# *Maelstrom*, a *Drosophila spindle*-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage

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## SUMMARY

A hallmark of germline cells across the animal kingdom is the presence of perinuclear, electron-dense granules called nuage. In many species examined, Vasa, a DEAD-box RNA helicase, is found in these morphologically distinct particles. Despite its evolutionary conservation, the function of nuage remains obscure. We have characterized a null allele of *maelstrom* (*mael*) and shown that Maelstrom protein is localized to nuage in a Vasa-dependent manner. By phenotypic characterization, we have defined *maelstrom* as a *spindle*-class gene that affects Vasa modification. In a nuclear transport assay, we have determined that Maelstrom shuttles between the nucleus and cytoplasm, which may indicate a nuclear origin for nuage components. Interestingly, Maelstrom, but not Vasa, depends on two genes involved in RNAi phenomena, *aubergine* and *spindle-E* (*spn-E*), for its nuage localization. Furthermore, *maelstrom* mutant ovaries show mislocalization of two proteins involved in the microRNA and/or RNAi pathways, Dicer and Argonaute2, suggesting a potential connection between nuage and the microRNA-pathway.

Supplemental data and movies available on-line

Key words: Oogenesis, Germ plasm, Nuage, *Drosophila*

## INTRODUCTION

How germline status is established and maintained in sexually reproducing organisms is a fundamental question in developmental biology. A conserved feature of germ cells in species across the animal kingdom is the presence of a distinct morphological element called nuage. Ultrastructurally, nuage appears as electron-dense granules that are localized to the cytoplasmic face of the nuclear envelope (reviewed by Eddy, 1975; Saffman and Lasko, 1999). Despite the breadth of nuage in the animal kingdom, we currently lack depth in understanding its function. In animals ranging from the nematode to vertebrates, the Vasa protein has been detected in these granules (reviewed by Raz, 2000). Both nuage and Vasa thus offer potential clues as to what makes a germ cell unique.

One system with high potential for understanding the role of nuage is *Drosophila*. In females, Vasa-positive germline granules are continuously present throughout the life cycle, taking one of two forms, nuage or pole plasm (Hay et al., 1988a; Lasko and Ashburner, 1990). Pole plasm, which contains polar granules, is a determinant that is both necessary and sufficient to induce formation of the germ lineage in early embryogenesis (Illmensee and Mahowald, 1974; Illmensee and Mahowald, 1976) (reviewed by Mahowald, 2001). In *Drosophila*, nuage is first detectable when primordial germ cells are formed; it persists through adulthood, where it is present in all germ cell types of the ovary (Mahowald, 1968; Mahowald, 1971a; Mahowald, 1971b).

In *Drosophila*, three proteins are known to localize to nuage: Vasa (Hay et al., 1988a; Lasko and Ashburner, 1990), Aubergine (Harris and Macdonald, 2001) and Tudor (Bardsley et al., 1993). The sequence or mutant phenotype of each gene suggests a role in post-transcriptional RNA function. Vasa is a DEAD-box RNA helicase (Hay et al., 1988b; Lasko and Ashburner, 1988; Liang et al., 1994) required for nurse cell-to-oocyte transport of several mRNAs critical to oocyte patterning (Styhler et al., 1998). Vasa is also required for efficient translation of several key proteins in oogenesis, and itself interacts both physically and genetically with a *Drosophila* homolog of yeast Translation Initiation Factor 2 (dIF2) (Carrera et al., 2000). Vasa is thus potentially implicated in translational control. Aubergine is a member of the RDE1 (for RNAi defective)/AGO1 (Argonaute1) protein family, homologs of which are required in both RNAi and developmental processes in diverse organisms (reviewed by Fagard et al., 2000; Carmell et al., 2002). Aubergine is required, during oogenesis, for efficient translation of Oskar (Wilson et al., 1996), which is pivotal in initiating pole plasm assembly (reviewed by Rongo and Lehmann, 1996). *Aubergine* is also required for RNAi in late oogenesis (Kennerdell et al., 2002). Tudor, a novel protein (Golumbeski et al., 1991), comprises ten copies of an ~120 residue motif (the 'Tudor Domain', pfam00567) (Callebaut and Mornon, 1997) present in several proteins involved or implicated in RNA-binding capacities (reviewed by Ponting, 1997). The domain has been suggested to mediate protein-protein interactions (Selenko et

al., 2001). *Drosophila* Tudor is required to mediate transfer of mitochondrial ribosomal RNAs from mitochondria to the surface of polar granules during pole cell formation in early embryogenesis (Amikura et al., 2001). A role for Tudor prior to pole plasm assembly, however, has not been described.

A*ubergine* and *vasa* are members of a larger group of female sterile mutants, the *spindle* (*spn*) class, which produces eggshells with variable anteroposterior (AP) and dorsoventral (DV) axis defects (Schupbach and Wieschaus, 1991; Tearle and Nüsslein-Volhard, 1987). In most of the characterized *spn* mutants, the etiology of these patterning defects has be traced to a failure in Gurken (a TGFα homolog) presentation in developing egg chambers (reviewed by Ray and Schupbach, 1996; Riechmann and Ephrussi, 2001). Each *spn* mutant also has a meiotic progression defect (Ghabrial et al., 1998): by stage 4 in a normal egg chamber, the DNA within the oocyte nucleus (germinal vesicle) condenses into a compact sphere called a karyosome (Smith and King, 1968) after successful meiotic recombination. Mutants in all *spn* genes fail to form a karyosome (Ghabrial et al., 1998; Gonzalez-Reyes et al., 1997; Styhler et al., 1998; Tomancak et al., 1998). The characterized *spn* genes fall into two groups: those whose gene products are directly required for recombinational DNA repair steps within the germinal vesicle [e.g. *okra* (*okr*), *spn-B* and *spn-C*] and those whose protein sequence or localization suggest indirect involvement in meiotic progression [e.g. *aubergine*, *vasa* and *spn-E*] (reviewed by Gonzalez-Reyes, 1999). The *spn* mutants, as a group, demonstrate that meiotic and patterning processes intersect during oogenesis.

In this paper, we identify and characterize a null allele of the *maelstrom* gene, which encodes a novel protein with a human homolog. The mutant displays each of the defects in oocyte development common to the *spindle*-class. We also demonstrate that Maelstrom localizes to nuage in a Vasa-dependent manner and that *maelstrom* is required for proper modification of Vasa. Through mutant analysis, we have begun to unravel genetic dependencies of nuage particle assembly.

## MATERIALS AND METHODS

### Fly strains

The wild-type *Drosophila melanogaster* strain used in this study was Oregon R. The following allelic combinations were used for immunolocalization and western (indicated by asterisks) analyses: *Maelstrom* (*mael*) stocks *mael^M391/Df (3L)79E-F** (Clegg et al., 1997); *mei-W68* stocks *mei-W68^1* (McKim and Hayashi-Hagihara, 1998) and [*mei-W68^1; mael^M391/Df(3L)79E-F*]; *Okra* (*okr*) lines *okr^AA/AG*, *okr^RU/Df(2L)JS17**, *okr^WS/Df(2L)JS17** (Ghabrial et al., 1998; Schupbach and Wieschaus, 1991); *aubergine* (*aub*) lines *aub^K86/N11*, *aub^QC42/N11*, *aub^HN2/N11*, *aub^QC42/HN2* and *nanos*-Gal4-driven Aubergine-GFP (Wilson et al., 1996); *spindle-A* (*spn-A*) spn-*A^050/057**; *spindle-B* (*spn-B*) lines *spn-B^056/153** and *spn-B^153/Df(3R)trxE12**; *spindle-C* (*spn-C*) lines *spn-C^422/Df(3L)pblX1*, *spn-C^422/660** and *spn-C^660/Df(3L)pblX1*; *spindle-D* (*spn-D*) lines *spn-D^150**, *spn-D^349** and spn-*D^150/349** (Gonzalez-Reyes et al., 1997; Tearle and Nüsslein-Volhard, 1987); *spindle-E* (*spn-E*) lines *spn-E^616/hlsΔ125** and *spn-E^hls3987/hlsΔ125* (Gillespie and Berg, 1995; Gonzalez-Reyes et al., 1997); *vasa*(vas) lines *vas^PH165** (a null allele), *vas^011/PH165* and *vas^014/PH165* (Liang et al., 1994; Styhler et al., 1998). All flies were aged 2-4 days at 25°C before analysis, with the exception of *mael^M391/Df (3L)79E-F* females, which were used at 2 days post-eclosion. Younger *maelstrom* females were used to minimize the number of ovarioles presenting age-dependent germline-depletion and germline-tumor class phenotypes (S. D. F. and H. R.-B., unpublished).

Southern analysis of *mael^M391/Df (3L) 79E-F* genomic DNA was carried out using standard procedures. We cloned the deletion junction by PCR, using 5′ primer M1 (TAC-TAG-TGC-TCA-GCA-ACG-CC), 3′ primer M3 (GGC-TTG-TAG-GAG-CTT-GAT-GC) and Vent DNA polymerase (New England Biolabs, Beverely, MA) for amplification. The resulting PCR product was subcloned and sequenced using standard techniques.

### Western blotting

Ovaries from 20 females were dissected in *Drosophila* Ringer's solution (EBR) (130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl2, 10 mM HEPES, pH 6.9) and processed as before (Clegg et al., 1997). Supernatant equivalent to the mass of a single wild-type ovary was loaded per well of 8% SDS-polyacrylamide gels (Laemmli, 1970). Equivalent loading was verified by Coommassie staining of duplicate lanes. After electrophoresis, gels were western blotted using standard protocols and probed with either affinity-purified anti-Maelstrom rabbit polyclonal (1:5,000) or anti-Vasa rat polyclonal (1:10,000). Immunoreactive bands were visualized with HRP-conjugated goat anti-rabbit (or anti-rat) secondary antibody (BioRad) at 1:10,000 and enhanced chemiluminescence using an NEN Renaissance kit (Dupont-NEN, Boston, MA).

### Immunocytochemistry

Previously (Clegg et al., 1997), ovaries were fixed for immunolocalization in 4% paraformaldehyde for 20 minutes. Here, ovaries were fixed in 6.2% formaldehyde for 5 minutes, as described previously (Li et al., 1994). Briefly, ovaries from groups of ten females were dissected in EBR and fixed for 5 minutes in 100 μl devitellinizing buffer under 600 μl n-heptane on a Clay-Adams nutator (Becton-Dickinson, Sparks, MD). Devitellinizing buffer is composed of 1 vol buffer B, 1 vol reagent grade formaldehyde (Fisher catalog number F79, which contains 10-15% methanol as a preservative) and 4 vol $H_2O$. Buffer B is 100 mM $KH_2PO_4/K_2HPO_4$, pH 6.8, 450 mM KCl, 150 mM NaCl and 20 mM $MgCl_2$. After fixation, ovaries were rinsed three times in PBS, three times in PBT (PBS plus 0.1% Triton X-100), then washed in PBT for 2 hours at room temperature. Ovaries were then dissected into individual ovarioles, washed for an additional 2-16 hours in PBT at 4°C and blocked in PBT-BSA (PBT plus 1% bovine serum albumin) overnight at 4°C. Ovaries were then incubated overnight at 4°C in PBT-BSA plus primary antibodies, followed by washing in PBT-BSA for 2-3 hours at room temperature with multiple changes. Secondary antibodies were diluted 1:500 in PBT-BSA, and incubation was carried out overnight at 4°C. Ovaries were subsequently washed in multiple changes of PBT-BSA, then PBT, each for 1-2 hours, at room temperature, then stained with DAPI (0.04 μg/ml). After several quick rinses in PBS over 15 minutes, ovaries were mounted in a solution of 1×PBS, 70% glycerol and 2% n-propyl gallate (Sigma, St Louis, MO).

We used the following primary antibodies: anti-ADD87 (an adducin-like protein) mouse monoclonal 1B1 (1:20) (Zaccai and Lipshitz, 1996), anti-alpha-Spectrin rabbit polyclonal (1:500) (Byers et al., 1987), anti-Argonaute1 rabbit polyclonal (1:100) (Kataoka et al., 2001), affinity-purified anti-Argonaute2 rabbit polyclonal (1:100) (Hammond et al., 2001), anti-Bicaudal-D mouse monoclonal (1:20) (Suter and Steward, 1991), anti-C(3)G guinea pig polyclonal (1:500) (Page and Hawley, 2001), affinity-purified anti-Dicer rabbit polyclonal (1:100) (Bernstein et al., 2001), anti-Gurken mouse monoclonal 1D12 (1:20) (Queenan et al., 1999), affinity-purified anti-Maelstrom rabbit polyclonal (1:50-100) (Clegg et al., 1997), anti-nuclear Lamin mouse monoclonals, ADL67 and ADL84 (1:20) (Stuurman et al., 1995), anti-Oskar rabbit polyclonal (1:500) (Paul Lasko), anti-Staufen rabbit polyclonal (1:500) (Daniel St Johnston) and anti-Vasa rat polyclonal (1:500) (Paul Lasko). We used Alexa

Fluor® (488, 568 or 633)-conjugated goat anti-rat, anti-mouse, anti-guinea pig or anti-rabbit IgG secondary antibodies (Molecular Probes, Eugene, OR).

### Nuclear transport assay

For the nuclear transport assay, ovaries from groups of ten females were dissected in EBR and incubated 20 minutes at ambient temperature on a nutator in 2 ml EBR with or without 50 nM Leptomycin B (Dr Minoru Yoshida, University of Tokyo). After incubation, ovaries were rinsed briefly in EBR and fixed for immunolocalization, as indicated above.

### Confocal microscopy

Confocal images were collected using an inverted Leica DMIRBE microscope equipped with Leica TCS SP/MP confocal and multiphoton attachment using Leica Confocal Software (LCS) version 2. For each experiment, no less than 150 ovarioles were examined, from which representative figures were prepared. Variability and penetrance are noted where relevant. Within individual mutant backgrounds (Fig. 5), Maelstrom levels proved to be rather variable. In order to compare nuage localization, we chose ovarioles in which Maelstrom levels were not reduced, relative to wild type (somatic Maelstrom staining served as an internal control for individual sections). By contrast, overall Vasa levels in the mutants were comparable with wild type. However, as the relative distribution of Vasa to nuage was slightly reduced, we set the gain in confocal sections of mutants such that peak signal values in mutants were comparable to peak signal values for the wild type. This results in an apparent relative increase in cytoplasmic levels of the protein in Fig. 5.

### Whole-mount in situ hybridization

RNA in situ hybridization to ovaries was performed using DIG-labeled DNA probes as previously described (Clegg et al., 1997). Light microscopy was performed on a Leitz DMRB with Nomarski differential interference contrast. Images were acquired with a model 3.2.0 Spot Insight color digital camera (Diagnostics Instrument, Sterling Heights, Michigan). We used Adobe Photoshop (Adobe Systems, Mountain View, CA) to crop and assemble all photographic images.

## RESULTS

### *mael^{M391}* is a null allele

*Maelstrom* allele M391 was isolated by imprecise excision of a P element from line P [w+lacZ] 11A4 (Clegg et al., 1997), which is inserted in the genomic region corresponding to the 5′UTR of the *maelstrom* gene (Fig. 1A). The sterility of *mael^{M391}*/*Df (3L)79E-F* females can be rescued by a transgene containing the 4.5 kb genomic region indicated in Fig. 1A (Clegg et al., 1997). Southern analysis of *mael^{M391}* genomic DNA (not shown) revealed that, in addition to the P element, about 1.2 kb of sequence 3′ to the element was lost in the excision event. We cloned the deletion junction by PCR, using primers predicted to flank the breakpoint. Sequencing of the resulting genomic fragment revealed a deletion of 1319 basepairs of genomic DNA, leaving a 17 bp P-element residue. Nucleotides corresponding to 124-1270 of the mRNA were thus deleted, resulting in the loss of 73% (codons 1-335) of the predicted coding sequence of *maelstrom* (Fig. 1B,C). Western analysis (Fig. 1G) and immunocytochemistry (Fig. 1D-F) show that *mael^{M391}*/*Df (3L)79E-F* ovaries contain no detectable Maelstrom protein. We conclude that *mael^{M391}* is a null allele of the *maelstrom* locus.

Homologs of Maelstrom can be identified in mosquito (*Anopheles gambiae*), honey bee (*Apis mellifera*), mouse and human. A ClustalW alignment (see http://dev.biologists.org/supplemental/) of the *Drosophila*, mosquito and human homologs shows 7.3% identity and 25.9% similarity shared between the three proteins. Fourth iteration Psi-blast searches
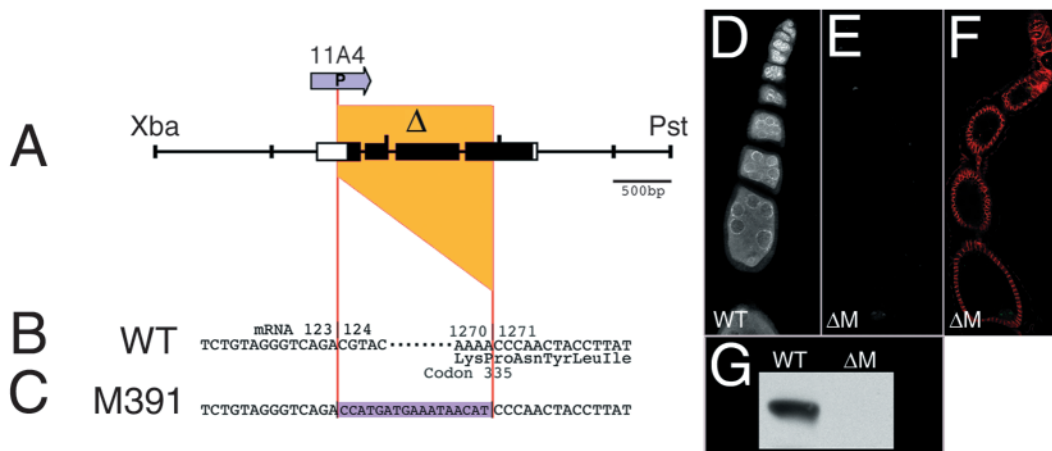


**Fig. 1.** *mael^{M391}* is a null allele. (A) Organization of the *maelstrom* gene. Exons are indicated by boxes in which coding regions are black and UTRs are white. The P [w+lacZ]11A4 insertion is indicated by a lavender arrow, and the region deleted (Δ) in *mael^{M391}* is ochre. (B) Wild-type genomic DNA sequence at the junctions of the deletion. The P element is inserted between basepairs corresponding to nucleotides 123/124 of the mRNA. The DNA encoding nucleotides encoding 129-1266 of the mRNA is indicated by (dots). (C) The imprecise P-element excision in *mael^{M391}* results in the deletion of 1319 basepairs of genomic DNA corresponding to nucleotides 124-1270 (including the initiation codon) of the mRNA. The residual P-element sequence is indicated by the purple background. (D) Maelstrom staining in a wild-type ovariole. Immunoreactive signal is predominant in cells throughout the germline. (E) Maelstrom or (F) Maelstrom (green) and Adducin (red) staining of a *mael^{M391}*/*Df (3L)79E-F* ovariole. Virtually all anti-Maelstrom immunoreactive signal is lost in the mutant. In the Western blot (G) probed with anti-Maelstrom antiserum, neither the full-length wild-type (left, at 50 kDa) nor potential truncated forms (not shown) of Maelstrom is detected in *mael^{M391}*/*Df (3L)79E-F* ovaries lane (right).

with the *Drosophila* homolog gives overall E-values of $e^{-151}$ (to the mosquito homolog, agCP12344) and $e^{-150}$ (to the human homolog, FLJ14904). In addition, a partial potential HMG-box is found in *Drosophila* Maelstrom (residues 2-50), whereas a canonical HMG-box is found in the human homolog (residues 5-65).

### *Maelstrom* is a *spindle*-class gene

As hypomorphic alleles of *maelstrom* showed AP and DV *spindle*-class-like defects in the developing oocyte (Clegg et al., 2001; Clegg et al., 1997), we wished to determine whether the *maelstrom* null (hereafter referred to as *maelstrom*) shared the meiotic progression defect common to the *spindle*-class mutants. Specifically, the *spn* mutants fail to form a karyosome (Ghabrial et al., 1998; Gonzalez-Reyes et al., 1997; Styhler et al., 1998; Tomancak et al., 1998), despite the apparently normal assembly of synaptonemal complexes within the oocyte

nucleus (Huynh and St Johnston, 2000). To this end, we examined meiotic progression in the oocyte nucleus (germinal vesicle) using synaptonemal complex component, C(3)G, to assess progression to synaptonemal complex formation; we used oocyte DNA morphology to assess progression to the karyosome stage (Fig. 2A-E). C(3)G is normally acquired by oocyte chromosomes in the germarium (Fig. 2A) and dissociated from DNA upon karyosome formation (Page and Hawley, 2001). In more than 90% of stage 2 or 3 *maelstrom* egg chambers, C(3)G signal is present and restricted to the oocyte (Fig. 2B), where it colocalizes with DNA in a morphology comparable with wild type (Fig. 2C). This suggests that meiosis has proceeded in the mutant to at least zygotene phase of prophase I. As reported for other *spn* mutants (Huynh and St Johnston, 2000), *maelstrom* ovaries show some delay in restriction of synaptonemal complexes to a single cell (data not shown). When the karyosome forms in
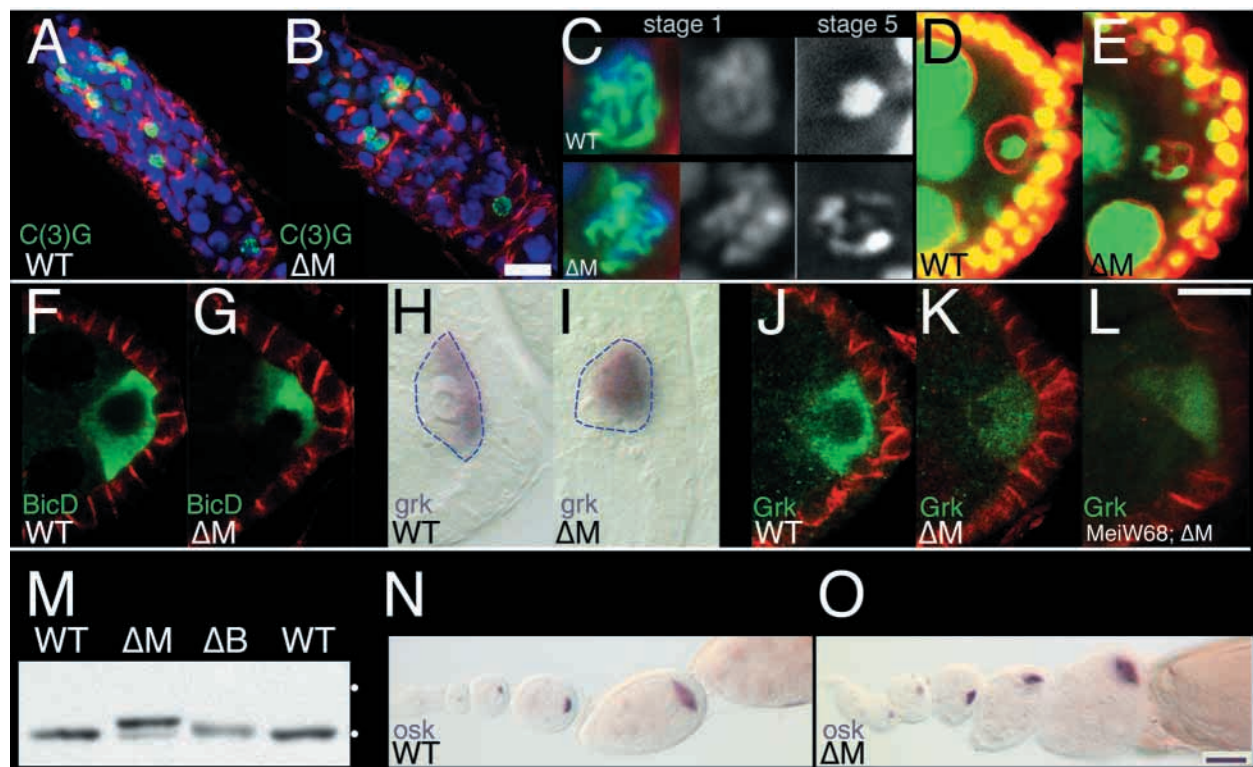


**Fig. 2.** *Maelstrom* is a *spindle*-class gene. (A) Distribution of synaptonemal complex component C(3)G (green) in a wild-type ovariole labeled with DAPI (blue) and for Adducin (red), which marks fusomes and cell membranes. C(3)G is normally acquired by germline chromosomes in region 1 of the germarium (top left) and is restricted to the oocyte nucleus by stage 1 (lower right). (B) The localization of C(3)G on chromosomes is unperturbed in most *maelstrom* null ovaries. (C) Magnified views of oocyte nuclei from wild-type and *maelstrom* oocytes. Top row (WT), wild type; bottom row (ΔM), maelstrom (left to right): stage 1 C(3)G (green channel) and DNA (blue channel); stage 1 DNA channel; and stage 5/6 DNA. Wild-type (D) and *maelstrom* (E) stage 5/6 oocytes labeled with DAPI (green) and for nuclear lamin (red). Chromosomes within the wild-type oocyte nucleus (D) have condensed to form the karyosome. A *maelstrom* stage 5/6 oocyte (E), in which the karyosome never forms. (F) Distribution of BicD (green) in a wild-type stage 5/6 oocyte. Follicle cells are labeled with α-Spectrin (red). BicD appears in a distinct gradient emanating from the posterior oocyte cortex. In *maelstrom* oocytes at this stage, BicD appears in a randomly localized focus (G) or unlocalized (not shown). Several RNAs, such as *gurken*, which localize to the posterior cortex of the wild-type stage 5/6 oocyte (H), are present at normal levels, but fail to localize in the *maelstrom* mutant (I) (oocytes indicated by broken blue lines). (J) Distribution of Gurken protein (green) in a wild-type stage 5/6 oocyte with follicle cells labeled for α-Spectrin (red). Gurken is present at either low (K) or undetectable (not shown) levels in both *maelstrom* (K) and *mei-W68[1]*; *mael[M391]/Df (3L)79E-F* (L) oocytes. (M) Western blot of Vasa in wild-type (WT), *mael[M391]/Df (3L)79E-F*(ΔM) and *spn-B[153]/Df(3R)trxE12\** (ΔB) backgrounds. In wild-type ovaries, Vasa runs as a ~72.4 kDa singlet. In *maelstrom*, Vasa runs as a doublet, the slower migrating band of which has a larger apparent mass (~74.4 kDa) than that of *spn-B* mutant. (MW markers are indicated on the right of the panel, the upper dot is at 83.6 kDa, and the lower dot is at 72 kDa). Nurse cell to the oocyte transport of *oskar* mRNA in *maelstrom* (O) ovarioles is comparable with wild-type (N). Scale bars: in L, 10 μm for A-D,F-L; in O, 50 μm for N,O.

wild-type oocytes (Fig. 2C, WT, stage 5; Fig. 2D), DNA within the germinal vesicle loses it association with C(3)G. Despite the dispersion of C(3)G within the oocyte nucleoplasm by stage 6, *maelstrom* oocytes never form karyosomes. Instead, the DNA shows a nuclear morphology distinct from both stage 1 and karyosome: forming variably distended loops and threads, often closely apposed to an invariably 'deflated' nuclear envelope (Fig. 2C, ΔM, stage 5; Fig. 2E). This DNA morphology, maintained in *maelstrom* through at least stage 10B, is similar to that described for other *spn* mutants (Ghabrial et al., 1998).

The axial patterning defects displayed by *maelstrom* hypomorphs (Clegg et al., 2001; Clegg et al., 1997) are fully penetrant in oocytes of the *maelstrom* null (Fig. 2F-K). AP axis determination in the *Drosophila* oocyte is a multistep process, the first known step of which is the establishment of microtubule-mediated cytoplasmic polarity in the stage 2 oocyte. This asymmetry, which is defective in *spn* mutants such as *spn-A*, *spn-B* and vasa (Pare and Suter, 2000; Styhler et al., 1998; Tomancak et al., 1998), is a likely prerequisite for efficient Gurken signaling from the oocyte to the follicle cells overlying the posterior oocyte. A number of RNAs and proteins accumulate in the posterior of the wild-type oocyte during stages 2-6 in a distribution that both requires and reflects the oocyte polarity in this interval. We assayed polarity in the oocyte indirectly by monitoring the localization of Bicaudal D (BicD) (Suter and Steward, 1991) and multiple RNAs including *grk* (shown), *osk*, *bicD* and *oo18 RNA binding* (*orb*) (not shown). In normal stage 5/6 oocytes, BicD forms a distinct gradient emanating from the posterior oocyte cortex (Fig. 2F). In *maelstrom* oocytes, although BicD is present at levels comparable with wild type (Fig. 2G), a wild-type gradient is not established. Instead, about half of stage 5/6 *maelstrom* oocytes show BicD in a diffuse or only vaguely polarized distribution (not shown). In the remaining oocytes, the marker forms a randomly localized focus within the ooplasm (Fig. 2G). Similarly, the normally polarized distribution of *grk* and other RNAs is lost in *maelstrom* oocytes (Fig. 2H,I). Gurken protein distribution in wild-type oocytes (Fig. 2J) is comparable with that of BicD, albeit more punctate in appearance. In *maelstrom* oocytes, not only is the gradient lost, but Gurken levels are either highly reduced (~50%) (Fig. 2K) or undetectable (~50%) (not shown). The Gurken defect is probably sufficient to account for the observed polarity defects in mid- to late-stage *maelstrom* oocytes, in which variety of polarity markers, including multiple mRNAs (e.g. *osk*) and proteins (including Staufen, Oskar, Vasa), fail to accumulate in the posterior ooplasm. Dorsal appendages are also invariably vestigial or absent in the *maelstrom* null (not shown). The failure in establishing AP polarity in the early oocyte, together with reduction in Gurken accumulation, the DV phenotypes of the null and hypomorph, and failure to proceed to karyosome stage collectively puts *maelstrom* in the *spindle*-class of mutants.
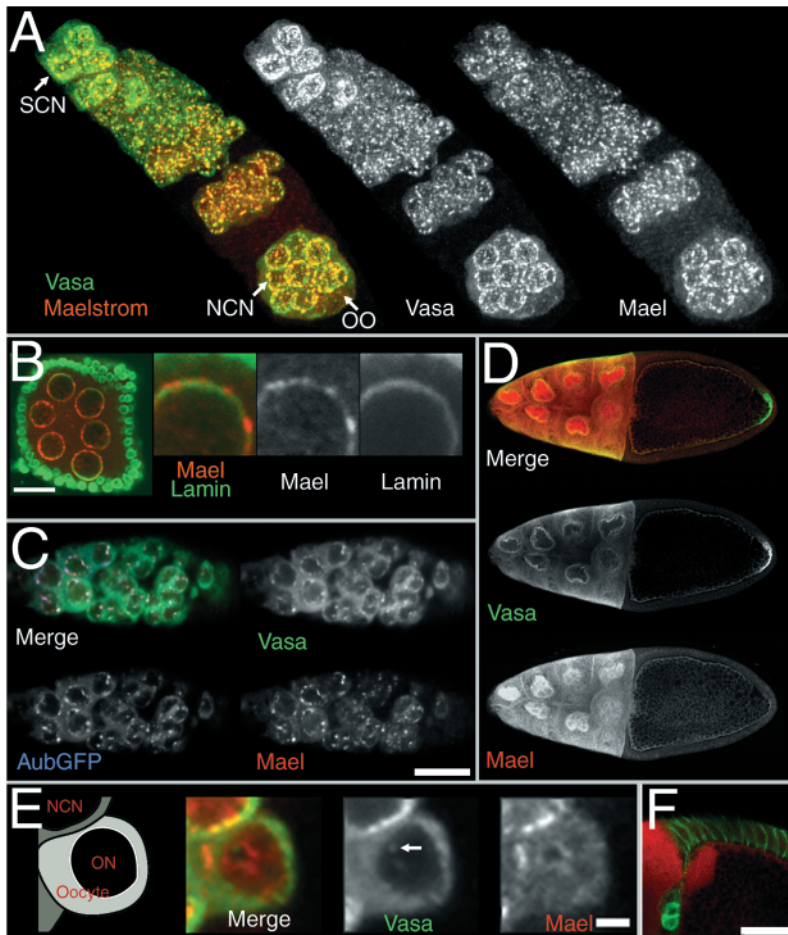


**Fig. 3.** Distribution of Maelstrom protein during oogenesis. (A) Left panel: three-dimensional projection of a partial confocal *z*-series stack showing a wild-type ovariole double-labeled (left) for Vasa (middle, green channel) and Maelstrom (right, red channel). Maelstrom colocalizes with Vasa in highly abundant perinuclear particles of all germline cells. Arrows indicate individual stem cell nuclei (SCN), nurse cell nuclei (NC) and an oocyte (OO). (B) From left to right: double labeling of Maelstrom (red) and nuclear Lamin (green) in a stage 4 egg chamber; enlargement of a single nurse cell nucleus; single Maelstrom and single Lamin channels. Maelstrom particles are closely apposed to the cytoplasmic face of the nuclear envelope. Scale bar: 10 μm. (C) An Aub-GFP germarium stained for Maelstrom and Vasa. Upper left: merged image of Vasa (green, upper right), Maelstrom (red, lower right) and Aub-GFP (blue, lower left) channels. Each discrete perinuclear particle labels for the three nuage components. Scale bar: 10 μm. (D) Localization of Vasa (green) and Maelstrom in a stage 10 egg chamber. Within nurse cells both proteins are present in nuage and cytoplasm. Maelstrom, unlike Vasa, shows no accumulation in the oocyte posterior. (E) Localization of Vasa and Maelstrom within a stage 2 oocyte nucleus. Panels from left to right: diagram of confocal field, with nurse cell nucleus (NCN) and oocyte nucleus (ON) indicated. Vasa (green in merged image) frequently localizes to intranuclear punctae (white arrow) ringed by concentrated Maelstrom (red in merged image). Scale bar: 2 μm. Anterior is towards the left in all panels. (F) Dorsal anterior corner of a stage 10B egg chamber labeled for 1B1 (green) and Maelstrom (red), which is present as diffuse staining in the oocyte nucleus. Scale bar: 20 μm.

The phenotypes of the double-strand break (DSB) repair-specific *spn* mutants (e.g. *spn-B*) can be suppressed by a mutation in *mei-W68* (Ghabrial and Schupbach, 1999). This locus encodes the *Drosophila* homolog of the Spo11 protein, which induces double-strand breaks in chromosomes, the initiating event required for subsequent steps in recombination (McKim and Hayashi-Hagihara, 1998). If DSBs do not occur, then genes normally required in the ensuing recombinational repair steps are not required. Thus, their absence will not be detected by the elements of the meiotic checkpoint, which responds to persistent unrepaired DSBs. To resolve the sphere of *maelstrom* function, we assessed genetic interaction between *mei-W68* and *maelstrom* by examining Gurken accumulation in early oocytes of *mei-W68-maelstrom* double mutant ovaries. If *maelstrom* were required only in recombinational repair, we would expect a suppression of the Gurken translation defect of the *maelstrom* null oocyte (Fig. 2K). We observe that the Gurken defect of *maelstrom* oocytes is not, in fact, suppressed by *mei-W68* (Fig. 2L), from which we conclude that *maelstrom* cannot only be required in a recombinational repair step.

How meiotic progression status in the oocyte nucleus is transmitted to effectors of oocyte patterning is a key, and largely unanswered, question. One candidate effector is Vasa, a target of the pachytene checkpoint, which displays a mobility shift in *spn-B* ovaries, in which the checkpoint is activated (Ghabrial and Schupbach, 1999). Interestingly, Vasa mobility is aberrant in *maelstrom* ovaries; two distinct species of Vasa protein are observed: a minor band with wild-type mobility and a species larger, curiously, than that reported for *spn-B* (Fig. 2M). Although the relationship to activated-checkpoint-Vasa is unclear, our data shows that *maelstrom* is required for proper Vasa modification (or processing). It is thus conceivable that any phenotype(s) of the *maelstrom* mutant could arise, indirectly, as a result of this Vasa modification. The apparent mass of Maelstrom, by contrast, was unchanged in *vasa* null (*vas^{PH165}*) background, and in alleles of each of the *spn* genes (A-E) and *okr* (not shown).

## Maelstrom localizes to Nuage

Previously, we reported that Maelstrom protein displayed no distinct subcellular localization within the germline (Clegg et al., 1997). We have since re-examined its localization using a 'lighter' fixation-based protocol (see Materials and Methods). As a result, we find that in addition to previously observed diffuse nuclear and cytoplasmic germline staining, much of Maelstrom localizes to highly abundant particles within germline cells (Fig. 3A; see http://dev.biologists.org/supplemental/). The frequency and distribution of Maelstrom particles were reminiscent of that previously described for nuage, to which Vasa localizes. Double labeling of Maelstrom and Vasa (Figs 3, 5; see http://dev.biologists.org/supplemental/) shows overlap in perinuclear germline granules from stem cells through stage 10 nurse cells. Double labeling of Maelstrom and a nuclear lamin shows that virtually all distinct Maelstrom particles are closely apposed to the cytoplasmic face of the nuclear envelope in nurse cells (Fig. 3B). Because *nanos*-GAL4-driven GFP-tagged Aubergine (AubGFP) was reported to localize to nuage in late stage egg chambers (Harris and Macdonald, 2001), we examined its localization in combination with Vasa and

Maelstrom immunostaining (Fig. 3C). Each discrete particle in the germarium and early egg chamber labels for Vasa, Maelstrom and AubGFP, a concordance that is also maintained in stages 7-10. (Owing to the discontinuous nature of the *nanos* driver, AubGFP is not highly expressed between approximately stages 3 and 6.) At the ultrastructural level, most nuage is lost from the oocyte by stage 1, prior to the formation of the karyosome (Mahowald and Strassheim, 1970). However, occasional particles of Vasa and Maelstrom can be detected in the ooplasm as late as stage 4 (Fig. 3E and data not shown). Although the most conspicuous localization of Maelstrom and Vasa is to nuage, each protein is also present within the nucleus and cytoplasm of all germline cells (Fig. 3A-D). Within the oocyte nucleus, both proteins localize to discrete regions in young egg chambers: in single confocal sections, Vasa often appears in discrete dot or dots, exclusive of, but adjacent to an 'aura' of concentrated Maelstrom (Fig. 3E). Maelstrom persists in the oocyte nucleus as diffuse staining through at least late stage 10B (Fig. 3F). After onset of pole plasm assembly (stage 8/9), Vasa accumulates in posterior region of the oocyte (Fig. 3D, Vasa panel) (Hay et al., 1988b; Lasko and Ashburner, 1990). Maelstrom, by contrast, never shows a posterior concentration in the ooplasm (Fig. 3D, Maelstrom panel). Although Maelstrom is present in the mature egg and early embryo, its distribution is again uniform at these stages (not shown). As neither the Maelstrom nor its RNA (not shown) show preferential posterior accumulation in the ooplasm, Maelstrom is the first described nuage component that is not also concentrated in pole plasm.

## Maelstrom shuttles between compartments

Because Maelstrom and Vasa are each present in the nucleus, nuage and cytoplasm of germline cells, it was of interest to determine whether either protein could transit between these compartments. We assayed nuclear shuttling utilizing Leptomycin B (LMB) (Hamamoto et al., 1983), a specific inhibitor of nuclear transport receptor, CRM1 (Exportin). CRM1 mediates nuclear export of substrates containing a leucine-rich nuclear export sequence (NES) in cells as diverse as yeast and human (Fornerod et al., 1997; Fukuda et al., 1997; Stade et al., 1997). *Drosophila* CRM1 has been shown to be mechanistically indistinguishable from its homologs in other systems, including its specific inactivation by LMB (Fasken et al., 2000). LMB treatment of *Drosophila* ovaries has a marked effect on Maelstrom protein localization within the germline (compare Fig. 4C with 4G and Fig. 4D with 4H), whereas Vasa protein shows only a slight redistribution (compare Fig. 4A with 4E and Fig. 4B with 4F). The effect is most pronounced in nurse cells and oocytes, where Maelstrom manifests a nuclear accumulation, with a corresponding depletion in cytoplasm (Fig. 4H). We surmise that Maelstrom must transit between cytoplasm and nucleus.

## Maelstrom is dissociated from nuage particles in *vasa, aubergine* and *spn-E* mutants

We do not know if the known nuage proteins act in a common pathway before their convergence in nuage particles. To begin to answer this question, we have sought to determine genetic dependencies for nuage particle assembly (Fig. 5). AubGFP has previously been shown to depend on *vasa* function for its nuage localization (Harris and Macdonald, 2001). We analyzed

Maelstrom and Vasa localization in wild-type, *maelstrom*, *vasa*, *aubergine* and *spn-E* backgrounds. Maelstrom protein levels proved to be quite variable among ovarioles of single mutant backgrounds. So, in order to compare localization, we examined individual ovarioles in which Maelstrom levels were not significantly reduced (see Materials and Methods). Maelstrom and Vasa localization in the wild-type ovariole is shown in Fig. 5 (parts A1 and A1′). Virtually no Maelstrom immunoreactive signal is present in the *maelstrom* (Fig. 5, part A2), whereas Vasa is largely maintained in nuage (Fig. 5, part
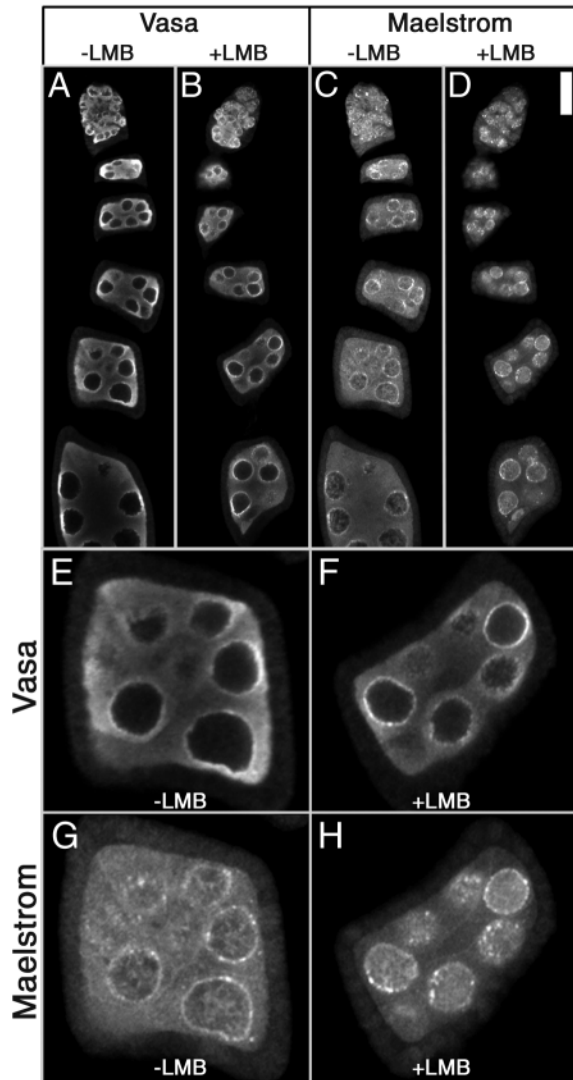


**Fig. 4.** Leptomycin B inhibits the nuclear export of Maelstrom. Ovaries were incubated for 20 minutes with or without 50 nM LMB, then stained for Vasa and Maelstrom. Two representative double-labeled ovarioles (A-D) and enlarged fields (E-H) are shown. The distribution of Vasa between nucleoplasm, nuage and cytoplasm of nurse cells is unperturbed during the 20 minute incubation without LMB (A, enlarged in E). There is a slight nuclear elevation of Vasa in LMB-treated samples (B, enlarged in F). Maelstrom maintains a normal distribution in the control ovariole (C, enlarged in G); upon LMB treatment (D, enlarged in H), Maelstrom is simultaneously reduced within cytoplasm of nurse cells and the oocyte, and enhanced within nuclei of the same cells. Anterior is towards the top in all panels. Scale bar: 20 μm in A-D.
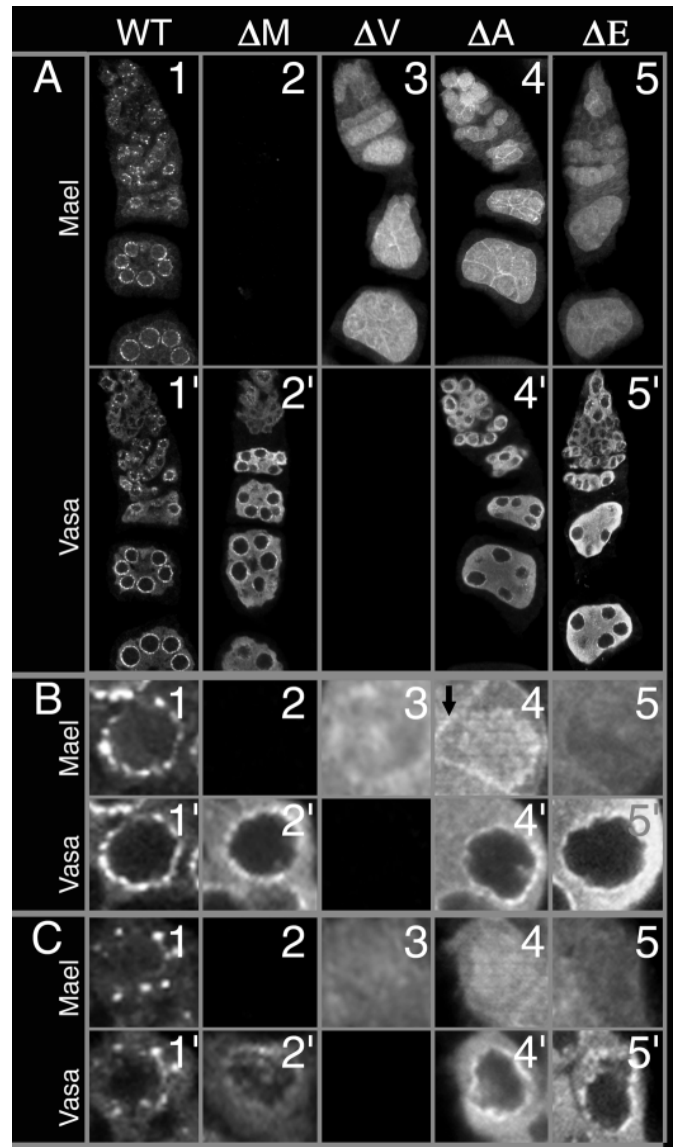


**Fig. 5.** Dependencies of nuage particle assembly. Maelstrom (A1) and Vasa (A1′) localization in wild-type, *aub*$^{HN2/N11}$ (A4, A4′) and *spn-E*$^{616/hls\Delta125}$ (A5,A5′) ovarioles. Maelstrom (A2) and Vasa (A2′) localization in *mael*$^{M391}$*/Df (3L)79E-F* ovarioles; and Maelstrom localization in *vasa* ovarioles (A3). (Vasa signal in *vasa* ovarioles was not examined in this experiment, but in similar experiments performed, no signal is detected.) (B,C) Enlargements of nurse cells (B) and stem cells (C) from the correspondingly numbered panels in A. (A1) Distribution of Maelstrom in a wild-type ovariole. The highest concentration of Maelstrom is in perinuclear granules, evident in the germline from stem cells and cystoblasts (enlarged in C1) to nurse cells (enlarged in B1). In the *maelstrom* ovariole, virtually no Maelstrom immunoreactive signal is present (A2); yet Vasa is largely maintained in nuage (A2′). Maelstrom protein level in the germline of *vasa* null ovaries (A3, B3, C3) shows little preferential distribution to perinuclear particles in any cell type. (A4) Maelstrom in an *aub*$^{HN2/N11}$ ovariole. Maelstrom's preferential localization to perinuclear particles is abolished. Instead, Maelstrom often accumulates on membranes between germline cells [B4 (black arrow), C4]. (A5) Maelstrom in a *spn-E*$^{616/hls\Delta125}$ ovariole. Maelstrom shows no preferential accumulation in perinuclear particles in any cell type (B5,C5). Differential localization of Vasa to nuage is largely maintained in *mael* (A2′, B2′,C2′), *aub*$^{HN2/N11}$ (A4′,B4′,C4′) and *spn-E*$^{616/hls\Delta125}$ (A5′,B5′,C5′) backgrounds. Anterior is towards the top in all panels.

A2′; Fig. 6D). By contrast, the perinuclear accumulation of Maelstrom is virtually absent in the *vasa* null (Fig. 5, part A3), suggesting that Maelstrom localization in nuage is Vasa dependent. We examined Maelstrom's distribution in several *vasa* point mutants, in the hope of correlating functional domains in the protein with nuage organizational function. Of particular interest were two *vasa* EMS alleles, *vas$^{011}$* and *vas$^{014}$*, each of which produces a protein devoid of RNA binding and unwinding activities (Liang et al., 1994). In both of these mutants, Vasa and Maelstrom colocalization in nuage is largely maintained (data not shown). We analyzed Vasa and Maelstrom localization in several allelic combinations of *aubergine*, as a null for this gene has not been described. Maelstrom (Fig. 5, part A4) and Vasa (Fig. 5, part A4′) localization is shown for a representative, *aub$^{HN2/N11}$* ovariole. Both *aub$^{HN2}$* and *aub$^{N11}$* alleles encode truncated proteins (Harris and Macdonald, 2001). In this and other *aubergine* mutant combinations, the normal concentration of Maelstrom in nuage is severely depleted in all germline cells (Fig. 5, part A4). Vasa is largely maintained in perinuclear localization in this mutant background, but the normally discrete particles are less obvious; instead, Vasa appears as a more uniform perinuclear band (Fig. 5A, part 4′).

*Spn-E* encodes a putative Dex/hD-box RNA helicase, required for proper localization of several oocyte-destined RNAs and proteins over the course of oogenesis (Gillespie and Berg, 1995; Pare and Suter, 2000). While the localization of Spindle-E in the ovary has not been determined, its involvement in both RNAi and oogenesis (Aravin et al., 2001; Stapleton et al., 2001; Kennerdell et al., 2002), like Aubergine, prompted its inclusion in our analysis. As with *aubergine* mutants, the concentration of Maelstrom in perinuclear particles is lost in strong *spn-E* allelic combinations, *spn-E$^{616/hlsΔ125}$* (Fig. 5A, part 5) and *spn-E$^{hls3987/hlsΔ125}$* (not shown). Vasa retains a perinuclear concentration in *spn-E* ovaries (Fig. 5A, part 5′), but as in *aubergine*, the normal particulate appearance of nuage is less pronounced. We extended our localization analysis to include the remaining members of the better characterized *spn*-class mutants, *spn-A*, *spn-B*, *spn-C*, *spn-D* and *okr*. Of particular interest was *spn-B*, which has been shown to modify Vasa as a consequence of meiotic checkpoint activation (Ghabrial and Schupbach, 1999). The dependency of Maelstrom on Vasa for its localization could, in principle, be affected if Vasa is aberrant. However, in multiple allelic combinations of well-characterized *spn* genes (*spn-B*, *spn-D* and *okr*) and uncloned *spn* genes (*spn-A* and *spn-C*), colocalization of Vasa and Maelstrom in nuage particles was unperturbed at all stages of oogenesis (not shown).

### The *maelstrom* and *vasa* nulls differ in their RNA phenotypes

Normally, a variety of RNAs required for proper oocyte function are transcribed, exported from nurse cell nuclei and transported to the oocyte. As this process is defective in *vasa* ovaries (Styhler et al., 1998), we wished to assess transport in *maelstrom* ovarioles, with the aim of resolving potential functions of individual nuage components. We found that transport of several of these mRNAs, including *oskar* (Fig. 2N,O), *orb* and *BicD* (not shown), is unaltered in *maelstrom* ovaries.

### RNAi/microRNA components are mislocalized in *maelstrom* egg chambers

The dissociation of Maelstrom from nuage particles in *aubergine* and *spn-E* backgrounds was intriguing in light of their requirement in RNAi in *Drosophila* spermatogenesis and late oogenesis (see Discussion). Importantly, proteins (or homologs) of RNAi pathway components also act in micro RNA (miRNA) processing (Grishok et al., 2001; Hutvagner et al., 2001). As miRNAs have been shown to regulate RNA translation, it is conceivable that miRNAs are assembled in RNP particles formed in nuage. In this setting, nuage could represent a step in the generation of specificity in translational control in the germline. To explore this potential relationship between nuage and RNAi/miRNA processing pathways, we examined the localization of additional RNAi components in wild-type and *maelstrom* ovaries. Argonaute1 and Argonaute2 are RDE1/AGO1 homologs required for RNAi in *Drosophila* (Hammond et al., 2001; Williams and Rubin, 2002). Dicer is the core RNase of RNAi in *Drosophila* (Bernstein et al., 2001); it is also required for production of the small RNA effectors of the RNAi and miRNA pathways in *C. elegans* (Grishok et al., 2001; Hutvagner et al., 2001). In vertebrate cell lines, Dicer is primarily cytoplasmic (Billy et al., 2001). In wild-type *Drosophila* ovarioles, Dicer (Fig. 6A,A′) and AGO1 (Fig. 6G,G′) appear uniform and cytoplasmic in nurse cell cytoplasm; AGO2 (Fig. 6E,E′) appears cytoplasmic but relatively more granular. In *maelstrom* ovaries, AGO1 distribution is relatively unperturbed (Fig. 6H,H′). However, AGO2 and Dicer are both dramatically mislocalized in *maelstrom* ovarioles. Beginning around stage 3, Dicer aggregates in discrete, often perinuclear foci in nurse cells (Fig. 6B,B′). AGO2 is observed in perinuclear regions of nurse cells (Fig. 6F,F′), which, by contrast, could colocalize with Vasa in nuage (Fig. 6D,D′).

### DISCUSSION

The failure of *maelstrom* oocytes to proceed to the karyosome stage, to establish cytoplasmic polarity and to accumulate Gurken qualifies the inclusion of *maelstrom* in the *spindle* class. Maelstrom is a component of *Drosophila* nuage and is required for proper modification (or processing) of a key nuage component, Vasa. Maelstrom is also present within the nucleus and cytoplasm of all germline cells, and can shuttle between these compartments in a CRM1-dependent manner. To understand better the cellular processes in which nuage is important, we have examined genetic dependencies for association of nuage components in particles. Of the known nuage-localizing proteins, Vasa appears to be a pivotal organizer or nucleator of nuage, whereas Maelstrom can be dissociated from nuage particles in *aubergine* and *spn-E* mutants. Furthermore, Dicer and AGO2 are mislocalized in the *maelstrom* background.

### Resolving the domains of function of spindle genes

The characterized s*pn* genes currently fall into two general classes: those that encode proteins that are likely to be directly involved in meiotic recombinational repair, such as *okr, spn-B* and *spn-C* (Ghabrial et al., 1998; Ghabrial and Schupbach, 1999); and those, such as *maelstrom* and *vasa*, whose mutant
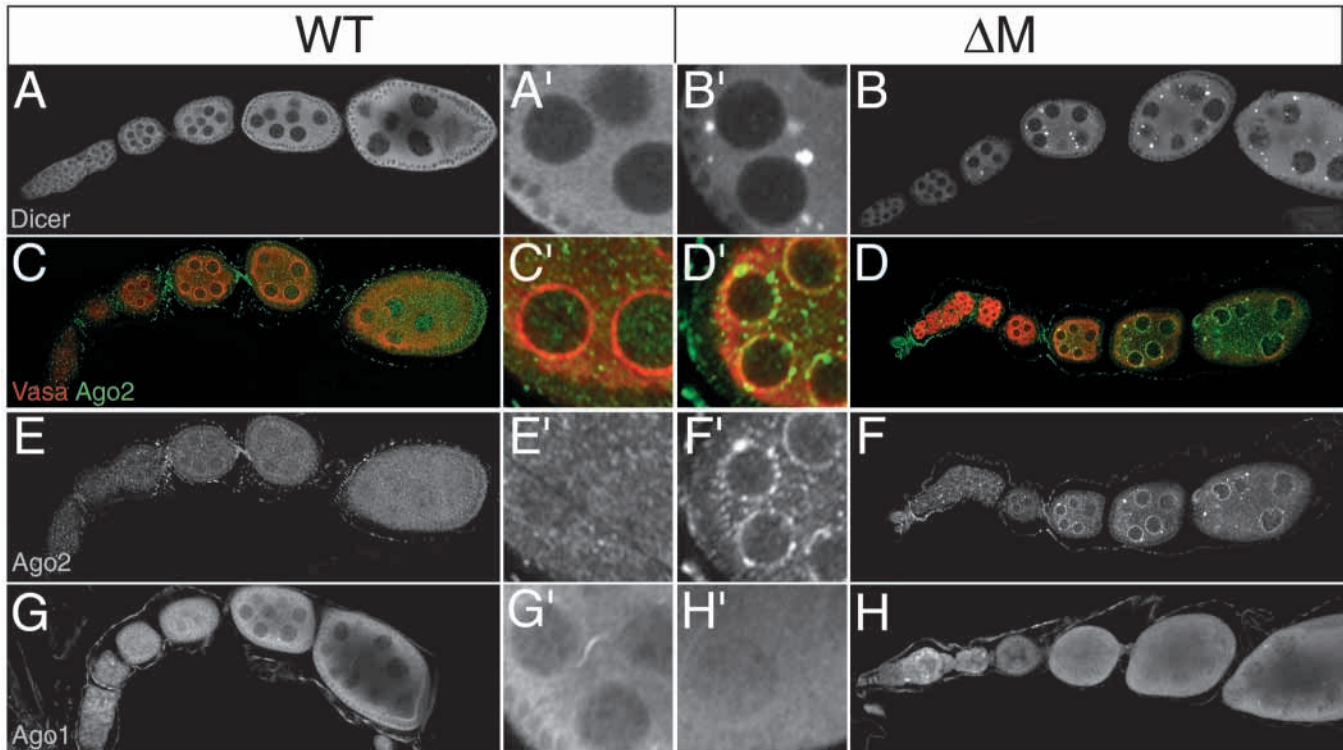
**Fig. 6.** *Maelstrom* is implicated in miRNA/RNAi pathways. Dicer in a wild-type ovariole (A, enlarged in A′) is ubiquitously expressed and primarily cytoplasmic in both germline and somatic cells. In *maelstrom* ovarioles (B, enlarged in B′), Dicer is ectopically localized to discrete punctae, which are frequently perinuclear in stages 3-9 nurse cells. A wild-type ovariole (C, enlarged in C′) labeled for Vasa (red channel) and AGO2 (green channel). AGO2 appears as small, irregular particles distributed randomly in the cytoplasm of germ cells. (E, enlarged in E′) Wild-type AGO2 channel alone. In *maelstrom* ovarioles (D, enlarged in D′), AGO2 (green channel) aggregates in perinuclear halos that overlap Vasa (red channel). (F, enlarged in F′) *maelstrom* AGO2 channel alone. AGO1 shows no discernable subcellular localization in either wild-type (G, enlarged in G′) or *maelstrom* ovarioles (H, enlarged in H′).

meiotic phenotype, protein sequence and/or localization suggest indirect roles. Work presented in this paper suggests that the *spn* mutants can be sorted by an additional criterion: those that are also required for nuage assembly (*vasa*, *aubergine*, *maelstrom* and *spn-E*) and those that are not (*spn-A*, *spn-B, spn-C, spn-D* and *okra*). Taken together, these data suggest that the Vasa-like group of *spn* genes are essential in general 'nuage activities' in all cells of the germline. The activity of the *spn-B*-class genes, which are involved in recombination or meiotic checkpoint, could represent one avenue through which to use or modulate existing nuage functions that are operative within the germline cyst as a whole. Such nuage-related processes, if inactivated or defective, could culminate in polarity and translational defects within the oocyte.

## Cell biology of nuage

Within the time frame of the life cycle of the female fly, pole plasm, in its mature form, is ephemeral (Mahowald, 1971b). Nuage, by contrast, is present in morphologically stable form from mid-embryogenesis through to late oogenesis in the adult (reviewed by Mahowald, 1971b). Nuage is thus a feature that is specific to established germ cells, perhaps reinstated by pole plasm components in early embryogenesis. Maelstrom is unique among previously identified *Drosophila* nuage components, which are also concentrated in pole plasm.

Maelstrom is thus the first identified nuage-specific component in *Drosophila*. Nuage is the most conserved form of Vasa-positive germline granule: it is present in germ cells of diverse organisms, including mammals, which do not use a pole plasm equivalent (reviewed by Saffman and Lasko, 1999). The cell biology of nuage, however, remains largely unexplored. As nuage components are also present in the nucleus and cytoplasm, these particles may represent a morphologically distinct form (or kinetic intermediate) of a nucleocytoplasmic continuum. We have demonstrated that Maelstrom can shuttle between nuclear and cytoplasmic compartments. The observation that Vasa showed only a slight redistribution to the nuclear compartment implies that these proteins are not necessarily always associated. This begs the question of whether components of nuage converge on and diverge from perinuclear particles from separate pathways or subcellular compartments. As more nuclear transport tools become available, we may better resolve how these particles are formed.

## Nuage may function in RNA processing

Within egg chambers, a number of mRNAs crucial for oocyte patterning are synthesized in nurse cells and transported to the developing oocyte (reviewed by Riechmann and Ephrussi, 2001). The oocyte represents a discrete compartment in the continuous cytoplasm of the germline cyst, and precise

spatiotemporal control of nurse cell-derived mRNA translation is crucial for proper development. Between transcription in nurse cell nuclei and their ultimate translation, oocyte-destined RNAs are likely to be associated with factors required to mediate both localization and translational control. Recent work has shown that heterogeneous nuclear ribonucleoproteins (hnRNP-proteins) associated with mRNAs during their transit from nucleus to cytoplasm can play key roles in mRNA localization and translational control (reviewed by Dreyfuss et al., 2002). Similarly, splicing factors have been shown to continue their association with mRNA into cytoplasm. And although the exchange of nuclear for cytoplasmic RNA-binding proteins has been demonstrated, the location and regulation of such exchange processes are poorly understood (reviewed by Shatkin and Manley, 2000). A working hypothesis is that nuage in the *Drosophila* germline (and other systems) functions in such an exchange process. In this capacity, nuage might be a platform for, or represent a kinetic intermediate of, cytoplasmic ribonucleoprotein (RNP) particle assembly (Fig. 7). The perinuclear localization of nuage and the observation that Maelstrom shuttles between compartments are consistent with this role (Figs 3, 4). Two additional lines of evidence support the proposition. The first is that nuage ultrastructurally interfaces with sponge bodies, which are highly abundant, RNA-rich particles present in the cytoplasm of nurse cells and the oocyte (Wilsch-Brauninger et al., 1997). Mounting evidence points to a role for the sponge body as a vehicle for transport of RNP complexes between nurse cells and the oocyte (Cha et al., 2001; Nakamura et al., 2001; Theurkauf and Hazelrigg, 1998; Wilhelm et al., 2000). Sponge bodies are also highly enriched for several regulatory proteins, including Exuperantia (Wilsch-Brauninger et al., 1997), DEAD-box RNA helicase Me31B (Nakamura et al., 2001) and cold shock protein YPS (Wilhelm et al., 2000), which can be purified as an RNase-sensitive RNP complex (Nakamura et al., 2001; Wilhelm et al., 2000). This complex, by purification or localization, is associated with numerous oocyte-destined RNAs, such as *oskar* and *bicoid* (Nakamura et al., 2001; Wilhelm et al., 2000). Ultrastructurally, the perinuclear fraction of sponge body particles appear to embed individual nuage particles (Wilsch-Brauninger et al., 1997), a morphological association that implicates the interface as one possible segment in what may be a continuous RNP assembly process (Fig. 7).

The second line of evidence is that three of the proteins present in, or required for, nuage particle assembly (Aubergine, Vasa and Spn-E) are also implicated in post-transcriptional RNA-related capacities. *Aubergine* mutants fail to translate Oskar efficiently, despite proper localization of *oskar* mRNA to the oocyte posterior (Wilson et al., 1996). Aubergine is concentrated in two distinct regions within germline cysts: in nuage and also in pole plasm (Harris and Macdonald, 2001). Thus, *aubergine* function may be required for proper Oskar translation before *oskar* mRNA is even transported to the oocyte posterior. Vasa is a DEAD-box RNA helicase, a class of proteins thought to act as RNA chaperones (reviewed by Tanner and Linder, 2001). *Vasa*-null egg chambers display a systemic failure in mRNA targeting. This could be attributable, in part, to the loss of interface of nuage with sponge bodies, as *vasa* null ovaries are devoid of nuage at the ultrastructural level (Liang et al., 1994). *spn-E* encodes a Dex/hD class (putative) RNA helicase. *spn-E* ovaries contain enlarged sponge bodies (Pare and Suter, 2000) and display defects in the localization of several normally oocyte-destined molecules, including *bicoid* RNA, as well as BicD(GFP) and Dynein Heavy Chain (Gillespie and Berg, 1995; Pare and Suter, 2000) (D. E. Gillespie, PhD thesis, University of Washington, Seattle WA, 1996). Each of these molecules is largely retained in mutant nurse cells, accumulating in the vicinity of ring canals. The specificity of the localization defect suggests an underlying defect in transport through ring canals, which is a discrete, microtubule-independent step in nurse cell-to-oocyte transport (Theurkauf and Hazelrigg, 1998). This defect together with the nuage assembly defect of *spn-E* are consistent with the hypothesis that nuage may function in formation of RNPs required for correct mRNA localization in the *Drosophila* germline.

## Nuage and the miRNA pathway

A tantalizing possibility for nuage is that it may function at some point in miRNA and/or RNP particle assembly processes (Fig. 7). This hypothesis is supported by the mislocalization of Dicer and AGO2 to perinuclear regions of germline cells in the *maelstrom* mutant (Fig. 6B,B′,F,F′). Such discrete redistributions of proteins could reflect an accretion of intermediate in a normally *maelstrom*-mediated step. A connection is further insinuated by the requirement of both
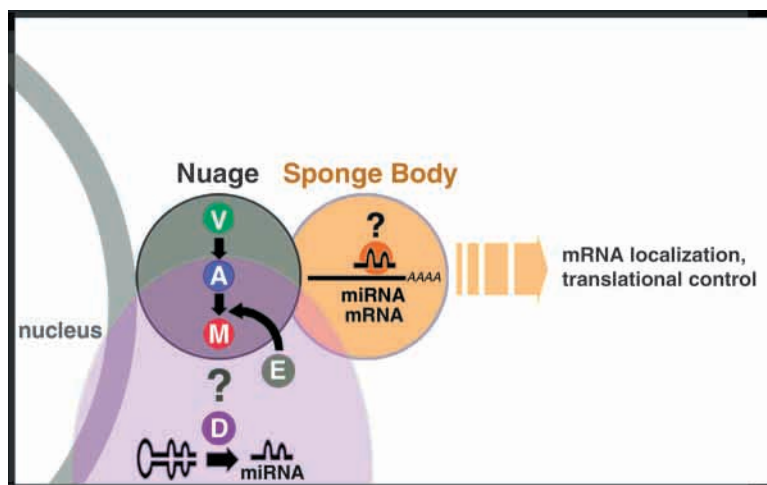


Fig. 7. Nuage is implicated in the miRNA pathway. Vasa (V, green circle), a pivotal organizer of nuage (large gray circle) is required to recruit (black arrows) both Aubergine (A, blue circle) and Maelstrom (M, red circle) into particles (large gray circle). In nurse cells, sponge bodies (orange circle) are ultrastructurally interfaced with nuage particles, and contain RNPs required for proper translation and localization of mRNAs. MicroRNA maturation or assembly is suggested (pink shading) in the nuage/sponge body interface by the requirement for both Spn-E (gray circle) and Aubergine for recruitment of Maelstrom to nuage, as well as the ectopic localization of Dicer (pink circle) and Argonaute2 (not shown) to the perinuclear zone. Either microRNA processing (bottom) or recruitment of miRNA-directed specificity factors (small darker orange circle in sponge body) into RNPs could occur in the vicinity of nuage.

*aubergine* and *spn-E* in the assembly of nuage particles. In mutants of both genes, Maelstrom is dissociated from perinuclear Vasa particles in all germline cells (Fig. 5). The dissociation is interesting because both *aubergine* and *spn-E* have a common requirement in double-stranded RNA-mediated gene silencing in both *Drosophila* spermatogenesis and late oogenesis (Aravin et al., 2001; Kennerdell et al., 2002; Stapleton et al., 2001). In the testis, *spn-E* is required for silencing of retrotransposons (e.g. *copia*) and both *spn-E* and *aubergine* are required for silencing of genomic tandem repeats (e.g. *Stellate*). Mutants in either gene relieve RNAi-mediated suppression of respective target genes. The fact that each is also required for proper mRNA localization or translation raises the possibility that these proteins could function as common components in the allied miRNA pathway. RNAi and miRNA are mechanistically related: each pathway processes a dsRNA substrate, using a common processing factor, Dicer, to generate the respective small RNA effectors of each pathway. In *C. elegans*, 24 RDE1/AGO1 homologs have been identified (Grishok et al., 2001). The studied homologs are required in either RNAi or miRNA processing, but in not both pathways (Grishok et al., 2001; Hutvagner et al., 2001). The *Drosophila* genome encodes only five RDE1/AGO1 homologs: Piwi, Aubergine, AGO1, AGO2 and AGO3 (Williams and Rubin, 2002), which may necessitate dual usage in both miRNA and RNAi pathways.

Recent reports suggest that hundreds of miRNAs exist in metazoans (Grosshans and Slack, 2002; Lagos-Quintana et al., 2002). These miRNAs are thought to be modulators of target mRNA translation, although additional functions have been hypothesized (reviewed by Ambros, 2001). Indeed, miRNAs might represent a common means of post-transcriptional regulation of gene expression in both vertebrates and invertebrates. It is known for many cell types, including neurons and oocytes, that translation and localization of mRNA is controlled by RNA-binding proteins. However, the specificity of this process is poorly understood. In some cases, a sequence-specific RNA binding protein is found to be involved (Crittenden et al., 2002; Wharton et al., 1998); in other cases a combinatorial action of many hnRNPs is proposed (Dreyfuss et al., 2002). The abundance of miRNAs raises the possibility that these small RNAs could generate the missing specificity: miRNAs bound to target mRNAs are predicted to form a loop structure that could be recognized by multiple RNA-binding proteins, allowing for assembly of a full RNP particle. In the context of oogenesis, miRNAs could provide an added level of control by conferring specificity through nucleation or regulated assembly of translational (and possibly localization) control factors on RNAs (see Fig. 7). The data presented in this paper suggest that nuage function may be involved in the miRNA or RNAi pathways. Future experiments should be aimed at determining the role of nuage components in miRNA precursor maturation or in assembly of mature miRNAs with their target mRNAs.

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