Live imaging of nuage and polar granules: evidence against a precursor-product relationship and a novel role for Oskar in stabilization of polar granule components

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Summary

Nuage, a germ line specific organelle, is remarkably conserved between species, suggesting that it has an important germline cell function. Very little is known about the specific role of this organelle, but in *Drosophila* three nuage components have been identified, the Vasa, Tudor and Aubergine proteins. Each of these components is also present in polar granules, structures that are assembled in the oocyte and specify the formation of embryonic germ cells. We used GFP-tagged versions of Vasa and Aubergine to characterize and track nuage particles and polar granules in live preparations of ovaries and embryos. We found that perinuclear nuage is a stable structure that maintains size, seldom detaches from the nuclear envelope and exchanges protein components with the cytoplasm. Cytoplasmic nuage particles move rapidly in nurse cell cytoplasm and passage into the oocyte where their movements parallel that of the bulk cytoplasm. These particles do not appear to be anchored at the posterior or incorporated into polar granules, which argues for a model where nuage particles do not serve as the precursors of polar granules. Instead, Oskar protein nucleates the formation of polar granules from cytoplasmic pools of the components shared with nuage. Surprisingly, Oskar also appears to stabilize at least one shared component, Aubergine, and this property probably contributes to the Oskar-dependent formation of polar granules. We also find that Bruno, a translational control protein, is associated with nuage, which is consistent with a model in which nuage facilitates post transcriptional regulation by promoting the formation or reorganization of RNA-protein complexes.

Key words: Nuage, Polar granules, *oskar*, *aubergine*, *vasa*, *Drosophila*

Introduction

Germline cells serve as the carriers of genetic information between generations. These cells segregate from the soma early in development and must maintain their identity until finally producing gametes in adults. Given the conserved role of germline cells in all sexually reproducing animals, some similarities in the form and components of these cells are expected. One seemingly universal structure is nuage, which appears in the germ cells of a broad spectrum of animals (reviewed in Eddy, 1975). Nuage was visualized by light and electron microscopy and appears as a dense, fibrous organelle that is not membrane-bound. It is often associated with mitochondrial clusters or is concentrated in the perinuclear cytoplasm and frequently in close proximity to nuclear pores. Nuage contains proteins, and several of the component molecules have been identified (below). It is widely believed that nuage embodies or contains the germ cell determinant, although the evidence is circumstantial and rests on its evolutionary conservation as well as the properties of mutants lacking nuage (Saffman and Lasko, 1999).

Nuage has been characterized most extensively in invertebrates. In *Drosophila*, a wide range of cell types were examined for the presence of nuage and it is limited exclusively to germline cells (Mahowald, 1971). A series of ultrastructural studies have traced nuage throughout the life cycle. Nuage first forms in primordial germ cells in the embryo, and persists there during larval and pupal development. Male gametes retain nuage through their differentiation, at least to the spermatocyte stage (Rasmussen, 1973). In the female germ line, nuage is lost from the oocyte when it becomes specified, but remains associated with the nuclei of the nurse cells until their contents is transferred to the oocyte (Mahowald and Strassheim, 1970).

The disappearance of nuage in the oocyte is followed, after an appreciable interval, by the accumulation of polar granules at the posterior pole of the oocyte (Mahowald, 1962). These granules are found in the germ plasm, a specialized region of the cytoplasm that contains the determinants of the germline (Mahowald, 2001). During cellularization early in embryogenesis the polar granules are largely sequestered into the pole cells, the progenitors of the germline, and begin to coalesce into a smaller number of large particles (Mahowald, 1968). With completion of the germ-band-extension phase of embryogenesis, the polar granule particles disappear and nuage appears (Mahowald, 1971). Some other animals, but not all, have a similar specialized cytoplasm at the site where germ

cells will form, suggesting that the use of germ plasm is a common strategy to reform the germline in embryogenesis.

Not surprisingly, given their related functions, nuage and polar granules have some components in common. The Vasa (Vas) and Tudor (Tud) proteins are highly enriched in nuage and in polar granules (Bardsley et al., 1993; Hay et al., 1988a; Hay et al., 1988b; Hay et al., 1990; Lasko and Ashburner, 1990). Notably, there are *vas* homologs expressed in the germline cells of a variety of animals (reviewed in Raz, 2000) and where examined, the distribution of the Vas protein is consistent with its presence in nuage. In *Drosophila*, the association of the Vas protein with nuage, originally detected by electron microscopy (Hay et al., 1988a), can also be monitored by immunofluorescence or fluorescent detection of the Vas:green-fluorescent-protein fusion protein (VasGFP) (expressed from a transgene) (Sano et al., 2002). Because all of these assays reveal characteristic particulate structures – either of nuage in nurse cells or of polar granules in oocytes and early embryos – the simpler fluorescence-based assays can be used as an alternative to electron microscopy. A third shared component is Aubergine (Aub), which was detected using a green fluorescent protein:Aub-fusion protein (GFPAub). Although no ultrastructural studies have directly demonstrated the association of Aub with nuage, the colocalization of GFPAub and Vas in perinuclear nuage validates the use of GFPAub as a nuage marker (Harris and Macdonald, 2001). Mutants lacking Vas or Aub display complete (*vas*$^{-}$) or partial (*aub*$^{-}$) loss of nurse cell nuage and both mutants fail to make polar granules (Findley et al., 2003; Harris and Macdonald, 2001; Liang et al., 1994; Schüpbach and Wieschaus, 1986).

The presence of shared components reinforces the notion that nuage and polar granules are closely related structures and raises the possibility of an ongoing cycle in which one is converted into the other. Efforts to address this possibility have focused on the transitions between the structures. Static images support a direct conversion of polar granules into nuage: both structures appear in the same perinuclear zones of the germline cells, the loss of polar granules is accompanied by the appearance of nuage, and contiguous regions of polar granules and nuage can be detected, which is consistent with a wave of transformation from polar granule to nuage (Mahowald, 1971).

The transition from nuage to polar granules is more complex. The two structures have very different locations and nuage would have to move from the nurse cells into the oocyte and then to the posterior pole to act as polar granules. Such a path is consistent with static images, which reveal nuage particles in the nurse-cell cytoplasm (Giorgi, 1976) within the ring canals that link nurse cells and the oocyte, and also within the ooplasm (Mahowald and Kambysellis, 1978; Wilsch-Brauninger et al., 1997) (for clarity, we refer to cytoplasmic bodies that contain nuage components as particles, whereas the large perinuclear punctae of nuage are termed clusters). Furthermore, the Oskar (Osk) protein, which is tightly localized to the posterior pole of the ooctye and is an essential component of polar granules (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995; Lehmann and Nüsslein-Volhard, 1986; Smith et al., 1992), can interact directly with Vas (Breitwieser et al., 1996) and could therefore serve as an anchor to which Vas-containing nuage particles are bound. Ectopic expression of Osk in the nurse cells has been reported to lead to cortical concentration of polar granule components in those cells (Riechmann et al., 2002), supporting the anchoring role.

Here we use live-cell imaging of GFP-tagged Vas and Aub to obtain a dynamic picture of the properties and movements of nuage. Our results show that fragments of nuage can indeed be displaced from the original site in the perinuclear zone of nurse cells and can move out into the cytoplasm, and also move through ring canals to the oocyte. However, these particles do not appear to become polar granules. Instead, our data are more consistent with a model in which Osk nucleates the formation of polar granules de novo. A possible role for nuage in assembling or reorganizing RNP complexes, particularly those involving localized or translationally regulated mRNAs, is supported by our identification of Bruno, which is associated with nuage and represses translation of two localized mRNAs.

Materials and Methods

Live imaging

Ovaries were removed from females expressing VasGFP or GFPAub, and individual egg chambers were teased apart with forceps under series-95 halocarbon oil onto a 22×45mm coverslip. The coverslip was transferred to the stage of a Leica TCS SPII inverted confocal microscope. 1024×1024 pixel images were collected using 40× or 63× HCX PL APO oil immersion objectives. Live images were collected at time intervals ranging from 0.82 to 60 seconds and for time periods up to 60 minutes. Images were analyzed using Leica software or Image J (National Institutes of Health). To determine whether polar granule formation occurs under these conditions, egg chambers were imaged 1 hour apart by performing a *z*-series scan through the oocyte posterior. The total fluorescence was measured in an area (the same area before and after time lapse) that encompassed all the polar granule material at the posterior. We determined the change in fluorescence over time by measuring the total posterior fluorescence in the *z*-section with the highest fluorescence and accounted for laser power fluctuations by normalizing these levels to the total fluorescence in a region with the same total area in the centre of the oocyte.

