollard, 1998; Romero et al., 2004). In addition to this, most Profilins also mediate exchange of adenine nucleotide bound to actin monomers. In many organisms, Profilin is found to bind with Formin homology (FH) proteins (Evangelista et al., 1997; Higgs, 2005; Machesky and Insall, 1998; Tanaka and Shibata, 1985).

The Drosophila FH protein Capu, has been shown to interact with Profilin through the proline rich FH1 domain of Capu, in a two-hybrid system (Manseau et al., 1996). In *Saccharomyces cerevisiae*, the FH protein Bni1p binds with Profilin (Evangelista et al., 1997). The interaction of Profilin with both actin and the poly-proline rich proteins is necessary for its function (Lu and Pollard, 2001). It is also known that Profilin accelerates actin elongation processes mediated by formins (Romero et al., 2004).
Mutations in the *chic* locus cause defects in mid oogenesis (Schupbach and Wieschaus, 1991). Mutants in *chic* show disrupted nurse cell cytoplasm transfer into the oocyte (Cooley et al., 1992). Throughout oogenesis there is cytoplasmic flow from the nurse cells into the oocyte carrying maternal RNAs and proteins. Later in oogenesis this flow to the oocyte becomes very rapid and due to this the oocyte doubles its volume in a short time (Mahajan-Miklos and Cooley, 1994). In *chic* mutant egg chambers, the nurse cells lack a proper cytoplasmic actin network that holds the nurse cell nuclei in place, thus resulting in blocking cytoplasmic flow. The incomplete flow of cytoplasm into the oocyte in *chic* mutants results from the fact that the nuclei are not held in position when the cytoplasmic flow starts and as a result they are pushed into the ring canals blocking the path of flow to the oocyte (Cooley et al., 1992). Eggs laid by *chic* mutant females are small probably because of this aberrant actin filament organization (Cooley et al., 1992; Schupbach and Wieschaus, 1991). *chic* egg chambers also have multinucleate nurse cells implicating defects in cytokinesis (Verheyen and Cooley, 1994).

**The actin and microtubule cytoskeleton in Drosophila ovary**

Ring canal actin and cytoplasmic actin bundles are the two populations of actin filaments in the Drosophila oocyte and nurse cells. The cytoplasmic actin bundles resemble parallel actin bundles of the brush border microvilli and hair cell
stereocilia (Bartles, 2000; DeRosier and Tilney, 2000). Filament cross linking proteins such as Quail has been shown to be required for bundling of these filaments and fascin is required to crosslink the filaments (Matova et al., 1999). Phalloidin staining of ring canals reveals a tight band of F-actin that appear to be organized into loosely packed parallel bundles through most stages of oogenesis (Tilney et al., 1996).

The organization of the microtubule cytoskeleton also plays a critical role in determinant localization. After the adoption of a posterior fate, the follicle cells send a signal back to the oocyte resulting in changes in the organization of the microtubule organizing center (MTOC) at the posterior pole, which changes the orientation of the microtubules in the oocyte (Theurkauf et al., 1992). Early on during oogenesis, microtubules are polarized with their plus ends at the posterior and the minus ends at the anterior end of the oocyte (Figure 1.4) (Theurkauf et al., 1992) thus facilitating the transport of nutrients from the nurse cells to the developing oocyte. By the time the anterior-posterior polarity of the oocyte is established the microtubules change their orientation such that the plus end is at the anterior and the minus end faces the posterior. Positioning of determinants such as bicoid RNA at the anterior pole and the oskar RNA at the posterior pole of the oocyte have been shown to be dependent on the proper organization of the cytoskeleton since, drugs that disrupt the microtubule cytoskeleton prevent proper localization of bicoid and oskar mRNA to their respective poles (Pokrywka and Stephenson, 1991; Schnorrer et al., 2000). Aberrations result in
misorganization of the microtubules with their plus ends now at the center of the oocyte and minus ends at both poles resulting in the mislocalization of the anterior and posterior pole components (Gonzalez-Reyes et al., 1995; Lane and Kalderon, 1993).

**capu spir and chic share phenotypes**

capu, spir and chic share numerous phenotypes. capu and spir are maternal effect loci that affect the organization of both the anterior-posterior and dorsal-ventral axes of the Drosophila egg and embryo (Manseau and Schupbach, 1989). The gene products they encode are necessary for the localization or stabilization of developmentally important molecules to specific regions within the cytoplasm of the developing egg. capu and spir mutant females produce embryos that are dorsalized, lack pole cells and abdominal segments (Manseau and Schupbach, 1989). Mutants in chic exhibit dorsal-ventral egg shell defects (Manseau et al., 1996).

capu, spir and chic are required for localizing key determinants in the oocyte during development. The localization of bicoi mRNA to the anterior of the oocyte is required for the establishment of the anterior pole (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988). This seems to be normal in capu spir and chic mutants (Manseau et al., 1996; Wellington et al., 1999). Localization of
molecular determinants to the posterior pole is required for the formation of the abdomen and pole cells (Bardsley et al., 1993; Ephrussi et al., 1991; Hay et al., 1988; Kim-Ha et al., 1991; St Johnston et al., 1991). Mutants in capu and chic fail to localize staufen protein and oskar mRNA to the posterior pole (Manseau et al., 1996). The oskar mRNA is mislocalized and Staufen localization seems to be normal in spir mutant oocytes (Wellington et al., 1999). The cytoskeleton has been implicated to play an important role in the localization of determinants to their respective poles.

The localizing defects seen in these mutants could arise from misregulation of the microtubule and actin cytoskeleton during oogenesis. A major microfilament based cytoskeletal movement occurs towards the end of oogenesis, when the nurse cells dump their contents into the oocyte (Gutzeit, 1986). This cytoplasmic dumping process is actin dependent as it is inhibited by drugs that depolymerize actin (Cooley et al., 1992; Gutzeit, 1986). During this rapid transfer of cytoplasm from the nurse cells into the oocyte microtubule-dependent cytoplasmic streaming, inhibitable by colcemid, occurs within the oocyte. It is thought that this movement within the oocyte is necessary to mix the oocyte cytoplasm with the cytoplasm coming from the nurse cells (Gutzeit, 1986). Coincident with ooplasmic streaming, microtubules bundle at the cortex of the oocyte (Theurkauf et al., 1992).
In *capu*, *spir* and *chic* mutants this microtubule-based cytoplasmic streaming and the bundling of the microtubules that accompanies streaming occurs prematurely during mid oogenesis (Emmons et al., 1995; Theurkauf, 1994; Wellington et al., 1999). Long, thick microtubules are seen wrapped around the cortex of the oocyte in *capu*, *spir* and *chic* mutants. In *chic*, the microtubules were longer than that seen in *capu* and *spir* and were sometimes found toward the central part of the oocyte rather than being restricted to the cortex like in *capu* and *spir* oocytes (Manseau et al., 1996; Theurkauf, 1994; Wellington et al., 1999). Treating wild-type egg chambers during mid-oogenesis with Cytochalasin D mimics the premature microtubule based cytoplasmic streaming and the abnormal microtubule distributions observed in *capu*, *spir* and *chic* mutant oocytes suggesting that actin probably plays an essential role in regulating the formation of the polarized microtubules (Emmons et al., 1995).

It is possible that this premature streaming sweeps determinants away from the posterior end of the developing egg before they are localized. This is supported by the observation that induction of cytoplasmic streaming in wild type oocytes during mid-oogenesis displaces Staufen protein from the posterior pole but the *gurken* mRNA in the dorsal anterior position is not mislocalized (Emmons et al., 1995).
capu, chic and spir have a similar effect on the microtubules and cytoplasmic streaming suggesting that this could be the primary cause of the localization defect. Despite the apparent dysfunction of the microtubule cytoskeleton, several lines of evidence presented above suggest that misregulation of the actin cytoskeleton results in the disruption of polarity in these mutants. However, the defect in the actin cytoskeleton that leads to premature microtubule streaming is unknown. Also, the mechanism of cause and effect of actin disruption in capu, spir and chic mutants is yet to be unraveled. What is also not known is if these loci have actin independent roles that interfere with development, their exact mechanism of cytoskeleton regulation, and if they are solely regulated by actin binding.
Figure 1.1 Drosophila oogenesis.

A) Diagram of a pair of Drosophila ovaries in the female reproductive system. B) Diagram of the ovariole with the germline stem cells and their progression to egg chambers. The follicle cells are somatic and surround the cystoblasts and the oocyte is then localized to the posterior pole of the egg chamber. C) Diagram of an egg chamber showing the anterior nurse cells and the posterior oocyte surrounded by the follicle cells.
Figure 1.1 Drosophila oogenesis
Figure 1.2 Localization of determinants during oogenesis.

A) Red area depicts the location of the posterior and the green area, the dorsal anterior determinants in a wild type oocyte during oogenesis. B) In capu or spír mutant mother, posterior determinants are absent and fail to localize to the posterior pole. Dorsal anterior determinants (green) are mislocalized along the anterior cortex of the oocyte.
Figure 1.2 Localization of determinants during oogenesis.
Figure 1.3 Domain organization of Formin and Spir proteins.

