The role of BicD, Egl, Orb and the microtubules in the restriction of meiosis to the *Drosophila* oocyte

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**SUMMARY**

The oocyte is the only cell in *Drosophila* that goes through meiosis with meiotic recombination, but several germ cells in a 16-cell cyst enter meiosis and form synaptonemal complexes (SC) before one cell is selected to become the oocyte. Using an antibody that recognises a component of the SC or the synapsed chromosomes, we have analysed how meiosis becomes restricted to one cell, in relation to the other events in oocyte determination. Although BicD and egl mutants both cause the development of cysts with no oocyte, they have opposite effects on the behaviour of the SC: none of the cells in the cyst form SC in BicD null mutants, whereas all of the cells do in egl and orb mutants. Furthermore, unlike all cytoplasmic markers for the oocyte, the SC still becomes restricted to one cell when the microtubules are depolymerised, even though the BicD/Egl complex is not localised. These results lead us to propose a model in which BicD, Egl and Orb control entry into meiosis by regulating translation.

Key words: Synaptonemal complex, Oocyte determination, Meiosis, Microtubule, Recombination, Early oogenesis

**INTRODUCTION**

In region 1 of the *Drosophila* germarium, a germline stem cell divides asymmetrically to produce a cystoblast, which then divides four times with incomplete cytokinesis to give rise to a cyst of 16 cells connected to each other by cytoplasmic bridges called ring canals (Spradling, 1993). During these divisions, a cytoplasmic structure called the fusome anchors one pole of each mitotic spindle, and therefore ensures that cells follow an invariant pattern of division (Lin et al., 1994; Lin and Spradling, 1995). This leads to the formation of a symmetric cyst containing two cells with four ring canals, two cells with three, four cells with two and eight cells with one. All these events define the region 1 of the germarium (King, 1970). The cyst then enters region 2a, where the two cells with four ring canals start differentiating as pro-oocytes, by entering meiotic prophase I and accumulating centrioles (King, 1970; Carpenter, 1975). These cells are referred to as pro-oocytes because one of them invariably becomes the oocyte and starts accumulating specific factors such as BicD, Egl, Orb and Cup proteins and *oskar* and *BicD* mRNAs (Suter et al., 1989; Wharton and Struhl, 1989; Ephrussi et al., 1991; Kim-Ha et al., 1991; Lantz et al., 1994; Keyes and Spradling, 1997; Mach and Lehmann, 1997). When the cyst goes from region 2a to 2b, it flattens to form a 1-cell thick disc that extends across the whole width of the germarium. Oocyte-specific factors are now concentrated into the oocyte and an MTOC is clearly seen in this cell (Theurkauf et al., 1993) whereas the ‘losing’ pro-oocyte reverts to a nurse cell fate. At the same time, somatic follicle cells start to migrate and surround the cyst. As the cyst moves down to region 3, it rounds up to form a sphere with the oocyte always lying at the posterior pole.

It is not known how one of the pro-oocytes is chosen to become the oocyte, but two models have been proposed. In the first, it is suggested that the oocyte is determined as early as the first division of the cystoblast, when one of the two daughters inherits the spectrosome, a small spherical precursor of the fusome (Lin et al., 1994; Theurkauf, 1994; Lin and Spradling, 1995). In support of this model, it has been shown that this first asymmetry is propagated by an asymmetric genesis of the fusome at each subsequent division (de Cuevas and Spradling, 1998). A second model is based on the symmetric behaviour of the two pro-oocytes until region 2a of the germarium. In this model, both cells would have the same probability of becoming the oocyte, but the two cells compete for this fate. It has been suggested that the deciding factor between the two could be their advancement into meiosis (Carpenter, 1975, 1994). The more advanced of the two cells wins the competition and becomes the oocyte, while the other cell reverts to the nurse cell fate.

*BicD* and *egl* are believed to act close to the determination step of the oocyte, because *BicD* null and *egl* mutant cysts contain no oocyte and 16 nurse cells, and all oocyte-specific markers fail to accumulate in one cell (Schüpbach and Wieschaus, 1991; Suter and Steward, 1991; Ran et al., 1994). In *egl* mutant flies, all cells of the cyst start to behave like pro-oocytes and reach the pachytene stage of meiosis, before they
revert to the nurse cell fate (Carpenter, 1994). This has led to the proposal that ‘meiosis entry factors’ or ‘oocyte determinants’ are initially present in all 16 cells of the cyst, and that Egl and BicD, which are part of the same protein complex, somehow act to concentrate these factors in the oocyte (Suter and Steward, 1991; Carpenter, 1994; Mach and Lehmann, 1997). One of the earliest markers for the oocyte is the formation in this cell of a Microtubule Organising Centre (MTOC), which nucleates a microtubule network that extends through the ring canals into the other cells of the cyst (Theurkauf et al., 1993). When this microtubule cytoskeleton is depolymerised by drug treatments, all known markers for the oocyte fail to accumulate in one cell, and the cysts develop 16 nurse cells and no oocyte, mimicking the BicD and egl phenotypes. Furthermore, the MTOC is never formed in BicD\textsuperscript{R26} cysts, and is not maintained in egl cysts. These observations suggest that the oocyte determinants are transported along microtubules into one cell to specify oocyte fate. BicD and Egl proteins are amongst the first proteins to localise to the oocyte and remain localised where the microtubules are most concentrated at all stage of oogenesis, supporting the idea that they play a role in either organising the microtubule cytoskeleton or in the transport process itself (Suter and Steward, 1991; Theurkauf, 1994; Mach and Lehmann, 1997).