GFPAub and VasGFP were photobleached by focusing on the region to be bleached, using the maximum possible zoom and then performing one or two scans with the 488 nm laser at maximum power. Fluorescence recovery in the bleaching target was monitored every 15 seconds and the average fluorescence levels in the bleached region were measured using Leica confocal software.

Immunostaining

Ovaries were either hand dissected into *Drosophila* Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES pH 6.9), fixed for 5 minutes in devitellinizing buffer (1 volume of 100 mM KH_2PO_4/KH_2HPO_4 pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM $MgCl_2$, 1 volume of 37% formaldehyde and 10% methanol, 4 volumes of H_2O) under heptane before teasing apart ovarioles using forceps as described (Findley et al., 2003), or ovaries were hand dissected in ice cold phosphate buffered saline (PBS), then disrupted using flame drawn pasteur pipettes before fixing in 4% paraformaldehyde in PBS for 20 minutes under heptane. Egg chambers were rinsed in PBS plus 0.1% Triton X-100 (PBST), then blocked in PBST, containing 3% normal goat serum before incubating with rabbit anti-Oskar (final dilution of 1/1000) or rat anti-Bru (final dilution of 1/500) overnight. After rinsing in PBST, egg chambers were incubated with Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories). Egg chambers were rinsed then mounted in Vectashield (Vector Labs).

Fly strains

Drosophila melanogaster w^{1118} flies were used as a wild-type strain.

The following transgenic fly strains were used: *osk-bcd 3′UTR* (Ephrussi and Lehman, 1992), *UAS-osk-k10 3′UTR*, *UAS-gfp-aub* (Harris and Macdonald, 2001), *UAS-bruno-gfp* (P.M.M., unpublished), *vasaGFP* (Breitwieser et al., 1996), *exu-gfp* (Wang and Hazelrigg, 1994), and *nanos* GAL4. Mutants were aub^{HN2}/aub^{QC42}, $spnE^{E616}/spnE^{3987}$, tud^{1}/tud^{1}, vas^{AS}/vas^{AS}, $aret^{QB}/aret^{QB}$, and $aret^{PA}/aret^{PD}$.

Results

Previous descriptions of nuage have relied on static images, obtained initially by electron microscopy and later by immunodetection methods once individual nuage components were identified. Recently, GFP-tagged forms of two nuage components – Vasa and Aubergine – have been described (Breitwieser et al., 1996; Harris and Macdonald, 2001; Sano et al., 2002), and these fusion proteins offer the possibility to obtain a dynamic picture of nuage properties in living egg chambers.

We used time-lapse confocal microscopy to monitor nuage in egg chambers of ovaries that had been dissected under halocarbon oil. In these preparations, individual egg chambers can be viewed but remain in contact with the fluids and material present in the intact ovaries. Egg chambers prepared in this manner continue to support physiological processes for at least 60 minutes, as revealed by ongoing cytoplasmic movements (Glotzer et al., 1997; Theurkauf and Hazelrigg, 1998) and transport and localization of mRNA (Cha et al., 2001; Glotzer et al., 1997). There appear to be no deleterious effects on nuage within this time period, because the perinuclear clusters of nuage components persist in normal numbers and size. By contrast, egg chambers that are dissected into a buffered solution, rapidly lose the perinuclear nuage clusters, such that very few remain after 20 minutes. Dissection of testes under the same conditions also allows live detection of nuage.

Perinuclear nuage clusters are highly perdurant yet exchange components

The perinuclear nuage clusters observed in fixed samples could be stable, they could be stable yet have the ability to exchange components with cytoplasmic pools, or they could be short-lived structures whose population size remains relatively constant because of similar rates of cluster formation and dissolution. Using VasGFP as the reporter, experiments were performed to distinguish among these options. In the first set of experiments we traced the perinuclear nuage clusters (see Fig. 1A for an example) in stage 6-9 egg chambers for periods of 11-47 minutes, for a collective time of 6 hours. From analysis of 21 egg chambers, almost all of the clusters that could be continuously tracked (i.e. did not move out of the focal plane) were present throughout the experiment and maintained a constant size and level of fluorescence, and a constant position relative to the nuclear envelope and to other nuage clusters (Fig.1, arrowheads). Thus the nuage clusters appear to be stable and occupy relatively fixed positions around the nuclei. However, in rare examples (3 of 21 egg chambers or ~3 of 2100 nuage clusters, assuming an average of 100 visible clusters per egg chamber) individual nuage clusters or layers of the perinuclear zone of nuage moved into the cytoplasm (Fig. 1, arrows). New clusters were not observed to replace the lost clusters during the time of observation.

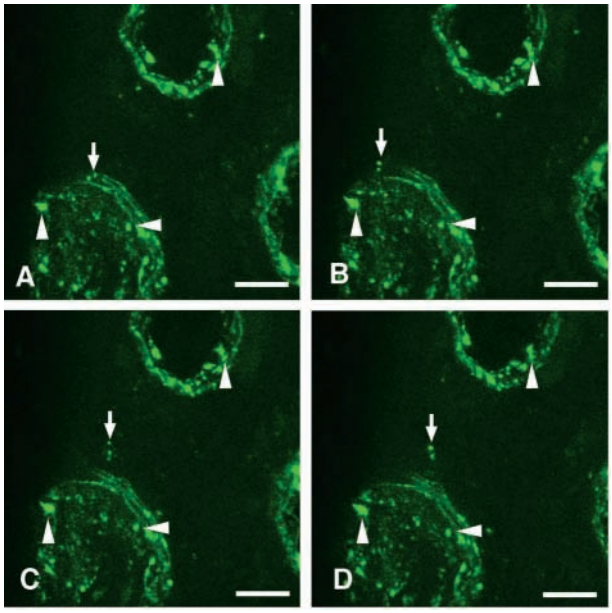

Fig. 1. Nuage clusters are stable. Panels A-D show VasGFP in the same live-egg chamber at 0, 4, 8 and 12 minutes after the start of imaging. Arrowheads indicate nuage clusters that are stable over this time period. Arrows indicate a nuage cluster that appears to detach from the nuclear envelope and move freely in the nurse cell cytoplasm. Note that this detachment was a very rare event. The sampling rate for this experiment was one scan every 2 minutes, and was 1-2 scans/minute for others. To address the possibility that some clusters are rapidly lost, followed by rapid reassembly of novel clusters at the same positions, some experiments were performed with sampling rates of up to 73 scans/minute, and the clusters appeared stable over 61 frames. Scale bars, 10 μm.

A second set of experiments was designed to determine whether the nuage clusters have a static organization, similar to that of ribosomal subunits, or whether they have a dynamic organization. We used fluorescence-recovery after photobleaching (FRAP) to distinguish between these options. For each experiment a part of an individual nurse cell, including a portion of the nucleus and a subset of the nuage clusters, was photobleached and reappearance of VasGFP fluorescence was monitored (under the conditions used, less than 20% of GFP fluorescence is restored within 1 hour after photobleaching, data not shown). In all experiments (n=20) we observed a rapid reappearance of nuage fluorescence, with a fluorescence-

Table 1. Fluorescence-recovery after photobleaching of GFPAub and VasGFP in nuage and polar granules from wild-type and mutant egg chambers

Bleaching target	$t_{1/2}$±s.d. (seconds)	n
VasGFP in nuage	59±27	20
VasGFP in nuage in aub^{HN2}/aub^{QC42}	50±12	10
VasGFP in nuage in $spnE^{E616}/spnE^{3987}$	48.5±15.8	10
GFPAub in nuage	35±8.45	10
VasGFP in spermatocyte nuage	57±15	8
VasGFP at posterior of stage 10 oocytes	210±49	8
VasGFP in posterior ooplasm	23±6	5
GFPAub at posterior of stage 10 oocytes	233±148	7

