Subcellular localization of Bic-D::GFP is linked to an asymmetric oocyte nucleus

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SUMMARY

Bicaudal-D (Bic-D) is essential for the establishment of oocyte fate and subsequently for polarity formation within the developing Drosophila oocyte. To find out where in the germ cells Bic-D performs its various functions we made transgenic flies expressing a chimeric Bic-D::GFP fusion protein. Once Bic-D::GFP preferentially accumulates in the oocyte, it shows an initial anterior localization in germarial region 2. In the subsequent egg chamber stages 1-6 Bic-D::GFP preferentially accumulates between the oocyte nucleus and the posterior cortex in a focus that is consistently aligned with a crater-like indentation in the oocyte nucleus. After stage 6 Bic-D::GFP fluorescent signal is predominantly found between the oocyte nucleus and the dorso-anterior cortex. During the different phases several genes have been found to be required for the establishment of the new Bic-D::GFP distribution patterns. Dynein heavy chain (Dhc), spindle (spn) genes and maelstrom (mael) are required for the re-localization of the Bic-D::GFP focus from its anterior to its posterior oocyte position. Genes predicted to encode proteins that interact with RNA (egalitarian and orb) are required for the normal subcellular distribution of Bic-D::GFP in the germarium, and another potential RNA binding protein, spn-E, is required for proper transport of Bic-D::GFP from the nurse cells to the oocyte in later oogenesis stages. The results indicate that Bic-D requires the activity of mRNA binding proteins and a negative-end directed microtubule motor to localize to the appropriate cellular domains. Asymmetric subcellular accumulation of Bic-D and the polarization of the oocyte nucleus may reflect the function of this localization machinery in vectorial mRNA localization and in tethering of the oocyte nucleus. The subcellular polarity defined by the Bic-D focus and the nuclear polarity marks some of the first steps in antero-posterior and subsequently in dorso-ventral polarity formation.

Key words: Drosophila melanogaster, Oogenesis, Differentiation, Polarity formation, Microtubule transport, Nuclear asymmetry, Nuclear positioning, Bicaudal-D, GFP
germarial stages have demonstrated that the centrioles from the pro-nurse cells migrate through the ring canals to the oocyte where they accumulate either at the anterior of the oocyte or between the germinal vesicle and the posterior end (Mahowald and Strassheim, 1970).

A microtubule dependent transport or anchoring system is at work repeatedly during oogenesis, functioning in oocyte determination, oocyte growth mediated by nurse cell to oocyte transport, polarity formation along the dorsal-ventral and antero-posterior axis, and localization of the oocyte nucleus (Koch and Spitzer, 1983; Theurkauf et al., 1993). Aside from microtubules, a number of genes have been shown to function in several or all of these processes, and it appears that their products use these microtubules to localize different cargo at different phases. Such requirements have been directly demonstrated for Bic-D, egl and DLis-1 (Mach and Lehmann, 1997; Ran et al., 1994; Suter and Steward, 1991; Swan et al., 1999; Swan and Suter, 1996). In addition, there is strong genetic evidence that Dynemin heavy chain (Dhc) and Glued (Gl) are also essential in these processes, implicating the negative-end directed microtubule motor dynein/dynactin (Swan et al., 1999).

At the time the oocyte nucleus gets localized to the dorso-anterior corner, DLis-1 is localized to the oocyte cortex in a microtubule independent manner (Swan et al., 1999). DLis-1 is essential for the subcellular localization of Dhc and Bic-D, and therefore seems to act upstream of Bic-D and dynein, potentially acting as a cortical anchor for the microtubule transport system. The cellular role of Bic-D in the localization process is not clear but based on its structure it could act as a linker molecule. Bic-D consists largely of coiled-coil domains that are likely to be involved in protein-protein interactions (Stuurman et al., 1999). Even though nuclear lamin interacts with Bic-D in the yeast two hybrid system, the only in vivo complex partner identified thus far for Bic-D is Egl (Mach and Lehmann, 1997).