The first visible sign of synapsis between homologous chromosomes appears in the second or third 16-cell cyst in region 2a, with the formation of a proteinaceous tripartite structure called the synaptonemal complex (SC) (King 1970; Carpenter, 1975). The SC initially forms in the two pro-oocytes only. These cells then progress to the pachytene stage, where the SC reaches its maximum length, and the two cells with three ring canals also enter meiosis and form a zygotene-like SC. Soon afterwards, the SC disappears from the cells with three ring canals, but the pro-oocytes contain an identical SC throughout the rest of region 2a. This structure then disappears from the ‘losing pro-oocyte’ when the cyst enters region 2b. The oocyte remains in meiosis, and the SC compacts to form the karyosome, before it disappears soon after the cyst leaves the germarium.

Although very little is known about the relationship between meiosis and oocyte selection, recent data suggest that the two processes are linked. The spindle (spn) group loci were originally identified because mutants in these genes disrupt several symmetry-breaking steps later in oogenesis, such as the positioning of the oocyte at the posterior of the cyst, and Gurken signalling to polarise the anterior-posterior and dorsal-ventral axes (González-Reyes and St Johnston, 1994; Gonzalez-Reyes et al., 1997). However, it has recently been found that two of these genes, spnB and okra, encode homologues of the yeast DMC1 and RAD54 proteins, which are both involved in repairing dsDNA breaks during meiotic recombination (Story et al., 1993; Kooistra et al., 1997; Ghabrial et al., 1998). Furthermore, the patterning defects of spnB, C and D mutants are suppressed by mei\textsuperscript{W68} mutants, which block the generation of the dsDNA breaks that initiate recombination, and also by mutations in mei-41, which is a component of the DNA damage checkpoint pathway (Ghabrial and Schüpbach, 1999). These results indicate that the primary defect in these mutants is a failure to repair the DNA breaks formed during recombination, and that the later phenotypes are consequence of the activation of the meiotic checkpoint pathway. In spn double mutant combinations and in strong alleles of spnC, there is a delay in the localisation of BicD protein to the oocyte, suggesting that the checkpoint also inhibits oocyte determination.

Up until now, it has only been possible to observe meiosis by following the SC in the electron microscope. Here we report the identification of an antibody that marks the formation of the SC. This has allowed us to follow the pattern of meiosis and the localisation of cytoplasmic markers for the oocyte at the same time, and therefore to correlate the nuclear and cytoplasmic events in oocyte determination in both wild-type and mutant cysts. These results challenge the simple microtubule transport model for the determination of the oocyte, and lead us to propose an alternative mechanism for how meiosis becomes restricted to one cell.

MATERIALS AND METHODS

Flies used during this study

The following mutants were used in the course of this study: C (3)G\textsuperscript{17}, C (3)G\textsuperscript{G88} and Df (3R) C (3)G\textsuperscript{2} (Gowen, 1933; King, 1970; gift from Scott Hawley); insc\textsuperscript{22} (Burchard et al., 1995), a protein null allele (W. Chia and R. Renkawitz-Pohl, 1999), egl\textsuperscript{R26} and egl\textsuperscript{W50} (Schüpbach and Wieschaus, 1991), BicD\textsuperscript{R26} (Mohler and Wieschaus, 1986), BicD\textsuperscript{5} and BicD\textsuperscript{D} (Ran et al., 1994), spnA\textsuperscript{050}, spnA\textsuperscript{057}, spnB\textsuperscript{056}, spnC\textsuperscript{094}, spnD\textsuperscript{440}, spnE\textsuperscript{168} and spnD\textsuperscript{P4A} (for more details see González-Reyes et al., 1997, Tearle and Nusslein-Volhadr, 1987), orb\textsuperscript{net} and orb\textsuperscript{P43} (Christerson and McKearin, 1994; Lantz et al., 1994) and vas\textsuperscript{PH165} (Styhler et al., 1998).

Generation of clones

We used the FLP/FRT technique to generate homozygous clones of insc\textsuperscript{22} and BicD\textsuperscript{5} (Chou and Perrimon, 1996), which were marked by the loss of a nuclear GFP. The FRT40A-GFP chromosome is a kind gift from Stefan Luschnig (Tubingen).

Drug treatment

Flies were starved for 5 hours and then fed with 200 μg/ml colcemid (Sigma) mixed with some dry yeast and sucrose. The flies were treated for 12, 16, 24, 48 and 72 hours, and then dissected immediately. The colcemid was changed every 24 hours to ensure that it remained active throughout the treatments.

