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Null alleles reveal novel requirements for *Bic-D* during *Drosophila* oogenesis and zygotic development

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SUMMARY

In the *Drosophila* ovary, the *Bicaudal-D* (*Bic-D*) gene is required for the differentiation of one of 16 interconnected cystocyte sister cells into an oocyte. A new class of *Bic-D*^{null} alleles reveals a novel requirement for *Bic-D* for zygotic viability. In the germ line, the null mutations show that developmental processes that take place in germarial region 1, even those that create asymmetry, are independent of *Bic-D* function. *Bic-D* is then required to establish oocyte identity in one cystocyte and is essential, not only for the oocyte-specific accumulation of all oocyte markers that we have tested so far, but also for the posterior migration of the oocyte. In addition, normal polarity amongst the nurse cells requires *Bic-D*, indicating that the creation of different nurse cell identities may depend on oocyte determination. Our results show that different processes in early oogenesis require different amounts of *Bic-D* in a process-specific way and certain later processes can proceed at low levels of *Bic-D*. This suggests that the patterning of the female germ line and the development of an oocyte depend on differential responses to a single activity that is capable of initiating distinct oogenesis processes and can establish different cell fates.

Key words: *Drosophila*, oogenesis, cell fate determination, RNA localization, cytoskeleton

INTRODUCTION

Cytoplasmic localization of determinative molecules can create the molecular asymmetries required for the establishment of differential cell fate during development. In the *Drosophila* ovary, such processes have been implicated in oocyte determination and the establishment of anterior-posterior polarity in the oocyte and embryo (Lasko, 1992). An ovary consists of individual ovarioles, which are subdivided into an anteriorly located germarium and a vitellarium. Each germarium contains 2-3 germ-line stem cells in the most anterior position (King, 1970; Mahowald and Kambysellis 1980; Wieschaus and Szabad, 1979). An asymmetric mitotic division of a germ-line stem cell gives rise to a new stem cell and a cystoblast which goes through four mitotic divisions. During these divisions, cytokinesis is incomplete, resulting in 16 cystocytes which remain interconnected through cytoplasmic bridges called ring canals (Meyer, 1961). A precise pattern of cell divisions invariantly leads to clusters of 16 cells in which the two 'oldest' cystocytes are each connected to four other cells, two cystocytes are connected to three others, four to two, and eight to one other cystocyte. It is always one of the two '4-ring canal cells' that differentiates into an oocyte, the other 15 develop into nurse cells (Brown and King, 1964). The germarium has been subdivided into three regions (Fig. 5A). The mitotic divisions that gives rise to the formation of the 16-cell cluster take place in the germarial region 1. In the anterior part of the germarial region 2 (region 2A), molecular differences such as oocyte-specific localization of RNAs start to become apparent between the oocyte and the nurse cells. In the posterior part of germarial region 2 (region 2B), the somatic follicle cells start to invaginate and to surround each cluster, a process that gives rise to the stage 1 egg chamber in germarial region 3.

Oocyte determination is thus dependent on a precise pattern of cell divisions and the generation of cellular diversity among the 16 interconnected cystocytes. For reasons of clarity, we use the term 'oocyte' or 'nurse cell' to refer to cells that possess one or more molecular or morphological characteristics of the respective cell type, and the term does not imply function. *Bicaudal-D* (*Bic-D*) and *egalitarian* (*egl*) are required for creating the cellular diversity. Recessive mutations in these genes cause all 16 cystocytes to differentiate into polyploid nurse cells (Schüpbach and Wieschaus, 1991; Mohler and Wieschaus, 1986). While the molecular analysis of *egl* has not been reported yet, the sequence of the *Bic-D* gene revealed that it encodes a protein with coiled coil domains and with sequence similarity to cytoskeletal proteins and molecular motors. The highest similarity was found between Bic-D and the tail domain of myosin heavy chain, intermediate filament proteins and kinesin, suggesting that Bic-D may act as a component of the cytoskeleton (Suter et al., 1989; Wharton and Struhl, 1989).

Bic-D is required to establish oocyte identity and is essential for the localization of specific RNAs and proteins to the presumptive oocyte as soon as this cell starts to develop a unique identity at the molecular level (Suter and Steward, 1991). The role of *Bic-D* in oocyte differentiation may therefore be to

localize oocyte factors that are initially present in all cystocytes to the presumptive oocyte. Two recessive *Bic-D* alleles, *Bic-D^{PA66}* and *Bic-D^{R26}*, block oocyte differentiation at the earliest steps and cause a 16-nurse-cell terminal phenotype. Nevertheless, certain later aspects of oocyte differentiation are initiated in both of these mutants and proceed normally for some time. For example, the transcripts of two different genes, *orb* and *oskar* (*osk*), specifically accumulate in the germarial region 2 oocyte in the wild type (Lantz et al., 1992; Kim-Ha et al., 1991; Ephrussi et al., 1991). *orb* RNA accumulation in the germarial oocyte is abolished by the *Bic-D^{PA66}* but not by the *Bic-D^{R26}* mutation and *osk* RNA accumulates in the young oocyte of both mutants. Furthermore, a process that takes place even later in oogenesis is not affected by the two *Bic-D* mutations: the presumptive oocyte moves to the posterior-most position in the cystocyte cluster in germarial region 3 where it remains until about pseudo-stage 3-4 (Wharton and Struhl, 1989; Suter and Steward, 1991; Lantz et al., 1992). Despite this progression into oogenesis, oocyte identity is subsequently completely reverted to nurse cell identity.

These observations of some aspects of oocyte differentiation in recessive *Bic-D* mutants indicate that either there are processes in oocyte differentiation that are independent of *Bic-D* function or, alternatively, the two recessive alleles contain sufficient residual *Bic-D* activity to promote certain *Bic-D*-dependent oocyte differentiation processes without giving rise to a functional oocyte. This second interpretation is only possible if the recessive alleles are not null alleles, and several lines of evidence indicated that they indeed cannot be true null alleles. (i) Both mutants make normal amounts of *Bic-D* RNA and protein and the mutant protein is of approximately normal size (Suter and Steward, 1991). (ii) Oogenesis proceeds further in *Bic-D^{R26}* than in *Bic-D^{PA66}*, suggesting that *Bic-D^{R26}* is not a null allele. (iii) Over the dominant *Bic-D* allele *Bic-D$^{71.34}$*, the *Bic-D^{PA66}* mutation causes female sterility while hemizygous *Bic-D$^{71.34}$* flies are fertile. Because *Bic-D^{PA66}* does not behave like a deficiency in this genetic background, it cannot be a null allele.

To determine which aspects of oocyte differentiation require *Bic-D*, we set out to isolate *Bic-D^{null}* alleles. In addition, since *Bic-D* is expressed virtually throughout development, null alleles may reveal further processes, potentially during zygotic differentiation, which are also dependent on *Bic-D* activity and localization of determinative molecules. In the work presented here, we took advantage of the phenotypic differences between the *Bic-D^{PA66}* allele and a deficiency and designed a positive selection screen to isolate *Bic-D^{null}* alleles. We verified at the molecular level that the new alleles are indeed nulls, characterized the phenotype of the null mutants and evaluated the role of *Bic-D* in oogenesis.

