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A Spectraplakin Is Enriched on the Fusome and Organizes Microtubules during Oocyte Specification in *Drosophila*

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Summary

Background: During *Drosophila* oogenesis a membranous organelle called the fusome has a key function in the establishment of oocyte fate and polarity, ultimately leading to the establishment of the major body axes of the animal. The fusome is necessary for the microtubule-driven restriction of markers of oocyte fate to the oocyte, but the mechanism by which the fusome organizes the microtubules is not known.

Results: We have identified the spectraplakin Short stop (Shot) as a new component of the fusome. Spectraplakins are giant cytoskeletal linker proteins, with multiple isoforms produced from each gene. Shot is the sole spectraplakin in *Drosophila*. The phenotype caused by the absence of Shot is not similar to that of other components of the fusome but instead is similar to the absence of the downstream components that interact with microtubules: the dynein/dynactin-complex-associated proteins Egalitarian and BicaudalD. Shot is required for the association of microtubules with the fusome and the subsequent specification of the oocyte in 16-cell cysts. Shot is also required for the concentration of centrosomes into the oocyte, a process thought to be independent of microtubules because it still occurs in the presence of microtubule depolymerizing drugs. This suggests that Shot may protect some microtubules from depolymerization and that these microtubules are sufficient for this process.

Conclusions: Shot provides the missing link between the fusome and microtubules within meiotic cysts, which is essential for the establishment of the oocyte. Shot associates with the fusome and is required for microtubule organization. We suggest that it does this directly, via its microtubule binding GAS2 domain.

Introduction

The establishment of the main body axes is a crucial aspect of the development of the body plan during embryogenesis. In *Drosophila*, these axes are already established in the egg prior to fertilization and therefore are generated as the egg is made, during oocyte development [1, 2]. Thus, to understand how the body plan is generated, one must determine the mechanism by which the progeny of the germ line stem cells, the cystoblasts and their progeny, first gain asymmetry. An early "symmetry-breaking" step in *Drosophila* oogenesis is the selection of the oocyte from the cyst of 16 cells arising from each cystoblast, and it is this step that is the focus of this work. Once the oocyte has been selected, reciprocal interactions between the oocyte and the surrounding layer of somatic cells, the follicle cell layer, lead to the establishment of the main body axes [3, 4].

The selection of the oocyte is a complex process, but an examination of the distribution of molecules involved and the characterization of mutants that affect the process have allowed it to be divided into a series of steps. The oocyte arises from a stem cell at the anterior tip of the germarium, which at each division produces a stem cell and a cystoblast. The cystoblast undergoes four divisions to produce a cyst of 16 cells. Due to incomplete cytokinesis, these cystocytes remain interconnected by cytoplasmic bridges, called ring canals. One of the 16 cells becomes the oocyte, whereas the other fifteen become supporting nurse cells. Due to the necessary topology of this process, two cells end up with four ring canals (being connected to each other and three other nurse cells), two cells with three, four cells with two, and eight cells with one ring canal. The oocyte has four ring canals, indicating that it is derived from one of the two cells with four ring canals; these cells are referred to as pro-oocytes. The number of ring canals is not sufficient to specify pro-oocyte fate, so another signal, which appears to be provided by an unusual organelle called the fusome, is also required [5, 6].

The fusome is a branched membranous structure in the cytoplasm that extends into all 16 cells of a cyst through the ring canals. It contains high levels of the cytoskeletal proteins α-Spectrin, β-Spectrin, Ankyrin, and an adducin-like protein encoded by the gene *hu-li tai shao (hts)*. The fusome appears to arise from a similar stem cell structure called the spectrosome. At each division of the stem cell, the fusome is partitioned asymmetrically between the stem cell and the cystoblast, with about one-third ending up in the cystoblast. At each subsequent division, the spectrosome/fusome provides an anchor for one end of the mitotic spindle and so is initially inherited by one of the daughters [7]. However, shortly after each division new fusome material appears, initially at the ring canal. It then extends into both cells and reconnects with the existing fusome to produce the continuous branched network [5]. The majority of fusome material is found in the two pro-oocytes, and it has been proposed that the cell that inherits the fusome in the first cystoblast division continues to have the most fusome material and thus becomes the oocyte [6]. However, in the absence of live imaging, this has yet to be confirmed. Genetic elimination of the fusome components Hts or α-Spectrin caused a number of defects: fusome structure was disrupted, the number of cystoblast cell divisions was abnormal, and neither the pro-oocytes nor the oocyte became specified [8, 9]. Thus, the fusome appears to play a central role in specifying oocyte cell fate.

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A number of molecular markers that reveal the progress in the oocyte cell fate decision have been identified, and their distribution suggests that two cells start down the road to an oocyte fate but that only one becomes an oocyte, whereas the others revert to a nurse cell fate [10]. This is first revealed by the appearance of the synaptonemal complex, which marks cells progressing through meiosis. It appears first in the two pro-oocytes, then in additional cells, and then becomes restricted to the oocyte [11]. As this occurs, several other proteins become concentrated in the pro-oocytes and are then restricted to the oocyte. Among these are three proteins that are essential for the selective concentration process, the products of the *oo18 RNA binding protein (orb), egalitarian (egl)* and *BicaudalD (BicD)* genes [11–15]. The concentration of these proteins to the two pro-oocytes and later the oocyte is prevented by microtubule inhibitors, suggesting that microtubules play an essential role (see Table 1; [16]). Mutations in the dynein heavy chain gene (*Dhc64C*), as well as in *orb, egl*, and *BicD*, all caused a block in this process, so that none of the proteins were concentrated and cysts containing 16 nurse cells rather than 15 nurse cells and an oocyte were produced [14, 17, 18]. BicD and Egl, which lack protein motifs indicative of a function, have been found to interact with the dynein/dynactin complex [19, 20], suggesting that they work with dynein to transport each other and additional proteins into the oocyte. The role of Orb, which is an RNA binding protein [21, 22], in this process is not yet clear. The association of microtubules with the fusome suggested a link between the fusome and the microtubule-dependent concentration of these proteins into the oocyte [23]. Furthermore, with time the microtubules become polarized, with the minus ends concentrated in the central region of the fusome. This region is likely to be within the two pro-oocytes and then the oocyte, such that the minus end-directed motor dynein and its cargo become concentrated there. This suggests the existence of a protein on the fusome that polarizes the microtubules, but this protein has yet to be identified. The known fusome components do not have microtubule binding activity, whereas the proteins Egl and BicD are not found on the fusome [19] and Dynein is only weakly associated with the fusome in a cell cycle-dependent manner in mitotic cysts [18].

Whereas these results support a model in which the fusome organizes the microtubules into a polarized array that concentrates key proteins into the oocyte, the concentration of centrosomes into the oocyte still occurs in the presence of microtubule inhibitors and in the absence of either Egl or BicD [11, 16, 24]. This suggested that there are two parallel mechanisms that are responsible for concentrating factors into the oocyte. However, in apparent contradiction, Dynein was found to be required for the concentration of all factors into the oocyte [24]. This suggests that the concentration of the centrosomes may depend on a pool of colchicine-resistant microtubules.

