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Genetic analysis of localization of a Bic-D::GFP fusion protein and identification of novel subcellular domains

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Submitted Friday, March 12, 1999

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the degree of Master of Science

Statement of originality

The results reported in section 4 of this thesis appear in print for the first time. The Bic-D::GFP fusion protein, the genetic analysis of its localization, and the suppressor isolated in the suppressor screen are original results produced by the author. The novel subcellular domains, namely the spool and the crater, have never, to the best of our knowledge, been described in the literature.

Abstract

Bicaudal-D (*Bic-D*) is essential in Drosophila for the establishment of oocyte fate and polarity within the developing oocyte. To study these processes we have engineered a chimeric Bic-D::GFP fusion protein which behaves like the endogenous Bic-D polypeptide. We have identified three genes which are required for the normal subcellular distribution of Bic-D::GFP: two genes predicted to encode RNA binding proteins (*egalitarian* and *orb*) *and Dynein heavy chain*. In particular, they affect Bic-D::GFP localization during the early germarial stages of oogenesis during which oocyte fate is established, or later when anterior-posterior polarity is initiated. Our results support the model that Bic-D acts in conjunction with mRNA binding proteins and a negative-end directed microtubule motor in localizing mRNAs. Throughout stages 1-6 of oocyte development, Bic-D::GFP accumulates in the oocyte in a strong posterior cortical focus, resembling a spool, that is aligned with a crater-like indentation in the oocyte nucleus. The aligned focus and crater reveal an early oocyte polarity and a previously undescribed asymmetric subcellular structure that may be involved in tethering the oocyte nucleus. Shape, positioning and orientation of the oocyte nucleus change around stage 6-7, concomitantly with a change in position of the Bic-D::GFP focus to the presumptive dorsoanterior corner. This re-orientation appears to anticipate the establishment of a new dorsoventral polarity in the oocyte and egg chamber. *Dhc* and *Bic-D* are both involved in the process of re-orientation of the oocyte nucleus and in polarity formation.

Résumé Français

Le gène*Bicaudal-D* (*Bic-D*) est essentiel pour la formation de l'oocyte et de sa polarité. Afin d'étudier ce processus, nous avons créé une protéine chimérique, Bic-D::GFP; selon certains critères, cette protéine fonctionne comme la protéine endogène. Deux gènes qui encodent des protéines interagissant directement avec des ARN, soit *egalitarian (egl)* et *orb*, ainsi que le gène encodant la chaîne lourde de la dynéine (*Dhc*) sont requis pour la distribution de Bic-D::GFP à l'intérieur de la cellule. Ces gènes sont requis plus précisément pendant les stades de l'oogenèse qui se déroulent dans le germarium, là où s'établit l'identité de l'oocyte et commence la formation de la polarité antéro-postérieure. Nos résultats suggèrent un modèle dans lequel Bic-D coopère avec des protéines interagissant directement avec des ARN et une protéine motrice se dirigeant vers le pole négatif des microtubules, afin de distribuer ces ARN. Pendant les stades de 1 à 6, Bic-D::GFP s'accumule intensément au cortex du pole postérieur de l'oocyte; cette accumulation prend la forme d'une bobine alignée avec une indentation dans le noyau de l'oocyte en forme de cratère. Cet alignement entre la bobine et le cratère révèle un nouvel aspect dans l'établissement de la polarité de l'oocyte ainsi qu'une structure cellulaire assymétrique n'ayant jamais été décrite dans la litérature à ce jour, qui joue peut-être un rôle dans le rattachement du noyau au cortex. La forme et la position du noyau de l'oocyte changent vers les stades 6-7, en même temps qu'un changement de position du point de plus intense accumulation de Bic-D::GFP vers l'extrémité présumée antéro-dorsale. Cette ré-orientation semble précéder l'établissement d'une nouvelle polarité dorso-ventrale dans l'oocyte et dans le syncytium. Bic-D ainsi que la chaîne lourde de la dynéine sont toutes deux impliqués dans le processus de ré-orientation du noyau de l'oocyte et la formation de la polarité.

1. Introduction

Most eucaryotic cells, whether they make up a simple unicellular organism or a complex metazoan, show some degree of morphological or molecular polarization. Polarization is the key to a great number of cellular processes: yeast buds apically or axially in response to environmental conditions; a daughter cell can differentiate from its mother cell; epithelial cells can have different basal and apical properties, and an egg cell can be endowed with the morphogen gradients that are the basis for the organismal body plan. Thus, cellular asymmetry is a common denominator of the birth, development and life of every eucaryote.

Cellular asymmetry is built through the vectorial transport of a variety of molecules, from RNAs to cytoskeletal elements. There are a number of cues which may help such cellular asymmetry to be established, for instance, in the correct orientation relative to the body plan of a multicellular organism. Cell-cell signaling between identical cells may result in the differentiation and the polarization of a subset of these cells. As well, a concentration gradient of a secreted, diffusible signal can differentially induce cell fate both proximally and distally (van der Wees et al., 1998). Asymmetrical distribution of RNA is also instrumental in carrying out the polarization program of the cell. In yeast, for example, the mechanism responsible for mating type switching between a mother cell and a daughter cell involves the actin-mediated asymmetric localization of the *ash1* mRNA to the daughter cell (Long et al., 1997; Takizawa et al., 1997).

RNA transport appears to be a conserved mechanism. Even within the same organism, the same RNA transport molecules may have, in different organ systems, seemingly unrelated functions. Staufen provides such an example in Drosophila. The *staufen* gene is both required in neurogenesis and oogenesis. Normal neurogenesis requires the asymmetric distribution of the Notch transmembrane receptor repressor Numb and the transcription factor Prospero between two daughter cells in response to the apical-basal polarity of the epithelial cells. The underlying molecular mechanisms behind this distribution, however, are just beginning to emerge. Both Prospero protein and *prospero* mRNA localization are dependent on *inscuteable* and *miranda* function, but the mRNA also requires the actin-dependent *staufen* function. Yet, while Numb and Prospero are not known to play a role in oogenesis, *staufen* is required for microtubule-dependent mRNA transport in Drosophila oogenesis (Ferrandon et al., 1994)} (Hirata et al., 1995; Broadus et al., 1998; Shen et al., 1998). Thus, *staufen* is required for mRNA transport in neurogenesis and oogenesis, but in one case for actin-dependent transport, and the other, microtubule-dependent transport.

Discoveries such as this one show how central mRNA transport is to polarization processes, and point out how little is known. The purpose of the work described in this thesis is to better understand some of the steps involved in building cellular asymmetry, and to find known and novel genes required for this process. In particular, we will be learning about the *Bicaudal-D (Bic-D)* gene, which plays a role in oocyte determination and RNA transport in Drosophila.

1.1. Drosophila oogenesis: a model system for transport and differentiation

Owing to the high level of conservation that exists between humans and an invertebrate such as Drosophila, we can study the cellular processes in one organism in order to learn about the other.

Drosophila is a genetically tractable model organism with only four chromosomes, highly developed genetics, well documented transposon-based mutagenesis and transformation, and a rapid life cycle. For these reasons, the fruit fly often paves the way for discovery in developmental biology.

Human genome sequencing and disease mapping continues to progress. We hope that when genes responsible for diseases are identified, a Drosophila homologue will already have been studied, thus hastening the pace of therapeutic research. Drosophila oogenesis has proven to be a useful model for a wide range of developmental processes, from cytoskeletal organization to cell-cell signalling.

The induction of Drosophila body axes and segmentation is governed by the asymmetric distribution of key maternal patterning determinants in the egg, a complex process which begins in the ovary. It is in the anteriormost portion of the ovary, called the germarium, that a cyst of 16 germline cells is formed, interconnected by large cytoplasmic bridges called ring canals. From this syncytium, one of the two cells which possess 4 ring canals will differentiate into a mostly transcriptionally inactive oocyte, while the remaining 15 cells become polyploid nurse cells with a function to supply the oocyte with materials for growth and development (Mahowald and Tiefert, 1969). Some of the molecules which are transported are bound for the anterior or the posterior of the oocyte, in order to give rise to the morphogen gradients required to generate the embryonic axes and segmentation.

Several important areas of investigation arise from these seemingly simple developmental steps. What are the events that lead to the establishment of cell identity? How are these key factors transported from the nurse cell to the oocyte? By what mechanism are they sorted into an anterior or a posterior localization pathway? How are they anchored? We have chosen to address these important

questions by focusing on a single molecule central to the establishment of oocyte fate and microtubule-based RNA transport in Drosophila oogenesis, Bic-D. Our experimental objectives were to observe the distribution pattern in real time or in sequence of the Bic-D::GFP fluorescent signal, find genes which affect this distribution pattern in a meaningful way, and identify new genes which genetically interact Bic-D by way of a suppressor screen.

In order to understand the broader context surrounding Bic-D, we will discuss in detail the role of the fusome, centrioles, microtubules, cell adhesion, and mRNA transport, and how each process may contribute to the spefication, growth and patterning of the oocyte. We will also discuss some of the Bic-D interacting genes that have been discovered in this laboratory by other students, because they provide valuable insights into the possible function of Bic-D. After a review of the literature, we will describe the methods employed in the experiments which were performed, the results which were obtained, and elaborate a discussion.

For reference, the early stages of oogenesis are outlined in Figure 1. The Drosophila ovary is composed of clusters of ovarioles. At the anteriormost tip of the ovarioles, 2 or 3 germline stem cell give rise to a succession of 16-cell cystocyte clusters. These clusters are interconnected by cytoplasmic bridges called ring canals, formed from incomplete cytokinesis. Approximately at the time of completion of the cystocyte cluster, a subset of RNAs accumulate in a single cell, which will become the oocyte. The other 15 cells will become nurse cells. After the oocyte reached the posterior of the cluster, the cluster, now called an egg chamber, exits the germarium to enter to vitellarium, where it will perform the rest of its growth. The nurse cells produce factors which are transported into the oocyte and begin to degenerate after stage 10 (not shown).

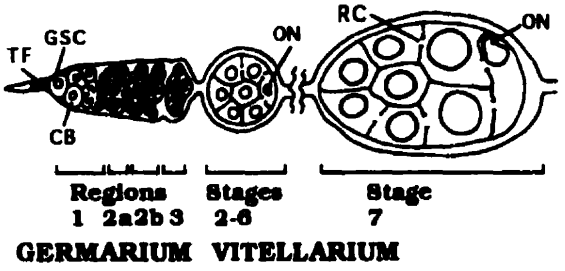

Figure 1. Schematic diagram illustrating early oogenesis of Drosophila
This diagram depicts the anterior part of a Drosohila ovariole. The germarium is shown on the left side. Region 1 contains the germline stem cells (GSC), which lie at the base of the somatic terminal filaments (TF), and their daughter cystoblasts (CB); it is also the site of the mitotic activity leading to the formation of 16-cell cystocyte clusters. As a result of incomplete cytokinesis the cystocytes in each cluster are interconnected by cytoplasmic bridges called ring canals (RC). In region 2, following cyst formation, organelles and certain RNAs and proteins accumulate in a single cell, the oocyte which becomes positioned at the posterior center of the egg chamber. In the vitellarium, from stage 2 to stage 6, the oocyte nucleus (ON) is at the center of the oocyte, and around stage 6-7, it is found at the antero-dorsal corner of the oocyte.

2. Literature review

We begin with an overview of some of the morphological and macromolecular features of the early stages of oogenesis, namely cystocyte cluster formation and oocyte determination and adhesion-mediated cell sorting. We will then move on to the more specific process of RNA transport, in which Bic-D is believed to play a role. Next, we examine some important steps occuring after the specification of oocyte fate pertaining to germinal vesicle migration and the patterning of the body axes, another process requiring Bic-D.

Introducing Bic-D itself, we will review in some detail what is known about its molecular characteristics, the various *Bic-D* mutants, and their respective phenotypes. Genes known to interact with *Bic-D*, whether from the literature or from results obtained in the Suter laboratory, will be discussed.

2.1. The fusome in cystocyte cluster polarity

Whether polarization of the 16-cell cluster occurs during or after cyst formation is still a controversial issue. At first, it was thought that oocyte specification followed cyst formation, from the observation that there are initially two cells with 4 ring canals which enter meiosis and form synaptonemal complexes. Because one cell reverts to nurse cell fate, it is possible that an asymmetry between the two pro-oocytes is instrumental in oocyte specification (Carpenter, 1975, 1994). Later, it became apparent that the fusome could potentially be a morphological basis for the building of polarity in the dividing cyst (Storto and King, 1989). The fusome is a specialized branched membranous organelle devoid of mitochondria and ribosomes. Branches span the ring canals in the cystocyte cluster. Molecular components of the fusome include Hu-li tai shao *(hts)*, α-spectrin and β-spectrin, Bam and ankyrin (Lin et al., 1994; de Cuevas et al., 1996). Of these, only *hts* and *α-spectrin*

encode essential components for fusome biogenesis, for mutations in either of these two genes results in abolition of the fusome. Such mutant cysts have no oocyte, but as they contain less than the full complement of 16 cells, the oocyte specification defect may be indirect. Dynein heavy chain is also believed to associate transiently with the fusome, consistent with its role in the orientation of the mitotic spindles relative to the fusome (McGrail and Hays, 1997). The complex pattern of fusome genesis, fusion and constriction results not only in an asymmetrical structure, but also one which causes the syncytium to form rosettes of cells of a predictable organization and hierarchy. Moreover, it is thought that the presumed oocyte always retains a larger allotment of fusomal material than the presumed future nurse cells (de Cuevas and Spradling, 1998). This suggests an involvement of the fusome in oocyte specification, but no direct evidence has been reported.

The reported association of Dhc with the fusome appears to be cell-cycle dependent. Mutants in *Dhc* do form some 16 cell cysts, but exhibit interesting defects in fusome formation: the fusome is fragmented, the mitotic spindles are misaligned relative to the fusome and detached from it. Moreover, the cysts do not form oocytes (McGrail and Hays, 1997). This suggests an involvement of the fusome in oocyte specification, but no direct evidence has been reported; in this regard, a discovery of an interaction between *Bicaudal-D (Bic-D)* or *egalitarian (egl)* , whose mutant phenotype is a failure of oocyte specification, with the fusome would be very meaningful.

2.2. A role for microtubule organization in oocyte specification.

Microtubules and microtubule organization have an essential function in oocyte specification. As soon as the cystoblast has completed the 4 rounds of incomplete divisions that give rise to the 16 cell cluster, in what appears to be region 2a of the germarium, mRNA and proteins

begin to accumulate in the oocyte (Bier, 1963). Also, the migration of nurse cell centrioles towards the oocyte is initiated in the same region. The centrioles, initially in a juxtanuclear position, move to the cell membrane and to the ring canals, and the centriole pairs lose their usual perpendicular arrangement. By stage 1 (germarial region 3), most centrioles have clustered into the oocyte. However, Mahowald and Strassheim (1970), who performed the sequential electron microscopy (EM) analysis that led to this discovery, admitting the tedious nature of the work, were only able to examine 8 16-cell clusters. In the 3 egg chambers observed where the centrioles had moved into the oocyte, 2 had the centrioles clustered at the posterior of the putative oocyte, while 1 had the centrioles clustered at the anterior. Validating these EM studies with immunofluorescence of α-tubulin, Theurkauf and colleagues (1992) have found that the MTOC is also initially found at the anterior of the oocyte, near the ring canals, and later found at the posterior about the time the egg chamber exits the germarium.