MATERIALS AND METHODS

Media and strains

Flies were reared on standard medium unless otherwise specified. Standard medium contained 120 g torula yeast, 280 g cornmeal, 300 g sugar, 36 g agar, 6 g methyl paraben, 32 g potassium sodium tartrate, 20 ml propionic acid and 2.6 l water. Apple juice agar plates were prepared as described by Wieschaus and Nüsslein-Volhard (1986). Geneticin fly medium was prepared from Ward's instant *Drosophila* medium (Ward's Natural Science Ltd.) and contained 800 μg/ml Geneticin (Gibco BRL). To select for geneticin resistance, animals were heat shocked every second day for 1 hour at 37°C to induce neomycin expression. Most fly strains have been described elsewhere (Suter et al., 1989; Lindsley and Zimm, 1992; Mohler and Wieschaus, 1986; Schüpbach and Wieschaus, 1991). The FLP-FRT stock *P[ry$^+$ hsp70:FLP] w^{1118}; P[ry$^+$ hsp70:neo FRT]40A* (Xu and Rubin, 1993) was obtained from the Bloomington stock center.

Mutagenesis

EMS was applied to *Drosophila* males as described by Grigliatti (1986). After the appropriate crosses (see Fig. 1), vials containing 25 females of the genotype *Bic-D$^{71.34}$/Bic-D^{PA66}* (EMS treated) were set up for fertility tests with males from the same cross. Offspring recovered from a test vial were re-tested. From lines that were fertile in the second test, two to four different sublines with a single balanced *Bic-D^{PA66}* chromosome were established. All novel *Bic-D* alleles complement *dorsal*, *l(2)HT-1* and *quail*, the genes neighboring the *Bic-D* locus (Steward and Nüsslein-Volhard, 1986), indicating that they do not contain an extended deficiency of the *Bic-D* region. To test whether the new alleles are suppressors or null mutations, the effect of the mutation was tested over the *Bic-D^{PA66}* and *Bic-D^{R26}* recessive alleles and over *Df(2L)TW119*. While flies containing the suppressor *Bic-D^{PA66}* double mutant chromosome over any of the recessive alleles are viable and fertile (Suter and Steward, 1991), the *Bic-D^{null}* alleles behave like deficiencies over *Bic-D^{PA66}* and *Bic-D^{R26}*, and these females are viable but sterile.

Phenotypic and molecular analysis

All eight *Bic-D^{null}* lines were crossed to *Df(2L)TW119*, the smallest deletion that removes the *Bic-D* locus. For the determination of the lethal phase, mothers were used in which the second chromosome is not balanced to avoid possible effects of the balancer on viability. Eggs were collected on apple juice-agar plates and the embryos were arranged in groups of 10 for easier counting. Freshly eclosed larvae were immediately transferred onto a fresh plate (10 larvae/plate) and the plates were counted at least twice a day until no further adults hatched.

The *Bic-D^{r5}* and *Bic-D^{r8}* lines were used in combination with the FLP-FRT system to induce germ-line clones of homozygous *Bic-D^{null}* cells (Golic, 1991; Xu and Rubin, 1993). To prevent uncovering other lethal or female sterile mutations on 2L, the chromosomal sequences distal to *Bic-D* (from *b* to the tip of 2L) were replaced with sequences from a strain that is homozygous viable and female fertile (*b pr cn wxt bw* chromosome). An FRT insertion in 40A, between *Bic-D* and the centromere of the second chromosome, was then recombined onto the *Bic-D^{null}* chromosomes. Candidate chromsomes, which may contain the FRT insert and the *Bic-D^{null}* allele, were identified based on their resistance to geneticin (G418) and on the presence of the *b* marker, which is closely linked to *Bic-D*. These candidate chromosomes were then tested for the presence of the *Bic-D^{null}* allele over *Df(2L)TW119*. An X chromosome containing the FLP recombinase gene under heat-shock control was finally crossed into this background. Second instar larvae of the genotype *P[ry$^+$ hsp70:FLP] w^{1118}/+; b Bic-D^{null} P[ry$^+$ hsp70:neo FRT]40A/+ Bic-D$^+$ P[ry$^+$ hsp70:neo FRT]40A* were heat shocked at 38°C for one hour to initiate the expression of FLP and mitotic recombination.

Sequencing of the mutant genomic DNA and RNA in situ hybridizations were done as described previously (Suter and Steward, 1991).

Histology and immunocytochemistry

Staining of ovaries with Hoechst was done as described by Schüpbach and Wieschaus (1991). YO-PRO (Molecular Probes Inc., Eugene, OR, USA) was used as a DNA dye for experiments involving confocal laser scanning microscopy. In this case, ovaries were dissected in Ringers and fixed for 10 minutes in 4% paraformaldehyde in PBST

(PBS + 0.2% Tween). After several rinses, samples were incubated with RNase A at a concentration of 100 μg/ml in PBST for 20 minutes, then washed twice in PBST for 10 minutes each and subsequently stained with 0.5-1 μM YO-PRO-1 in PBST for 10 minutes. The staining was followed by three PBST washes for 15 minutes each and the ovaries were mounted in 50-70% glycerol.

For double stainings with antibodies and YO-PRO-1, ovaries were dissected into Ringers and fixed in 200 μl freshly prepared PP (4% paraformaldehyde in PBS, supplemented with 0.2% Tween after cooling down the fixative), 20 μl DMSO and 600 μl heptane on a rocking plate for 20 minutes. The samples were rinsed three times, washed twice for 5 minutes with PBST and blocked three to four times for 60 minutes in 1× PBSBTT (PBS, 0.2% Tween, 0.1% Triton, 1% BSA). During the first blocking period, RNase A was added to 100 μg/ml to remove RNAs that would bind YO-PRO. Samples were stained with anti-Bic-D antibody 1B11 (1:20 dilution in PBSBTT) overnight at 4˚C and 2 hours at room temperature. The samples were rinsed 3 to 4 times, washed three times for 20 minutes with PBST and then incubated in the dark with 50- to 100-fold diluted Texas-Red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) as described for the primary antibody. Three PBST rinses were then followed by a 20 minute incubation with 1 μM YO-PRO-1 in PBST. After two more rinses and two 20 minute washes with PBST, the ovaries were mounted in 75% glycerol and inspected under the confocal microscope.

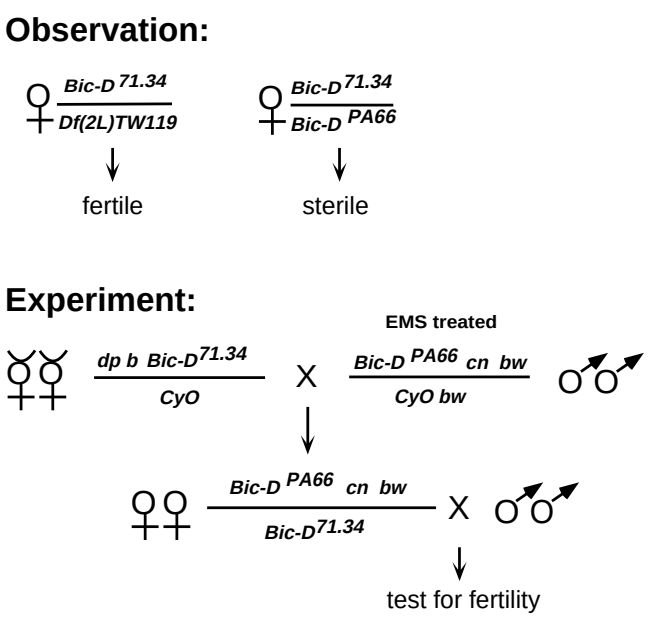


Fig. 1. Screen for *Bic-D*^{null} alleles. *Bic-D*$^{71.34}$: dominant maternal effect allele of *Bic-D* giving rise to bicaudal embryos; *Bic-D*^{PA66}: recessive oogenesis allele of *Bic-D* causing the 16-nurse-cell phenotype; *Df(2L)TW119* deficiency for the *Bic-D* region. Females of the genotype *Bic-D*$^{71.34}$/*Df(2L)TW119* are fertile while *Bic-D*$^{71.34}$/*Bic-D*^{PA66} are sterile. Based on this observation males containing a *Bic-D*^{PA66} chromosome were treated with EMS to induce null revertants of this allele. *Bic-D*^{PA66} males were crossed to *Bic-D*$^{71.34}$ females and in the next generation females containing the mutagenized *Bic-D*^{PA66} chromosome over the *Bic-D*$^{71.34}$ chromosome were tested for fertility.