Several other proteins that are concentrated into the oocyte are required for the next step in the process, the maintenance of oocyte fate. For example, in the absence of Par-1, concentration of the oocyte-specific proteins occurs normally, but the oocyte fate is lost and the cell reverts back to nurse cell fate, also resulting in egg chambers containing 16 nurse cells [25, 26].

In summary, analysis of mutant phenotypes of genes required for the specification of the oocyte has revealed three steps in the process: (1) forming the fusome and going through the four incomplete cell divisions, (2) concentrating a set of proteins initially into the two pro-oocytes and then specifically into the oocyte, and (3) maintaining oocyte cell fate. A link between steps 1 and 2, whereby a fusome component organizes the microtubule machinery that concentrates proteins specifically into the oocyte, has yet to be identified. Here, we present evidence that the cytoskeletal linker protein Short stop performs this role.

The gene *short stop* (*shot*) is the sole spectraplakin gene in *Drosophila* and encodes a number of exceptionally large cytoskeletal linker proteins that share similarity to proteins in the spectrin and plakin superfamilies (for review see [27]). All isoforms that have been identified to date contain a microtubule binding domain at the C terminus, and some isoforms contain an actin binding domain at the N terminus [28–32]. These two cytoskeletal-interacting domains can be separated by as much as 8000 amino acids of intervening sequence containing either spectrin repeats or both plakin and spectrin repeats. Analysis of the spectraplakin mutant phenotypes in both invertebrates and vertebrates has indicated that spectraplakins are essential for changes in cell shape and tissue integrity [27, 32–34].

Here we describe the role of Shot in the specification of the oocyte and present evidence that it is a component of the fusome that provides the missing link between the fusome and the microtubule-dependent consolidation of oocyte fate in meiotic cysts.

Results

Shot Localizes to the Fusome and Is Required for Oogenesis

While we were examining the role of spectraplakins in the integrity of the epithelial follicle cell layer [32], we noticed that Shot was found on the fusome and that germ line clones of a *shot* null allele failed to develop fully. These observations suggested that Shot had an important function in oocyte development, and this function warranted further analysis. We characterized the Shot isoforms expressed in the *Drosophila* germline by using antibodies raised against different domains of the protein (Figure 1A). The isoforms of Shot that localized to the fusome contained both the spectrin (Figures 1C and 1D) and the plakin repeats (Figure 1E). Shot colocalized with α-Spectrin (Figure 1C′), an integral component of the fusome [9]. Like α-Spectrin, Shot was detected within the spectrosome and on the fusome within germarium regions 1–3 (see Figures 1B and 1C for nomenclature of stages). The staining of α-Spectrin becomes diminished in region 2b-3 (Figure 1C′), which suggests that the fusome begins to disintegrate at this stage [6]. However, whereas α-Spectrin was barely detectable, Shot was still strongly present in structures that looked like fusome remnants in region 3 of the germarium and even in the newly budded stage 2 egg

Table 1. Comparison of Molecular Phenotypes of Mutations Affecting the First Steps in Oocyte Determination

Mutation	Structural fusome components[a]	BicD	Egl	Orb	Dhc	Shot	microtubules (colcemid treatment)
Localization of affected component	fusome [9]	cytoplasm	cytoplasm	cytoplasm	cytoplasm and fusome in region 1, 2a	fusome	highly concentrated on fusome [23]
Number of germ cells/oocyte?	aberrant [9]	16 nurse cells (NC), no oocyte (OO) [14]	16 NC, no OO [17]	16 NC, no OO [17]	16 NC, no OO[b] [18]	16 NC, no OO	16 NC, no OO [16]
Fusome intact?	no [9]	yes [8]	yes [8]	yes	no[c] [18, 24]	yes	yes [24]
Synaptonemal complex (SC)?	n.d.	no SC present[d] [11]	SC present in all cells [11]	SC present in all cells [11]	no SC present [24]	random[e]	SC lost [11]
Microtubules associated with fusome? MTOC in region 2b?	no [7, 23]	no [16]	no[g]	n.d.	n.d.	no	no [16]
Centrosome migration?	n.d.	yes [24]	yes [24]	n.d.	no [24]	no	ye [24]
Orb, BicD, Egl localized?	in *hts*[f]: no orb [7]	no orb [14, 19] localization	no [19]	no	no [18]	no	no [39]

[a] α-Spectrin, hts, ankyrin.

[b] 16 nurse cells and no oocyte are found in a hypomorphic allele, whereas in a strong allele cysts contain only 8, 4, or 2 nurse cells because dynein is required on the mitotic spindle during the synchronous mitotic divisions.

[c] Fusome disintegrates from region 2b onward.

[d] In a null allele.

[e] In no, two, or more than four cells, but always lost in region 2b.

[f] Present in region 2a, but restriction was lost in region 2b/3.

[g] In egl^{WU50}/Df: no [7], in egl^{WU50}/egl^{R26}: microtubules are present on the fusome in region 2a, but there is no MTOC later on [16].

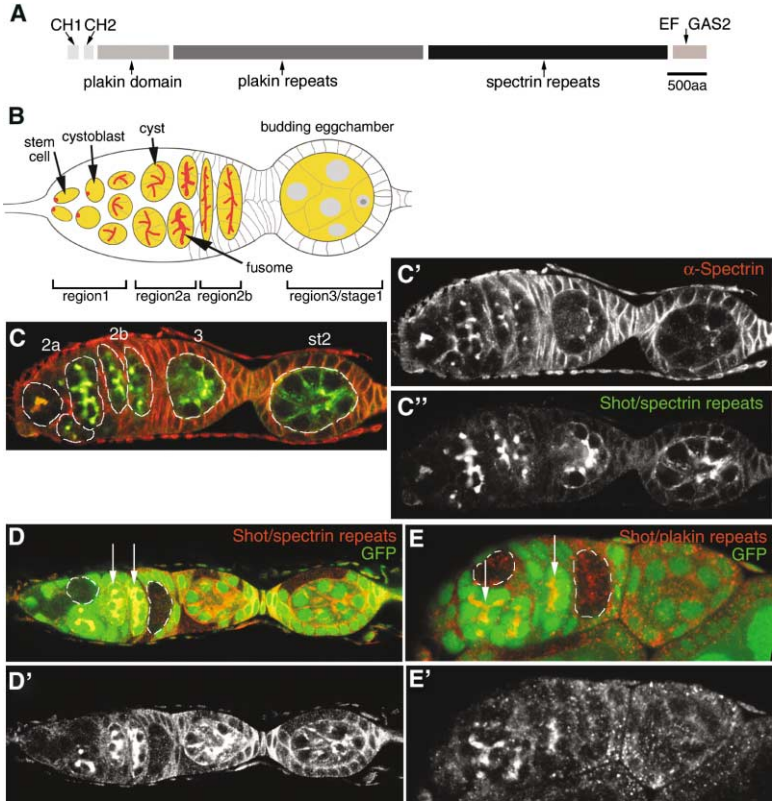

Figure 1. Shot Localizes to the Fusome during Oogenesis

(A) Schematic of the various protein domains that can be incorporated into isoforms of the spectraplakin Shot. From N terminus to C terminus, the longest isoform would contain two calponin-homology-type actin binding domains (CH1 and CH2), a plakin domain found in all plakin family members, a plakin repeat domain, a spectrin repeat domain, and a domain that contains a set of calcium binding EF hands plus a GAS2 domain and can bind microtubules [41, 42].