A) Formins contain the well conserved Formin Homology (FH) domains, the proline rich FH1, and the FH2 domains involved in actin nucleation and a weakly conserved FH3 domain important for localization of some formins. They also may contain a Rho binding domain (RBD) and the Diaphanous Autoregulatory Domain (DAD) (see text). B) Spir proteins have three SPEM (spir Pem-5) domains, an RBD, four WH2 Wiskott-Aldrich Homology involved in actin binding, the FYVE zinc finger domain and the Jun N-terminal kinase binding domain (JNKBD).
Figure 1.3 Domain organization of Formin and Spir proteins.
Figure 1.4 Microtubule bundling seen in capu and spir mutants.

A) Diagram of stage 8 wild type oocyte shows polarized microtubules with their plus ends pointed towards the posterior pole. This facilitates transportation of determinants to the posterior via plus ended microtubule motors (arrow). B) Egg chamber of capu or spir mutant mother shows microtubules bundled around the cortex which prevents directional determinant localization.
Figure 1.4 Microtubule bundling seen in capu and spir mutants.
CHAPTER 2

CAPU AND CHIC REGULATE YOLK PROTEIN BIOGENESIS DURING DROSOPHILA DEVELOPMENT

Background

Endocytosis is a cellular process by which membranes and macromolecules are internalized and sorted by cells. Endocytic trafficking is essential in many cellular processes such as nutrient uptake, membrane lipid and protein recycling, synaptic vesicle recycling and growth factor receptor regulation during development (Brodin et al., 2000; Di Fiore and De Camilli, 2001; Goldstein et al., 1979; Mukherjee et al., 1997; Roth and Porter, 1964). A role in endocytosis, vesicle movement in particular, has been shown for the members of the formin family of proteins (Evangelista et al., 2002; Gasman et al., 2003; Randazzo, 2003). Formins function to regulate the cytoskeleton during processes such as cell polarization, cytokinesis and signaling (Castrillon and Wasserman, 1994; Chang et al., 1997; Emmons et al., 1995; Leader and Leder, 2000; Lynch et al., 1997; Wallar and Alberts, 2003).

Based on sequence homology formins have been subdivided into three subfamilies, the formin, the Bni1 and the Diaphanous subfamilies (Figure 2.1)
(Zeller et al., 1999). Bni1 and the Diaphanous subfamilies have roles in endocytosis. Studies on a Diaphanous subfamily formin, the human Diaphanous hDia2C, have shown localization to early endosomes (Gasman et al., 2003). Expression of this formin along with RhoD in HeLa cells causes alignment of endosomes along actin cytoskeleton and also has an effect on endosome motility, suggesting a new role for this formin in endosome function (Gasman et al., 2003; Randazzo, 2003). Another study on double mutants in the budding yeast formins, Bni1 and Bnr1P of the Bni subfamily has been shown to be required for the polarized accumulation of Myo2, the actin cable motor and Sec4p, a RabGTPase to the growing bud tip, suggesting a role for the yeast formins in polarized vesicle transport (Evangelista et al., 2002). These studies, done in unicellular systems or in vitro may not clearly indicate the role of formins in endocytosis, in multi-cellular systems. Also it is not clear if these formins also play roles in regulating other endocytic functions. Although it is useful to analyze the role of endocytosis in unicellular organisms such as the fission yeast, S. cerevisiae, much remains to be learned about endocytosis in multicellular organisms, especially in the context of cell-cell communication and the role of the actin cytoskeleton interacting proteins in endocytic regulation.

My studies in the Drosophila formin cappuccino (capu), a member of the formin subfamily (Emmons et al., 1995) identifies a novel function for capu in endosome biogenesis and for the first time identifies a role for the formin subfamily of
proteins in regulation of endosome biogenesis. Specifically, this work identifies a role for formins in the endocytosis of yolk during oogenesis. Yolk is internalized into the oocyte from the insect hemolymph, by receptor mediated endocytosis after being synthesized in the fat bodies (Flickinger and Rounds, 1956; Raikhel and Dhadialla, 1992). Yolk uptake during Drosophila oogenesis is an excellent model to study the mechanisms and molecules involved in endocytosis, since endocytosis is essential during oogenesis (Kress, 1982) and the power of genetic dissection can be used to identify critical regulators of this process. Using mutant analysis and designer transgenes, I also find that the interaction of chic with capu is necessary for the role of chic in yolk granule biogenesis.

The Endocytic pathway

During the process of receptor mediated endocytosis, cells bring in proteins, lipids and other types of ligands attached to the plasma membrane via receptors (Goldstein et al., 1979). The endocytic system comprises various morphologically heterogeneous compartments called endosomes. Endosomal compartments are smooth membrane systems consisting of tubular vesicular structures (Hopkins, 1983). The endocytosed molecules are first delivered to the early endosomes, which are found primarily close to the plasma membrane (Goldstein et al., 1979). From the early endosomes, the internalized proteins, lipids and receptors are either recycled back to the plasma membrane for re-use or targeted to the late
endosomes and then to the lysosomes for degradation. The early endosome serves as a major sorting station where decisions are made to send internalized molecules back to the plasma membrane or to the lysosome for proteolytic degradation (Mukherjee et al., 1997; Mullock et al., 1994). Lysosomes are electron dense membrane bound organelles that contain many hydrolytic enzymes that function in an acidic pH environment (Schmid et al., 1989). The early endosomes, late endosomes and lysosomes have different internal pHs and also differ in their protein composition (Mukherjee et al., 1997). The early and late endosomes are also characterized by the association of different small GTPases of the Rab family (Pfeffer, 2003). The endocytic pathway culminates with the degradation step that occurs in the lysosomal compartments (Kornfeld and Mellman, 1989). In contrast to the general endocytic pathway where the internalized ligands are typically degraded in the lysosomes, some endocytic processes such as the uptake of yolk proteins differ in that they are not degraded but stored and utilized during embryogenesis (Fagotto, 1995).

**The Drosophila oocyte as an endocytic cell**

During early oogenesis in Drosophila, a rapid uptake of yolk protein occurs, beginning at stage 8 of oogenesis (Cummings and King, 1970; Mahowald, 1972). The yolk protein made in the fat bodies of the insects is secreted into the hemolymph and then taken up by the oocyte (Flickinger and Rounds, 1956). The uptake of yolk into the oocyte occurs by receptor mediated endocytosis (Dhadialla
and Raikhel, 1991) (Figure 2.2). Yolk is taken up by the vitellogenin receptor, yolkless, in Drosophila (Mukherjee et al., 1997). Oogenesis consists of 14 stages, according to the staging of oocyte growth (Cummings and King, 1970; Flickinger and Rounds, 1956). The first seven are the pre-vitellogenic stages, and stages 8 to 10 are the vitellogenic stages and stages 11 to 14 are mainly involved in the maturation of the egg and formation of egg coats. At the completion of vitellogenesis, the oocyte grows rapidly as a result of the addition of the nurse cell cytoplasm. The endocytosed yolk initially accumulates in vesiculotubular structures similar to early endosomes (Postlethwait and Giorgi, 1985; Raikhel and Lea, 1986) which then coalesce to form primary yolk bodies analogous to late endosomal structures (Richard et al., 2001; Schonbaum et al., 2000). It is then stored as yolk granules or yolk platelets, which are modified lysosomes. In contrast to the general endocytic pathway where the internalized ligands are degraded in the lysosomes, yolk proteins are stored in yolk granules for later use during embryogenesis (Pasteels, 1966).

The pH of the yolk granules seems to play a key role in regulating the degradation of yolk. The relatively high pH of yolk granules, during embryogenesis, drops close to lysosomal pH (Fagotto, 1995). The acidic pH of the lysosomes aids degradation. They contain hydrolase, enzymes that are needed for the degradation of yolk. Degradation does of the yolk granules does not start until the embryos reach a particular stage of development and factors such as pH and
enzymatic latency seem to be involved in this regulation (Pasteels, 1966).
Although only little is known about yolk usage and degradation in Drosophila, studies in other organisms have shown that, yolk granules are initially neutral or lightly acidic, making it hard for any degradation to occur. They later on become more acidic as they progress into embryogenesis, which triggers degradation of yolk (Fagotto, 1990; Fagotto and Maxfield, 1994a; Fagotto and Maxfield, 1994b; Mallya et al., 1992). Studies in Xenopus have also shown that degradation of yolk platelets during early embryogenesis occurs by the fusion of the yolk platelets with late endosomes containing lysosomes (Komazaki and Hiruma, 1999). The Drosophila oocyte makes an excellent system to study endocytosis and vesicular trafficking of yolk as this process is crucial for oogenesis where transported nutrients are vital for the growth of the oocyte. An essential role for endocytosis in yolk uptake and biogenesis has been indicated by studies with the temperature-sensitive shibire mutants. shibire encodes dynamin, a molecule that plays an essential role in the internalization step of endocytosis (Chen et al., 1991; Grigliatti et al., 1973; van der Bliek and Meyerowitz, 1991). In oocytes, shibire mutants at the non-permissive temperature show disappearance of endosomal compartments consisting of tubules and small yolk spheres, leading to an arrest of yolk uptake (Tsuruhara et al., 1990).
Regulation of cytoskeleton by Capu

capu mutant egg chambers have abnormal distribution of microtubules and premature microtubule-based cytoplasmic streaming suggesting that the microtubules act directly or indirectly to regulate the cytoskeleton during polarity establishment (Emmons et al., 1995; Theurkauf et al., 1992). capu shows genetic and physical interaction with Drosophila chickadee (chic), which encodes the actin binding protein, Profilin (Manseau et al., 1996). The formin homology1 (FH1) domain of members of the formin family interact with Profilin (Evangelista et al., 1997; Higgs, 2005; Tanaka and Shibata, 1985). More recently Profilin has been shown to accelerate actin assembly by formins (Kovar et al., 2003; Romero et al., 2004). Mutants in Profilin that are unable to bind the proline rich FH1 domain have no effect on enhancing formin mediated nucleation (Pring et al., 2003; Sagot et al., 2002b). This suggests that the FH1-Profilin interaction is essential for the increased nucleation of actin filaments by formins.

