Rtnl1 is enriched in a specialized germline ER that associates with ribonucleoprotein granule components

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Summary

During oogenesis in *Drosophila* an organelle called the fusome plays a crucial role in germline cyst development and oocyte selection. The fusome consists of cytoskeletal proteins and intracellular membranes and, whereas many cytoskeletal components have been characterized, the nature and function of the membrane component is poorly understood. I have found the reticulon-like 1 (Rtnl1) protein, a membrane protein resident in the endoplasmic reticulum (ER), to be highly enriched in the fusome. In other *Drosophila* tissues Rtnl1 marks a subset of ER membranes often derived from smooth ER. During oogenesis, Rtnl1-containing membranes are recruited to the fusome by the cytoskeletal components and become concentrated into the forming oocyte. On the central part of the fusome, which is contained within the future oocyte and also at later stages in the growing oocyte and the nurse cells, Rtnl1-containing membranes colocalize with components of ribonucleoprotein complexes that store translationally repressed mRNAs. As the ER is actively transported into the oocyte, this colocalization suggests a role for the Rtnl1-containing subdomain in anchoring the ribonucleoprotein complexes within and/or transporting them into the oocyte.

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Introduction

In many organisms oogenesis proceeds in a cyst of interconnected germ cells (de Cuevas et al., 1997). In *Drosophila melanogaster*, one cell out of a cluster of 16 cells is singled out as the oocyte, whereas the others assume the fate of supporting nurse cells. The mechanism of oocyte determination at an early stage of oogenesis also contributes to the establishment of the later anterio-posterior body axis of the animal (Riechmann and Ephrussi, 2001).

Drosophila oogenesis proceeds in ovarioles within the female ovary, each comprising a chain of progressively older egg chambers or cysts. Each cyst of 16 cells is formed within the germarium (a structure located at the anterior tip of each ovariole) by four synchronous divisions, each followed by incomplete cytokinesis between the daughter cells. Thus, the cells remain connected by cytoplasmic bridges surrounded by ring canals. A cytoplasmic structure called the fusome stretches through the ring canals into all cells of a cyst. The fusome has an essential role in the selection of the oocyte and, thus, the generation of the major body axis (Lin and Spradling, 1995). It arises from a precursor structure in the stem cell, the spectrosome. During each stem cell division, the daughter cell that will form the cystoblast inherits one-third of the spectrosome/fusome (de Cuevas and Spradling, 1998). In the next division, the fusome remains in the mother cell but a new fusome ‘plug’ appears in the ring canal between the two forming cystocytes and fuses with the existing fusome. This process is repeated in each division and, thus, the original cystoblast appears to contain the largest part of the fusome in the 16-cell cyst. This inheritance of the largest part of the fusome has been proposed to be the earliest determining factor in the selection of the oocyte, marking this cell from the very first division (de Cuevas and Spradling, 1998).

After the 16-cell cyst is established, the fusome recruits a stable array of microtubules that are polarized with their minus-ends pointing into the future oocyte. This cytoskeletal array is essential for the directed transport of cell-fate determinants, such as the proteins Orb, BicaudalD and Egalitarian, into the future oocyte (Suter et al., 1989; Suter and Steward, 1991). Also, many mRNAs are selectively transported into the oocyte and are anchored there, usually in the form of ribonucleoprotein (RNP) complexes that contain machinery to prevent precocious translation during transport (Lantz et al., 1994; Nakamura et al., 2001; St Johnston, 2005). Although most of the cytoskeletal components of the fusome are disintegrating by the time that oocyte-specific proteins start to accumulate in the oocyte in region 2b of the germarium (see Fig. 2A for a scheme of regions in the germarium), some markers of oocyte cell fate appear to colocalize with the largest part of the fusome – which will eventually be contained in the oocyte – at earlier stages (Cox and Spradling, 2003; Grieder et al., 2000).

The fusome itself is composed of a membranous and a cytoskeletal part. Of those, the cytoskeletal part is better understood at a molecular level. The components of the cytoskeletal part include α-Spectrin, β-Spectrin, Ankyrin, the Adducin-like protein Huli tai-shao (Lin et al., 1994) and the spectraplakin Shot (Röper and Brown, 2004). Mutations in α-Spectrin or Hts disrupt the formation of the fusome, the synchrony and number of divisions and prevent oocyte formation (de Cuevas et al., 1996). The membranes of the fusome have ultrastructurally been described as membrane vesicles and tubules that resemble the endoplasmic reticulum (ER), associated with electron-dense matter and excluding ribosomes and mitochondria (Lin et al., 1994). Only one integral membrane protein has been described in a recent study that localizes to fusomal membranes: the ER translocon channel protein Sec61α. A GFP-fusion protein of Sec61α marks equally the fusome and all of the rough ER at some stages of cyst development in the germarium (Snapp et al., 2004). The same study showed that ectopic expression of a GFP-fusion protein of lysozyme with an engineered ER-retention signal (KDEL), LysGFP-KDEL, also localized to the fusome lumen in germaria. Thus, the fusome membranes appear to be composed of or derived from ER membranes.

The only mutations reported to selectively affect the fusomal membranes are mutations in the cytoplasmic protein Bag of marbles (Bam). Null-mutations in *bam* disrupt cyst formation at a very early stage and lead to overproliferation of the stem cells, and – hence the name – the formation of tumorous egg chambers (McKearin and Ohlstein, 1995). In *bam* mutants the cytoskeletal part of the fusome is present in the form of spectrosomes or dumbbell-shaped fusomes in two-cell cysts; however, when investigated by electron microscopy (EM) the cisternae of the membranous part of the fusome are strongly reduced. Therefore, although we have some idea of what the cytoskeletal components contribute to the fusome (e.g. Shot organizing the microtubules), the nature and role of the membranes is unclear.

One speculation is that the fusomal membranes provide a scaffold for the cytoskeletal structure to be assembled upon and the cytoskeleton then polarizes the transport within each cyst. ER membranes may have taken on the role of a scaffold simply because of their availability and abundance within each cell. Cytoskeletal proteins such as Spectrins, Adducin and Ankyrin are known to be recruited to the plasma membrane to form a submembranous cytoskeleton (Bennett and Gilligan, 1993), but they can also be recruited to intracellular organelles, such as the ER and the Golgi (Devarajan et al., 1996). Alternatively, the fusome membranes may play a more direct and instructive role during cyst formation and oocyte determination.

Here, I have addressed the nature of the fusomal membranes and their relation to the cytoskeletal components of the fusome through analysis of the distribution of ER components that have been tagged with green fluorescent protein (GFP) by a gene trap approach (Morin et al., 2001). This revealed that the ER-resident protein reticulon-like 1 (Rtnl1) is a highly selective component of fusomal membranes. Rtnl1 is the sole predicted functional member of the reticulon protein family in the fly. Reticulons, in particular the vertebrate Rtn4 and the two yeast reticulons, have recently been shown to be specific for tubular rather than sheet-like or cisternal ER structures and have been proposed to function in the formation of these structures in conjunction with the protein DP1/Yop1p (Voeltz et al., 2006). Reticulons are hairpin-forming transmembrane proteins (see scheme in Fig. 2B), and the fly reticulon was found to be localized to specialized forms of the smooth ER in several different cell types, such as the sarcoplasmic reticulum (SR) in muscles. The fusomal membranes marked by Rtnl1 become increasingly concentrated in cysts in the germarium throughout cyst maturation and, at stage 1 of oogenesis, are very concentrated in the oocyte. Data presented here indicate a role for the fusome membranes and other membranes labeled by Rtnl1-GFP in the germline in localizing and transporting RNP complexes.