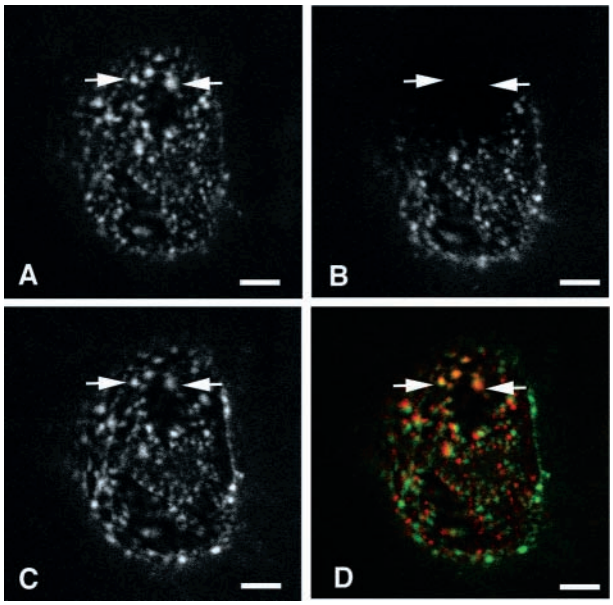

Fig. 2. Nuage clusters are stable yet exchange components with cytoplasmic pools. Panels A-C show VasGFP fluorescence in the same region of a stage-8 egg chamber at different phases of a FRAP experiment. (A) The initial distribution of nuclear envelope-attached nuage clusters; (B) immediately after photobleaching; (C) after 10 minutes recovery. (D) is an overlay of panels A (red) and C (green) that reveals the rapid recovery of fluorescence in stable nuage clusters (arrows). Scale bars, 4 μm.

recovery half-time ($t_{1/2}$) of 59 seconds (Fig. 2, Table 1). New VasGFP fluorescence appeared both in the relatively uniform perinuclear zone of nuage and in the perinuclear clusters. These clusters reappeared at the same positions of the original clusters, suggesting that there is exchange of VasGFP between cytoplasmic pools and stable nuage clusters. VasGFP in nuage of spermatocytes displays similar protein dynamics by FRAP (fluorescence-recovery $t_{1/2}$=57 seconds) (Table 1). To confirm that the reappearance of VasGFP fluorescence in nuage represented recruitment of additional VasGFP and not recovery of fluorescence of the photobleached VasGFP, entire egg chambers were photobleached; as expected, there was little recovery of fluorescence within the 10-minute time-frame of the bleaching experiments.

Just as observed for VasGFP, GFPAub in nurse-cell nuage can rapidly be exchanged with cytoplasmic pools, having a half-time of fluorescence recovery of 35 seconds after photobleaching (Table 1).

Our results with live imaging of perinuclear nuage reveal properties not apparent from analysis of fixed samples. The nuage clusters are dynamic structures in which at least a subset of components can rapidly be exchanged with cytoplasmic pools, yet the clusters themselves are stable with respect to both position and size. The extremely low rate of cluster detachment and our failure to detect dramatic changes in cluster size, argues that exchange of nuage components between the perinuclear clusters and cytoplasmic pools represents an exchange from existing clusters and not the release or dissolution of one cluster and its replacement with a new cluster at the same position.

Properties and movements of nuage particles in the cytoplasm

Nuage appears not only around the nurse cell nuclei, but also in cytoplasmic particles. Others have speculated that the cytoplasmic nuage particles derive from the perinuclear nuage, and are intermediates in the assembly of polar granules at the posterior of the oocyte (Liang et al., 1994; Styhler et al., 2002). Our results now show that cytoplasmic particles can indeed arise from the perinuclear nuage, although the low rate of particle formation by this mechanism suggests that particles also form by other means (see below). To learn more about potential roles for the cytoplasmic nuage particles, we characterized them by live imaging.

We monitored the cytoplasmic nuage particles primarily using GFPAub. The particles in the nurse-cell cytoplasm of stage-9 oocytes had an estimated average diameter of 0.52 μm and ranged from less than or equal to 230 nm (the theoretical limit of resolution by the objective used) to 1.02 μm (n=48), similar to the values obtained for nuage particles in ultrastructural studies (e.g. 0.19 to 0.47 μm) (Wilsch-Brauninger et al., 1997). This suggested that GFPAub particles are equivalent to the sponge body-associated cytoplasmic nuage observed via electron microscopy. Some of the particles were stationary and others moved (Fig. 3A,C). Particle movements were rapid (mean for the motile class is 123 nm/second, n=23, s.d.=63 nm/second, range 79 nm/second to 376 nm/second), and the velocities are slower than those observed for particles containing the protein Exuperantia (Exu) fused to GFP (ExuGFP) (250 to 2400 nm/second)(Theurkauf and Hazelrigg, 1998).

Particles in the nurse cell cytoplasm are sometimes concentrated near the ring canals that connect nurse cells to the oocyte, and occasionally near ring canals connecting nurse cells to other nurse cells (Fig. 3B; data not shown). Consequently, there must be preferential movements toward the ring canals that connect the nurse cells with the oocyte or particles must be captured near the ring canals. The latter interpretation is more probable for two reasons. First, the cytoplasmic particle movements appear to be random, with no obvious preference for movement towards the ring canals (Fig. 3A). Second, most particles throughout the bulk of the nurse cell cytoplasm are often more active in their movements than particles near the ring canals (Fig. 3C), suggesting that the latter class might be anchored or in some manner restricted in movement. Indeed, particles near the ring canals appear to be embedded in a membranous network, as seen by the exclusion of free cytoplasmic GFPAub fluorescence (Fig. 3D). This is very similar to observations made about the movements of ExuGFP (Theurkauf and Hazelrigg, 1998), which is associated with sponge bodies in the nurse cells (Wilsch-Brauninger et al., 1997). ExuGFP in the perinuclear region and near ring canals is relatively immobile whereas individual ExuGFP particles in the cytoplasm display rapid random motions throughout the nurse cell. We have also observed these movements and noticed that the ExuGFP in the perinuclear region is often in what appears to be a connected network (Fig. 3E). Because some nuage particles have been detected by electron microscopy within sponge bodies, it seems possible that the less mobile particles are sequestered within sponge bodies, while the rapidly moving particles are not.