The microtubule bundling and microtubule based cytoplasmic streaming phenotypes seen in capu mutants are also seen in spir and chic mutant oocytes (Manseau et al., 1996; Wellington et al., 1999). Studies have shown that Spir is an actin binding protein (Kerkhoff et al., 2001; Wellington et al., 1999) that can...
bind to Capu in a two-hybrid system and \textit{in vitro} (James, 2001; Wellington et al., 1999). In addition to the microtubule misregulation, \textit{chic} also shares the posterior localization defects seen in \textit{capu}. \textit{chic} mutants fail to localize \textit{staufen} and \textit{oskar} mRNA to the posterior pole of the oocyte (Manseau et al., 1996). Thus strong genetic interactions between \textit{capu} and \textit{chic} indicate a common mechanism of regulation.

Although we know that \textit{capu} and \textit{chic} act to regulate the cytoskeleton and are also required for the localization of determinants in the oocyte, the exact role of \textit{capu} and \textit{chic} during oogenesis is not known. My studies reveal a role for Capu and Chic in endosome regulation during yolk granule biogenesis and indicate that the interaction of Chic with Capu is necessary for this regulation during Drosophila oogenesis.

\textbf{Results}

\textbf{Loss of function mutation in \textit{capu} results in enlarged yolk granules}

Mutations in \textit{capu} lead to defects in localization of posterior determinants. We speculated if a defect in \textit{capu} mutant oocytes may be involved in the localization role of Capu. As a first step toward understanding the function of \textit{capu}, we performed ultrastuctural analysis of the Drosophila oocyte. We expected that this
study might reveal any macromolecular changes that could contribute to the mutant phenotypes seen in capu oocytes. Stage 7-10 capu mutant oocytes were fixed for electron microscopy and observed. Strong mutant alleles of capu EE15 and capu G7 reveal strikingly large electron dense vesicles in the oocyte starting with stage 8 during oogenesis (Figure 2.3). These granules were not observed until stage 7 and started appearing only from stage 8 onward. Past literature on ultrastuctural studies in Drosophila oocytes have identified these large electron dense vesicles to be yolk granules (Giorgi and Jacob, 1977; Tsuruhara et al., 1990). Also, we know that vitellogenesis, the process of yolk protein uptake by the oocyte, begins at stage 8 during oogenesis (Mahowald, 1972). The yolk granules observed in capu mutant oocytes were large in comparison to similar stages of wild type oocytes going through the process of yolk uptake. Yolk uptake is one of the most dramatic examples of endocytosis known and it is possible that capu is involved in regulation of yolk granule biogenesis.

To verify that these electron dense vesicles are lysosome-like yolk granules, we labeled capu mutant oocytes with Lysotracker, a dye that stains acidic compartments. We found dramatically large-sized acidic vesicles in capu mutant oocytes compared with wild type (Figure 2.4). Because the Drosophila oocyte contains large amounts of modified lysosomes in the form of yolk spheres (Fagotto, 1995), the acidic compartments likely correspond to yolk spheres that function as nutritional storage organelles of protein and carbohydrates.
We next wanted to quantify the granules, to see if there is a significant difference in the number and volume of these granules in capu compared to wild type. Quantification revealed a 50% reduction in the number of yolk granules in capu oocytes when compared to wild type (Figure 2.5A). Also, a distribution of vesicle size in a histogram indicates that in capu mutants the vesicles are skewed towards larger size compared to wild type. The number of vesicles with larger volumes is insignificant in wild type, whereas in capu mutants more than 30% of vesicles are larger than 3.75 μ, the largest vesicle volume observed in wild type (Figure 2.5B). The decrease in number of granules together with increased volume indicates a possible defect in the biogenesis of yolk granules. The decrease in yolk granule number along with increase in size suggests that abnormal fusion may contribute to the mutant phenotype. We next wanted to see if this phenotype is due to the loss of function of capu itself and not due to some strain background modifiers.

The large yolk granule phenotype is due to capu loss of function

To ensure the yolk phenotype we see in capu mutants is due to mutation in capu itself and not due to genetic modifiers, we performed complementation analysis (Figure 2.6A-D). In this analysis, single mutant copy of capu over a wild type chromosome that is a capu heterozygote is expected to have smaller, wild type
like yolk granules while a *capu* mutant chromosome over a deficiency that uncovers *capu* is expected to have enlarged yolk granules. DIC imaging of the *capu* heterozygote oocytes revealed wild type like yolk granules whereas *capu* in the presence of a deficiency that uncovers *capu* showed enlarged yolk granules as observed in *capu* mutants. This result indicates that the strikingly large yolk granules seen in *capu* mutant oocytes is due to *capu* loss of function mutation and not an artifact from strain backgrounds. Thus, this large yolk granule phenotype in *capu* mutants suggests that Capu may function to regulate yolk granule biogenesis.

Large vesicles seen in *capu* could arise for various reasons. One possible reason is that there is internalization of large yolk vesicles to begin with at the stages when vitellogenesis occurs. The second possibility for the biogenesis defect is there is increased endocytosis of yolk during oogenesis. A third possible reason could be that internalization of yolk is normal but post internalization defects such as abnormal fusion leads to large yolk granules. The final possibility we considered is a direct or an indirect effect of the actin cytoskeleton on the yolk endocytic machinery through the interaction of Capu with the cytoskeleton.
The biogenesis defect is not due to internalization of large yolk granules

Our results indicate that mutants in capu show a defect in biogenesis of yolk granules. One possible reason for how this could occur is by internalization of large vesicles during the stages at which the yolk protein is endocytosed into the oocyte. To see if large vesicles are endocytosed during oogenesis, we compared stage 8 and stage 10 oocytes of capu mutants with that of the wild type (Figure 2.7 A-F). When stage 8 and older oocytes were observed, we found that in the capu mutants the vesicle size increased as the stages progressed, while in the wild type, the increase was not significant, indicating that in capu, post internalization steps after endocytosis of yolk and not internalization itself, may be affected. It is possible that capu regulates the trafficking after internalization. Examination of much older stages revealed the presence of huge vesicles in the mutants, thus enhancing the possibility of a post internalization defect.

Kinetics of early endocytosis is not affected in capu mutants

To see if the defect in capu mutants is due to increase in yolk protein endocytosis, we looked at the uptake of an endocytic tracer, dextran conjugated to a fluorophore. Unless taken up by an endocytic process, dextran conjugates are
membrane impermeable. It is possible that increased uptake of yolk protein leads to accumulation of excess yolk in the oocyte and the extra amount of yolk spheres fuse with one another resulting in enlarged vesicles. To test if increased endocytosis could lead to the defect, capu mutant oocytes were exposed for a specific short time point (t=30s) to dextran, allowing enough time for the tracer to be taken up by the early endosomes. This revealed that capu mutants do not exhibit any difference in uptake of dextran at these early time points compared to wild type (Figure 2.8A-B). Moreover it suggests that the endosomes are accessible by the tracer. When the oocytes were pulsed with dextran for 5 min, it was taken up by the large endosomes in capu mutants (Figure 2.8C-D). While we did not observe a difference in endocytosis at earlier time points, imaging of egg chambers at later time points show dextran localized in large vesicles in capu mutants. It is possible that dextran is trafficked into large vesicles during endocytosis or dextran containing vesicles fuse shortly after internalization. These results suggest that kinetics of early endocytosis of yolk may not be the cause for the formation of large yolk granules.

**Abnormal fusion and large endosomes**

To test the possibility that post internalization defects such as abnormal fusion and other defects in the endosome machinery is a cause for the large yolk granule phenotype, we performed a pulse chase experiment with the endocytic tracer,
dextran. The ovaries were pulsed with two fluorescently tagged dextran molecules, one attached with the fluorophore rhodamine and the other attached to the fluorophore Alexa 647. A pulse and chase of these two dextran molecules normally should not lead to mixing of these fluorophores until very late in the endocytic pathway. However, if abnormal fusion of vesicles or defects in processing of late endosomes occurs in capu mutants, it should lead to the mixing of the fluorophores. To test this, ovaries were pulsed first with rhodamine dextran for 1 or 3 min. and washed. The second tracer, Alexa 647 dextran or other dextran conjugates such as Alexa 488 or Fluorescein were added and pulsed for about 4-5 min. and chased for 2 min. and the ovaries were fixed in the cold. I also performed the reverse of this experiment, where I first pulsed with Alexa 647 and dextran conjugates and chased with Rhodamine dextran to eliminate any dye preference. I was able to image the pulsing with one dye using both fluorophores but was unable to observe good fluorescence of both dyes after pulsing and chasing. This problem persisted even after changing possible variables like the fluorophores themselves and time of pulsing and chasing and buffer conditions, preventing me from reaching any conclusion on the role of capu in the regulation of endocytic fusion that may be involved in the large yolk granule defect.
Actin and Large vesicles

We next tested the possibility of direct or an indirect effect of the actin cytoskeleton on the yolk endocytic machinery. We asked if disruption of actin mimics the large vesicle phenotype observed in capu mutants. Cytochalasin D, a drug that caps actin filaments, thus preventing assembly and thereby disrupting actin dynamics, has previously been shown to mimic the cytoplasmic streaming phenotype seen in capu mutants (Manseau et al., 1996). Wild type ovaries were exposed either for 5 or 10 min to Cytochalasin D at a concentration of 10 µg/ml, similar to that which induced cytoplasmic streaming (Manseau et al., 1996). We did not observe any changes in vesicle morphology. Latrunculin A (latA) is a drug that causes actin filament disassembly, by sequestering actin monomers (Coue et al., 1987). I tried to disrupt actin using a combination of these drugs, Cytochalasin D followed by latA treatment. Still, I did not observe an effect on vesicle size. In an effort to increase the time of drug exposure that was sufficient to cause actin disruption, the flies were fed overnight and the egg chambers observed under microscope at various time points to determine the effect of actin disruption on yolk granule formation. We still did not observe any changes in vesicle morphology (Figure 2.9A-B). Rhodamine-phalloidin staining of cytochalasin D treated oocytes also did not show a change in distribution of actin
compared to control oocytes (Figure 2.9C-D). We were unable to conclude on the effect of actin disruption on vesicle size during oogenesis since our experimental design was insufficient to cause disruption of actin.