(B) shows a schematic germarium at the anterior end of an ovariole. Cystoblasts at the anterior end of the germarium in region 1 divide four times with incomplete cytokinesis to give rise to a cyst of 16 cells interconnected by ring canals, and the fusome extends through these canals (red in [B]). In region 3 newly formed eggchambers bud off the germarium.

(C–C″) Staining for the spectrin repeats of Shot ([C″] and green in [C]) colocalized with α-Spectrin ([C′] and red in [C]), a core component of the fusome, until α-Spectrin staining disappears in region 3, whereas Shot labeling persisted for longer.

(D) The fusome labeling with the antibody against the spectrin repeats ([D′] and red in [D]) was absent in germline clones of *shot*^{3}, a protein null allele.

(E and E′) An antibody against Shot's plakin repeats also labeled the fusome, and labeling was abolished in *shot*^{3} clones. Arrows point to wild-type cysts, and dotted lines mark mutant cysts in (D–E′).

chambers (Figures 1C and 1D). *shot* mutants are embryonic lethal when homozygous, so we used mitotic recombination to generate clones of germline cells that were homozygous for *shot* mutants. The staining with the anti-Shot plakin repeat and anti-Shot spectrin repeat antibodies was abolished in germ line clones of *shot*^{3}, a protein null allele (Figures 1D and 1E; [32]), confirming that the labeling of the fusome reflects the presence of Shot. Thus, we have identified the spectraplakin Shot as a new component of the fusome.

The germline clones of the null allele *shot*^{3} produced egg chambers, but they did not progress beyond stage 7 of oogenesis (Figure 2A). We therefore examined whether the *shot* mutant phenotype is similar to that of the other fusome components, which are characterized by aberrant patterns of divisions in the cysts and disintegration of the fusome. We found that *shot* mutants lacked these phenotypes, placing *shot* function downstream of these components. Thus, each *shot*^{3} egg chamber contained 16 nuclei (Figures 2B–2F), indicating that the mitotic divisions generating each cyst had occurred. The pattern of divisions was also normal, as revealed by the number of ring canals in each cell (data not shown). Examination of the integral fusome components Hts (Figures 3A, 3B, 3E, and 3F), α-Spectrin (Figures 3C, 3D, and 3G), β-Spectrin, and Ankyrin (data not shown), in *shot*^{3} mutant germline clones demonstrated that fusome structure and persistence appeared normal. In addition, the fusome/spectrosome was still distributed asymmetrically in early stem cell/cystoblast divi-

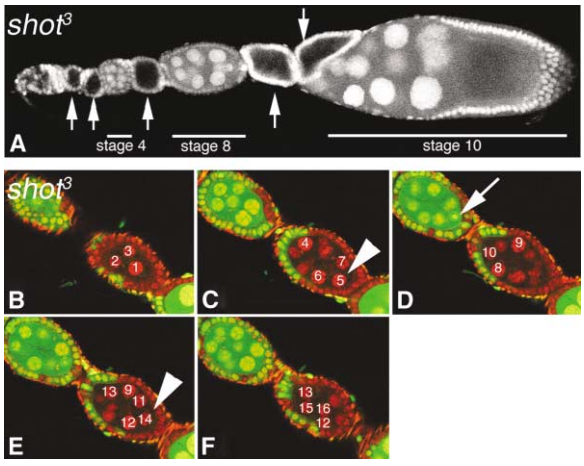

Figure 2. *shot*^{3} Germline Clones Do Not Develop and Arrest around Stage 7 of Oogenesis

(A) An ovariole containing germline clones of *shot*^{3} homozygous mutant cells is marked by the absence of nuclear green fluorescent protein (GFP; arrows). Germline clones arrested around stage 7.

(B–F) Confocal z scan through a *shot*^{3} mutant (no nuclear GFP, green) and a wild-type egg chamber. Propidium iodide was used for labeling nuclei (red). Note that the mutant egg chamber contains 16 identical polyploid nurse cell nuclei, even at the posterior position of the egg chamber where the oocyte should be localized (arrowheads in [C] and [E]), whereas the wild-type egg chamber contains a highly condensed oocyte nucleus at its posterior (arrow in D).

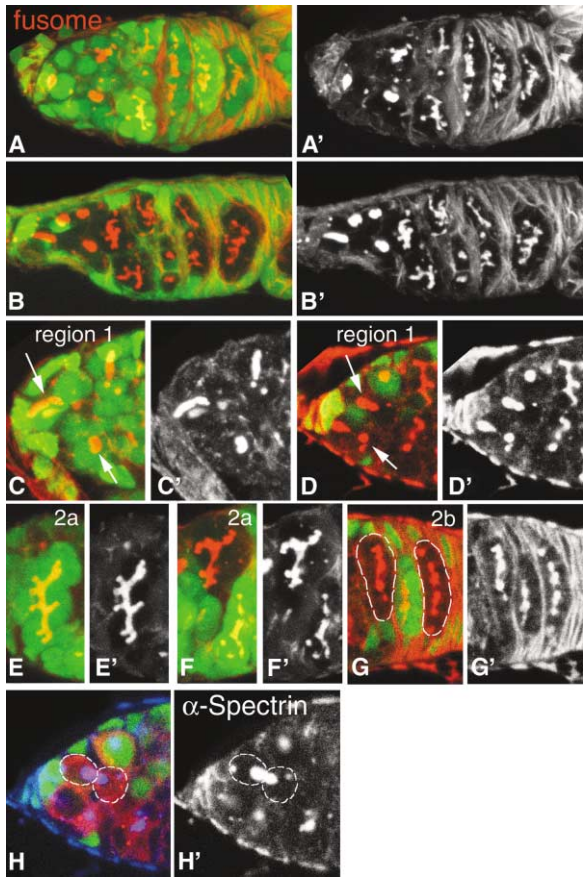

Figure 3. The Fusome Is Intact in *shot*^{3} Mutant Cysts and Is Distributed Asymmetrically

To analyze whether fusome structure is affected in *shot*^{3} mutant cysts, marked by the absence of GFP, we labeled germaria with antibodies against two integral fusome components: α-Spectrin and Hts. (A and B) Stacks of confocal z series through the whole germarium. In *shot*^{3} germline clones (B), fusomes ([B′] and red in [B]) were indistinguishable from those in wild-type cysts ([A′] and red in [A]). (C–G) Stacks of confocal z series showing that fusomes in region 1 (C, C′, D, and D′), region 2a (E, E′, F, and F′) and region 2b (G and G′) were indistinguishable between wild-type and mutant cysts (arrows in [C] and [D] point to wild-type and mutant cysts, respectively; mutant cysts are outlined by a dashed line in [G]). (A, A′, B, B′, E, E′, F, and F′) Hts. (C, C′, D, D′, G, and G′) α-Spectrin. (H and H′) In *shot*^{3} mutant stem cells located at the anterior tip of the germarium, the fusome was distributed asymmetrically between the stem cell and the cystoblast, with approximately two-thirds remaining in the stem cell (α-Spectrin: [H′] and blue in [H]; lack of GFP marks mutant clones, and red outlines cell circumferences).

sion, with the larger portion being found in the stem cell after the division (Figure 3H). These results indicate that Shot is not required for the stability of the fusome, nor is it required in the cystoblasts for the fusome's early mitotic functions, such as the anchoring of the mitotic spindles via one spindle pole. Instead, it may be required for a later function of the fusome.