Results

Membranes of the ER form part of the fusome and concentrate into the oocyte during oogenesis

To test whether the fusome membranes share features with ER membranes, ovarioles were stained with an antibody that recognizes the HDEL-peptide, a sequence conferring ER-retention to lumenal proteins (Napier et al., 1992). This antibody labeled part of the fusome within the germarium (Fig. 1A, arrows; see also scheme in Fig. 2A) and showed that the

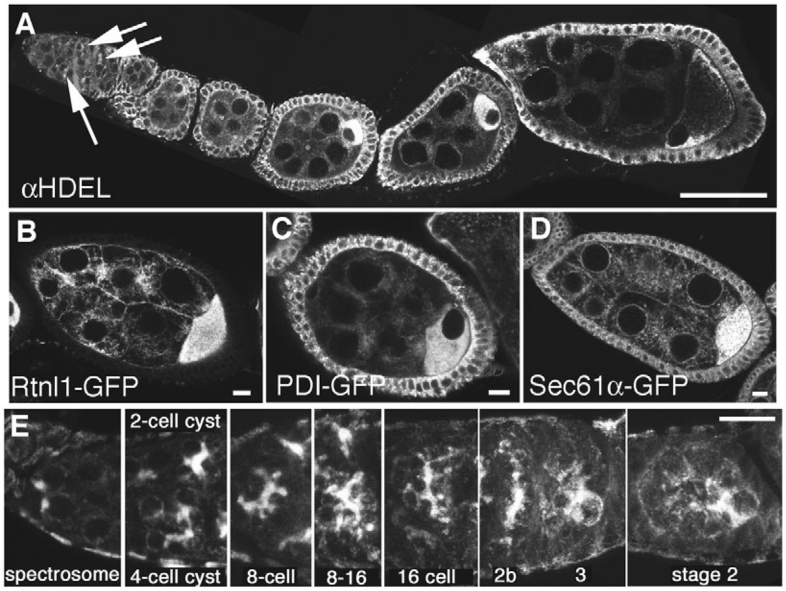

Fig. 1. The ER concentrates in the oocyte during oogenesis and is derived from fusome membranes representing a specialized ER. (A-D) The ER accumulates in the oocyte during oogenesis, as shown using an antibody against (A) the ER-retention signal HDEL and three different GFP-tagged ER proteins: (B) Rtnl1-GFP, (C) protein disulfide isomerase (PDI) and (D) translocon channel subunit Sec61α (Sec61-αGFP). Arrows indicate HDEL labeling of the fusome in the germarium. Note that only Rtnl1-GFP is specifically enriched in the germline and mostly absent from the somatic follicular cell layer. (E) Concentration of Rtnl1-GFP in the fusome during different stages of cyst maturation in the germarium (for a scheme of stages within a germarium see Fig. 2A). Bars, 50 μm (A), 10 μm (B-E).

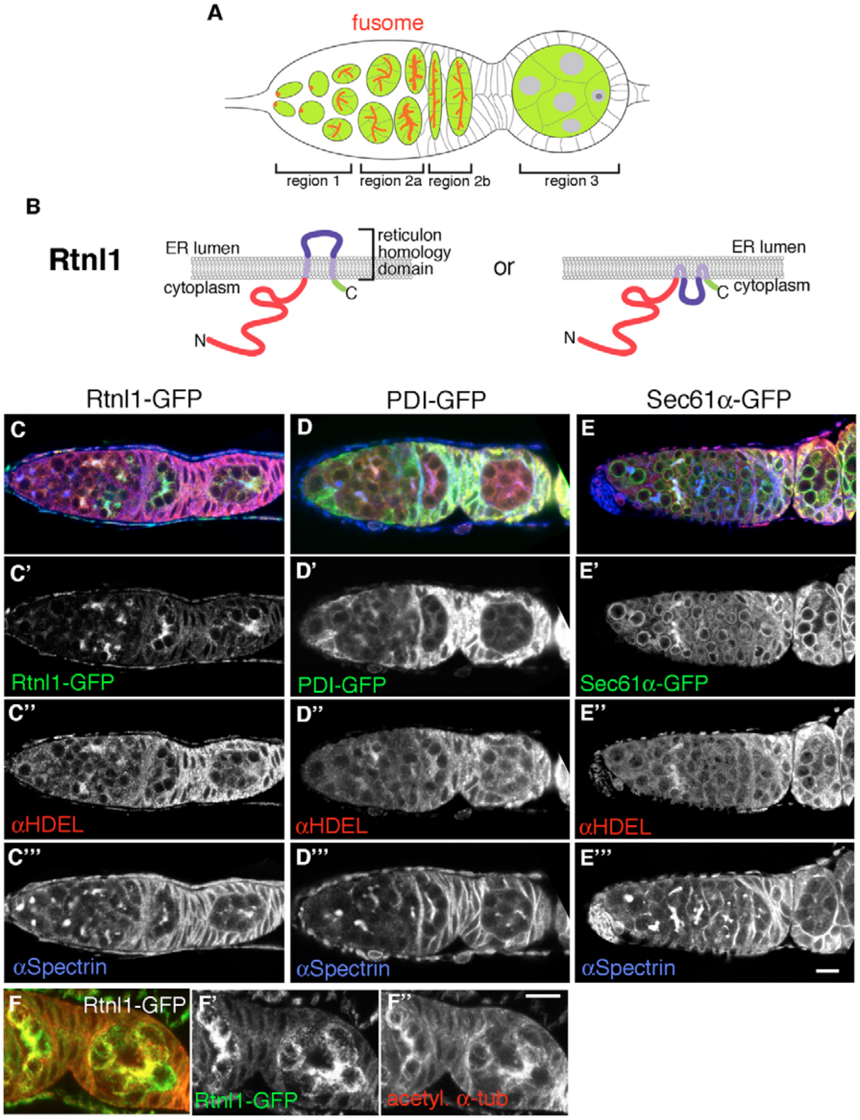

Fig. 2. The ER-membrane protein Rtnl1 is a marker of fusomal membranes during oogenesis. Examination of the fusome in the germaria during oogenesis reveals that the transmembrane protein Rtnl1 is highly enriched in fusomal membranes and very specific for the germline compared with PDI-GFP and Sec61α-GFP. (A) Scheme of a germarium. (B) Scheme of Rtnl1, showing the two proposed topologies for reticulon proteins. (C-C''',D-D''', E-E''') Comparison of Rtnl1-GFP, PDI-GFP and Sec61α-GFP, respectively, with antibody staining for HDEL (C'',D'',E'') and α-Spectrin (C''',D''',E'''). (F-F'') Comparison of Rtnl1-GFP with labeling for acetylated α-tubulin that marks the stable microtubule array, which is associated with the fusome and points into the oocyte (Röper and Brown, 2004). Note that Rtnl1 becomes very concentrated in the oocyte and at its posterior in region 3 of the germarium. Bars, 10 μm.

ER is strongly concentrated in the oocyte throughout oogenesis, as has been noticed previously (Morin et al., 2001). To establish whether this antibody accurately reflected the ER distribution during oogenesis, three ER-resident proteins were examined: the lumenal protein disulfide isomerase (PDI), a transmembrane subunit of the translocon channel Sec61α and the membrane protein Rtnl1. GFP-tagged versions of these proteins, generated by exon-trapping, were used (Morin et al., 2001). Because the GFP-encoding exon was incorporated into the genomic locus of all three proteins, the GFP fluorescence highlights endogenous expression and localization patterns of each protein. All three ER proteins were highly concentrated within the oocyte (Fig. 1B-D) but, in contrast to PDI and Sec61α that were also very strongly expressed within the somatic follicle cells, Rtnl1 appeared to be almost exclusively expressed within the germline cells during oogenesis (Fig. 1B). Rtnl1-GFP was also particularly enriched on the fusome at all stages of cyst maturation within the germarium (Fig. 1E, but see also below and Fig. 4).

The ER-membrane protein Rtnl1 is highly enriched in fusomal membranes during oogenesis

To address whether fusome membranes were indeed composed of or derived from ER membranes, the localization of all three GFP-ER markers within the germarium was compared with labeling for the ER-retention peptide HDEL and cytoskeletal markers of the fusome (Fig. 2). Rtnl1 was present in the fusome throughout the germarium (Fig. 1E and Fig. 2C) and, when compared with the cytoskeletal fusome component α-Spectrin, it appeared to increase in intensity until region 3 when it becomes highly concentrated in the oocyte (Fig. 2C and Fig. 4A, see scheme in Fig. 2A for nomenclature of regions within the germarium). By contrast, the anti-HDEL antibody, PDI-GFP and Sec61α-GFP labeled the fusome most strongly in region 2a (Fig. 2C'',D-D'',E-E''). Rtnl1 also colocalized with the stable microtubule array on the fusome that can be highlighted with an antibody against acetylated α-tubulin (Fig. 2F). Thus, the fusome membranes contained components typical for the ER and might therefore be derived from the common ER in the cystocytes. Although all ER markers analyzed labeled the fusome at some stage in the germarium, only Rtnl1-GFP seemed to be specifically concentrated in the fusome.

Rtnl1 is a component of the smooth ER in various different cell types

As the GFP-trap ER components showed strikingly different levels of expression between germline and somatic cells, and also only partially labeled the same structures during oogenesis, their expression and localization was analyzed in a variety of tissues during embryogenesis. As described previously, PDI-GFP labeled a perinuclear ER within the embryonic syncytial blastoderm (Bobinnec et al., 2003), and both Rtnl1-GFP and Sec61α-GFP were found to be associated with these structures (Fig. 3A and C, respectively). However, compared with PDI and Sec61α, Rtnl1 showed a strikingly different localization within the central nervous system (CNS) and in muscles. Rtnl1-GFP very strongly labeled the axonal tracts within the embryonic CNS (Fig. 3D), whereas both PDI-GFP and Sec61α-GFP could only be found in the neuronal cell bodies but were virtually excluded from axons (Fig. 3E,F). In embryonic body-wall muscles, Rtnl1 highlighted a reticular network, stretching throughout the muscles, that is characteristic of the SR (Fig. 3G); the same was observed within ovarian muscles (data not shown). PDI-GFP and Sec61α-GFP strongly labeled the ring-like perinuclear ER of the syncytial muscle nuclei and only weakly labeled the SR (Fig. 3H,I). In stage 14 embryonic epidermis all three proteins were within the perinuclear ER, although Rtnl1-GFP was present at lower levels (Fig. 3K-M).

