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Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during *Drosophila* oogenesis

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SUMMARY

Embryonic patterning in *Drosophila* is regulated by maternal factors. Many such factors become localized as mRNAs within the oocyte during oogenesis and are translated in a spatio-temporally regulated manner. These processes are controlled by *trans*-acting proteins, which bind to the target RNAs to form a ribonucleoprotein (RNP) complex. We report that a DEAD-box protein, Me31B, forms a cytoplasmic RNP complex with oocyte-localizing RNAs and Exuperantia, a protein involved in RNA localization. During early oogenesis, loss of Me31B causes premature translation of oocyte-localizing RNAs within nurse cells, without affecting their transport to the oocyte. These results suggest that Me31B mediates translational silencing of RNAs during their transport to the oocyte. Our data provide evidence that RNA transport and translational control are linked through the assembly of RNP complex.

Key words: *Drosophila*, Maternal RNA, RNA transport, RNA masking, RNP complex, Translational control

INTRODUCTION

Cell polarity is often generated by asymmetric distribution of mRNAs (St Johnston, 1995; Bashirullah et al., 1998). Such mRNAs act as the localized source for translation, and allow specific proteins to be synthesized in the subcellular region where they are required. As these mRNAs are synthesized in the nucleus and travel through the cytoplasm to their final destinations, their translation must be silenced during their localization.

Drosophila oogenesis provides an excellent model system with which to study translational regulation of localized mRNA. The *Drosophila* egg chamber is made up of 16 germline cells surrounded by a monolayer of somatic follicle cells (Spradling, 1993). The 16 cells are generated from a single germline cell by four successive mitotic divisions. As cytokinesis is incomplete during these divisions, the cells remain connected to each other through specialized cytoplasmic bridges called ring canals. The oocyte is determined from one of 16 germline cells, while the remaining 15 cells differentiate into nurse cells. Nurse cells synthesize large amounts of RNAs and proteins, which are transported to the developing oocyte through ring canals.

The mRNAs encoding determinants for embryonic polarity such as *bicoid* (*bcd*), *oskar* (*osk*) and *nanos* (*nos*) are synthesized in nurse cells, transported to the oocyte and localized within the oocyte during oogenesis. Translation of these mRNAs is silenced during their localization, and is activated when and where the protein is required. It has been reported that premature or ectopic translation of these mRNAs causes severe developmental defects (Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Smith et al., 1992; Kim-Ha et al., 1995; Dahanukar and Wharton, 1996; Gavis et al., 1996). For example, translation of *osk* mRNA is repressed during its posterior localization in the oocyte by an RNA-binding protein, Bruno, which binds to a repeated sequence in the 3′ untranslated region (3′UTR), or Bruno response elements (BREs) (Kim-Ha et al., 1995; Webster et al., 1997). When BRE-mutated *osk* mRNA **(***oskBRE*$^{-}$**)** is expressed in oogenesis, the Osk protein is produced prematurely in oocytes (Kim-Ha et al., 1995). The premature translation of *oskBRE*$^{-}$ mRNA leads to a maternal-effect lethal phenotype. Similarly, translation of unlocalized *nos* mRNA in embryos is repressed by Smaug protein, which binds to the regulatory element in the 3′UTR. Ectopic translation of unlocalized *nos* mRNA causes a defect in anteroposterior axis formation (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996, 1999; Dahanukar et al., 1999). Thus, localization-coupled translational control of these mRNAs is an essential mechanism for embryonic patterning.

Although the translational repression of unlocalized mRNAs in oocytes and embryos has been well analyzed, little is known as to how these mRNAs are translationally silenced during their transport from nurse cells to the oocyte. In egg chambers that express *oskBRE*$^{-}$ mRNA, Osk protein is present throughout the oocyte, yet is undetectable in nurse cells (Kim-Ha et al., 1995). Furthermore, additional introduction of a mutation in the *osk* 5′UTR causes a more

pronounced premature translation of a reporter gene within nurse cells than BRE$^-$ alone (Gunkel et al., 1998**)**. This suggests that BRE-mediated translational repression is not the only mechanism to prevent premature translation of *osk* mRNA.

Several elegant studies in diverse organisms have shown that localized RNAs are organized into particles, which move through the cytoplasm in a microtubule-dependent manner (Ainger et al., 1993; Ferrandon et al., 1994; Bertrand et al., 1998). This suggests that localization of RNAs are controlled by *trans*-acting proteins, which bind these RNAs to form a ribonucleoprotein (RNP) complex. One of the candidates for such *trans*-acting proteins in *Drosophila* oogenesis is Exuperantia (Exu). Green fluorescent protein (GFP)-tagged Exu protein forms cytoplasmic particles, which move to the oocyte in a microtubule-dependent manner (Wang and Hazelrigg, 1994; Theurkauf and Hazelrigg, 1998). Genetic studies have shown that Exu is essential for the initial step of *bcd* mRNA localization to the anterior of the oocyte (St Johnston et al., 1989). A recent study has further shown that Exu is also required for proper localization of *osk* mRNA at the posterior pole of the oocyte (Wilhelm et al., 2000). Exu is highly concentrated in distinctive RNA-rich regions in the cytoplasm of nurse cells and oocytes, called the sponge bodies (Wilsch-Bräuninger et al., 1997). These observations have led to the proposal that Exu-containing particles are specialized RNP complexes that are involved in the transport of maternal RNAs to the oocyte.

Similarly, *trans*-acting proteins for translational control of maternal mRNAs may be assembled into a cytoplasmic RNP complex. This complex could be visualized, like Exu, as particles using light microscopy. To isolate such proteins, we conducted a visual screen with a GFP-cDNA expression library. Here, we report that a putative RNA-binding protein, Maternal expression at 31B (Me31B; De Valoir, 1991), forms cytoplasmic particles during oogenesis. Me31B also forms a complex with Exu in an RNA-dependent manner, and is concentrated in the sponge bodies. Furthermore, we show that several oocyte-localizing RNAs are colocalized with Me31B during their transport from nurse cells to the oocyte. In early egg chambers that lack Me31B, at least two mRNAs in the particles, *osk* and *Bicaudal-D* (*BicD*) mRNAs, are prematurely translated in nurse cells, though the transport of these RNAs to the oocyte is Me31B independent. Our data suggest that Me31B plays an essential role in translational silencing of oocyte-localizing mRNAs during their transport to the oocyte.

MATERIALS AND METHODS

Drosophila stocks

Fly stocks were raised at 25°C on standard cornmeal and agar medium. *P[w$^+$ gfp::exu]* line (Wang and Hazelrigg, 1994) was a gift from Dr P. Lasko (McGill University, Montreal, Canada). *P[w^{+mc}=lacW]l(2)k06607^{k06607}/CyO* (Spradling et al., 1999), *hsFLP; CyO/Sco*, *FRT40A; ry*, and *ovo^{D-2L} FRT40A/CyO* lines were obtained from Bloomington stock center.