To better understand the function of Bic-D and the cell biology of the various localization processes we analyzed the subcellular localization of a Bic-D::GFP fusion protein in living oocytes. The results revealed a subcellular compartment in the oocyte that seems to be involved in tethering and re-orienting the germinal vesicle, a process implicated in directing the establishment of polarity. By analyzing the genetic requirement for the establishment and positioning of this compartment and for the proper Bic-D localization patterns, several genes were found to contribute to normal patterning. These genes are good candidates for additional components of the patterning pathway and we discuss the role of these genes with respect to their position in the pathway. In addition, the molecular nature of the products encoded by these genes sheds light on the nature of the localization process.

MATERIALS AND METHODS

Fly stocks and techniques

Drosophila melanogaster OregonR flies were used as a wild-type strain. Flies were raised on standard corn-meal agar medium at room temperature. The following alleles were used for this study: Dhc-5-2, Dhc-6-6 and Dhc-6-12 (Gepner et al., 1996), spn-A1, spn-B1, spn-D2, spn-E1, spn-F1 (Tearle and Nüsslein-Volhard, 1987), eglPV27, eglPV35, eglPV29, eglPV12 (Schüpbach and Wieschaus, 1991), orbF343, orbF305 (Lantz et al., 1994). Observations in a Bic-D8 background were made using w'; F1[w+ Bic-D::GFP (201)], Bic-D8. Observations in a Bic-D8 background used w'; P[w+ Bic-D::GFP (201)] Bic-D8 cn bw flies either homozygously or over Df(2L)T1W119. Colchicine feeding of flies was done under the following conditions: a size 0 paintbrush was dipped once 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-D8 strain was used for recombination with eglPV27 and crossing with spn-E, orbF343 or Dhc alleles, as well as for all confocal observation unless otherwise noted.

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 into 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 ATT AAG ATA AGA TCA 3′. The BamHI fragment from GFP S65T cloned in pRSET (Heim et al., 1994) was inserted in this unique BglII site and a clone with the correct orientation was selected. To obtain the 5′ part of the Bic-D gene, a 3.5 kb genomic Bic-D fragment containing the 5′ sequences of Bic-D and extending to the HindIII site in the first intron (H+ in Fig. 2 by Suter et al., 1989) was cloned into pBSKS+. This 5′ end plasmid was cut with Xhol and Scal (in the vector), partially-filled-in at the Xhol overhang with dTTP, 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 that 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 in the KpnI and NotI sites of a modified pCaSpeR transformation vector. The fusion gene was finally introduced into flies by P-element mediated transformation.

Microscopic observations

For light microscopy, ovaries were dissected and mounted in PBS for immediate observation under a Zeiss Axioplan microscope. For confocal observations (Leica CLSM), ovaries were dissected in PBS and mounted in PBS on 60 mm coverslip. Ovarioles were detached from the ovary and extending slightly beyond the polyadenylation site was cloned into 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 ATT AAG ATA AGA TCA 3′. The BamHI fragment from GFP S65T cloned in pRSET (Heim et al., 1994) was inserted in this unique BglII site and a clone with the correct orientation was selected. To obtain the 5′ part of the Bic-D gene, a 3.5 kb genomic Bic-D fragment containing the 5′ sequences of Bic-D and extending to the HindIII site in the first intron (H+ in Fig. 2 by Suter et al., 1989) was cloned into pBSKS+. This 5′ end plasmid was cut with Xhol and Scal (in the vector), partially-filled-in at the Xhol overhang with dTTP, 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 that 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 in the KpnI and NotI sites of a modified pCaSpeR transformation vector. The fusion gene was finally introduced into flies by P-element mediated transformation.

RESULTS

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. 1B). 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. The minigene also included, in the 3′ flanking sequence, 240 base pairs beyond the most distal polyadenylation site. The S65T...
GFP expression in oogenesis

GFP sequence (Heim et al., 1994) 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 amino acids GDPPAEF immediately to the amino-terminal end of the GFP polypeptide and the amino acids GS at the carboxy-terminal end of the chimeric protein.