**Loss of function mutations in chic result in enlarged yolk granules**

To understand the role of capu in yolk biogenesis, we looked at proteins that interact with capu. Studies of these proteins may be able to shed light on the role of capu in this process. capu has been shown to physically and genetically interact with chic, which encodes Profilin, an actin binding protein (Manseau et al., 1996). Mutants in chic have phenotypes similar to capu. It would be interesting to see if mutants in chic also share the yolk granule phenotype with capu.

To this end, we performed ultrastructural studies in chic mutant oocytes. Electron microscopy studies on chic mutant oocytes undergoing vitellogenic stages revealed large yolk granules, similar to that seen in capu mutant oocytes (Figure 2.10). Quantification showed that, like capu mutant oocytes, the number of yolk containing vesicles was reduced by nearly 40% in chic compared to wild type (Figure 2.11A). Also, distribution of vesicle size in a histogram indicated that the vesicle size in chic was skewed towards larger size (Figure 2.11B). The number of vesicles with larger volume is insignificant in wild type, whereas in chic
mutants more than 30% of vesicles are larger than $3.75^3$, the largest vesicle volume in wild type. The ability of chic to induce endosome enlargement is phenotypically similar to that described in capu mutants. The sizes of these enlarged endosomes were comparable to those induced by capu. We consistently found that the large vesicles induced by capu and chic were significantly larger than in wild type.

To ensure the yolk phenotype we see in chic mutants is due to mutation in chic itself and not due to genetic modifiers, we performed complementation analysis. DIC imaging of the chic heterozygote oocytes revealed wild type like yolk granules whereas chic in the presence of a deficiency that uncovers chic showed enlarged yolk granules as seen in chic mutant oocytes (Figure 2.12A-D). This result indicates that the strikingly large yolk granules seen in chic mutants, is due to chic loss of function mutation and not an artifact from strain backgrounds.

**Interaction of Profilin with Capu is necessary for yolk granule biogenesis**

Since capu and chic share the vesicle phenotype it is interesting to speculate if these proteins act as a complex to regulate yolk granule biogenesis. Profilin is known to bind formins, the FH1 domain of formins has been shown to bind Profilin (Chang et al., 1997). Also, Profilin accelerates actin elongation processes
mediated by formins (Romero et al., 2004). It has been previously shown in our laboratory that interaction of chic with capu is necessary for Profilin function (Andrea Wellington, personal comm.). Mutations in residues critical in Profilin for formin binding did not rescue the dorsalized egg phenotype of chic mutants, indicating that the interaction with capu is necessary for Profilin function (Figure 2.13A, B).

Since chic mutants have the large yolk granule phenotype I asked whether the interaction of chic with capu is essential for yolk granule biogenesis. To do this, I introduced a wild type chic transgene or a chic transgene defective in Capu binding, into a chic mutant background. If Profilin functions to regulate yolk granule biogenesis by binding to capu, the chic transgene defective in Capu binding should not be able to rescue the large granule phenotype in chic mutants. We observed that while the wild type chic transgene rescued the large vesicle phenotype in chic mutants, a chic transgene that is defective in Capu binding was unable to rescue the chic mutant phenotype (Figure 2.13C-E). To ensure that the chic transgene encodes the Profilin protein similar to wild type transgene, we performed a Western blot to look for levels of Profilin protein in the chic transgene defective in Capu binding compared to a wild type chic transgene. I observed that the level of Profilin in the chic mutant transgene was comparable to the wild type chic transgene in a chic mutant background (Figure 2.13F). This indicates that Profilin binding to capu is necessary for yolk granule biogenesis.
Since spir also shows phenotypes similar to capu and chic and shows interaction with actin, I looked at yolk granules in spir mutant oocytes. spir mutants showed a moderately large yolk granule phenotype, but complementation tests failed to uncover the defect to the spir locus. A deficiency that uncovers spir, in the presence of a spir mutation did not show the large vesicle phenotype, whereas mutants homozygous for spir loci did show the large vesicle defect. It is possible that the Bloomington deficiency stocks for spir had genetic modifiers that interfered with the mutant phenotype. We also looked at RhoA, a protein that also interacts with capu. Mutations in rhoA did not lead to a large yolk granule phenotype, indicating that capu interaction with chic may specifically affect yolk biogenesis among other functions of capu.

**Discussion**

Biogenesis of yolk granules is an essential process during Drosophila oogenesis. My study identifies a novel role for capu and chic in the regulation of yolk granule biogenesis and also implicates the formin multigene family in the endocytosis of yolk during oogenesis. While the Diaphanous and the Bni1 subfamily of the formins have been shown to be required for vesicle movement, until now a role in endocytosis has not been shown for the formin subfamily of formin proteins to
which Cappuccino belongs. Our studies here identify a new function for the formin subfamily in the regulation of endocytic trafficking. We also show binding of Profilin, an actin regulating protein, encoded by chic, to Capu is necessary for regulating the size of yolk vesicles. Our results suggest that the large yolk granules observed in capu and chic loss of function mutants result from defects in endocytic trafficking events post-internalization. This is supported by our tracer uptake assays and studies using differential interference contrast microscopy.

**Large endosomes and vesicle fusion**

The increase in the size of the yolk granules as the stages of oogenesis progress and the decrease of the number of granules in capu and chic mutant oocytes suggest that the defect most likely arises by fusion of internalized vesicles that contain yolk. This strikingly large endosome phenotype has been observed in other systems with overexpression studies. Overexpression of a constitutively active form of the GTPase Rab, which functions in the maintenance of early endosomes, leads to enhanced endosome fusion resulting in enlarged endosomes (Barbieri et al., 1996; Ceresa et al., 2001). While endosome fusion events are affected by GTP hydrolysis defective Rab5, kinetics of endocytosis is not affected, thus indicating that endosome geometry is not critical for sorting efficiency during trafficking.
Overexpression of LGP85, a glycosylated transmembrane protein found on the membranes of lysosomes and late endosomes in COS cells, induces the formation of abnormally large endosome-like structures (Kuronita et al., 2002). Studies have shown that the transport of cargo from these structures is blocked resulting in accumulation of free cholesterol. Co-expression of a dominant-negative form of Rab5b, a GTPase that functions in endosomal/lysosomal membrane trafficking, inhibits the effect of LGP85, suggesting a role for Rab5b in the formation of large endosomes (Kuronita et al., 2002). LGP85 probably regulates the biogenesis of endosomes/lysosomes by interacting with components of the vesicle fission/fusion machinery such as Rab5b.

Mutations in capu and chic lead to enlarged yolk granules and the proteins they encode may play a key role in regulating the lysosomal compartment. One possible intermediary for this regulation is the actin cytoskeleton. Actin is known to play a role in endocytosis in several systems, especially in yeast and also in mammalian cells (Jeng and Welch, 2001; Munn, 2001). Recent studies have established a link between actin nucleating proteins and proteins involved in endocytosis. For example, the actin nucleating mammalian protein, Abp1 interacts with the proline rich domain of dynamin, a protein essential for endocytosis (Kessels et al., 2001). It is postulated that this interaction may contribute to force generation which results in pinching off of vesicles during
internalization. Capu is a formin, a class of proteins newly identified to have an actin nucleating activity. It is possible that such an activity in Capu plays a role in regulating components of the vesicle fusion machinery.

Profilin, encoded by chic, is an actin binding protein that may regulate the actin cytoskeleton and vesicle biogenesis. Also, both capu and chic show microtubule dependent cytoplasmic streaming phenotypes that can be mimicked by disruption of the actin cytoskeleton. While these results argue that disruption of actin may lead to defects in endosome biogenesis, I did not observe any change in vesicle size after exposure of Drosophila oocytes to the actin depolymerizing drug, cytochalasin D. While I was unable to also observe disruption of actin after drug exposure, studies from mammalian cells indicate that during endocytosis actin filaments only play an accessory role and not an essential role (Fujimoto et al., 2000). This suggests that interaction of Profilin and Capu to other proteins and to each other may be more important in regulation of yolk biogenesis, than their interaction with the actin cytoskeleton. While the efficiency of endocytosis of yolk by itself may not be the cause of the patterning defects in capu and spir, the presence of these large vesicles may impede directional transport of determinants to the posterior pole and thus could lead to indirect effects of Capu and Profilin on determinant localization. Also, I did not see a large vesicle phenotype that mapped to the spir locus, while spir shares several other phenotypes with capu and chic. This strengthens the possibility that the
interaction between Capu and Profilin in actin independent functions may be contributing to the regulation of yolk granule biogenesis.