The localization of Rtnl1 in both CNS and muscles suggests that it may preferentially be associated with specializations of the smooth ER, i.e. non-translocating ER that is free of ribosomes. Axons use smooth ER as a Ca^{2+} store that is needed for excitation (Henkart, 1980). The SR of muscles, derived from the smooth ER, is also a Ca^{2+} store essential for muscle excitation and contraction. PDI, as an enzyme associated with protein folding in the rough ER (Krijnse-Locker et al., 1994; Oprins et al., 1993), and Sec61α, as component of the translocon channel (Knight and High, 1998), both appeared to label the rough ER in all cell types but were largely excluded

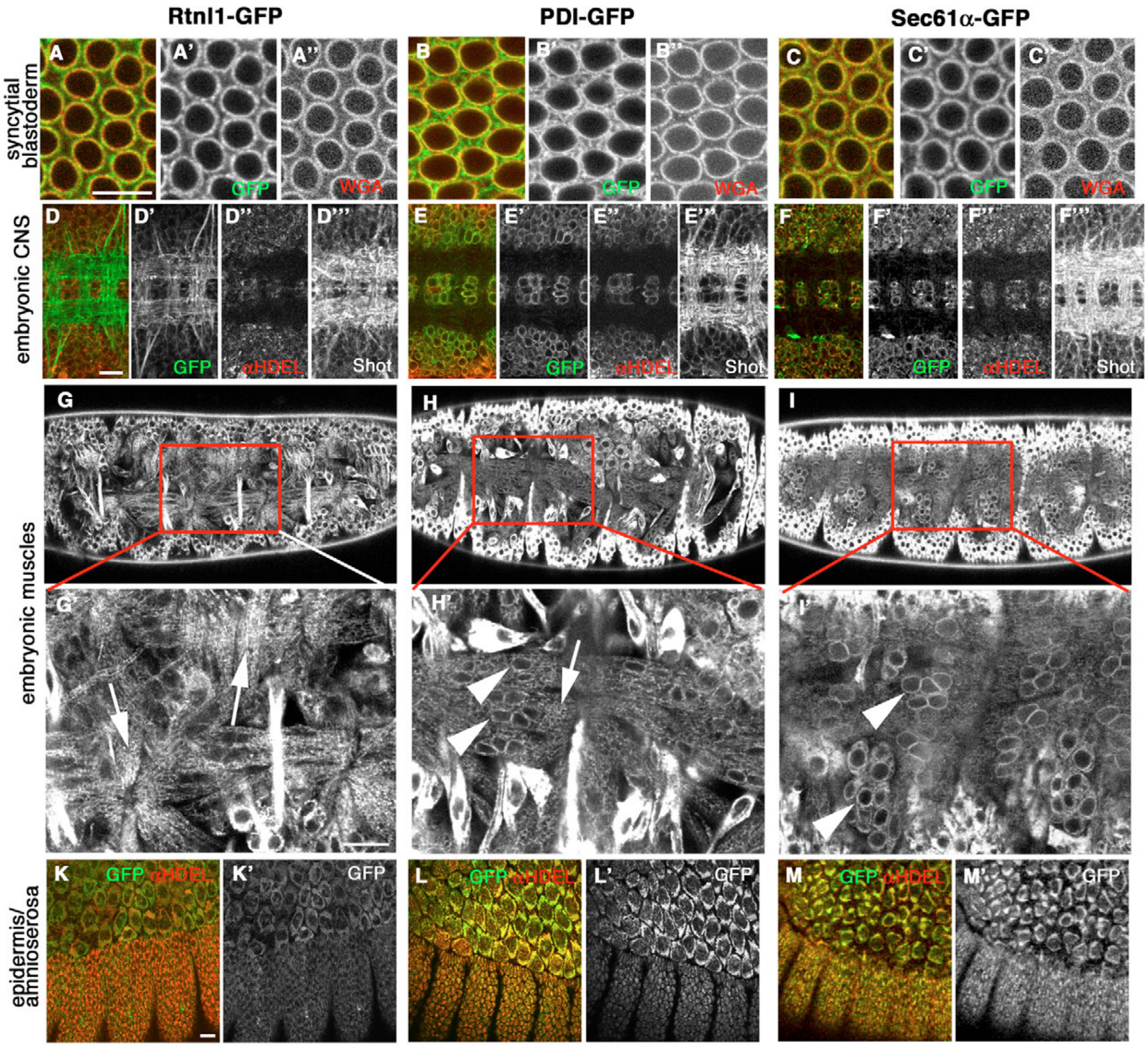

Fig. 3. Comparison of Rtnl1-GFP, PDI-GFP and Sec61α-GFP in various embryonic tissues during development. (A-M') The labeling of Rtnl1, PDI and Sec61α by exon-trapping with GFP shows the endogenous expression patterns of all three proteins. All highlight similar ER structures in the early syncytial embryo before cellularization (A-C) and later within the embryonic epidermis (K-M, shown is the epidermis-amnioserosa interface), although levels differ in stage 14 embryos. Note the striking differences in expression within the CNS (D-F) where Rtnl1-GFP (D) is strongly concentrated in the axonal tracts but both PDI-GFP (E) and Sec61α-GFP (F) are completely absent. Rtnl1-GFP is enriched in the SR (a specialization of smooth ER) of embryonic muscles (G), whereas PDI-GFP and Sec61α-GFP strongly mark the perinuclear rough ER in muscles (H and I, respectively). G'-I' show higher magnifications of the areas indicated by red boxes in G-I. Arrows in G' and H' point to the SR, arrowheads in H' and I' point to the nuclear envelope. Note that the bright staining in G'-I' is the labeling within the embryonic epidermis and not the muscle. Bars, 10 μm.

from the specializations of ER containing high levels of Rtnl1. Therefore, because Rtnl1-GFP was localized to specializations of the smooth ER in the embryo, this suggests that the fusome is also a specialized form of smooth ER.

The cytoskeleton recruits the membranes to the fusome

To address whether the membrane components of the fusome provides a scaffold for the recruitment of the cytoskeletal components of the fusome, I determined which component was recruited first to the fusome. The timing of association of the cytoskeletal components was compared with Rtnl1-GFP as the fusome developed. The cytoskeletal components appeared to be recruited prior to the membranes. The cytoskeletal and membrane part of the fusome showed an inverse intensity of fluorescence labeling in the germarium (Fig. 4). Whereas cytoskeletal components, such as Hts (Fig. 4A'',B'') and α-Spectrin (Fig. 4E''), were initially very concentrated in regions 1 and 2 but their labeling intensity then rapidly declined, Rtnl1-GFP levels, although already detectable on spectrosomes and fusomes in region 1, increased substantially in regions 2 and 3 (Fig. 4A',B',E' and also Fig. 1D). In addition, in fully branched fusomes of 16-cell cysts in region 2b, the cytoskeleton (marked by Hts) appeared to be in the core of the fusome structure, whereas the membranes (marked by Rtnl1) seemed to be recruited both throughout and surrounding the cytoskeletal core (Fig. 4C,D). This is especially obvious in animated 3D-reconstructions of *z*-stacks that cover the whole thickness of the germarium (see supplementary material Movie 1). The increase in Rtnl1 and, hence, fusomal membranes is in agreement with a previous finding from EM analysis, showing that the density of the vesicular material in fusomes increases as cysts mature (Lin et al., 1994; McKearin and Ohlstein, 1995). Only one cytoskeletal component, the spectraplakin Shot, showed a distribution very similar to Rtnl1 (Fig. 4F). This might reflect that, in contrast to the other cytoskeletal components, Shot itself is dispensable for fusome assembly and is recruited onto the fusome at a later stage to recruit the polarized microtubule array (Röper and Brown, 2004).

To test whether the cytoskeletal fusome, including components such as Hts and α-Spectrin, is necessary to assemble the fusome membranes Rtnl1-GFP localization was analyzed in *hts*^{1}, the loss-of-function allele of Hts (Lin et al., 1994). *hts*^{1} mutant germaria have cysts with a maximum of four cystoblasts, show loss of α-Spectrin labeling and seem to lack any structure resembling the fusome (EM analysis). In *hts*^{1} germaria Rtnl1-GFP was only diffusely localized in cystoblasts (Fig. 5A compared with 5B) and did not label any structure reminiscent of the fusome. Conversely, the only known mutation that affects the fusomal membranes but not the cytoskeleton is a null mutation of the cytoplasmic protein Bam. In *bam*$^{\Delta 86}$ mutant germaria, the cysts fail to progress beyond the cystoblast/two-cell stage, and contain only spectrosomes and small dumbbell-shaped fusomes marked by Hts; by EM the density of vesicular material is reduced (McKearin and Ohlstein, 1995). I found that, in *bam*$^{\Delta 86}$ mutant germaria Rtnl1-GFP was present on both spectrosomes and small fusomes, but the intensity of labeling appeared to be reduced (Fig. 5C).

These results suggest that the cytoskeletal components of the fusome are necessary to initially recruit the fusomal membranes. As the membranes still accumulated at a time when the cytoskeletal fusome began to diminish it appears that,

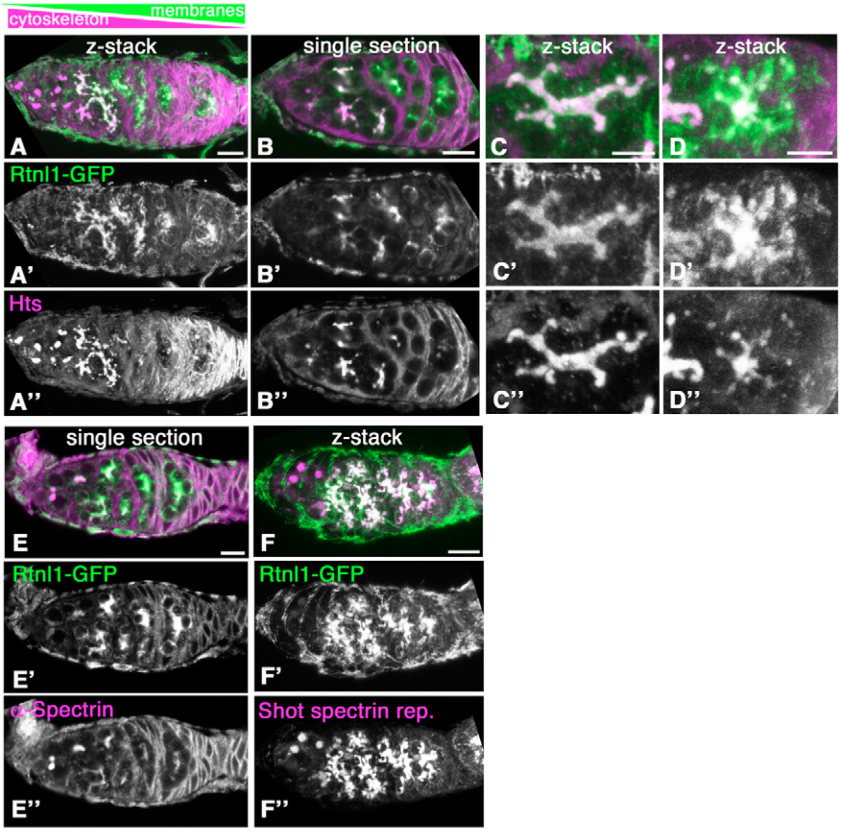

Fig. 4. The cytoskeleton recruits the membranes to the fusome. (A-F'') Comparison of Rtnl1-GFP as a specific marker for the fusomal membranes with cytoskeletal components of the fusome: *z*-stack (A) or single confocal section (B) comparison of Rtnl1 (green in A and B and as a single channel in A' and B') with the adducin-like protein Hts (purple in A and B and as a single channel in A'' and B''). Note that Hts is concentrated on spectrosomes and early fusomes and then rapidly declines in intensity during region 2b, respectively, whereas Rtnl1 becomes increasingly stronger with progress of the forming cyst through the germarium. (C,D) Two fusomes in early and later region 2b, demonstrating the increase in membrane fusome over cytoskeletal fusome. Note that the membranes (marked by Rtnl1-GFP in green in C and D and as a single channel in C' and D') are found throughout and also surrounding the structure labeled with the cytoskeletal fusome marker Hts (purple in C and D and as a single channel in C'' and D''). (E) α-Spectrin (purple in E and as a single channel in E'') appears similar to Hts in its decline in intensity over the germarium compared with Rtnl1-GFP (green in E and as a single channel in E''), whereas Shot, F, remains very strong all throughout the germarium (purple in F and as a single channel in F''). Bars, 10 μm (A,B,E,F), 5 μm (C,D).

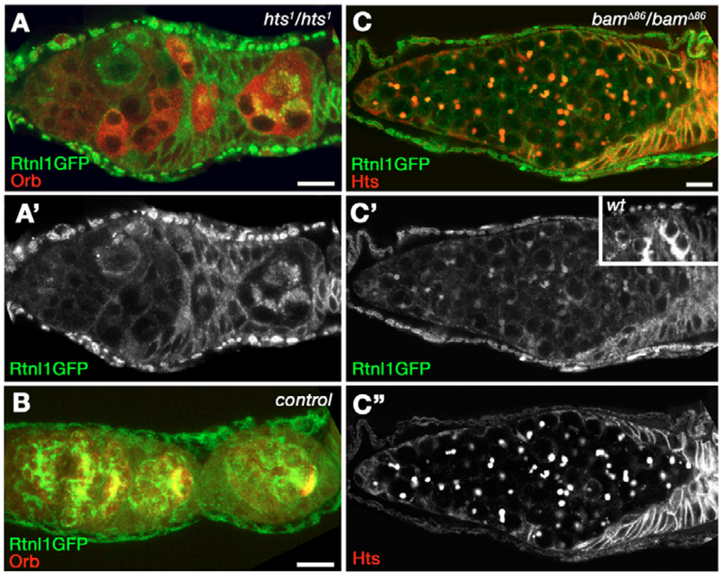

Fig. 5. Fusomal membranes are absent in *hts*^{1} and present in *bam*$^{\Delta 86}$ mutants. (A,A') *hts*^{1}-mutant germaria that do not contain a cytoskeletal fusome (Lin et al., 1994) do not show any concentration of Rtnl1-GFP (green in A and as a single channel in A') in cysts in the germarium (cysts are labeled by Orb-staining in red in A). (B) Control germarium with the usual concentration of Rtnl1-GFP (green) on the fusome and Orb concentration into the oocyte (red). (C-C'') In *bam*$^{\Delta 86}$-mutant germaria, which only contain spectrosomes and dump-bell shaped fusomes (McKearin and Ohlstein, 1995) marked by Hts (red in C and as a single channel in C''), Rtnl1-GFP associates with these fusomes (green in C and as a single channel in C'). Inset in C' shows Rtnl1-GFP in a wild-type germarium scanned at identical intensity to C'. The amount of Rnl1-GFP appears reduced. Bars, 10 μm.

after an initial recruitment, the further accumulation of membrane is now cytoskeleton-independent, leading to the inverse concentration seen. Thus, the role of the ER/fusome membranes is not to simply provide the scaffold for the cytoskeleton. Instead, fusomal membranes are actively recruited, suggesting that they serve a more instructive role during cyst maturation and oocyte selection than previously thought.

Recruitment of fusomal membranes does not depend on Shot, Egl or microtubules – but concentration of Rtnl1 into the oocyte does

The fusome is essential to recruit a stable array of polarized microtubules, and these fusome-bound microtubules and associated motors are needed to concentrate cell fate determinants into the oocyte (Ran et al., 1994). I, therefore, wanted to address the following: (1) how are fusome membranes affected by mutations that impact on the polarized microtubule array and (2) how are they affected by the complete depolymerization of microtubules when using colchicine. In germaria containing germline cells that lack Shot and, therefore, the stable microtubule array on the fusome (Röper and Brown, 2004), Rtnl1 was still associated with the fusome (Fig. 6A). Thus, the presence of Shot and the stable microtubule array on the fusome is not required to recruit fusomal membranes. Constraints of the experiment prevented the examination of Rtnl1 levels in Shot-mutant cysts compared with wild-type cysts, because the germline clones were generated using a GFP-marked wild-type chromosome; therefore, Rtnl1-GFP could not be detected in the control cysts. Germaria of loss-of-function mutants of the dynactin-associated protein Egl, using the allelic combination *egl*^{WU50}/*egl*^{RC12} (Huynh and St Johnston, 2000) have been shown to still contain a fusome with components such as Hts and α-Spectrin (Lin et al., 1994). Germaria from animals of this allelic combination had fusomes that contained Shot and also had stable microtubules, revealed by the distribution of acetylated α-tubulin (Fig. 6B). Rtnl1 was found on apparently normal fusome structures but, in most cases, it failed to become concentrated into one cell in region 3 of the germarium (Fig. 6C). In flies of this allelic *egl* combination, determinants of oocyte fate (such as Orb) also failed to become concentrated in one cell (Fig. 6D). It has previously been demonstrated that cytoskeletal components of the fusome, including core components, such as Hts and α-Spectrin but also Shot, are not affected when microtubules are depolymerized using colchicine (Bolivar et al., 2001; Röper and Brown, 2004). I wanted to address whether disruption of all microtubules in the germarium affected the fusomal membranes marked by Rtnl1. After treatment with colchicine for 40 hours Orb was mislocalized in all cysts (Fig. 6E''), indicating that microtubule-based transport into the oocyte was disrupted. Under these conditions Rtnl1-GFP was still found to be concentrated on fusome-like structures in region 2 (Fig. 6E,E'); however, these structures looked disorganized compared with wild-type fusomes. This was especially obvious in 3D-reconstructions of confocal *z*-stacks covering the whole germarium (data not shown). Also, in region 3 of the germarium, Rtnl1-GFP was not found to be concentrated within one cell. Taken together, these data suggest that, although some microtubules are necessary for the maintenance of proper fusomal membrane structure, the stable microtubule array on the fusome is not involved in the recruitment of fusomal membranes. Rather, it is needed for the later transport into – and the concentration within – the oocyte of membranes that contain Rtnl1.

Orb, Me31b and Trailer Hitch colocalize with Rtnl1 on both the fusome-ER and the oocyte-ER

In region 2b of the germarium, Orb always colocalized with the central part of the fusome (Fig. 7). This has been noticed in a previous study, which also showed that the Cup protein and the mRNAs encoding *orb* and *oskar* also localized to this central fusome (Cox and Spradling, 2003). This analysis showed that the association with the fusome was lost when the cell-fate determinants moved to the posterior pole of the oocyte. Comparing Orb with the membranes of the fusome marked by Rtnl1, I found that Orb continued to be associated with the fusome membranes while both translocated to the posterior pole (Fig. 7A,B). Surprisingly, the colocalization

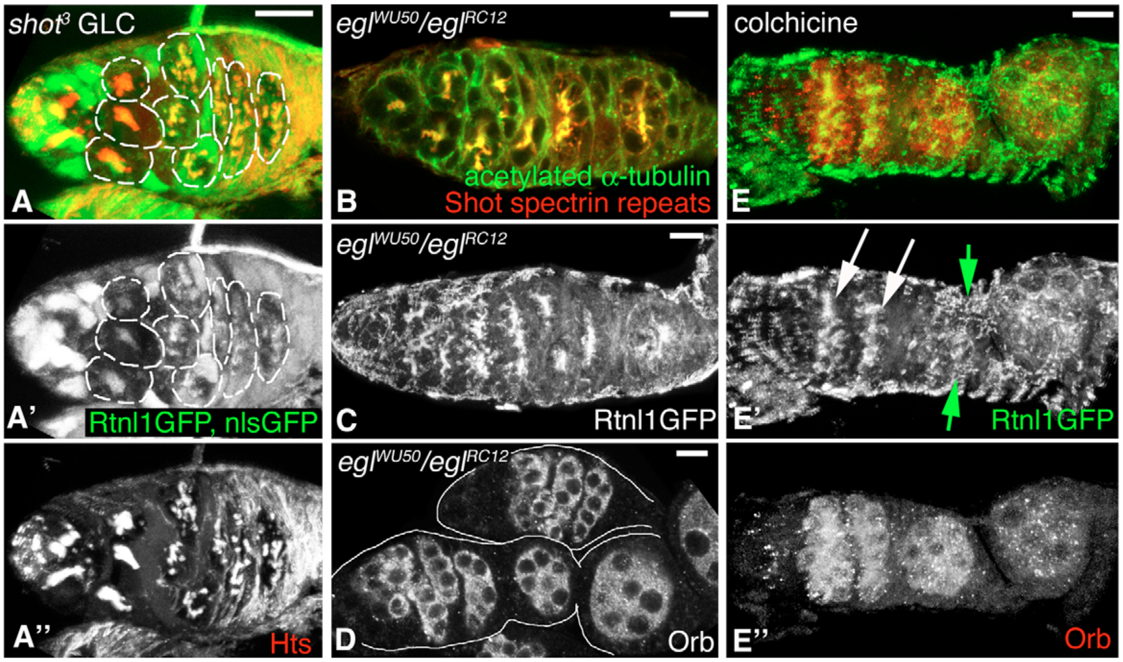

Fig. 6. The recruitment of fusomal membranes does not depend on Shot, Egl or microtubules, but the concentration of Rtnl1 into the oocyte does. (A-A'') In germline clones of a null allele of shot (*shot*^{3}) Rtnl1-GFP is still localized to the fusome, but does not concentrate into the oocyte. Germline clones are marked by the absence of GFP (green in A and as a single channel in A'; clones are indicated by the dotted lines). Rtnl1-GFP is also shown in green in A and also in the single channel in A'. As the germline clones lack GFP, Rtnl1-GFP distribution can be analyzed in these, but cannot be directly compared with adjacent wild-type germline cells. The cytoskeletal fusome is marked by Hts (red in A and as a single channel in A''). (B-D) In a heteroallelic combination of *egl* reported to be a null mutant, *egl*^{WU50}/*egl*^{RC12}, Shot (red in B) still localizes to the fusome and recruits the stable microtubule array marked by acetylated α-tubulin (green in B), although Orb is completely mislocalized in all cysts (D). In this allelic combination, Rtnl1-GFP is recruited to the fusome at wild-type levels (C), but in the majority of cases the accumulation into the oocyte in region 3 of the germarium fails. (E-E'') In the absence of microtubules in the germarium (by treatment of female flies with colchicine) Orb is completely mislocalized (red in E and as a single channel in E''), though some Rtnl1-GFP (green in E and as a single channel in E') still accumulates in the region of the fusome (white arrows), but it fails to concentrate in the oocyte. A-A'',C and E-E'' are stacks of confocal sections through the whole germarium, B and D are thick sections through the central portion of the germarium. Green arrows in E' point to the muscle sheet surrounding each ovariole that is also labeled by Rtnl1-GFP and partly visible in the projection. Bars, 10 μm.

between Orb and Rtnl1 within the oocyte continued throughout egg-chamber maturation until stage 8 (later stages could not be analyzed reliably due to antibody penetration problems). Both Orb and Rtnl1 were found in punctate structures scattered throughout the oocyte cytoplasm (Fig. 7C,D).

mRNAs that are specifically localized to the oocyte are translationally silenced during transport and until the stage that their expression is needed (for a review, see Johnstone and Lasko, 2001). Me31b is a DEAD-box protein involved in translational silencing of mRNAs, that has previously been shown to colocalize in particulate structures with *oskar*, *BicD*, *bcd*, *nos*, *pgc*, *gcl* and *orb* mRNAs (RNAs that are specifically concentrated in the oocyte) and the proteins Exu and Yps, both involved in mRNA localization during oogenesis (Nakamura et al., 2001). A recent study showed that Me31b and the associated proteins Trailer Hitch and Cup appeared to localize to the ER in nurse cells (Wilhelm et al., 2005). Me31b and Trailer Hitch are constituents of ribonucleoprotein complexes that contain the translationally silenced RNAs (Albrecht and Lengauer, 2004; Anantharaman and Aravind, 2004; Nakamura et al., 2001; Wilhelm et al., 2005). In early oogenesis, Me31b colocalized with both Orb and Rtnl1 on the central part of the fusome in the germarium, and together with these two other proteins concentrated in the oocyte in region 3 (Fig. 8A). Trailer Hitch also colocalized with Me31b on this central fusome portion (see supplementary material Fig. S1A). By contrast, Me31b did not colocalize with Sec61α-GFP and PDI-GFP in the germarium, where the latter labeled the fusome strongest in region 2a, before its association with Me31b (see Fig. 8B for Sec61α-GFP; PDI-GFP, data not shown). I found that both Me31b- and Trailer Hitch-labeled foci colocalized with accumulations of Rtnl1-GFP in both nurse cells (Fig. 8D) and oocyte until stage 8 (Fig. 8C). Sec61α-GFP and PDI-GFP were strongly concentrated within the oocyte but, whenever accumulations of either Sec61α-GFP or PDI-GFP were observed within the oocyte, they did not colocalize with Me31b- and Orb-containing foci in the oocyte (see Fig. 8G for Sec61α-GFP and Fig. 8I for PDI-GFP). However, within the nurse cells Me31b (and Trailer Hitch; see supplementary material Fig. S1C,D) was also found in the structures that contained Sec61α-GFP (Fig. 8H) and PDI-GFP (Fig. 8K). But, in contrast to Rtnl1GFP, both were not more concentrated in these foci than the surrounding labeled structure.

Thus, in agreement with recent findings (Wilhelm et al., 2005) Me31b, Trailer Hitch and Orb, and thus RNP complexes, were found to localize to a subdomain of the ER. The data presented here indicate that this subdomain is marked by Rtnl1-GFP. In addition to the colocalization in nurse cells, the results presented here show that the RNP complexes are already colocalized with the Rtnl1-labeled ER early in the germarium, on the central part of the fusome that is located within the forming oocyte. Throughout maturation of egg chamber and oocyte, RNP complexes marked by Orb, Me31b and Trailer Hitch remained associated with the Rtnl1-marked ER within the oocyte.

Hypomorphic and null mutations in *rtnl1* are viable and fertile

Restriction of Rtnl1 to the germline during oogenesis and also its striking concentration in fusomal membranes suggested that the protein itself has a function in the generation or maintenance of fusomal membranes, or the anchoring of RNP complexes to these membranes. Mutant alleles were generated by EMS-induced mutagenesis of the GFP-exon-trapped Rtnl1 strain and scored for the loss and/or mislocalization of GFP. A mutation, *rtnl1*^{1} – that biochemically appeared to be a truncated protein – was isolated (Fig. 9B). Flies carrying this mutation were viable and fertile, as were flies carrying a null mutation in *rtnl1* (*rtnl1*^{null}) that was generated by imprecise excision of a *P* element and led to the deletion of the whole reticulon-homology domain (Wakefield and Tear, 2006).

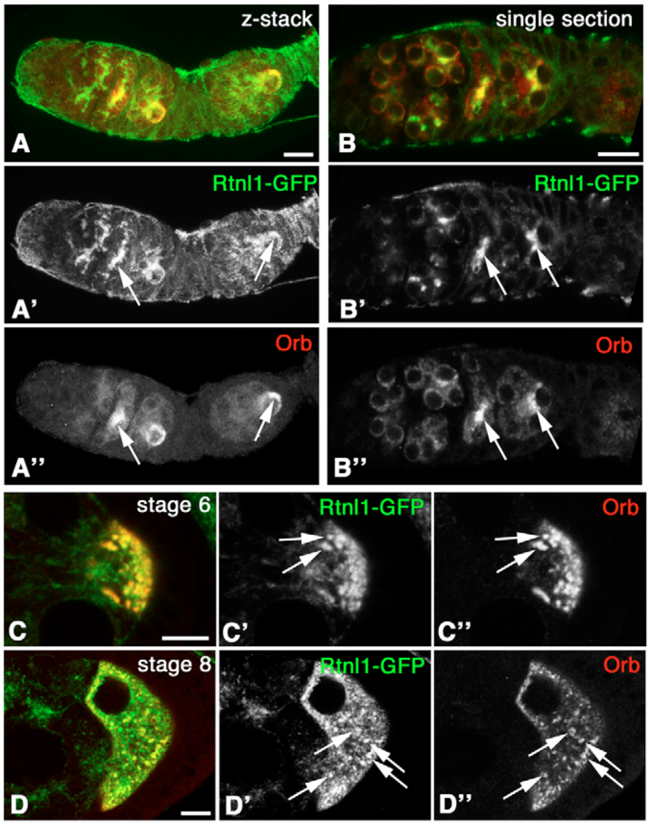

Fig. 7. Orb colocalizes with Rtnl1 on both the fusome-ER and the oocyte-ER. (A-B'') The RNA-binding protein Orb (red in A and B and as a single channel in A'' and B'') colocalizes with Rtnl1-GFP (green in A and B and as a single channel in A' and B') on the central portion of the fusome. This is visible in both projections of confocal *z*-stacks (A-A'') and also single confocal sections (B-B''). Arrows point to the central portion of the fusome in the germarium and the posterior crescent of Orb and Rtnl1-GFP in a budding egg chamber. (C-D'') Orb and Rtnl1-GFP also colocalize in punctate structures in oocytes of stage 6 (C-C'') and stage 8 (D-D'') egg chambers. Rtnl1-GFP is shown in green in C and D and as a single channel in C' and D', Orb is in red in C and D and as a single channel in C'' and D''. Arrows in C',C'',D' and D'' point to colocalizing foci. Bars, 10 μm.

In germaria of *rtnl1*^{1} or *rtnl1*^{null} flies the fusomal membranes were still present when stained for HDEL (data not shown), and the fusome cytoskeleton labeled with either Hts (data not shown) or Shot (Fig. 9C,D) appeared normal. However, both Orb and Me31b were less concentrated on the central part of the fusome in region 2b and appeared to translocate to the posterior of the oocyte in region 3 less efficiently (Fig. 9C,D). Nevertheless, both always accumulated within one cell once egg chambers budded off the germarium, and oocyte maturation appeared to progress normally. Thus, although Rtnl1 provides a useful marker of fusomal membranes, the deletion of the protein itself only mildly affected the association of RNP complexes with the fusome ER.

Discussion

The analysis of Rtnl1 and fusomal membranes shows that Rtnl1 is a highly enriched component of fusomal membranes, identifies these membranes as a specialized smooth ER and suggests a connection between fusomal ER, oocyte ER, and the localization and transport of RNP complexes within the germ cells.

Fusome cytoskeleton versus membranes

The strong labeling of fusomal membranes by Rtnl1-GFP allowed the direct comparison of the two parts of the fusome: the cytoskeletal components (such as Hts, α-Spectrin and Shot) and the membranes. During cyst formation and maturation in the germarium, the membranes and core cytoskeletal components of the fusome show an inverse concentration: the cytoskeletal part decreases, whereas the membranes become concentrated. This relationship and the analysis of mutants in the cytoskeletal component *hts*, that abolish all cytoskeletal components and the membranes of the fusome, suggest that the cytoskeletal part of the fusome is essential to recruit the membranes. In contrast to the core cytoskeletal components, such as Hts and α-Spectrin, the membranes of the fusome marked by Rtnl1-GFP do not disintegrate in regions 2b and 3 in the germarium. Rather, they continue to accumulate and become highly concentrated in the oocyte in region 2b, concomitant with the accumulation of cell fate determinants in the oocyte. The decrease in the amount of cytoskeletal components such as Hts and α-Spectrin suggests that the recruitment is non-stoichiometric and, after an initial recruitment that is dependent on Hts, the further accumulation of membrane and growth of the fusome is cytoskeleton-independent. The recruitment of fusomal membranes is independent of factors such as Egl, microtubules or Shot. However, these factors are required to concentrate membranes marked by Rtnl1-GFP into the oocyte.

A specialized germline ER is involved in the localization of RNP complexes

The active recruitment of fusomal membranes by the cytoskeleton suggests that the membranes are recruited to serve