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 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 blot analysis (Fig. 1C).

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, suggesting that the discrepancy between the Bic-D localization pattern in fixed tissue and that of Bic-D::GFP in living tissue is not an artefact of, for instance, GFP aggregation (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 the ones produced by a single copy of wild-type Bic-D could 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.

**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. Low levels of fluorescence becomes visible in the female germ line already at the germarium tip, in region 1. In this part of the germarium the germline cells are mitotically active; dividing stem cells give rise to another stem cell and to a differentiating daughter cell called cystoblast. This cell divides further to form the 16 cell cystocyte clusters which will later become the germ line part of the egg chamber.

In the anterior part of germarial region 1, the overall signal intensity is very low and the cytoplasmic Bic-D::GFP signal accumulates preferentially in a structure whose size (approximately 2 μm), spherical shape, and localization pattern in germ line stem cells and cystoblasts appear to correspond to the spherical fusome (spectrosome; small arrows in Fig. 2A,A'). The fusome is a cytoplasmic structure rich in membrane skeletal proteins such as Spectrin, Adducin-like proteins.
(Hts), Bam and Ankyrin (de Cuevas et al., 1996; Lin and Spradling, 1995; Lin et al., 1994; McKearin, 1997; McKearin and Ohlstein, 1995). During the remainder of the mitotic stages in region 1 and in region 2a Bic-D::GFP shows a more uniform cytoplasmic distribution (Fig. 2A-B). Occasionally the fluorescent signal is seen to accumulate in the center of the cluster and in an array of dot-like foci of greater fluorescence that is then often arranged concentrically around this central accumulation (large arrow in Fig. 2A,B). The central cyst accumulation indicates that Bic-D::GFP may be fusome associated during this period. Consistent with this interpretation, a more uniform cytoplasmic distribution of the Bic-D::GFP signal is seen in hts mutants that do not form a proper fusome (data not shown).

This distinct central accumulation with the additional dot-like foci is apparent around the time the cysts move from region 1 to region 2a, and the dot-like foci are only seen in a subset of the clusters that show the central accumulation. 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 crucial transient event in oocyte differentiation because mutations in genes that interact with Bic-D in oocyte differentiation seem to stabilize these foci (see below). Based on their position, these foci could either correspond to the MTOCs or to ring canals. The latter have recently been shown to be the site of the formation of new fusome during early interphase (de Cuevas and Spradling, 1998). Because the Bic-D::GFP foci are disrupted during fixation, in vivo markers for the two organelles need to be developed before we can discriminate between these two possibilities.

**orb and egl are required for Bic-D subcellular localization in the germarium**

The orb and egl genes both play essential roles in oocyte differentiation and they are required for the normal localization of the Bic-D protein in germlar region 2b and 3 (Lantz et al., 1994; Mach and Lehmann, 1997; Suter and Steward, 1991). We therefore revisited the role of these two genes in the localization of Bic-D::GFP during the initial phase of germ line differentiation. The pattern of central Bic-D::GFP fluorescence seen in cystocyte clusters of wild-type region 2a is abolished in eglPV27 mutants. Furthermore, dot-like foci appear in random positions in the cytoplasm and they persist into region 2b (Fig. 2C). This phenotype is strongest in PV27 and PR29 alleles and it contrasts the wild type where the foci are concentrically organized and disappear before the cysts reach region 2b. Another gene that is required for the differentiation of an oocyte is the putative transcription factor stonewall (stwl; Clark and McKearin, 1996; but lack of stwl does not affect the Bic-D::GFP distribution in the germarium, suggesting that this gene may act downstream of Bic-D or in a different pathway (data not shown).

**orbF343** ovaries consist mainly of 8-cell cystocyte clusters (Lantz et al., 1994). In germlar region 1 of these ovaries Bic-
D::GFP distribution is similar to wild type. In pseudo-region 2a, however, it accumulates in variously sized foci with discrete margins and the rest of the cytoplasm shows reduced Bic-D::GFP fluorescence (Fig. 2D,E). The foci do not appear to have a particular orientation with respect to the cell cluster and they fade away at the posterior of these rudimentary ovaries. Unlike the foci observed in *egl*, the ones in *orb* vary in size and are nested in what appears to be a depression of the nuclear membrane (Fig. 2E). Previous antibody stainings did not reveal these structures, suggesting that they are not preserved under the fixation conditions.