RESULTS

Isolation and molecular characterization of *Bic-D* null alleles

The experimental strategy used to isolate EMS induced potential null alleles of *Bic-D* is outlined in Fig. 1. This screen is based on the observation that the recessive *Bic-D*^{PA66} allele causes sterility over one of the dominant, double abdomen forming, *Bic-D* alleles (*Bic-D*$^{71.34}$) while a deficiency over the same dominant allele is female fertile. Because a knock out revertant of the *Bic-D*^{PA66} allele should have the same effect as a deficiency, it should be possible to isolate null alleles by mutagenizing *Bic-D*^{PA66} chromosomes and selecting for fertility amongst the females that contain the dominant allele over the mutagenized *Bic-D*^{PA66} allele. We tested 6,500 females containing a mutagenized *Bic-D*^{PA66} chromosome and found that 14 of these females produced offspring. We succeeded in establishing lines containing the new mutation from eight of them. Of these, seven turned out to be null alleles and one a suppressor. The suppressor mutation has been described earlier (Suter and Steward, 1991) and we will focus here entirely on the null alleles that we have named *Bic-D*$^{r5\ to\ r11}$.

The newly isolated *Bic-D* alleles behave like *Bic-D* deficiencies over the dominant *Bic-D*$^{71.34}$ allele. For this reason and because their parental *Bic-D*^{PA66} chromosome behaves like a deficiency in all other heteroallelic combinations, the new alleles are good candidates for null alleles. To find out whether they also qualify for null alleles at the molecular level, we determined the molecular nature of the mutations by sequencing the *Bic-D*$^{r5\ to\ r11}$ alleles. The results of the sequence analysis are shown in Fig. 2. Because these novel *Bic-D* alleles have been isolated as knock out revertants of the *Bic-D*^{PA66} allele, all seven novel *Bic-D* alleles contain the *Bic-D*^{PA66} mutation in codon 40 (Suter and Steward, 1991). In addition, we found that in six of the seven alleles, a single nucleotide substitution created a termination codon (*Bic-D*$^{r5\ to\ r10}$, Fig. 2) while the mutation in *Bic-D*^{r11} is predicted to change a lysine into a methionine residue in a heptad repeat domain.

Consistent with the sequencing results, a mixture of five monoclonal anti-*Bic-D* antibodies recognizes very low levels of polypeptides of the predicted molecular mass on western blots containing extracts from *Bic-D*$^{r7\ to\ r11}$ flies (summarized in Fig. 2B). In *Bic-D*$^{r5\ to\ r6}$, however, no immunopositive bands could be detected possibly because these early terminated polypeptides may not contain any epitopes recognized by the monoclonal antibodies. In most or all of these *Bic-D* mutants, the mutations cause a premature termination of the Bic-D protein and a strong reduction in levels of this mutant protein. Based on the genetic and the biochemical characterization, the newly isolated *Bic-D*^{null} alleles appear to be true null alleles. We will therefore refer to these new *Bic-D* alleles as the *Bic-D*^{null} mutations and we will use the term oogenesis alleles for the other two recessive *Bic-D* alleles *Bic-D*^{PA66} and *Bic-D*^{R26}, which are hypomorphic and specifically interfere with oogenesis.

Bic-D^{null} mutations are recessive lethals

To assess the *Bic-D*^{null} phenotype, heterozygous *Bic-D*^{null} mutants were crossed to flies heterozygous for a deficiency of the *Bic-D* locus. One quarter of the offspring from this cross should contain the *Bic-D*^{null} mutation over the deficiency and therefore show the *Bic-D*^{null} phenotype. When offspring from

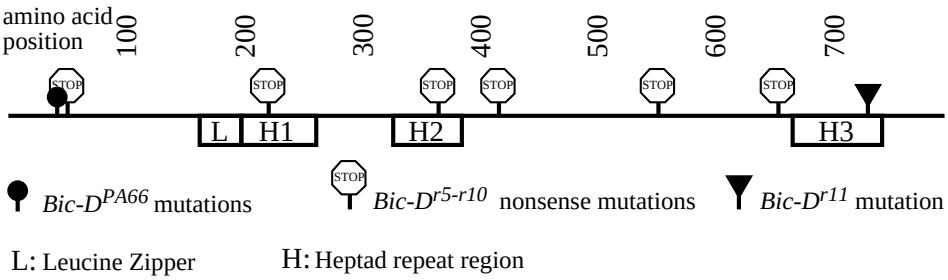
this cross were raised on standard food at 29°C, no hemizygous *Bic-D*^{null} adult hatched, indicating that the *Bic-D*^{null} phenotype is zygotic lethality (Table 1). When flies were raised at 18°C, however, all *Bic-D*^{null} alleles gave rise to a small number of *Bic-D*^{null} adults. Depending on the line, the frequency with which we recovered such adults ranged from 0.6-6.6% of the expected *Bic-D*^{null} flies (n=200-300; Table 1). With the possible exception of *Bic-D*^{r10}, this frequency does not correlate with the length of the truncated *Bic-D* protein. However, the frequency depends on the genetic background because in a parallel experiment three sublines of *Bic-D*^{r5} that differ in their genetic background gave rise to 0.4, 1.7 and 5.8% of expected *Bic-D*^{null} adults. All these *Bic-D*^{null} flies died within a day of hatching.

The frequency of *Bic-D*^{null} adults can be significantly increased by raising the flies on apple juice-agar medium supplemented with live yeast paste. Under these conditions larvae hatched from up to 98% of the eggs, 90% developed up to the pupal stage and 34-76% of the expected *Bic-D*^{null} hemizygous adults eclosed (Table 1). Such null flies are weak and their bodies are often somewhat smaller. Their behavior is generally lethargic and uncoordinated, and they usually die within two or three days after eclosion. The null mutations clearly show that the *Bicaudal-D* gene is required for zygotic viability while the genetic background and environmental factors such as food and temperature affect the lethal phase.

Bic-D^{null} ovaries are composed of 16-nurse-cell egg chambers

A major objective of this work was to determine the first step during oocyte differentiation that requires *Bic-D* function. To find out what the *Bic-D*^{null} phenotype in the ovary is, wild-type and all *Bic-D*^{null} ovaries were stained with fluorescent DNA dyes to examine the polyploidization of the germ-line nuclei. In the wild-type egg chambers, a diploid oocyte nucleus is clearly visible as a small fluorescent object and the nurse cell nuclei start differing in their DNA content in stage 4-5 (Fig. 3A). In contrast, the *Bic-D*^{null} ovaries contain only egg chambers with 16 polyploid nuclei, which all have approximately the same size and staining intensity indicating that they have a similar DNA content (Fig. 3B,C). These results show that, while *Bic-D* is required for the

A



B

Allele	Codon position	Codon alteration	Type of alteration	Apparent M_r of *Bic-D* polypep.	Abundance of *Bic-D* polypep
Bic-D^{r5}	050	AAG > TAG	termination	nd	nd
Bic-D^{r6}	220	CAA > TAA	termination	nd	nd
Bic-D^{r7}	363	CAG > TAG	termination	46×10^3	reduced
Bic-D^{r8}	416	TGG > TAG	termination	50×10^3	reduced
Bic-D^{r9}	551	CAG > TAG	termination	68×10^3	reduced
Bic-D^{r10}	653	AAG > TAG	termination	75×10^3	reduced
Bic-D^{r11}	730	AAG > ATG	K > M	96×10^3	reduced

Fig. 2. Molecular characterization of the *Bic-D*^{null} mutants. (A) Schematic representation of the Bic-D protein showing the localization of the *Bic-D*^{null} mutations. All *Bic-D*^{null} alleles also contain the *Bic-D*^{PA66} mutation Ala>Val in codon position 40 in addition to the null mutation. (B) Molecular nature of the *Bic-D*^{null} mutations and their effect on the Bic-D protein. Single nucleic acid changes cause in six mutants premature termination of *Bic-D* translation product while the seventh mutation leads to a single amino acid substitution. All *Bic-D*^{null} mutations also appear to destabilize the mutant Bic-D protein. nd, polypeptide was not detected with anti Bic-D antibodies.

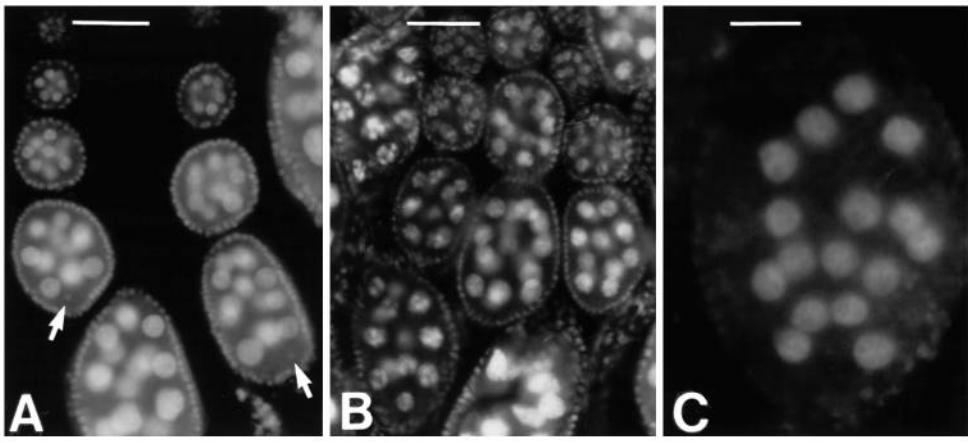


Fig. 3. The ovarian null phenotype of *Bic-D*. Ovaries stained with the nuclear dye Hoechst. (A) In the wild-type egg chambers the small diploid oocyte nucleus is visible (arrows) and the polyploid nurse cell nuclei vary in size and staining intensity starting around stage 5. Scale bar represents 35 μm. (B) In hemizygous *Bic-D*$^{r5\ to\ r11}$ ovaries, no diploid germ cell nuclei are visible in older egg chambers and no oocyte develops. Scale bar represents 50 μm. (C) All 16 germ cell nuclei of the hemizygous *Bic-D*^{r8} egg chamber are polyploid and all nuclei have the same size and stain with the same intensity, suggesting that all have developed into the same type of nurse cell. Scale bar represents 20 μm.

Table 1. Dependence of zygotic viability on food and temperature*

	Bic-D^{null} alleles						
Growth conditions	r5	r6	r7	r8	r9	r10	r11
Corn meal, 29°C† (N§ > 200)	0	0	0	0	0	0	0
Corn meal, 18°C† (N§ > 200)	5.8	4.4	1.3	0.7	0.6	6.6	0.9
Apple juice, 22°C†† (N§ > 21)	52	40	52	34	38	76	44

*The data in the table show the percentage of *Bic-D*^{null} hemizygotes (*Bic-D*^{null}/*Df(2L)TW119*) which survive to adulthood.
§Expected number of *Bic-D*^{null} progeny from each cross.
†Cross: *Bic-D*^{null} *cn (bw)/CyO bw*×*Df(2L)TW119 cn bw/CyO bw*.
††Cross: *Bic-D*^{null} *cn (bw)/+*×*Df(2L)TW119 cn bw/CyO bw*.

establishment of oocyte identity in one of the 16 interconnected cystocytes, it is dispensable for the establishment of a 16-cystocyte cluster.

Oocyte differentiation requires *Bic-D* in the germ line

Bic-D is not only expressed in the germ line but also in the somatic tissue (Suter et al., 1989). It is therefore not known in which tissue type the gene is required for oocyte differentiation. To determine the cellular requirement for *Bic-D*, we made germ-line clones of homozygous *Bic-D*^{null} cells. Ovaries potentially containing such clones were dissected and double stained for Bic-D protein and DNA. *Bic-D*^{null} egg chambers were identified as the ones that show only background staining with anti Bic-D antibodies. Fig. 4A shows an overview of such an ovary stained for the Bic-D protein. One of the egg chambers shows only background levels of Bic-D staining. Optical sections through this egg chamber were taken with the confocal microscope at different levels to analyze the morphology of every germ cell nucleus. Fig. 4B shows the superimposed optical sections of this egg chamber, which contains only 16 polyploid nurse cells and no oocyte. Further inspections of two additional Bic-D protein-negative egg chambers revealed that the *Bic-D*^{null} germ-line clones give rise to 16-nurse-cell egg chambers without an oocyte as do egg chambers from *Bic-D*^{null} flies. The *Bic-D* gene is therefore required in the germ line for determination and differentiation of an oocyte.

oskar and *orb* RNAs do not localize in *Bic-D*^{null} ovaries

In the absence of *Bic-D*, none of the cystocytes becomes an oocyte; but, similar to the *Bic-D* oogenesis mutants, the null mutants may still develop some oocyte features in one of the 4-ring canal cells. A number of mRNAs have been described to accumulate specifically in the germarial oocyte of a wild-type female (Kim-Ha et al., 1991; Ephrussi et al., 1991; Suter and Steward, 1991; Golumbeski et al., 1991; Lantz et al., 1992; Yue and Spradling; 1992; Dalby and Glover, 1992). We showed previously that the two recessive oogenesis alleles, *Bic-D*^{PA66} and *Bic-D*^{R26}, prevent the oocyte-specific accumulation of some but not all of these markers. Starting in germarial region 2, *osk* RNA accumulates in early *Bic-D*^{PA66} and *Bic-D*^{R26} oocytes (Fig. 5A; Suter and Steward, 1991) and *orb* RNA accumulates in germarial oocytes of *Bic-D*^{R26} mutants (Fig. 5C; Lantz et al., 1992). To find out whether this remaining polarity is *Bic-D* independent or whether the residual *Bic-D* activity in these ovaries is sufficient for the accumulation of *osk* and *orb* RNA, we analyzed the distribution of *osk* and *orb* RNA in *Bic-D*^{null} ovaries. Ovaries from *Bic-D*^{null} flies express *osk* and *orb* RNA but do not show any preferential accumulation of either RNA in a single cell, the RNA remains evenly distributed amongst the

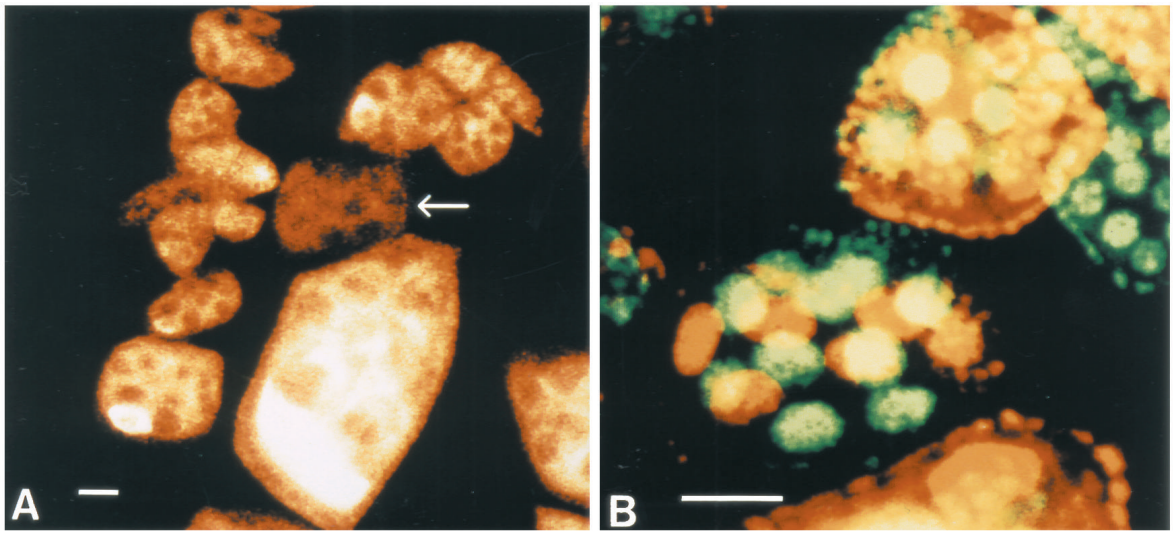


Fig. 4. Egg chambers that are *Bic-D*^{null} in the germ line do not contain an oocyte. Confocal laser scanning micrographs of an ovary containing *Bic-D*^{r8}/*Bic-D*^{r8} clones in the germ line. The ovary was double stained for the Bic-D protein and for DNA. The scale bars represent 20 μm. (A) Distribution of Bic-D protein, the arrow points to an egg chamber that shows only background fluorescence. (B) The same Bic-D protein-negative egg chamber stained with the DNA stain YO-PRO to reveal polyploidization of the germ cell nuclei. To visualize superimposed nuclei, the fluorescence in the optical sections which show the upper 10 nuclei is shown in green and the fluorescence in the lower sections in orange. All 16 germ cell nuclei are polyploid.

cystocyte sister cells (Fig. 5B,D). These and our earlier results (Suter and Steward, 1991) show that *Bic-D* is required for the oocyte-specific accumulation of at least four different mRNAs. The accumulation of *osk* and *orb* RNA requires only low levels of *Bic-D* activity because the activity present in the hemizygous *Bic-D*^{PA66} and *Bic-D*^{R26} mutants is sufficient.

Bic-D is essential for the posterior localization of the germarial oocyte

During normal oogenesis, the oocyte moves to the most posterior position in the 16-cystocyte cluster in germarial region 2B. As is evident by the accumulation of *osk* RNA in the posterior most germ-line cell in young *Bic-D*^{PA66} (and *Bic-D*^{R26}) egg chambers (Fig. 5A, this work; Suter and Steward, 1991), this positioning occurs normally in the two recessive oogenesis mutants even though this cell develops into a nurse cell and not into an oocyte. Conceivably, the posterior migration of the oocyte may be independent of *Bic-D* even though the migration occurs later than oocyte cell fate determination. Alternatively, the process may depend on *Bic-D* but the low levels of *Bic-D* activity found in the oogenesis mutants may induce sufficient oocyte differentiation to initiate posterior localization. To differentiate between these two possibilities, we wanted to find out where the pro-oocytes are localized in the *Bic-D*^{null} egg chambers.

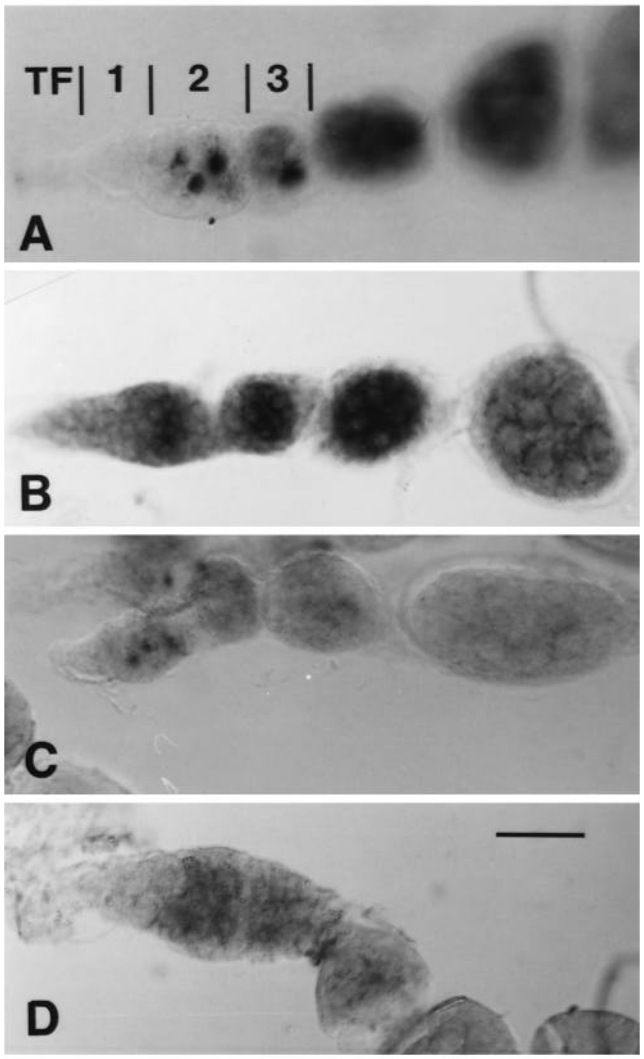


Fig. 5. Distribution of osk and orb RNA in Bic-D^{null} ovaries. Whole-mount in situ hybridizations to ovaries. Scale bar represent 20 μm. (A) Localized *osk* transcripts in *Bic-D*^{PA66}/*Df(2L)TW119* ovaries. (B) *osk* RNA is uniformly distributed in the germ line of the *Bic-D*^{r8}/*Df(2L)TW119* and *Bic-D*^{r10}/*Df(2L)TW119* females. (C) Localized *orb* transcripts in *Bic-D*^{R26}/*Df(2L)TW119* ovaries. (D) Uniform distribution of *orb* RNA in *Bic-D*^{null}/*Bic-D*^{null} ovaries. The following heteroallelic combination were analyzed: *Bic-D*^{r8}/*Bic-D*^{r10}, *Bic-D*^{r5}/*Bic-D*^{r9}, *Bic-D*^{r5}/*Bic-D*^{r11}, *Bic-D*^{r7}/*Bic-D*^{r9}.

The absence of *Bic-D* prevents the earliest molecular aspects of oocyte differentiation and therefore molecular oocyte markers are not available to identify unambiguously the cell of interest amongst the 16 cystocytes. However, it is possible to identify the two 4-ring canal cells by examining the size and position of the ring canals connecting the cystocytes. The ring canal built during the first mitotic division of the cystoblast is the oldest one, contains more actin and has a bigger diameter than all the subsequently formed ones, which are progressively smaller. Because the oocyte always differentiates from one of the two cystocytes with four ring canals, the largest ring canal serves as a marker for these two cells (Koch and King, 1969).

We stained wild-type and *Bic-D*^{null} ovaries with rhodamine-labeled phalloidin to visualize the ring canals (Warn et al., 1985), and analyzed them with the confocal laser scanning microscope. The results of this experiment are shown in Fig. 6. When the 16-cell cysts are arranged in a disk-shape structure in germarial region 2, the ring canals are lined up in a plane perpendicular to the ovariole in both the wild type and the *Bic-D*^{null} mutants (Fig. 6A,B). At this stage, the largest ring canals are localized in the center of the disk-shaped clusters in both the wild-type and *Bic-D*^{null} ovaries (see arrow Fig. 6A,B). In the following developmental stages of the wild type, the largest ring canal moves to the posterior end of the 16-cell cluster. This process starts in the posterior part of germarial region 2 and the oocyte reaches a clearly evident posterior location in germarial region 3 (Fig. 6C). In this stage 1 egg chamber, the four ring canals connecting the wild-type oocyte to four nurse cells occupy positions close to the posterior end where they are oriented parallel to the surface of the most posterior cell (the oocyte) with their axes pointing towards this cell.

In the *Bic-D*^{null} mutants, this reorganization of the ring canals does not occur. The largest ring canal does not move to the posterior end of the cluster and is instead found in apparently random positions in the posterior half of the egg chamber and we never observe four ring canals focusing onto a single cell (Fig. 6D). These results show that *Bic-D* is essential for the spatial organization of the germ-line cells; it is, however, not required for establishment or maintenance of ring canals with different diameters because the *Bic-D*^{null} egg chambers contain the correct number of 15 ring canals and these show the same size distribution as the wild-type ones (compare Fig. 6E and F).

In wild-type oogenesis, there is one more process in germarial region 1 that creates differences between the cystocytes. The characteristic mitotic division pattern gives rise to

cystocytes with different numbers of ring canals ranging from one to four. To investigate whether *Bic-D* is essential for the establishment of this asymmetry, the egg chamber shown in Fig. 6F was reconstructed from serial optical sections to show the connections between the different nurse cells (Fig. 7). In these *Bic-D*^{null} mutants, the normal connections between the cystocytes are established, indicating that *Bic-D* is not required for the establishment of these early differences. Therefore, asymmetries that are established during the mitotic divisions in the germarial region 1 do not require *Bic-D* and are the only polarities that are apparent in the *Bic-D*^{null} egg chambers.

DISCUSSION

Formation of 16-germ-cell clusters with normal ring canals is independent of *Bic-D*

The newly isolated *Bic-D* alleles appear to be true null alleles according to genetic and molecular criteria. Their phenotypic analysis shows that *Bic-D* is not required for the first steps in oogenesis, the mitotic divisions of the cystoblast which take place in germarial region 1. *Bic-D*^{null} egg chambers contain 16 polyploid germ cells (Fig. 3B,C) and the correct number of ring canals with the same range in diameter and apparent actin content as the wild-type ring canals (Fig. 6). Most notably, even the asymmetric distribution of ring canals, which occurs during the mitotic divisions, is normal as judged from a reconstruction of an older egg chamber (Fig. 7). Therefore, all developmental processes that take place in the germarial region 1 appear to be independent of zygotic *Bic-D*. This phenotype contrasts with that of *hu-li tai shao* (*hts*), another mutation that prevents the formation of an oocyte in most but not in every cyst (Yue and Spradling, 1992). Mutations in *hts* seem to affect primarily the mitotic division pattern in germarial region 1: *hts* mutations reduce the number of germ cells per egg chamber and prevent the incorporation of actin filaments into the ring canals.