We next examined whether oocyte fate was specified normally in the absence of Shot. In *shot*^{3} mutant cysts all of the nuclei, including the two at the posterior of the eggchamber, appeared polyploid (Figures 2B–2F; arrowheads in C and E). This indicated that all germ line cells had adopted a nurse cell fate; none had the highly condensed appearance of a wild-type oocyte nucleus arrested in prophase I of meiosis (Figure 2D, arrow). We confirmed this by examining two proteins, Orb and BicD, that are normally specifically concentrated in the oocyte. In wild-type ovarioles, Orb and BicD begin to accumulate in region 2a of the germarium and are restricted to the prospective oocyte in region 2b (Figures 4A and 4D). In *shot*^{3} mutant germline clones, Orb and BicD failed to become restricted to a single cell of the cyst (Figures 4B, 4C, and 4E), and so did Dynein (data not shown). This places *shot* mutants in the same category as the mutations *BicD, orb, egl*, and *Dhc64C*, affecting step 2 in the process of oocyte specification. In germline cysts lacking components that mediate the next step, the maintenance of oocyte fate, Orb and BicD are initially localized normally. This localization did not occur in the absence of Shot, showing that Shot must act before this step. Thus, Shot is a component of the fusome but has a mutant phenotype characteristic of the next step in the process of oocyte determination.

We next sought to place the function of Shot within the context of the other genes known to function in the selection of the oocyte. The phenotypes of each of these genes have unique aspects and are distinguishable by their effect on the synaptonemal complex and the migration of the centrosomes into the oocyte (see Table 1).

Once the 16-cell cyst has formed in region 2a, the two cells of a wild-type cyst with four ring canals progress through meiotic prophase more quickly than the other cystocytes and accumulate synaptonemal complexes, identified by a synaptonemal complex component, C(3)G (Figure 4F; [35]). Several cells with three ring canals in region 2a follow to accumulate synaptonemal complexes temporarily, before further progression through meiosis is restricted to the two pro-oocytes in region 2b and the oocyte only in region 3 (Figure 4F). In *egl* mutant cysts the synaptonemal complex was present in all 16 cells, whereas in *BicD* mutant cysts it was completely absent [11]. In *shot*^{3} mutant cysts, we observed a more variable phenotype, in which the synaptonemal complex accumulated in none or two or more cells all throughout region 2a, but this accumulation failed to refine onto one cell only and was always lost in region 2b (Figures 4G–4H), indicating that all cells had exited meiosis. The loss of restriction of the synaptonemal complex in region 2b-3 has also been observed after drug-induced depolymerization of microtubules, indicating that microtubules are necessary not for cells to enter meiosis but rather to maintain the pro-oocytes and oocyte in meiosis [11]. Under these conditions, i.e., depolymerized microtubules, Egl and BicD are dispersed, but nonetheless the initial accumulation of the synaptonemal complex is fine, indicating that Egl and BicD have an earlier function that is not dependent on their restriction to the two pro-oocytes [11]. Because loss of Shot not only abolished BicD localization but also affected the assembly of the synaptonemal complex in a way that was distinct from both *egl* and *BicD* mutant cysts, Shot appeared to influence both late and early functions of these proteins.

After playing a role in anchoring the mitotic spindles to the fusome, cystocyte centrosomes are associated

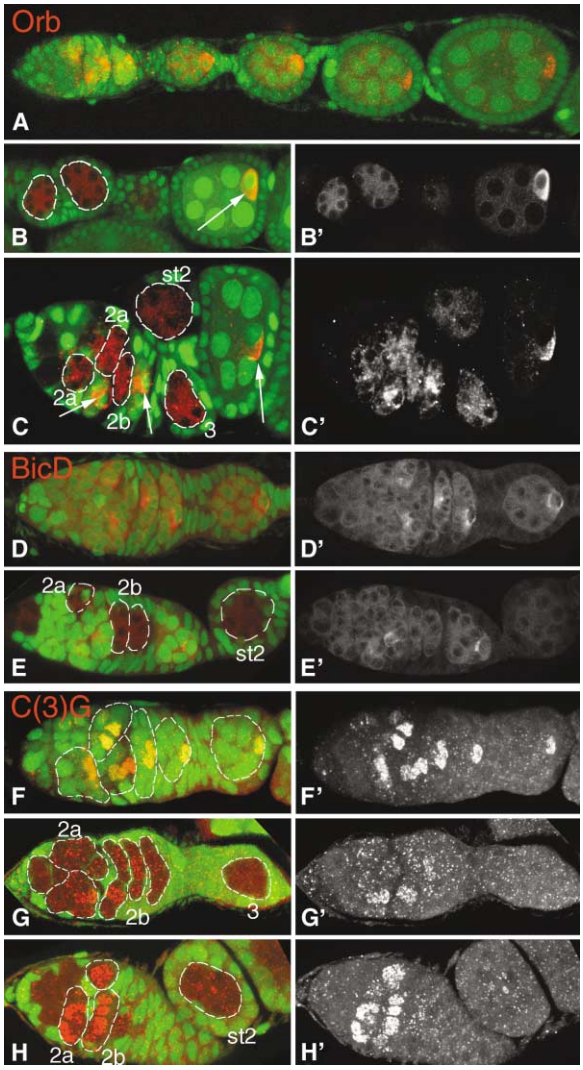

Figure 4. *shot*^{3} Germline Clones Fail to Specify an Oocyte

Germline clones of *shot*^{3} were revealed by the absence of nuclear GFP (green); other markers are shown in red and white. Boundaries of individual cysts are marked by dashed lines. (A–C) Orb, a marker of oocyte fate, becomes concentrated at the anterior of the oocyte in region 2b and translocates to the posterior in region 3 in wild-type ovarioles ([A] red). In *shot*^{3} mutant cysts Orb failed to accumulate within one cell (B, B′, C, C′). Later egg chambers are depicted in (B) and (B′), whereas a germarium is shown in (C) and (C′). Arrows point to Orb staining in wild-type cysts; regions of the germarium are indicated in (C). (D and E) Like Orb, BicD accumulates in the oocyte in wild-type eggchambers (D and D′), but this restriction failed in *shot*^{3} mutant cysts (E and E′). (F–H) In wild-type cysts, C(3)G, a component of the synaptonemal complex and marker of meiosis, accumulates in 2–4 cells in region 2a before it becomes restricted to the oocyte only in region 2b (F and F′). In *shot*^{3} mutant cysts, cells entered meiosis as indicated by C(3)G staining (G, G′, H, and H′), but the number of cells entering meiosis appears aberrant, with sometimes more than four and sometimes none. By region 3 all mutant cysts have lost C(3)G.

with fusome arms in region 2a and migrate along the fusome into the oocyte in region 2b, where they first localize to the anterior and subsequently translocate to the posterior together with other oocyte fate markers, such as Orb and BicD [26]. Curiously, the migration