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

In early germarial region 2b, the Bic-D::GFP signal is prominently enriched in the anterior of every cell of the cyst. It is not until mid region 2b that we start seeing the signal preferentially accumulate in a single cell. At this point Bic-D::GFP does not fill the entire cytoplasmic compartment of the oocyte but accumulates strongly in a bright focus anterior to the presumptive germinal vesicle (Fig. 3A, arrow). In later germarial stages, late in region 2b and in region 3, we can observe what appears to be the dynamic establishment of antero-posterior polarity within the oocyte. The fluorescent focus, which has the appearance of a globe, is nested in a depression in the nuclear surface. Once the focus moves away from the anterior position, it can be seen either lateral or posterior to the germinal vesicle. Around the time the cystocyte cluster enters germarial region 3 and becomes a stage 1 egg chamber, the focus is consistently seen in the anterior position (arrowhead in Fig. 3A), indicating that it has moved around the nucleus to assume the posterior location (Fig. 3B) in which it stays until stage 6. This process constitutes the earliest sign of posterior polarization within the oocyte and the accumulation of Bic-D::GFP seems to coincide with the position of the microtubule minus end (Theurkauf et al., 1992).

*maelstrom (mael)* is essential for the posterior localization of the microtubule minus ends between oocyte nucleus and posterior end (Clegg et al., 1997). In *mael* mutants the microtubule minus ends are found in the center of the oocyte, anterior to the oocyte nucleus, and it has been argued that *mael* plays a role in polarizing the oocyte. If Bic-D::GFP indeed localizes to microtubule minus ends, we would expect Bic-D::GFP to also accumulate in the center of the oocyte during mid oogenesis. Indeed, in *mael* mutant ovaries we consistently find the Bic-D::GFP signal in a strong focus in the oocyte center (Fig. 3C). Similarly, Bic-D::GFP localization anterior to the germinal vesicle is seen in the *spindle (spn)* mutants *spn*-A and *spn*-B (Fig. 3D,E). *mael*, *spn*-A and *spn*-B are therefore required for the posterior relocalization of Bic-D::GFP.

Because *Bic-D* interacts with the negative-end directed dynein/dynactin microtubule motor (Swan et al., 1999), we were interested to find out whether *Dhc* is required for the subcellular localization of Bic-D::GFP. *Dhc* mutants are homozygous and hemizygous lethal, but there exist viable heteroallelic combinations which are female sterile (McGrail and Hays, 1997). Ovaries from *Dhc*6-6/*Dhc*6-12 females display a range of phenotypes; interesting in this context is the failure to differentiate an oocyte. In these 16 nurse cell 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; Fig. 3F). *Dhc* is therefore
essential for the posterior localization of the Bic-D::GFP focus. This is particularly interesting since immunolocalization of Dhc in Bic-DR26 ovaries also shows Dhc localization to a focus anterior of the pseudo germinal vesicle (Li et al., 1994). Therefore, 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 cortex. This mutual requirement indicates that the two genes closely interact with each other.

**A nuclear crater is oriented towards the Bic-D::GFP focus and the MTOC**

The Bic-D::GFP focus is continuously nested in a polarized nuclear depression with the appearance of a ‘crater’. The crater features a concave face with sharply defined margins from its appearance in the germarium to stage 6 (Fig. 3B). The crater is not only apparent with fluorescent microscopy but can also be seen with Nomarski microscopy in living ovaries of OregonR flies (Fig. 3G). The DIC analysis suggests that the crater is formed by the nuclear membrane and that its width can reach up to three-quarters of the nuclear diameter.

From stage 1 to stage 6 the posterior Bic-D::GFP focus is found in close association with both the nuclear crater and the oocyte cortex. Two lines of evidence suggest that the Bic-D::GFP focus is associated with the MTOC. Firstly, experiments with incomplete microtubule depolymerization have identified small tubulin positive foci that resemble this Bic-D::GFP focus both in shape and in subcellular localization (Fig. 7C in Theurkauf et al., 1992). Secondly, immunolocalization of the Dhc polypeptide, which is expected to move towards the microtubule minus ends, is seen in a similar posterior focus in wild-type oocytes (Li et al., 1994). To learn more about the relationship between this Bic-D::GFP accumulation and the MTOC, we tested whether the focus 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 focus under the confocal microscope. Even after prolonged observations of up to two hours, the presence of the posterior Bic-D::GFP focus 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 a posterior Bic-D::GFP focus nor a nuclear crater (Fig. 3H). Therefore the posterior Bic-D::GFP focus is resistant to short treatment with colchicine but sensitive to prolonged treatment. This microtubule dependence may indicate that Bic-D or components of the anchoring mechanism continuously need to become localized in a microtubule dependent way.

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

Using Bic-D::GFP, we have observed early evidence of secondary axis formation in the vitellarium that indicates that the process is initiated as early as stage 6 or 7. The posterior Bic-D::GFP focus is present until stage 6. From stage 7 to stage 10 we observe Bic-D::GFP fluorescence between the oocyte nucleus and the presumptive antero-dorsal oocyte cortex which is adjacent to the nurse cells (Fig. 4A-C). The stage 8 accumulation of Bic-D::GFP (Fig. 4C) is similar to the pattern of a Nod:β-gal chimeric reporter protein (Clark et al., 1997), indicating that Bic-D::GFP again seems to 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.

Live analysis of oocyte nucleus re-localization was not possible using regular confocal microscopy; but DIC microscopy showed that the nucleus movement is not steady but consists of phases of rapid movement, interrupted by phases during which the nucleus either remains in its position or drifts into a different direction. This indicates that the directional forces responsible for nucleus repositioning act on the oocyte nucleus in bursts. During this process the nucleus can show remarkable changes in shape and once it reaches the dorso-anterior position it has assumed a new shape (Figs 4C, 5).

**Function of spn-E in subcellular distribution and nurse cells to oocyte transport**

Bic-D::GFP becomes localized to different cellular compartments in the course of oogenesis. Our studies identified genes required to terminate one specific distribution pattern and genes required to establish a new specific localization pattern. The establishment of specific distribution patterns must involve subcellular transport of proteins. Even though spn-E does not affect Bic-D mRNA localization (Gillespie and Berg, 1995), it affects the distribution of the Bic-D::GFP

![Image](image-url)

Fig. 4. Dorso-ventral polarity and Bic-D transport. (A-C) Bic-D::GFP focus at the time of re-localization and re-orientation of the germinal vesicle from posterior to dorsoanterior. (A) A second focus of Bic-D::GFP is present in what may be the dorso-anterior corner (arrowhead) while the nuclear crater still faces towards posterior (arrow). (B) Bic-D::GFP focus and nuclear crater have also been observed aligned facing in an intermediate direction (arrow). (C) Optical section through the Bic-D::GFP signal (arrow) associated with the dorso-anterior side of the stage 8 oocyte nucleus. Note that the nuclear shape has changed. (D) spn-E mutation causes abnormal aggregation of Bic-D::GFP in the nurse cells (arrowhead) and anterior localization presumably at the ring canal in the oocyte (arrow). Scale bars represent 10 μm, some nurse cell nuclei (ncn), oocytes (oo), and follicle cells (fc) are labeled.
protein. Beginning approximately in stage 6, Bic-D::GFP accumulates in aggregates in spn-E nurse cells (Fig. 4D). 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. 4D). This effect is much the same as spn-E’s effect on the distribution of bcd mRNA which is also retained in a punctate pattern in the nurse cells and at the anterior of the oocyte (Gillespie and Berg, 1995). Immuno-electron-microscope analysis of spn-E mutants indicates that spn-E causes the formation of enlarged sponge bodies and that Bic-D does indeed accumulate in these enlarged RNA containing particles (M. Wilsch-Brauninger, personal communication). spn-E therefore has an essential function in organizing Bic-D containing particles in the nurse cells, in translocating them to the oocyte and in localizing them to the proper oocyte compartment. The hyper-aggregation in the nurse cells is not seen in hemizygous spn-A, spn-B, spn-D, and spn-F ovaries.