As a whole, these observations suggest that centriole migration is a complex, multiple step process, but we do not know whether it has any consequence on oocyte specification. Detailed sequential electron microscopic analysis of *Bic-D* and *egl* mutant ovaries, which do not form oocytes, would be of value, since it would allow us to determine whether these genes are essential for centriole migration. Also, the lack of an antibody that binds centriolar proteins in region 2 has hampered research in this area. Such an antibody would permit the use of less labour-intensive histochemical techniques.

Also unknown is the means by which centrioles migrate. Does this movement require any of the known components of the cytoskeleton? This question may remain unanswered for some time, but some recent results provide some valuable insights. Myosin Va was found to be enriched in MTOCs in interphase, and in the mitotic

asters of dividing cells in a variety of cell types, including HeLa cells, fibroblasts, and mouse melanocytes. This association was found to be preserved in detergent-extracted cells, showing that the interaction does depend on membranes (Wu et al., 1998). This suggests that membranes may be important for microtubule organization. Indeed, *β_H-spectrin* mutations have been identified in a screen for suppressors of *Bic-D^{PA66}* (Nguyen, T. D. pers. comm., 1996).

A microtubule organizing center (MTOC) is believed to play a role in oocyte specification, since an MTOC can sometimes be found in a single cell of the 16 cell cyst in region 2b (Theurkauf et al., 1993). It is not known whether this cell represents the future oocyte, but it is likely since in later stages, a prominent MTOC is always found in the oocyte. The ends of microtubules extend from this MTOC through the ring canals into the nurse cells, and form a polarized network which could support the microtubule-dependent transport of RNAs and other factors from the nurse cells into the oocyte.

2.3. RNA transport in oocyte specification

Now that we have seen the physical organization that may influence oocyte specification and oocyte positioning, we follow with a widespread molecular process, which is key to the building of oocyte fate: RNA transport.

More is known about RNA transport in middle stages of oogenesis than in the earlier stages. Because RNA transport is likely to employ similar mechanisms throughout these stages, we will look at the best known example in Drosophila oogenesis, that of *oskar (osk)* RNA. Some mRNAs are transported via specific sequences, which frequently form specific secondary structures such as hairpins and loops. Some of these structures are recognized by specific RNA binding proteins (Kim-Ha et al., 1995). Staufen protein associates with the 3'UTR of *bicoid (bcd)* and *osk* mRNA to form particles that move in a

microtubule-dependent manner, at two separate stages of development (Ferrandon et al., 1994). The posteriorly localized *osk* RNA 3'UTR contains separable regions for transport; one of transport to the oocyte, and another for transport to the posterior of the oocyte (Kim-Ha et al., 1993). During its transport, *osk* mRNA is translationally repressed by the Bruno protein, the product of the *arrest* locus (Webster et al., 1997). Bruno is a translational repressor which interacts physically with the positive translational regulator Vasa, showing at the very least that RNA transport is a tightly regulated multiple step process (Webster et al., 1997).

Until recently, it was thought that RNA transport was dependent on either actin microfilaments or microtubules. Recent experiments now unveil that some of the protein components involved in RNA localization can participate in both microtubule or actin dependent transport in the nurse cells (Theurkauf and Hazelrigg, 1998).

2.4. The cadherin-mediated positioning of the oocyte is an asymmetry-breaking step in antero-posterior axis polarization.

The normal positioning of the oocyte at the posterior of the egg chamber is a process which is was recently found to require cell adhesion molecules (Godt and Tepass, 1998). This positioning is abolished in *Bic-D*^{null} flies as well - the two cells with four ring canals are randomly positioned in the cyst (Ran et al., 1994). Because *Bic-D* is required in the germline for oocyte positioning, it may be required for the oocyte specific accumulation of the molecules that will allow the oocyte to be recognized by the follicle cells. Several elegant experiments have recently shown both a somatic and germline requirement for the hemophilic cadherin adhesion complex of DE-cadherin, Armadillo and α-catenin (Gonzalez-Reyes and St Johnston 1998; Godt and Tepass, 1998). In *DE-cadherin*$^{+}$/*DE-cadherin*$^{-}$ chimæric follicular epithelia, it

was found that the oocyte was consistently juxtaposed against the follicle cells expressing DE-cadherin; this is the first reported in vivo example of a cell sorting process dependent on differential cell adhesion (Godt and Tepass, 1998). This finding is rich in implications for the field of cancer research, where it has been long known that invasive cancer cells are often marked by a down-regulation of cadherin-based cell adhesion (reviewed in Davies et al., 1998, and in Stein et al., 1998).

In the follicle cells, DE-cadherin interacts with the embryonic segment polarity gene *armadillo*, which encodes β-catenin, a membrane-associated protein which doubles as a bipartite transcription factor with the HMG-box protein Tcf when stimulated by the reception of an extracellular signal such as the one encoded by *wingless*, suggesting the involvement of an intracellular cascade in response to positional information for oocyte positioning. Notable is DE-cadherin accumulation to the posterior cortex of the oocyte, which occurs while Egl is still concentrated at the anterior of the oocyte in region 2b clusters; Egl localizes to the posterior cortex early on as well, but it appears that this accumulation lags behind that of DE-cadherin (Godt and Tepass, 1998; Mach and Lehmann, 1997). We have already mentioned the transient accumulation of centrioles and localization of the MTOC at the anterior of the oocyte at the same stage.

In addition to Egl and the MTOC, Orb protein, and Bic-D::GFP (Figure 4a) first appear in the 16-cell cluster at the anterior of the oocyte, and also soon move to the posterior (Mach and Lehmann, 1997; Theurkauf et al., 1992; Lantz et al., 1994). Testing for posterior polar follicle cell (PPFC) expression of DE-cadherin in *Bic-D*^{null} flies, which misposition the pseudo-oocyte within the egg chamber, may provide compelling evidence: if the follicle cells do express DE-cadherin, this would show that the follicle cells are pre-patterned early on to adhere

to the oocyte independent of *Bic-D*-mediated oocyte specification; if they fail to express DE-cadherin, we could safely conclude that oocyte specification, and cystocyte cluster-autonomous positioning of the oocyte, induces or stabilizes DE-cadherin expression in the PPFC.

While Godt and Tepass' (1998) results would favor the first alternative, which is a pre-patterning of the follicle cells, it is still possible to explain their results by the following alternative hypothesis: the oocyte, failing to induce *DE-cadherin* expression in the *DE-cadherin*$^-$ cells of the chimæric follicular epithelium, continues to shift position within the egg chamber until it encounters a group of cells which have the genetic capability to respond to the inductive signal *(DE-cadherin*$^+$*)*.

2.5. Germinal vesicle migration and patterning of the body axes

During oocyte growth, the germinal vesicle undergoes a predictable pattern of migration which is essential to the establishment of the Drosophila body axes.

H^3-uridine incorporation and autoradiographic electron microscopy studies of oogenesis has shown that the oocyte nucleus (also called the germinal vesicle) is not entirely transcriptionally inactive. Incorporation of the label appeared to begin at stages 7-8, most definitely at stage 9, and quite strongly in stage 10A. Because the oocyte nucleus does not have nucleoli, it is believed that the incorporation is not due to the synthesis of ribosomal RNA. Prior to stage 7-8, the oocyte nucleus diameter varies between two and three microns. During stage 8, the nucleus begins to enlarge, growing to 5.5 microns by stage 9. After stage 10A is completed, it shrinks back to 3.5 microns (Mahowald and Tiefert, 1970). The significance of this variation in size is not known.

What could this burst of germinal vesicle transcriptional activity in the germinal vesicle be? Good candidates would certainly include

gurken (grk) RNA; it is distributed in a cap just above the oocyte nucleus.

grk is a very important player in the establishment of polarity within the oocyte. Indeed, the same signal is important both for antero-posterior and dorso-ventral patterning (Ray and Schupbach, 1996). The induction of the dorsal follicle cell fate requires the activity of *grk* in the germline and *torpedo (top)* in the soma. *grk* encodes a product with similarity to the transforming growth factor (TGF)-α and *top* is the homolgue of the epidermal growth factor (EGF). It is therefore probable that Grk is a secreted ligand for the follicle cell receptor. Furthermore, its secretion depends on *cornichon*, whose yeast homologue has been shown to mediate export of secreted cargo from the ER (Powers and Barlowe, 1998). *grk* also plays a role in the induction of posterior polar follicle cells (PPFC). Failure of this induction results in the PPFC to persist in the default border cell fate, which are normally restricted to the anterior polar epithelium. In normal oogenesis, the PPFC signal back to the germline in order to induce the cytoskeletal rearrangements that will result in the making of a secondary axis in the oocyte. In this rearrangement, the microtubules, which are then organized at the posterior pole of the oocyte, become concentrated at the anterior instead. In the *grk* mutant, failure of PPFC fate induction and subsequent failure of PPFC-to-germline signalling, seems to prevent microtubule re-organization; the MTOC in the mutant egg chambers is found in the center of the oocyte, and the germinal vesicle fails to migrate to the antero-dorsal corner (Gonzalez-Reyes et al., 1995).

This process of cellular differentiation is at the core of our investigations in this thesis, and *Bicaudal-D* the molecule whose function we try to better understand.

2.6. Bicaudal-D

2.6.1. Molecular and phenotypic characteristics

Bic-D is essential for the specification of one of the cystocytes as an oocyte. Loss-of-function mutations in *Bic-D* result in the production of egg chambers containing 16 nurse cells and no oocyte (Mohler and Wieschaus 1986; Suter et al., 1989). They also abolish the transport of all known ribonuclear oocyte markers (Suter and Steward, 1991; Ran et al., 1994).

In this era of molecular biology, giant steps have been made in genome sequencing and expressed sequence tag cloning. This has created a great amount of work to be done in the elucidation of protein functions, and of the protein domains that underlie these functions. Since the cloning of the *Bic-D* gene nearly ten years ago, scientists have attempted to pinpoint what it is that Bic-D is doing on a molecular level, with moderate success. Bic-D may, for instance, play a role in centriole migration; in the organization and stabilization of microtubules; in the anchoring of determinants; in the transport of RNAs involved in microtubule organization and stabilization; or, more specifically, in intra-oocyte transport.

Analysis of the Bic-D amino acid sequence revealed several potential and evolutionarily conserved coiled-coil domains which could be involved in protein-protein interactions (Wharton and Struhl, 1989; Suter et al., 1989; Baens and Marynen, 1997). One of these protein-protein interactions is of Bic-D with itself. In fact, Bic-D is a homodimeric protein, consisting of two parts: a thin rod-shaped part of about 32 nm in length, hinged on a thicker rod-shaped part of about 26 nm in lenght (Stuurman et al., 1999). Another documented protein-protein interaction is with Egalitarian; this is confirmed

through co-immunoprecipitation experiments (Mach and Lehmann, 1997).

Several *Bic-D* mutants are available for study and are qualitatively different. They are discussed below.

2.6.1.1. Null mutants

Bic-D^{null} flies have revealed a zygotic requirement for viability. Flies raised on standard food at 29°C revealed *Bic-D*^{null} flies to be zygotic lethal; however, lethality was delayed at 18°C. These *Bic-D*^{null} died within a day of hatching. Raising the flies on apple-juice agar supplemented with live yeast paste greatly increased the frequency of adult flies, which could live for up to three days. The flies were small, weak, uncoordinated and lethargic. In the ovaries, neither *orb* nor *osk* RNA accumulated in a single cells. Also, inferring from the position of the 4-ring canals, one of which would become the oocyte in wild-type ovaries, the pseudo-oocyte was mispositioned within the 16-cell cluster. *Bic-D* is therefore required for the posterior localization of the pro-oocyte (Ran et al., 1994).

2.6.1.2. *PA66* mutation

Flies bearing this mutation have a 16 nurse-cell phenotype and are sterile, but unlike the null mutations, the pseudo-oocyte is properly positioned within the cystocyte cluster. (Suter and Steward, 1991). Genetically, this allele behaves either as a hypomorph or antimorph. The *PA66* mutant protein does not affect the formation of the Bic-D/Egl complex, but causes Egl to accumulate in a perinuclear punctate pattern (Mach and Lehmann, 1997). This may represent the "stalling" of a hypothetical mRNA localization machinery; other workers have

found that in the absence of microtubules, mRNA aggregated perinuclearly in myocytes (Perhonen et al., 1998).

2.6.1.3. *R26* mutation

In this mutation, the mutant Bic-D protein hyperaccumulates to the presumptive oocyte during the early stages of oogenesis, especially at the anterior of the cell, yet the oocyte also fails to differentiate further. This hyperaccumulation is independent of *Bic-D* RNA. The RNA does not preferentially accumulate to a single cell in region 2a in *R26*, yet the protein begins to hyperaccumulate at this early stage (Suter and Steward, 1991). The *R26* mutation also causes a similar hyperaccumulation of the Egl protein, and thus does not affect the formation of the Bic-D/Egl complex (Mach and Lehmann, 1997).

In *R26*, Orb protein accumulates at the posterior of the cyst, in a graded fashion, and as the pseudo egg chamber matures, it concentrates to the presumptive oocyte, with the highest levels found at the anterior of the cell (Lantz et al., 1994); this is similar to the distribution pattern of Dhc in the same mutants (Li et al., 1994), arousing speculation that the *R26* mutation results in failure of dynein-dependent intra-oocyte localization of a complex that may include Orb.

2.6.1.4. *Bic-D* gain-of-function alleles

The dominant *Bic-D* mutations give rise to the bicaudal phenotype. The embryos have reduced head structures or a double-abdomen structure. This is attributable to the mislocalization of *osk* mRNA to the anterior of the oocyte (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). The mutant Bic-D protein hyperaccumulates at the anterior of the oocyte during later oogenic stages (Wharton and Struhl, 1989).

2.6.1.5. *BicD*^{mom}

A heat-shock driven *Bic-D* transgene in a *Bic-D*^{null} background has allowed the examination of the role played by Bic-D in oogenesis beyond oocyte determination. The system permitted the initial stages of oogenesis to proceed under heat shock induction of the *Bic-D* transgene. Upon cessation of heat shock treatment, a drastic reduction in Bic-D protein levels in the more developed egg chambers is found. In addition to demonstrating a continued role for *Bic-D* in RNA transport, this experiment revealed a requirement for *Bic-D* in oocyte growth during early oogenesis, while transport is believed to be largely microtubule-dependent. An unexpected effect was the drifting of the germinal vesicle in the ooplasm (Swan and Suter 1996).

2.6.2. Homologues of Bic-D

In higher eucaryotes, homologues of Bic-D have been found in *C. elegans*, mouse, and humans. While the human and mouse Bic-D are highly homologous through the whole protein sequence, the *C. elegans* homologue distinguishes itself by a divergent C-terminal containing a non-polar sequence, 27 amino acids in length, as predicted by P-SORT (http://psort.nibb.ac.jp:8800/) and the transmembrane domain predictor SOSUI (www.tuat.ac.jp/~mitaku/adv_sosui). This domain is evenly divided by a pair of prolines. The significance of the diproline motif is obscure. There is, however, some evidence that in the membrane environment, as opposed to an aqueous environment, proline is a helix stabilizer rather than a helix breaker (Li et al., 1996). Many other proteins feature single predicted transmembrane (TM) domains at their C-terminal, such as Bax, a pre-apoptosis protein localized to cytoplasmic membranes including mitochondria, whose non-polar C-term is

necessary for its insertion into mitochondria following an apoptotic signal (Goping et al., 1998), ER-membrane resident lumenal proteins such as UDP glucoronosyltransferase (Meech and Mackenzie, 1998), and the homohexameric sarcoplasmic Ca2+ release channel RyR (Bhat et al., 1997). Curiously, removing the TM domain of some of these single-TM domain proteins does not affect their function, presumably because their association with the membrane only needs to be transient. This was found to be the case of the signal-recognition particle receptor β-subunit (Oog at al, 1998). The fact that the evolutionary distant nematode has evolved a version of *Bic-D* with a predicted transmembrane domain may simply reflect that increased membrane affinity enhances Bic-D function in that organism, but that it is dispensible in others.

One of the three human Bic-D homologues, called Bic-D1, is located on chromosome arm 12p 13.3-11.2 (Rauch et al., 1996; Baens and Marynen, 1997). Do Bic-D1 mutations cause disease in humans? One interesting human disease gene which has been mapped to this region is acrocallosal syndrome. This human developmental anomaly features an absence or underdevelopment of the corpus callosum, a deep C-shaped brain structure which unites the two hemispheres. The autosomal recessive syndrome also includes craniofacial abnormalities, polydactyly, loss of muscle tone (hypotonia) and mental retardation (Human Genome Project web site http//www.ornl.gov). Because of *Bic-D*'s genetic interaction with *DLis-1* (A. Swan., pers. comm.), a gene involved in cortical neuronal migration, there is a possibility that acrocallosal syndrome may represent the human phenotype of a Bic-D1 mutation.

Another gene with a phenotype similar to acrocallosal syndrome shows that the disease can result from the disruption of a pathway which does not appear to be related to Bic-D. This gene is responsible for Greig cephalopolysyndactyly syndrome (GCPS), and bears so many similarities to acrocallosal syndrome they were once believed to be allelic to one another; recent evidence show that they are not (Brueton et al., 1992). The GCPS gene has been cloned and found to encode Gli3, a putative zinc-finger transcription factor, and a homologue of the fly gene *cubitus interruptus (ci)* (Kang et al., 1997; Wild et al., 1997). This suggests the involvement of *hedgehog* signaling and *Bmp* genes in the pathway. Since no evidence exists that *Bic-D* interacts with this pathway, the case for the human homologue of *Bic-D* being implicated in acrocallosal syndrome is at the moment unconvincing.

Other disease loci are for testicular germ cell tumors, mapped to 12p11.1-p12.1, and acute lymphoblastic leukaemia (ALL), mapped to 12.p12.3 (Mastert et al., 1998).

2.7. Genes and molecules which interact with Bic-D.

2.7.1. Microtubules.

Even though evidence points to an interaction between *Bic-D* and the microtubule cytoskeleton (Suter and Steward, 1991; Swan and Suter, 1996), the nature of these interactions is unknown; *Bic-D* may be involved in microtubule organization, microtubule-based transport, or both. Feeding flies colchicine, a microtubule depolymerizing agent, prevents formation of an oocyte and results in a 16 nurse cell egg chamber similar to that observed in *Bic-D* loss-of-function alleles (Koch and Spitzer, 1983). Further genetic evidence that polarized transport is involved in oocyte differentiation comes from the observation

that certain allelic combinations of *Dhc* have a 16 nurse cell phenotype, indicating that *Dhc* is essential for oocyte differentiation (McGrail and Hays, 1997).

Although *Bic-D* and *egl* mutants fail to organize microtubules in the cystocyte clusters (Theurkauf et al., 1993), colchicine feeding results in Bic-D::GFP (this work) and Egl de-localization (Mach and Lehmann, 1997). There may therefore be a three-way mutual requirement between microtubule organization, *Bic-D* and *egl*.

After oocyte specification, *Bic-D* and microtubules continue to be essential for the same processes, namely oocyte growth, development of proper dorsal-ventral polarity in the follicle cell epithelium and posterior patterning in the oocyte. The same studies also demonstrated a requirement for both microtubules and *Bic-D* in stage 7 and subsequent stages of oogenesis for the positioning of the oocyte nucleus (Koch and Spitzer, 1983; Swan and Suter, 1996). During these stages of wild-type oogenesis, the oocyte nucleus is found at the antero-dorsal corner of the oocyte. This localization is a crucial step in setting up dorso-ventral polarity of the egg chamber and the embryo because the position of the oocyte nucleus defines the dorsal side (Schüpbach and Roth, 1995). Lack of *Bic-D* or disruption of microtubules cause the oocyte nucleus to drift in the ooplasm (Koch and Spitzer, 1983; Swan and Suter, 1996).

It was observed that in *Bic-D*^{mom} ovaries, as well as in ovaries treated with colchicine, the normal perinuclear particle clustering did not occur, giving the nurse cell cytoplasm a more uniform appearance. In addition, particulate transport through ring canals occurred faster than in wild-type (A. Swan, pers. comm., 1996). This suggested a *Bic-D* and microtubule dependent slowing of ring canal transport, perhaps due to some

unknown molecular sorting mechanism, or a reorganization of the transporting complex.

The in vivo analysis of GFP::exu cytoplasmic transport has revealed a complex and seemingly multi-step process, where particles employ alternatingly microtubule-dependent and microtubule independent steps. Theurkauf and Hazelrigg (1998) revealed the existence of 3 distinct populations of microtubules within the nurse cells themselves, responsible for 1) rapid particle movement within bulk cytoplasm and 2) perinuclear particle clustering and particle accumulation at the ring canal junctions, two *Bic-D* dependent processes (A. Swan, pers. comm., 1996). Particle passage through the ring canals themselves appear to be resistant to both actin and microtubule inhibitors separately, but it is not known whether a simultaneous inhibition would still allow directional transport through the cytoplasmic bridges. In other words, can passage use either microtubules or actin, or is it independent of these cytoskeletal elements? If there are indeed several different microtubule populations in the egg chamber, it would be useful to repeat the classical colchicine experiments with a newly developed microtubule inhibitor specific for dynamically unstable microtubule. This inhibitor is the magnesium salt of ilimaquinone, named 201-F (Poüs et al., 1998).

Nurse cell to oocyte transport occurs in two steps: 1) early transport is microtubule-based and 2) late transport is actin/myosin based. *Bic-D*^{mom} flies are probably deficient only the microtubule-based phase of transport because growth catches up during the actin-myosin stages. *Bic-D* does not appear to affect the polarity of microtubules, but it may affect their stability (Swan and Suter, 1996).

2.7.2. Sponge bodies

Recent electron microscopic observations have revealed the existence of sponge-like subcellular structures found in nurse cells and oocytes. These structures, consisting of ER-like cisternae embedded in electron-dense material, are enriched in Exu protein. Sponge bodies are believed to play a role in the transport of maternal products, such as *bcd* mRNA, and may itself be mobile within the egg chamber, although only indirect evidence has been offered for this (Wilsch-Brauninger et al., 1997).

2.7.3. Egalitarian

The *egalitarian (egl)* phenotype closely overlaps loss-of-function *Bic-D* phenotypes. The sequence analysis of *egl* was not as informative as researchers had hoped. The predicted protein product is largely hydrophilic, bears no homology to any other known product, save a puzzling exonuclease domain (Moser et al., 1997) and several poly-Q, poly-N and poly-S repeats of unknown function. There are no documented null alleles of *egl*, so it is not known whether a theoretical egl^{null} would, like $Bic\text{-}D^{null}$, also have a defect in oocyte positioning. Bic-D forms a complex with Egl, and mutations in *egl* can prevent this complex from occurring (Mach and Lehmann, 1997). Egl may also have an effect on transport. Carpenter's (1994) EM work has suggested that the *egl* phenotype may reflect a general failure of transport, as shown by the lack of orientation of mitochondria in the cystocyte clusters.

In *egl* mutants, Orb protein does concentrate to a subset of the cells in the cluster, but later fails to concentrate to one cell. Instead, it becomes uniformly distributed throughout the chamber (Lantz et al., 1994).

Electron microscopic sections of wild-type cystocyte clusters feature mitochondria which are oriented towards the ring canals and clustering in their vicinity, as well as others which are probably passing through ring canals. *egl* may have a generalized effect on transport: such orientation and clustering of mitochondria does not occur in *egl* mutants (Carpenter, 1975). The MTOC is also affected. Anti α-tubulin immunolabelling of microtubules has shown that although an MTOC initially forms in a single cell of cystocyte clusters, it is not maintained (Theurkauf et al., 1993).

We will now discuss the implications of *egl*'s predicted exonuclease domain in RNA transport. The domain belongs to the exonuclease D family, which is implicated in the digestion of single strand RNA, for example, precursor tRNAs after they are processed by endoribonucleases (Moser et al., 1997). Other members of this family include the Werner syndrome 3'-5' exonuclease gene (Huang et al., 1998), whose product co-purifies with a DNA replication complex, is homologous to the RecQ DNA helicase and is required for genomic stability. Werner's syndrome is characterized by premature aging (Lebel and Leder, 1998). The Werner's syndrome product localizes to the nucleoli of transcriptionally active cells, where it seems to play a role in rRNA transcription (Gray et al., 1998).

In the Drosophila ovary, it would not be unreasonable to assume that such an exonuclease would be more likely found in the nucleus, or perinuclearly, where RNAs are likely to undergo processing. Egl, however, is cytoplasmic, and it is transported. A common behaviour of the exonucleases is the removal of one or more bases until it reaches a stable base-pair stem (Moser et al., 1997). We have already mentioned that many transported RNAs

are recognized by stem-loop sequences, and that these sequences are known to impede ribosomal scanning. If Egl posseses exonuclease activity, it may serve to cleave the localization signal for efficient translation. Removal of such a stem-loop, however, would require additional enzymatic activity, such as double-strand endonuclease or RNA helicase. Alternatively, Egl may play a role in deadenylation. In Xenopus, for instance, exonucleolytic degradation of poly (A) tails is involved in the translational silencing of maternally supplied RNAs (Korner et al., 1998).

2.7.4. Spindle genes

Spindle mutants are charaterized by a severe ventralization phenotype and do not produce dorsal appendages, resulting in a spindle-shaped egg. All six spindle genes are epistatic, and appear to be redundant genes within a single pathway affecting the state of DNA condensation in the germinal vesicle, the dorsalization pathway, and oocyte determination. The cloning of two of these genes has been reported: *spn-E* encodes a DE-H box RNA-dependent helicase (Gillespie and Berg, 1995) and *spn-B* is homologous to the double-strand break repair yeast proteins. Indeed, *spn-B* and *spn-D* have been shown to greatly facilitate meiotic recombination (Ghabrial et al., 1998). The ventralization phenotype may be a result of failure of a hypothetical vectorial export of the *grk* message from the germinal vesicle, or checkpoint-mediated arrest induced by germinal vesicle disorganization; the oocyte determination defect is intriguing, and consists of the two 4 ring-canal cells adopting oocyte fate.

In the wild-type, the two cells with four ring canals in the cystocyte cluster initially enter meiosis and form synaptonemal

complexes (Carpenter, 1975). Gonzales-Reyes (1997) has proposed that in the spindle mutants, the two germinal vesicles persist in this meiotic state. Must oocyte specification involve a step where one of these cells is chosen at the expense of the other? Different researchers offer contradictory evidence. Gonzalez-Reyes's (1997) interpretation of the two-oocyte phenotype is that the choice of one of these two cells as the oocyte is delayed in spindle mutants. More recently, de Cuevas (1998) suggested that a transient ring canal blockage, a trivial mechanical by-product of fusome biogenesis, would temporarily allow accumulation of oocyte markers in two cells, but that once the blockage was lifted, markers would accumulate to one cell.

The two oocytes and fourteen nurse cell phenotype has an intruiguing feature: *osk* mRNA accumulates in the two cells, the true oocyte as well as the pseudo-oocyte, while Bic-D and Egl have never been observed in anything but a single cell in the same mutants, and always in the same cell that accumulates yolk (Gonzalez-Reyes et al., 1997). This has two implications. First, this shows that there is something unusual about *osk* RNA localization, because it can accumulate in a secondary oocyte where Bic-D and Egl do not accumulate. Secondly, even if two nuclei in the cluster have the characteristics of a germinal vesicle, Bic-D and Egl appear to respond to some other unknown cue, or quantitatively different cue, to accumulate in only one of these cells. Yet mutants in the spindle genes, while having no recorded effect on cytoskeletal organization, disrupt Bic-D localization in mid-oogenesis (Figure 5d). They also have enlarged sponge bodies (M. Wilsch-Brauninger, pers. comm. 1997). These pleiotropic effects suggest that the *spindle* genes may have roles that go beyond meiotic recombination.

2.7.5. Dynein heavy chain.

Cytoplasmic dynein is a large hetero-oligomeric complex of over 9 protein subunits. The dynein heavy chain component of this complex is made up of a globular N-terminal domain with ATPase activity and a coiled-coil stalk, the site of homodimer interactions. Dynein is a minus-end directed microtuble-dependent motor, and functions in the transport of vesicles and vesicular organelles such as the Golgi apparatus (Vallee, 1993; Goodson et al., 1997).

RNA transport in the egg chamber is known to occur in a microtubule-dependent manner, and in the direction of microtubule minus-ends, yet dynein has never been directly implicated in RNA transport in any system to date. However, no data concerning RNA accumulation in the particular *Dhc* heteroallelic combination giving rise to the cystocyte cluster mitotic defect has been published so far.

Further genetic evidence that polarized transport is involved in oocyte differentiation comes from the observation that certain allelic combinations of *Dynein heavy chain (Dhc)* have a 16 nurse cell phenotype, indicating that *Dhc* is essential for oocyte differentiation (McGrail and Hays, 1997). One must be careful in interpreting this 16 nurse cell phenotype, as it may arise indirectly from the general fusome and mitotic spindle dismorphology seen in these cystocyte clusters, and not necessarily from a failure in the oocyte specification pathway itself.

2.7.6. *DLis-1.*

A gene isolated in a screen for enhancers of *Bic-D* encodes for DLis-1, a protein containing WD40 repeats. Lis-1, its human homologue, is the causative gene for Miller-Dieker lissencephaly, a cerebral cortex malformation giving the brain

surface a smooth appearance, lacking normal sulci and gyri, and the cause of severe mental retardation. The defect is believed to involve faulty cell migration (Reiner et al., 1993). It was found that the WD40 repeats in Lis-1 bind to β-spectrin in vitro, and that it co-purifies with tubulin as well as, not surprisingly, Platelet-Activating Factor Acetyl Hydrolase-1 (PAFAH-1) and PAFAH-2. Lis-1 is in fact the non-catalytic subunit of the platelet-activating factor acetyl-hydrolase 1b (Wang et al., 1995). The tubulin co-purification is likely to be of biological significance, as Lis-1 reduces the rate of microtubule catastrophies in vitro (Sapir et al., 1997).

Lis-1 also has a homologue in *Aspergillus nidulans*, called NudF. Mutations in NudF cause a defect in nuclear migration; this process requires both dynein heavy and light chains, actin-related protein 1 (Arp1), $p150^{Glued}$, microtubules and NudC, which is also involved in cell wall secretion (Chiu et al., 1998) and whose mammalian homologue localizes to the Golgi apparatus in mammalian cells (Morris and Yu-Lee, 1998). Suppressor screens have unveiled a perplexing pattern of genetic interactions between the known members of the pathway. NudA (which encodes for dynein heavy chain in Aspergillus) is a suppressor of NudF (Willins et al., 1997). Wild-type NudA may stabilize microtubules (Wang et al., 1995). A mutation in α-tubulin, which causes destabilization of microtubules, suppresses NudA, NudC NudF and NudG. Treatment with benomyl, which similarly destabilizes microtubules, has the same suppression pattern as the mutation in α-tubulin (Willins et al., 1995). The stabilization of microtubules appears to play a pivotal role in the pattern of suppression of the nuclear migration pathway in Aspergillus.

There is further evidence that the stabilized/destabilized state of microtubules may modulate separate intracellular transport pathways. Another Aspergillus mutant, apsA, shows a nuclear migration defect during sterigmata formation (a specialized sexual structure) and was found to be an 183 kD homologue of the yeast Num1p, a protein which associates with the cell cortex in S/G2 phase and affects microtubule function as well (Fischer and Timberlake; 1995). Num1 is enhanced in yeast by α-tubulin and β-tubulin mutations. Num1p has a $Ca2^{+}$ binding EF hand domain, and pleckstrin homology domains believed to be involved in its targeting to the cortex (Farkasozsky and Kuntzel; 1995).

What could be the role of *DLis-1* at the molecular level? Lis-1 has been found to suppress microtubule dynamics when interacting with tubulin; in vitro microtubule dynamic measurements indicated that physiological concentrations of Lis-1 reduced the rate of microtubule catastrophic events, resulting in a net increase in the maximum length of microtubules (Sapir et al., 1997).

Some experiments inspired from research done with mammalian cells could be attempted in Drosophila. For example, rat granule cell migration was observed in vitro on laminin-coated layers. The process was found to be inhibited by PAF analogs which show receptor-antagonistic activity (CV-6209, CV-3988, and carbamoyl PAF). The inhibition was reversible upon a wash with a buffer (Adachi et al., 1997). It would be interesting to see whether these inhibitors could have a effect on germinal vesicle attachment on Drosophila cultured ovaries.

2.7.7. *orb*

The *orb* gene product contains domains matching that of RRM-family of RNA-binding proteins (Lantz et al., 1992). The protein is highly homologous to the Xenopus cytoplasmic polyadenylation element binding (XCPEB), an RNA binding protein that interacts with the maturation-type cytoplasmic polyadenylation element (CPE) to promote polyadenylation and translational activation of maternal mRNAs. Although *orb* has not been shown to have polyadenylation function in Drosophila, the mouse homologue of CPEB has been recently isolated in a search for factors that could mediate the cytoplasmic polyadenylation of mouse *c-mos* mRNA, a serine/threonine kinase required for oocyte maturation. The mCPEB mRNA is detected in oocytes, testis, and kidney (Gebauer and Richter, 1996).

Both *orb* RNA and Orb protein are localized in oogenesis, and these patterns are virtually indistinguishable from that of *Bic-D*. There is very little *orb* message until the 8-cell cyst stage, where levels increase and become very high by the time the 16-cell cyst is completed. Message distribution is cytoplasmic, but not uniform, and as the cysts continue to develop, the protein accumulates in one cell. Orb protein preferentially accumulates at the posterior cortex of the oocyte, until stage 8, when it begins to accumulate at the anterior cortex.

orb is essential for cyst formation, and 16-cell cysts are rarely formed in severe mutants. Most cysts arrest at the 8-cell stage. *Bic-D* and *egl* seem to be required later, because they both consistently form 16-cell cysts. However, *Bic-D* and *egl* are required for the localization of *orb* RNA to the presumptive oocyte in cystocyte development.

Mutations in *Bic-D* result in aberrant Orb localization, and the effect varies according to the allele of *Bic-D*. In the *Bic-D*^{PA66} mutant, Orb does not accumulate to the pro-oocyte efficiently. In the *R26* mutant, where the defective Bic-D protein does accumulates to a single cell which fails to fully differentiate into an oocyte, Orb accumulates at the anterior of the oocyte (Lantz et al., 1994), just like Dhc protein in the same mutant (Li et al., 1994)

Conversely, *orb* appears to be required for Bic-D localization (Figure 3c and 3d). In the older germarial cysts, anti-Bic-D indirect immunofluorescence staining has also revealed unequal distribution of the Bic-D protein into irregular subsets of cells within the cystocyte clusters (Lantz et al., 1994).

2.8. Objectives

We have now reviewed some of the most salient morphological, molecular and genetic details of oocyte specification in Drosophila. We have learned of the central and complex role *Bic-D* plays in this process. In addition, we have described the other molecules which interact with Bic-D.

We now outline the experimental objectives which we devised as a means to learn more about Bic-D function:

(1) observe the distribution pattern in real time or in sequence of the Bic-D::GFP fluorescent signal;

(2) find out how mutations in other genes involved in oocyte determination or microtubule-dependent transport affect the localization pattern of the Bic-D::GFP fusion protein;

(3) find out whether *Bic-D* interacts genetically with *Dynein heavy chain;*

(4) learn more about the dynamics of oocyte nucleus migration;

(5) perform a P-element mediated mutagenic screen of 10 000 mutagenized chromosomes in order to identify suppressors of Bic-D; and

(6) characterize genetically and phenotypically the single suppressor which arose from this screen; (7) generate new alleles of the suppressor through excision of the P-element insert.

3. Methods

3.1. Fly stocks and techniques

Drosophila melanogaster OregonR flies were used as a wild-type strain. Flies were raised on standard corn-meal agar media at room temperature. The following alleles were used for this study: Dhc^{3-2}, Dhc^{6-6} and Dhc^{6-12} (Gepner et al., 1996), $spn\text{-}A^{1}$, $spn\text{-}B^{1}$, $spn\text{-}D^{2}$, $spn\text{-}E^{1}$, $spn\text{-}F^{1}$ (Tearle and Nüsslein-Volhard, 1987), egl^{PV27}, egl^{WU50}, egl^{PR29}, egl^{RC12}, egl^{PB23} (Schüpbach and Wieschaus, 1991), orb^{F343}, orb^{F303} (Lantz et al., 1994), $mael^{1}$ (Clegg et al., 1997), and *stwl* (Clark and McKearin, 1996). Observations in a *Bic-D*$^{+}$ background were made using w^{-}; $P[w^{+}$ *Bic-D::GFP*$^{(201)}]$ *Bic-D*$^{+}$. Observations in a *Bic-D*^{null} background used w^{-}; $P[w^{+}$ *Bic-D::GFP*$^{(201)}]$ *Bic-D*^{r8} *cn bw* flies either homozygous or over *Df(2L)TW119 cn bw*. Colchicine feeding of flies was done under the following conditions: a size 0 paintbrush was dipped in a 20 mg ml^{-1} stock solution of colchicine in DMSO, and used to spread colchicine over standard fly media in a vial. After food surface drying, 3 day old flies were introduced into the vial and their ovaries were dissected after 48 hours. The w^{-}; *Bic-D::GFP*$^{(201)}$, *Bic-D*$^{+}$ strain was used for recombination with egl^{PV27} and crossing into the *spn-E*, orb^{F343} or *Dhc* background, as well as for all confocal observations unless otherwise noted.

3.2. Construction of *P(w+ Bic-D::GFP)* transgenic animals

A 4.2 kb EcoRV fragment starting at the 3' end of the first *Bic-D* intron and extending slightly beyond the polyadenylation site was cloned in pBSKS^{+}. Site-directed mutagenesis was performed in order to

introduce a BglII site before the termination codon of *Bic-D* using the primer 5' GCC AAT CCA TTC GGA GAT CTT AAC ATA AGA TCA 3'. The Bam HI fragment from GFP S65T cloned in pRSET (Heim et al., 1995), was inserted in this unique BglII site and a clone with the correct orientation was selected. To restore the full *Bic-D* coding sequence, the 3.5 kb genomic *Bic-D* fragment containing the 5' sequences of *Bic-D* and extending to the HindIII site in the first intron was cloned into pBSKS$^+$ (H* in Figure 2 by Suter et al., 1989). This 5' end plasmid was cut with XhoI and ScaI (in the vector), partially filled-in at the XhoI overhang with dTTP and dCTP and Klenow, and subsequently cut with NotI which excised the 5' end sequence from the vector. This fragment was gel purified and inserted in the 3'Bic-D-GFP plasmid which had been first cut with BamHI, partially filled in with dGTP and dATP, and then digested with NotI. The KpnI and NotI fragment of the resulting fusion gene was then inserted into the KpnI and NotI sites of a modified pCaSpeR transformation vector. The fusion gene was then introduced into flies by P-element mediated transformation.

3.3. Microscopic observations

For light microscopy, ovaries were dissected and mounted in PBS for immediate observation under a Zeiss Axioplan microscope. For confocal observations (Leica Diaplan), ovaries were dissected in halocarbon oil 200, and placed in a drop of this oil in the center of a 22 mm x 60 mm coverslip. Ovarioles were detached from the ovary and carefully dragged against the glass until each whole ovariole was in contact with the glass. The coverslip was then turned over, its edges

stuck to microscope slide stubs, and the sample was observed in the free hanging drop of halocarbon oil.

It was noticed during confocal sessions that the distribution of Bic-D::GFP remained unchanged for at least four hours, inasmuch as the ovary was well covered in oil. Four hours allow for sufficient time to observe the effect of anoxic conditions in the cultured ovary (Theurkauf and Hazelrigg, 1998). Because the inverted oil drop sags over time, the ovarioles at the edge of the oil drop would sometimes become so thinly covered in oil as to begin to look glassy by light microscopy, perhaps due to dessication. In the case of these ovarioles, the fusion protein would take on a characteristic distribution pattern consisting of very small bright particles in an otherwise very dark cytoplasm, accompanied by accumulation to the cortical and perinuclear areas. We have confirmed that the pattern results from dessication by simulating similar conditions of dessication, and observing the resulting Bic-D::GFP distribution by fluorescence microscopy. The conditions were simulated by spreading the ovary on the coverslip in a much smaller pool of oil, and comparing to ovaries spread under the usual quantity of oil. Due to the obviously artifactual nature of the pattern, it is not considered in this study.

3.4. Screen for suppressors of *Bic-D*^{PA66}

The screen was performed using a modified P-element with direct terminal repeats, the lacZ gene for staining purposes and the mini-white gene as a positive marker for transformation. Females with ammunition chromosomes carrying 4 copies of this P-element are

crossed to males carrying the transposase gene Δ2-3 and associated marker *Sb*. The crossing scheme is outlined below.

X *P[w⁺]*/ X *P[w⁺]* ; *Bic-D^{PA66} cn* / *b CyO*; +/+ **X** *w*/*Y*; *Bic-D^{PA66}cn*/*CyO* ; *Δ2-3 Sb* / *TM3Ser*

⇩

w /*w*; *b Df(2L)TW119 cn bw* / *b CyO* ; +/+ **X** X *P[w⁺]* / Y; *Bic-D^{PA66} cn* /*CyO* ; *Δ2-3 Sb* / +

The female progeny from the last cross was tested in bulk for strong supressors generating viable offspring. Using the females allows us to include the X chromosome in the screen; on the other hand we cannot select for hopping events. In parallel, males selected for P-element mobilization were crossed to w/w ; *b Df(2L)TW119 cn bw* / *b CyO* and their *Bic-D^{PA66} cn*/ *b Df(2L)TW119 cn bw* female progeny were tested for fertility. This way, over 10 000 mutagenized chromosomes were screened.

4. Results

4.1. Genetic analysis of subcellular Bic-D localization

4.1.1. A Bic-D::GFP fusion provides functional Bic-D activity and reflects the distribution pattern of endogenous Bic-D.

In making the Bic-D::GFP construct, we wished to preserve as much of the endogenous genomic environment of the *Bic-D* gene as possible (Fig. 2 *a*). We have used a *Bic-D* mini gene containing approximately 1.2 kb of 5' flanking sequence but lacking the central 6.5 kb of the 7.7 kb long first intron (Suter et al., 1989). The minigene also included, in the 3' flanking sequence, 240 base pairs of genomic DNA beyond the most distal polyadenylation site. The S65T GFP sequence (Heim et al., 1995) was then inserted before the *Bic-D* translational stop codon. As a by-product of this insertion, accompanying polylinker sequences are predicted to result in the addition of the heptapeptide GDPPAEF immediately to the amino-terminal end of the GFP peptide and the amino acids GS at the carboxy-terminal end of the chimeric protein (Fig. 2 *a*).

In order to ensure that the GFP signal accurately reflects the localization pattern of the wild type Bic-D protein, we have used a combination of three different criteria: (1) the protein fusion product must be stable to ensure that the GFP fluorescence originates from the whole chimeric protein; (2) the distribution pattern of the chimeric protein in fixed tissue must overlap the domain of immunologically detected wild-type Bic-D

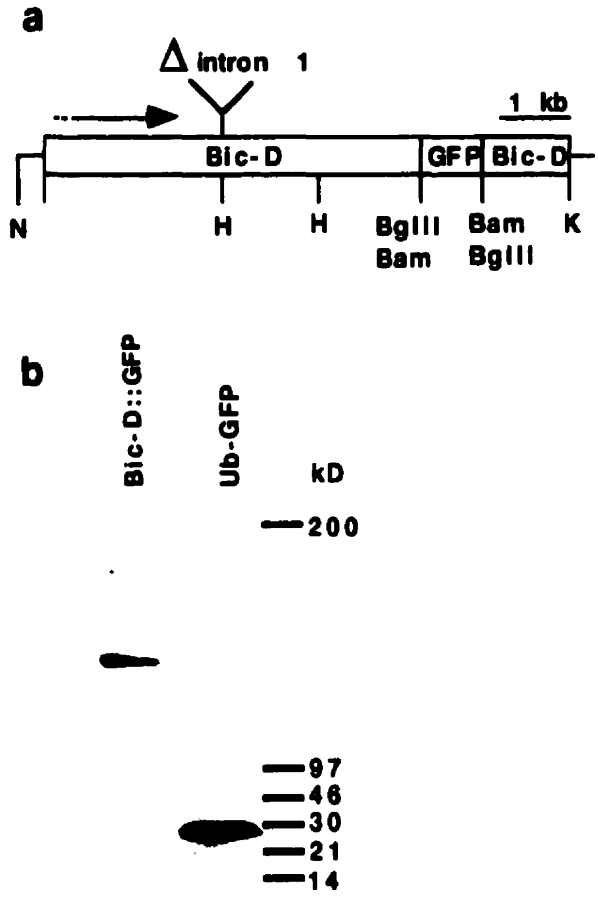

Figure 2. Bic::GFP fusion gene and protein

(*A*) Structure of the Bic-D::GFP fusion gene and the predicted primary transcript (box). A *Bic-D* mini gene in which the central 6.5 kb of the 7.7 kb long first intron were deleted at the position indicated (Δintron 1). The S65T GFP sequence (Heim et al., 1995) is inserted before the Bic-D translational stop codon (GFP). (*B*) Anti-GFP immunoblot of ovary extract. The proteins were separated in an SDS-PAGE, transferred on nitrocellulose paper and probed with a polyclonal anti-GFP antiserum. Note that the gel is a step gradient; the concentration above the 97kDa mark is 6%, and below 15%. The left lane contains fly ovary extracts from a line carrying a *Bic-D::GFP* transgene, and the right lane ovary extracts from a fly that contains GFP fused to a nuclear localization sequence only (Davis et al., 1995). No degradation product was detected.

protein and (3) the chimeric *Bic-D::GFP* must be biologically active.

We first tested whether the chimeric protein remained as a unit in the cell. Indeed, we did not detect degradation of Bic-D::GFP by Western analysis (Fig. 2 *b*). Secondly, we established that in fixed tissue, the distribution of the Bic-D::GFP fluorescence signal shows the same pattern as the native protein as detected by immunocytochemistry (data not shown). In our third test, the fusion gene must rescue *Bic-D*^{null} flies. We found that even levels of fusion protein that are lower than those produced by a single copy of wild type *Bic-D* could fully restore viability and fertility in *Bic-D*^{null} flies, indicating that the chimeric protein is biologically active. The fulfillment of the three above-mentioned criteria indicates that the GFP signal correctly reflects the distribution of wild-type Bic-D protein, and that the chimeric protein behaves like the wild type protein.

4.1.2. Bic-D::GFP in the germarium.

In vivo observations with Bic-D::GFP allow us to observe the distribution pattern of the Bic-D protein in the germarium with unprecedented definition. The earliest detectable accumulation of the fusion protein in the female germ line occurs near the germarium tip, in region 1. Region 1 of the germarium is an area where the germline cells are mitotically active. It is in this region that the germ line stem cells and their differentiating daughters, the cystoblasts, divide to form the 16 cell cystocyte

clusters (Fig. 1). In the anterior part of germarial region 1, where the overall signal intensity is low, the cytoplasmic Bic-D::GFP signal accumulates preferentially to a structure whose size (approximately 2 μm), spherical shape, and localization pattern in germline stem cells and cystoblasts corresponds to the properties of the spherical fusome (spectrosome) (small arrow in Fig. 3 *a*). The fusome is a cytoplasmic structure rich in membrane skeletal proteins such as spectrin, adducin-like Hts protein, Bam protein and ankyrin (Lin et al., 1994; Lin and Spradling, 1995; de Cuevas and Spradling, 1996; McKearin and Ohlstein, 1995; McKearin, 1997).

During the remainder of the mitotic stages in region 1 Bic-D::GFP shows a uniform cytoplasmic distribution. Once the 16 cell cluster is formed in region 2a, the fluorescent signal from the chimeric protein is seen to accumulate in the centre of the cluster and in an array of dot-like foci of greater fluorescence that is often seen arranged concentrically around this central accumulation (large arrow in Fig. 3 *a*). The central cyst accumulation indicates that Bic-D::GFP may be fusome associated during this period. This distinct localization pattern is only seen in region 2a and in particular the dot-like foci are seen only occasionally. Even though this could mean that this particular subcellular distribution of Bic-D::GFP may be disrupted during or even caused by the preparation of the ovaries for confocal microscopy, it seems more likely that it is a transient event because loss of specific gene activities seem to stabilize this distribution pattern (see below).

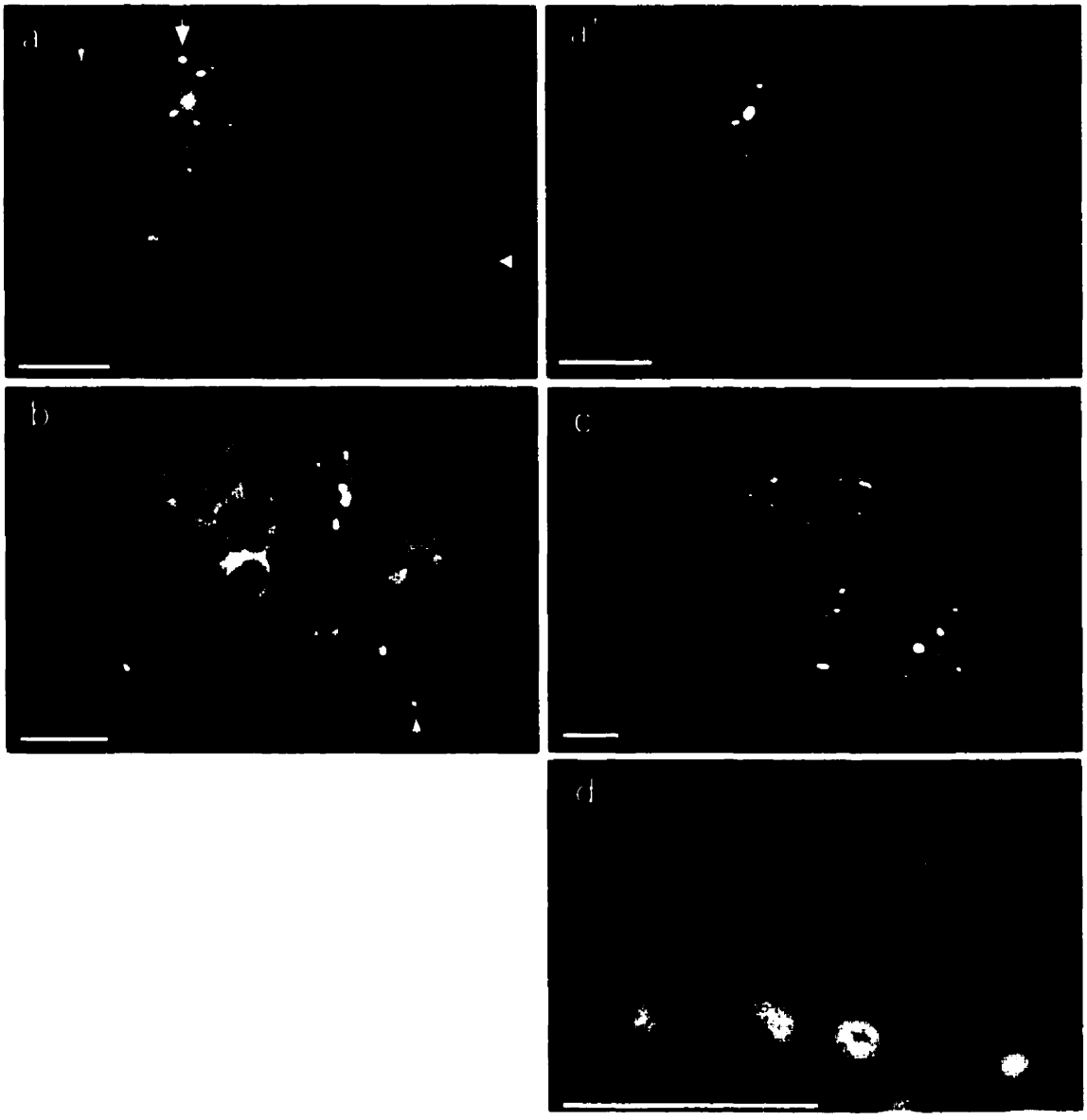

Figure 3. Distribution of Bic-D::GFP in wild type and mutant germaria
(*A*) Composite confocal image of 15 z stacks spaced 1 micron apart. In region 1 the signal concentrates in spheres in the anterior of the stem cells or cystoblasts (small arrow). In late region 1 or early region 2a the signal concentrates towards the centre of the cystocyte cluster with dot-like foci adjacent to this central region of accumulation (large arrow). Starting in region 2b we observe preferential accumulation in the oocyte (arrowhead). (*A'*) Single plane confocal image of the same germarium shown in (*A*). (*B*) Single plane confocal image of an egl^{PV27} mutant germarium. Bic-D::GFP fluorescence does not show central cystocyte accumulation, and the dot-like foci are disorganized. (*C*) Composite confocal image of 15 z stacks spaced 1 micron apart of an orb^{F343} mutant. Irregularly-shaped foci with discrete margins accumulate in the cystocyte clusters beyond region 1. These are seen with greater magnification in (*D*). Scale bars represent 10 μm.

4.1.3. *orb* and *egalitarian* are required for Bic-D subcellular localization in the germarium.

We wanted to see which of the well characterized female-sterile genes are essential for the different patterns of subcellular localization of Bic-D::GFP in the germarium. There is evidence that *Bic-D* and *egl* act at the same step in establishing oocyte fate because loss-of-function mutations in either *Bic-D* or in *egl* lead to a 16-nurse-cell phenotype (Schüpbach and Wieschaus, 1991) and because Egl forms a complex with Bic-D protein (Mach and Lehmann, 1997). In order to see how Bic-D protein localizes in the absence of *egl* function, we examined the distribution of Bic-D::GFP in egl^{PV27}. We found that the pattern of central Bic-D accumulation in region 2a was abolished and that the dot-like foci appear in random positions in the cytoplasm as opposed to being concentrically organized (Fig. 3 *b*). In wild-type germaria these dot-like foci are present in region 2a, but disappear before the cysts reach region 2b. In the *egl* mutant, however, they persist into region 2b. We conclude that *egl* function is essential for the concentric orientation of the Bic-D::GFP dot-like foci within the cystocyte cluster, and for regulating the temporal aspects of this particular pattern of accumulation.

The other mutant which affects Bic-D::GFP distribution in an informative way is *orb*. orb^{F343} ovaries consist mainly of 8-cell cystocyte clusters (Lantz et al., 1994). The severe *orb* allele *F343* has a dramatic effect on Bic-D::GFP localization. In region 1 of these ovaries, Bic-D::GFP distribution is similar to wild-type, but

further downstream in pseudo-region 2a, it accumulates in variously sized foci with discrete margins, while the rest of the cytoplasm shows reduced Bic-D::GFP fluorescence (Figs. 3 *c, d*). The foci do not appear to have a particular orientation with respect to the cell cluster and at the posterior of these rudimentary ovaries, they fade away and eventually disappear. Unlike the foci observed in *egl*, the foci in orb^{F343} vary in size and are nested in what appears to be a depression of the nuclear membrane.

4.1.4. Bic-D::GFP reveals that antero-posterior polarity of the oocyte is established in the germarium.

To more accurately describe the dynamic events occurring in region 2b, we further classify the cystocyte clusters in this small region as early, mid and late clusters. In early region 2b, the Bic-D::GFP signal is prominently enriched in the anterior of every cell of the cyst, but still not enriched in a single cell. It is not until mid region 2b that the signal is preferentially enriched in a single cell. At this time Bic-D::GFP does not fill the entire cytoplasmic compartment of the oocyte but manifests itself as an anteriorly localized globe (Fig. 4 *a*, black arrow).

In later germarial stages, we can observe what appears to be the dynamic establishment of antero-posterior polarity within the oocyte. Initially, in mid region 2b, the Bic-D::GFP signal accumulates strongly in a bright focus anterior to the presumptive germinal vesicle. This globe appears to be nested in a depression of the nuclear surface. Later in region 2b, this focus can be either lateral or posterior to the germinal vesicle, giving

the impression that it migrates around the oocyte nucleus until it reaches the posterior of the oocyte at the time the cystocyte cluster enters germarial region 3 and becomes a stage 1 egg chamber (Fig. 4 *a*, white arrows). This process constitutes the earliest sign of polarization within the oocyte itself.

4.1.5. Two novel subcellular domains which reveal cellular asymmetry, the spool and the crater.

Throughout the apparent migration of the oocyte nucleus, the globe is continuously nested in a polarized nuclear depression with the appearance of a "crater." The crater features a very regular concave face with sharply defined margins from its appearance in the germarium to stage 7 (Figs. 4 *b*, *c*, *d*, and *e*). The width of the crater may reach up to three quarters of the nuclear diameter. We visualized the crater with Nomarski optics microscopy in living ovaries of OregonR flies, and it appears to be formed by the nuclear membrane (Fig. 4 *c*).

Another subcellular domain is discovered in close association with the crater. It is revealed by a posterior Bic-D::GFP signal, from stage 1 to stage 5, in the shape of a broad cylinder with a core of less intense staining, much like a spool (Fig. 4 *b*). The distal end of the spool appears to be closely associated with the oocyte cortex, while its proximal end points towards the crater. Two lines of evidence suggest that spool shape and position are associated with the MTOC. Firstly, experiments with incomplete microtubule depolymerization have identified small tubulin positive foci that resemble the spool both in shape and in subcellular localization (Fig. 7c in

Theurkauf et al., 1992). Secondly, immunolocalization of the Dhc peptide, which is expected to move towards the microtubule minus ends, is seen in a spool-like posterior focus in wild type oocytes (Li et al., 1994). To learn more about the relationship between Bic-D::GFP accumulation at the spool and the MTOC, we tested whether the spool is resistant to a short and mild treatment with colchicine which causes the depolymerization of microtubules. We pre-incubated ovaries in colchicine for 30 minutes and observed the effects on the spool under the confocal microscope. Even after prolonged observations of up to two hours, the presence of Bic-D::GFP at the spool was resistant to colchicine exposures even at elevated concentrations (from 50 μM to 5 mM) and under conditions which cause depolymerization of microtubules as seen by the release of Tau::GFP (Micklem et al., 1997) from the microtubules into a diffuse cytoplasmic pattern (data not shown). However, flies fed colchicine for 24 hours have drifting germinal vesicles, and neither spool nor crater (data not shown). Therefore the spool is resistant to short treatment with colchicine but sensitive to prolonged treatment.

4.1.6. Changes in oocyte nucleus positioning, orientation and shape mark the beginning of secondary axis specification during stage 6-7.

Using Bic-D::GFP, we observed evidence of secondary axis formation in the vitellarium that indicates that the process is initiated as early as stage 6. As described above, the spool is

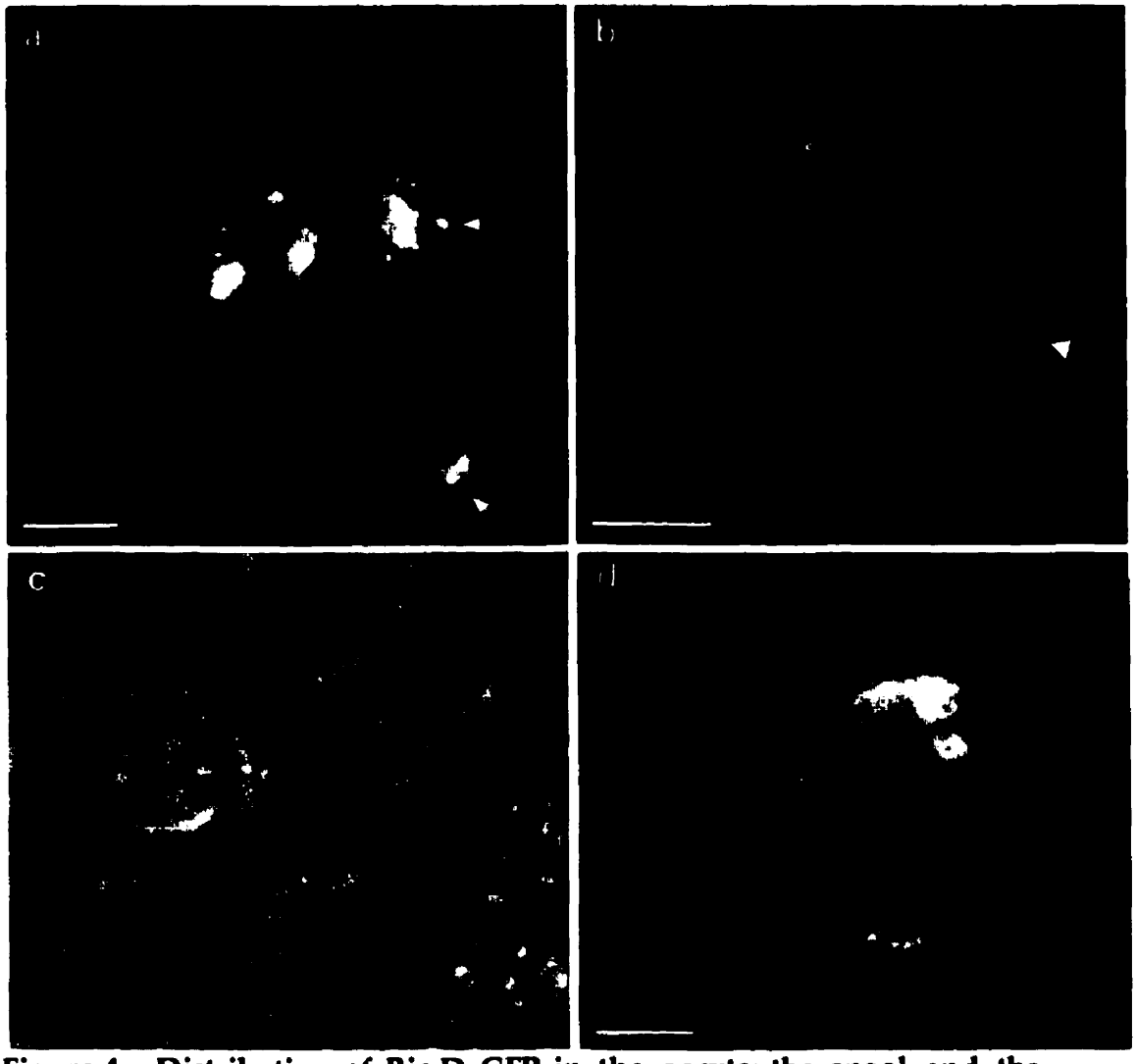

Figure 4. Distribution of Bic-D::GFP in the oocyte; the spool and the crater

(*A*) Dynamic changes in oocyte polarity in late germarial stages. Initially the Bic-D::GFP focus is observed at the anterior (black arrow). This focus is found in a lateral position in an older oocyte (horizontal arrow) and at posterior of the oldest egg chamber in the image (upward arrow). (*B*) The spool (arrow) abuts the posterior polar follicle cells. The spool has not been observed to reach into the crater. (*C*) Light microscopy of Oregon-R egg chambers showing the crater (arrow) in a stage 6 egg chamber. (*D*) Bic-D::GFP focus (potentially the remnants of the spool) at the time of re-localization and re-orientation of the germinal vesicle.

localized posteriorly during stage 5. The late stage 9accumulation of Bic-D::GFP is similar to the pattern of a Nod:βgal chimeric reporter protein (Clark et al., 1997), indicating that Bic-D::GFP may accumulate at the minus ends of microtubules. Concomitantly with this re-localization and re-orientation of the

Bic-D::GFP focus, we also observe a change in shape and orientation of the oocyte nucleus.

In an attempt to learn more about the dynamics of this re-orientation we tried to visualize intermediate stages of this process. Even though we have not been able to visualize the movement of the spool in real time from its posterior localization to its dorso-anterior place, we found individual egg chambers that had Bic-D::GFP foci both on the antero-dorsal and in some intermediate position between the posterior and the antero-dorsal side. In these cases we also observed, aligned with the second Bic-D focus, an additional depression in the shape of the stage 6 oocyte nucleus which gives it a distorted appearance (data not shown). This observation would indicate that the transition from the posterior orientation of the oocyte nucleus to the dorso-anterior one is a discontinuous one. On the other hand, we observed a small proportion of stage 6-7 oocytes in which the oocyte nucleus contained a single crater facing in different intermediate directions between posterior and dorso-anterior. In such cases a Bic-D::GFP focus was also evident between the crater and the oocyte cortex. These apparently intermediate stages may indicate that during this re-orientation the oocyte nucleus rotates from facing posteriorly to facing towards dorso-anterior and that at the same time the associated spool migrates around the oocyte nucleus from its posterior to its dorso-anterior localization. In these cases it appears that the distal end of the spool migrates along the cortex while the spool apex always points towards the crater giving the appearance that

the germinal vesicle and its crater rotate as if pulled by the migrating spool (Fig. 4 *d*).

Consistent with the variability in Bic-D::GFP distribution during this transition, live analysis of the process of oocyte nucleus relocalization showed that this process is also variable at the level of DIC microscopy. While in some specimens the oocyte nucleus seemed to migrate through the centre of the oocyte, it moved along the cortex in other specimens and the nucleus would sometimes even retain its shape in the process.

4.1.7. Requirement for functional Dynein heavy chain for the localization of Bic-D::GFP and the germinal vesicle

Because of the possibility that Bic-D may interact with a negative-end directed microtubule motor, we were interested to find out whether *Dynein heavy chain* (*Dhc*) is required for the subcellular localization of Bic-D::GFP. *Dhc* mutants are homozygous lethal, but there exist several viable heteroallelic combinations which are female sterile. Ovaries from the $Dhc^{6\text{-}6}/Dhc^{6\text{-}12}$ heteroallelic combination display a range of phenotypes. First, we observed egg chambers that do not make an oocyte and show a 16 nurse cell phenotype. In these egg chambers Bic-D::GFP still accumulates in a single cell into a strong focus, but this focus remains at the anterior of the nucleus into the vitellarial stages (pseudo stage 5; Figure 5 *a*). This was particularly interesting since immunolocalization of Dhc in $Bic\text{-}D^{R26}$ ovaries also shows that Dhc localizes to a focus anterior of the pseudo germinal vesicle (Li et al., 1994). This means that there is a mutual requirement for *Bic-D* and *Dhc* for the

localization of the other's protein product to the posterior of the oocyte. This mutual requirement may indicate that the two proteins closely interact with each other or that they are involved in a feedback loop. Furthermore, because Dhc is a component of the minus-end directed microtubule motor dynein, this observation strengthens the view that a negative-end directed microtubule motor is involved in *Bic-D* dependent localization processes.

Second, in less severely affected Dhc^{6-6}/Dhc^{6-12} egg chambers, oocytes are made and egg chambers develop beyond stage 6. We found in our confocal observations that in these mutants the oocyte nuclei frequently become misplaced at the time when they should appear at the antero-dorsal cortex (stage 7 and later; Figs. 5 *b, c*). When these egg chambers have progressed beyond stage 6, the spool-like Bic-D::GFP staining and the crater are usually absent, and the germinal vesicle is localized centrally in the oocyte (Fig. 5 *b*). *Dhc* therefore plays an essential role in building or stabilizing the spool-like Bic-D::GFP staining and the crater, and in localizing the oocyte nucleus to the dorso-anterior cortex. We also observe hyper-accumulation of Bic-D::GFP in the oocyte in four areas at the oocyte-nurse-cell interface and along the entire anterior cortex of the oocyte, suggesting that Bic-D gets transported to the oocyte ring canals but accumulates at the place where it enters the oocyte from the nurse cells. Figure 5 *b* shows a single plane optical section through two such foci, and Fig. 5 *c* shows Bic-D::GFP lining the entire length of the anterior cortex. This different requirement for *Dhc* suggests that intercellular

transport to the oocyte and intra-oocyte transport are different processes and that the latter requires *Dhc*.

4.1.8. Genetic interaction between *Bic-D* and *Dhc*

Because of the role that *Bic-D* and *Dhc* play in each other's localization within the oocyte, we wanted to see whether they genetically interact. We used the highly penetrant dorsal appendage fusion phenotype in the hemizygous *Bic-D*$^{71.34}$ allele as an assay. We found that both heterozygous *Dhc* alleles tested could radically alter the distribution of the dorsal appendage phenotypes (see Table 1). There is a shift in the distribution of dorsal appendage phenotype from the fused category to the intermediate and normal categories. *Dhc* mutants therefore act as enhancers of this *Bic-D* phenotype.

Table 1 Genetic interaction between Bic-D and Dhc - effect on dorsal appendage phenotype

dorsal appendages	normal		interme-diate		fused		short		none	
	n	%	n	%	n	%	n	%	n	%
Bic-D$^{71.34}$/*Bic-D*^{r5}; +/TM3*Ser*	10	3	59	16	289	79	6	2	2	1
Bic-D$^{71.34}$/ *Bic-D*^{r5}; +/*Dhc*$^{6-12}$	296	46	196	31	108	17	28	4	14	2
Bic-D$^{71.34}$/ *Bic-D*^{r5}; +/TM3*Ser*	13	17	19	25	22	29	2	3	19	25
Bic-D$^{71.34}$/ *Bic-D*^{r5}; +/ *Dhc*^{66}	91	42	69	32	40	18	12	6	4	2

4.1.9. Mutations in *spn-E* disrupt Bic-D::GFP localization in the nurse cells and oocytes.

spn-E mutations have a ventralization phenotype similar to *Bic-D*^{mom} flies (Gillespie and Berg, 1995; Swan and Suter, 1996) and cause the *bicoid* (*bcd*) mRNA to be retained in a punctate,

rather than a diffuse, pattern in the nurse cells and at the anterior of the oocyte. Spn-E has homology to DE-H box RNA-dedendent helicases and it seems to be involved in some aspect of RNA localization (Gillespie and Berg, 1995). Even though *spn-E* does not affect *Bic-D* mRNA localization (Gillespie and Berg, 1995), it affects the distribution of Bic-D::GFP in much the same way that it affects the distribution of *bcd* mRNA. Beginning approximately in stage 6, Bic-D::GFP signal accumulates in aggregates in *spn-E* nurse cells (Fig. 5 *d*). In these mutants Bic-D::GFP aggregates are often seen around the nurse-cell oocyte interface (ring canals) and along the anterior cortex of the oocyte (Fig. 5 *d*). Immunoelectron-microscopic analysis of *spn-E* mutants indicates that they contain enlarged sponge bodies and that Bic-D does indeed accumulate in these enlarged RNA containing particles (M. Wilsch-Brauninger, pers. comm., 1997). *spn-E* is therefore required for the normal translocation of Bic-D::GFP to the oocyte and for its localization within the oocyte.

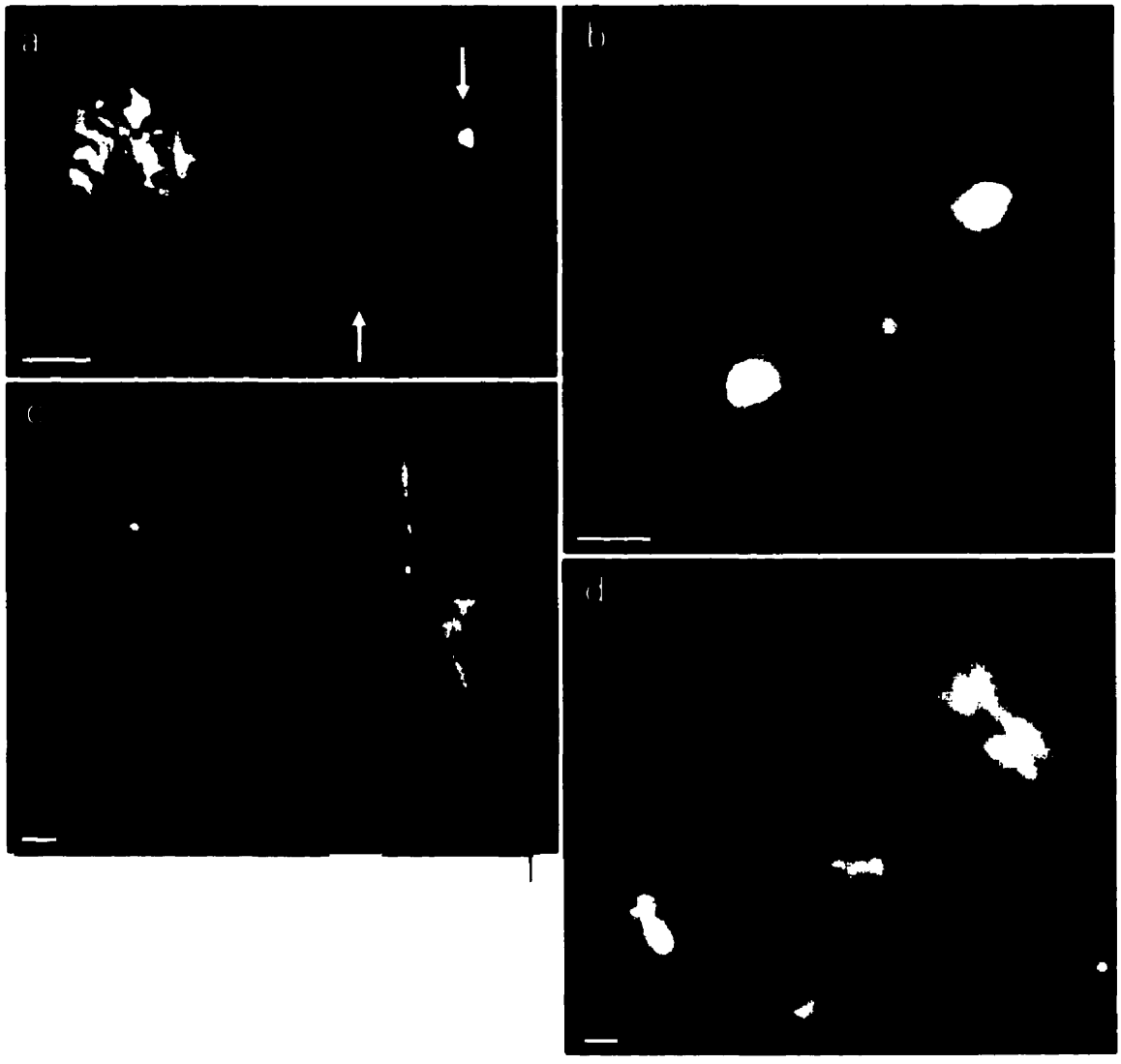

Figure 5. Bic-D::GFP in *Dynein heavy chain* and *spn-E* mutants

(A-C): Dhc^{6-6}/Dhc^{6-12} have ovarioles consistently showing one of the following phenotypes: (*A*) the Bic-D::GFP focus is localized at the anterior of the oocyte nucleus at a stage where it should be posterior (arrows). (*B*) Ectopic accumulation of the Bic-D::GFP signal in the vicinity of what appears to be the ring canals. Notice that the axis of the crater is in line with the additional strong Bic-D::GFP fluorescence despite the germinal vesicle mislocalization. In egg chambers that develop to this stage the oocyte nucleus is not associated with the cortex but is found in the centre of the oocyte. (C) In this hypomorphic heteroallelic combination of *Dhc* mutants, Bic-D::GFP consistently accumulates at the anterior end of the oocyte, and the oocyte nucleus is frequently found in the centre of the late stage oocytes. In addition, usually neither spool nor crater are visible, but we often observe the perinuclear nurse cell staining. (D) *spn-E* mutation causes abnormal aggregation of Bic-D::GFP in the nurse cells and anterior localization in the oocyte. Scale bars represent 10 μm.

4.1.10. Other genes influencing Bic-D::GFP localization

4.1.10.1. maelstrom

Mutations in *maelstrom (mael)* disrupt the normal asymmetric distribution of markers along the antero-posterior axis and the migration of the oocyte nucleus (Clegg et al., 1997). Like *grk* mutants (Gonzalez-Reyes et al., 1995) *mael* mutants also fail to induce posterior follicle cell fate. Thus the target cells retain the default anterior follicle cell identity, and like *grk*, the stage 7-8 microtubule rearrangement fails to occur normally, and the MTOC is found at the center of the oocyte. *mael* mutants ectopically localize several mRNAs in early oocytes. Also, the germinal vesicle often fails to migrate, and consequently dorsal appendages are either fused or lost (Clegg et al., 1997), and does not feature a crater (data not shown).

In wild-type egg chambers, Bic-D::GFP appears to localize at the minus-ends of microtubules. It is known that *mael* is required for microtubule organization. In *mael* mutants the minus ends are generally found in the center of the oocyte. Therefore, if Bic-D indeed really accumulates to the minus-ends, it should accumulate in the center of *mael* mutant egg chambers.
Our confocal observations of Bic-D::GFP confirm that Bic-D accumulates in the center of *mael* mutant oocytes (data not shown), and thus at the microtubule minus-ends.

4.1.10.2. stonewall

stonewall (stwl) has similarity to transcription factors and the protein it encodes is nuclear. It has a 16 mutant nurse-cell phenotype, but unlike Bic-D it is not fully penetrant (only 50%). However, the oocytes that do form undergo some degree of endoreplication, are thus are not fully differentiated (Clark and McKearin, 1996). In the germarium, the distribution pattern of

Bic-D::GFP did not differ from the wild-type. We have also found that these oocytes do have a spool and crater of normal appearance. None of the *stwl* pseudo-egg chambers appeared to progress beyond stage 2.

4.2. A potent enhancer of *Bic-D^{PA66}*

4.2.1. A suppressor screen recovers a potent enhancer locus of *Bic-D^{PA66}*; genetic mapping of the locus

A P-element screen of over 10 000 chromosomes yielded a single 2nd chromosome dominant suppressor of *Bic-D^{PA66}*, SUPB. Further tests made one year after the suppressor was isolated revealed that the original insert had lost all suppressing properties - but we generated a number of excision lines which showed a dramatic and complete dominant enhancement of *Bic-D^{PA66}* heterozygotes. We will describe the steps taken to map this suppressor and some interesting genetic interactions with other loci.

Recombination mapping was therefore performed between Tft (2-53.6) and the P-element insert, carrying the w^+ marker. Scoring 294 flies, the insert was found to be 41 cM from Tft, while the Tft marker is only 0.7 cM from Bic-D. In light of the unusually strong enhancement, these data excludes the possibility that the P-element insert disrupts Bic-D itself. These data narrowed the search to the cytological intervals 24-26 (corresponding to 2-12.6), or 56-58 (corresponding to 2-94.6). We then proceeded to more precisely map the insert, as well as one of the lethal excision lines we generated, using a non-complementation test over a collection of deficiencies covering a large proportion (approximately 80%) of the entire 2nd chromosome. We thus identified a deficiency in region 58 {Df(2R)X58-5} which failed to complement the original insert for

female fertility. Using a set of deficiencies with different breakpoints in this interval, we further refined the mapping to 58E5-F5 (Table 2).

The SUPB/ Df(2R)X58-5 flies laid eggs with an interesting phenotype (Figure 6). They laid a variety of abnormal eggs ranging from half-sized round eggs with small, horn-like dorsal appendages to normal size eggs with missing or weak chorion and reduced dorsal appendages. Control flies +/Df(2R)X58-5 and Df(2R)X58-5/SM5 laid normal eggs. Because the mutant phenotype resembles that of fs(2)Wi42, a female sterile gene which is uncovered by Df(2R)X58-5, we tested whether this mutation was allelic to SUPB, and if it dominantly suppressed *Bic-D*^{PA66}. It did not suppress.

While verifying that there were no other elements on the chromosome which could account for the interaction between *Bic-D* and its suppressor, we found a pattern of second site non-complementation between Df(2R)X58-5 and Df(2L)TW119 (the deficiency which uncovers *Bic-D*), and between Df(2R)X58-5 and *Bic-D*^{null}. Trans-heterozygotes displayed the small rounded egg phenotype described above (Figure 6), female sterility, uncoordinated leg movements, wings held up (like a butterfly's folded wings), as well as rough eyes with disordered arrays of ommatidia.

Table 2 Deficiency mapping of the Bic-D interactor (BI-58EF). The shaded area represents region where the interactor is found.

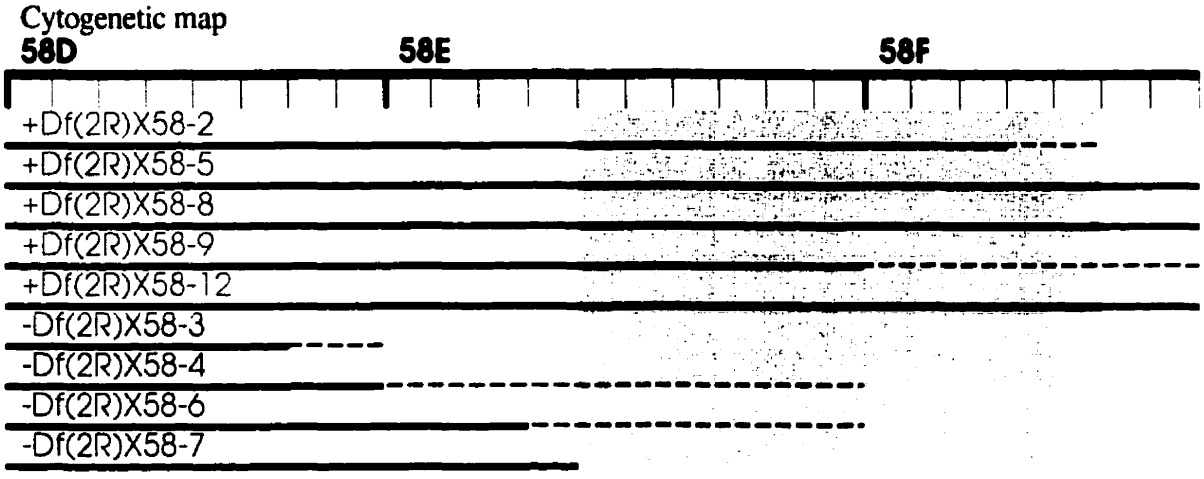

Figure 6: Rounded egg pheno-type with small dorsal appendages

This phenotype is observed with the following trans-heterozygous combinations: Df(2R)X58-5/SUPB, Df(2R)X58-5/Df(2L)TW119, Df(2R)X58-5/*Bic-D*^{PA66}, Df(2R)X58-5/*Bic-D*^{null}, and Df(2R)X58-5/SUPB excision lines. This egg is from Df(2R)X58-5/SUPB flies.

4.2.2. New alleles of SUPB generated through imprecise excision of the P-element show novel phenotypes

As SUPB itself is homozygous viable and has no discernable phenotype, we have attempted to generated stronger alleles using imprecise excision of the P-element. Out of the 8 excision lines created, 1 was both male and female fertile, 3 were male sterile but female fertile, and the remaining 4 were homozygous lethal. This high frequency (7/8) of phenotypically detectable imprecise excision suggests that the P-element is in a sensitive genomic segment, perhaps even in a coding region.

The male sterile flies were investigated with simple testis squashes under Nomarski optics. We have found that in all three male sterile lines, homozygous males produce very few

sperm bundles. The sperm heads are still together, but the flagella are short, rough textured, irregular, and kink easily (not shown).

All excision lines were tested for dominant enhancement for *Bic-D*^{PA66}. We have found that trans-heterozygotes for any of the lethal lines and *Bic-D*^{PA66}, just like a *Bic-D*^{PA66} homozygote, showed a completely penetrant 16 nurse-cell phenotype. Interestingly, a nuclear migration phenotype was observed when the excision line SUPBX44 was trans-heterozygous with Df(2R)X58-5 (Figure 7). This line also exhibits a dominant tiny egg phenotype with palmate dorsal appendages which is seen in 23% of eggs laid (Figure 8).

Table 3 Properties of SupB insertion and excision lines

	SupB	*SupBX 45*	*ms(2) SupBX 40*	*ms(2) SupBX 42*	*ms(2) SupBX 46*	*l(2) SupBX 41*	*l(2) SupBX 43*	*l(2) SupBX 48*	*l(2) SupBX 44*
homozygous phenotype	+	+	male sterile	male sterile	male sterile	lethal	lethal	lethal	lethal
heterozygous phenotype	+	+	+	+	+	+	+	+	145/625 tiny eggs
insert or excision / Df(2R)X58-5	rounded eggs*	+	lethal	rounded eggs*	rounded eggs*	rounded eggs*	rounded eggs*	rounded eggs*	rounded eggs*
insert or excision / *SupB*	+	+	+	+	+	lethal	lethal	lethal	lethal
insert or excision / *Bic-D*^{PA66}	+	+	+	+	+	16 nurse cells	16 nurse cells	16 nurse cells	16 nurse cells

\+ viable and fertile phenotypes.

* The flies with rounded eggs also had female sterility, uncoordinated leg movements, wings held up (like a butterfly's folded wings), as well as rough eyes with disordered arrays of ommatidia.

A

B

Figure 7: Migration of oocyte nucleus is affected in SUPB119/Df(2R)X58-5 ovaries.

(A) Stage 8 ORE-R control egg chamber showing the germinal vesicle at the antero-dorsal corner. (B) Stage 8 SUPB119/Df(2R)X58-5 egg chamber showing retention of the germinal vesicle at the posterior of the oocyte. The germinal vesicle features a well defined crater facing the posterior of the oocyte, also an abnormal finding for this developmental stage

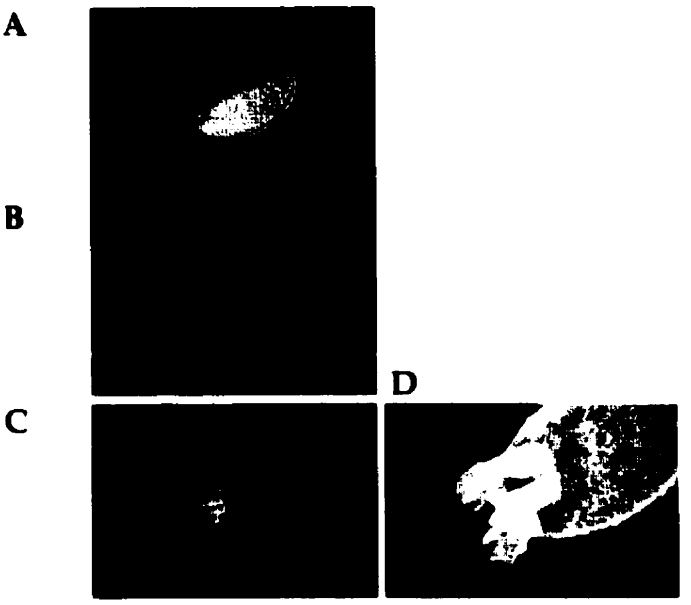

Figure 8: l(2)SUPBX44 flies have a dominant small egg phenotype with 23% penetrance.

(A) ORE-R control shows normal size of egg. (B) lateral view and (C) dorsal view of tiny eggs from l(2)SUPBX44 heterozygous flies.(D) close up of dorsal appendages in (C)

5. Discussion

Now that we have reviewed the literature and described our own experimental results, we will try to incorporate these results into the greater body of knowledge. We will see how these observations help to understand some of the finer aspects of oocyte specification and transport. We will discuss the implications of a requirement for *orb*, *egl* and *Dhc* function in Bic-D::GFP localization. Although they are important, the SUPB genetic studies will be discussed only briefly.

5.1. Bic-D and oocyte specification

Bic-D is essential for oocyte specification. The establishment of oocyte fate is a multistep process which seems to involve microtubule dependent transport of factors from the nurse cells to the future oocyte (Suter and Steward, 1991; Koch and Spitzer, 1983; Theurkauf et al., 1993). Accumulation of oocyte-specific factors can be detected in germarial region 2a, soon after the 16-cell cystocyte cluster is formed. Because of the requirement for *Bic-D* in the transport of oocyte-specific factors, the Bic-D::GFP distribution pattern in region 1 and region 2 of the germarium is relevant to the role of *Bic-D* in the process of oocyte specification. There is little evidence for transport of oocyte factors in germarial region 1 but in region 2a, around the time when the polar microtubule array connecting the cystocytes becomes visible (Theurkauf et al., 1993), the centrioles and mRNAs become localized to the oocyte (Mahowald and Strassheim, 1970, Suter and Steward, 1991). Bic-D::GFP fluorescence at the centre of the cystocyte cluster in this germarial region resembles the fusome and microtubule arrays during this stage, and the Bic-D::GFP foci in each cystocyte (Figs. 3 *a*, 3 *a'*) seem to lie between these arrays and the cystocyte nuclei in most specimens. This distribution pattern suggests that Bic-D may temporarily associate with the fusome and the microtubules and it is consistent with a

function for *Bic-D* in nurse cell to oocyte transport and in providing a link between the fusome or microtubule array and the cystocyte nuclei.

Like *Bic-D*, *egl* is required for the accumulation of mRNAs in the oocyte and for the differentiation of an oocyte (Suter and Steward, 1991; Schüpbach and Wieschaus, 1991; Mach and Lehmann, 1997). EM studies provided further evidence that polarized flow or transport between cystocytes of mitochondria is defective in *egl* mutants (Carpenter, 1994). *egl* is essential for the regular organization of Bic-D::GFP distribution and for the transition in Bic-D::GFP accumulation from a single focus per cell to a central accumulation in the cyst, because in *egl* mutants the accumulation of Bic-D::GFP into foci in pseudo region 2 is irregular, and Bic-D::GFP does not concentrate to the center of the cystocyte cluster (Fig. 3 *b*). Instead, the dotted distribution of Bic-D::GFP persists as far as pseudo-region 3. It is tempting to causally relate the lack of intercystocyte transport and the failure of Bic-D foci to accumulate in the centre of the cystocyte cluster.

5.2. Nurse cell transport vs. intra-oocyte transport

Intra-oocyte transport is likely to be qualitatively different from nurse-cell to oocyte transport (Theurkauf and Hazelrigg, 1998). At the nurse-cell oocyte boundary, a subset of macromolecules, perhaps those containing critical RNAs, must both be transported within the nurse cells, and then within the oocyte, they must respond to the resulting change in environment. These macromolecules, which are capable of being transported by a variety of pathways within the nurse cells, now must rely on a single pathway. This pathway includes*Dhc64C*, *Bic-D* and microtubules. Bic-D and microtubules are also required to slow down the passage of macromolecules to the ring canals, as colchicine treatment and *Bic-D*^{mom} ovaries show greatly increased flow through the

ring canals (A. Swan, pers. comm., 1996). In the oocyte environment, the transported macromolecules may arrest temporarily prior to continuing intra-oocyte transport, perhaps to switch transport system, perhaps to associate with different molecules.

5.3. RNA binding proteins play essential roles in the distribution of Bic-D protein

Homology analysis of the Egl sequence predicts that it possesses an exonuclease domain, and thus may play a role in RNA processing (Moser et al., 1997). Interestingly, another potential RNA binding protein, Orb, seems to be involved in the distribution of Bic-D in the same germarial stage. In *orb* mutants Bic-D::GFP also accumulates in a dot-like pattern, but the Bic-D::GFP foci are qualitatively and quantitatively different from those seen in wild type or *egl* mutants (Fig. 3 *c, d*). They are of variable size, have discrete edges, and there is a very low level of fluorescence outside the foci. Given that the Orb protein is likely involved in RNA processing, how could it affect Bic-D protein distribution? A potential explanation that would accommodate previous results by Lantz et al. (1994) is that Bic-D and Orb proteins could be involved in the localization of the same factors. The absence of *orb* activity in the *orb* mutant may then result in aggregation of Bic-D::GFP in the place in the cell where *orb* RNA binding is required for Bic-D to perform its function.

Later in oogenesis, mutations in another gene that encodes a polypeptide with homology to a family of RNA-binding proteins, *spn-E*, also lead to the appearance of Bic-D::GFP aggregation (Fig. 5d). The hyperaggregation of Bic-D::GFP in *spn-E* mutants (Fig. 5d) closely resembles the pattern of *bicoid* (*bcd*) RNA mislocalization in such

ovaries reported by Gillespie and Berg (1995). In wild type ovaries *bcd* RNA accumulates in subcellular compartments that have been described at the electron microscopic level as sponge bodies (Wilsch-Brauninger et al., 1997). Because Exu protein, an RNA binding protein which is essential for the localization of *bcd* mRNA is also present in sponge bodies, it appears that these particles may be involved in *bcd* mRNA transport. It is therefore possible that Bic-D accumulates in similar or even the same particles as *bcd* mRNA and Exu protein and that these sponge bodies are enlarged in *spn-E* mutants. A similar interpretation was reached independently by other researchers: immunoelectron microscopy studies showed that Bic-D hyperaggregates into enlarged sponge bodies in *spn-E* mutants (M. Wilsch-Brauninger pers. comm., 1997). The fact that Bic-D has been found to localize to subcellular structures which have also been found to contain potential RNA-binding proteins and localized mRNAs, and the fact that three genes that appear to encode RNA-interacting proteins are all essential for the normal subcellular distribution of the Bic-D protein provide strong cytological and genetic evidence supporting the suggested direct role of Bic-D in localizing mRNAs.

5.4. Role of nucleus orientation and positioning in creating polarity

Our data indicates that the establishment of antero-posterior polarity in the oocyte is gradual and posterior localization of Bic-D::GFP starts to become apparent at the end of germarial region 2b (Fig. 4*a*). The single focus of Bic-D::GFP accumulation in what appears to be the presumptive oocyte is first seen anteriorly of its nucleus, nesting in a crater-like depression in the nuclear envelope. Both the crater and the

associated Bic-D::GFP focus can later be seen in lateral positions and, at the end of germarial development, they both are positioned posterior to the nucleus in egg chamber stage 1 (germarial region 3). This gives the impression that they migrate in synchrony around the oocyte nucleus (Fig. 4 *a*) or that the Bic-D::GFP focus migrates around the oocyte nucleus which turns or rotates in synchrony with the Bic-D::GFP migration. This apparent intracellular movement is also one of the cellular processes that requires the negative-end directed microtubule motor *Dhc*, because in the *Dhc* mutant, Bic-D::GFP remains focused at the anterior of the oocyte.

During stages 6-7 shape and orientation of the oocyte nucleus change from facing towards posterior to facing in a dorso-anterior direction. At the same time the perinuclear Bic-D::GFP focus disappears from the posterior end and appears in a new position at the antero-dorsal cortex (Figs. 4 b, c, d, e). The variability we have observed in the transition from one state to the other as well as the fact that we have not yet been able to continuously observe this transition in a single oocyte using the confocal microscope has prevented us from identifying the mechanism of the re-orientation. Nevertheless, the results from the Bic-D::GFP distribution combined with the ones from the DIC analysis of germinal vesicle migration have some important implications for the examination of the initiation of dorsal ventral polarity. Because this migration occurs while the oocyte is still small (stage 6-7), the nucleus becomes anchored in the dorso-anterior corner before the oocyte starts its rapid growth in stage 8-9 (Figs. 4 c, d). Timelapse video microscopy demonstrated that the migration distance of the oocyte nucleus to the dorso-anterior corner is surprisingly short,

equivalent to the width of the oocyte nucleus. The re-orientation of the oocyte nucleus in stages 6-7 and the concomitant repositioning of the Bic-D::GFP focus may play an important role in establishing dorso-ventral polarity in the egg chamber. This process is reminiscent of the process of translocation of the Bic-D::GFP focus from its anterior to its posterior position in the germarial oocyte which may be involved in defining antero-posterior polarity. In both cases, a re-orientation of the oocyte nucleus and the establishment of a new asymmetry around the nucleus appears to anticipate the establishment of the new egg chamber polarity. We know that *Bic-D* is required for both these processes; *Bic-D*^{null} flies also fail to localize the oocyte to the posterior of the cluster, while *Bic-D*^{mom} flies fail to properly establish dorso-ventral polarity and they fail to retain the oocyte nucleus at the antero-dorsal corner (Ran et al., 1994; Swan and Suter, 1996). One of the important questions these results pose is what the function of these subcellular structures may be, and how the accumulation of Bic-D::GFP in this compartment relates to this function. Their position and appearance during development could be consistent with a function in mRNA localization and in oocyte nucleus positioning, but they are also consistent with tethering first at the posterior end and, after the transition in stage 6-7, at the dorso-anterior end of the oocyte cortex. Interestingly, several studies on positioning and migration of fungal nuclei in *Neurospora crassa*, *Aspergillus nidulans* and *S. cerevisiæ* have implicated the microtubule motor dynein as well as components of the dynactin complex in similar nuclear migration processes (Plamann et al., 1994; Tinsley et al., 1996; Beckwith et al., 1995). It therefore seems that *Drosophila* uses an evolutionarily conserved

cellular mechanism which may couple mRNA transport to nuclear localization in order to initiate the formation of polarity in the egg chamber.

This suggests the question of how such a coupling of mRNA transport and a microtubule-dependent process such as germinal vesicle migration would occur. Although we have no direct evidence of such a process in Drosophila, we have found an interesting example that demonstrates that coupling of microtubule stability and mRNA localization does exist in mammalian cells. A microtubule-binding protein, MAP1a, has an effect on microtubule dynamics (Pedrotti et al., 1996) and interacts with RNAs. MAP1a was identified as a component of an RNA complex (DeFranco et al., 1997). This interaction could be important in the regulation of translation. Indeed, MAP1a light chain 3 has been reported to bind a specific RNA - that of fibronectin - and enhance its translation (Zhou et al, 1997). The finding of MAP1a in an RNA complex suggests that the complex is associated with microtubules. MAP1a's role in stabilizing microtubules does not require a direct physical contact between the two molecules; indeed, in neuronal cells, mRNA (Bassell et al., 1994) and MAP1a (Shiomura and Hirokawa, 1987) are found not directly on microtubules, but between them.

We will now discuss the possible functions for the spool and the crater within our own experimental context and that of other findings.

5.4.1. The spool

The spool, where Bic-D::GFP and Dhc64C (Li et al., 1994) accumulate, may be made of highly stable microtubule bundles; indeed, Theurkauf's (1993) partial microtubule destabilization experiments

show this is likely. It may represent the site where the coupling of mRNA transport and microtubule stabilization occurs, or a transient accumulation of Bic-D::GFP at the end of the transport process.

5.4.2. Mechanisms of nuclear migration in the slime mold

Like the Drosophila germinal vesicle, the nucleus of the slime mold *Dictyostelium discoideum* must also undergo migration. Neujahr and colleagues (1998) have attempted to elucidate the relationship between myosin, microtubules, centrioles and nuclear migration. They also have observed a "crater" in the migrating nucleus, and they refer this nuclear shape as the "heart-shaped body." This invagination of the nuclear surface is produced by the centrosome within it. The movement of the nucleus occurs in the direction opposite the invagination. A few guiding microtubules begin at the centrosome, continue over the nucleus, and are presumably anchored in the cortex, while the majority of microtubules lagging away from the nuclear depression like a comet's tail. These "comet" microtubules are dragged and bent as the centrosome changes direction. Nuclear motion is saltatory, because the guiding microtubules are subject of polymerization and depolymerization dynamics, causing jerky changes in nuclear direction as the nucleus switches "tracks." This is similar to our own time-lapse video microscopic observation of germinal vesicle migration in Drosophila, where the orientation of the crater was a poor predictor of migration directionality, and where migration was clearly saltatory in nature. However, such an array of microtubules, and guiding microtubules, have never been observed. Injection of rhodamine-conjugated tubulin into egg chambers may allow us to visualize microtubule dynamics in vivo, providing microtubule

dynamics could allow incorporation of the label to all relevant microtubule subpopulations, and require videotaping equipment capable of capturing such a fluorescent signal.

Also in the slime mold model of nuclear migration, based on experiments in myosin null cells and measurements of centrosomal speed, it is believed minus-end directed motors are anchored to the plasma membrane or bound to the cortical network of actin filaments which is subtended by spectrin at the cortex. Cytochalasin D inhibits centrosomal motility in this system, suggesting the involvement of the actin network in the anchorage of minus-end directed motors.

5.5. SUPB, enhancer of *Bic-D*^{PA66}; further investigations

As many as seven quaking-related genes have been cloned from the 58EF region alone. Quaking-related genes contain maxi-KH domains which are believed to link RNA metabolism to cell signalling (Fyrberg et al., 1998). These quaking-related genes are also related to the gld-1 gene, required in *C. elegans* for oocyte differentiation and promotion of meiosis (Kadyk and Kimble, 1998). In mice, mutations in the *quaking* gene result in defects in the incorporation of myelin basic protein and proteolipids into myelin, and the males are sterile, due to a defect in spermiogenesis (Bennett et al., 1971). The quaking-related genes in 58EF are good candidate genes for being the site of the SUPB P-element insertion, and as no Drosophila mutants are known yet, they are open for scientific investigation.

Our preliminary results show that the lethal excision lines of SUPB, which are strong enhancers of *Bic-D*^{PA66}, should be further studied. For instance, it would be interesting to find out whether Bic-

D::GFP localization is disrupted in a heterozygous lethal SUPB excision line, especially *l(2)SUPBX44*, which displays a dominant small egg phenotype and a nuclear migration phenotype in the presence of the Df(2R)X58-5 chromosome. Germline clones of these excision lines may also reveal a role in oogenesis for the affected gene(s).

At the molecular level, expression patterns may show that a male specific transcript is affected in the male sterile lines. Ovary RNA in situ hybridization may show whether the transcript localizes to the oocyte, and, further into the future, antibodies may be used to reveal the localization pattern of a protein in the ovary.

5.6. Towards a molecular model for Bic-D function

In observing the distribution pattern of the Bic-D::GFP fluorescent signal, we have identified two novel subcellular domains, the spool and the crater. Through our search to identify mutations in other genes involved in oocyte determination or microtubule-dependent transport, which affect the localization pattern of the Bic-D::GFP fusion protein, we have found that proteins with putative roles in mRNA metabolism affect Bic-D::GFP localization, as well as Dynein heavy chain. One way to explain the influence of these proteins on Bic-D localization is that the whole complex may need to be assembled as the messages exit the nucleus. Failing this, as may happen in the *orb* and *egl* mutants, the complex may be stalled in the cytoplasm and fail to associate with the cytoskeleton and/or the transport machinery. The coupling of microtubule stabilization and RNA transport could occur at several levels: 1) increased activity of dynein transporting RNAs may stabilize microtubules, and 2) the dynein transported RNA complex may also include microtubule stabilizing proteins, perhaps involving an interaction with the membrane and then stabilize the microtubules

Furthermore, we have consolidated these data by characterizing a genetic interaction between *Bic-D* and *Dynein heavy chain*. Both our confocal and DIC video microscopic observations have helped us learn more about the dynamics of oocyte nucleus migration.

Our P-element mediated mutagenic screen of 10 000 mutagenized chromosomes in order to identify suppressors of Bic-D has "hit" a gene that appears to be very promising. One, a male-sterile locus, does not interact genetically with Bic-D but may represent an interesting tool to learn more about spermatogenesis. The lethal locus, which is the strong enhancer of Bic-D, has been characterized genetically and phenotypically, does indeed appear to be a very promising area of investigation for the future.

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7. Acknowledgements

First, I thank my supervisor, Beat Suter, for his continued support and scientific guidance. I am equally endebted to Robert Levine and Rebecca Kellum for their valuable input to my graduate committee.

I am grateful as well to past and present lab mates Jian Chen, Alexandru Ecovoiu, Francis Gagnon, Sirine Hijal, Jeff Kwan, Stephane Larochelle, Judit Pandur, Emily Patrick, Thuy Nguyen, Andrew Swan and James Velema for helpful discussions, technical advice and sharing of reagents, but most of all for providing a congenial atmosphere for scientific enquiry, humour and gossip. I also thank, from the Lasko Lab and the Genome Lab, Sheida Bonyadi, Heather Butler, Rashmi Chikarmane, Oona Johnstone, Pascal Lachance, Sylvia Levine and Sylvia Styhler, for their friendship and their help. Thanks to Paul Lasko, Akira Nakamura, Ursula Stochaj for additional guidance, to Robert Lamarche for teaching how to use the confocal laser microscope, and to Marco DiFruscio for believing my suppressor results, and pursuing the project. Thanks to my husband, Lawrence Joseph, for critical reading of the manuscript, and encouraging all my educational endeavours, and guiding me through the process; and to my son, Bertrand, for the motivation.

I also gratefully acknowledge receipt of research material: Trudi Schüpbach, Dennis McKearin and Thomas Hays for fly stocks, Ursula Stochaj for anti-GFP antibodies, and Roger Tsien for the pRSET GFP plasmid DNA. I would also like to thank Judit Pandur for virgin collection and transformation, Chris Chong for recombining the Bic-D::GFP insert to the five *egalitarian* alleles, as well as Cate and Lee for the fly food.

This work was supported by funds to B. S. from the National Science and Engineering Research Council of Canada.