Construction of GFP-cDNA fusion library

A germline-specific *vas* promoter is located within ~2 kb upstream of the *vas* transcription start site (A. N. and P. Lasko, unpublished). This genomic region was isolated from λ phage clone v3.04 (Lasko and Ashburner 1988). The *P[w$^+$ P$_{vas}$-gfp]* plasmid was constructed in pCaSpeR2 (Thummel and Pirrotta, 1992) by multiple subcloning steps, and contains the *vas* promoter, 5′ UTR and first 16 amino acids coding region of *vas*, enhanced green fluorescent protein (*egfp*, Clontech), *vas* 3′ UTR and the subsequent ~550 bp of the 3′ flanking region. This vector has unique *Xho*I and *Not*I sites between the 3′ end of *egfp* and the termination codon. Detailed construction procedure will be provided upon request.

Ovaries from *y w* females were used as the RNA source for library construction. Poly(A)$^+$ RNA (5 μg) was used to generate cDNA with Superscript Plasmid System kit (Gibco BRL). This procedure yielded cDNA fragments with a *Sal*I adapter at the 5′ end and oligo-dT/dA-*Not*I at the 3′ side. *Not*I digested cDNAs were ligated into *Xho*I/*Not*I-digested *P[w$^+$ P$_{vas}$-gfp]* plasmid. An unamplified cDNA library was generated from 2.2×10^6 independent clones. Of 168 random clones tested, all clones contained an insert and the average insert size was estimated to be 2.0 kb with a range of 0.1-6.0 kb.

P element-mediated germline transformation was performed according to a standard method (Spradling and Rubin,1982), using *y w* embryos as recipients. Ovaries from transformed females were hand-dissected, fixed in 4% paraformaldehyde in PBS (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl) and mounted in 50% glycerol in PBS. Spatial distributions of GFP signals in ovaries were observed under a laser confocal microscope (Leica TCS-NT) with an Ar/Kr laser unit. The cDNA insert of the fusion gene was amplified by PCR from genomic DNA of the transformant, and the nucleotide sequence was determined using PCR product as template.

Antibody generation

The N-terminal half (Met2-Gln294) of Me31B-coding region was subcloned into pProExHTa (Gibco BRL) to produce a His_6-tagged Me31B protein. The coding region of Me31B cDNA was amplified by PCR with primers 5′-CCCCCATGGATATGACTGAAAAGTTA-AATTCTGG-3′ and 5′-CCCCTCGAGTTATTTGCTAACGTTGC-CCTCC-3′ using an EST clone LD27473 (Berkeley *Drosophila* Genome Project) as a template. Amplified cDNA fragment was then digested with *Nco*I and *Pst*I, and ~0.9 kb fragment was ligated into pProExHTa. Histidine tagged Me31B protein was expressed in *Escherichia coli* BL21 cells by IPTG induction. After centrifugation at 5000 ***g*** for 10 minutes, *E. coli* cells were treated with Bugbuster regent (Novagen) containing 100 μg/ml of lysozyme and 10 μg/ml of DNase I. Me31B protein was recovered as an insoluble form, solubilized with 6M guanidine-HCl in buffer A (100 mM sodium phosphate pH 8.0, 10 mM Tris-HCl, 0.5 M NaCl) and purified with Ni-NTA agarose resin (Qiagen). Purified protein was loaded on 10% SDS-polyacrylamide gels and protein bands were visualized with 0.2 M KCl on ice. Me31B band was cut and dialyzed extensively against water. About 1 mg of the purified protein was emulsified with Titer Max Gold adjuvant (CytRx) and injected into two rabbits. Boosting was carried out with the same amount of protein for 4 weeks intervals. Being boosted twice, antisera from two rabbits both recognized a single ~50 kDa band on immunoblot of ovary extract. Antisera were further affinity purified with the protein immobilized on PVDF membrane (Millipore). The affinity-purified antibodies from antisera from two rabbits showed identical staining pattern in wild type ovaries.

Fluorescence in situ hybridization

Whole-mount in situ hybridization to ovaries was performed as described (Kobayashi et al., 1999) with several modifications. Hybridized signals were detected with mouse anti-digoxygenin monoclonal antibody (Roche), followed by Alexa568 conjugated anti-mouse IgG (Molecular Probes). The ovaries were double stained with an affinity-purified rabbit anti-Me31B antibody and Alexa488-conjugated anti-rabbit IgG. The ovaries were mounted in Vectashield

(Vector Laboratories) and observed under a laser confocal microscope (Leica TCS SP2).

Immunostaining

Immunostaining of ovaries were carried out as described (Kobayashi et al., 1999). Primary antibodies and antisera used were mouse anti-BicD 1B11 (Suter and Steward, 1991), rabbit anti-Exu (Wilsch-Bräuninger et al., 1997), rabbit anti-GFP (Clontech), mouse anti-GFP 3E6 (Wako Pure Chemicals, Osaka, Japan), rabbit anti-Me31B, rabbit anti-Osk (Kim-Ha et al., 1995), and rabbit anti-Yps (Wilheim et al., 2000). Secondary antibodies used were Alexa488-conjugated anti-rabbit IgG, Alexa568-conjugated anti-rabbit IgG, Alexa568-conjugated anti-mouse IgG (Molecular Probes), FITC-conjugated anti-mouse IgG (Jackson Immuno Research), biotinylated anti-rabbit IgG (Vector Laboratory) and 15 nm gold-conjugated anti-rabbit IgG (Biocell). To visualize cell boundaries and nuclei, ovaries were incubated with Texas Red-X phalloidin and Hoechst 33258 (Molecular Probes), respectively.

Immunoprecipitation of Me31B and Yps

Immunoprecipitation was performed as described by Wilheim et al. (Wilheim et al., 2000) with slight modifications. Affinity-purified antibodies (5 μg) or normal rabbit IgG (Santa Cruz Biotechnology) were crosslinked to ~50 μl of protein G sepharose 4B Fast Flow beads (Sigma) with dimethyl pimelimidate at a concentration of 25 mM in 0.2 M triethanolamine pH 8.0. After the beads were washed in 0.2 M ethanolamine pH 8.0, they were pre-eluted with 0.2 M glycine-HCl pH 2.7. The beads were then equilibrated with PBS.

Ovaries from *y w* females were homogenized on ice in DXB-50 (25 mM Hepes-KOH pH 6.8, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 0.1% Triton X-100) containing complete protease inhibitor cocktail EDTA free (Roche), and centrifuged at 10000 ***g*** for 20 minutes. The supernatant was pre-absorbed twice in ~100 μl of protein G sepharose beads for 1 hour at 4°C. The protein concentration of the extract was then adjusted to 10 mg/ml and 200 μl of the extract was immunoprecipitated with the antibody-coupled beads for overnight at 4°C on a rotator. To evaluate RNase sensitivity for the interaction between Me31B and Yps-Exu complex, 20 μl of 10 mg/ml RNase A was added during this incubation period. The beads were washed with DXB-50. Bound proteins were then eluted with 0.2 M glycine-HCl pH 2.7. The eluate was immediately neutralized with 1 M Tris-HCl pH 8.0, and residual beads were removed by passing through a spin column (Samprep C02-LH; Millipore). The samples were then precipitated with trichloroacetic acid. The precipitates were suspended in 40 μl of sample buffer and boiled for 5 minutes. 10 μl of denatured samples were run on a 10% SDS-polyacrylamide gel and transferred onto PVDF membrane (Millipore). The membrane was then probed with the primary antibodies. Proteins were detected by an ECL system (Amersham-Pharmacia Biotech).

Generation of *me31B*-null alleles

l(2)k06607 is a viable *placw* insertion at 31B (Spradling, 1999) but the chromosome carried separable lethal mutation(s). Homozygous viable and fertile lines were generated by meiotic recombination between chromosomes with and without *k06607* insertion. Excision lines were generated by crossing virgin *k06607* females with males possessing *Δ2–3* transposase. From 360 independent excision events, 351 white-eyed balanced stocks were established. These were checked by PCR to determine whether they carried a deletion within *me31B* locus. Breakpoints of the three deletion lines were determined by a direct sequencing of the PCR products. To generate germline clones, *FRT40A* was introduced on $me31B^{\Delta 1}$ and $me31B^{\Delta 2}$ chromosomes. They were crossed with *hsFLP*; *gfp-vas FRT40A* or *hsFLP*; ovo^{D} *FRT40A/CyO* males. To induce mitotic recombination in proliferating germ cells, progeny were heat shocked twice at 37°C for 2 hours during early pupal stage. Germline clones in *hsFLP/w*; $me31B^{\Delta}$ *FRT40A/vas-gfp FRT40A* females were identified by loss of anti-Me31B or anti-GFP staining. The two *me31B* mutations, $me31B^{\Delta 1}$ and $me31B^{\Delta 2}$, showed identical phenotypes.

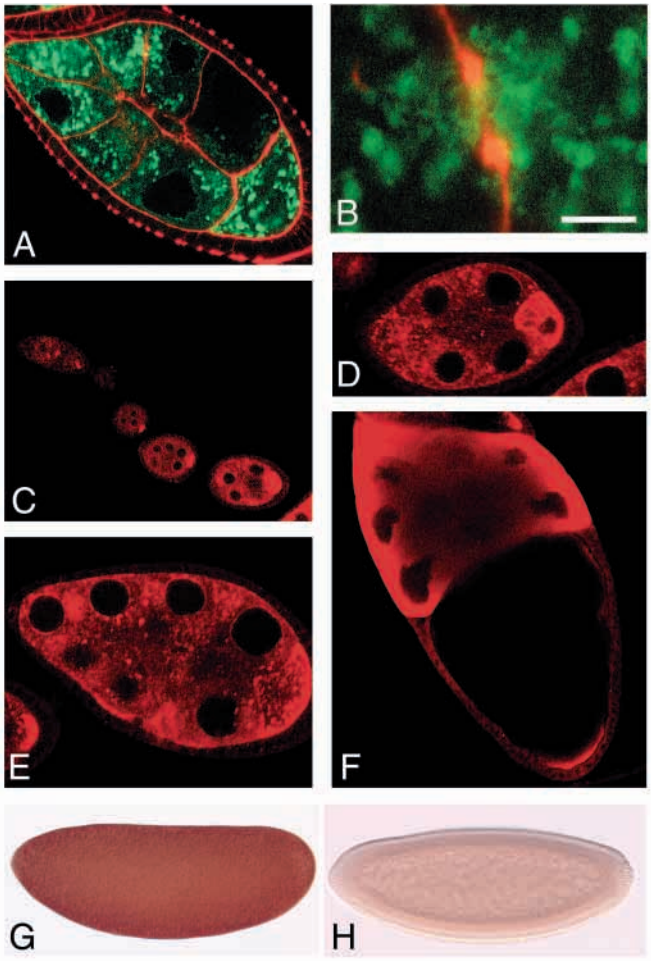

Fig. 1. Distribution of Me31B protein. (A) A confocal micrograph of stage 6 egg chamber expressing GFP-Me31B (green). The egg chamber is stained with phalloidin (red). GFP-Me31B signals show cytoplasmic particles in both nurse cells and the oocyte. (B) Higher magnification to highlight a ring canal. GFP-Me31B particles appear to pass through ring canal. (C-F) Distribution of endogenous Me31B. Wild-type ovaries were immunostained with an affinity-purified anti-Me31B antibody. Endogenous Me31B also distributed in a granular pattern in the cytoplasm of nurse cells and oocytes. Note that the antibody failed to permeate well in nurse cells in stage 10 egg chamber (F). (G) Cleavage embryos immunostained with anti-Me31B antibody. Me31B is uniformly distributed. By the cellular blastoderm stage (H), Me31B signal becomes undetectable. Scale bar in B: 5 μm.

Transgenic constructs for a rescue of *me31B* mutants

A ~13.7 kb genomic DNA containing an entire *me31B* locus was isolated from a *Drosophila* genomic DNA library in λFIXII (a gift from B. Suter, McGill University, Montreal, Canada). A 9 kb *Sal*I fragment, which contains the entire *me31B* mRNA-coding region and ~4.5 kb of upstream promoter region, was subcloned into pCaSpeR4 to yield the *P[w⁺ me9k]* plasmid. This $me31B^{+}$ transgene and two deletion derivatives were used as substrates for P element-mediated transformation. At least three independent transformed lines were checked for their ability to rescue $me31B^{\Delta}$ phenotype.

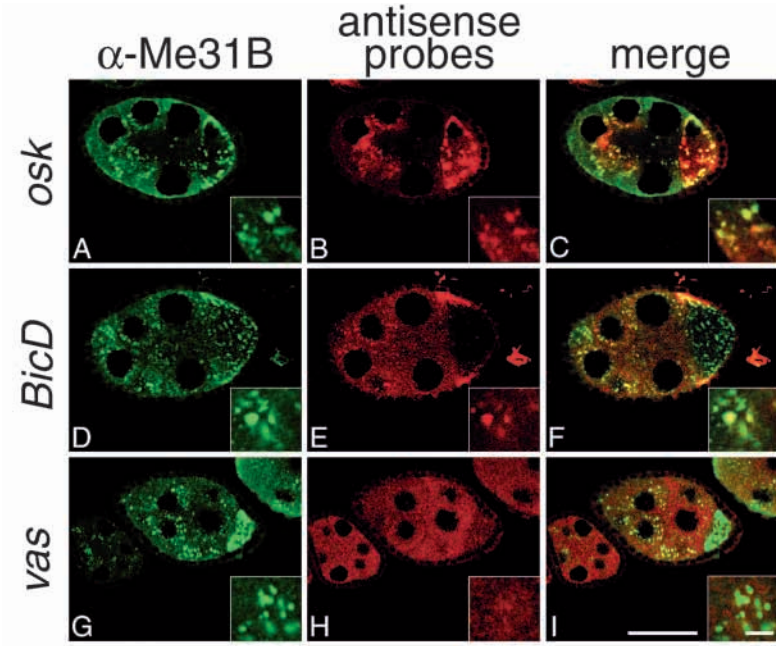

Fig. 2. Colocalization of Me31B with oocyte-localizing RNAs. Wild-type ovaries were double stained for Me31B (green) and *osk* (A-C), *BicD* (D-F) and *vas* (G-I) mRNAs (red). Higher magnification in the cytplasmic region of the nurse cells were also shown (insets). *osk* and *BicD* mRNAs show particulate signals that overlap with Me31B signals (C,F). In contrast, *vas* mRNA signal does not appear to colocalize with Me31B-containing particles (I). Scale bars, 50 μm and 5 μm (inset).

RESULTS

A DEAD-box protein, Me31B, forms cytoplasmic particles in germline cells during oogenesis

We conducted a visual screen with an ovarian GFP-cDNA library, in which fusion genes are expressed in germline cells during oogenesis. We generated transgenic flies with this library and identified proteins that distribute in a granular pattern during oogenesis. Screening ~3000 independent lines, we isolated one, in which GFP signals were detected as cytoplasmic particles during oogenesis (Fig. 1A). The particles were dispersed in the cytoplasm of both nurse cells and oocytes but never detected within nuclei. We frequently observed the particles passing through ring canals (Fig. 1B), suggesting that the particles are assembled in nurse cell cytoplasm and transported to the oocyte.

The cDNA from this line was identified as *me31B* (De Valoir et al., 1991). In the cDNA fusion, almost the entire coding region of *me31B*, which lacks only the first four codons, was fused in frame with that of *gfp*. Me31B, a DEAD-box protein and therefore a putative ATP-dependent RNA helicase, has been isolated as a gene expressed extensively during oogenesis (De Valoir et al., 1991). Me31B is a part of an evolutionally conserved DEAD-box protein group, which includes human RCK/p54 (71% identical), *Xenopus* Xp54 (73%), *Caenorhabditis elegans* C07H6.5 (76%), *Schizosaccharomyces pombe* Ste13 (68%) and *Saccharomyces cerevisiae* Dhh1 (68%). Furthermore, Me31B is phylogenetically close to two evolutionally conserved proteins, eIF4A and Dbp5/Rat8p but far from Vasa, which functions in germline development (data not shown).

To examine distribution of the endogenous Me31B, we generated antibodies that specifically recognized Me31B. As shown in Fig. 1C-F, the distribution pattern of endogenous Me31B was identical to that of GFP-Me31B. No detectable signal in somatic follicle cells was observed at any stages of oogenesis. Me31B was first detected at a low level in germarium region 2B, where the signal was concentrated in the pro-oocytes (Fig. 1C). The signal remained concentrated in the oocyte until mid-oogenesis (Fig. 1D,E). In early egg chambers, a low level Me31B signal was detected in nurse cell cytoplasm. In both nurse cells and oocytes, the signal appeared to be granular. Me31B signals in nurse cell cytoplasm became more evident from stage 5-6, when Me31B expression was drastically increased (Fig. 1D,E). In addition, Me31B was frequently enriched around nurse cell nuclei. Later, Me31B accumulates at the posterior pole of stage 10 oocytes (Fig. 1F). However, this posterior accumulation was transient, as revealed by uniform distribution of the signal in cleavage embryos (Fig. 1G). By cellular blastoderm stage, Me31B became undetectable in the entire embryonic region (Fig. 1H). No zygotic expression of Me31B was detected during embryogenesis (data not shown).

Me31B colocalizes with oocyte-localizing RNAs

Because Me31B is probably an RNA-binding protein that is transported to the oocyte, we next asked whether Me31B formed a complex with oocyte-localizing RNAs. We first examined colocalization of *osk* mRNA with Me31B. As has been reported previously (Ephrussi et al., 1991; Kim-Ha et al., 1991), *osk* mRNA starts to accumulate in oocytes from germarium region 2B, with the concentration of *osk* increasing over time. Posterior accumulation of *osk* mRNA in the oocyte began from stage 8 onwards. By fluorescent in situ hybridization, *osk* mRNA exhibited particulate signals in the cytoplasm of both nurse cells and oocytes, and was frequently concentrated around nurse cell nuclei (Fig. 2B). This distribution pattern of *osk* mRNA was essentially identical to that of Me31B **(**Fig. 2C**)**, with colocalization present until *osk* mRNA localized to the posterior pole of stage 10 oocytes (data not shown).

We also examined colocalization of Me31B with other RNAs. We double stained ovaries for Me31B and *BicD* mRNA. In early egg chambers, *BicD* mRNA also produced particulate signals, and appeared to localize in Me31B-containing particles (data not shown). This colocalization became apparent from stage 5-6, when *BicD* mRNA expression was elevated (Fig. 2D-F). The oocyte-localizing RNAs examined (*bcd*, *nos*, *oo18 RNA-binding* (*orb*), *Polar granule component* (*Pgc*) and *germ cell-less* (*gcl*)) all produced particulate signals in the cytoplasm of both nurse cells and oocytes, and colocalized with Me31B (data not shown). In contrast, *vasa* (*vas*) mRNA, which is not specifically transported to the oocyte (Hay et al., 1988; Lasko and Ashburner, 1988), did not appear to be colocalized with Me31B (Fig. 2G-I). These results

indicate that Me31B forms cytoplasmic particles that contain oocyte-localizing RNAs.

Me31B is colocalized with Exu and Yps

We next examined whether Me31B and Exu were colocalized, because distribution of GFP-Exu fusion protein was very similar to that of Me31B (Wang and Hazelrigg, 1994). GFP-Exu was concentrated to the oocytes from as early as stage 1, and transiently accumulated at the posterior pole of stage 10 oocytes (Wang and Hazelrigg, 1994; data not shown). From stage 5-6 onwards, particulate GFP-Exu signals in the cytoplasm became apparent (Fig. 3A; Wang and Hazelrigg, 1994). To compare the distribution between Exu and Me31B directly, we immunostained ovaries expressing GFP-Exu with an anti-Me31B antibody. We found that almost all GFP-Exu particles contained Me31B (Fig. 3A-C). Colocalization between Exu and Me31B throughout oogenesis was further confirmed by immunostaining of ovaries expressing GFP-Me31B with an anti-Exu antiserum (Fig. 3D-F).

We next tested the colocalization of Me31B with Ypsilon Schachtel (Yps), which has been shown to be an Exu-binding protein in oogenesis (Wilhelm et al., 2000). In nurse cells and oocytes, Yps signals were observed in GFP-Me31B-positive cytoplasmic particles (Fig. 3G-I). These results indicate that Me31B, Exu and Yps are all components of a cytoplasmic RNP complex in nurse cells and oocytes.

Exu has been reported to be highly concentrated in a specialized RNA rich regions in the cytoplasm of nurse cells and oocytes, called the sponge bodies (Wilsch-Bräuninger et al., 1997). Sponge bodies have been proposed to be involved in the localization of RNAs in the oocytes. We examined whether Me31B was also concentrated in the sponge bodies using immunoelectron microscopy (Fig. 3J). Me31B signals were detected in distinctive cytoplasmic regions, consisting of string-like materials embedded in an electron dense mass. The regions were in close proximity to mitochondria but not surrounded by membrane. All of these features completely satisfied the reported criteria of the sponge body (Wilsch-Bräuninger et al., 1997). Therefore, we conclude that Me31B is highly enriched in the sponge bodies, and that the sponge body is the region where cytoplasmic RNPs for oocyte localization are concentrated.

Me31B forms a complex with Exu and Yps in an RNase-sensitive manner

To characterize Me31B-containing particles in detail, the particles were immunoprecipitated with an anti-Me31B antibody from extracts of wild-type ovaries. The resulting immunoprecipitates were separated by SDS-PAGE and probed with antibodies against Me31B, Yps and Exu. We also tested

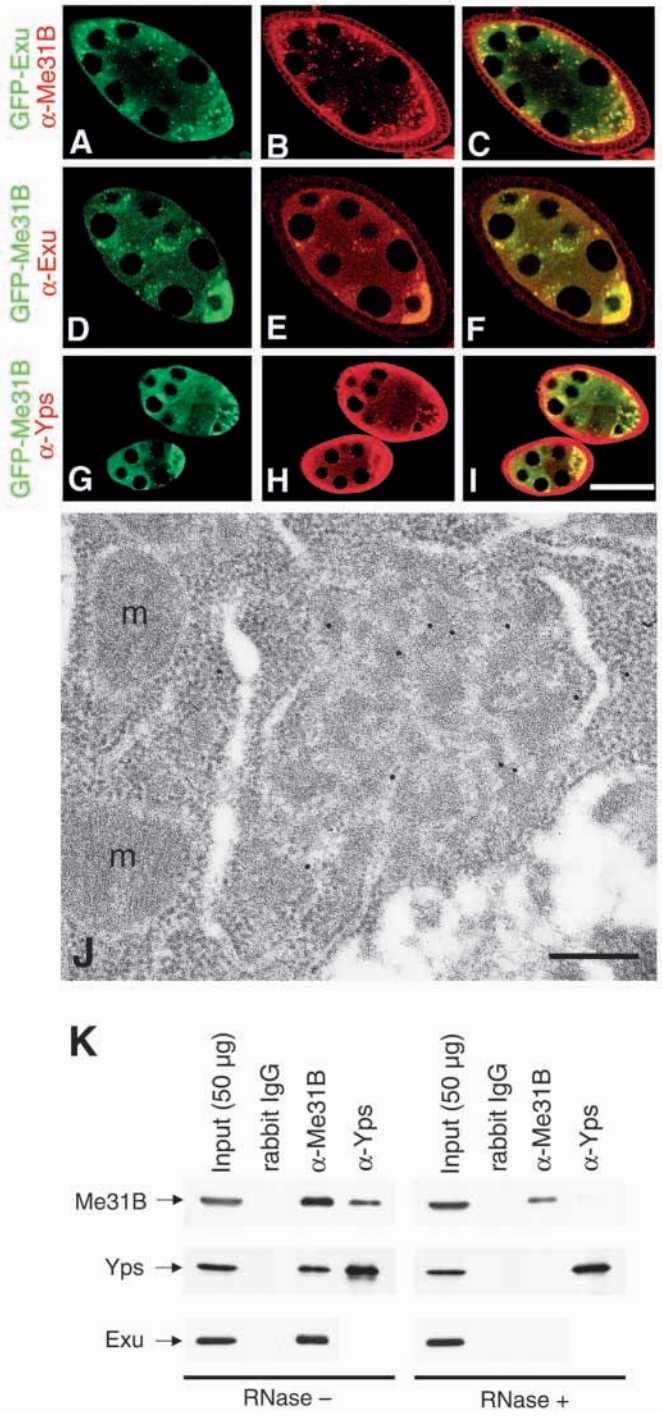

Fig. 3. Me31B forms a complex with Exu and Yps, and is concentrated in the sponge body. (A-C) A stage 6 egg chamber expressing GFP-Exu (green) was stained for Me31B (red). Me31B colocalizes with Exu. (D-F) A stage 6 egg chamber expressing GFP-Me31B (green) was stained for Exu (red). Exu is colocalized with GFP-Me31B. (G-I) Stage 4 and stage 5 egg chambers expressing GFP-Me31B were stained for Yps (red). Yps is colocalized with GFP-Me31B. Note that Yps is expressed extensively in follicle cells that surround germline cells. Hence, the antibody failed to permeate well into the interior of germline cells. (J) An immunoelectron-micrograph of a section for Me31B. The section shown is the cytoplasm of a nurse cell in a stage 9 egg chamber. Me31B signals are enriched in a distinctive cytoplasmic region, which is not surrounded by membrane. We detected 390 signals for Me31B in 356 μm^2 areas in sections of nurse cell cytoplasm. Among these, 331 signals (84.9%) were in these distinctive regions, which occupied only ~57 μm^2 (~16%) in the sections. m, mitochondrion. (K) Interaction between Me31B and Exu-Yps complex. Me31B and Yps were immunoprecipitated from ovary extracts in the absence (left) or presence (right) of RNase. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, and probed with antibodies against Me31B, Yps and Exu. In the presence of RNase A, Me31B-Yps and Me31B-Exu coimmunoprecipitation is diminished (right). Scale bar: 50 μm in A-I; 200 nm in J.

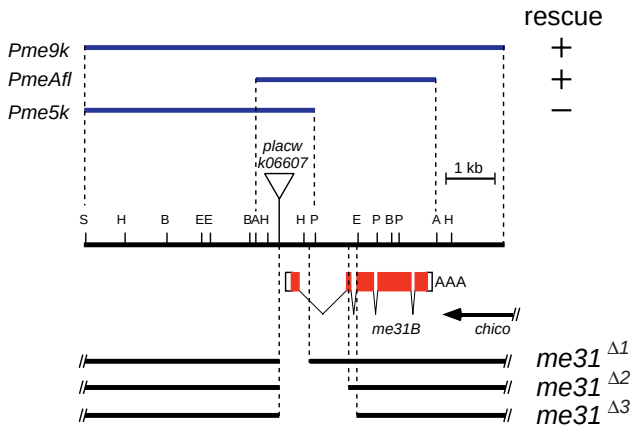

Fig. 4. Genomic organization of *me31B* locus. A P element insertion, *k06607*, is located ~100 bp upstream of *me31B*. By mobilizing the P element, three partial deletions within *me31B* locus (*me31B*$^{\Delta}$) were isolated. Lethality associated with *me31B*$^{\Delta}$ chromosomes was rescued by the two transgenes (*pme9k* and *PmeAfl*) but not by *Pme5k*. A, *Afl*II; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I.

whether Me31B was coimmunoprecipitated with Yps. As shown in Fig. 3K, Yps and Exu were coimmunoprecipitated with Me31B, and Me31B was coimmunoprecipitated with Yps. Control experiments showed that none of these proteins was coimmunoprecipitated with a normal rabbit IgG.

It has been reported that Exu binds directly to Yps, and that these proteins coimmunoprecipitate several unknown proteins in an RNase-sensitive manner (Wilhelm et al., 2000). We therefore examined the effect of RNase treatment on the nature of the Me31B-Exu/Yps complex. In the presence of RNase, Me31B-Yps and Me31B-Exu, coimmunoprecipitation was diminished (Fig. 3K). These results indicate that Me31B forms a complex with Yps and Exu via RNA.

me31B is an essential gene

To investigate the function of Me31B during oogenesis, we identified *me31B* mutations. We found that a P element insertion, *k06607*, is located ~100 bp upstream of the 5′ end of *me31B* (Fig. 4; Spradling et al., 1999). By mobilizing the *k06607* insertion, we isolated three partial deletions of *me31B* (*me31B*$^{\Delta 1}$, *me31B* $^{\Delta 2}$ and *me31B* $^{\Delta 3}$). All the deletions started from the original P element insertion point, and their other breakpoints were within the *me31B*-coding region (Fig. 4).

The three *me31B*$^{\Delta}$ lines were all recessive lethal. They did not complement each other, and were also lethal in *trans* to a deficiency, *Df(2L)J2*, which deletes the entire *me31B* locus (De Valoir et al., 1991; data not shown). Homozygous *me31B*$^{\Delta}$ individuals died during the second- or third-instar larval stages without expressing any discernible morphological defects. The lethality associated with the *me31B*$^{\Delta}$ chromosome was rescued by introducing a genomic DNA (*Pme9k*), which contains the entire *me31B* locus and ~4.5 kb of the upstream region (Fig. 4). A 3.7 kb *Afl*II/*Afl*II fragment (*PmeAfl*), including only ~700 bp upstream and 12 bp downstream of the *me31B* mRNA-coding region, also complemented the *me31B* mutations. In contrast, *Pme5k*, which deletes most of *me31B* locus from *Pme9k*, failed to rescue the lethality. From these results, we conclude that the three *me31B*$^{\Delta}$ mutations affect only *me31B* function, and that *me31B* is a vital gene.

Me31B is essential for oogenesis

To analyze the *me31B* function in oogenesis, we used the FLP-DFS mitotic recombination system (Chou and Perrimon, 1996) to produce *me31B*$^{-}$ germline clones. None of the egg chambers homozygous for *me31B* mutations completed oogenesis (Fig. 5). Most *me31B*$^{-}$ egg chambers degenerated during mid-oogenesis (Fig. 5D-F). In these egg chambers, cell membranes of germline cells were collapsed, and ring canals and nurse cell nuclei were concentrated to form large aggregates within egg chambers. Nurse cell nuclei in these egg chambers were then fragmented into small pieces. Occasionally, we found egg chambers that progressed as far as stage 10 (Fig. 5G,H). In these egg chambers, oocytes did not grow normally, and the nurse cell nuclei were displaced posteriorly relative to the

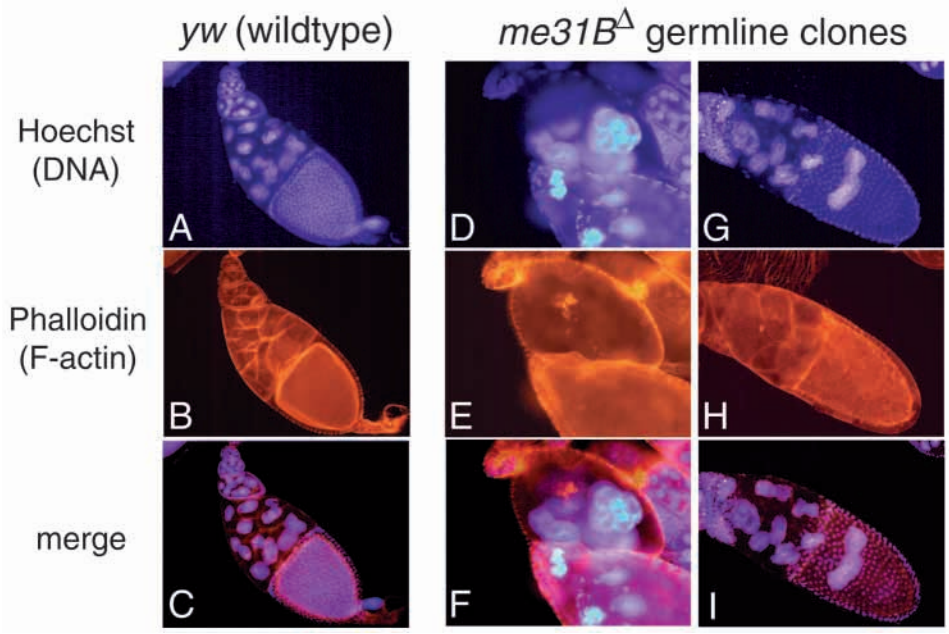

Fig. 5. Me31B is essential for oogenesis. Wild-type (A-C) and *me31B*$^{-}$ (D-I) ovaries were stained with Hoechst (A,D,G) and phalloidin (B,E,H). (D-F) A typical *me31B*$^{-}$ egg chamber, in which cell membranes of germline cells were collapsed. Ring canals and nurse cell nuclei in these egg chambers form aggregates. (G-H) A stage 10 *me31B*$^{-}$ egg chamber showing nurse cell nuclei invading the region normally occupied by the oocyte.

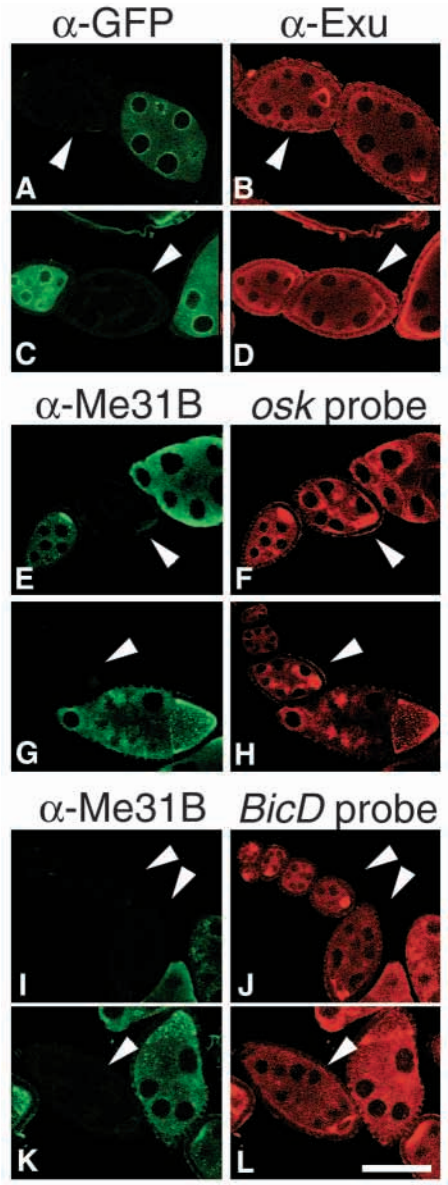

Fig. 6. Me31B is dispensable for the transport of Exu, and *osk* and *BicD* mRNAs to the oocyte. Arrowheads indicate *me31B*$^{-}$ egg chambers, which were identified by loss of Vas-GFP (A-D) or Me31B (E-L) signals. In early egg chambers, Exu (B,D), and *osk* (F,H) and *BicD* (J,L) mRNAs are all transported to the oocyte even in the absence of Me31B (arrowheads). Particulate signals in nurse cell cytoplasm were detectable in these *me31B*$^{-}$ egg chambers. Scale bar: 50 μm.

anterior border of columnar follicle cells, which normally surround the oocyte at stage 10 (Fig. 5A-C).

Me31B is dispensable for the transport of the associated molecules to the oocytes

The complicated and redundant phenotypes we observed in *me31B*$^{-}$ egg chambers in mid-oogenesis are unlikely to be the primary effect of loss of *me31B* function. We examined earlier phenotypes of *me31B*$^{-}$ egg chambers using a FLP/FRT system to generate homozygous germline clones that are marked by the loss of Vas-GFP fusion protein (see Materials and

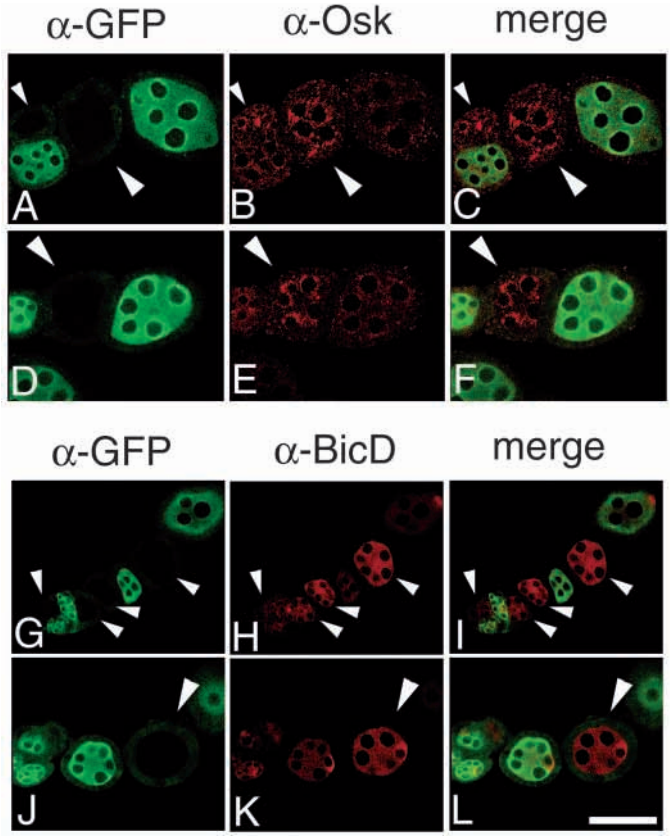

Fig. 7. Osk and BicD signals in nurse cell cytoplasm are increased in *me31B*$^{-}$ egg chambers. Arrowheads point to *me31B*$^{-}$ egg chambers, which are identified by loss of Vas-GFP signal (green in A,D,G,J). In these *me31B*$^{-}$ egg chambers, Osk (red in B,C,E,F) and BicD proteins (red in H,I,K,L) in nurse cell cytoplasm are both detected at higher levels than in *me31B*$^{+}$ egg chambers on the same optical sections. Scale bar: 50 μm.

Methods). Based on Hoechst and phalloidin staining, *me31B*$^{-}$ egg chambers were morphologically normal until stage 4-5. From stage 6 onwards, oocytes in *me31B*$^{-}$ egg chambers failed to grow normally. At this stage, these egg chambers began to degenerate. In early *me31B*$^{-}$ egg chambers, Exu signal was concentrated to the oocytes (Fig. 6B, arrowheads). We next examined distribution of *osk* and *BicD* mRNAs in *me31B*$^{-}$ egg chambers. As shown in Fig. 6C-F, both *osk* and *BicD* mRNAs also accumulated in the oocytes of *me31B*$^{-}$ egg chambers until the chambers degenerated (arrowheads). Particulate signals for these RNAs were detectable in nurse cell cytoplasm in these egg chambers. These results indicate that Exu, *osk* and *BicD* mRNAs can be transported to the oocyte even in the absence of Me31B. We conclude that in early egg chambers, Me31B is dispensable for the transport of the molecules that form a complex with Me31B.

Me31B is essential for the translational silencing of *osk* and *BicD* mRNAs during their transport to the oocyte

We next examined whether loss of Me31B affects translation of *osk* and *BicD* mRNAs. We immunostained ovaries with an anti-Osk antiserum (Fig. 7A-F). Although *osk* mRNA is expressed during almost all stages of oogenesis, its translation

is repressed to keep Osk protein level very low during early oogenesis (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). In *me31B*$^{-}$ egg chambers, Osk signal was significantly increased compared with that in the neighboring *me31B*$^{+}$ egg chambers (Fig. 7B,E).

A similar increase of BicD signal in *me31B*$^{-}$ egg chambers was more evident (Fig. 7G-L). In wild-type egg chambers, BicD protein, like *BicD* mRNA, is highly concentrated in the oocytes starting from germarium region 2B (Wharton and Struhl, 1989; Suter and Steward, 1991). In the egg chambers lacking *me31B*, increased BicD signal was detected in nurse cell cytoplasm (Fig. 7H,K). These results suggest that loss of Me31B in germline cells causes derepression of *osk* and *BicD* mRNA translation during their transport to the oocyte.

DISCUSSION

In this report, we have identified Me31B as a new component of cytoplasmic RNP particles that function in *Drosophila* oogenesis. The particles contain several oocyte-localizing RNAs, suggesting that these RNAs are transported to the oocyte with Me31B through the formation of an RNP complex. Consistent with this, the particles also contain Exu, a factor involved in the localization of *bcd* and *osk* mRNAs to the anterior and the posterior pole of the oocyte, respectively. We have further shown that Me31B is required for translational silencing of two mRNAs in the particle: *osk* and *BicD* mRNAs. This is, to our knowledge, the first evidence for a link between transport and translational silencing of localized RNAs through the assembly of an RNP complex.

Me31B forms RNP particles with oocyte-localizing RNAs

We have shown that Me31B is integrated in large cytoplasmic particles containing many oocyte-localizing RNAs and Exu (Figs 2, 3; data not shown). This suggests that each Me31B-containing particle is an aggregate of numerous RNP complexes that are composed of different RNA species. It has been shown that mRNA localization during *Drosophila* oogenesis is microtubule dependent (Pokrywka and Stephenson, 1995) and that GFP-Exu particles move to the oocyte in a microtubule-dependent manner (Theurkauf and Hazelrigg, 1998). Thus, it is likely that the particles act as vehicles for RNA transport from nurse cells to the oocyte. We speculate that RNA transport to the oocyte is operated by a common mechanism, which involves assembly of an RNP complex, integration of each RNP complex into large aggregates and targeting of the aggregates to the oocyte along microtubule networks.

These RNAs usually have *cis*-regulatory elements for oocyte localization in their 3′UTR (Bashirullah et al., 1998). *Trans*-acting protein(s) required for oocyte localization would bind these elements to form a link between localizing RNAs and microtubule networks. Such protein(s) should be able to bind target RNAs in the absence of Me31B, as the transport of *osk* and *BicD* mRNAs to the oocyte is Me31B independent (Fig. 6). It has been shown that Exu is required for proper localization of *bcd* and *osk* mRNAs within oocytes (St Johnston et al., 1989; Wilhelm et al., 2000), and that it is able to bind RNA in vitro (Macdonald et al., 1995). Therefore, Exu might bind to the *cis*-regulatory elements in oocyte-localizing RNAs and promote their transport. Me31B forms a complex with Exu and Yps in an RNA-dependent manner (Fig. 3K), whereas binding between Exu and Yps is direct (Wilhelm et al., 2000). This suggests that Me31B and the Exu-Yps complex bind different regions of the same RNA molecule. These data lead us to speculate that the assembly of a cytoplasmic RNP complex is achieved by binding of functionally different proteins to discrete regions of an oocyte-localizing RNA.

It remains unanswered where Me31B is assembled to form an RNP complex. It has been shown that shuttling hnRNP proteins remain associated with several localized RNAs, even in the cytoplasm (Hoek et al., 1998; Cote et al., 1999; Lall et al., 1999; Norvell et al., 1999), suggesting that cytoplasmic RNPs could be nucleated within the nucleus. Our results show that Me31B is frequently enriched around the nuclear envelope in the nurse cell cytoplasm but is never detected within nuclei (Fig. 1). Thus, we favor the idea that Me31B is assembled into RNP complexes containing oocyte-localizing RNAs in the perinuclear region of the nurse cell cytoplasm. It is notable that in yeast and mammalian cells, Dbp5p/Rat8p, a DEAD-box protein that is phylogenetically closed to Me31B, is also highly enriched in the perinuclear region, and is required for mRNA export from the nucleus (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999). Although the *Drosophila* counterpart of Dbp5 has not yet been characterized, similar distribution of the two related proteins suggests an important link between nuclear RNA export and assembly of cytoplasmic RNP complexes. The perinuclear region would be the site where nuclear RNP complexes are reorganized into a cytoplasmic form, in which additional proteins involved in RNA transport and translational silencing are incorporated.

Me31B is involved in translational silencing of oocyte-localizing RNAs

Significant accumulation of Osk and BicD proteins in nurse cells of *me31B*$^{-}$ egg chambers suggests that Me31B plays a role in translational silencing of these mRNAs during their transport to the oocyte. Alternatively, Me31B may regulate protein turnover such that these protein levels remain low in nurse cells. Both ideas are formally possible, but we favor the former one, because *osk* and *BicD* mRNAs are colocalized with Me31B during their transport from nurse cells to the oocyte. Interestingly, among DEAD-box proteins found in the *Drosophila* genome, Me31B is most similar to eIF4A, which is essential for translation initiation (Merrick and Hershey, 1996). Sequence similarity between the two proteins suggests that Me31B might antagonize the function of eIF4A such that translation of oocyte-localizing RNAs is silenced.

Egg chambers that lack *me31B* degenerate during mid-oogenesis (Fig. 5). As Me31B colocalizes with many oocyte-localizing RNAs other than *osk* and *BicD* mRNAs, Me31B is likely to silence translation of these RNAs. Therefore, *me31B*$^{-}$ phenotypes in mid-oogenesis could be caused simply by translational derepression of many mRNAs in the particles. Similar pleiotropic effects, including germ cell degeneration during mid-oogenesis and invading of nurse cell nuclei into the oocyte, has been observed in mutations for *vas*, which encodes a germline-specific DEAD-box protein implicated in translational activation of several maternal mRNAs

(Markussen et al., 1995; Rongo et al., 1995; Dahanukar and Wharton, 1996; Gavis et al., 1996; Styhler et al., 1998; Tinker et al., 1998; Tomancak et al., 1998; Carrera et al., 2000). We propose that translational control plays a fundamental and widespread role to advance oogenesis. Nevertheless, it is still an unanswered question whether Me31B has another function in oogenesis. Isolation of weak mutations of *me31B* may reveal additional functions, if any, of Me31B.

In *Xenopus* oocytes, Xp54, a protein homologous to Me31B, has been identified as a major integral component of stored RNP particles (Ladomery et al., 1997), in which maternal mRNAs are masked from translation, degradation and polyadenylation/deadenylation (Spirin, 1996). Although the function of Xp54 in the RNP particles remains elusive, RNA masking is thought to involve the structural rearrangement of an RNP complex into a condensed form. Therefore, Xp54 may function to unwind the secondary structures of mRNAs to facilitate binding of RNA-packaging proteins such as FRGY2 (Ladomery et al., 1997; Matsumoto and Wolffe, 1998; Sommerville, 1999). Interestingly, Yps, a component of Me31B-containing particles, shares a conserved sequence motif with FRGY2 (Thieringer et al., 1997; Matsumoto and Wolffe, 1998; Wilhelm et al., 2000). Although the function of Yps in the particles remains to be analyzed, these observations suggest that similar proteins mediate the translational silencing during oogenesis between two diverse organisms.

Me31B is highly concentrated in distinctive cytoplasmic regions, the sponge bodies (Fig. 3J). A similar electron-dense structure known as the mitochondrial cloud has been found in *Xenopus* oocytes (Heasman et al., 1984; Wilsch-Bräuninger et al., 1997). Numerous maternal mRNAs that are involved in the axis determination and germ plasm assembly, co-migrate with this structure to localize the vegetal cortex in growing oocytes (Kloc et al., 1993; Ku and Melton, 1993; Forristall et al., 1995; Kloc and Etkin, 1995; Houston et al., 1998; Hudson and Woodland, 1998; Mowry and Cote, 1999; MacArthur et al., 2000; Pannese et al., 2000). It has recently been shown that some of the RNA components in the mitochondrial cloud are translationally silenced during oogenesis and translated only after the onset of embryogenesis (MacArthur et al., 1999; Houston and King, 2000). These data strongly suggest that the sponge body and the mitochondrial cloud are related cytoplasmic structures in terms of morphology and function, although it has not been determined whether they contain related protein components. Further analysis of composition and the role of the maternal RNP complexes in *Drosophila* and *Xenopus* will uncover the molecular mechanism of how localization and translation of maternal mRNAs is spatio-temporally regulated.

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