Ultrastructural studies have also revealed nuage in ring

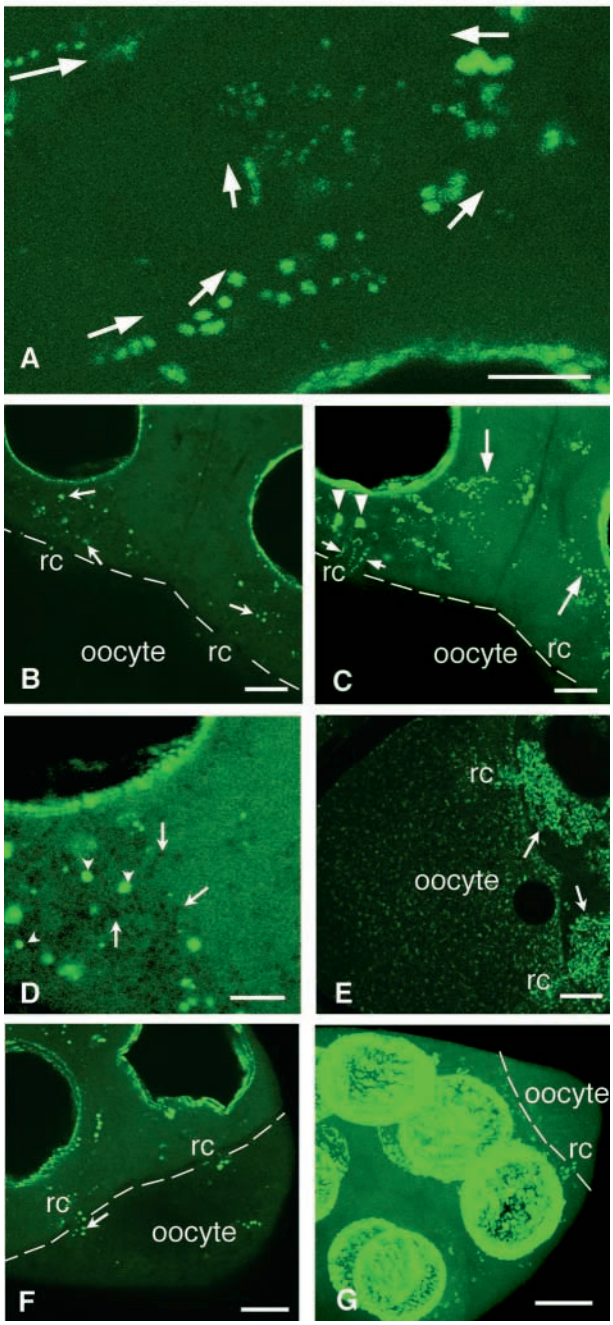

Fig. 3. Nuage particle movement in nurse cells. All panels show GFPAub except E, where ExuGFP particles are detected. The dashed lines in B, C, F, and G delineate the oocyte-nurse cell boundaries. (A) Representative movements of GFPAub particles in a nurse cell of a stage-9 egg chamber. A time projection of ten images (each taken 6 seconds apart) is shown, with the arrows aligned next to moving particles to indicate the direction and distance of migration. A ring canal connecting to the oocyte is out of the field to the top right of the image. (B) Nuage particles concentrated near the ring canals of a stage-9 egg chamber. The ring canals (rc; large gaps in dashed line) connect the nurse cells to the oocyte. The particle movements in this field are shown in the time projection in C, which represents 15 images captured at 6 second intervals. The small arrows indicate particles that are traversing the ring canals into the oocyte, the arrowheads indicate relatively immobile particles near the ring canals, and the large arrows indicate rapidly moving particles in other cytoplasmic regions. (D) Exclusion of GFPAub from regions of the cytoplasm. Structures appearing circular in cross section lack cytoplasmic GFPAub (arrows) and are sometimes clustered near ring canals along with nuage particles (arrowheads). (E) Clustering of relatively immobile ExuGFP structures near ring canals. This field was imaged for 10 minutes and showed very little movement of the particles (data not shown). (F,G) Onset of nurse-cell to oocyte transport at stage 8. For the stage-8 egg chamber in F, a much smaller data set was collected by imaging a single focal plane at one-minute intervals for four repetitions. In this and other experiments, particles were easily detected in passage through ring canals (arrow). For the stage-7 egg chamber in G, a large fraction of all possible particle movements were tracked by imaging at multiple focal planes and repeating the imaging at one-minute intervals for 20 repetitions. The data are presented as a *z*-series and time projection, and reveal no transport of particles into the oocyte. Scale bars, 5 μm (A,D), 8 μm (C), 10 μm (B,F,G), and 20 μm (E).

canals during vitellogenic stages of oogenesis, which suggests transport of nuage through ring canals into these oocytes (Mahowald and Kambysellis, 1978; Wilsch-Brauninger et al., 1997). Using either VasGFP or GFPAub as a marker, we observed many examples of particles entering the oocyte through the ring canals of stage-8 to -10 egg chambers (Fig. 3C,F). The particles move unidirectionally through ring canals into stage-9 oocytes at an average speed of 122 nm/second (n=21, range is from 72-261 nm/second), which is similar to the rates observed for the majority of ExuGFP-containing particles passing through ring canals (100-133 nm/second) (Theurkauf and Hazelrigg, 1998), although, unlike ExuGFP particles, they travel at the same speed in ring canals as in the nurse-cell cytoplasm. This transfer is restricted earlier in oogenesis, because very few particles pass through the ring canal into the oocyte during stages 1-6, even though cytoplasmic nuage particles are present in close proximity to the ring canals in the nurse cells adjacent to the oocyte (Fig. 3G). The absence of nurse cell to oocyte transport before stage 7 was previously reported in an analysis of the movements of unlabeled cytoplasmic particles (Bohrmann and Biber, 1994). Despite the general lack of transport into oocytes at the early stages, the oocytes do contain a few particles. Thus, either the restriction of transport is not absolute or particles can be assembled de novo within the ooplasm from the pool of soluble components.

Differential effects of *vas*, *aub* and *spnE* mutants on perinuclear and cytoplasmic nuage

Perinuclear nuage contains, in addition to Vas and Aub, the Maelstrom (Mael), and Gustavus (Gus) proteins (Findley et al., 2003; Styhler et al., 2002). We have identified a further component, Bruno (Bru), a protein that acts in translational repression of *osk* and *gurken* (*grk*) mRNAs (Kim-Ha et al., 1995; Filardo and Ephrussi, 2003). By immunolocalization (Fig. 4A) and expression of a GFP-tagged version of this protein (Fig. 4D), we find that Bru is concentrated in perinuclear clusters, similar to the distribution of known nuage components. Double labelling experiments with GFPAub confirmed that Bru colocalizes with nuage (Fig. 4A-C). However, Bru is also present at high levels in the cytoplasm, raising the question of whether the colocalization reveals an

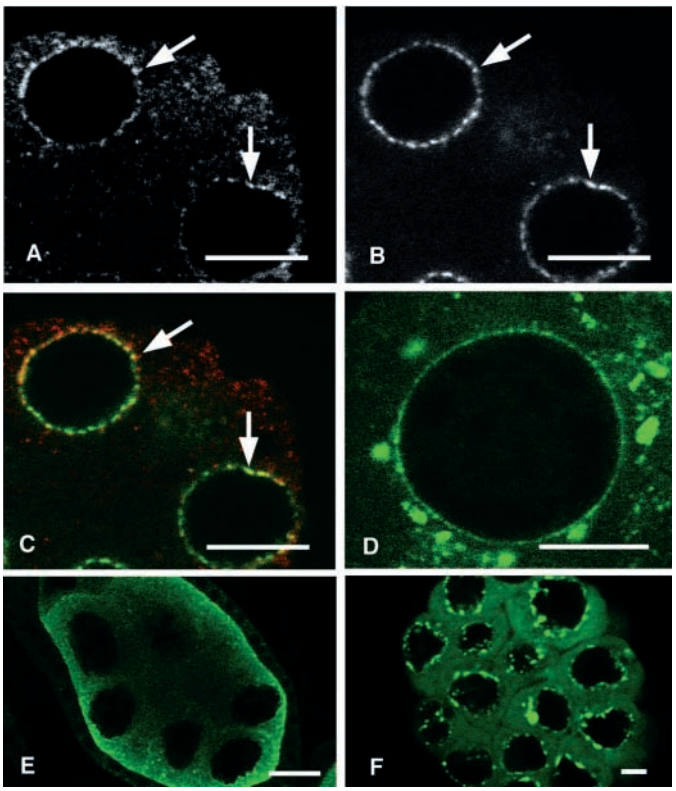

Fig. 4. Bruno is a nuage component. Immunostaining shows (A,E) Bru protein, (B,F) GFPAub and (D) BruGFP. (C) Bru staining in red and GFPAub in green. Bru protein can be found partially concentrated in particles around the nucleus (A, and red in C), where it colocalizes with GFPAub (B, and green in C). The colocalization of these molecules is not complete, suggesting some heterogeneity in the nuage. BruGFP is also found in these foci (D). This concentration is largely abolished in vas^{AS}/vas^{AS} nurse cells (E). Nuage, visualized with GFPAub, does not appear disrupted in $aret^{QB}/aret^{QB}$ pseudo-egg chambers (F), or in the nurse cells of the weaker allelic combination of $aret^{PA}/aret^{PD}$ (data not shown). Scale bars, 10 μm (A-D and F), 20 μm (E).