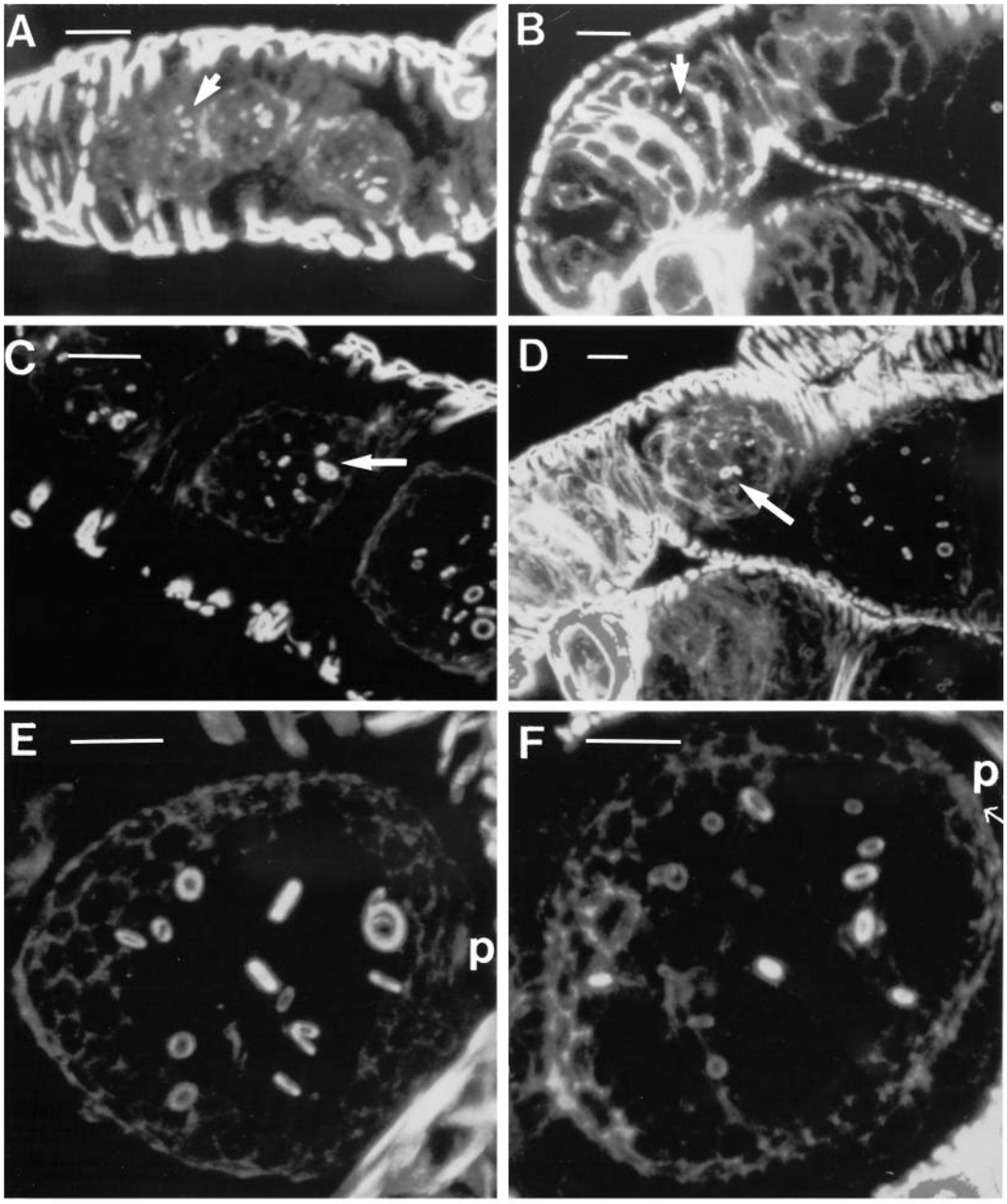


Fig. 6. Ring canals in wild-type and *Bic-D*^{null} ovaries. Ovaries from wild type and hemizygous *Bic-D*^{r5} mutants were stained with rhodamine-conjugated phalloidin to reveal the actin rich ring canals. Optical sections taken at different levels with the confocal laser scanning microscope were superimposed such that the germ-line staining is visible. Scale bars represent 5 μm. (A) Wild-type germarial region 2 and 3. (B) Germarial region 1 and 2 from hemizygous *Bic-D*^{r5} ovaries. (C) Wild-type germarial region 3, the arrow points to the largest ring canal and the group of four ring canals focusing onto the posteriorly localized oocyte. (D) Germarial region 3 from hemizygous *Bic-D*^{r5} ovaries, the arrow points to the largest ring canal which is not localized to the posterior end of the egg chamber and is not part of a group of four ring canals. (E) Wild-type stage 2-3 egg chamber. p: posterior end of the egg chamber. (F) Pseudo-stage 3 egg chamber from hemizygous *Bic-D*^{r5} females. The posterior end is indicated (p).

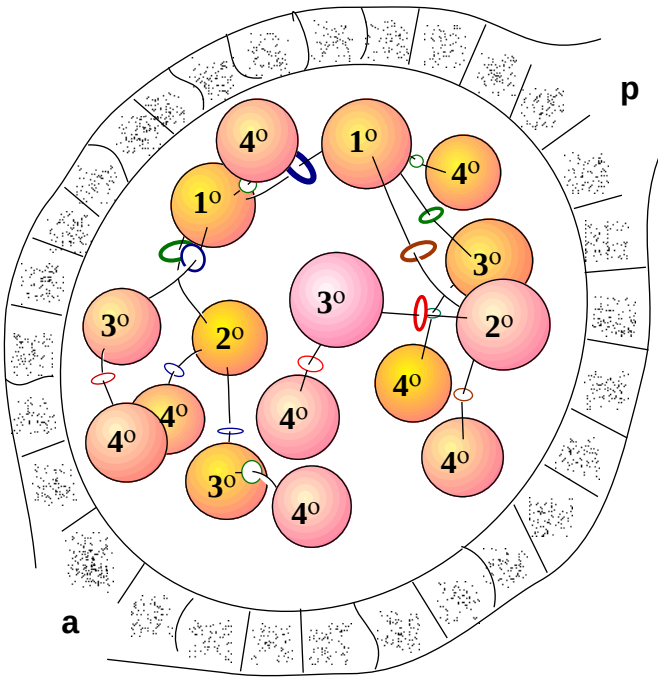


Fig. 7. Reconstruction of a *Bic-D*^{null} egg chamber. The phalloidin-stained egg chamber shown in Fig. 6F was used to reconstruct schematically a hemizygous *Bic-D*^{r5} egg chamber. The individual confocal images which show optical sections through different levels of the egg chamber were analyzed to find out how the individual cells are interconnected with each other. The balls schematically represent the approximate position of the center of each cell. The two 4-ring canal cells are labeled as 1°, the two 3-ring canal cells as 2°, the 2-ring canal cells as 3°, and the 1-ring canal cells as 4°. Ring canals are indicated as open ovals, lines connecting two cells through a ring canal indicate cells that are linked by a ring canal. The anterior and posterior ends of the egg chamber are indicated as 'a' and 'p'.

Function of *Bic-D* in patterning the germarial germ line

We have established that the first phase of oogenesis that requires zygotic *Bic-D* is the patterning of the cluster of 16 interconnected cystocytes into one oocyte and 15 nurse cells with different cellular identity, a process that involves the specific accumulation of a number of different mRNAs in the differentiating oocyte. As indicated by the 16-nurse-cell phenotype, *Bic-D* is also essential for keeping the oocyte in meiosis and it is required for the posterior localization of the oocyte in the egg chamber (Fig. 6). Taken together, *Bic-D* is essential for all early signs of oocyte differentiation.

The observations that the posterior positioning of the oocyte and the *osk* RNA accumulation occur in the oogenesis alleles *Bic-D*^{PA66} and *Bic-D*^{R26} (Suter and Steward, 1991), and that both processes are abolished in the *Bic-D*^{null} mutants (this work), strongly suggests that the two different cellular processes share some common mechanisms. While mRNA accumulation in the oocyte may be the primary process mediated by *Bic-D*, accumulation of a mRNA that gets localized in the wild type and the two oogenesis mutants may be essential for the migration of the oocyte to the posterior end of the cystocyte cluster.

Bic-D and nurse cell polarity

In wild-type oogenesis, polarity is not only established between the oocyte and the nurse cells but also amongst the different nurse cells within a vitellarial egg chamber. Starting in egg chamber stage four or five, the nurse cell nuclei differ in their polyploidy in such a way that the nurse cells closer to the oocyte reach a higher degree of polyploidization than the ones further away (Fig. 3A). In later stages, nuclear volumes in a single cyst can differ up to a factor of three (Brown and King, 1964). This gradient of nurse cell identity could either become established as an integral part of the mitotic division pattern in the germarial region 1 which gives rise to presumptive nurse cells with one, two, three or four ring canals, or it could become fixed by a redistribution of factors in the 16-cystocyte cluster as a response to oocyte determination. Consistent with the second hypothesis, we did not observe any polarity in nurse cells in *Bic-D*^{null} mutants that contain pseudo egg chambers that are old enough for this polarity to become apparent (Fig. 3).

Different mechanisms of RNA localization and their implications

A number of different RNAs and proteins become localized to the oocyte during the germarial stages of oogenesis and serve as early molecular markers for oocyte differentiation (Kim-Ha et al., 1991; Ephrussi et al., 1991; Suter and Steward, 1991; Golumbeski et al., 1991; Lantz et al., 1992; Yue and Spradling, 1992; Dalby and Glover, 1992). The results of the analysis of their distribution in wild-type ovaries and in the different ovaries that do not make an oocyte led to interesting insights into the mechanisms that specify oocyte cell fate in *Drosophila*. While some mRNAs (*Bic-D*, *K10*, Suter and Steward, 1991) accumulate in the wild-type oocyte but in neither of the *Bic-D* oogenesis mutants, another accumulates in the wild-type and in *Bic-D*^{R26} oocytes only (*orb*, Lantz et al., 1992). A third class (*osk* RNA) accumulates in the oocyte of the wild type and the *Bic-D*^{R26} and *Bic-D*^{PA66} mutants but does not accumulate in a single cell in the *Bic-D*^{null} (Ephrussi et al., 1991; Kim-Ha et al., 1991; Suter and Steward, 1991; Fig. 5A,B, this work).

The differences by which various RNAs become localized to the young oocyte may reflect their dependence on different transport or anchoring mechanisms. Alternatively, the different RNAs may utilize the same localization system but the interactions between the various mRNAs and the localization system may require different levels of *Bic-D* activity. This second model appears more attractive in the context of what we know about the mechanisms of oocyte differentiation. Several observations indicate that oocyte differentiation is a gradual process rather than a single molecular on/off switch. The earliest oocyte markers progressively accumulate in a single cystocyte, indicating that the building up of oocyte identity depends on the continuous transport of oocyte factors to this cystocyte (Suter and Steward, 1991). The accumulation of different mRNAs in the oocyte becomes apparent at different stages of oogenesis. *Bic-D* RNA is the first one to localize to the oocyte, followed by *orb* mRNA and later on *osk* RNA. The oocyte-specific accumulation of *K10* RNA follows considerably later in egg chamber stage 2 (Suter and Steward, 1991; Lantz et al., 1992; Cheung et al., 1992). Similarly, the

different morphological oocyte characteristics are established during different phases of oogenesis. For instance, the reorganization of the microtubule arrays that connect oocytes and nurse cells occurs in germarial region 2A, the 4-ring canal cell that does not become the oocyte loses its synaptonemal complexes late in region 2B and the oocyte takes up the most posterior position in germarial region 3 (Theurkauf et al., 1993; Carpenter, 1975). As exemplified by the number of different arrests of oocyte differentiation observed in the different *Bic-D* and in the *egl* alleles, oogenesis can be reverted at a number of different steps. Furthermore, the progression of later oogenesis events does not necessarily require that earlier ones have taken place. In the oogenesis mutant *Bic-D*^{PA66}, the early oocyte-specific accumulation of *Bic-D* and *orb* RNA is blocked, but nevertheless later events such as oocyte-specific accumulation of *osk* RNA and the movement of the oocyte to the posterior end of the egg chamber are initiated and proceed through the first vitellarial stages (Suter and Steward, 1991; Lantz et al., 1992; Fig. 5A, this work).

In a setting of a gradually progressing patterning mechanism, levels of oocyte activity vary between the cells of a cyst. Similarly, levels of these activities probably also change in a given cell during the course of oogenesis. Different oogenesis events, which respond to the same oocyte activity, could be initiated independently of each other at different stages if they require different levels of oocyte activity. An additional level of control that may add to the dynamics and plasticity of the patterning mechanism could be that some of the molecules and processes that depend on oocyte activities increase their responsiveness to these factors over time and thus respond to lower levels of oocyte factors at later stages. Both mechanisms have to be postulated to explain the entire set of different phenotypes observed. In such a situation, *osk* RNA would appear to be very sensitive to *Bic-D* and oocyte factors and it would have a high affinity to the localizing system. *K10* RNA, in contrast, may be an example of a factor that depends on high levels of *Bic-D* activity and may increase its responsiveness during oogenesis. *K10* RNA is expressed in the germarium but does not specifically accumulate in the oocyte until the egg chamber leaves the germarium. This localization does not take place in the hypomorphic oogenesis mutants (Suter and Steward, 1991; Cheung et al., 1992). If such a differential responsiveness is indeed crucial for oogenesis, one gradient of information could promote different early oogenesis events and induce different cell fates, and it could explain such puzzling phenomena as why both 4-ring canal cells still remain in meiosis (Koch et al., 1967; Carpenter, 1975) while oocyte markers preferentially accumulate in a single cystocyte.

A cytoplasmic transport mechanism that is capable of asymmetrically localizing macromolecules amongst the cystocytes would probably depend on an asymmetric organization of a cytoskeleton that provides for a directed transport of oocyte factors into the oocyte. An asymmetrically organized cytoskeletal structure is in place at the time when the different molecules become localized to the oocyte in germarial region 2A. In a subset of the germarial region 2A cysts, the microtubule cytoskeleton reorganizes and forms bundles that extend from the oocyte into the other cystocytes (Theurkauf et al., 1993). It is tempting to speculate that this microtubule system serves as the track for the transport of some of these RNAs. Concentrations of microtubule drugs that dissociate this cytoskeleton also prevent the maintenance of oocyte-specific accumulation of mRNAs and cause the formation of egg chambers containing no oocyte but 16 nurse cells (Koch and Spitzer, 1983; Theurkauf et al., 1993). A number of additional observations also suggest that *Bic-D* and the microtubule cytoskeleton interact in localizing RNAs and oocyte factors to one of the four ring canal cells. *osk* mRNA accumulation in the oocyte is not only less affected than other RNAs by reductions in *Bic-D* activity, it is also less sensitive to microtubule disruption (Theurkauf et al., 1993; Fig. 5A,B, this work). This remarkable correlation suggests that the microtubule cytoskeleton and *Bic-D* may collaborate in the localization of *osk* RNA. In addition, *Bic-D* is required for the reorganization or the stability of this microtubule cytoskeleton: In the *Bic-D* oogenesis mutants, *Bic-D*^{PA66} and *Bic-D*^{R26}, no reorganized microtubule network was found and most RNAs do not accumulate in the pro-oocyte (Theurkauf et al., 1993; Suter and Steward, 1991). However, the analysis of *osk* RNA distribution also points to the possibility that there may be another cytoskeletal system that is involved in RNA localization. The initial oocyte-specific accumulation of *osk* RNA is normal in the *Bic-D* oogenesis mutants, even though no stable reorganization of the microtubule cytoskeleton is observed in them. This indicates that the localization of *osk* RNA is either accomplished by a different localization system or that levels of microtubules which could not be visualized are sufficient for the localization.

The zygotic null phenotype

The *Bic-D*^{null} phenotype is zygotic lethality and *Bic-D*^{null} flies die as pupae or young adults. An interesting aspect of the *Bic-D*^{null} phenotype is that, even though the phenotype is fully penetrant and null animals invariantly die, the lethal period varies considerably with environmental conditions and genetic background. This is surprising because a clean phenotype may be expected to result from a complete lack of a gene. However, dependence on temperature or other environmental conditions is not unique to *Bic-D* and flies, it has also been described in *Caenorhabditits elegans* where there is precedence for genes and developmental processes that are temperature sensitive either in the wild type or in the absence of a certain gene (Capowski et al., 1991; and references therein).

In agreement with the zygotic lethality is the finding that *Bic-D* is not only expressed during oogenesis but also at virtually all developmental stages (Suter et al., 1989). An interesting task for the future will be to find out which zygotic processes require *Bic-D* function and whether these processes rely on *Bic-D*-dependent localization of determinants similar to the determination of an oocyte. The zygotic processes that require *Bic-D* not only offer a possibility to unravel new aspects of the cytoplasmic localization processes, which may be simpler to address in those systems, but they may also show whether similar localization mechanisms are also used in patterning cells that do not share an extended common cytoplasm as the cystocytes or the syncytial preblastoderm embryo.

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