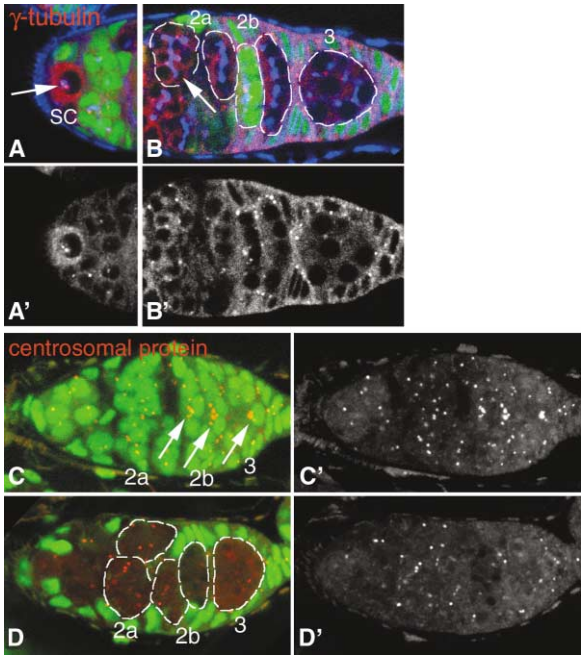

Figure 5. The Centrosomes Do Not Migrate along the Fusome in the Absence of Shot

In *shot*^{3} mutant cysts the centrosomes fail to migrate along the fusome to concentrate in the oocyte but are dispersed throughout the cyst. (A and B) In *shot*^{3} mutant stem cells (arrow in [A]) or cysts in region 2a (arrow in A), centrosomes marked by γ-tubulin ([A′], [B′], and red in [A] and [B]) associated with the fusome, but they did not accumulate within one cell of the cyst. (C and D) Another marker of centrosomes (J. Raff; personal communication) was strongly accumulated in the oocyte in regions 2b and 3 in wild-type cysts ([C′] and red in [C]), whereas in *shot*^{3} mutant cysts the centrosomal protein did not accumulate within one cell but was dispersed ([D′] and red in [D]).

process is not inhibited by microtubule-depolymerizing drugs, and during the migration these centrosomes do not nucleate microtubules [24]. The concentration of the centrosomes in the oocyte is disrupted in *Dhc64C* mutant cysts but not in *orb, egl, BicD* mutant cysts [24]. In *shot*^{3} mutant germline clones, centrosomes were associated with the fusome in the stem cells (Figure 5A, arrow) and in region 2a (Figure 5B, arrow), but in contrast to findings in wild-type cysts (Figure 5C), a concentration of centrosomes was not detected in region 2b or 3 (Figures 5B and 5D). When detectable, centrosomes appeared to be spread throughout the cyst (Figure 5D). Thus, from these results Shot appears to function most similarly to Dynein, suggesting that its ability to interact directly with microtubules may be key to its function in the germline.

Shot Colocalizes with Microtubules and Is Required for Their Recruitment by the Fusome

Shot can bind microtubules with its C-terminal GAS2 domain [31], and all isoforms examined to date contain this domain. In the absence of an antibody specific for the GAS2 domain, we used in situ hybridization to confirm that *shot* mRNAs encoding this domain were ex-

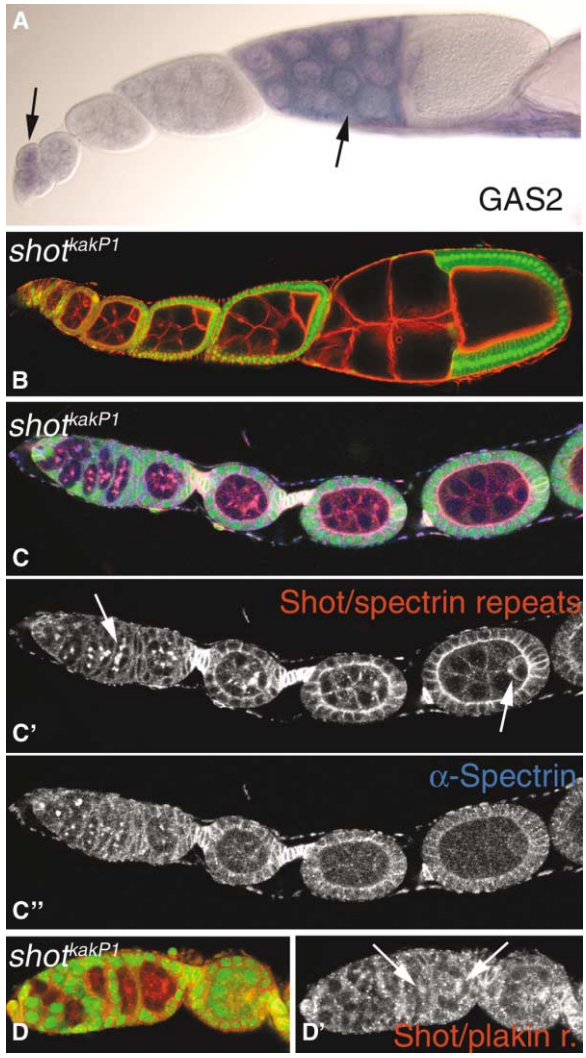

Figure 6. The Actin Binding Domain of Shot Is Not Needed for Oocyte Specification

(A) The microtubule binding GAS2 domain of Shot is expressed in the germarium (arrow), as shown by whole mount in situ hybridization. (B) Germline clones of the allele *shot*^{kakP1}, which abolishes expression of Shot isoforms containing the actin binding domain, proceeded normally through oogenesis and specified an oocyte. Phalloidin staining (red in [B]) of clones revealed that mutant egg chambers (lacking GFP, green) are indistinguishable from wild-type. (C, C′, and C″) In *shot*^{kakP1} mutant cysts, labeling against Shot's spectrin repeats ([C′] and red in [C]) still colocalized with labeling against α-Spectrin ([C″] and blue in [C]) on the fusome. Arrows point to the fusome in a cyst and to the accumulation of spectrin repeat staining in a stage 4 egg chamber that is still present in *shot*^{kakP1} mutant cysts. (D and D′) In *shot*^{kakP1} mutant cysts, labeling against Shot's plakin repeats is still present on the fusome ([D′] and red in [D]; arrows point to fusomes).

pressed in the germline (Figure 6A). We do not have a mutant that specifically eliminates this domain, but we were able to do the converse and test whether the actin binding domain is important for the function of Shot in the germline. Germline clones of the *shot*^{kakP1} allele, which eliminates the expression of isoforms of Shot containing the full actin binding domain [28, 32], showed no defect in oocyte determination but developed egg chambers with 15 nurse cells and one oocyte that developed fully and could be fertilized (Figure 6B and data not shown). In these *shot*^{kakP1} germline clones, other Shot isoforms containing the spectrin (Figure 6C′) and the plakin repeats (Figure 6D′) were localized normally to the fusome (compare Figures 6C′ and 6C″).