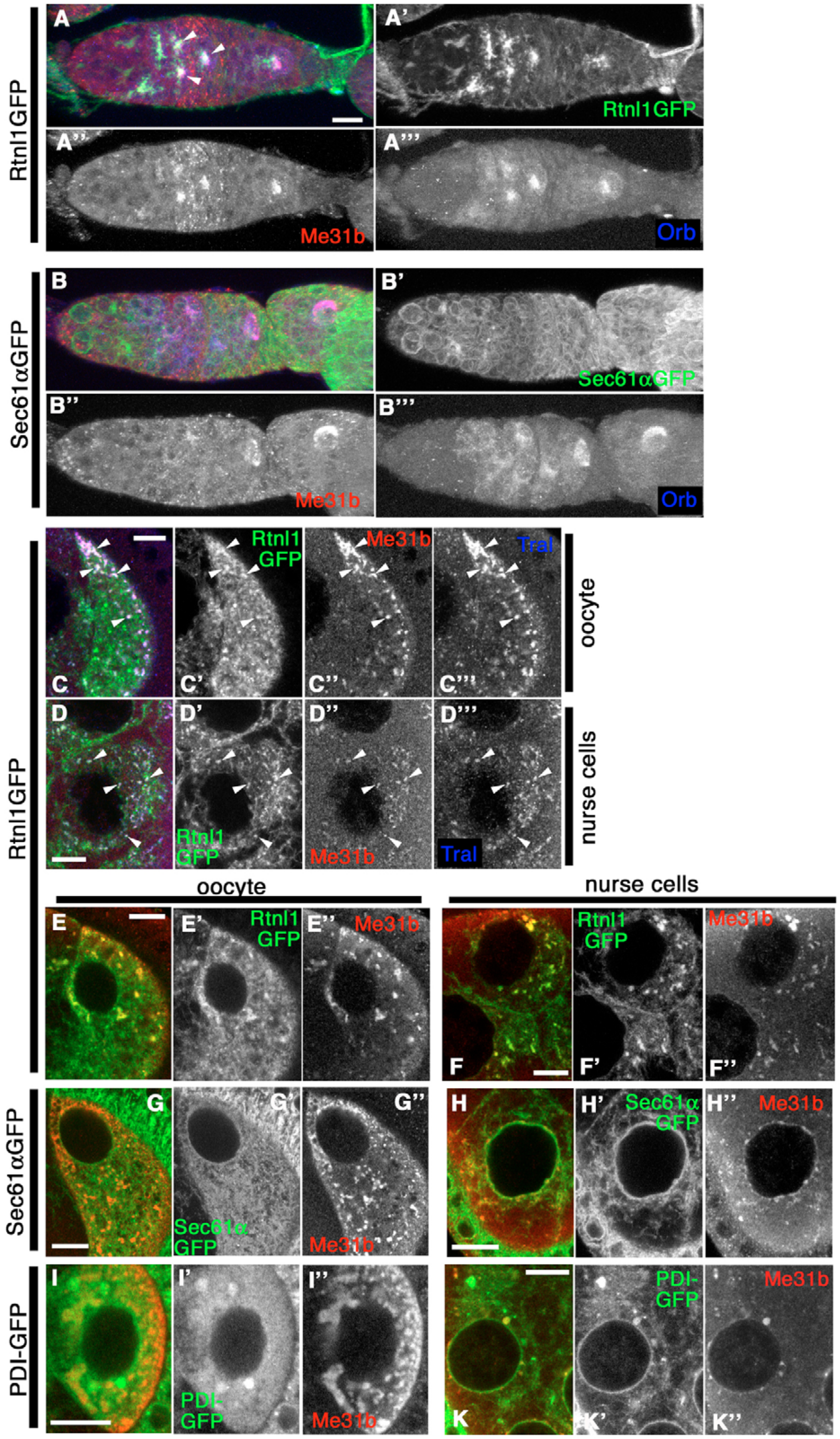

Fig. 8. Rtnl1GFP colocalizes with components of RNP complexes on both the fusome-ER and the oocyte-ER. (A-A’”) The DEAD-box protein Me31b, which is involved in translational silencing of mRNAs and a component of RNP complexes, colocalizes with Rtnl1-GFP and Orb on the central portion of the fusome in germaria. Shown is a *z*-stack projection of a germarium. Rtnl1GFP is green, Me31 is red, and Orb is blue in A, with the respective single channels shown in A’-A’”. Arrowheads in A point to the colocalization on the central portion of the fusome. (B-B’”) Sec61α-GFP shows strongest labeling of the fusome in region 2a, where neither Orb nor Me31b are concentrated yet, and no further colocalization within the germarium is observed. Sec61α-GFP is green, Me31 is red, and Orb is blue in B, with the respective single channels shown in B’-B’”. (C-F) Rtnl1-GFP, Me31b and also Trailer Hitch (Tral), another component of RNP complexes, colocalize in punctate structures in the oocyte (C-C’” for Rtnl1GFP versus Me31b and Trailer Hitch, and E-E” for Rtnl1GFP versus Me31b alone) and nurse cells (D-D’” for Rtnl1GFP versus Me31b and Trailer Hitch, and F-F” for Rtnl1GFP versus Me31b alone). Shown are single confocal sections of a stage 6-7 egg chamber. In all composites Rtnl1GFP is in green, Me31b is in red and Tral is in blue. Arrowheads in C and D point to some of the colocalizing foci of Rtnl1GFP, Me31b and Tral. (G-K) Comparison of the localization of Me31b and Sec61α-GFP and Me31b and PDI-GFP. Both Sec61α-GFP and PDI-GFP are strongly concentrated in the entire oocyte and do not show foci of stronger labeling where Me31b is concentrated into foci (G for Sec61α-GFP and I for PDI-GFP). Within the nurse cells, Me31b foci colocalize with labeling for Sec61α-GFP and PDI-GFP (H for Sec61α-GFP and K for PDI-GFP). In all composites Sec61α-GFP and PDI-GFP are in green, Me31b is in red. Bars, 10 μm.

another function than being a passive scaffold during cyst formation and oocyte selection. The colocalization of Rtnl1-GFP enriched ER with components of RNP complexes in both the germarium and the oocyte indicates a potential role for this subdomain of the ER in anchoring or even transporting these complexes. A colocalization between RNP complexes and the ER has recently been described in both *Drosophila* nurse cells (Wilhelm et al., 2005) and in the *Caenorhabditis elegans* embryo (Squirrell et al., 2006), although neither study identified the part of the ER the RNP complexes localize to. My findings extend the description of this colocalization during *Drosophila* oogenesis to early stages in the germarium and also to the oocyte itself, and they show that Rtnl1 is a marker for this subdomain of the ER. Why use the ER as a site

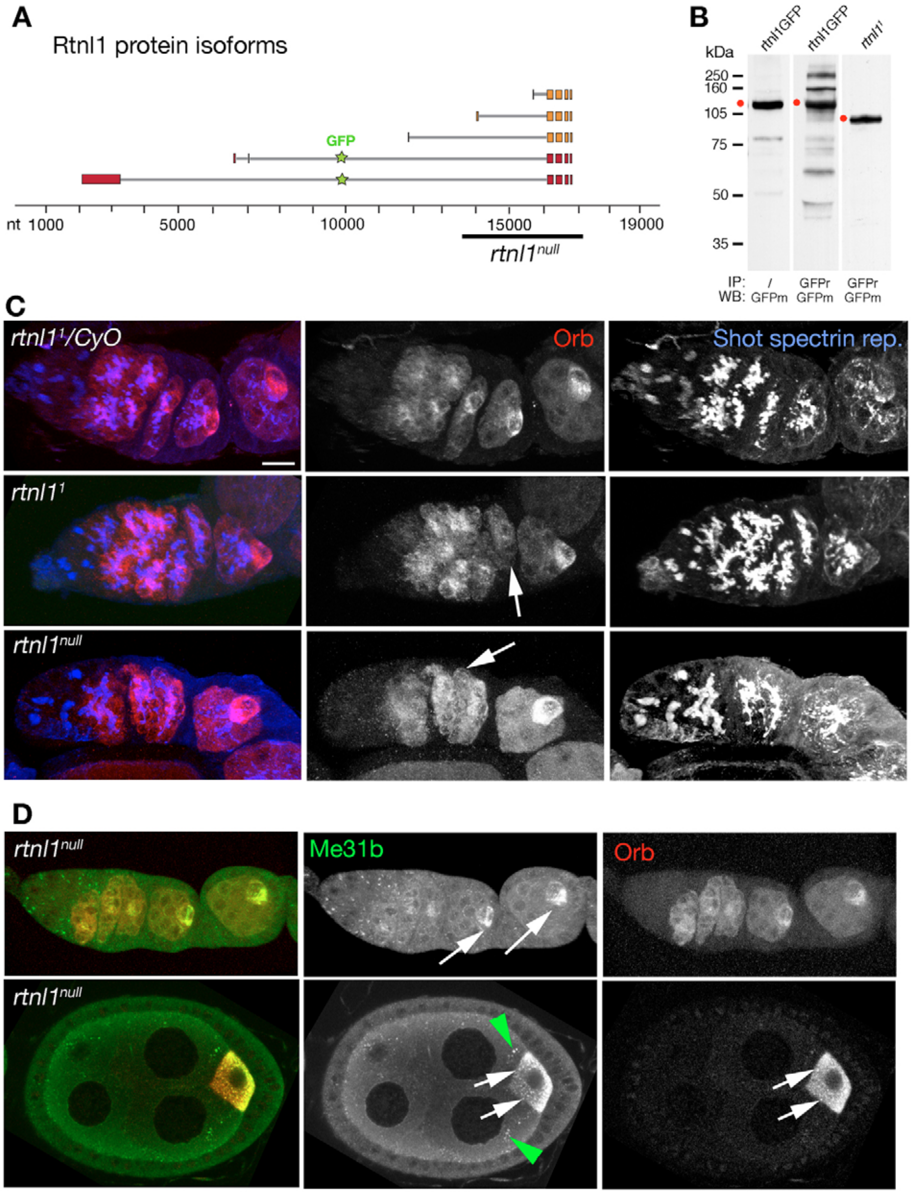

Fig. 9. Flies carrying a hypomorphic or a null mutation in *rtnl1* show only a mild phenotype in cyst maturation during oogenesis. (A) Scheme of potential Rtnl1 protein isoforms; red and orange boxes show protein-encoding exons. The green stars indicate the position where the GFP-exon is inserted and, thus, only the two largest protein isoforms are labeled in the Rtnl1-GFP line. The black bar indicates the deletion in the *rtnl1^{null}* allele that eliminates all of the reticulon-homology domain encoding the membrane-association domain of the protein (Wakefield and Tear, 2006). (B) The *rtnl1^{1}* allele, generated by F1-EMS mutagenesis screen (see Materials and Methods) appears to result in expression of a truncated protein. (Left) Western blot of Rtnl1-GFP exon-trap ovaries in the left panel; (right) immunoprecipitation with anti-GFP antibody followed by western blotting of Rtnl1-GFP exon-trap and *rtnl1^{1}* ovaries. Red dots indicate the main Rtnl1-GFP protein band. (C) Compared with control germaria, germaria of both homozygous *rtnl1^{null}* and *rtnl1^{1}* mutant females show a less tight concentration of Orb on the fusome, and also sometimes even cysts completely lacking detectable concentration of Orb (arrows). Nonetheless, budding egg chambers in region 3 always have Orb concentrated within one single cell. Shown are *z*-stack projections through the whole germarium. Orb is in red and as a single channel shown in white in the middle panels, Shot, marking the fusome, is in blue and as a single channel shown in white in the right panels. Bar, 10 μm. (D) Germaria of homozygous *rtnl1^{null}* mutant females still show concentration of Me31b into the oocyte in budding egg chambers, although similar to Orb, the concentration on the fusome is less tight (top panel, arrows point to budding egg chambers). In later egg chambers, Orb and Me31b still colocalize in foci within the oocyte (bottom panels, white arrows) and Me31b still accumulates in foci within the nurse cells (bottom panels, green arrowheads). Top panel is a *z*-stack projection through a whole germarium, bottom panel is a single confocal section. Me31b is in green and as a single channel shown in white in the middle panels, Orb is in red and as a single channel shown in white in the right panels.

to localize and anchor RNP complexes? Within the germarium, the mode of fusome growth during the four synchronous divisions leads to the largest part of the fusome being located within the cell that originated the first division; this cell will become the future oocyte. Localization of cell fate determinants, such as RNA and associated proteins, to the fusome in general and especially to this largest part of the fusome will ensure their localization in the forming oocyte. As the fusome membranes do not break down at the end of cyst formation but rather begin to concentrate in the oocyte, the association with these membranes could also provide a means of transport into the oocyte. The fact that colocalization of RNP-complex components with a subdomain of the ER persists into later stages might again indicate a function of the ER in both anchorage and transport: throughout oogenesis the ER (using as markers not only Rtnl1-GFP but also PDI-GFP, Sec61α-GFP and anti-HDEL staining) concentrates increasingly within the oocyte, and the accumulation of the GFP-tagged ER proteins in front of ring canals leading into the oocyte is very suggestive of an active transport process (Bobinnec et al., 2003). This accumulation of membrane in the oocyte is needed to allow for the dramatic increase in plasma membrane surface area of the oocyte itself during later stages of oogenesis, and also to provide membrane storage for the early stages of embryogenesis (where membranes are needed for the cellularization of the initially syncytial embryo). Thus, RNP complexes that need to be segregated into the oocyte might 'hitch hike' on the Rtnl1-GFP marked ER to ensure their transport. A similar function has been proposed for a membranous structure, termed sponge body, that is found in egg chambers and colocalizes with RNP complex components, such as the protein Exuperantia and RNAs (Wilsch-Bräuninger et al., 1997). Although these authors comment that there is only superficial resemblance to fusomes and doubt that the sponge bodies could be derived from fusome, the conspicuous colocalization of RNP-complex components with Rtnl1-GFP in both the fusome and later the nurse cells and oocyte suggests that the sponge bodies are indeed modified fusomal membranes that lack the cytoskeletal aspect of the fusome.

As Rtnl1 appeared to be a membrane component highly concentrated in the fusome and in part of the ER in the oocyte at later stages of oogenesis, I tested whether the protein itself is important for either the generation or maintenance of these membrane structures or for the anchoring of RNP complexes to them. Two different *rtnl1* mutants, carrying an allele with a potential truncation of Rtnl1 or a null allele (see Fig. 9) (Wakefield and Tear, 2006), were both viable and fertile. This is in agreement with a recent study showing a redundant function for vertebrate and yeast reticulon-like proteins in the formation of tubular ER structures; an effect on ER morphology was only observed after both yeast reticulons and the associated protein Yop1p had been eliminated (Voeltz et al., 2006). Thus, ablation of Rtnl1 alone did not affect fusomal membrane recruitment or function. A *Drosophila* orthologue of Yop1p exists but is not yet characterized, so future studies may reveal a redundant function of Rtnl1 and *Drosophila* Yop1 in the germline.

Taken together, Rtnl1-GFP appears to be a marker of ER specializations derived from smooth ER. Its strong concentration within the fusome identifies the fusomal membranes as a specialization of the ER. These specializations could be built up by tubular structures in accordance with Voeltz and colleagues (Voeltz et al., 2006). Moreover, it has recently been proposed that smooth ER consists of tubules and rough ER is sheet-like, and that these structural differences are important for ER function (Shibata et al., 2006). The subdomain of the ER highlighted by Rtnl1-GFP in the female germline appears to anchor RNP complexes that contain translationally silenced mRNAs, and these complexes might even use this ER as a means of moving into and concentrating within the oocyte. The colocalization of Rtnl1-marked ER with Me31b and Trailer Hitch (that both have been linked to RNA-silencing complexes affecting oskar and BicD RNAs) also suggests that these Rtnl1-containing membranes are precursors or indeed part of the sponge bodies and maybe also the large silencing complexes found at the posterior of the oocyte at later stages. It would be interesting to determine whether several classes of RNP complexes exist that differ in their content of mRNAs, e.g. general oocyte-localized mRNAs versus asymmetrically localized mRNAs. Only a specific disruption of the Rtnl1-containing membranes will show whether their main function is to transport and anchor the RNP complexes or whether the membranes are also needed for other fusome-dependent processes in the germline.

Materials and Methods

Fly husbandry

Rtnl1-GFP, PDI-GFP and Sec61α-GFP fly lines were obtained from Fly Trap GFP Protein Trap Database (Morin et al., 2001) (http://flytrap.med.yale.edu/), the *rtnl1*^{null} flies were a kind gift from Guy Tear (Wakefield and Tear, 2006). The *rtnl1*^{1} allele was generated by treating homozygous rtnl1-GFP flies with EMS using standard procedures and scoring for absence of GFP fluorescence in F1 heterozygous larvae. To depolymerize microtubules, female flies were starved for 6 hours and then fed colchicine in yeast paste for 40 hours to induce depolymerization of microtubules in their ovaries.

Immunofluorescence and confocal microscopy

Embryos were collected on apple-juice plates at the indicated stages of development and processed for immunofluorescence using standard procedures. Ovaries were dissected from well-fed females and processed for fluorescence using standard procedures. The rat anti-Me31b and rabbit anti-Trailer-Hitch antibodies were a kind gift from Akira Nakamura (Nakamura et al., 2001), the mouse αHDEL antibody (2E7) a kind gift from Hugh Pelham (Napier et al., 1992). Secondary antibodies used were Alexa-Fluor-488-coupled (Molecular Probes) and Cy3- and Cy5-coupled (Jackson ImmunoResearch Laboratories Inc.). Samples were embedded in Vectashield (Vector Laboratories). Confocal images were obtained using a Radiance 2000 Confocal Microscope (Bio-Rad, Hemel Hempstead, UK) and an Olympus Fluoview 1000. Confocal laser, iris and amplification settings in experiments comparing intensities of labeling were set to identical values. Confocal pictures were assembled in Adobe Photoshop, *z*-stacks and *z*-stack projections, and 3D-reconstructions of *z*-stacks were assembled using Imaris (Bitplane).

Immunocytochemistry

Embryos were collected overnight, dechorionated in 50% bleach for 3 minutes and rinsed in water. Embryos were homogenized in at least five times their volume of solubilization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) in a glass homogenizer on ice. The lysates were incubated on ice for 30 minutes and centrifuged for 10 minutes at 13,000 ***g*** at 4°C. The upper lipid layer that formed after centrifugation was removed and the clear lysate used for immunoblotting and immunoprecipitations.

For each immunoprecipitation, 400 μl of lysate diluted to 1 ml in solubilization buffer were incubated with the primary antibody (1:500 for rabbit anti-GFP; Abcam) overnight at 4°C. Immunocomplexes were collected by incubation with proteinA-Sepharose. Proteins were eluted from the beads by boiling in sample buffer. Western analysis was performed as described in (Röper et al., 2000). Mouse anti-GFP antisera were diluted 1:3000. Antibodies were revealed by chemoluminescence using the Amersham western blotting detection reagents (Amersham, Little Chalfont, UK).

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