To monitor the progression of cysts through the germarium in the presence of colcemid, we measured the number of postgermarial cysts with 16 nurse cells and no oocyte at each time point, by staining with Oligreen (Molecular Probes) to label the polyplody nuclei of the nurse cells. We found an average of 1.3 cysts with no oocyte per ovariole after 24 hours of treatment (n=20); 2.7 cysts/ovariole after 48 hours (n=26) and 4 cysts/ovariole after 72 hours (n=21). Thus, the cysts still leave the germarium at a constant rate in the presence of colcemid, with a new cyst emerging every 18 hours.

Staining procedures

Antibody stainings, rhodamine-phalloidin staining and in situ hybridisations were performed as previously described (St Johnston et al., 1991; Gonzalez-Reyes and St Johnston, 1994). We used the rabbit anti-Insc antiserum to label the SC at 1/100 dilution (Kraut et al., 1994) and rabbit anti-Insc antiserum to label the SC at 1/1000 dilution (Kraut et al., 1994) and rabbit anti-Insc antiserum to label the SC at 1/1000 dilution (Kraut et al., 1994) and mouse anti-Orb (Lantz et al., 1994) at 1/20 dilution and mouse anti-Orb (Lantz et al., 1994) at 1/100 dilution. Secondary antibodies conjugated to Cy5, FITC and Alexa-568 were used at 1/200 dilution (Molecular Probes).
RESULTS

A marker for the synaptonemal complex

In the course of a study on the role of inscuteable (insc) during oogenesis, we found that the insc antibody recognises a nuclear structure that is present in some of the germ cells of in regions 2a to 3 of the germarium (Fig. 1A). However, this staining does not disappear in germline clones of protein null allele, insc22, indicating that it is due to a cross-reaction of the antibody (Knirr et al., 1997). Nevertheless, the staining pattern is very reminiscent of that expected for a component of the synaptonemal complex, and we therefore decided to analyse it further, since this would be the first marker identified for this structure in Drosophila.

Several lines of evidence indicate that the antiserum does indeed label the synaptonemal complex or a component associated with its formation. Firstly, the nuclear staining colocalises with DNA (data not shown), and has a morphology that corresponds exactly with the observed behaviour of the SC in electron micrographs (Carpenter, 1975). The staining is dotty in very early region 2a when the SC starts to form, becomes more thin and thread-like when the chromosomes are fully synapsed, and then becomes more compact in region 3, when the meiotic chromosomes condense to form the karyosome (Fig. 1A,B). Secondly, this structure first appears at the stage when the cysts enter into meiosis. The mitotic cysts in region 1 of the germarium express Bam protein, but this disappears after the final division when the cysts move from region 1 to region 2a of the germarium. The nuclear staining is only detectable in cysts that no longer show any Bam expression, indicating that it labels a postmitotic structure (Fig. 1C). Thirdly, the spatial distribution of the signal within the cyst precisely follows that described for the SC at the EM level (Carpenter, 1975). As described in more detail below, the signal first appears in two cells in early region 2a and spreads to four cells per cyst in the middle of 2a, before it is restricted to two cells, and finally to one cell in region 2b (Fig. 1A,B). As a final experiment, we stained ovaries from females that are mutant for C (3)G, since these are the only characterised mutants that completely abolish the formation of the synaptonemal complex at the electron microscope level (King, 1970). C (3)G has recently been found to encode the fly homologue of yeast Zip1 and mammalian SCP1, which are components of the transverse filament of the SC, and the effects of the C (3)G mutation on the SC are therefore likely to be direct (Szauter and Hawley, personal communication). The nuclear structure stained by this antibody is absent in C (3)G1/ C (3)G1 and C (3)G68/Df C (3)G2 cysts, even though the localisation of Orb protein to the oocyte occurs normally (Fig. 1D). Thus, the antibody acts as a marker for the formation of the SC. Although we do not know the molecular nature of the epitope recognised,
it maybe a component of the SC itself or some factor associated with the synapsed meiotic chromosomes.

A detailed analysis of the behaviour of the SC in comparison to that of cytoplasmic markers for oocyte determination, such as Orb and Bic-D proteins, reveals a number of distinct steps in the restriction of oocyte fate to one cell (Fig. 1E,E'). The SC first appears in early region 2a cysts in the nuclei of two cells, which are presumably the pro-oocytes. The punctate appearance of the SC suggests that they are at the zygotene stage of meiotic prophase 1. The next one or two cysts per gerarium have four cells in synapsis. Two of these cells have four ring canals (the pro-oocytes) and contain an almost continuous SC, typical of the pachytene stage, while the two cells on either side, presumably the cells with three ring canals, contain a zygotene-like SC. In middle of region 2a, the SC disappears from the two cells with three ring canals, but the two pro-oocytes still have complete SCs, and accumulate Orb and Bic-D proteins. Soon afterwards, Orb and Bic-D become concentrated in only one of these cells, providing the first sign that this pro-oocyte has been selected to become the oocyte. However, the SC still appears identical in both pro-oocytes at this stage (n=62). The SC disappears from one pro-oocyte as the cyst enters region 2b, and the cell that remains in meiosis is always the one that has already accumulated Orb or Bic-D. Finally, SC becomes more compact in region 3 and a hole forms in its middle, before it disappears soon after the cyst leaves the gerarium.

This comparison of the behaviour of nuclear and cytoplasmic markers for the oocyte reveals two important features about how oocyte fate becomes restricted to one cell. Firstly, the two pro-oocytes are already different from the other 14 cells in the cyst in early region 2a, as they both start to form SC at this stage. Bic-D and Orb only accumulate in these cells in mid 2a, about two cysts further down the gerarium. Secondly, Orb and Bic-D become restricted to the oocyte before any sign of oocyte identity can be deduced from the behaviour of the SC.

**BicD and egl have opposite effects on the synaptonemal complex formation**

Mutations in egl and BicD result in the formation of cysts with 16 nurse cells and no oocyte (Suter et al., 1989; Schüpbach and Wieschaus, 1991; Suter and Steward, 1991; Carpenter, 1994; Mach and Lehmann, 1997). Furthermore, Egl and BicD are part of the same protein complex, suggesting that they act together in the specification of oocyte identity (Mach and Lehmann, 1997). An EM analysis of the egl phenotype has shown that all 16 cells in a cyst enter meiosis, reach full pachytene and then revert to the nurse cell fate (Carpenter, 1994). Since the behaviour of the SC in BicD mutants has not been analysed at the EM level, we took advantage of our marker for this structure to examine whether these mutants have the same effect on meiosis as egl mutants.

We first examined the behaviour of the SC in egl mutant females, and observed that the SC forms with equal intensity in all 16 cells of the cyst in early region 2a, in agreement with the EM data (Fig. 2A) (Carpenter, 1994). All of the cells appear to be fully synapsed, but the SC looks somewhat thinner than in wild type (data not shown). The SC is not maintained, however, and disappears before the cysts reach region 2b.

Homozygotes for a hypomorphic mutation in BicD, BicD-R26, show a similar but weaker phenotype to egl mutants. More than four cells per cyst enter meiosis, but the cysts still...
retain a graded distribution of the SC, with the highest levels in the two pro-oocytes (Fig. 2B). The SC sometimes becomes restricted to these two cells in region 2b/3, but eventually disappears in region 3. Since BicD null alleles are semi-lethal, we examined the phenotype produced by the complete absence of BicD protein using the FLP/FRT system to generate homozygous germline clones that are marked by the loss of the nuclear GFP (Ran et al., 1994; Chou and Perrimon, 1996). While adjacent heterozygous cysts show a normal pattern of SC staining, almost no staining is visible in the nuclei of the mutant cysts (Fig. 2C-F). In some cases, a weak and diffuse staining is detected in the nuclei of all cystocytes in early region 2a, but this never shows the thread-like distribution that is typical of the SC. Thus, the BicD null mutation produces the opposite phenotype to egl mutants. All 16 cells behave identically in each case, but none of the cells form SC in the absence of BicD, whereas all of them reach the pachytene stage when Egl is removed.

**Microtubules are not required for the restriction of the synaptonemal complex to the oocyte**

The microtubule cytoskeleton is required for all known events in oocyte determination, since microtubule depolymerising drugs block the localisation to the oocyte of cytoplasmic factors, such as BicD protein and oskar mRNA, and lead to the development of cysts with 16 nurse cells (Theurkauf et al., 1993). We therefore examined whether microtubules are also required for the restriction of SC to one cell, by feeding adult females with high concentrations of the microtubule-depolymerising drug, colcemid, for various lengths of time. To test if this drug was disrupting the progression of the cysts through region 2 and 3, we monitored the production of cysts with 16 nurse cells and no oocyte, and found that a new cyst leaves the gerarium every 18 hours throughout the drug treatment (see Materials and Methods). Thus, the cysts still move posteriorly through the germarium at a constant rate in the presence of colcemid, although this is slightly slower than the 12 hours between cysts reported for untreated germinaria (King, 1970). However, this treatment does inhibit the production of new 16-cell cysts, presumably because microtubules are required for the mitoses that give rise to the cysts, and region 1 therefore shrinks during the treatment (Fig. 3E).

As previously observed by Theurkauf, the depolymerisation of the microtubules completely blocks the accumulation of BicD protein and oskar mRNA in the oocyte, and this is also the case for Orb protein (Fig. 3). In contrast, the SC still becomes restricted to a single cell of the cyst in region 2b of the gerarium after 16, 24, 48 and even 72 hours of treatment (Fig. 3A-C and data not shown). Furthermore, although cysts with two or more cells in synapsis are common in ovaries that have been treated for 16 hours, they are rarely seen after 24 or 48 hours.

Since region 2a usually contains 3-5 cysts, it takes between 56 and 90 hours for a newly formed 16-cell cyst to reach region 2b, where the SC is restricted to one cell. Thus, the cysts in region 2b after 24, 48 or 72 hours were in region 2a when the colcemid was added, and had several cells in synapsis. The SC can therefore become restricted to one cell in the absence of an intact microtubule cytoskeleton. The SC has disappeared from the cysts by the time that they enter region 3, however, indicating that microtubules are required for the maintenance of the oocyte identity.

**Meiotic and patterning defects in the spindle mutants**

Mutations in several of the *spindle* genes disrupt meiotic recombination, leading to the activation of a meiotic checkpoint pathway that inhibits a number of steps in the differentiation and patterning of the oocyte. In double mutant combinations, and in strong alleles of *spnC*, the first visible patterning defect is a delay in the localisation of BicD and Orb proteins to the oocyte (Gonzales-Reyes et al., 1997; Ghabrial and Schüpbach, 1999). We therefore performed double stainings for Orb and SC in homozygotes for mutants in each of the five *spn* loci, as well as in several double mutant combinations, to determine whether the nuclear events in oocyte determination are also affected (Fig. 4).

**Fig. 3.** The microtubules are not required to restrict the SC to the oocyte. (A) A wild-type gerarium after 16 hours of colcemid treatment. The SC (red) can be seen in several cells in the cysts in region 2a, and in one cell in region 2b, but is not maintained in region 3. Orb protein (green) is completely delocalised at all stages. (B,C) Wild-type gerarium after 24 and 48 hours of colcemid treatment stained for SC (red) and BicD protein (green) (B) and SC (red) and DNA (green) (C). More than 70% of the germinaria contain a cyst with the SC localised to one cell in region 2b. In region 2a, cysts with more cells in meiosis gradually disappear, since the treatment disrupts the formation of new cysts in region 1. (D,E) oskar mRNA distribution in untreated germinaria (D), and germinaria exposed to colcemid for 16 hours (E). The drug treatment prevents the localisation of oskar mRNA to the oocyte, indicating that the microtubules have been depolymerised.
In contrast to wild-type ovaries, where the SC is always restricted to the oocyte by region 2b, all of the *spn* mutants show a significant delay in this process, with cysts with more than one cell in synapsis in region 3 of the germarium and in stage 1 and stage 2 of the vitellarium (Fig. 4, Table 1). However, the chronology of oocyte determination is conserved, since Orb always accumulates in one cell before the SC (Fig. 4A,C). This phenotype is enhanced in the double mutant combinations, and is markedly more penetrant in the single mutants than the delay in the localisation of BicD and Orb to the oocyte (González-Reyes and St Johnston, 1998). Furthermore, in the strongest mutants, such as *spnC* homozygotes, cysts with four cells in meiosis are often found in region 2b of the germarium (Fig. 4C,D). This defect represents the earliest detectable phenotype of the *spn* mutants, and indicates that the meiotic checkpoint preferentially inhibits the nuclear steps in oocyte determination. Although they have very strong effects on the pattern of SC staining, the *spn* mutants do not affect the morphology of this structure, consistent with the conclusion of McKim et al. (1998) that SC formation precedes recombination.

*vasa* has been classified as a spindle-related gene because null alleles cause very similar defects in the positioning of the oocyte and in Gurken signalling (Styhler et al., 1998; Tinker et al., 1998; Tomancak et al., 1998). However, we found that the *vasa* null phenotype is different from that of the *spn* mutants in the germarium (Fig. 4E). Most mutant cysts show either a normal restriction of the SC to one cell, or no SC at all. Rare cysts show two cells in synapsis in region 2b, but most of the cysts have lost the SC by the time they reach region 3.

**Fig. 4.** *spindle* mutants delay in the restriction of the SC to the oocyte. (A) *spnA*057. A pachytene-like SC (red) is still present in both pro-oocytes in region 2b, and persists in region 3 although Orb (green) is localised to the oocyte by this stage. (B) *spnD*349. Both pro-oocytes still contain SC in region 2b, in the weakest phenotype shown by the single *spindle* mutants. (C) *spnD*349A057 double mutant, showing the SC in three cells in region 2b. (D) *spnC*094. Four cells contain SC in region 2b. Note the misplaced oocyte in region 3. (E) *vasa*PH165. These cysts show a variety of phenotypes, but the most common is a normal progression through meiosis until region 2b, when the SC often disappears from the oocyte.

**Fig. 5.** Orb is required for the oocyte determination. (A) *orb*mel homozygote. Both the SC (red) and Orb protein (green) are present in both pro-oocytes in region 2b. This delay in oocyte determination sometimes results in misplaced oocytes, as shown by the cyst in region 3. (B) *orb*F343. An Orb protein null mutant stained for SC. The cysts fail to go through the last mitotic division and contain only eight cells, but the SC can be detected in all nuclei. A projection of several confocal sections shows that all eight cells (presumably of the same cyst) have formed SC in the lower cyst.
Table 1. Frequency of cysts at each stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
<th>Stage 2</th>
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<tr>
<td></td>
<td>2 cells in synapsis</td>
<td>≥2 cells in synapsis</td>
<td>1 cells in synapsis</td>
<td>≥2 cells in synapsis</td>
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<tr>
<td>Wild type</td>
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<td>2 (1)</td>
<td>100 (31)</td>
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<td>47 (21)</td>
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<td>83 (20)</td>
<td>0 (0)</td>
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<td>76 (63)</td>
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<tr>
<td>SpnD349A057</td>
<td>54 (22)</td>
<td>46 (19)</td>
<td>0 (0)</td>
<td>100 (73)</td>
</tr>
</tbody>
</table>

Values are the percentage of cysts with the number of cells in synapsis indicated at the top of the column (n).

Although this phenotype is hard to interpret, it suggests that Vasa acts both before and after the restriction of the SC to one cell, but that it does not affect the same process as the spn genes.

In orb−−−− cysts all the cells enter meiosis

Some orb mutant alleles produce misplaced oocytes and defects in A/P and D/V axis formation that are similar to those caused by spn mutants (Christerson and McKearin, 1994; Lantz et al., 1994). The strongest alleles, orbF343 or orbΔcen, block the final division of the cystocytes to produce 8-cell cysts that usually degenerate. The weakest allele, orbmel, fails to localise osk and gurken mRNAs, giving rise to eggs with A/P and D/V axis defects.

Double staining for Orb and the SC shows that, like the spindle mutants, orbmel delays the restriction of the SC to one cell: both pro-oocytes can still be in synapsis and contain equal concentrations of Orb protein in region 2b (Fig. 5A). Although this allele has not previously been reported to affect oocyte positioning, this delay in oocyte determination can also give rise to misplaced oocytes, as it does in the spindle mutants (lower cyst in Fig. 5A).

In orbF343 homozygous germainia, all of the germ cells in region 2a seem to form a complete SC (Fig. 5B). Most mutant cysts degenerate in region 2, but we occasionally find clusters of cells in region 3, and these are presumably single cysts that have not degenerated, since they always contain eight cells. In these cases, all eight nuclei show strong SC staining typical of the pachytene stage. This phenotype is more reminiscent of a egl mutant cyst than a spindle cyst, and indicates that Orb protein is required to restrict meiosis to a subset of cystocytes.

DISCUSSION

The role of the microtubules in early oogenesis

Up until now, all known markers for oocyte differentiation that have been examined have required an intact microtubule cytoskeleton to become restricted to one cell in the cyst, but the synaptonemal complex provides the first exception to this rule. A cyst can progress through the normal pattern of SC localisation to one cell in the presence of high concentrations of colcemid. Although it is possible that the restriction of SC to one cell is mediated by a population of microtubules that are refractory to depolymerisation, we believe that this is unlikely because the colcemid concentrations that we used are much higher than those required to block the accumulation of all cytoplasmic factors in the oocyte. Thus, these data strongly suggest that the restriction of the SC to the oocyte as it moves from mid-region 2a to region 2b is independent of microtubules.

Although a 72 hour colchicine treatment depolymerises microtubules for long enough for a cyst to move from early region 2a to region 2b, we cannot exclude a role for the microtubules before these stages. For example, microtubule transport may be required in region 1 or early region 2a to set up an asymmetry that ultimately leads to the microtubule-independent restriction of the SC to the oocyte later on. If such a transport system exists, however, it must be different from the one that localises BicD, Orb and Egl proteins and oskar mRNA, because these localisations are completely abolished by colcemid treatment. Furthermore, the microtubule cytoskeleton of the cyst does not seem to be polarised until the middle of region 2a, when the first signs of intracellular transport appear with the migration of the centrioles (Mahowald and Strassheim, 1970; Theurkauf et al., 1993).

Although the microtubules are dispensable for the restriction of SC to one cell in region 2b, they are required for the maintenance of the SC as the cyst enters region 3. A clear MTOC can first be unambiguously identified in one cell in region 2b, and this microtubule network therefore seems to be necessary to stabilise oocyte fate. Microtubule- based transport must begin earlier in cyst development, however, since BicD, Orb and Egl proteins accumulate in a microtubule-dependent manner in both pro-oocytes in mid-region 2a and then the oocyte in late region 2a.

The initial asymmetry and the role of BicD, egalitarian and orb

Unlike the microtubules, BicD, orb and egl mutations disrupt all steps in the restriction of the SC to one cell, and this leads to two important conclusions. Firstly, BicD and Egl must have a function that is independent of microtubules, even though they are required for the establishment or maintenance of the MTOC in the oocyte (Theurkauf et al., 1993). Secondly, this function of BicD, Egl and Orb does not depend on their own localisation to the oocyte, since all three proteins are completely delocalised after colcemid treatments, yet the SC still becomes restricted to one cell.

Although both BicD and egl mutations give rise to cysts in which all 16 cells appear identical, they have different effects on the behaviour of the SC itself. In BicD null germine clones, none of the cells form a detectable SC, whereas all cells reach the full pachytene stage in egl mutants. Mach and Lehmann (1997) found that BicD and Egl are part of the same protein complex, and it is therefore surprising that they have opposite
phenotypes. Since the hypomorphic allele BicD<sup>R26</sup> gives a phenotype that is more similar to egl, one possible explanation for this difference is that the egl mutations still retain some residual activity. However, this appears not to be the case, as egl<sup>WU50</sup> has been described as a protein null, and the allelic combination that we examined (WU50/RC12) gives the same phenotype as WU50/Df (Carpenter, 1994; Mach and Lehmann, 1997). In light of these data, we favour the alternative explanation that BicD and Egl have different functions. BicD is required to enhance SC formation in the cells that normally enter meiosis, whereas Egl functions to repress SC formation in the other cells of the cyst. The strongest mutations in orb have a very similar effect on SC formation to egl mutants, suggesting that Orb protein is also involved in this repression. Given the colocalisation of Orb with Egl and BicD, it will be interesting to determine whether it is part of the same protein complex, as suggested by Mach and Lehmann (1997).

The discovery that the restriction of SC to one cell requires neither microtubules nor the localisation of BicD, Egl and Orb raises the question of how this asymmetry arises. It has previously been suggested that BicD and Egl function in the transport of meiosis promoting factors and oocyte determinants from the future nurse cells into the oocyte. Although this could still be the case if this transport occurs either very early in region 2a or along some non-microtubule cytoskeletal network, such as actin, this model cannot easily explain why BicD and egl mutations have opposite effects on SC formation. We therefore prefer an alternative model in which BicD, Egl and Orb are required to interpret a pre-existing asymmetry that is set up in region 1 (Theurkauf, 1994; Lin and Spradling, 1995). The divisions that give rise to the cyst are asymmetric with respect to the fusome, and recent data strongly suggest that this structure, or some factor associated with it, somehow marks the future oocyte (de Cuevas and Spradling, 1998). If this is correct, this unidentified mark could regulate the BicD/Egl complex, so that it performs different functions in the different cells of the cyst. For example, the Egl-dependent activity of the complex could repress SC in the cells that do not inherit the factor, and the BicD-dependent activity could enhance its formation in the cells that do, thereby explaining the different phenotypes of the null mutations in the two genes. It is interesting to note that BicD protein is phosphorylated, and that mutations that disrupt this phosphorylation give rise to egg chambers with 16 nurse cells (Suter and Steward, 1991). Thus, this post-translational modification could be responsible for the spatial regulation of the activity of the BicD/Egl complex.

Although our results suggest that BicD and Egl have functions that are independent of the microtubules, the nature of this activity is unclear. However, a number of lines of evidence suggest that these proteins may be involved in translational control. Firstly, BicD was originally identified because two single amino acid changes in the gene produce a dominant bicaudal phenotype in which oskar mRNA is misexpressed at the anterior of the oocyte (Mohler and Wieschaus, 1986; Suter et al., 1989; Wharton and Struhl, 1989; Ephrussi et al., 1991; Kim-Ha et al., 1991). Since Oskar translation is normally repressed unless the RNA is localised to the posterior pole, these mutant BicD proteins must not only trap oskar RNA at the anterior, but also relieve translational repression. Mutations in egl suppress the BicD gain-of-function phenotype, while extra copies of egl enhance it, indicating that the ectopic translation of oskar mRNA requires the formation of the BicD/Egl complex (Mohler and Wieschaus, 1986; Mach and Lehmann, 1997).

The second argument for a role of BicD and Egl in translational control comes from the discovery that orb null mutations give a very similar phenotype to egl mutants. Orb protein, which contains two RNA-binding motifs, has recently been shown to associate with the 3'UTR of oskar mRNA, and is required for its efficient translation (Lantz et al., 1992; Chang et al., 1999). Similarly, the Xenopus Orb homologue, CPEB, binds to elements in the 3'UTRs of a number of mRNAs, and induces the polyadenylation and translational activation of these mRNAs during oocyte maturation (Hake and Richter, 1994). Furthermore, the Spisula solidissima homologue plays a role not only in translational activation, but also in repression, since it binds to masking elements in the 3'UTRs of cyclin mRNAs to prevent their translation before fertilisation (Minshall et al., 1999; Walker et al., 1999). Thus, Orb functions as a regulator of translation, and can act as both a repressor and an activator in other species. This raises the possibility that the BicD/Egl complex exerts different effects in the cells of the cyst by controlling the inhibitory and activating functions of Orb. For example, Orb could repress the translation of factors required for SC formation in the future nurse cells, and activate their translation in the pro-oocytes and oocyte. If this model is correct, the selection of the oocyte would occur by a similar mechanism to the other asymmetries that are generated later in oogenesis, which are also based on the translational regulation of asymmetrically localised mRNAs, such as bicoid, gurken and oskar (van Eeden and St Johnston, 1999).

**Meiosis and oocyte development**

The behaviour of the SC indicates that the determination of the oocyte occurs in two steps. The two pro-oocytes must have been selected by early region 2a, because they already behave differently from the other 14 cells of the cyst at this stage, but the development of the cyst remains symmetric until the end of 2a, when BicD and Orb disappear from the losing pro-oocyte. Carpenter (1975) has proposed that the choice between the two pro-oocytes could depend on competition between these cells as they progress through meiosis, with the cell that is more advanced becoming the oocyte and then inhibiting its neighbour. However, our results argue against this model. Firstly, cytoplasmic factors, such as BicD and Orb, are concentrated in one cell before there is any visible difference between the SCs in the two pro-oocytes. Secondly, the cytoplasmic aspects of oocyte determination occur normally in C (3)G mutants, which completely lack the SC (Fig. 1D), and in meiW68 mutants (data not shown), which fail to initiate meiotic recombination (McKim et al., 1998). Thus, any competition between these two cells must be independent of SC formation and recombination.

Although meiosis is not required for oocyte determination, it can clearly influence this process, as demonstrated by the results on the spn genes. Several lines of evidence indicate that mutations in spnB, C and D disrupt the repair of dsDNA breaks during meiotic recombination, activating a checkpoint pathway that inhibits gurken mRNA translation and the formation of the karyosome (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999). Our results strongly suggest that this checkpoint also
inhibits the determination of the oocyte, as the SC becomes restricted to one cell much later than in wild type in these mutants. This phenotype also allows us to narrow down the time when recombination occurs. This process cannot begin until the SC forms in early region 2a, but the double-strand DNA breaks have to be repaired before the two cells with three ring canals exit meiosis, since this stage is delayed in spnC mutants, indicating that the checkpoint pathway has already been activated.

Activation of the meiotic checkpoint causes a change in the mobility of Vasa protein, leading to the suggestion that the patterning defects seen in spn mutants result from the inhibition of Vasa by this pathway (Ghabrial and Schüpbach, 1999). Our results show that the SC becomes restricted to one cell at the normal time in most vasa mutant cysts. Thus, the delay in oocyte determination in spn mutants cannot be a consequence of the inhibition of Vasa, suggesting that the checkpoint pathway has additional targets that control oocyte selection.

**A time-course for early oogenesis**

One problem in the study of cyst development in region 2 has been the difficulty in ordering the various developmental events that occur in this region. Using this marker for the SC, we have been able to follow the behaviour of this structure relative to the localisation of cytoplasmic factors like Orb and BicD, and to correlate these with the data from EM studies on the behaviour of the SC, and the centrioles (King, 1970; Mahowald and Strassheim, 1970; Carpenter, 1975). On the basis of this comparison, we can distinguish a number of distinct stages in the restriction of oocyte fate to one cell, which are depicted in Fig. 6 for a typical germarium: (1) The first cyst in region 2a (number 1) shows no sign of SC, but Bam protein has already disappeared. (2) The two pro-oocytes reach the zygotene stage of meiosis in early region 2a, and start to form SC (red) (number 2). (3) Soon afterwards, the two cells with three ring canals also form SC. The SC in the pro-oocytes has reached its maximum length, indicating that they have reached the pachytene stage. The dsDNA breaks generated during recombination must have already been repaired, since the meiotic checkpoint can arrest the pattern of SC staining at this stage. EM data also suggest that intracellular transport begins at this point, since the first signs of the migration of the centrioles (blue) towards the pro-oocytes can be seen when the two cells with three ring canals are in meiosis, and this may correlate with the first appearance of a focus of microtubules in the cyst in the middle of region 2a (Mahowald and Strassheim, 1970; Carpenter, 1975; Theurkauf et al., 1993). (4) The SC disappears from the two cells with three ring canals in middle of region 2a, but the two pro-oocytes still have complete SCs (number 4). Orb and Bic-D (green) start to accumulate in the pro-oocytes at this stage. The centrioles have migrated to either side of the largest ring canal, which connects the two pro-oocytes, and the first signs of ‘nutrient streaming’ appear, since elongated mitochondria can be seen inside the ring canals in electron micrographs. (5) All of the steps in cyst development so far are symmetric relative the largest ring canal, and the first asymmetry becomes evident in cysts numbers 5 and 6, when Orb and Bic-D become concentrated in one cell. The centrioles also start to move into the oocyte, and the largest ring canal is presumably open, because mitochondria can now be seen inside it. However, both pro-oocytes still contain an identical intact SC at this stage. (6) As the cyst enters region 2b, one pro-oocyte loses its SC and reverts to the nurse cell pathway of development (number 7). The pro-oocyte that remains in meiosis and becomes the oocyte is always the cell that has already accumulated Orb and Bic-D. The cytoplasm of the oocyte now contains all of the centrioles, BicD and Orb proteins, and an obvious MTOC, which nucleates microtubules (blue) that extend into the other 15 cells of the cyst. Thus, both the nucleus and cytoplasm of the oocyte are clearly different from the other cells of the cyst by this stage. Immediately afterwards, the oocyte starts to behave differently from the other cells in the cyst, as it moves to the posterior during the transition between region 2b and region 3 (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998). At the same time, the karyosome forms, and the SC becomes more compact, before disappearing soon after the cyst leaves the germarium.

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**Fig. 6.** A diagram summarising the different steps in the restriction of oocyte fate to one cell in region 2 of the germarium.
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