We further confirmed the functioning of Shot with microtubules rather than actin in the germline by examining the relative distribution of Shot, actin, and microtubules in more detail. Comparing Shot localization within the germarium to α-tubulin (revealed by staining for acetylated α-tubulin; Figures 7A and 7B) and actin (labeled with phalloidin; Figure 7A‴) showed good colocalization of Shot with acetylated α-tubulin on the fusome and no colocalization of Shot with the actin-rich ring canals in the germarium. Acetylation of α-tubulin is a modification associated with stable microtubules but not with dynamic structures such as mitotic spindles [36–38]. In accord with this, acetylated microtubules were not detected on the spindles in the early stages of cystoblast divisions and were first detected in region 2a, when the cysts have completed their divisions, and the acetylated microtubules clearly localized to the fusome (Figure 7B′). Particularly strong colocalization of Shot and acetylated microtubules was detected in region 3 structures that appeared to be fusomal remnants concentrated around the oocyte (Figure 7B″). Even later, in egg chambers of stage 3–4, Shot colocalized with microtubule structures and bundles labeled with acetylated tubulin (Figure 7C). These results suggest that the key function of Shot is to mediate the association of microtubules with the fusome in meiotic cysts. Because these microtubules later polarize toward the oocyte [23] and allow the concentration of proteins into the oocyte, a failure in microtubule organization could account for the defects in the absence of Shot. We therefore examined the microtubules in *shot* mutant germline clones.

Microtubules are highly concentrated around the fusome from region 2a on (Figures 7B, 8A, 8B, and 8C; [23]), and in region 2b and 3 a microtubule organizing center (MTOC) is visible in the oocyte as a high concentration of microtubules within a broad spot (Figures 8A, 8B, and 8C, arrows). This organization and concentration of microtubules was lost in *shot*^{3} mutant germline clones; microtubules failed to concentrate around the fusome (Figures 8D and 8F), and an MTOC did not form in region 2b or 3 (Figures 8E and 8G). In fact, already the initial association of stable microtubules with the fusome in region 2a was lost in *shot*^{3} clones (Figure 8H). In addition, the overall levels of acetylated microtubules were drastically reduced (Figures 8C and 8E). In support of Shot's linker function between the fusome and microtubules in meiotic cysts, Shot was still associated with the fusome after drug-induced depolymerization of microtubules (Figure S1). Thus, after completion of the mitotic divisions, Shot is required for microtubule organization by the fusome.

In summary, we have shown that the spectraplakin Shot is a fusome component that is essential for the organization of the microtubule cytoskeleton within the meiotic cysts. Consistent with this, microtubules fail to assemble in the absence of Shot, and the oocyte fails to become specified. In addition, the migration of the

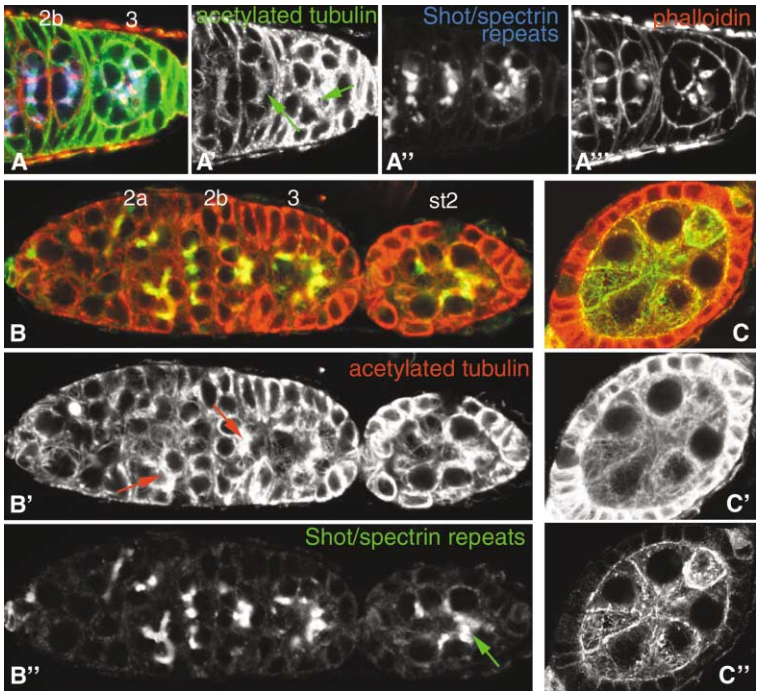

Figure 7. Shot Colocalizes with Microtubules on the Fusome and in Early Egg Chambers

(A–A‴) Shot ([A″] and blue in [A]) strongly colocalized with microtubules (labeled with an antibody against acetylated α-tubulin, a modification that accumulates in stable microtubules; [A′] and green in [A]) on the fusome (green arrows in [B′]), whereas phalloidin ([A‴] and red in [A]) highlighted the actin-rich ring canals through which the fusome stretches into all cells of a cyst. (B–B″) From early region 2a onward, Shot ([B″] and green in [B]) colocalized with the fusomal microtubules ([B′] and red in [B]). Arrows point to a fusome in region 2a and region 2b. Note the strong colocalization of Shot and acetylated tubulin on presumptive fusome remnants in the budded egg chamber (green arrow in [B″]). (C–C″) Even in egg chambers of stage 4, Shot ([C″] and green in [C]) still colocalized with acetylated tubulin ([C′] and red in [C]) on microtubule-like fibers or bundles of microtubules all throughout the nurse cells.

centrosomes into the oocyte, a process that was thought to be independent of microtubules, fails to occur. One likely explanation of these results lies in the ability of Shot to stabilize microtubules and render them resistant to microtubule-depolymerizing drugs [41, 42]. Thus, we hypothesize that such resistant microtubules are sufficient for transporting the centrosomes and that in the absence of Shot they fail to form.

Discussion

The restriction of oocyte fate to a single cell within a cyst of 16 cells is a crucial process during oogenesis in *Drosophila*. This is the first event to result in asymmetry in each cyst, and this asymmetry will later be amplified and translated into the two embryonic axes. A number of players in this process have been identified, but the molecular mechanism that leads to the selection of a single cell as the oocyte has still to be fully elucidated. We have identified the spectraplakin Shot as a new player in this process and propose that it provides a previously missing component required for this mechanism.

Shot is a component of the fusome, and the fusome itself has been implicated in most processes important for oocyte determination: (1) The fusome is asymmetrically inherited by the cystocytes, and it has been proposed that the cell that inherits more fusome in the first division of the cystoblast will be the future oocyte [6]; (2) the fusome organizes the microtubule cytoskeleton within each cyst [23], apparently so that oocyte-specific components can be transported into and concentrated within the oocyte; and (3) the centrosomes appear to migrate along the fusome into the oocyte, where they later contribute to the establishment of the body axes within the oocyte [1, 24]. We propose that Shot is recruited to the fusome by binding to a component of the fusome, where it contributes to the organization of the microtubules, so that Dhc, Egl, and BicD can transport factors to enrich them in the oocyte.

The analysis of mutant phenotypes is consistent with the placement of Shot between the structural fusome components and the second level of components, Dhc, Egl, BicD, and Orb, in the genetic pathway of oocyte determination (see Table 1 and Figure 9). Two particular phenotypes, the distribution of the synaptonemal complex and the migration of centrosomes, allow discrimination between these components. The differing requirement of these components for initiation of meiosis, as revealed by the synaptonemal complex, has lead to the idea of the balance between activating (Dhc and BicD) and repressing (Orb and Egl) activities [11]. The finding that *shot* has a variable effect on the appearance of the synaptonemal complex has therefore not aided our efforts to place Shot within the pathway. In contrast, the finding that Shot and Dhc are the only components required for centrosomal migration places them at the top of the pathway because they are the only ones required for the transport of all known oocyte-specific components.

