The KASH domain protein MSP-300 plays an essential role in nuclear anchoring during *Drosophila* oogenesis

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Abstract

During late stages of *Drosophila* oogenesis, the cytoplasm of nurse cells in the egg chamber is rapidly transferred ("dumped") to oocytes, while the nurse cell nuclei are anchored by a mechanism that involves the actin cytoskeleton. The factors that mediate this interaction between nuclei and actin cytoskeleton are unknown. MSP-300 is the likely *Drosophila* ortholog of the mammalian Syne-1 and -2 and *C. elegans* ANC-1 proteins, contained both actin-binding and nuclear envelope localization domains. By using an antibody against C-terminus of MSP-300, we find that MSP-300 is distributed throughout the cytoplasm and accumulates at the nuclear envelope of nurse cells and the oocyte. A GFP fusion protein containing the C-terminal region of MSP-300 is also sufficient to localize protein on the nuclear envelope in oocytes. To eliminate the maternal gene activity during oogenesis, we generated homozygous germ-line clones of a loss-of-function mutation in *msp-300* in otherwise heterozygous mothers. In the mutant egg chambers that develop from such clones, cytoplasmic dumping of nurse cells is severely disturbed. The nuclei of nurse cells and the oocyte are mislocalized and the usually well-organized actin structures are severely disrupted. These results indicate that maternal MSP-300 plays an important role in actin-dependent nuclear anchorage during cytoplasmic transport.

Introduction

During *Drosophila* oocyte development, the cystoblast undergoes four mitotic divisions to form a cyst of 16 cells within each egg chamber, and these 16 daughter cells remain connected by stable intercellular bridges called ring canals. A cell with four ring canals is selected to be the oocyte, while the rest become polyploid nurse cells. Both the oocyte and the nurse cells are encapsulated by a monolayer of approximately 1000 somatic follicle cells. Nurse cells provide the oocyte with the vast majority of its cytoplasmic components via a process called cytoplasmic transport (Robinson and Cooley, 1997; Spradling, 1993).

Cytoplasmic transport consists of two dynamically distinct but continuous processes: a slow and highly selective transport phase at early stages and a fast "dumping" phase at late stages (Robinson and Cooley, 1997). Dumping initiates at stage 10B, during which the remaining nurse cell cytoplasm is transferred into the oocyte through ring canals in about 30 min (Spradling, 1993). After dumping, nurse cell nuclei and other remnants undergo apoptosis outside of the oocyte. One challenge the nurse cells must handle during the cytoplasmic transport, especially in the dumping phase when maximum force is generated, is to anchor their nuclei in a proper position to prevent them from clogging the narrow ring canals. A commonly accepted model is that during dumping, the actin

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structure changes dynamically to form a halo surrounding the huge nucleus, thus keeping it approximately at the center of the nurse cell and away from the ring canals (Guild et al., 1997; Robinson and Cooley, 1997; Spradling, 1993). However, the specific mechanism, including key factors at nuclear envelope, for anchoring the nurse cell nuclei has not been revealed.

Both microtubule-mediated and actin-mediated mechanisms are involved in nuclear anchorage and migration (Morris, 2000; Starr and Han, 2003; Tran et al., 2001). Mechanisms involved in microtubule systems have been extensively studied. For instance, a connection between the microtubule organization center (MTOC) and the nucleus mediated by the Klarsicht (Klar) protein has been shown to play a critical role in nuclear migration and positioning during *Drosophila* photoreceptor cell development (Fischer et al., 2004; Mosley-Bishop et al., 1999; Patterson et al., 2004).

The analysis of *C. elegans* ANC-1 and UNC-84 proteins has shed some light on an actin-involved nuclear positioning mechanism (Hedgecock and Thomson, 1982; Malone et al., 1999; Starr and Han, 2002). ANC-1, a large protein of more than 8000 amino acids, has two calponin-homology domains in its N-terminus that are capable of binding actin filaments both in vitro and in vivo. The C-terminal region of ANC-1 contains a conserved KASH domain (for <u>K</u>larsicht/<u>A</u>NC-1/<u>S</u>yne-1 <u>H</u>omology), which has been shown to be critical for the nuclear envelope localization for a number of KASH domain containing proteins in different organisms (Apel et al., 2000; Fischer et al., 2004; Mislow et al., 2002; Mosley-Bishop et al., 1999; Padmakumar et al., 2004; Starr and Han, 2002; Zhang et al., 2001; Zhen et al., 2002). Interestingly, genetic experiments have demonstrated that the nuclear envelope localization of the *C. elegans* KASH protein ANC-1 requires the function of the nuclear envelope SUN protein UNC-84 (Starr and Han, 2002). Proteins homologous to ANC-1 in mammals (Syne-1, also known as Myne-1, Nesprin-1, Enaptin, and CPG2; and Syne-2, also known as Myne-2, Nesprin-2, and Nuance) and *Drosophila* (MSP-300) have been previously identified (Apel et al., 2000; Cottrell et al., 2004; Grady et al., 2005; Mislow et al., 2002; Padmakumar et al., 2004; Rosenberg-Hasson et al., 1996; Starr and Han, 2002, 2003; Volk, 1992; Zhang et al., 2001, 2002; Zhen et al., 2002). Like ANC-1, all these proteins are huge and contain both the N-terminal actin-binding calponin domains and the C-terminal KASH domain. The KASH domain containing C-terminal fragments of both Syne-1 and Syne-2 is sufficient for the localization at nuclear envelope (Apel et al., 2000; Grady et al., 2005; Mislow et al., 2002; Padmakumar et al., 2004; Zhang et al., 2001; Zhen et al., 2002). Recent work indicated that Syne-1 functions to tether the synaptic nuclei in muscle cells to neuromuscular junctions (Grady et al., 2005).

A mutation in the *Drosophila msp-300* (<u>muscle-specific protein 300</u>) gene, *msp-300^{sz-75}*, causes larval lethality. Mutant flies are defective in myogenesis and embryonic elongation (Rosenberg-Hasson et al., 1996; Volk, 1992). By using an antibody against middle part of the protein, MSP-300 was shown to express in muscle precursors at muscle–ectoderm and muscle–muscle attachment sites associated with *Drosophila* muscle myofibrillar network (Rosenberg-Hasson et al., 1996).

The *msp-300* gene was the first KASH domain containing gene identified (Volk, 1992). Several differently spliced transcripts were also predicted by the *Drosophila* database. However, the C-terminal end of the gene, which is separated from the rest of *msp-300* by a 45 kb intron, was only identified recently (Starr and Han, 2002; Zhang et al., 2002). The mechanism of MSP-300 function in myogenesis is not clear and its roles in other developmental processes, such as oogenesis, have not yet been investigated.

In this report, we examine the cellular localization and function of MSP-300 during oogenesis. By mosaic analysis of *msp-300^{sz-75}* germ-line clones, we show that MSP-300 is a key factor in regulating the actin cytoskeleton and nuclear anchorage during the cytoplasmic dumping stage of oogenesis.

Materials and methods

Fly stock

The *w^{1118}* strain was used as the wild type control and as the host strain for conventional germ-line transformation experiments. *msp-300^{sz-75}* was provided by T. Volk (Rosenberg-Hasson et al., 1996); *T155-Gal4 UAS-FLP* (*T155UF*) (Duffy et al., 1998) and *[histone-GFP]62A* were gifts from D. Harrison at the University of Kentucky. The balancer chromosome *CyO, hs-Hid* was kindly provided by R. Lehmann at New York University Medical Center. Other lines used in this report were all provided by the *Drosophila* Genetics Center at University of Indiana at Bloomington. *BM615(Dp(2;1);Df(2L)sc19-1/SM6B)* was used to do the phenotype rescue experiment. Both *Dp(2;1)B19* and *Df(2L)sc19-1* cover the whole *msp-300* locus. BM615 males were crossed with *MSP-300^{sz-75}/CyO,arm-GFP* virgin flies, and their progeny with the genotypes of *Dp(2;1)B19/w; Df(2L)sc19-1/MSP-300^{sz-75}*, *Dp(2;1)B19/w; MSP-300^{sz-75}/SM6B*, and w/Y; *MSP-300^{sz-75}/Df(2L)sc19-1* were phenotypically characterized.

Construction of transgenic flies expressing a GFP::MSP-300(KASH) fusion protein

The last 1021 bp coding sequence of *msp-300* that contains the KASH domain was amplified from EST CK00024 (GenBank entry AA142252) (supplied by Invitrogen) by PCR and inserted into the *Kpn*I and *Bam*HI site of the pUASP vector (Rorth, 1998). A GFP coding sequence from plasmid pEGFP-C1 (Invitrogen) was then inserted upstream of the *msp-300* sequence, resulting in an in-frame cDNA sequence encoding a GFP::MSP-300(KASH) fusion protein. This construct was injected into *w^{1118}* embryos to obtain transgenic animals. A *nanos-Gal4* strain (Van Doren et al., 1998) was used to induce GFP::MSP-300(KASH) expression during oogenesis.

Generation of germ-line clones

The FLP-DFS system (Chou and Perrimon, 1992, 1996) was used to generate homozygous *msp-300^{sz-75}* germ-line clones, and also used for rescuing germ-line clones with *Dp(2;1)B19*. Virgin females *y w hs-FLP^{12}; msp-300^{sz-75} FRT40A/CyO, hs-Hid* were crossed with w/Y; *ovo^{D1} ovo^{D1} FRT40A/CyO, hs-Hid* males or *Dp(2;1)B19/Y; ovo^{D1} ovo^{D1} FRT40A/SM6B* males, respectively, and eggs laid within 1 day were collected. Third instar larvae were heat-shocked in a 37°C water bath for 90 min to induce FRT-mediated mitotic recombination (Xu and Rubin, 1993). All females generated were of the genotype *y w hs-FLP^{12}/w; msp-300^{sz-75} FRT40A/ovo^{D1} ovo^{D1} FRT40A* or *Dp(2;1)B19/y w hs-FLP^{12}; msp-300^{sz-75} FRT40A/ovo^{D1} ovo^{D1} FRT40A*, respectively. They were crossed with wild type males in fresh vials for 3 days before sampling the ovaries. In the control experiment, *P[w+]31E* was used instead of *msp-300^{sz-75}*. For visualizing the oocyte nuclei, *[Histone-GFP]62A* (*his-GFP*) was used to generate females of the genotype *y w hs-FLP^{12}/w; msp-300^{sz-75} FRT40A/ovo^{D1} ovo^{D1} FRT40A; his-GFP/+* .

Generation of mutant follicle cell clones

Mutant follicle cell clones were generated using the FLP/FRT technique described in Xu and Rubin (1993). T155-Gal4 induced follicle-cell-specific expression of FLP recombinase leads to homozygous clones in follicle region in egg chambers efficiently (Duffy et al., 1998). Female flies of the genotype $msp-300^{sz-75}$ FRT40A/ubi-nGFP FRT40A; T155UF/+ were generated and dissected for staining after an incubation in fresh vials for 3 days.

Preparation of anti-MSP-300 antibody

The same $msp-300$ fragment used to generate $GFP::msp-300(KASH)$ transgene was cloned into pQE30 (Qiagen) or pGEX-2T (Amersham Pharmacia Biotech) to make $6xHis:msp-300(KASH)$ or $GST:msp-300(KASH)$ fusion genes, respectively. Proteins were expressed in E. coli strain BL21 codon plus (Stratagene) and purified with Ni-NTA (Qiagen) or glutathione sepharose 4B beads (Amersham Pharmacia Biotech) according to manufacturers' protocols. Polyclonal antibodies were raised by immunizing 200 g female Sprague–Dawley rats (Harlan, Indianapolis, IN) with 100 mg of purified 6xHis:MSP-300 fusion protein in Freund's adjuvant for four times at 3-week intervals. Crude serum was produced from the blood 10 days after the final boost. 0.5 ml of crude serum from the best responder was affinity purified against 5 mg of GST:MSP-300(KASH) fusion protein on a 0.5 ml Affi-Gel 15 (Bio-Rad, Hercules, CA) column (Harlow and Lane, 1999).

Quantitative real-time PCR and genomic DNA sequencing

Total RNAs were isolated from homozygous $msp-300^{sz-75}$ or w^{1118} wild type larvae at L2 and L3 stages using TRIzol Reagent (Invitrogen). RNA samples were treated with DNaseI. First-strand cDNA was synthesized using an RT-PCR kit (v3.0, TaKaRa). Real-time PCR was performed using MX3000 instrument (Stratagene) and SYBR Green I Dye following the standard protocols. For identifying the molecular lesions of $msp-300^{sz-75}$, genomic DNA was isolated from homozygous mutant larvae and PCR amplified using $msp-300$ primers prior to sequencing. The detailed primer sequences are available upon the request.

Immunofluorescence analysis of egg chambers and microscopy

Ovaries were dissected in ice cold Ringer's Solution to release the egg chambers and fixed following standard protocols (Roberts, 1998). They were then washed in PBS several times and resuspended in PBS with 1% Triton X-100 for 1 h at room temperature. Blocking was performed by incubating the samples at room temperature in PBST (0.4% Triton X-100 in PBS) with 5% NGS (Normal Goat Serum, Santa Cruz) for at least 1 h. Samples were then incubated overnight with 1:50 diluted MSP-300 polyclonal antibody or 1:20 diluted htsRC antibody (Robinson et al., 1994) in PBST with 1% NGS at 4°C, and washed several times with PBST. TexasRed-conjugated Goat anti-rat IgG (Santa Cruz) or TRITC conjugated Goat anti-mouse IgG (Rockland) was then applied at 1:500 dilution in PBST with 1% NGS for 2 h in the dark before further treatments, respectively. The number of ring canals was statistically analyzed by t test.

For actin/DNA visualization, fixed ovaries were washed in PBST and stained for 30 min with FITC-phalloidin (Sigma). The samples were washed several times with PBST and stained for 10 min with DPAI before they were mounted in anti-fading solution for photographing.

Samples were visualized and photographed by using a Leica DMIRE2 Microscope with bright field, Rhodamine, Fluorescein, or UV channels. Confocal images were taken with a Leica TCS-NT Confocal System.

Results

MSP-300 protein is localized in the cytoplasm and at the nuclear envelope of several cell types in Drosophila ovaries

To investigate the expression and localization of MSP-300 in egg chambers, we raised an antibody against the C-terminal segment of MSP-300, including its KASH domain. This antibody recognized MSP-300 in wild type ovaries, but failed to do so in the ovaries from $msp-300^{sz-75}$ germ-line clones (Fig. 1). This result is consistent with claim that $msp-300^{sz-75}$ is a loss-of-function mutation (see below). Comparison of the staining signals of $msp-300^{sz-75}$ mutant and wild type ovaries indicates a high expression level of MSP-300 in wild type egg chambers. Consistent with the fact that follicle cells are heterozygous in genotype in $msp-300^{sz-75}$ germ-line clones, we detect no difference in antibody staining in follicle cells between wild type and mutant ovaries.
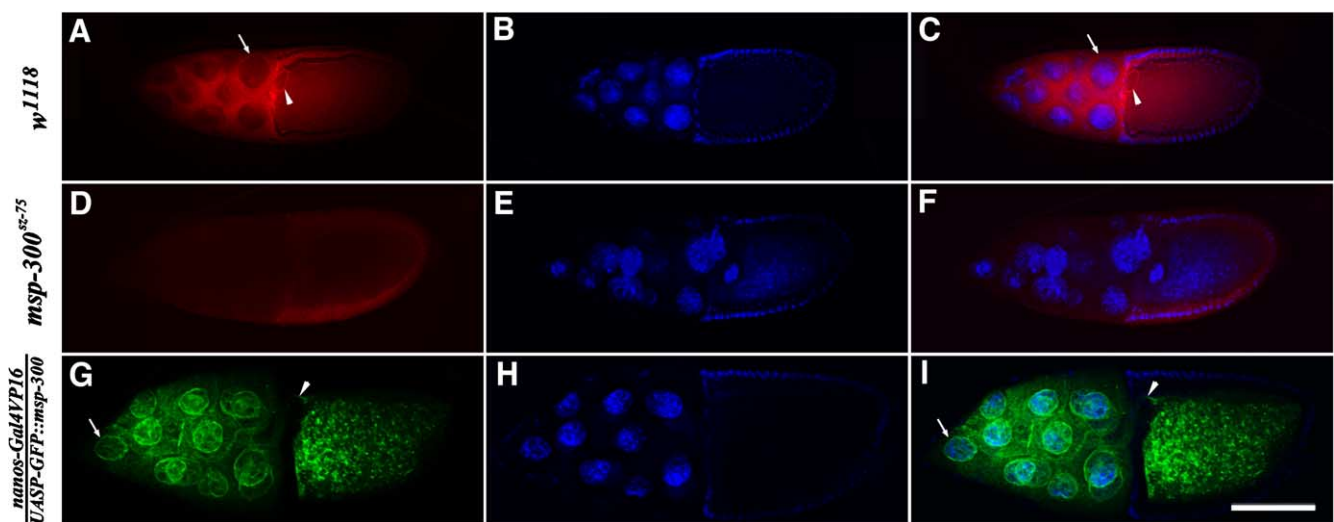

Fig. 1. Localization of endogenous MSP-300 and a transgenic GFP::MSP-300(KASH) fusion protein in Drosophila egg chambers. (A–F) Staining using anti-MSP-300 antibody to determine the protein localization in egg chambers. (A) w^{1118} ovary stained with anti-MSP-300 antibody. Accumulation of MSP-300 at the oocyte nuclear envelope is indicated (arrowhead). In the nurse cells, MSP-300 is localized to the cytoplasm and the nuclear envelope (arrow indicates the nuclear envelop of a nurse cell). (B) DAPI staining of the same egg chamber as in panel (A). (C) Merged image. (D–F) Antibody staining, DAPI staining, and merged images of an $msp-300^{sz-75}$ GLC egg chamber. (G–I) $nanos-Gal4/UASP-GFP::msp-300(KASH)$ egg chamber. $nanos-Gal4$ induced GFP::MSP-300(KASH) fusion protein is localized at the nurse cell nuclear envelope (G, arrow) and the oocyte nuclear envelope (G, arrowhead). (H) DAPI staining of the same egg chamber. (I) Merged image. All egg chambers in the images are oriented with anterior to the left. Scale bar, 100 μm.

MSP-300 localization was detected in oocytes, nurse cells, and follicle cells. In oocytes, localization of the protein at nuclear envelope was clearly detected (Fig. 1A). In nurse cells, MSP-300 was mainly localized in the cytoplasm. However, higher levels of staining were observed at the perinuclear region of nurse cells than in the cytoplasmic region (Fig. 1A), indicating that some MSP-300 localizes at the nuclear envelope of the nurse cells. Antibody staining in the nucleoplasm of germ cells was not above background. These data suggest that the primary function of MSP-300 protein is carried out in the cytoplasm and/or at the nuclear envelope.

Transgenically expressed C-terminal MSP-300 is localized at the cytoplasm and nuclear envelope of germ cells

To investigate whether the KASH domain is sufficient to localize protein at the nuclear envelope, we constructed a *UASP-GFP::msp-300(KASH)* transgene and expressed it in *Drosophila* germ cells. As shown in Fig. 1G, the GFP fusion protein was predominantly localized at the nuclear envelope in nurse cells and was also clearly detected at the nuclear envelope and in the cytoplasm of oocytes. This result is consistent with the idea that the KASH domain is responsible for the nuclear envelope localization of MSP-300 and that this localization might be critical for the protein function.

Maternally supplied MSP-300 protein is required for oogenesis and embryogenesis

msp-300^{sz-75} is a loss-of-function allele as suggested by previous genetic studies (Rosenberg-Hasson et al., 1996; Volk, 1992). This was confirmed by a rescue experiment using a duplication *Dp(2;1)B19* that covers the entire *msp-300* locus (see Materials and methods). We found that both *Dp(2;1)B19/w; msp-300^{sz-75}/SM6B* and *Dp(2;1)B19/w; msp-300^{sz-75}/ Df(2L)sc19-1* females displayed a wild type phenotype.

We also sequenced about 50% of coding region of *msp-300^{sz-75}* mutant genomic DNA, including N-terminal and C-terminal region, and identified a single G/C to A/T transition that resulted in a nonsense mutation at codon 2154 (Fig. 2A), truncating 80% of the protein. *Msp-300* has been predicted to produce several alternatively spliced transcripts (www.flybase. net) and it has never been shown that the KASH domain exon actually connects to the exon in which the *msp-300^{sz-75}* mutation resides. We performed real-time PCR using the primers specific for the C-terminal end segment of the *msp-300* RNA containing the KASH domain showed a 2-fold decrease in its level (Fig. 2B), suggesting that at least half of the mutated transcripts were degraded by the nonsense-mediated decay mechanism. In addition, given the strong maternal effect of this gene (see below), we expect that at least
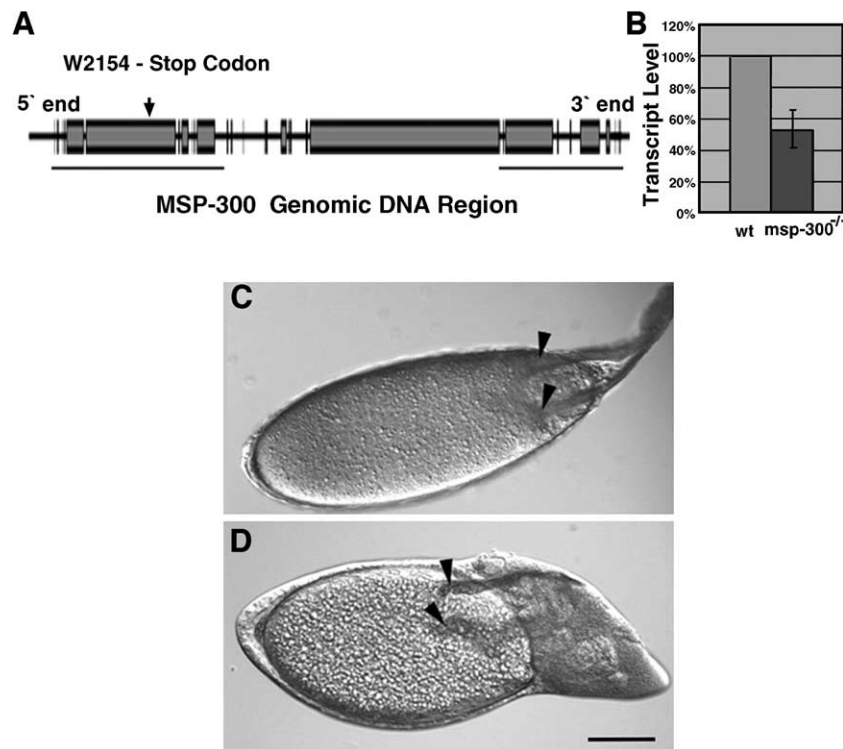

Fig. 2. (A) Schematic illustration of the MSP-300 genomic region and the location of the *msp-300^{sz-75}* lesion. The genomic regions underlined were sequenced to identify the mutant lesion. Please note that *msp-300* has been predicted to produce several differently spliced transcripts that have not been verified (www.flybase.net). (B) Quantitative RT-PCR results showing the decrease in transcript(s) level in *msp-300^{sz-75}* mutants. The level of the *msp-300* transcript containing the KASH domain was determined by its relative level to the actin transcript in both strains. Since the potential alternatively spliced transcripts may exist in differential abundances at different developmental stages, it is possible that the RT-PCR data for RNA in ovaries differs to some extent from that in the larvae. (C, D) Egg chambers in *msp-300^{sz-75}* GLC females display a dump-less phenotype. Nomarski images of egg chambers at stage 13 from a wild type control (*w^{1118}*) ovary (C) and from an *msp-300^{sz-75}* GLC mutant ovary (D). Arrowheads indicate the dorsal appendage structures. The cytoplasm of nurse cells retained at a late stage in the mutant egg chamber. Anterior is oriented to the right in both images. Scale bar, 100 μm.

Table 1
Frequency of Dump-less phenotype in *msp-300* GLC egg chambers

Genotype	Dump-less phenotype (%)	Normal dumping phenotype (%)	Total number
msp-300[sz-75]/+	2	98	n = 66
msp-300[sz-75] GLC	88	12	n = 211
P[w+] GLC	0	100	n = 58

a significant portion of the PCR products are derived from the maternal wild type transcript. Thus, our real-time PCR result implies that a large percentage of the KASH domain-containing *msp-300* transcripts include sequences upstream codon 2154. These results of genetic and molecular analysis of the *msp-300*[sz-75] mutation strongly support the claim that it is a loss-of-function mutation, and support the idea that the exon contains the *msp-300*[sz-75] mutation and the KASH domain containing exons contribute to the same protein at least some of the time.

Homozygous *msp-300*[sz-75] mutants produced by heterozygous females die as larvae. Therefore, a potential role of MSP-300 during earlier stages of development would be masked by maternal gene activity. To obtain oocytes and embryos that have no maternal wild type *msp-300* gene product, we generated homozygous *msp-300*[sz-75] clones in the female germ-line using the FRT/FLP system (see Materials and methods). As a negative control, we used a P element insertion line *P[w+]31E* in place of the *msp-300*[sz-75] mutation in the same procedure.

Females with homozygous *msp-300*[sz-75] germ-line clone (*msp-300*[sz-75] GLC) laid a small number of eggs with abnormal morphology. Compared with wild type eggs, the vast majority of these mutant eggs were significantly shorter, while the others had a severely shrunken appearance (data not shown). None of the mutant eggs developed normally. Moreover, dorsal appendages (DA) of most *msp-300*[sz-75] GLC eggs were shorter than those of wild type eggs (data not shown). To verify that the defects were indeed caused by MSP-300 mutation, we also did the rescue experiment with *Dp(2;1)B19* using the germ-line mosaic analysis (see Materials and methods). We found that the GLCs with *Dp(2;1)B19* developed normally, the dump-less defect and other defects (see below) observed in homozygous *msp-300*[sz-75] germ-line clones were completely rescued by the duplication (data not shown). These results indicate that maternal MSP-300 plays an essential role during oogenesis and embryogenesis.

Cytoplasmic transport in msp-300[sz-75] *mutant egg chambers is disrupted*

Msp-300 products are essential for the cytoplasmic dumping process. Egg chambers derived from *msp-300*[sz-75] GLC were examined by light microscopy. No obvious morphological defects were observed from samples prior to stage 10 (data not shown). However, severe defects were first visible in mutant egg chambers at stage 10B when dumping begins. In wild type animals, the oocyte is approximately one-half the volume of the egg chamber at stage 10A. During the

progression of cytoplasmic dumping, the volume of the oocyte increases while the size of the nurse cell cluster shrinks (Guild et al., 1997; Robinson and Cooley, 1997). During stage 13, dorsal appendages begin to be synthesized and elongate. At stage 14, the nurse cells disappear (Fig. 2C). In contrast, 88% of late egg chambers from *msp-300*[sz-75] GLCs had significant amounts of cytoplasm retained in the nurse cells (n = 211, Table. 1). Many of the post-stage 13 egg chambers, categorized by the appearance of the dorsal appendages, had a nurse cell to oocyte volume ratio similar to the stage 11 egg chambers of the control flies (Fig. 2D), suggestive of a dump-less phenotype Thus, we conclude that rapid cytoplasmic transport from the nurse cells to the oocyte in stage 10B is severely disturbed in *msp-300*[sz-75] mutant egg chambers.
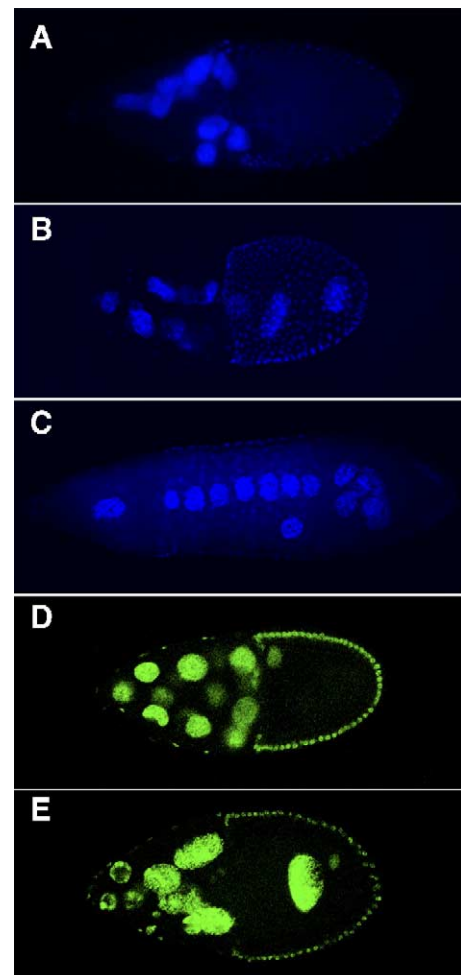

Fig. 3. Nurse cell and oocyte nuclei are often mislocalized in *msp-300*[sz-75] GLC mutant egg chambers. (A–C) DAPI staining images showing nurse cell nuclei. (A) Nurse cell nuclei cluster together (arrowhead). (B) Nurse cell nuclei invade into the oocyte; (C) nurse cell nuclei at post-stage 13 were dramatically mislocalized, spreading through the entire egg chamber. (D–E) Confocal images showing GFP fluorescence driven by a *histone* promoter. (D) A wild type control egg chamber at stage 10. The arrow indicates an oocyte nucleus localized at the dorsal anterior cortex. (E) An *msp-300*[sz-75] GLC egg chamber at stage10. The arrow indicates an oocyte nucleus mislocalized to the posterior region of oocyte. Scale bar, 100 μm.

Nuclear positioning in nurse cells and the oocyte is disrupted in msp-300^{sz-75} GLC egg chambers

In wild type egg chambers, the nucleus remains near the center of each nurse cell from stage 10B to stage 11, when the rapid flow of cytoplasm into the oocyte is occurring. In the msp-300^{sz-75} GLC egg chambers, nurse cell nuclei appear to be positioned normally prior to stage 10B, but then became disorganized when dumping started (Fig. 3). They often formed nuclear clusters (Fig. 3A), and invaded the oocyte (Fig. 3B). Totally, in 92% ($n = 304$) of the egg chambers, nurse cell nuclei mislocalized in late stage msp-300^{sz-75} GLC egg chambers. Among the egg chambers with mislocalized nuclei, a small percent (4%, $n = 280$) displayed a more severe phenotype: the nurse cell nuclei not only invaded into the oocyte but also ranged as a line (Fig. 3C). These data indicate that the nuclear anchorage of nurse cell was disrupted in msp-300^{sz-75} mutant egg chamber.

In the egg chambers of wild type flies, the oocyte nucleus is invariably localized to the dorsal anterior cortex (Fig. 3D). By using a *[histone-GFP]* reporter, we observed that the positioning of the oocyte nucleus was often disrupted in the msp-300^{sz-75} GLC egg chambers. In 85% of mutant egg chambers ($n = 59$), oocyte nuclei displayed a mispositioning defect as many of them moved to the posterior part of the oocyte during or after stage 10 (Fig. 3E). This result may suggest that the anchorage of oocyte nuclei was also disrupted in the msp-300^{sz-75} mutant egg chamber. However, it is possible that the defect of oocyte nucleus is a secondary consequence of defects during nurse cell dumping that may damage the membrane and generate mechanical forces on oocyte nucleus.

Actin structure of nurse cells is disrupted in msp-300^{sz-75} GLC egg chambers

Nurse cell cytoplasmic dumping is an actin-dependent process; the well-organized actin structures are necessary for not only anchoring the nuclei, but also generating and/or relaying the force for dumping (Spradling, 1993). MSP-300 may function to connect the nucleus to the actin cytoskeleton and play a critical role in regulating actin organization. We thus examined actin organization in the msp-300 mutant egg chambers.

In wild type, the major F-actin containing structures in the nurse cells prior to stage 10 are the subcortical actin that associates with the plasma membranes and the intercellular ring canals. Starting at stage 10B, thick transverse microfilament bundles are formed gradually in nurse cells, extending from the plasma membrane toward nuclei. Finally, actin-cage like structures form around each nurse cell nucleus and prevent them from entering the oocyte during the fast dumping phase. FITC-phalloidin staining detected severe defects of actin structures in nurse cells of msp-300^{sz-75} GLC egg chambers (Fig. 4). At stage 10, actin bundle-like microfilaments were formed, but in disorganized architectures, they extended from the plasma membrane but did not stretch out far enough to build up actin-cages around the nuclei (Fig. 4D). Thereby, the actin structures were severely disrupted after the fast dumping process in mutant egg chambers (Fig. 4F). These results indicate that MSP-300 is
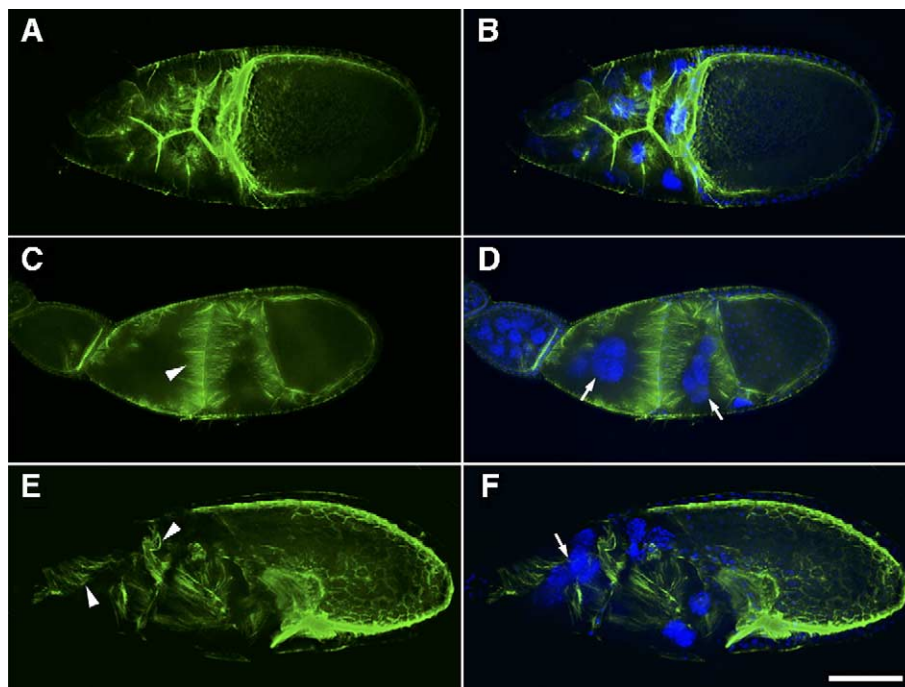

Fig. 4. Actin structure in nurse cells is abnormal in msp-300^{sz-75} GLC egg chambers. FITC-phalloidin staining or DAPI/FITC-phalloidin merged images of egg chambers at stage 10 from wild type control females (w^{1118}) (A, B) and msp-300^{sz-75} GLC females (C–F), in which subcortical actin structures are drastically disorganized in the mutant egg chamber after stage 10. Arrowheads indicate disorganized actin bundles in nurse cells. Multinucleated nurse cells (arrows) are often visible. Scale bar, 100 μm.

essential for the organization of the actin cytoskeleton that is required for nuclear anchorage and cytoplasmic dumping.

Ring canals are the intercellular channels between different nurse cells, as well as between nurse cells and the oocyte. By staining with a ring canal-specific marker, an htsRC antibody, we observed that at the early stages the structure and number of ring canals in msp-300^{sz-75} GLC egg chambers appeared normal (Fig. 5G). However, the number of ring canals at stage 10 was dramatically reduced from an average of 15 in wild type to an average of 6 in the mutant (n = 43) (Fig. 5). This result indicates that the MSP-300 is not necessary for the formation of ring canals but is required for maintaining the intercellular structures.

Nuclear positioning of follicle cells also display minor defects in msp-300^{sz-75} mutant egg chambers

Somatic derived follicle cells surrounding the oocyte and nurse cells play a supporting role during oogenesis (Spradling, 1993). During the dumping phase, abundant F-actin is located subcortically in both nurse cells and follicle cells. Therefore, nurse cell regression could be driven by the contraction of the follicle cells, the nurse cells, or both (Robinson and Cooley, 1997). We thus examined the phenotypes of msp-300^{sz-75} homozygous clones in follicle cells. To distinguish the mutant cells from wild type ones, nuclear localized GFP protein driven by the $ubiquitin$ promoter was used as a reporter on the opposite chromosome from msp-300^{sz-75}. Follicle cell number in mutant clones appeared normal compared to the surrounding wild type cells (Fig. 6). However, when compared with nuclei in wild type clones, a few nuclei with abnormal localization and shape were observed in mutant clones (Fig. 6E). Nuclei at the edge of the mosaic clones were irregularly spaced and some nuclei showed irregular sizes. These results may suggest that MSP-300 plays a role in anchoring the follicle cell nuclei. In wild type egg chambers, follicle cells change their shape as they stretch over the growing oocyte (Spradling, 1993). Therefore, the proper position of the nucleus may play a role in actin reorganization and the shape change. However, because the eggs derived from follicle cell mosaic egg chambers developed into adults normally, the defects in these mutant follicle cells alone did not cause a drastic physiological effect during oocyte maturation and embryogenesis.

Discussion

MSP-300 is necessary for cytoplasmic dumping during Drosophila oogenesis

In this paper, we showed that the maternal gene products of msp-300 were essential for proper transport of the cytoplasm from nurse cells to the oocyte during the late stages of oogenesis. We provided evidence that the MSP-300 likely provides the critical linkage between nurse cell nuclei and the actin cytoskeleton that is required for cellular integrity during cytoplasm dumping.

Dump-less mutations can be classified into five groups: (1) mutations that cause blockage or structural defects of ring canals ($chickadee$; $fascin$; $quail$; $kelch$) (Cant et al., 1994; Cooley et al., 1992; Guild et al., 1997; Mahajan-Miklos and Cooley, 1994a,b); (2) mutations that disrupt the ability of egg chamber to generate forces for cytoplasmic transport ($spaghetti$–$squash$) (Wheatley et al., 1995); (3) mutations that abolish nurse cell apoptosis which is required for initiating the dumping process (dcp-1) (McCall and Steller, 1998); (4) mutations that alter the actin cytoskeleton integrity ($armadillo$) (Peifer et al., 1993; White et al., 1998); and (5) mutations in genes that regulate the expression or activity of genes involved in the first four categories ($lark$) (McNeil et al., 2004). Analysis of the actin organization indicated that the msp-300 mutants have phenotypes similar to the severe $armadillo$ mutants. In mutants of both genes, multinucleated nurse cells resulting from cell membrane breakdown, disorganized actin structures, and abnormal ring canals were commonly observed (White et al., 1998). However, the misplacement of the oocyte observed in the $armadillo$ mutant egg chambers was never observed in the msp-300
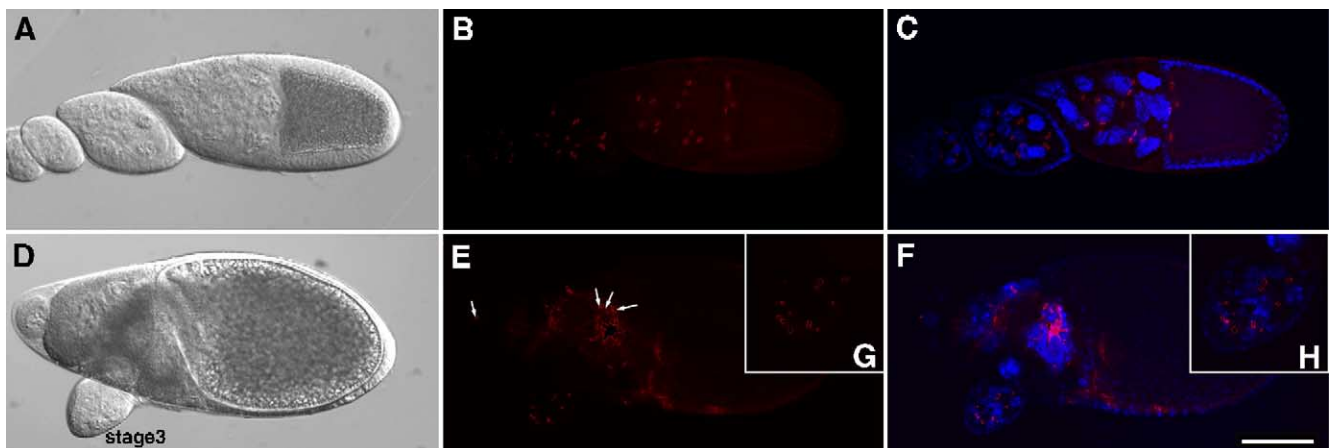

Fig. 5. The maintenance, but not the formation, of ring canal structures, is disrupted in msp-300^{sz-75} GLC egg chambers. Nomarski (A, D) htsRC antibody staining (B, E) and DAPI/htsRC merged images (C, F) of a control (w^{1118}) egg chamber (A–C) and an msp-300^{sz-75} GLC egg chamber (D–F). Arrows indicate the remaining ring canals in a stage 10 msp-300^{sz-75} GLC egg chamber. Note that the number of ring canals at this late stage is drastically decreased. Scale bar, 100 μm. (G, H) High magnification images of a stage 3 mutant egg chamber, indicating the normal number of ring canals at that stage.
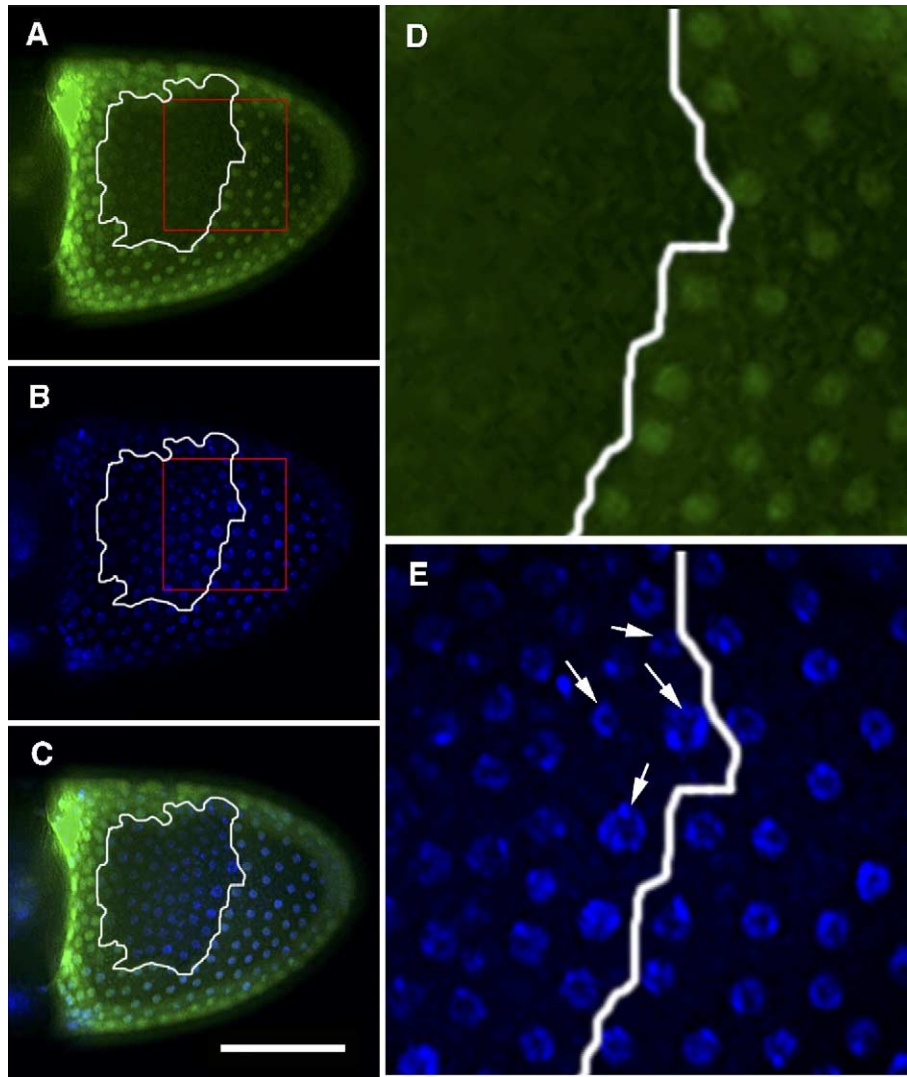
Fig. 6. Follicle cell nuclei display slight defects in their positions and shapes in *msp-300*[sz-75] mosaic egg chambers. (A) Mitotic recombination induced formation of a follicle cell mosaic egg chamber. GFP protein was used as a reporter to indicate *msp-300*(+) genotype. The white line marks the boundary between the GFP positive and negative follicle cells, GFP-negative cells are homozygous for the *msp-300*[sz-75] mutant allele. (B) DAPI staining of the same egg chamber. (C) Merged image of panels A and B. Scale bar, 100 μm. (D and E) High magnification images of red-lined box. Arrows indicate representative irregularly spaced or shaped follicle cell nuclei.

mutants. This suggests that the major pathways for establishing and maintaining the polarity of egg chambers, such as *De*cadherin–catenins pathway that is interrupted in the *armadillo* mutant (Oda et al., 1997; Robinson and Cooley, 1997), are not affected in *msp-300* mutants.

MSP-300 plays an important role in anchoring the nuclei of germ cells during oogenesis

MSP-300 belongs to a family of KASH domain containing proteins that have been shown to connect nuclei to the cytoskeleton (Starr and Han, 2003). Functional study of ANC-1 in *C. elegans* and cell biology analyses in both mammals and *C. elegans* have provided a solid basis to predict a role for MSP-300 in nuclear anchorage or movement. The significance of studying the function of MSP-300 in *Drosophila* is at least two-folds. First, the phenomena and functions of nuclear positioning during animal development are largely

unexplored. Although it had been suspected that nuclear anchorage plays an important role during *Drosophila* oogenesis and embryogenesis, no mechanisms have been revealed. The study presented in this paper represents a significant step in characterizing such a mechanism. Second, MSP-300 is structurally more similar to the mammalian Syne proteins than it is to ANC-1, because huge stretches of the middle part of MSP-300 and Syne proteins consist of typical spectrin repeats rather than the novel coiled-coil structure in ANC-1 protein. In *C. elegans*, the function of ANC-1 in nuclear positioning is well characterized by loss-of-function mutations. Studies of Syne proteins in mammals have so far been mostly limited to cell biology characterizations: genetic studies using mutants are lacking. Therefore, exploring the functions of MSP-300 using *Drosophila* genetics will have important implications for understanding the function of this gene family in mammals.

Several results in this study demonstrated that MSP-300 is a key player for tethering nuclei in nurse cells and the oocyte.

Furthermore, MSP-300 likely functions through the actin network. First, C-terminal antibody staining showed that MSP-300 is distributed throughout the cytoplasm and accumulates preferentially at the nuclear envelopes of nurse cells and the oocyte, indicating that the primary function of MSP-300 is carried out in the cytoplasm and/or at the nuclear envelope. Similar antibody staining results have also been observed for ANC-1 (Starr and Han, 2002) and Syne-1/2 (Apel et al., 2000; Zhang et al., 2001; Zhen et al., 2002). Second, during cytoplasmic transport, the nuclei of nurse cells and the oocyte were mislocalized in the *msp-300^{sz-75}* mutant egg chambers, suggesting that MSP-300 is essential for anchoring these nuclei. Third, actin organization in *msp-300^{sz-75}* mutants is severely disrupted. Proteins in the ANC-1/MSP-300/Syne family all contain two actin-binding domains in their N-terminal ends (Rosenberg-Hasson et al., 1996; Starr and Han, 2002; Volk, 1992; Zhang et al., 2002; Zhen et al., 2002). Previous work has also shown that MSP-300 co-precipitates with actin from an embryonic extract and co-localizes with actin filaments in vivo (Volk, 1992). Therefore, MSP-300 likely plays a key role in nuclear anchorage during oogenesis, likely by physically connecting nuclei to the actin cytoskeleton. Although the above model is supported by studies on similar proteins in other systems, the results described in this paper may also be consistent with alternative models. For example, MSP-300 might play a role in organizing the actin cortex or the cytoplasmic actin bundles. The nuclear anchorage defect would be the consequence of disruption of these functions. Additionally, MSP-300 might also have a function in nuclear architecture that is important for the attachment of nuclei to actin bundles.

Overexpression of the C-terminal KASH domain of ANC-1 in *C. elegans* and Syne-1 in mouse causes strong dominant negative mutant phenotypes (Grady et al., 2005; Starr and Han, 2002). This dominant negative effect is likely caused by competition for nuclear docking sites between the ectopically expressed KASH domain and endogenous ANC-1. Intriguingly, the expressed GFP∷MSP-300(KASH) fusion protein did not cause any obvious dominant negative defects during *Drosophila* egg chamber development. It is possible that the binary expression system we used fails to express the protein at a sufficiently high level to compete out the endogenous protein at the nuclear envelope. Alternatively, the docking sites for KASH domain proteins at the nuclear envelope in the fly system may not be limiting. It has also noted that no dominant negative effect on eye development is observed when the C-terminal region containing the KASH domain of Klarsicht is expressed in *Drosophila* (Fischer et al., 2004).

Subcellular localization of MSP-300

The staining result using the C-terminal end antibody indicates that only a small amount of protein seems to concentrate at the nuclear envelope. In contrast, the GFP∷MSP-300(KASH) fusion protein produced from a transgene is predominantly localized to the nuclear envelope of nurse cells. This difference may suggest that the spectrin repeats and N-terminal actin-binding domains are responsible

for the strong cytoplasmic retention of the endogenous MSP-300. Alternatively, there may be isoforms of the protein that lack the C-terminal end KASH domain but still contain some of C-terminal region to be recognized by the antibody.

In conclusion, the phenomena and functions of nuclear positioning during animal development are largely unexplored. Although it had been suspected that nuclear anchorage plays an important role during *Drosophila* oogenesis and embryogenesis, no mechanisms have been revealed. The study presented in this paper represents a significant step in characterizing such a mechanism.

References

Apel, E.D., Lewis, R.M., Grady, R.M., Sanes, J.R., 2000. Syne-1, a dystrophin- and Klarsicht-related protein associated with synaptic nuclei at the neuromuscular junction. J. Biol. Chem. 275, 31986–31995.

Cant, K., Knowles, B.A., Mooseker, M.S., Cooley, L., 1994. *Drosophila* singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. J. Cell Biol. 125, 369–380.

Chou, T.B., Perrimon, N., 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. Genetics 131, 643–653.

Chou, T.B., Perrimon, N., 1996. The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. Genetics 144, 1673–1679.

Cooley, L., Verheyen, E., Ayers, K., 1992. chickadee encodes a profilin required for intercellular cytoplasm transport during *Drosophila* oogenesis. Cell 69, 173–184.

Cottrell, J.R., Borok, E., Horvath, T.L., Nedivi, E., 2004. CPG2: a brain- and synapse-specific protein that regulates the endocytosis of glutamate receptors. Neuron 44, 677–690.

Duffy, J.B., Harrison, D.A., Perrimon, N., 1998. Identifying loci required for follicular patterning using directed mosaics. Development 125, 2263–2271.

Fischer, J.A., Acosta, S., Kenny, A., Cater, C., Robinson, C., Hook, J., 2004. *Drosophila* klarsicht has distinct subcellular localization domains for nuclear envelope and microtubule localization in the eye. Genetics 168, 1385–1393.

Grady, R.M., Starr, D.A., Ackerman, G.L., Sanes, J.R., Han, M., 2005. Syne proteins anchor muscle nuclei at the neuromuscular junction. Proc. Natl. Acad. Sci. U. S. A. 102, 4359–4364.

Guild, G.M., Connelly, P.S., Shaw, M.K., Tilney, L.G., 1997. Actin filament cables in *Drosophila* nurse cells are composed of modules that slide passively past one another during dumping. J. Cell Biol. 138, 783–797.

Harlow, E., Lane, D., 1999. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hedgecock, E.M., Thomson, J.N., 1982. A gene required for nuclear and mitochondrial attachment in the nematode *Caenorhabditis elegans*. Cell 30, 321–330.

Mahajan-Miklos, S., Cooley, L., 1994a. Intercellular cytoplasm transport during *Drosophila* oogenesis. Dev. Biol. 165, 336–351.

Mahajan-Miklos, S., Cooley, L., 1994b. The villin-like protein encoded by the *Drosophila* quail gene is required for actin bundle assembly during oogenesis. Cell 78, 291–301.

Malone, C.J., Fixsen, W.D., Horvitz, H.R., Han, M., 1999. UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during *C. elegans* development. Development 126, 3171–3181.

McCall, K., Steller, H., 1998. Requirement for DCP-1 caspase during *Drosophila* oogenesis. Science 279, 230–234.

McNeil, G.P., Smith, F., Galioto, R., 2004. The *Drosophila* RNA-binding protein Lark is required for the organization of the actin cytoskeleton and Hu-li tai shao localization during oogenesis. Genesis 40, 90–100.

Mislow, J.M., Kim, M.S., Davis, D.B., McNally, E.M., 2002. Myne-1, a spectrin repeat transmembrane protein of the myocyte inner nuclear membrane, interacts with lamin A/C. J. Cell Sci. 115, 61–70.

Morris, N.R., 2000. Nuclear migration. From fungi to the mammalian brain. J. Cell Biol. 148, 1097–1101.

Mosley-Bishop, K.L., Li, Q., Patterson, L., Fischer, J.A., 1999. Molecular analysis of the klarsicht gene and its role in nuclear migration within differentiating cells of the *Drosophila* eye. Curr. Biol. 9, 1211–1220.

Oda, H., Uemura, T., Takeichi, M., 1997. Phenotypic analysis of null mutants for DE-cadherin and Armadillo in *Drosophila* ovaries reveals distinct aspects of their functions in cell adhesion and cytoskeletal organization. Genes Cells 2, 29–40.

Padmakumar, V.C., Abraham, S., Braune, S., Noegel, A.A., Tunggal, B., Karakesisoglou, I., Korenbaum, E., 2004. Enaptin, a giant actin-binding protein, is an element of the nuclear membrane and the actin cytoskeleton. Exp. Cell Res. 295, 330–339.

Patterson, K., Molofsky, A.B., Robinson, C., Acosta, S., Cater, C., Fischer, J.A., 2004. The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the *Drosophila* eye. Mol. Biol. