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α-spectrin is required for germline cell division and differentiation in the *Drosophila* ovary

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SUMMARY

During *Drosophila* oogenesis, developing germline cysts are spanned by a large cytoplasmic structure called a fusome, containing α-spectrin and the adducin-like product of the *hu-li tai shao (hts)* gene. We found that fusomes contain two additional membrane skeletal proteins: β-spectrin and ankyrin. *hts* was shown previously to be required for cyst formation and oocyte differentiation; the role of the fusome itself, however, and the organization and function of its other components, remains unclear. Using the FRT/FLP recombinase system to generate clones of α-spectrin-deficient cells in the ovary, we have shown that α-spectrin is also required for cyst formation and oocyte differentiation, but that its role in each process is distinct from that of Hts protein. Furthermore, α-spectrin is required for these processes in germline cells, but not in the follicle cells that surround each cyst. We have also found that the organization of membrane skeletal proteins is more dependent on α-spectrin in the fusome than at the plasma membrane in other cells. Our results suggest that the fusome and its associated membrane skeleton play a central role in regulating the divisions and differentiation of cyst cells.

Key words: spectrin, membrane skeleton, oogenesis, *Drosophila*, fusome

INTRODUCTION

The process of cyst formation has been studied extensively in insects (Büning, 1994). In the *Drosophila* ovary, cyst formation begins when a germline stem cell divides asymmetrically to produce a new stem cell and a cystoblast (Fig. 1A). The cystoblast, which immediately begins a program of cell division distinct from that of its sibling stem cell, divides exactly four more times to form a cluster of sixteen cells, called cystocytes. Because cytokinesis is incomplete at each division, the cystocytes remain connected by stable intercellular bridges known as ring canals. Following cyst formation, despite the fact that they share a commom cytoplasm, one of the cystocytes differentiates into an oocyte while the other fifteen develop as nurse cells. Various morphological and genetic studies suggest that polarized transport of mRNAs and other cytoplasmic components through the ring canals, probably along microtubules, is essential for oocyte differentiation (Mahowald and Strassheim, 1970; Koch and Spitzer, 1983; Suter and Steward, 1991; Theurkauf et al., 1992, 1993). The mechanisms controlling cyst formation, however, are not understood at the molecular level.

A large cytoplasmic structure called a fusome is associated with cyst formation in *Drosophila* and other insects (Telfer, 1975). The fusome was first described in the ovary of a diving beetle, *Dytiscus marginalis* (Giardina, 1901), and later in electron micrographs of *Drosophila* ovaries, where it appears as a distinctive region of cytoplasm rich in fibrils and vesicles but excluding mitochondria and most ribosomes (Mahowald, 1971; Storto and King, 1989). Stem cells and cystoblasts contain a large sphere of fusomal material, termed a spectrosome by Lin and Spradling (1995). During the four cystocyte mitoses, one pole of each spindle associates with the fusome, and following each mitosis, as the spindles disaggregate, additional fusomal material accumulates in their place. Thus, by the fourth division, the fusome forms one large branched structure that extends through the ring canals into all the cells in a cyst. The association of spindle poles with the fusome has been proposed to mediate the specific branching pattern of interconnections between cells that characterizes these cysts (Telfer, 1975).

Cysts are formed in an assembly-line fashion in the germarium, at the anterior tip of an ovariole. The germarium is conventionally divided into three regions (Fig. 1B). Region 1 contains 2-3 germline stem cells, cystoblasts, and mitotically active cysts of 2, 4 or 8 cells. In region 2, somatic follicle cells migrate in from the surface of the germarium and completely surround each 16-cell cyst, forming an egg chamber. Follicle cells are produced from a pair of somatic stem cells, which are located at the surface of the germarium in region 2 (Margolis and Spradling, 1995). Completed egg chambers subsequently

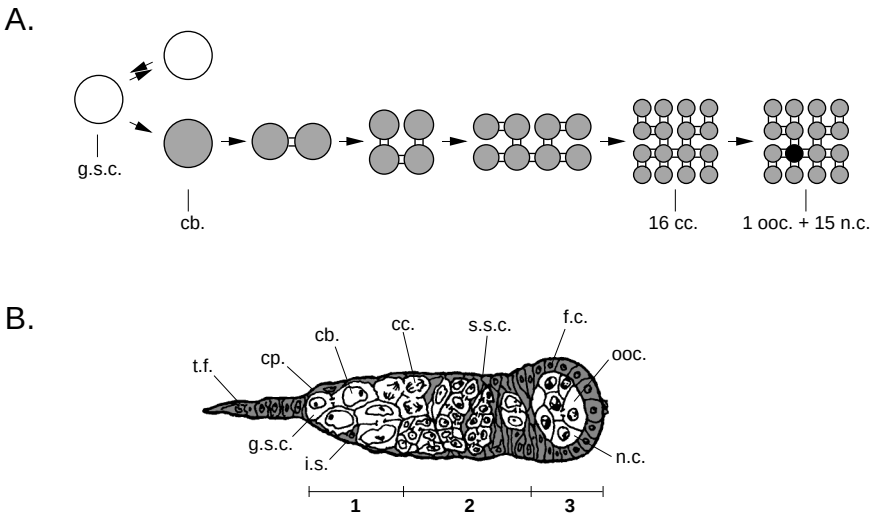

Fig. 1. Schematic diagrams of cyst formation and the germarium. (A) The pattern of germline cell divisions, from germline stem cell to differentiated 16-cell cyst, is illustrated. (B) The organization of cells in the germarium is shown in a drawing adapted from Margolis and Spradling (1995). Anterior is to the left. The germarium contains germline cells, shown here in white, and somatic cells, shown in grey. Germline cell types include germline stem cells (g.s.c.), cystoblasts (cb.), cystocytes (cc.), oocytes (ooc.), and nurse cells (n.c.). Somatic cell types include quiescent terminal filament cells (t.f.), cap cells (cp.), and inner sheath cells (i.s.), and mitotically active somatic stem cells (s.s.c.) and follicle cells (f.c.).

bud off from the germarium in region 3. Fusomes are present throughout region 1, but begin to regress as soon as a cyst stops dividing, in region 2, and disappear soon after. By region 3, the oocyte can be distinguished from its sibling nurse cells.

Nothing was known of the molecular composition of the fusome until recently, when two membrane skeletal proteins, α-spectrin and the adducin-like hu-li tai shao (Hts) protein, were shown to be fusomal components (Lin et al., 1994). Antibodies raised against these proteins stain the submembranous region of most cell types, as expected, but in stem cells and dividing cysts they specifically stain the spectrosome and fusome. Furthermore, Hts protein was shown to be required for cyst formation and oocyte differentiation. Flies bearing female sterile mutations in the *hts* gene produce cysts with a reduced number of cells and a reduced frequency of oocyte formation (Yue and Spradling, 1992). *hts*^{1} ovaries also completely lack a fusome: staining of fusomes with anti-Hts protein and anti-α-spectrin antibodies is abolished, and the distinctive cytoplasm that characterizes the fusome is missing in electron micrographs (Lin et al., 1994; Yue and Spradling, 1992). These studies showed that at least one membrane skeletal component of the fusome is required for cyst formation and oocyte differentiation. But whether Hts protein is required for these processes in the fusome in germline cells, or at the plasma membrane in other cells, was unclear. In addition, the role of other fusome components remained unknown.

The spectrin-based membrane skeleton has been characterized most extensively in erythrocytes, where its structure and function can be analyzed without interference from other cytoskeletal elements or organelles. Visualized by EM as an organized assembly of proteins, it is composed of a lattice of α- and β-spectrin and short actin filaments, which are attached to each other and to the plasma membrane by a handful of accessory proteins (reviewed by Bennett, 1990; Bennett and Gilligan, 1993). Membrane attachment is provided by ankyrin, which associates with β-spectrin, and by protein 4.1; these proteins in turn associate with integral membrane proteins including the anion exchanger and glycophorin C. Protein 4.1, as well as adducin, protein 4.9 (dematin), tropomyosin and tropomodulin, may also mediate the association of spectrin with actin, based on immunolocalization and in vitro binding experiments. The membrane skeleton is thought to function in erythrocytes by modulating the mechanical properties of the plasma membrane and by influencing the dynamic behavior of integral membrane proteins.

Proteins closely related to components of the erythrocyte membrane skeleton have been found in most other cell types in vertebrates, as well as in invertebrates, higher plants and *Dictyostelium*; however, their organization and function in these cells are more complex (Bennett and Gilligan, 1993). One novel function is in organizing discrete, specialized domains of plasma membrane, such as basolateral domains or sites of cell-cell contact in epithelial tissues (reviewed in Nelson, 1992; Shiel and Caplan, 1995). Moreover, in nonerythroid cells, membrane skeletal proteins are not always restricted to the plasma membrane. For example, isoforms of β-spectrin and ankyrin have been found in the ER and Golgi apparatus in a variety of cell types and may be coupled to Golgi organization and function (Beck et al., 1994; Devarajan et al., 1996). Also, in some populations of T lymphocytes, spectrin and ankyrin can occur in non-membrane bound aggregates away from the cell periphery, and may play a role in signal transduction within these cells (Lee et al., 1988; Gregorio et al., 1994). Understanding how membrane skeleton proteins are organized within the fusome and how they relate to fusomal membranous vesicles may reveal new roles of these proteins, and shed light on how the fusome functions in the development of germline cells.

To elucidate the organization and function of the fusome and its associated membrane skeleton, we have identified three more of its membrane skeletal components. We have also examined the role of one of its components, α-spectrin, in cyst formation and oocyte differentiation. Our results show that α-spectrin is required for these processes in germline cells but not in follicle cells, and indicate that the fusome and its associated membrane skeleton play a central role in regulating the formation of cysts.

MATERIALS AND METHODS

Drosophila strains and culture

All flies were maintained at 22-25°C on standard medium. The strains used to generate *α-spec*$^{-}$ clones are described briefly in the Results, and more fully in Lee et al. (1997). Parental flies were grown in vials

for 2 days and then removed; the vials, containing 0- to 2-day old larvae, were then heat shocked at 37°C for 30 minutes in a circulating water bath. Ovaries were dissected out of adult flies 1-3 days after eclosion.

Antibodies

Two antibodies were used to visualize α-spectrin: rabbit antibody 354 (referred to as 905 by Byers et al., 1987 and Pesacreta et al., 1989), which was used at a 1:200 dilution, and mouse monoclonal antibody 323 (referred to as M10-2 by Byers et al., 1987 and Pesacreta et al., 1989); cell culture supernatants from this clone were used at a 1:1 dilution. β-spectrin was labeled using rabbit antibody 337 (referred to as 089 by Byers et al., 1989) at a 1:200 dilution. All α- and β-spectrin antibodies were provided by E. Brandin and D. Branton. β_H-spectrin was labeled using rabbit antibody 243 (Thomas and Kiehart, 1994), which was a gift from G. Thomas, at a 1:500 dilution. Hts protein was visualized using a mouse polyclonal antibody generated by K. Cant and L. Cooley and described by Lin et al. (1994); this antibody was used at a 1:200 dilution. Anti-D4.1 monoclonal antibodies 566.9C and 615.16B (Fehon et al., 1994) were a gift from R. Fehon and were used at a 1:500 dilution. Affinity-purified rabbit anti-ankyrin antibodies, described by Dubreuil and Yu (1994) and Dubreuil et al. (1996), were a gift from R. Dubreuil and were used at a 1:100 dilution. Cell culture supernatants from the mouse monoclonal antibody 1B1 (Zaccai and Lipshitz, 1996) were a gift from M. Zaccai and H. Lipshitz and were used at a 1:1 dilution. This antibody was raised against a portion of the characterized Hts protein, but appears to recognize a related protein (see Results). Anti-phosphotyrosine antibody PY-20 was purchased from ICN Biomedicals and used at a dilution of 1:1000.

TRITC- or FITC-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and used at a 1:200 dilution.

Immunostaining and fluorescence microscopy

Ovaries were dissected in saline solution and teased apart to allow better penetration of the antibodies. They were fixed for 10 minutes at room temperature in Buffer B (16.7 mM KH_2PO_4/K_2HPO_4, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$, pH 6.8) plus 5% formaldehyde (Ted Pella Inc.), washed for 30 minutes in PBT (PBS: 10 mM NaH_2PO_4/Na_2HPO_4, 175 mM NaCl, pH 7.4, plus 0.1% Triton X-100), incubated for 30 minutes in PBT-NGS (PBT plus 5% normal goat serum; Jackson ImmunoResearch Laboratories), and then incubated overnight at 4˚C or room temperature in PBT-NGS containing primary antibodies at the appropriate dilution. Ovaries were then washed for 2 hours in PBT-BSA (PBT plus 0.2% BSA), incubated for 30 minutes in PBT-NGS and then for 4 hours in PBT-NGS containing fluorophore-conjugated secondary antibodies. The stained ovaries were washed in PBT for 2 hours at room temperature or overnight at 4˚C, rinsed in PBS, and mounted in PBS plus 50% glycerol and 2.5% 1,4-diazabicyclo-[2.2.2]octane (DABCO; Sigma) for microscopy.

To visualize nuclei, ovaries were incubated before the final wash either in 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1 μg/ml in PBT for 6 minutes, or in propidium iodide at 5 μg/ml in PBT for 20 minutes. (To remove RNA prior to propidium iodide staining, 0.4 mg/ml RNase A was added to the PBT-BSA wash step.) Actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes) at 0.4 U/ml in PBS for 20 minutes. All steps were performed at room temperature unless otherwise noted.

Stained ovaries were examined by epifluorescence and confocal microscopy on a Zeiss Laser Scan Microscope.

RESULTS

Identification of new fusome components

To determine the membrane skeletal protein composition of the fusome, we stained ovaries with antibodies shown previously to recognize other *Drosophila* membrane skeletal proteins, and examined them by immunofluorescence microscopy. Two isoforms of β-spectrin have been identified in *Drosophila*, β- and β_H-spectrin, and antibodies specific for each have been generated (Byers et al., 1989; Thomas and Kiehart, 1994) and used to study the distribution of each protein in fixed embryos (J.K.L., unpublished; Thomas and Kiehart, 1994). When wild-type ovarioles were labeled with anti-β-spectrin antibodies (Fig. 2A), we observed a staining pattern similar to that seen with anti-α-spectrin antibodies (Lin et al., 1994). In the

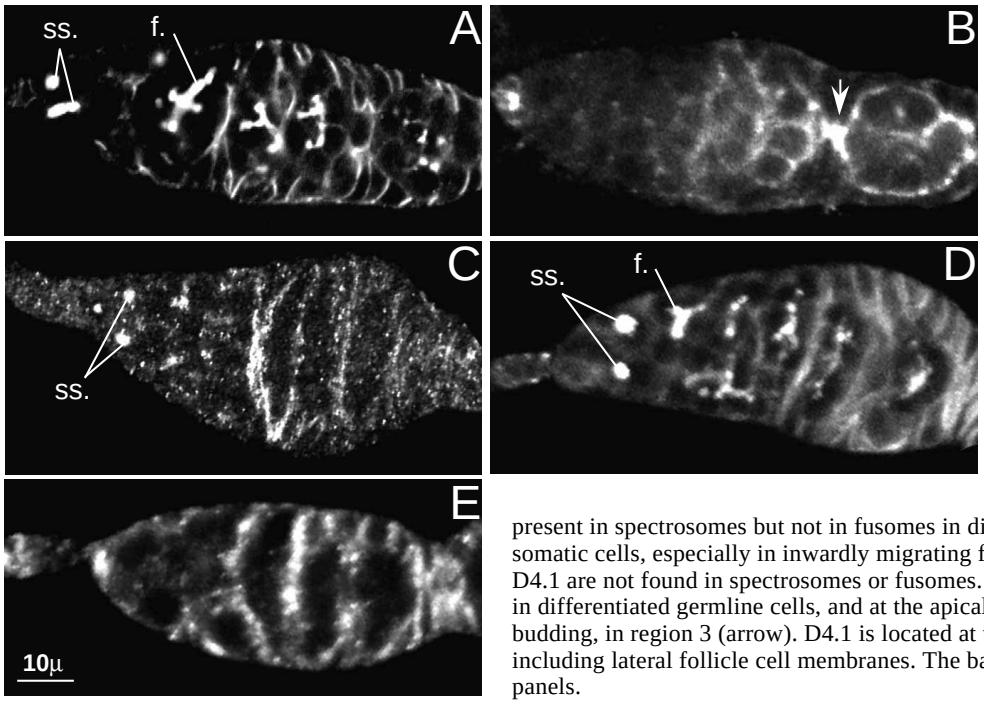

Fig. 2. Wild-type germaria stained with antibodies raised against β-spectrin (A), β_H-spectrin (B), ankyrin (C), or D4.1 (E), or with mAb 1B1 (D) (see text), and visualized by confocal microscopy. A and B are single confocal sections; C-E are stacks of confocal images. Anterior is to the left. β-spectrin and the protein recognized by mAb 1B1 are present in spectrosomes (ss.) and fusomes (f.) in germline stem cells and dividing cysts, and at the membrane in somatic cells, including terminal filament, inner sheath, and follicle cells. (Only the most anterior fusome has been labeled in each panel.) Ankyrin appears to be present in spectrosomes but not in fusomes in dividing cysts; it is also present in somatic cells, especially in inwardly migrating follicle cells in region 2. β_H-spectrin and D4.1 are not found in spectrosomes or fusomes. β_H-spectrin is present at the membrane in differentiated germline cells, and at the apical end of follicle cells involved in budding, in region 3 (arrow). D4.1 is located at the membrane in somatic cells, including lateral follicle cell membranes. The bar in E shows the magnification for all panels.

germarium, spectrosomes and fusomes in germline stem cells and cysts were strongly labeled, and also the submembranous regions of terminal filament and inner sheath cells. Beginning in region 2, the submembranous regions of follicle cells were strongly labeled, and by region 3 nurse cell and oocyte membranes as well. Furthermore, like α-spectrin and Hts protein, β-spectrin was present only at very low levels at the membranes of germline stem cells and dividing cystocytes. Anti-β_H-spectrin antibodies, however, do not stain fusomes or spectrosomes (Fig. 2B). In the germarium, strong staining is seen in the submembranous regions of terminal filament and cap cells, nurse cells and oocytes, and at the apical end of follicle cells, especially those involved in budding in region 3 (see arrow in Fig. 2B). These observations suggest that fusomes contain only a single isoform of spectrin, composed of α and β subunits.

A single isoform of ankyrin has been identified in *Drosophila* (Dubreuil and Yu, 1994), and antibodies raised against it have been shown to label ankyrin in *Drosophila* S2 tissue culture cells (Dubreuil et al., 1996). In the germarium, these antibodies stained spectrosomes heavily only in stem cells; fusomes in dividing cysts were stained very weakly, if at all, above background levels (Fig. 2C). This staining pattern was verified by double-labelling germaria (data not shown) with anti-ankyrin antibodies and a monoclonal antibody, mAb 1B1, that heavily stains both fusomes and spectrosomes (see below). Anti-ankyrin antibodies also strongly labeled inwardly migrating follicle cells in region 2, and labeled more weakly the membranes of other follicle and somatic cells.

Another antibody that we tested is mAb 1B1, which was raised against a portion of Hts protein (residues 466-804) expressed and purified from *E. coli* (Zaccai and Lipshitz, 1996). This antibody recognizes a single band on western blots that is still present at high levels in *hts*^{1} flies, and that is not recognized by previously described anti-Hts protein antibodies (T. Smith-Leiker and L. Cooley, personal communication). A high level of staining is also present in *hts*^{1} ovaries examined by immunofluorescence microscopy. Therefore, mAb 1B1 appears to recognize a distinct Hts-related protein that differs in size from previously described isoforms of Hts. In wild-type ovarioles, the staining pattern of mAb 1B1 is similar to that seen with anti-Hts protein antibodies (Lin et al., 1994). Spectrosomes, fusomes, and the submembranous regions of nurse cells, oocytes, and follicle cells were strongly stained, and the protein was present only at low levels in the submembranous regions of stem cells and cystocytes (Fig. 2D).

We also stained ovaries with antibodies raised against the *Drosophila* homolog of protein 4.1, D4.1, which is encoded by the *coracle* gene and associates with septate junctions of epithelial cells (Fehon et al., 1994). In the germarium, these antibodies strongly label the submembranous regions of terminal filament and cap cells, inner sheath cells, and follicle cells, but they do not label spectrosomes or fusomes (Fig. 2E). In the rest of the ovariole, stalk cell and lateral follicle cell membranes were also heavily stained (data not shown). Two other members of the protein 4.1 superfamily in *Drosophila*, Dmoesin and Dmerlin, have been localized in ovarioles (McCartney and Fehon, 1996). Dmoesin is found at the membrane in follicle cells, and Dmerlin localizes to punctate structures at the membrane and in the cytoplasm of germline cells, but neither one is found in fusomes.

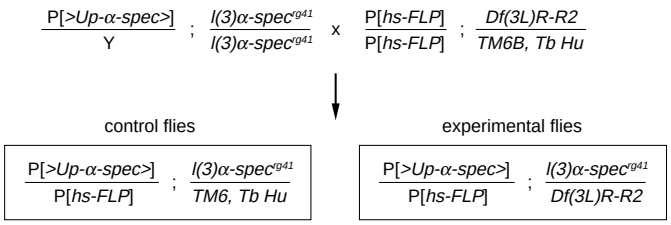

Fig. 3. The fly strains used to generate clones of α-spectrin-deficient (*α-spec*$^-$) cells. *l(3)α-spec*^{rg41} is a null allele of the gene encoding α-spectrin; flies that are null for α-spectrin die as first instar larvae (Lee et al., 1993). *Df(3L)R-R2* is a deficiency that uncovers this gene. *[>Up-α-spec>]* is a wild-type α-spectrin minigene, driven by the *Drosophila* ubiquitin promoter and flanked by FLP recombinase target sites (FRT's); this minigene fully rescues the lethality of flies that are null for α-spectrin. *[hs-FLP]* is a FLP recombinase minigene, driven by the *Drosophila* hsp70 promoter. Upon induction of FLP recombinase via heat shock, the α-spectrin minigene can be excised and *α-spec*$^-$ clones can be produced in the ovaries of experimental flies.

Generation of *α-spec*$^-$ clones

Mutations in the gene encoding α-spectrin were previously identified by Lee et al. (1993). Flies bearing a null allele of this gene, *l(3)α-spec*^{rg41}, die as first instar larvae. Thus, to examine its phenotype in the ovary, we had to generate clones of α-spectrin-deficient cells (*α-spec*$^-$ clones) using the heat shock-inducible FLP recombinase system (Golic and Lindquist, 1989). The strains of flies used to generate clones are described briefly in Fig. 3, and more fully in Lee et al. (1997). To induce FLP expression and clone formation, experimental and control flies were heat shocked as first instar larvae and their ovaries were dissected out 1-3 days after eclosion.

Because our heat shock protocol limited the time of clone induction to a brief period during the first instar when ovaries contain only a small number of precursor cells, we expected that any *α-spec*$^-$ clones recovered in the germline and follicle cells of adult egg chambers would be the product of *α-spec*$^-$ stem cells that developed from these precursors. The numbers and locations of stem cells in the adult ovary have been determined by clonal analysis (Schüpbach et al., 1978; Margolis and Spradling, 1995). Each ovariole contains approximately 2-3 germline stem cells and 2 somatic stem cells, which give rise to follicle cells. Thus, single stem cell clones should be restricted either to germline cells or to follicle cells, and to only a portion (one third or one half) of each cell type. The frequency of clone induction with this system is high, however, and some first instar precursor cells may give rise to more than one adult stem cell, so we also expected to find a much smaller number of ovarioles containing multiple stem cell clones, in which all of one or both cell types is *α-spec*$^-$.

To identify *α-spec*$^-$ clones, we stained ovaries with anti-α-spectrin antibodies and examined them by immunofluorescence microscopy. Our expectations of clone induction were confirmed by what we saw: in most ovarioles, clones were restricted to a portion of one cell type; some ovarioles lacked α-spectrin in all germline or follicle cells; and in a few ovarioles, α-spectrin was missing in both cell types. No clones were seen in ovaries from control flies, or in experimental flies that had not been heat shocked.

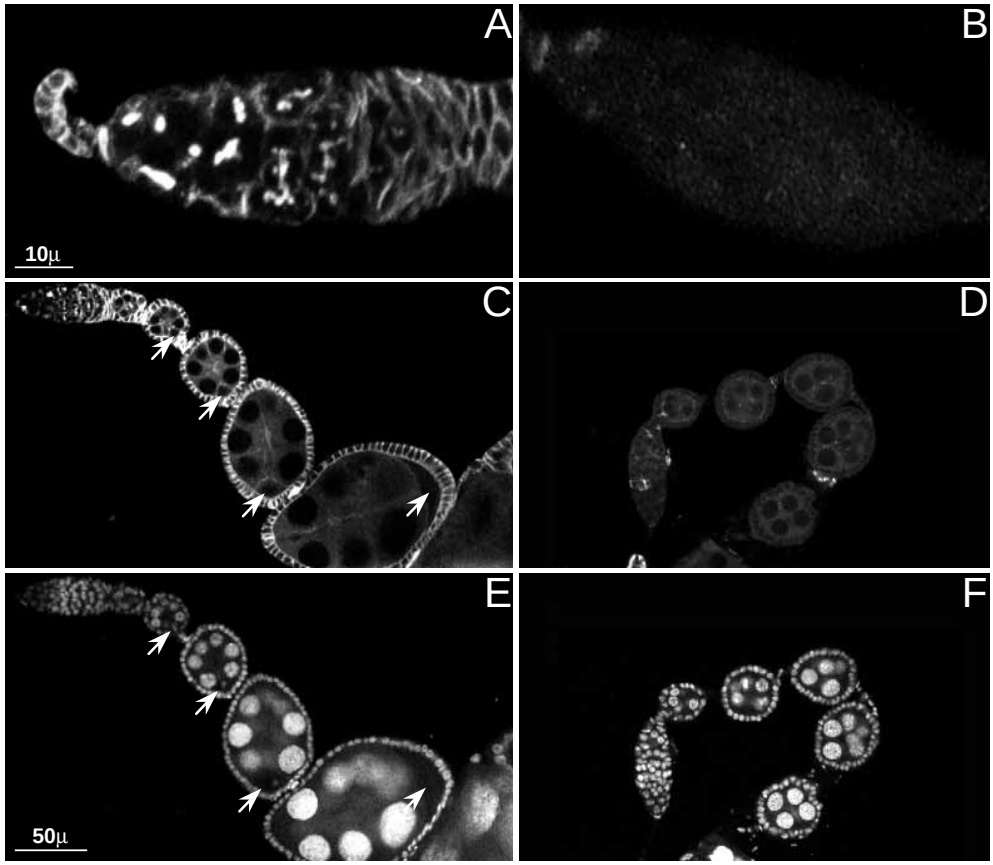

Fig. 4. Cyst formation is defective in *α-spec*⁻ ovarioles. Germaria (A-B) and ovarioles (C-F) are stained with anti-α-spectrin antibodies (A-D) or with propidium iodide to label DNA (E-F). Anterior is to the left in each panel. (A,C,E) In control ovarioles, which have retained α-spectrin in all cells, each cyst contains an oocyte and 15 nurse cells. (Not all cells are visible in the plane of focus of each image.) Oocytes (arrows) can be distinguished by their characteristic nucleus and/or by their uptake of yoke in later stages. α-spectrin is present in fusomes and at the membrane in oocytes, nurse cells, and somatic cells in these ovarioles. (B,D,F) In *α-spec*⁻ ovarioles, which are missing α-spectrin in both germline and follicle cells, most cysts contain fewer than 16 cells and do not contain an oocyte. α-spectrin staining is absent from fusomes and follicle cell membranes in these ovarioles. The bar in A shows the magnification for A and B; the bar in E for C-F.

α-spectrin is required in germline cells for cyst formation

To analyze the phenotype of α-spectrin loss in the ovary, we compared *α-spec*⁻ ovarioles, which were missing α-spectrin in both germline and follicle cells, to control ovarioles, which retained α-spectrin in both cell types. In control ovarioles, cyst formation looked completely normal; control egg chambers invariably contained 15 nurse cells and 1 oocyte, and progressed normally through oogenesis (Fig. 4A,C,E). In addition, in germaria that retained α-spectrin in both tissues, dividing cysts contained fusomes that were indistinguishable from wild-type fusomes. In *α-spec*⁻ ovarioles, however, cyst formation was inevitably disrupted (Fig. 4B,D,F). *α-spec*⁻ egg chambers almost always contained fewer than 16 cells and often lacked an oocyte, and most appeared to degenerate before completing oogenesis. α-spectrin staining was completely gone from fusomes and cell membranes in these ovarioles. These results show that α-spectrin is required for cyst formation and oocyte differentiation.

To determine if the defects in cyst formation were caused by loss of α-spectrin from germline cells, follicle cells, or both, we examined ovarioles that were missing the protein in only one of these cells types. In ovarioles that lacked α-spectrin only in germline cells, cyst formation was still severely disrupted; egg chambers contained fewer than 16 cells and often lacked an oocyte (Fig. 5A,C,E). α-spectrin staining in these ovarioles looked normal in follicle cells but was absent from fusomes and cell membranes in germline cells. In ovarioles that lacked α-spectrin only in follicle cells, however, α-spectrin staining in fusomes was indistinguishable from wild-type, and cyst formation appeared to proceed completely normally (Fig. 5B,D,F). Egg chambers in these ovarioles always contained 15 nurse cells and 1 oocyte. Furthermore, *α-spec*⁻ follicle cells appeared to be able to migrate in and pinch off cysts completely normally. These egg chambers often degenerated before completing oogenesis, however, presumably because of defects in follicle cell behavior in later stages (see Lee et al., 1997). These results show that α-spectrin is required only in the germ line for cyst formation and oocyte determination.

To characterize the cyst formation defects more fully, we counted the number of nurse cells and oocytes in egg chambers with *α-spec*⁻ germline cells. Several conclusions can be drawn from these data (Fig. 6; Tables 1, 2). First, whether or not they included an oocyte, *α-spec*⁻ cysts almost always contained an even number of cells. Also, oocytes were not seen in egg chambers that contained fewer than 8 cells and only rarely in

Table 1. Average cyst cell number in wild-type and mutant cysts

	Cyst genotype		
Cell type	Wild type	*α-spec*⁻*	*hts*[1]†
Cystocytes	16	8.4	4.3
Oocytes	1	0.18	0.01

1028 *α-spec*⁻ cysts were scored in this experiment.
*The genotype of the follicle cells surrounding each *α-spec*⁻ cyst was not determined.
†Data from Yue and Spradling (1992).

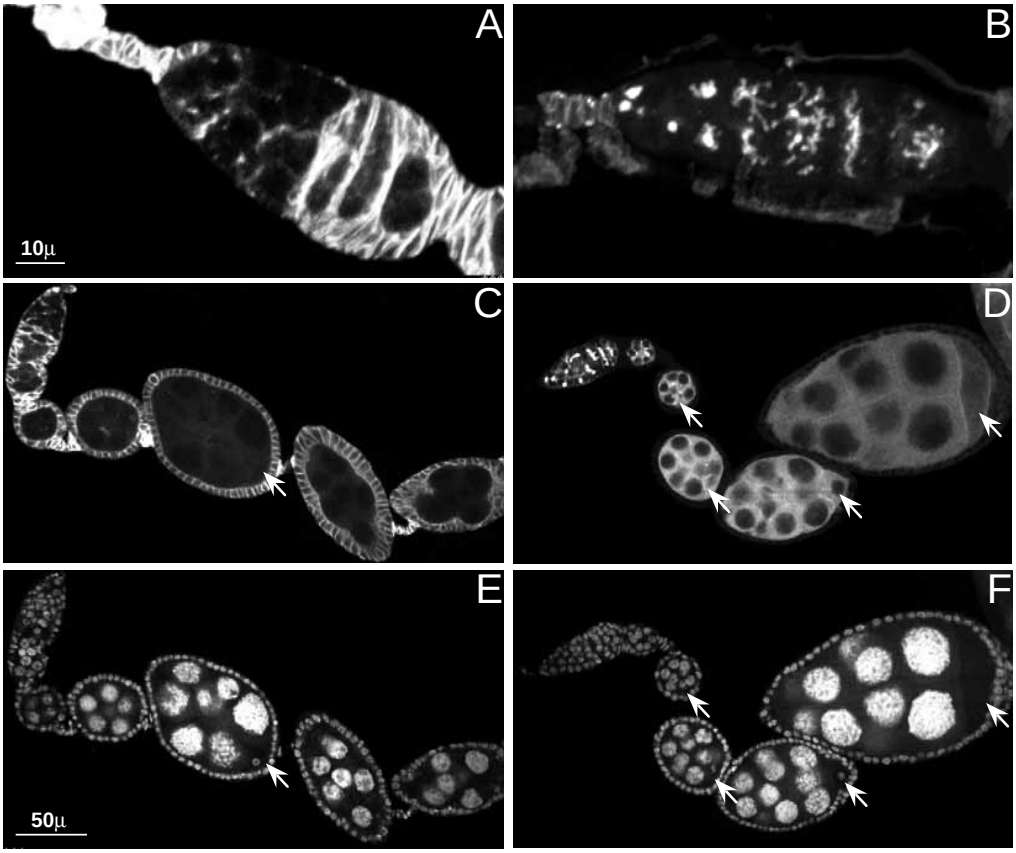

Fig. 5. Cyst formation requires α-spectrin only in germline cells. Germaria (A-B) and ovarioles (C-F) are stained with anti-α-spectrin antibodies (A-D) or with propidium iodide to label DNA (E-F). Anterior is to the left in each panel. (A,C,E) In ovarioles that are missing α-spectrin only in germline cells, most cysts contain fewer than 16 cells and do not contain an oocyte. Only one of the egg chambers in C and E contains an oocyte (arrow). In these ovarioles, α-spectrin is missing from fusomes and the membranes of germline cells, but is present in follicle cells. In region 1 of the germarium in (A), α-spectrin is also present in the inner sheath cells; this staining is not in fusomes. (B,D,F) In ovarioles that are missing α-spectrin only in follicle cells, each cyst contains an oocyte (arrows) and 15 nurse cells. (Not all cells are visible in the plane of focus of each image.) In these ovarioles, α-spectrin is present in fusomes and at the membrane in germline cells, but is missing from follicle cells. The bar in A shows the magnification for A and B; the bar in E for C-F.

those with fewer than 10 cells. Second, the number of cells per cyst and the frequency of oocyte formation were the same regardless of the presence or absence of α-spectrin in the surrounding follicle cells. In other words, cyst formation was unaffected by the phenotype of the follicle cells. And third, the phenotype of α-spectrin loss in the germ line is less severe than the loss of Hts protein; a more detailed comparison of the phenotypes is presented in the Discussion.

Another defect was occasionally seen in egg chambers that lacked α-spectrin in the germ line (data not shown). In some egg chambers with oocytes that developed to later stages of oogenesis, nurse cell cytoplasm was not transferred normally into the oocyte, resulting in a weak 'dumpless' phenotype. A similar defect was also seen in *hts*^{1} ovaries (Yue and Spradling, 1992).

Ring canals are normal in *α-spec*$^{-}$ cysts

Ring canals are formed by the sequential recruitment of proteins to arrested cleavage furrows between cystocytes (Robinson et al., 1994). In wild-type ovaries, they are composed of two layers: an outer rim that is laid down upon arrest of the cleavage furrow, adjacent to the plasma membrane, and an actin-rich inner rim, which accumulates shortly after the mitotic divisions of the cyst are complete. The *hts* gene encodes a component of the inner rim, and *hts*^{1} cysts have defective ring canals that fail to accumulate actin (Robinson et al., 1994; Yue and Spradling, 1992). To determine if α-spectrin is required for ring canal formation, we

Table 2. The effect of follicle cell genotype on average cyst cell number in *α-spec*$^{-}$ cysts

	Follicle cell genotype		
Cell type	*α-spec*$^{+}$	*α-spec*$^{-}$	*α-spec*$^{+}$ and *α-spec*$^{-}$
Cystocytes	8.7	8.4	8.3
Oocytes	0.21	0.17	0.17

Between 84 and 161 cysts were scored for each class.

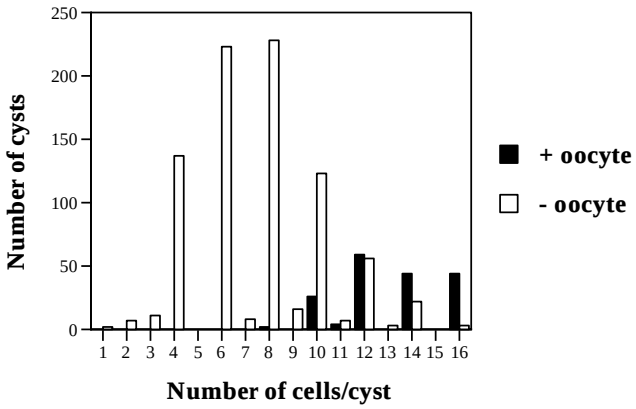

Fig. 6. Histogram of cell number in *α-spec*$^{-}$ cysts. Cysts that include an oocyte are shown in black; those that lack an oocyte are shown in white. The presence or absence of α-spectrin in the follicle cells surrounding each cyst was not scored. In about 2% of cysts, the presence of an oocyte was uncertain; these data are not included on the histogram.

stained ovaries containing *α-spec*⁻ clones with an anti-phosphotyrosine antibody, which labels at least one component of the outer rim (Robinson et al., 1994). Bright, distinct rings of staining were seen in *α-spec*⁻ germline cells that were similar to those seen in wild-type egg chambers (Fig. 7A,B). We also examined *α-spec*⁻ egg chambers stained with rhodamine-conjugated phalloidin to visualize filamentous actin. Although the level of staining at the membranes of *α-spec*⁻ germline and follicle cells was slightly reduced, the staining pattern in ring canals was similar in *α-spec*⁻ and wild-type egg chambers (Fig. 7C,D). We conclude from these results that ring canal formation is normal in the absence of α-spectrin.

In some mutants, follicle cells envelope more than one cyst, forming composite egg chambers. To determine the number of cysts within *α-spec*⁻ egg chambers, we compared the numbers of ring canals and cells (data not shown). The number of ring canals in each egg chamber was always one less than the number of cells, as in wild-type egg chambers (which always contain 15 ring canals, connecting 16 cells). This finding suggests that each *α-spec*⁻ egg chamber contains a single cyst.

Analysis of other membrane skeletal proteins in *α-spec*⁻ clones

In *hts*[1] germaria, fusomes in germline cells

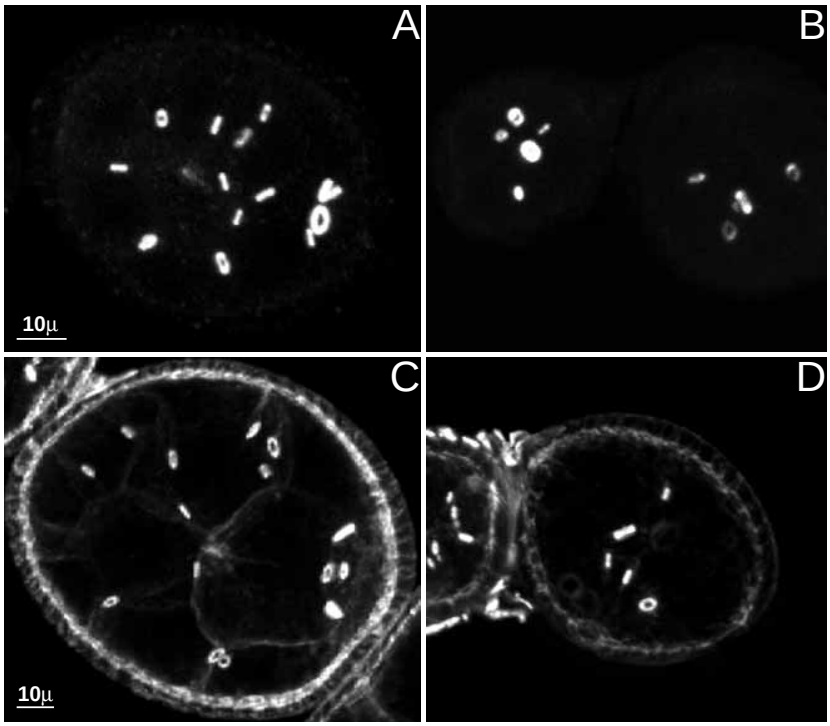

Fig. 7. Ring canals are normal in *α-spec*⁻ cysts. Control (A,C) and *α-spec*⁻ (B,D) egg chambers are stained with anti-phosphotyrosine antibodies (A-B) or with rhodamine-phalloidin (C-D), which labels filamentous actin. (A,C) Control egg chambers each contain 15 ring canals, interconnecting 16 cells. (B,D) In both panels, *α-spec*⁻ egg chambers each contain 5 ring canals, which stain as brightly as wild-type ring canals. The bar in (A) shows the magnification for A and B; the bar in C for C-D.

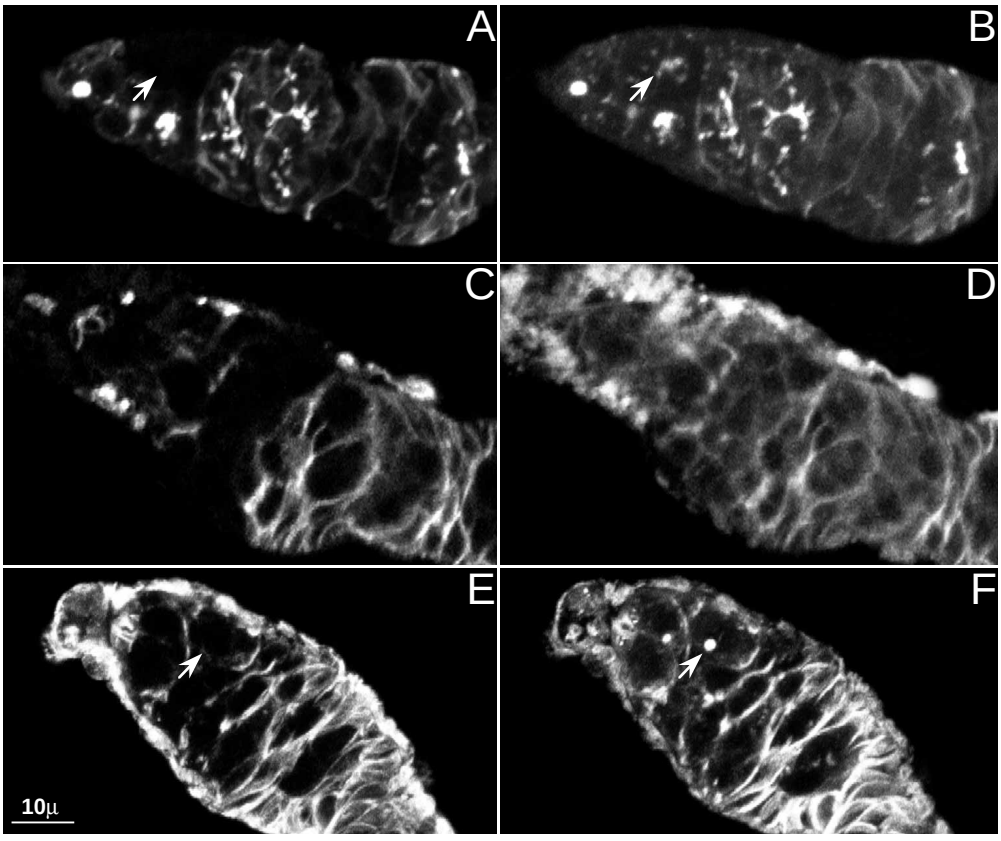

Fig. 8. Distribution of membrane skeleton proteins in *α-spec*⁻ germline cells. Germaria are stained with anti-α-spectrin antibodies (A,C,E) or with anti-Hts protein antibodies (B), anti-β-spectrin antibodies (D), or mAb 1B1 (F). Anterior is to the left. (A,B) In region 1, one cyst is *α-spec*⁻. α-spectrin is completely gone from its fusome, but some Hts protein is retained (arrows). (C,D) In a germarium that is missing α-spectrin in germline cells, no α- or β-spectrin can be detected in fusomes. In region 1, both proteins are present in inner and outer sheath cells; this staining is not in fusomes. (E,F) In this germarium, α-spectrin is missing from all germline cells, and none can be detected in fusomes. The protein recognized by mAb 1B1, however, is retained in a small patch in one of the cysts in region 1 (arrows). The bar in E shows the magnification for all panels.

appear to be completely absent: no Hts protein or α-spectrin can be detected there, though α-spectrin remains at the membrane in follicle cells (Lin et al., 1994). To determine if fusomes are missing in *α-spec*$^-$ cysts, we stained ovaries containing *α-spec*$^-$ clones with antibodies against other fusome components. In most *α-spec*$^-$ cysts, β-spectrin, Hts protein, and the protein recognized by mAb 1B1 were completely absent from fusomes (Fig. 8A-F). With mAb 1B1 and anti-Hts protein antibodies, however, strong staining was sometimes retained in small round patches in dividing cysts. Moreover, anti-Hts protein antibodies occasionally stained larger structures that resembled wild-type fusomes, although the amount of Hts protein in the *α-spec*$^-$ fusomes was reduced. These results suggest that some fusomal material is often retained in *α-spec*$^-$ cysts.

We also examined the staining patterns of mAb 1B1, anti-β-spectrin and anti-Hts protein antibodies in early stage follicle cells that were missing α-spectrin. In contrast to the results in germline cells, all three proteins were retained at the membrane in follicle cells (Fig. 9A-F). The amount of β-spectrin was slightly reduced, but Hts protein and the protein recognized by mAb 1B1 appeared to be present at levels similar to those in wild-type cells. Taken together, these observations suggest that membrane skeletal proteins are organized differently in fusomes than at the membrane in other cell types, because in the absence of α-spectrin, other membrane skeletal proteins are often missing from fusomes but are retained at the membrane in follicle cells.

DISCUSSION

Membrane skeletons with distinct protein compositions are present in the germarium

In erythrocytes, the membrane skeleton modulates the physical properties of the plasma membrane. In other cells, it appears to have additional functions that include the establishment of discrete membrane domains – not only in the plasma membrane but also in at least one internal organelle, the Golgi complex. The distribution of particular membrane skeletal proteins and their isoforms varies both within and between cell types, presumably reflecting differences in their functions.

Our studies highlighted a number of *Drosophila* membrane skeletons with distinct protein compositions in developing germline cysts and their surrounding somatic cells. We examined in particular the fusome, a germline-specific organelle containing small membrane-bound vesicles whose contents are unknown. Proteins related to most of the known components of the erythrocyte membrane skeleton have now been localized to the fusome, including α-spectrin, β-spectrin, ankyrin, and the adducin-like Hts protein (Fig. 2; Lin et al., 1994). Actin is present in fusomes from several Hymenoptera and Coleoptera species (Büning, 1994, and references therein), and probably is also found in *Drosophila* fusomes (Hawkins et al., 1996; A. C. S., unpublished). Members of the protein 4.1 superfamily, however, appear to be absent. Antibodies specific for D4.1, encoded by the *coracle* gene, did not label the fusome (Fig. 2E), and similar results were seen for the two other known members of this family in *Drosophila*, Dmoesin and Dmerlin (McCartney and Fehon, 1996). Although a previously undescribed 4.1 protein may reside within the fusome, these results raise the possibility that 4.1 proteins are not required for associations of membrane skeleton proteins.

Most fusome components are also found in stem cell spectrosomes, the apparent precursors of fusomes, and persist until fusomes regress in region 3. Ankyrin, however, is found preferentially in spectrosomes (Fig. 2C), suggesting that the fusomal membrane skeleton undergoes structural changes as cysts form and mature. Spectrosomes contain many fewer vesicles than fusomes in dividing cysts (Lin et al., 1994; McKearin and Ohlstein, 1995); perhaps the increase in the number of vesicles in fusomes is related to the decrease in ankyrin. These structural differences suggest that spectrosomes play a somewhat different role than fusomes.

Our studies also revealed several instances of somatic cells that appear to contain specialized membrane skeletons. For

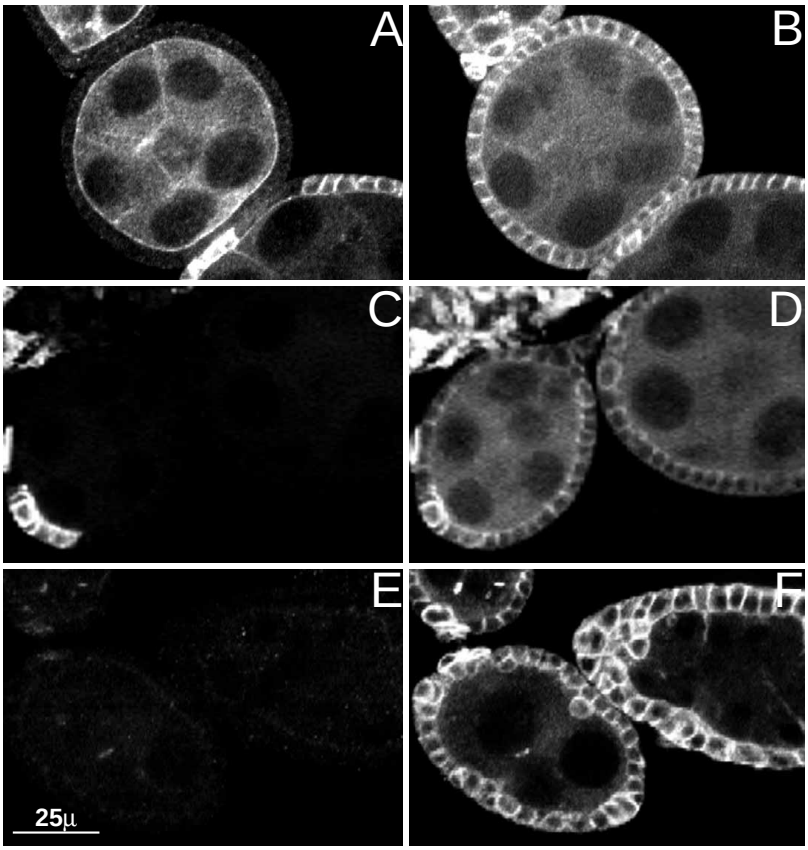

Fig. 9. Distribution of membrane skeleton proteins in *α-spec*$^-$ follicle cells. Egg chambers are stained with anti-α-spectrin antibodies (A,C,E) or with anti-Hts protein antibodies (B), anti-β-spectrin antibodies (D), or mAb 1B1 (F). α-spectrin is missing from most or all of the follicle cells in each panel, but Hts protein, β-spectrin, and the protein recognized by mAb 1B1 are retained. The amount of β-spectrin, however, appears to be slightly reduced in *α-spec*$^-$ cells. In (C-F), α-spectrin is also missing from germline cells. The bar in E shows the magnification for all panels.

example, terminal filament and cap cells have high levels of D4.1 and β_H-spectrin (Fig. 2B,E). These cells express *hedgehog* and *wingless* and appear to communicate with germline and somatic stem cells (Lin and Spradling, 1993; Forbes et al., 1996a,b). β_H-spectrin is also enriched at the apical ends of follicle cells located between cysts in regions 2 and 3 (Fig. 2B). These cells are in the process of migrating and changing shape as they pinch off new egg chambers, which is consistent with the proposed role for β_H-spectrin in regulating cell shape changes and morphogenetic movements (Thomas and Kiehart, 1994). Other proteins, especially ankyrin, appear to be enriched in inwardly migrating follicle cells in region 2 (Fig. 2C). Finally, D4.1 is present in lateral follicle cell membranes but appears to be absent from apical/basal ones. These differences in composition suggest that membrane skeletons are specialized for particular functions such as communication, migration and cell shape changes.

Role of α-spectrin in membrane skeletons

Our studies gave new insights into the function of α-spectrin at the plasma membrane and in the fusome. α-spectrin is a major component of the membrane skeleton, and yet its loss from the plasma membrane did not appear to cause severe cellular defects, at least in the early stages of oogenesis. Follicle cells still divided, changed shape, migrated, differentiated and signalled extensively with adjacent cells. In these cells, loss of α-spectrin did not severely disrupt the membrane skeleton, as demonstrated by the relatively normal distributions of β-spectrin and Hts protein (Fig. 9). Thus, it is possible that β-spectrin or other membrane skeletal proteins are able to compensate for the loss of α-spectrin at the plasma membrane. More severe defects were observed in the membrane skeleton and behavior of follicle cells in later stages of oogenesis; these are the subject of another paper (Lee et al., 1997), however, and will not be discussed here.

In contrast, α-spectrin is necessary for maintaining the distribution of other membrane skeletal proteins in the fusome. In *α-spec*$^-$ cysts, only a small amount of these proteins could still be seen in place of the fusome (Fig. 8). How membrane skeletal proteins are organized in the fusome, however, remains to be determined. Because the vesicles characteristic of wild-type fusomes are completely gone in *hts*^{1} cysts (Lin et al., 1994), we propose that membrane skeletal proteins are associated with these vesicles and are necessary to maintain their location or structure. For example, they might form a matrix that prevents the vesicles from dispersing throughout the cytoplasm; a similar role has been proposed for α-spectrin associated with secretory vesicles in chromaffin cells (Perrin and Aunis, 1985; Perrin et al., 1987) and in sea urchin eggs (Fishkind et al., 1990). They might also surround the vesicles with a plasma membrane-like skeleton and be necessary to maintain their integrity.

Function of the fusome

Previous work on *hts* mutations suggested that the fusome might play an essential role in cyst formation and oocyte differentiation (Lin et al., 1994). But this work did not rule out the possibility that defects in *hts* mutant cysts were caused indirectly by abnormal somatic cells, which were also affected by the mutation. We have shown here that *α-spec*$^-$ germline cells produce cysts with a reduced number of cells and a reduced frequency of oocyte formation. The strict germline autonomy of these defects reinforces the conclusion that membrane skeleton proteins are required for cyst formation only in germline cells, where they reside almost entirely in the fusome.

Exactly what role the fusome and its component vesicles play has yet to be established with certainty. Fusomes are not required to block cytokinesis or to initiate ring canal formation (Lin et al., 1994). Instead, the picture of fusome function emerging from our study of *α-spec*$^-$ clones and previous work on *hts* mutations (Lin et al., 1994) is that fusomes have two functions, one in cyst formation and one in oocyte determination.

Fusome reduction may limit synchrony to cell pairs

α-spec$^-$ cysts had a reduced number of cells, suggesting that fusome-deficient cystocytes divide fewer times than wild-type cells. Moreover, the synchrony characteristic of wild-type cystocyte divisions must also be altered, since the number of cells in each cyst showed no tendency to cluster around powers of 2. *α-spec*$^-$ cysts usually had an even number of cells, however, and a similar trend was reported previously in some *hts* mutations (Yue and Spradling, 1992). The simplest explanation for these observations is that, in the absence of a fusome, cystocyte cell cycles are synchronized only between cell pairs, rather than throughout the cyst. Thus if one cell divides, a partner will as well, but the other cells in the cyst will not necessarily divide. These findings suggest that the fusome has a function in coordinating the cell cycles of cystocytes.

Fusome reduction interferes with oocyte formation

Since strong *hts* alleles virtually abolish oocyte formation, the fusome almost certainly plays an important role in determining or maintaining the oocyte cell fate. Loss of germline α-spectrin also reduced oocyte formation. The ability of small cysts to form oocytes, however, appears to differ when they are generated by lack of α-spectrin or by reduced Hts protein. Weak *hts* alleles produced cysts as small as 6 cells that sometimes had an oocyte and the majority of 8-cell cysts contained an oocyte (Yue and Spradling, 1992). In contrast, very few 8-cell and only half of 12-cell *α-spec*$^-$ cysts had an oocyte (Fig. 6).

In *hts*^{1} cysts, which lack a fusome, differential accumulation of oocyte-specific mRNAs in a single cell is disrupted (Yue, 1992). Thus, the fusome was proposed to organize and polarize the cyst cytoskeleton to ensure that one cell receives materials transported through the ring canals from the remaining 15 cells (Lin et al., 1994; Lin and Spradling, 1995). The polarized microtubules that extend from the oocyte into adjacent nurse cells (Theurkauf et al., 1992) are likely to be an important component of this transport, since their disruption blocks oocyte formation (Koch and Spitzer, 1983; Theurkauf et al., 1993). Thus, the different abilities of Hts- and α-spectrin-deficient cysts to form oocytes may reside in the way their microtubule cytoskeletons are affected. The nature and amount of residual membrane skeleton in the fusome might influence microtubule establishment. Alternatively, there may be differences in the residual number of fusomal vesicles between Hts- and α-spectrin-deficient cysts. Critical materials involved in oocyte formation might reside within these vesicles, and be transported to the oocyte during fusome breakdown. In the mutant cysts, decreased vesicle production, stabilization or transport might reduce the concentration of vesicles within any single cyst cell below a threshold necessary for oocyte formation.

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