The requirement for Shot in the organization of microtubules was just apparent after the cystocytes completed their synchronous divisions. Thus, Shot did not bind the centrosomes that nucleate spindle microtubules to the fusome, nor did the absence of Shot affect the highly dynamic microtubules in dividing cysts. This differential effect of Shot on centrosomes and microtubules is likely due to the differing characteristics of mitotic and meiotic cysts: (1) early centrosomes actively nucleate microtubules, whereas the migrating centrosomes do not [24], indicating a molecular difference between them; (2) microtubules in mitotic cysts are

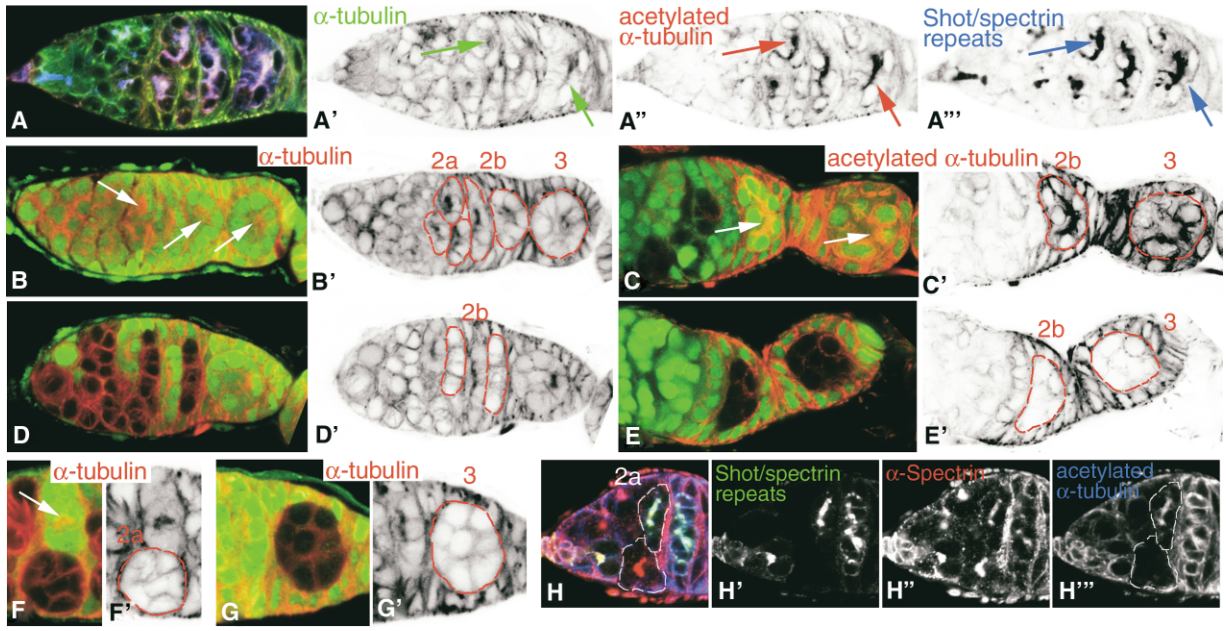

Figure 8. The Polarized, Fusome-Associated Microtubule Cytoskeleton of a Cyst Is Disrupted in the Absence of Shot

Germaria from wild-type and *shot*^{3} mutant cysts (marked by the absence of nuclear GFP, green) were labeled with an antibody against α-tubulin and acetylated α-tubulin. (A) Beginning in region 2a, stable microtubules ([A″] and red in [A]) are associated with the fusome (marked by the spectrin repeats of Shot; [A‴] and blue in [A]), and as of region 2b an MTOC can clearly be identified as a dense accumulation of microtubules in the vicinity of the two pro-oocytes and, later, in the vicinity of the oocyte. Note the stronger accumulation of stable, i.e., acetylated microtubules (A″) on the fusome compared to overall microtubule levels (A′). In *shot*^{3} mutant cysts, the microtubules failed to focus onto a single cell (dotted lines in [D′], [F′], and [G′] for regions 2b, 2a, and 3, respectively; compare to control in [B′]). (C and E) Acetylated α-tubulin ([C′] and red in [C]) marked the strongly concentrated fusome-associated microtubules around the oocyte in regions 2b and 3 (arrows in [C]). This accumulation could not be detected in *shot*^{3} mutant cysts (dotted lines in [E]; [E′] and red in [E]), and the overall level of acetylated α-tubulin staining seemed to be reduced. Single color panels are shown as inverse images in (A)–(G). (H) In early region 2a, stable microtubules (H‴) begin to associate with the fusome (marked by α-Spectrin [H″] and Shot spectrin repeats [H′]) in the wild-type top outlined cyst. In *shot*^{3} mutant cysts (bottom outlined cyst), stable microtubules failed to make the initial association with the fusome.

highly dynamic and have to reassemble into spindles in synchronous cycles, whereas we show that fusome-associated microtubules are stable.

It has not been possible to definitely position Dhc and Shot relative to each other within the pathway. Shot is the only one of these second-level proteins that is clearly part of the fusome at all stages in the germarium. In addition, the localization of Shot on the spectrosome and early fusome, prior to the accumulation of microtubules on the fusome, suggests that Shot is responsible for the organization of microtubules rather than that it binds to them once they have been recruited to the fusome. This was also supported by the fact that upon colchicine-mediated depolymerization of microtubules, Shot still localized to the fusome. We therefore think that the most likely scenario is that Shot is required for recruiting microtubules to the fusome and that Dhc is required for transporting the oocyte-specific factors along them into the oocyte (Figure 9). This is in agreement with the loss of Dynein accumulation in the oocyte in the absence of Shot, which is probably a secondary effect caused by the absence of polarized microtubules with their minus ends concentrated in the oocyte. The one inconsistency with this view is that Dhc is required for the integrity of the fusome [18, 24], whereas Shot is not, suggesting that microtubule organization or transport plays an essential role in the stability of the fusome, independent of Shot function.

The only other protein known to localize to the fusome and affect microtubule stability is Par-1. However, the loss-of-function phenotype of Par-1 is less severe than loss of Shot: the association of microtubules with the fusome appears normal, and markers of oocyte fate become restricted to one cell but then fail to translocate from the anterior to the posterior cortex of the oocyte; consequently, oocyte fate is lost [26, 39].