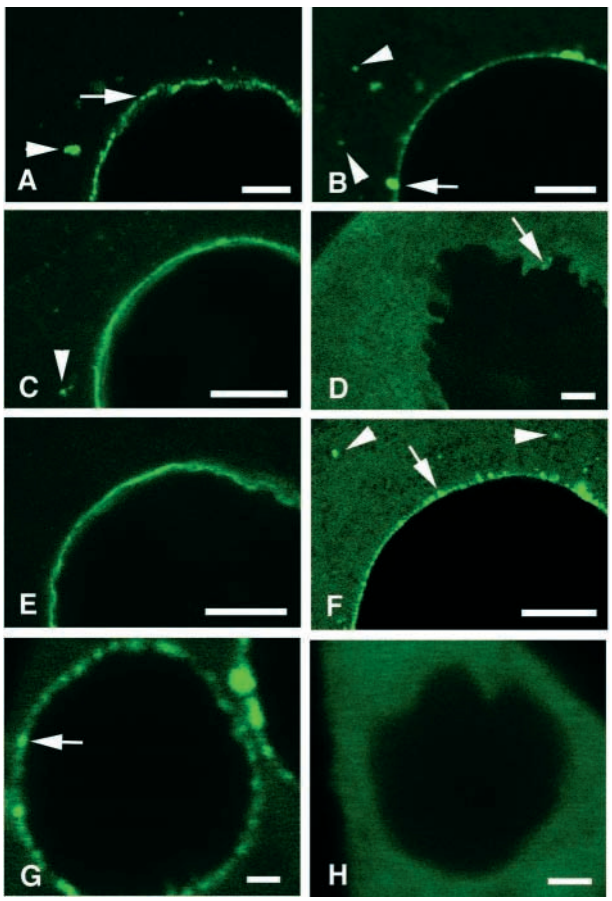

Fig. 5. Nuage clusters and particles in mutants. (A,C,E) Immunostaining of VasGFP. (B,D,F-H) Immunostaining of GFPAub. In wild-type nurse cells the pattern of VasGFP (A) and GFPAub (B) includes perinuclear nuage clusters (arrows) and cytoplasmic particles (arrowheads). Perinuclear nuage appears smoother in aub^{HN2}/aub^{QC42} than in wild type, but nuage particles are unaffected (C). Very few nuage clusters and particles remain in vas^{AS}/vas^{AS} nurse cells (D). In the $spnE^{E616}/spnE^{3987}$ mutant nuage particles are lost and the perinuclear nuage is smoother (E). Nuage clusters and particles appear wild type in $tudor^{1}$-mutant nurse cells (F). Nuage in wild-type spermatocytes is readily detectable using GFPAub (G), and is completely disrupted in vas^{AS} males (H). Scale bars, 1 μm (G,H), 5 μm (A,D), 10 μm (B,C,E,F).

association with nuage or simply reflects random overlap of an abundant protein with the more narrowly distributed nuage. Evidence that Bru is specifically associated with nuage comes from analysis of Bru distribution in *vas* mutants: as for other nuage components, the perinuclear clusters of Bru are strongly reduced (Fig. 4E). Given this identification of Bru as a nuage-associated protein, we included *arrest* (*aret*) mutants (the *aret* gene encodes Bru) (Schüpbach and Wieschaus, 1991; Webster et al., 1997) in a genetic analysis of nuage. The other genes tested were *vas*, *tud*, *aub* and *spindle E* (*spnE*), each of which encodes a nuage component, has been shown to be required for nuage formation, or both (Bardsley et al., 1993; Findley et al., 2003; Harris and Macdonald, 2001; Liang et al., 1994).

To better characterize the perinuclear nuage defects seen in static images and to extend the analysis to include cytoplasmic nuage particles, we used live imaging. GFPAub was used as the nuage marker to test the role of *vas*, *aret* and *tud*, and VasGFP was used to test the roles of *aub* and *spnE*. The live imaging confirmed, for the most part, the basic observations from analysis of fixed samples. In *vas* mutants perinuclear nuage is almost completely absent (Fig. 5D), with only a few nuage clusters visible. Loss of *spnE* activity has a less extreme effect: the perinuclear nuage clusters are largely missing, but a perinuclear zone of VasGFP remains (Fig. 5E). Consistent with the results by using fixed samples, the persistent perinuclear zone of VasGFP is qualitatively different from wild type, appearing almost completely uniform and lacking any visible discontinuities (Fig. 5E). Similar results were obtained with the *aub* mutant, except that the VasGFP perinuclear clusters remained present up to stage 8 of oogenesis, after which they disappeared (Fig. 5C). In *aret* and *tud* mutants (Fig. 4F and Fig. 5F) we detected no significant alteration of perinuclear nuage.

In mutants whose perinuclear VasGFP is uniform ($spnE^{-}$ and later stage aub^{-}), the protein undergoes rapid exchange with cytoplasmic pools, just as for VasGFP in perinuclear clusters of wild-type egg chambers. In photobleaching experiments the

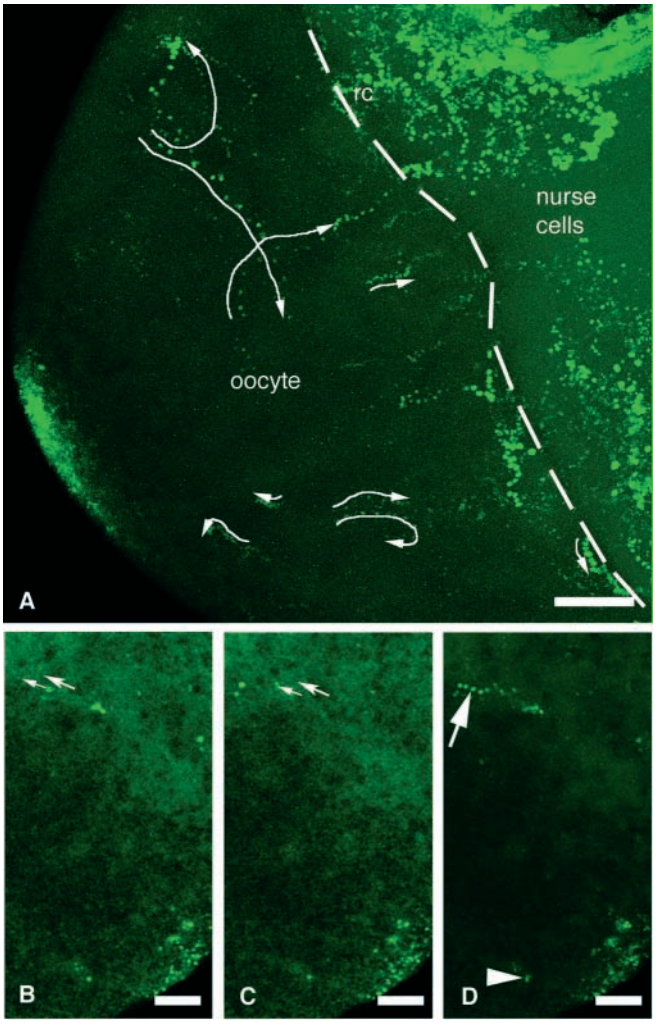

Fig. 6. Nuage-particle movements in the oocyte. (A) To track oocyte-particle movements, GFPAub was imaged in a live stage-8/9 egg chamber for 60 minutes (Δt=1 minute) in multiple *z*-planes, with the data shown as a combined *z*-axis and time series projection. The arrows are positioned next to a subset of the moving particles and show the direction and path of movements. (B-D) GFPAub in different regions of a single egg chamber, images were taken from a series of scans performed at one-minute intervals. B and C are the first and last scans of the series and show that the movement of nuage particles follow general cytoplasmic movements. The arrows show the direction of and the distance covered by a nuage particle (small arrow) and a yolk platelet/vesicle (large arrow) in this time interval. The yolk platelet/vesicles (which appear black because they lack cytoplasmic GFPAub fluorescence) provide a simple means of tracking general cytoplasmic movements. D shows that particle movements near the posterior pole of the oocyte particle movements are restricted. The arrow shows the rapid movement of a nuage particle at the anterior (the same particle whose movement is shown by the small arrows in B and C), whereas a particle close to the posterior (arrowhead) is relatively immobile. These results are typical of those obtained in multiple additional experiments. The dashed line in A delineates the oocyte nurse-cell boundary. Scale bars, 7 μm (B-D) and 10 μm (A).

fluorescence-recovery half-time is 50 seconds in *aub*$^{-}$ and 48.5 seconds in *spnE*$^{-}$, similar to the $t_{1/2}$=59 seconds for wild type.

Cytoplasmic nuage particles are affected differently in the *vas*, *aub* and *spnE* mutants. The *vas* and *spnE* mutants have few or no cytoplasmic nuage particles (Fig. 5D,E). By contrast, *aub* mutants have no dramatic reduction in the abundance of cytoplasmic nuage particles (Fig. 5C), even at times well after the disappearance of perinuclear nuage clusters at stage 8, and the particles have a fairly typical size distribution (mean=423 nm, s.d.=189, *n*=37, range=<240 nm to 1182 nm). These particles do not simply represent the default appearance of VasGFP, as they are absent in the *spnE* mutant (Fig. 5E). Thus, it seems unlikely that perinuclear nuage clusters are required for the formation of cytoplasmic nuage particles, a conclusion consistent with the observation that cytoplasmic particles are produced only infrequently by detachment of perinuclear nuage clusters.

We also examined the consequences of loss of *vas* activity in the male germ line. Just as in nurse cells, Vas appears to be concentrated in nuage in spermatocytes (Hay et al., 1988a) (Fig. 5G). However, *vas* mutant males are fertile (Lasko and Ashburner, 1988) (data not shown). Given the crucial role for Vas in the nuage of other cell types, either male nuage must differ in this requirement or nuage is not essential in the male germ line for fertility. To distinguish between these possibilities we tested *vas*^{AS} spermatocytes for the presence of nuage, using GFPAub as a marker. Although GFPAub was present in the cytoplasm, there were no visible perinuclear nuage clusters (Fig. 5H), indicating that nuage does not form in the *vas* mutant and is therefore not required for spermatocyte function. An alternate and less probable interpretation is that a rudimentary form of nuage, lacking Aub, is present and is sufficient to provide a minimal requirement for nuage in males.

Accumulation of polar granule components at the posterior pole of the oocyte

Our demonstration that cytoplasmic nuage particles are moved from nurse cells into the oocyte is consistent with current models positing that these particles become localized at the posterior pole of the oocyte as polar granules (Styhler et al., 2002). To test the model we asked whether it is possible to trace individual nuage particles moving to the posterior of the oocyte. We were unable to reliably track VasGFP particles in the oocyte, but the GFPAub particles have a higher fluorescence intensity and could be detected persistently over long periods of time.

Particles that entered the oocyte between stages 8 and 10 initially moved unidirectionally and away from the ring canal for short distances. Subsequently, individual particles were behaving differently: some were stationary, whereas many others continued to move – but in various directions (Fig. 6A). Among the latter group the average speed was 21.6 nm/second (*n*=25), ranging from 8.8-49 nm/second, an order of magnitude slower than the speed of nuage particles in nurse cells. Some of the particles in the oocyte moved towards the posterior, others moved towards the lateral cortex and some moved back towards the anterior (Fig. 6A). Although such patterns of movement could be consistent with travel along microtubules, the paths taken by many of the particles paralleled the movements of the bulk cytoplasm (as revealed by the movements of anonymous cellular structures, possibly vesicles

or yolk platelets, that are visualized by their exclusion of cytoplasmic GFPAub) (Fig. 6B-D). Moving particles were observed travelling unidirectionally for long distances, sometimes half the width of the oocyte (Fig. 6A), but we were unable to track the movement of any single particle from its entry into the oocyte to a position near the posterior pole. These results argue against a directed transport system in which nuage particles are delivered to the oocyte and then directed specifically to the posterior pole. They instead suggest that most movements are not directional and simply reflect microtubule-dependent cytoplasmic flow (see also Palacios and St Johnston, 2002).

Even in the absence of directed posterior transport the particles still could become concentrated via a capture mechanism at the posterior pole, where they then appeared as polar granules. We therefore examined particle movements in the posterior region of stage-8 to -9 oocytes, when polar granules first appear. We focused on egg chambers with low but detectable levels of posterior polar granules, and which therefore had just begun the process of polar granule formation. Compared with particles in the anterior ooplasm, those near the posterior pole showed very little movement (Fig. 6D). From a total of 483 minutes (~8 hours) of observation in 14 stage-8 to -9 egg chambers, only a single particle was seen to move for a significant distance toward the posterior pole in the posterior third of the oocyte. Nevertheless, we assume that polar granule material must have been accumulating during the experiments, because there is usually a dramatic assembly of polar granules during stages 8 and 9, a developmental period of about 12 hours. Even if this assembly occurs in a sporadic fashion (something not suggested by any previous analysis), such that there might be no accumulation of polar granules within a single 2-hour period, there should have been significant polar granule assembly in the combined 8 hours of viewing. To test this prediction we performed control experiments in which the polar granules were imaged at the beginning and the end of a 45- to 60-minute interval, but not at intermediate times so as to avoid the photobleaching that is inevitable during multiple confocal scans. In nine egg chambers, we found that the total fluorescence at the posterior increased an average of 32%, confirming that polar granule assembly does occur under the experimental conditions.

Additional evidence that polar granule components can accumulate at the posterior of the oocyte independently of any detectable particle addition, was obtained from photobleaching experiments (Fig. 7). Stage-9 oocytes were photobleached in a zone at the posterior pole that included part of the crescent of polar granules. The photobleaching eliminated fluorescence from GFPAub in the polar granules and from GFPAub in the ooplasm, including GFPAub in particles and the pool of GFPAub that normally appears at uniform low levels throughout the cytoplasm of nurse cells and the oocyte. After photobleaching, we found that GFP fluorescence was rapidly reacquired in the posterior ooplasm ($t_{1/2}$=23 seconds) and less rapidly in the crescent of polar granules ($t_{1/2}$=233 seconds). A similar rate of exchange was observed for VasGFP in the polar granules of stage-10 egg chambers ($t_{1/2}$=210 seconds). In no case did we observe any GFPAub particles arriving at the posterior pole or becoming incorporated into the crescent of polar granules during the fluorescence recovery periods.

Our results suggest that the polar granules are not simply nuage particles that have been moved from the nurse cells. Instead, it appears that polar granules are assembled de novo, using some of the same proteins that are also present in nuage. Furthermore, polar granules differ significantly from nuage in that incorporation of new components is significantly slower, suggesting a less dynamic structure.

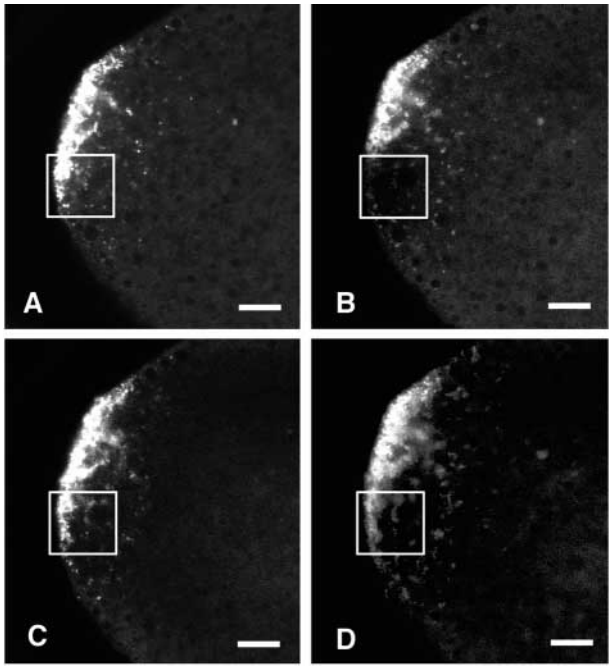

Fig. 7. Incorporation of cytoplasmic GFPAub into polar granules. GFPAub fluorescence in polar granules and in the cytoplasm was monitored before (A), immediately after (B), and 10 minutes after (C) photobleaching. (D) is a projection of 11 images (Δt=1 minute) that follow the recovery of GFPAub at the posterior. Large nuage particles were not detected to arrive at the posterior and could therefore not account for the recovery of fluorescence (D). The white box indicates the region of photobleaching. Scale bars, 10 μm.

Oskar mediates nucleation of cytoplasmic particles and stabilizes GFPAub in the oocyte

Formation of polar granules at the posterior pole of the oocyte requires Oskar (Osk) protein, while nuage appears normal in *osk* mutants (Lasko and Ashburner, 1990). Thus the involvement of Osk clearly distinguishes polar granules and nuage. How Osk acts in polar granule formation is largely unknown at the molecular level, but it can interact with Vas and Staufen (Stau) proteins (Breitwieser et al., 1996) and it is thought to provide a localized cortical anchor for binding or recruitment of polar granule components, perhaps in the form of nuage particles (Styhler et al., 2002; Vanzo and Ephrussi, 2002; Webster et al., 1994). Osk is not sufficient for the nucleation of polar granules as it requires Vas: mutants of *vas* that disrupt nuage also disrupt polar granules (data not shown) (Liang et al., 1994; Schüpbach and Wieschaus,1991; Lehmann and Nüsslein-Volhard, 1991).

Normally, the Osk protein appears only at the posterior pole of the oocyte, and this restriction underlies the formation of polar granules exclusively at that location: mislocalization of

Osk to the anterior cortex results in the formation of ectopic polar granules at the anterior of the oocyte (Ephrussi and Lehmann, 1992). We used two transgenes that direct synthesis of wild-type but mislocalized Osk to further address the question of whether polar granules arise from recruitment of nuage particles, or from recruitment of nuage components.

One mislocalized form of Osk is provided by *osk-bcd* transgenes, in which the *osk* 3′ UTR is replaced by the *bcd* 3′ UTR. This exchange of UTRs redirects the hybrid-*osk* mRNA, just like *bcd* mRNA, to the anterior of the oocyte from stage 8 onwards (Ephrussi and Lehmann, 1992). Removal of the *osk* 3′ UTR also eliminates translational control elements, thus allowing the hybrid mRNA to be translated significantly before the endogenous *osk* mRNA, at the earlier stages of oogenesis when the hybrid *osk* mRNA is concentrated within the oocyte but not yet restricted to the anterior (Wilson et al., 1996). The Osk protein made at this time is present throughout the oocyte and concentrated cortically (Fig. 8B).

When Osk protein is expressed precociously in the *osk/bcd* flies, there is a dramatic increase in the number of GFPAub particles, present in the oocytes of pre-stage-8 egg chambers. However, there is no striking elevation of particle number in the nurse cells (Fig. 8C-E). This superabundance of particles in the oocyte could be caused by increased particle transport from the nurse cells, by enhanced particle formation in the oocyte, or both. As noted above, nurse cell to oocyte transport of nuage particles is rare before stage 7 in wild type, and the examination of *osk/bcd* ovaries reveals no significant increase (Fig. 8F). Thus, the many GFPAub particles observed in *osk/bcd* early-stage oocytes arise not from elevated transport of nuage particles, but rather from de novo particle assembly. Osk could act directly to nucleate particle formation, and the similar distribution patterns of Osk protein and GFPAub particles are consistent with such a role.

Osk protein can also be expressed ectopically using the upstream activator sequence (UAS)/GAL4 system (Brand and Perrimon, 1993) and an *osk* cDNA in which the 3′ UTR has been replaced with that from the *fs(1)K10* gene, *UAS-osk-K10* (Riechmann et al., 2002). Unlike mis-expression from the *osk/bcd* transgene, which is largely or completely limited to the oocyte, *UAS-osk-K10* in combination with the nosGAL4VP16 driver (Van Doren et al., 1998) (a combination we call *UASosk* for simplicity) directs high levels of Osk protein in both the oocyte and the nurse cells (Riechmann et al., 2002) (Fig. 9A,B). We examined the distribution of GFPAub in the *UASosk* ovaries and found two dramatic effects on cytoplasmic particles that contained nuage components: the number of cytoplasmic particles was greatly elevated, and these particles often formed large clumps (Fig. 9C,D). Not surprisingly, the zones of clumped nuage particles were also sites where Osk protein was concentrated, and many of the individual particles also contained Osk (Fig. 9E-G, short arrows). However, some cytoplasmic particles lack Osk (Fig. 9E-G, long arrows). The number of particles of this class was approximately the same than the number of cytoplasmic nuage particles in wild-type nurse cells. Perinuclear nuage is not detectably altered by the ectopic production of Osk protein (Fig. 9C,D) and Osk was not strongly associated with perinuclear nuage (Fig. 9E-G, arrowheads).

Two observations – the exclusion of Osk from perinuclear nuage clusters, and the persistence of cytoplasmic nuage particles that lack Osk – strongly suggest that bona fide nuage cannot interact with Osk. Thus, the many additional cytoplasmic particles that appear as a consequence of ectopic Osk expression are most probably formed de novo and not converted directly from nuage.

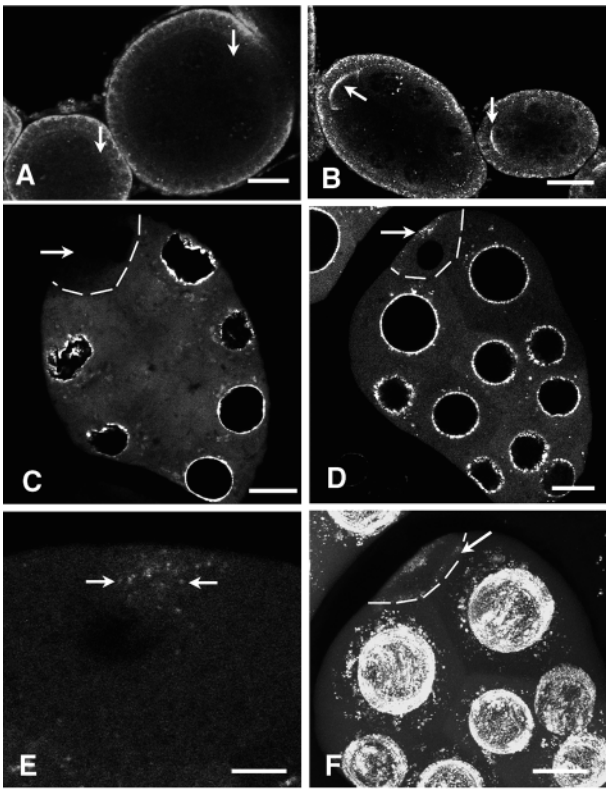

Fig. 8. Oskar nucleates the formation of exogenous GFPAub particles in the early oocyte. Osk protein in wild-type egg chambers (A) and in flies expressing *osk-bcd3′UTR* (B). Arrows in B indicate ectopic Osk protein expressed from the *osk-bcd 3′UTR* transgene, arrows in A show wild-type oocytes with no Osk staining (the follicle cell staining is non-specific). (C-F) GFPAub fluorescence in wild-type (C) and *osk-bcd 3′UTR* flies (D-F). Wild-type oocytes contain very few GFPAub particles (arrow in C), but those with the *osk-bcd 3′ UTR* transgene have abundant particulate GFPAub fluorescence (arrows in D,E), as well as a significantly enhanced level of cytoplasmic GFPAub. The image in F is a combined *z*-axis and time projection of a live stage-7 *osk-bcd 3′ UTR* egg chamber that has been imaged at 70-seconds intervals for 14 frames, showing that very few nuage particles can be tracked entering the early oocyte, just as in wild type. Dashed lines in C, D, and F delineate the oocyte-nurse cell boundaries. Scale bars, 5 μm (E), 15 μm (D,F), 17 μm (B), 20 μm (C) and 23 μm (A).

Discussion

The widespread presence of nuage in germ line cells raises two important and related questions, neither of which has yet been answered: What is the significance of their conservation? And what is the molecular role of nuage in these cells? In *Drosophila*, two types of function, not mutually exclusive, have been proposed for nuage. In one model nuage has been suggested to serve as a precursor to polar granules, a view initially based on ultrastructural similarities of the two

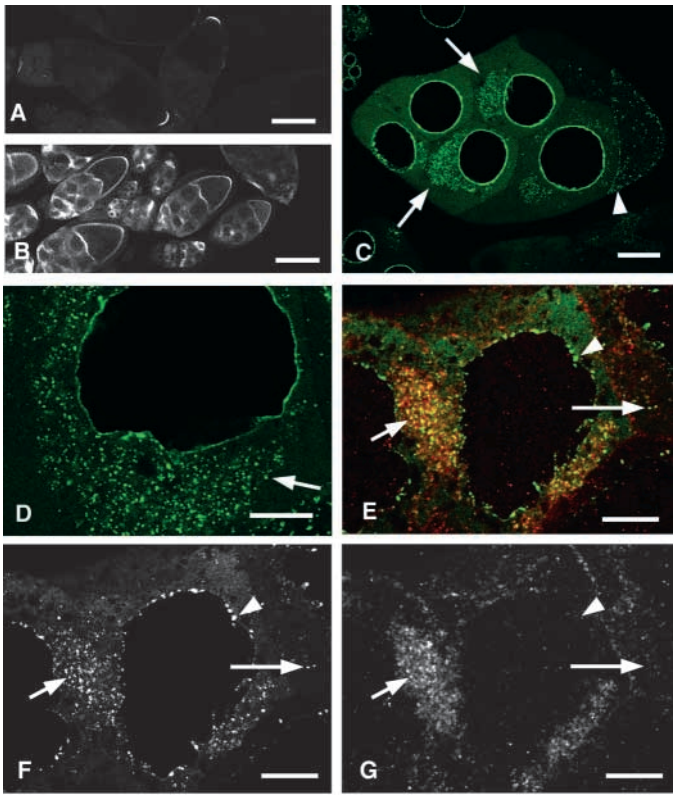

Fig. 9. Oskar protein promotes ectopic particle formation in nurse cells. Osk protein in wild-type (A) and *UASosk* egg chambers (B). Ectopic Osk protein is present at the cortex of oocytes and in nurse cells of *UASosk* egg chambers. (C,D) Distribution of GFPAub in *UASosk* egg chambers. Clumps of GFPAub particles are often found in the nurse cells of these egg chambers (arrows in C,D), and the level of GFPAub fluorescence is often enhanced. (E-G) The extent of colocalization of GFPAub and Osk was monitored by double labeling of *UASosk* egg chambers. Osk (G) and GFPAub (F) are merged in E, with Osk in red and GFPAub in green. The clumps of GFPAub particles colocalize with regions of concentrated Osk protein (arrows in E-G). Most particles in these clumps contain GFPAub and Osk (yellow particles in E, short arrows in E-G), some particles contain mostly Osk protein (red particles in E), others only contain GFPAub (long arrows in E-G), particularly those attached to the nuclear envelope (arrowheads in E-G). Osk protein was distributed throughout the nurse cells, but was not found to be strongly associated with the nurse-cell cortex, in contrast to the strong cortical association within the oocyte (B,G). Scale bars are 10 μm (D-G), 20 μm (C) and 100 μm (A,B).

organelles (Mahowald, 1968; Mahowald, 1971) and supported by the identification of shared components (Hay et al., 1988a; Bardsley et al., 1993; Harris and Macdonald, 2001). Another possible role for nuage is based on its position at the periphery of the nucleus, at or near nuclear pores. Specifically, nuage might act in some aspect of remodelling RNPs when RNAs are exported from the nucleus (Findley et al., 2003). Our analysis of the movements and genesis of nuage particles provides two arguments against the first model. First, the rate of release of perinuclear nuage clusters in the nurse cells is very low, much lower than expected if the clusters form polar granules. Second, we were unable to detect any nuage particles arriving at the posterior pole of the oocyte and becoming incorporated into polar granules. An additional observation that argues against a model where nuage is a precursor for polar granules, is the presence of cytoplasmic nuage particles in *aub* mutants, despite the fact that these mutants do not assemble polar granules. However, this evidence does not exclude the first model, because the nuage particles in the mutant might not be fully functional. A third argument is provided by the evidence that Osk cannot interact with nuage, leaving de novo assembly of polar granules as the only reasonable option. Overall, our results strongly suggest that nuage is not the precursor to polar granules, and we believe that the shared features are simply indicative of similar biochemical activities, rather than a precursor-product relationship.

Our data do not directly test the model that nuage might function as a transition zone in the movements of mRNAs from the nucleus to the cytoplasm, where RNP components might be exchanged or otherwise modified. However, we have identified new properties of nuage, and these relate to possible functions. First, we find that Bruno, an RNA binding protein that acts as a translational repressor of *osk* and *grk* mRNAs, is associated with nuage. This extends the correlation of nuage components with factors that act in some aspect on mRNA localization or translational control. Of the previously identified nuage components, Vas and Gus are involved in the regulation of *grk* mRNA localization and translation (Styhler et al., 2002; Styhler et al., 1998; Tinker et al., 1998; Tomancak et al., 1998), Aub is required for efficient translation of *osk* mRNA (Wilson et al., 1996) and has also been implicated in RNAi (Kennerdell et al., 2002), and *mael* mutants display defects in the early stages of mRNA localization (Clegg et al., 1997). Moreover, *spnE*, which is necessary for normal nuage formation, is required for the localization of multiple mRNAs and acts in RNAi (Findley et al., 2003; Gillespie and Berg, 1995; Kennerdell et al., 2002). Thus, every known nuage component has a role in one or more types of post-transcriptional control of gene expression.

The second property of nuage reported here, is the remarkably dynamic composition of perinuclear nuage clusters, despite their relatively fixed positions around the nucleus. This is in contrast to studies showing that general protein exchange is slow in mouse nuage (Eddy, 1975). The rapid exchange of both Vas and Aub, the two proteins tested, suggests that the clusters are staging sites where these, and presumably additional proteins, become associated with other molecules and move off into the cytoplasm. Much like shuttling-proteins that escort RNAs in their travels from the nucleus to the cytoplasm, there might be a class of proteins that interact in nuage with newly exported RNAs and then facilitate post-transcriptional control events that occur in the cytoplasm. By this model nuage could be an organelle that concentrates and thus potentiates the activity factors normally present in all cells, but that must be especially active in germline cells because of their intensive reliance on post-transcriptional controls of gene expression.

Osk and the transition from nuage to polar granules

We have argued that nuage from the nurse cells is not used for polar granule assembly in the oocyte, yet these two subcellular structures clearly share components and may well have similar

activities. One feature that clearly distinguishes polar granules from nuage is the presence of Osk protein. Under normal circumstances Osk is never in contact with nuage, because an elaborate set of post-transcriptional control mechanisms serves to prevent Osk accumulation in the nurse cells and to restrict the distribution of Osk protein within the oocyte to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1995; Kim-Ha et al., 1991; Markussen et al., 1995; Rongo et al., 1995; Vanzo and Ephrussi, 2002; Webster et al., 1994). The presence of Osk at this single location provides the cue for the assembly of polar granules, and misdirection of Osk to other sites in the oocyte leads to ectopic polar granule formation (Ephrussi and Lehmann, 1992; Smith et al., 1992). Thus Osk is generally viewed as an anchor for the recruitment of the factors that form polar granules. Given our finding that polar granules are significantly more stable that perinuclear nuage clusters, it might be that Osk not only recruits other factors, but also strengthens their interactions. A further and unanticipated property of Osk was revealed in our studies in which Osk was expressed precociously throughout the oocyte. Under these conditions GFPAub levels are substantially elevated in the oocyte. Two general explanations are possible. First, Osk might stimulate the rate of transfer of GFPAub from the nurse cells to the oocyte. Such a model is not supported by any known property of Osk, and we were unable to detect any increase in the rate at which GFPAub particles move into the oocyte. Furthermore, GFPAub levels in the oocyte are enhanced even before the onset of known nurse cell to oocyte movements in the cytoplasm, and so Osk would have to dramatically alter the properties of the egg chamber under this model. Second, Osk could stabilize a normally labile pool of GFPAub in the oocyte. In the simplest form of this model, stabilization would occur as a consequence of the assembly into complexes, which could include factors other than Osk and GFPAub. This model appears to be most compatible with our data. In addition, such a model provides a possible explanation for the curious association of the Fat facets (Faf) protein, a deubiquitinating enzyme, with pole plasm (Fischer-Vize et al., 1992). The role of Faf could be to stabilize one or more polar granule components, thereby enhancing the growth of polar granules.

The restriction of Osk protein to the posterior pole of the oocyte is known to be important for limiting the spatial distribution of posterior body patterning activity (Ephrussi and Lehmann, 1992; Smith et al., 1992; Webster et al., 1994). By analogy, this restriction might also be important for allowing normal assembly and function of nuage in nurse cells, if Osk can compete with nuage for their shared components. To evaluate this possibility, we examined ovaries in which Osk was allowed to accumulate in the nurse cells as well as the oocyte. Osk does indeed nucleate the formation of large bodies in the nurse cell cytoplasm, but the presence of these bodies does not appear to limit the amount of perinuclear nuage. Notably, we observed no Osk in association with perinuclear nuage, which appears not to be affected by the ectopic Osk. The Osk protein can interact directly with Vas in the two-hybrid assay in yeast (Breitwieser et al., 1996), and so its failure to associate with perinuclear nuage – the regions of greatest Vas concentration – in nurse cells is notable. One interpretation is that the site of Vas binding to Osk is blocked when it is in nuage. This fits with the model in which Osk protein nucleates polar granule formation not from nuage particles themselves, but from individual nuage components or subassemblies.

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