*capu* and *chic* are required for oogenesis and female fertility. While the exact mechanism that lead to female infertility in the offspring of these mutants are not known, it is also not clear to what extent the large yolk granule phenotype contributes to infertility. My work has identified that both *capu* and *chic* mutations lead to this phenotype and that the interaction between Capu and Profilin is necessary for Profilin function in yolk biogenesis. These results suggest that Capu and Profilin interact as a complex, probably along with other proteins in the vesicle fusion machinery to regulate yolk granule biogenesis. Although we present clear evidence for formin and Profilin function together in yolk biogenesis, the molecular mechanisms of this regulation are still to be determined.
Figure 2.1 The formin multigene family.

Based on sequence homology of the FH2 domain of the formin family of proteins, they have been subdivided into the a) Formin/cappuccino subfamily b) the Diaphanous subfamily and c) the Bni1 subfamily.
Figure 2.1 The formin multigene family.
Figure 2.2 Schematic of the pathway of yolk granule biogenesis in Drosophila oocytes.

Yolk is internalized by the yolkless receptor and goes through the endocytic pathway in the oocytes and is stored in yolk granules which are modified lysosomes.
Figure 2.2 Schematic of the pathway of yolk granule biogenesis in Drosophila oocytes.
Figure 2.3 Enlarged yolk granules in *capu* mutants.

Electron micrographs of stage 10 oocyte egg chambers in wild type (A) and *capu* EE15 (B) mutants. The electron dense structures are yolk containing vesicles and are enlarged in *capu* mutants. Scale bar= 5µ.
Figure 2.3 Enlarged yolk granules in capu mutants.
Figure 2.4 The enlarged compartments in *capu* mutant oocytes are accessed by Lysotracker.

Confocal images showing Lysotracker staining. Small acidic compartments are accessed by Lysotracker in wild type (A) and strikingly large acidic compartments are accessed by the dye in *capu* mutant oocytes (B).
Figure 2.4 The enlarged compartments in capu mutant oocytes are accessed by Lysotracker.
Figure 2.5 The number of yolk granules is decreased and volume increased in capu mutants compared to wild type.

Quantification of vesicle number and size. The number of vesicles present in the egg chambers of capu mutants (53 ±3) are decreased compared to wild type (106±4), n=4 stage 10 oocytes (A). The number of vesicles with larger volumes is insignificant in wild type, whereas in capu mutants more than 30% of vesicles are larger than 3.75 cubic μ, the largest vesicle volume observed in wild type (B).
Figure 2.5 The number of yolk granules is decreased and volume increased in capu mutants compared to wild type.
Figure 2.6 The large yolk granule phenotype is due to *capu* loss of function. Differential interference contrast (DIC) imaging of oocytes (A-D). *capu*EE15 heterozygotes shows wild type like yolk granules (A) whereas *capu*EE15 homozygote shows large yolk granules (B). *capu*EE15 transheterozygous with another allele, *capu*G7, also shows the mutant phenotype (C). The large yolk granule phenotype is also uncovered by a deficiency that uncovers *capu* over *capu*EE15 (D).
Figure 2.6 The large yolk granule phenotype is due to capu loss of function.
Figure 2.7 Vesicle size increases during oogenesis in *capu* mutants.

The vesicle size is similar in stage 8 (A), stage 10 (B) and mature oocytes (C) of wild type egg chambers. In *capu* mutant egg chambers, vesicle size increases as the stages progress from stage 8 (D) to stage 10 (E) to more mature oocytes (F) indicating that defective internalization may not be the cause of enlarged yolk granules.
Figure 2.7 Vesicle size increases during oogenesis in *capu* mutants.
Figure 2.8 Endocytic tracer uptake.

Confocal images of oocytes exposed to a short pulse of $t=30s$ of the endocytic tracer Dextran, show no difference in kinetics of tracer uptake between wild type and $capu$ mutant egg chambers (A and B). A longer pulse of $t=5min$ shows that tracer is localized to different sized vesicles, small endosomes in wild type (C) and by the larger endosomes in $capu$ mutant oocytes (D).
Figure 2.8 Endocytic tracer uptake.
Figure 2.9 Exposure to cytochalasin D does not cause large vesicle defect. Wild type oocytes of females fed with cytochalasin D for 24 hours (B) shows no change in vesicle morphology to that of the control oocytes (A). Rhodamine phalloidin staining of actin does not show difference in actin distribution in wild type oocytes of females fed with cytochalasin D (D) compared to control oocytes (C).
Figure 2.9 Exposure to cytochalasin D does not cause large vesicle defect.
Figure 2.10 Enlarged yolk granules in chic mutants.
Electron micrographs of stage 10 oocyte egg chambers in wild type (A) and chic 1320 (B) mutants. Yolk containing vesicles are enlarged in the mutants, similar to that seen in capu mutants. Scale bar= 5µ.
Figure 2.10 Enlarged yolk granules in chic mutants.
Figure 2.11 The number of yolk granules is decreased and volume increased in *chic* mutants compared to wild type.

Quantification of vesicle number and size. The number of vesicles present in the egg chambers of *chic* mutants (68 ±7) are decreased compared to wild type (106±4), n=4 stage 10 oocytes (A). The number of vesicles with larger volumes is insignificant in wild type, whereas in *chic* mutants more than 30% of vesicles are larger than 3.75 _3, the largest vesicle volume observed in wild type (B).
Figure 2.11 The number of yolk granules is decreased and volume increased in chic mutants compared to wild type.
Figure 2.12 The large yolk granule phenotype is due to *chic* loss of function. Differential interference contrast (DIC) imaging of oocytes (A-D). *chic*<sub>1320</sub> heterozygotes shows wild type like yolk granules (A) whereas *chic*<sub>1320</sub> homozygote shows large yolk granules (B) *chic*<sub>1320</sub> transheterozygous with another allele, *chic*<sub>221</sub>, also shows the mutant phenotype(C). The large yolk granule phenotype is also uncovered by a deficiency that uncovers *chic* over *chic*<sub>1320</sub> (D).
Figure 2.12 The large yolk granule phenotype is due to *chic* loss of function.
Figure 2.13 Interaction of Profilin with Capu is necessary for Profilin function in endosome biogenesis.

(A) Highlighted in boxes are amino acid positions 120 and 126 in Profilin known to be responsible for Profilin’s interaction with formin family members. These residues were mutated to alanines and transgenes were made and analyzed. While the wild type transgene shows interaction with both Capu and actin, a mutant transgenic line for chic, chic4B shows no Capu binding while showing normal actin binding (B) (Andrea Wellington). Expression of this Capu binding defective transgene in the presence of chic1320/chic221 mutation is unable to rescue the large vesicle phenotype (E) seen in chic1320/chic221 mutant oocytes (C). D, shows a wild type transgene in a chic mutant background. This lack of rescue may not be due to lack of Profilin expression (F) since the chic4B mutant transgene contributes Profilin in a chic1320/chic221 mutant background (lane 4) compared to chic1320/chic1320 (lane 2) and chic1320/ chic221 (lane 3) from western blots performed from ovary extracts. chic221 is a null allele and chic1320 is a hypomorph. Wild type (lane 1) and wild type transgene in chic1320/chic221 mutant background (lane 5) Profilin expression are also shown in the western blot.
Figure 2.13 Interaction of Profilin with Capu is necessary for Profilin function in endosome biogenesis.
CHAPTER 3

A GENOME-WIDE DEFICIENCY SCREEN TO IDENTIFY INTERACTORS OF CAPU AND SPIR

Background

Genetic studies have revealed a vital role for the Drosophila genes capu and spir during oogenesis. Studies of the mutant phenotypes have demonstrated a requirement for their gene products in the localization of determinants during Drosophila development. While such studies have identified defects in the cytoskeletal architecture and suggest that capu and spir alter microtubule distribution in the oocyte during oogenesis, the molecular role of capu and spir during development is not clear. Specifically, the molecular partners of capu and spir are yet to be identified that could shed light on a pathway for regulation of the cytoskeleton by capu and spir during oogenesis.

We know that mutations in capu and spir alter microtubule distribution (Emmons et al., 1995; Theurkauf et al., 1992; Wellington et al., 1999) and this phenotype can be mimicked in wild type oocytes by treating ovaries with Cytochalasin D, a drug that depolymerizes actin cytoskeleton (Manseau et al., 1996). It is not clear whether disruption of actin polymerization or altered
microtubule distribution causes the typical cytoplasmic streaming phenotype. Furthermore it is not known whether capu and spir regulate the microtubule cytoskeleton directly or through actin cytoskeleton or if they regulate both in parallel.

A key advantage of a model system is the ability to perform genetic screens to identify components of pathways and the role of each gene that is involved in such pathways. In Drosophila, genome-wide screens can be carried out to identify loci that modify specific phenotypes that are clearly discernible (St Johnston, 2002). Here, a sensitized background is selected and can be used to search for chromosomal regions that make the phenotype worse or better. One powerful way is to use sets of chromosomal deletions that can be used to screen a wide extent of the Drosophila genome at low resolution and subsequently use finer tools to identify loci in those deleted chromosomal regions to identify individual genes that modify the specific phenotype that is analyzed. A beautiful exhibition of this approach is an analysis of embryonic segmentation that identified several zygotic gene requirements during development (Zusman and Wieschaus, 1985).

To gain a better understanding of capu and spir function during oogenesis, we designed and conducted a deficiency screen to identify novel interactors of capu and spir using an available set of chromosomal deletions that is organized as a “deficiency kit,” a set of about 170 fly stocks that include deletions that cover
nearly 80% of the euchromatic genome (BLOOMINGTON DROSOPHILA STOCK CENTER, 2001).

Deficiency screens are based on the knowledge of a phenotype and screening the genome for deficiencies which, when doubly heterozygous with a gene of interest will exhibit a phenotype of interest. This is called second-site non-complementation, where mutant alleles in two different genes result in a mutant phenotype when doubly heterozygous at both loci, and also carries a wild type copy of each allele. This indicates that the gene products function in the same pathway and probably function as a complex. Such screens have been able to successfully identify genetic components of various pathways in Drosophila (St Johnston, 2002).

In Drosophila, the germplasm becomes localized in the posterior of the embryo and forms the pole cells, the progenitors of the germ line. Polar granules in germplasm are electron-dense structures and have been proposed to contain factors essential for pole cell formation. The information necessary for the formation of pole cells is provided maternally and is localized to the posterior pole of the developing egg (St Johnston et al., 1991). Localization of molecular determinants such as vasa protein, oskar mRNA and Staufen, tudor and nanos to the posterior pole is required for the formation of the pole cells and abdomen (Bardsley et al., 1993; Ephrussi et al., 1991, 1991; Hay et al., 1988; Kim-Ha et al.,
Mutants in *capu* fail to localize Staufen and oskar mRNA and *spir* mutants fail to localize oskar mRNA to the posterior pole resulting in females that produce embryos that lack pole cells and abdominal segments (Manseau et al., 1996; Wellington et al., 1999). The proper development of the pole cells and abdomen is achieved by the localization of these gene products to the posterior pole of the developing oocyte. In *capu* and *spir* mutants these gene products are produced but they are not localized properly to the posterior pole, suggesting that *capu* and *spir* are required either for the transport or for localization of these molecules to the posterior pole. Females homozygous for mutations in *capu* and *spir* lay embryos with no pole cells.

Studies from our lab have shown that Capu and Spir physically interact with the Rho family GTPases (James, 2001). The finding that *capu*+/+ *rhoA* and *spir*+/+ *rhoA* double heterozygous females also lay embryos lacking pole cells (Figure 3.1) allowed us to search for other second site mutations that will show a lack of pole cells when heterozygous with *capu* or *spir*. Pole cells are the progenitors of germ cells and when the embryos from these sterile adults were analyzed for the presence of pole cells it turned out that the adults were lacking pole cells. About 80% or more of the eggs laid by females heterozygous for *capu* and *rhoA* were lacking pole cells indicating a strong interaction between *capu* and *rhoA*. The interaction seen between *spir* and *rhoA* was not as strong as that seen with *capu*. 

1991; St Johnston et al., 1991).
spir showed much stronger interaction with cdc42 and rac1. This genetic interaction between capu or spir with rhoA indicates that they are acting in a similar pathway. Studies from the lab have also shown that Capu and Spir physically interact with the Rho family GTPases and Capu preferentially interacts with rhoA in the two hybrid and in vitro assays confirming the genetic interaction (James, 2001).

We designed a deficiency screen using the lack of pole cells as a phenotype. I screened the Drosophila genome for deficiencies that when doubly heterozygous with capu or spir would produce embryos with no pole cells. This tells us that a gene in that deficiency region is an interactor of capu or spir. We used this synergistic phenotype of embryos with no pole cells as a basis for this second site non-complementation analysis using chromosomal deficiencies and mutant analysis to identify interactors of capu and spir.

The Drosophila deficiency stock collection of about 170 deficiencies of chromosomes I, II and III that uncover about 70-80% of the genome were used to create double heterozygotes with capu and spir to identify regions of the genome that interact with these genes. The deficiency kit was screened for deficiencies that when doubly heterozygous with capu or spir produced females that are agametic. Absence of gametes will indicate that a gene or genes in that deficiency is interacting with capu and spir, suggesting that it is involved in the
same pathway as capu and spir. Instead of looking at the pole cells in the eggs of the double heterozygote, I looked for presence or absence of gametes in the next generation. Since pole cells are the progenitors of germ cells, the progeny of these females would be gameteless. With the sequencing of the Drosophila genome complete and the genome being highly annotated, it is possible to identify rapidly genes in a particular deficiency of interest and use smaller deficiencies to narrow down the size of second-site non-complementing region and ultimately identify the interacting gene. Possible candidate genes include cytoskeletal components, formin interactors, signaling GTPases, female sterility and MAPK signaling components.

**Results**

**Screen Design**

I selected the same mutations for capu and spir that were used in the earlier capu, rhoA and spir, rhoA interaction (James, 2001). Homozygous flies mutant for capu or spir were crossed to the deficiency to be tested. The Bloomington Drosophila deficiency stock kit is a collection of approximately 170 large deficiencies which I used for the crosses (Figure 3.2). In the F1 generation, females, carrying heterozygous capu or spir with the test deficiency were selected. These flies which are now double heterozygotes for capu or spir and the
test deficiency were crossed to wild type males. The females resulting from this cross were dissected to screen for the absence of the ovaries. I dissected at least 20 females for every cross and calculated the percent females that showed absence of ovaries for each cross. Deficiencies that showed higher than 50% lack of ovaries were considered strong and selected for further examination.

A Primary Screen for capu interactors identifies the chromosomal region 49C1-50D2

capu mutant females were crossed individually with the 170 available chromosomal deficiencies (Table 3.1). About 20 females that were capu/+;deficiency/+ were dissected from each cross to screen for absence of the ovaries. This screen identified a chromosomal region, 49C1-50D2 on chromosome 2 which when deficient as in Deficiency Df(2R)CX1 doubly heterozygous with capu, produced a high percentage of females with no ovaries. Nearly 90% of the females were found to lack ovaries. I was able to observe repeatedly a strong interaction with this region, suggesting the possibility that capu interacts strongly with a gene in this chromosomal interval. Control crosses performed with wild type siblings heterozygous for the deficiency show no defect in ovaries indicating that the presence of one copy of the capu mutation and one chromosome with this deficiency causes the lack of ovaries. This interacting
deficiency was a large chromosomal region consisting of about 230 genes. Hence we decided to narrow further this region using smaller deficiencies.

**A secondary screen for capu interactor narrowed the interacting interval to chromosomal region 50A-50D2**

From the primary screen, I identified that a chromosomal region, 49C1-50D2 that interacted strongly with capu. To narrow further the large interacting interval, smaller deficiencies in the region were used. Crosses were performed similar to the primary screen to test for an interaction with capu and we found none of the small deficiencies to show an interaction with capu, while three larger deficiencies showed moderate interaction with capu (Figure 3.3). This narrowed down the interacting region to 50A-50D2.

Some of the smaller deficiencies that I used map to 50A-50D2. It is possible that these smaller deficiencies do not overlap and hence do not entirely cover the larger deficiency, thus missing certain regions of the chromosome. In order to test this, we performed complementation analysis on the smaller deficiencies. If the deficiencies overlapped, then deficiencies that lie next to each other, when crossed should lead to lethality due to non-complementation. If there is no overlap in the deficiencies, one chromosome is able to complement the other deficient one and thus lead to viability when crossed to each other. When tested, I
found that deficiencies within the chromosomal region 50A-50D2 did not overlap. There are about 128 genes in this region and it is possible that \textit{capu} interacts with loci in this region. We were unable to get other small deficiencies or duplications for this interval. Due to unavailability of tools, we were unable to narrow down the loci that interacts with \textit{capu}. I did not pursue other interacting regions because they showed a far lower synergism and also focused more of my time with the \textit{spir} interacting deficiencies, as described below.

\textbf{A primary screen for \textit{spir} interactors identifies the chromosomal region 55A-55F}

\textit{spir} mutant females were crossed individually with the 170 large deficiency regions (Table 3.1) available from the Drosophila deficiency kit from the Bloomington Drosophila stock center, to screen rapidly for an interacting interval. Crosses were performed similar to the ones described for the primary screen for \textit{capu} interactors. Total of 20 females that were \textit{spir}/+;\textit{deficiency}/+ were dissected from each cross to screen its interaction with the \textit{spir} loci and tested by analyzing for absence of ovaries.

A chromosomal region 55A-55F deleted in Deficiency \textit{Df(2R)PC4} lying on the second chromosome showed a strong interaction with \textit{spir}. 70\% of the females doubly heterozygous for \textit{spir} and this chromosomal region had no ovaries
indicating a strong interaction with spir. Control crosses performed with wild type siblings heterozygous for the deficiency show no defect in ovaries indicating that the presence of one copy of the spir mutation and one chromosome with this deficiency causes the lack of ovaries.

This region consisted of about 222 genes and we consistently found a strong interaction with this region. Hence we decided to narrow further the interacting interval with smaller deficiencies that would cover the large interacting region.

**A secondary screen for spir interactors narrowed the interacting interval to 55A-55C1**

spir interacted strongly with a region of chromosome 2; 55A-55F. This was a large region so smaller deficiency regions available from the stock center and other Drosophila research labs were used to help narrow down this interacting interval (Figure 3.4). These small deficiencies were crossed to spir similar to the crosses described previously to test for an interaction (see Figure 3.2). From these crosses we observed that small deficiencies in the region 55A-55C1 showed an interaction with spir, while deficiencies lying outside this region, 55C2-55F did not show an interaction with spir. This suggests that the interacting locus lies within the region of 55A-55C1. This region consists of 91 genes of which 47 genes were mapped to the sequence and 44 genes were not mapped to sequence. I
tested several potential candidate genes lying within this region, 55A-55C1, using hypomorphic mutations and P-element insertions for interaction with spir (Table 3.2). None of the tested mutants or P-element insertion lines interacted with spir. Since mutations were not available for many genes in this narrowed interacting interval, we used knowledge from the literature and studies on spir from the lab to pick possible candidate genes. Genes that are part of the cytoskeleton, signaling GTPases, female sterility and MAPK signaling components were listed for study.

Amongst the candidate genes in the interacting region the gene, Mapmodulin seemed to be an interesting candidate. Mapmodulin binds to microtubule associated proteins (MAPs) and is thought to inhibit the rate of MAP2 binding to the microtubules, allowing a path for organelles translocating along the microtubules. (Ulitzur et al., 1997). During the early development of Drosophila mutants in spir exhibit premature microtubule-dependent cytoplasmic streaming, a process that mixes the cytoplasm of the oocyte with the incoming cytoplasmic materials from the nurse cells. The oocyte also has gross defects in the microtubule cytoskeleton suggesting a possible role for spir in the regulation of the microtubule cytoskeleton. It is possible that mutations in mapmodulin may potentially enhance spir phenotypes. Mapmodulin also plays a role in the transport of mannose 6-phosphate receptors from endosomes to the trans-golgi network suggesting a role in vesicle transport (Itin et al., 1999). Interestingly,
Spir co-localizes with Rab11 in NIH 3T3 cells. Rab11 GTPases are involved in the transport process from the transgolgi and in recycling processes. A dominant negative Spir blocks exocytosis of a viral protein indicating that Spir could be an effector of Rab GTPases and is involved in vesicle transport (Kerkhoff et al., 2001). Based on this knowledge of spir function during oogenesis and its role in vesicle transport and the role of mapmodulin in microtubule based processes and vesicular transport, it is possible that mapmodulin interacts with spir.

Since mutations were not available for the mapmodulin loci, we looked for available P-element insertions in nearby genes. We found a line that was inserted 5kb away from mapmodulin, in a gene called three rows. I used a strategy (Figure 3.5) that would make localized excision of the P-element. This excision could take away a chunk of DNA from adjacent chromosomal regions, thereby possibly creating a deletion in our gene of interest, mapmodulin. I tested 70 excision lines for interaction with spir in the lack of ovaries phenotype and did not identify an interaction. P-element excision itself is a random process (Ryder and Russell, 2003) and it is possible that while excision may have occurred, mapmodulin may have been left untouched by the excision events.

Thus, from my several deficiency crosses, I was able to narrow down an interactor for spir to a small region of chromosome 2 but due to unavailability of
genetic and molecular tools, I ended my search to identify interacting deficiencies for \textit{capu} and \textit{spir}.

\textbf{Discussion}

From a deficiency screen that we designed, I identified 2 chromosomal regions, 49C1-50D2 to interact strongly with \textit{capu} and 55A-55F to interact strongly with \textit{spir}. I was able to further narrow down this interacting region to 50A-50D2 in \textit{capu} and 55A-55C1 in \textit{spir}. While the phenotype that I used to search for interacting deficiencies was strong enough to identify regions of the chromosome that show interaction with \textit{capu} and \textit{spir}, due to lack of tools, I was unable to identify a gene that interacted with either \textit{capu} or \textit{spir}.

One major problem that I came to realize during the screen is the heterogeneity of the different genetic backgrounds of the deficiency kit stocks themselves and also the smaller deletions that I subsequently used to narrow down the interacting region. I observed that the lack of ovaries phenotype was highly variable even when a smaller deficiency showed interaction with either \textit{capu} or \textit{spir}, in the chromosomal region of a larger deficiency that I originally screened. Also, the available deficiencies for certain regions (such as region 50A-50D2) did not cover the whole larger deficiency and thus produced gaps in my
complementation tests. Due to unavailability of tools, I was unable to map the cytological interacting region to a smaller interval to identify interacting loci.

Subsequently, several studies have been published that would allow screening of well defined deficiencies that cover most of the euchromatic genome. There are several advantages to using these deficiency sets compared to the one available from the BLOOMINGTON DROSOPHILA STOCK CENTER in 2001. First, recent studies have used tranposon based strategies to design custom chromosomal aberrations in Drosophila (Parks et al., 2004; Ryder et al., 2004; Thibault et al., 2004). A great advantage with these strategies is an ability to chose the background of the strain where one is going to make deletions compared to the heterogeneous collection of deletions that were isolated by the Drosophila community that are part of the “deficiency kit.” Recent studies have isolated the deficiencies in an isogenic strain that permits a uniform genetic background that decreases sensitivity to modifier effects that are highly prevalent in the genome. Secondly, since these studies were based on transposon mobilization, their precise end points are mapped with base-pair resolution compared to the genetic and cytological mapping of the deficiency kit, where the resolution is between 50-100 kb. This best possible resolution in the newly available kits affords precise fine mapping of interacting deficiencies and to easily identify interacting genes. This is because the coverage of the genome is ~95% and the ability to make point mutant deletions that can be screened in the same way as the larger deletions
makes it highly likely that an interacting large deficiency will be sufficiently covered to single gene resolution by the ability to make even base pair deletions by custom design.

The deficiency screen identified other interacting regions for both capu and spir which showed weak to moderate interactions. We selected the best interacting region for capu and spir because they showed consistently strong interaction of the lack of ovaries phenotype.

Further studies to narrow down the interacting deficiencies of capu and spir should involve using particular genomic deletions that map to the cytological region of interest from collections such as DrosDel P-element collection. After precise molecular mapping, generation of smaller deletions that will resolve the interacting region to single genes could identify molecular regulators of capu and spir function in oogenesis.
Figure 3.1 Pole cells are absent in embryos from *capu* or *spir/+ rhoA/+* mothers. A) A wild type embryo shows pole cells at the posterior pole during cellularization. B) An embryo from a *spir +/- rhoA* female during cellularization with no pole cells at the posterior pole.
Figure 3.1 Pole cells are absent in embryos from *capu* or *spir/+ rhoA/+* mothers.
Figure 3.2 Schematic of the deficiency screen design.

Virgin females heterozygous for alleles with intermediate strength of *capu* and *spir* are crossed to deficiency stock males. The F1 progeny females are selected for absence of the respective balancers and are thus double heterozygotes for *capu* or *spir* and the deficiency. These females will lay eggs that lack pole cells if the deficiency interacts with *capu* or *spir*, which is analyzed in the F2 females by scoring for ovaries.
Figure 3.2 Schematic of the deficiency screen design.
Figure 3.3 Deficiency $Df(2R)CX1$ interacts with $capu$.

Females doubly heterozygous for $capu$ and $Df(2R)CX1$ give rise to female offspring that show a severe ovary absent phenotype. In an effort to narrow down the interacting region (blue), smaller deficiencies in the region were crossed and as indicated, 3 deficiencies showed interaction with $capu$ (dotted line) and several did not (red), that indicated the interacting region to be 50A to 50D2. The three smaller deficiencies in that region did not overlap and the gaps in the deficiency coverage resulted in the smallest interacting region as large as 50A-50D2.
Figure 3.3 Deficiency Df(2R)CX1 interacts with capu.
Figure 3.4 Deficiency \( Df(2R)PC4 \) interacts with \( spir \).

Females doubly heterozygous for \( spir \) and \( Df(2R)PC4 \) give rise to female offspring that show a severe ovary absent phenotype. In an effort to narrow down the interacting region (blue), smaller deficiencies in the region were crossed and as indicated, 2 small deficiencies and a large deficiency showed interaction with \( spir \) (dotted line) and three did not (red), that indicated the interacting region to be 55A1-55C1.
Figure 3.4 Deficiency $Df(2R)PC4$ interacts with $spir$. 
Figure 3.5 Schematic of the P-element Excision strategy to excise *mapmodulin* locus.

Due to lack of mutations in the *mapmodulin* gene, an excision strategy was used to make mutations in the *mapmodulin* locus, by P-element excision of a nearby gene *three rows*, 5 kb downstream of the *mapmodulin* gene. A transposase source was crossed to the *three rows* P-element. The transposase results in excision of the P-element and in the next generation the transposase was selected against and crossed to *spir* heterozygote and tested for the ovaries absent phenotype as described earlier.
Figure 3.5 Schematic of the P-element Excision strategy to excise *mapmodulin* locus.
Table 3.1 List of deficiencies tested for the screen.

<table>
<thead>
<tr>
<th>Stock #</th>
<th>Kit</th>
<th>Symbol</th>
<th>Breakpoints</th>
</tr>
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<tbody>
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<td>5705</td>
<td>DK1</td>
<td>Df(1)EA2.8</td>
<td>4F5;5A13</td>
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<tr>
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<td>DK1</td>
<td>Df(1)dc51</td>
<td>5C3;10.6C3;12</td>
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<td>Df(1)EKL10</td>
<td>16A2;15C7;10</td>
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<td>DK1</td>
<td>Df(1)E25</td>
<td>15C3;15A4;6</td>
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Table 3.2 Summary of interactions of *spir* with chromosomal region 55A-55F.
CHAPTER 4

FUTURE PERSPECTIVES

My dissertation research has identified a role for the formin protein *cappuccino* and its interacting protein Profilin in yolk granule biogenesis during Drosophila oogenesis. While it is clear that they play a role in the formation of yolk granules in the oocyte, the mechanisms of yolk granule regulation by *capu* and *chic* were not identified by my studies. Further experiments are needed to clarify this important issue which has implications in regulation of actin cytoskeleton in the developing oocyte and may be of interest to cell biologists looking for ways to regulate the process of endocytosis, which has recently been shown to have tumor suppressor functions.

While my experiments failed to resolve the exact nature of the endocytic defect, whether a defect in increased vesicle fusion events, or decreased fission events cause the defects that lead to large yolk granules in *capu* mutants, more advanced techniques in microscopy are now available to address such questions. The advent of two-photon laser scanning microscopy (TPLSM) has given access to low intensity high resolution laser scanning that may avoid the bleaching of the fluorescent dyes that was a frequent problem in my experiments with conventional laser confocal microscopy. Also, TPLSM works very well with live
imaging and is less toxic to tissues during the microscopy process and thus may yield more reliable data than by other means of imaging.

My experiments also did not resolve the role of actin cytoskeleton in regulation of endocytosis by \textit{capu}. To better understand the effect of Capu on actin, first, use of a wide array of antibodies to actin may inform us about the exact nature of the actin cytoskeleton in \textit{capu} mutants. Second, use of GFP actin expressing flies and application of actin depolymerizing drug immediately before imaging dissected ovaries and using TPLSM to image actin in the oocyte will clearly indicate whether the endocytic defect is related to actin depolymerization. Third, the role of other cytoskeletal elements such as microtubules can be evaluated by use of drugs to target microtubules and imaging the oocytes using TPLSM.

My experiments indicate a new role for the formin subfamily of formin proteins, to which \textit{capu} belongs, in endocytosis. Additionally, the requirement for \textit{capu} binding for proper \textit{chic} function in endocytosis suggests that interaction with actin and actin binding proteins may be a key mediator of \textit{capu} function. It will be useful to find other interactors of \textit{capu} and \textit{chic} in vesicle biogenesis, to inform us about the role of actin in endocytosis, an important cellular process that may be aberrant in signaling responses during carcinogenesis.
My deficiency screen to identify interacting proteins for \textit{capu} and \textit{spir} did not identify interacting loci while it identified interacting regions because it is possible that BLOOMINGTON DROSOPHILA STOCK CENTER coverage of deficiencies are not complete enough to map out the interacting regions from the screen. Strategies to overcome these problems are, first, recent studies have used tranposon based strategies to design custom chromosomal aberrations in Drosophila (Parks et al., 2004; Ryder et al., 2004; Thibault et al., 2004). These methods use an isogenic strain background and highly precise mapping of the deletions that enable rapid identification of interacting loci from a deficiency screen. Second, these methods can make deletions by custom design and thus the labor and time involved in conducting a screen can be reduced by using only selected regions of the genome, with the confidence that nearly 95% of the genome is covered by these advanced methods to generate deficiencies.

The experiments mentioned above should identify additional genes that interact with \textit{capu} during oogenesis and also clarify the mechanism of yolk endocytosis regulation by \textit{capu} and profilin. This knowledge will contribute to a new understanding of cytoskeletal regulation during oogenesis in Drosophila.
APPENDIX A: MATERIALS AND METHODS

Fly Stocks

Flies were raised on medium consisting of instant food, agar and oatmeal supplemented with yeast. All stocks were maintained at 25°C under uncrowded conditions. The capu, chic and spir stocks were from the Manseau lab stock collection. The Bloomington Drosophila Stock center provided us with the first, second and the third chromosome deficiency kit as well. capuG7 and spirPJ56 were the capu and spir stocks used in the deficiency screen. Smaller deficiencies for the capu interacting region, Df49C1-49E6, Df50A-50B, Df50C, Df 50C21-50D5, Df50A-50E, Df49D3-50A3, Df48D3-50C6, Df49A4-49F1 and Df49A4-49D;E and the spir interacting region, Df54E8;55C1, Df55A1;55C1, Df55C1;56B2, Df55E2;56C11, Df55D2;56B2, 54F2-56A1 and the mutant stocks Hsf, Pcl, pabp, lolal, Dgp-1, fj, Su(hh)II, nw, mama, hall and the P-element insertions at 55A1-F3, 55A and 55C1-2 were obtained from the Bloomington Drosophila stock center. Other stocks were obtained from the Drosophila research community.

Dissection of Ovaries

Ovaries for the deficiency screen were obtained by letting the flies feed on for 12hrs in fly food vials powdered with yeast to stimulate oogenesis. The ovaries were then hand dissected in Ringers Saline.
**Cross Scheme**

Deficiency stocks from the first, second and third chromosome deficiency kits were crossed with *capu* and *spir* to generate *capu* or *spir/+; deficiency/+ F1 females. The F1 females carrying the chromosomal deficiency were identified by the presence of dominant markers on the balancer chromosomes. Some deficiency stocks were sick and did not survive. The F1 females that are *capu* or *spir/+; deficiency/+ F1 females from each cross were then mated with males, and the females resulting from this cross were collected a total of about n=20 were then fed yeast for at least 12 hours to stimulate oogenesis and then the ovaries were dissected in Ringers Saline. Deficiencies that showed higher than 50% lack of ovaries were selected for further analysis.

**Electron Microscopy**

We followed the procedure from (Giorgi et al., 1993) for preparing electron microscope sections on drosophila ovaries. Flies were dissected in Ringers solution and immediately fixed for 2hrs in 5% Glutaraldehyde, 4% Formaldehyde in 0.1M cacodylate buffer at pH 7.2 at 4 C. Post-fixation was carried out for 2hrs in 1% osmium tetroxide in 0.1M cacodylate buffer at pH 7.2. Ovaries were dehydrated in a graded series of alcohols and embedded in Epon-Araldite. Polymerization was achieved by 3 days of incubation at 60C. Thin sections of both wild type and mutant oocytes were prepared using an ultramicrotome and were then stained and observed under a Jeol electron microscope.
Yolk granule Quantification

For quantification of vesicle number, and size distribution, electron micrographs of wild type and mutant stage10 egg chambers were used for measuring the yolk granules using the Metamorph software. The EM images were scanned and imported into metamorph and similar areas for analysis were selected for both wild type and mutant. The number of granules in each area was counted and the volume was calculated using diameter of these granules using region tools of metamorph.

Endocytic Tracer Studies

Oocyte uptake studies were performed by incubating ovaries Ringer’s saline containing 1mg/ml Rhodamine-Dextran 10,000 MW; dextran, Alexa Fluor 647, 10,000MW; dextran, fluorescein, 10,000MW and 10micro Molar of Lysotracker Red (Molecular Probes). Ovaries were then washed with ice cold Ringers to wash the tracer and fixed with a cold fixative. Internalization occurs minimally at low temperatures, using cold Ringers and fixatives, controls the amount of tracer being internalized after the specified time point. The ovaries were then mounted with vectashield and images were captured using confocal microscopy.
**Differential Interference Contrast (DIC) Imaging**

Ovaries were prepared by dissecting in Ringer’s saline and then transferred to eppendorf tubes containing 100 _l deveitellinizing buffer (1 volume Buffer B, 1 volume 36% Formaldehyde, and 4 volumes water) and 600 _l heptane was added and agitated gently for 10 minutes. The solution was removed and the ovaries were rinsed with PBS 3 times, and the egg chambers were mounted using Aquapolymount. Images were acquired and analyzed on a Nikon fluorescence microscope equipped with Nikon laser scanning confocal head using simple PCI software. 1µm optical sections were collected with a 60X objective and projected on to a single plane.

**Drug Treatment**

Ovaries were dissected and soaked in Ringers containing 10 _g/ml of the drug, Cytochalasin D, for short times (5 or 10 minutes) and then fixed and observed for DIC microscopy. For feeding experiments, the flies were initially starved and Cytochalasin D was fed up to 24 or 48 hours mixed in yeast paste. Treatments involving Latrunculin A and Cytochalasin D were done consecutively with 50µM LatA followed by 20µg/ml of CytD. The ovaries were then dissected and observed at various time intervals after feeding.
**Actin Staining**

Ovaries were dissected and fixed in 4% formaldehyde in PBS and 0.05% Tween for 20 minutes. Following fixation, ovaries were washed three times in PBS-Tween, 10 minutes each wash. The ovaries were then permeabilized in PBS-Tween plus 1% Triton X-100 for one hour and then stained with 3 units of Rhodamine phalloidin and imaged using confocal microscope.

**Western Blotting**

Ovarian protein extracts were subjected to SDS-PAGE and gels were electroblotted onto Hybond-PVDF (Amersham, Arlington Heights, IL), blocked with 5% nonfat dry milk powder in PBS/0.05% Tween 20, and incubated with the Profilin antibody (chic 1J obtained from the developmental studies hybridoma bank) and used on Western blots at a concentration of 1:500. Detection was done with an HRP-conjugated secondary antibody (Cappel) and developed with an ECL detection system (Amersham, Arlington Heights, IL), according to the manufacturer’s directions.
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