How does Shot fulfil the role of an essential mediator between the fusome and the fusome-associated polarized microtubule cytoskeleton in meiotic cysts? Shot contains a bona fide microtubule binding domain, the GAS2 domain, in its C terminus [31, 40, 41]. This domain is likely to be present on the fusome because all described Spectraplakin isoforms, including those of Shot, contain the GAS2 domain, and we could detect cytoplasmic RNAs containing this domain in the germarium. Thus, Shot could bind the fusome via one of its other domains, leaving the GAS2 domain free to bind, stabilize, or bundle microtubules in the vicinity of the fusome. In accordance with this, loss of Shot leaves the fusome unaffected but abolishes fusome-associated and polarized microtubules. There are at least three ways that Shot could contribute to the organization of the microtubules within the meiotic cysts. Shot could be involved in the nucleation of microtubules, particularly because neither the centrosomes nor γ-tubulin plays a role in the nucleation of microtubules on the fusome after comple-

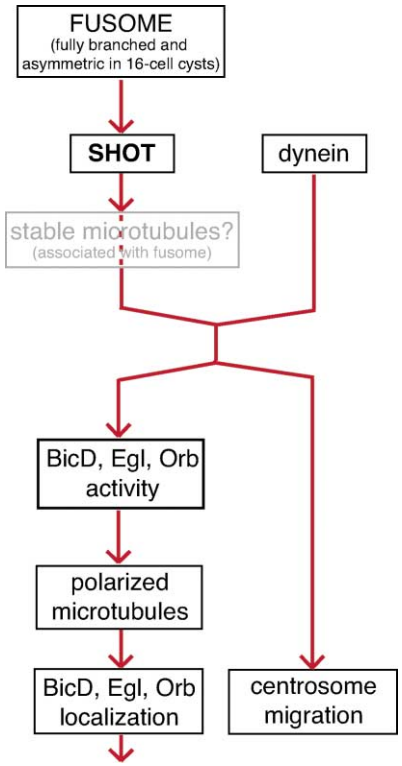

Figure 9. Model of Shot Action on Microtubules in Meiotic Cysts

Shot acts downstream of integral fusome components but associates with the fusome and helps to link the fusome to potentially stabilized microtubules in meiotic cysts. Shot gives the same loss-of-function phenotype of 16 nurse cells and no oocyte as found in mutations for BicD, Egl, Orb, and hypomorphic mutations of Dhc, but Shot appears to act most similarly to Dhc within the meiotic cysts; both of these not only affect concentration of factors in the oocyte but also abolish centrosome migration into the oocyte. The figure was adapted from [24].

tion of mitoses [24]. This would be a novel role for Shot; in most other cases it is functioning in combination with other microtubule-organizing centers. Alternatively, given the ability of the GAS2 domain of spectraplakins to bundle and stabilize microtubules [41, 42], this could be Shot's function on the fusome. However, Shot appears on the fusome prior to accumulation of microtubules, so it is not recruited to the fusome solely by interaction with microtubules, as is also indicated by its absence from the mitotic spindles. The idea of a population of microtubules that are stabilized by Shot and resistant to depolymerization by colcemid helps to explain a previously anomalous result: that centrosome migration into the oocyte still occurs in the presence of colcemid [24]. Because both Shot and Dhc are required for this, it suggests that this is still a microtubule-dependent process but can occur on colcemid-resistant, stable microtubules. Because Egl- and BicD-dependent concentration of each other and Orb into the oocyte is blocked by colcemid, the stable microtubules seemingly cannot provide a sufficient track to transport them. This may be because the number of molecules to be transported is greater relative to the number of centrosomes or because the rate of diffusion out of the oocyte is greater. Alternatively, two distinct mechanisms may exist for microtubule-dependent transport into the oocyte.

In addition to being required for the accumulation of stable microtubules, Shot could also assist in polarizing the microtubules so that components can be transported into the oocyte. Such a role would be consistent with recent observations on the effect of Shot in axon outgrowth. Mutations in *shot* were picked up in a screen that analyzed neuronal morphogenesis in *Drosophila* [33]. *shot* mutants showed defects in axonal microtubule polarity, and these defects led to mixed polarity of neurons in the axons that in the wild-type situation have the plus ends pointing distally. This possible function is difficult to analyze because the loss of Shot abolishes all fusome-associated microtubules in the 16-cell cysts.

How does Shot being the link between the fusome and the polarized microtubule cytoskeleton help to explain how oocyte fate is restricted to one cell only? Starting with the first division of the cystoblast, the fusome is inherited asymmetrically, potentially leaving the future oocyte with the largest portion of fusome once the 16 cell stage is reached [6]. Shot would act in establishing the fusome-associated microtubule-cytoskeleton in meiotic cysts, and slight differences in the distribution of factors that depend on the microtubule cytoskeleton could then be amplified over the process of cyst maturation from region 2a to region 3, leading to the selective enrichment of proteins first in the two pro-oocytes and later the oocyte. At a time when most structural fusome markers such as α-Spectrin have disappeared, in region 3 and stage 1–2 of oogenesis, Shot is still highly enriched on structures that could be fusome remnants. Shot was still more concentrated toward the oocyte and strongly colocalized with the highly polarized microtubules, thus again placing it physically between the fusome and the microtubules, with the fusome acting as a template to mold this highly polarized microtubule cytoskeleton.

Conclusions

The spectraplakin shot is the only member of a class of proteins with similar loss-of-function or partial-loss-of-function phenotypes during oogenesis (this class includes Dynein heavy chain, the dynein/dynactin-complex associated proteins Egl and BicD, and the RNA binding protein Orb) to associate with the fusome and directly bind to microtubules. In this paper we have demonstrated that Shot is the missing link between the fusome and the polarized microtubule cytoskeleton in meiotic cysts and is essential for oocyte specification and establishment of the embryonic axes.

Experimental Procedures

Fly Strains and Induction of Clones

For the induction of FLP-FRT clones of *shot* mutant cells in the germline, the following strains were used: *ywhsFLP;FRTG13 shot*^{3}/*FRTG13 2xUbi::GFP* or *ywhsFLP;FRTG13 shot*^{kakP1}/*FRTG13 2xUbi::GFP*. Offspring of the right genotype were heat shocked for 2 hr at 37°C on two consecutive days around late second to third larval instar. Adult females emerging were dissected 10–12 days after the heat shock.

Immunofluorescence and Confocal Microscopy

Ovaries were dissected in PBS, fixed for 10 min in 4% formaldehyde in PBS (or 8% formaldehyde in PBS to preserve microtubules), and

permeabilized and blocked in PBS containing 0.5% BSA and 0.3% Triton X-100 (PBT). Primary and secondary antibody incubations took place overnight at 4°C in PBT. Antibodies used were anti-plakin repeats (rabbit, 1:200; [32]), anti-spectrin repeats (guinea pig, 1:400; [29]), anti-α-Spectrin (rabbit, 1:400) [43]), anti-β-Spectrin (rabbit, 1:300; [43]), anti-hts (mouse, 1:10; Developmental Studies Hybridoma Bank), anti-Orb 4H8 (mouse, 1:250; Developmental Studies Hybridoma Bank), anti-BicD (rat, 1:1000 [13]), anti-C(3)G (guinea-pig, 1:500; [35]), anti-α-tubulin-FITC (mouse, 1:200; Sigma), anti-acetylated α-tubulin (mouse, 1:500; Sigma), anti-γ-tubulin (mouse, 1:50, Sigma), and an antibody against a centrosomal protein (rabbit, 1:1000; J. Raff, personal communication). After antibody staining, the ovaries were mounted in Vectashield. Images were acquired on a Biorad 1024 or Biorad Radiance confocal microscope and assembled in Adobe Photoshop.

In Situ Hybridization

Ovaries were dissected and fixed as described for immunofluorescence. Fixed ovaries were transferred to 100% methanol and rehydrated in methanol/PBT. Hybridization was performed with the GAS2-specific RNA-probe as described in [32].

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