DISCUSSION

Bic-D and oocyte specification

Bic-D is essential for oocyte specification. The establishment of oocyte fate is a multistep process that seems to involve microtubule dependent transport of factors from the pro-nurse cells to the future oocyte (Koch and Spitzer, 1983; Suter and Steward, 1991). 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 beginning in region 2a, around the time when the polar microtubule array connecting the cystocytes becomes visible (Theurkauf et al., 1993), the centrioles and mRNAs move to the oocyte (Mahowald and Strassheim, 1970; Suter and Steward, 1991). While most of Bic-D::GFP appears to be cytoplasmic, we observe stronger Bic-D::GFP fluorescence at low frequency in the center of the cystocyte cluster, suggesting that Bic-D temporarily associates with the fusome. In a subset of these cysts we observe one additional Bic-D::GFP focus per cell (Fig. 2A,A’). In most specimens these foci seem to lie between the central signal and the cystocyte nuclei, suggesting that Bic-D also associates with the MTOCs or the newly forming fusome in the ring canals. Because we have not been able to observe this specific Bic-D distribution pattern in fixed ovaries, and because there are no in vivo markers available yet for these subcellular structures, we will only be able to definitely identify the site of accumulation of these Bic-D::GFP foci once the appropriate in vivo markers become available.

The temporally restricted accumulation of Bic-D::GFP into one focus per cell may play an important role in oocyte determination because egl and orb mutants block oocyte differentiation at the point when Bic-D::GFP accumulates in such foci (Fig. 2C-E).

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 of mitochondria between cystocytes may be defective in egl mutants (Carpenter, 1994). 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. 2C). egl is therefore 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. It is likely that the failure of Bic-D::GFP foci to re-localize to the center of the cystocyte cluster is also caused by a defect in intercystocyte transport.

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

Computer analysis of the Egl sequence predicts that it possesses an exonuclease domain (Moser et al., 1997). Because Egl is a cytoplasmic protein, it may therefore interact with RNA. Lack of egl leads to the Bic-D::GFP hyper-accumulation described above. Interestingly, another potential RNA binding protein, Orb (Lantz et al., 1992), 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 strong single focus in each cystocyte, but the resulting foci differ from those seen in wild-type or egl mutants (compare Fig. 2C with D,E). They are of variable size, have discrete edges, and there is 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 RNA-binding proteins, spn-E, also lead to the appearance of Bic-D::GFP aggregation (Fig. 4D). The hyper-aggregation of Bic-D::GFP in spn-E mutants (Fig. 4D) closely resembles the pattern of bicoid (bcd) RNA mislocalization in such ovaries reported by Gillespie and Berg (1995). In wild-type ovaries bcd RNA and Exu RNA-binding protein accumulate in subcellular compartments that have been described at the electron microscope level as sponge bodies (Wilsch-Brauninger et al., 1997). It is therefore possible that Bic-D accumulates in similar or even the same particles as bcd mRNA or Exu protein and that these sponge bodies are enlarged in spn-E mutants. This interpretation is also supported by immunoelectron microscope studies that showed directly that Bic-D hyper-aggregates into enlarged sponge bodies in spn-E mutants (M. Wilsch-Brauninger, personal communication). In wild-type oogenesis, sponge bodies may therefore be the particles in which Bic-D protein translocates to its target site of accumulation.

Bic-D localizes to subcellular structures that contain potential RNA-binding proteins and localized mRNAs. In addition, three genes that appear to encode RNA-binding proteins are all essential for the normal subcellular distribution of the Bic-D protein. Together these results provide strong cytological and genetic evidence in support of a direct role of Bic-D in localizing mRNAs.

Nuclear crater and the role of nucleus orientation and positioning in initiating polarity formation

The striking oocyte nuclear indentation that is associated with the posterior MTOC and the posterior focus of Bic-D::GFP (Fig. 3A,B) has parallels in an indentation described for the sperm nucleus (Fuller, 1993; Tokuyasu, 1974). During spermatid elongation microtubules concentrate on one side of the nucleus and this place then forms a concavity similar to the crater in the oocyte nucleus. Later on the microtubules migrate out of the spermatid crater and associate with the nuclear membrane on the convex portion of the nucleus and the nucleus then regains some of its convexity. A process similar to this may be at work during the re-localization of the oocyte nucleus to the dorsoanterior cortex which coincides with the re-organization of the oocyte microtubules. During this process the oocyte nucleus also changes its shape again (compare Figs 3B and 4C; Fig. 5).

In a number of different situations, Bic-D::GFP shows preferential accumulation in cellular locations in which microtubule minus ends are thought to be localized (Clark et al., 1997; Clegg et al., 1997; Li et al., 1994; Theurkauf et al., 1992): the posterior Bic-D::GFP focus in wild-type stages 1-6, the localization in the center of the oocyte in the same stages in mael (Fig. 3C), and the dorso-anterior oocyte localization in stages 7 and older (Fig. 4C; Swan et al., 1999). The microtubule minus end accumulation suggests that Bic-D either performs its function in this place or that it is transported there by a negative-end directed microtubule motor. The mutual requirement for Bic-D and Dhc for each other’s protein localization (Fig. 3F; Li et al., 1994) is consistent with both models.

The establishment of antero-posterior polarity in the oocyte appears to be marked by the posterior localization of Bic-D::GFP that starts to become apparent towards the posterior most region of the germarium (Fig. 3A). The single focus of Bic-D::GFP accumulation in the presumptive oocyte is first seen anterior of its nucleus. In egg chamber stages 1-6 the crater and the associated Bic-D::GFP focus are found at the posterior side of the nucleus. This relocalization to the posterior is also a cellular processes that requires a negative-end directed microtubule motor and, in addition, mael and spn genes (Fig. 3C-F).

During stages 6-7 the shape and orientation of the oocyte nucleus change from facing posteriorly to facing towards dorso-anterior (Figs 3B,C,G, 4A-C, 5). At the same time the perinuclear Bic-D::GFP focus disappears from the posterior end and appears more diffusely in a new position at the dorsoanterior cortex (Fig. 4A-C). Because this migration happens already at the time when 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, and the migration distance of the oocyte nucleus to the dorso-anterior corner is surprisingly short, equivalent to the width of the oocyte nucleus (Fig. 4A,B). The re-orientation and repositioning of the oocyte nucleus plays an important role in establishing dorso-ventral polarity in the egg chamber. The repositioning of Bic-D::GFP in 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 oocyte identity and in initiating antero-posterior polarity. In both cases, a re-orientation of the oocyte nucleus and the establishment of a new asymmetry around the nucleus appear to anticipate the establishment of the new oocyte and egg chamber polarity.

The nuclear asymmetry of the oocyte may be the basis for the polarized secretion of the Grk signal to the adjacent follicle cells, because this TGFα-like molecule is expressed from the oocyte nucleus (Saunders and Cohen, 1999). During the time the crater in the oocyte nucleus faces towards posterior, Grk first instructs the adjacent posterior polar follicle cells about their fate (Gonzalez-Reyes et al., 1995; Ray and Schüpbach, 1996; Roth et al., 1995). When the oocyte nucleus becomes localized to the dorso-anterior corner, Grk is secreted again along the axis of the newly polarized nucleus and the Bic-D::GFP focus to instruct the adjacent dorso-anterior follicle cells. Proper initiation of polarity would in this case not only depend on proper subcellular positioning of the nucleus but also on its proper orientation, an area of research that has not received much attention, yet.

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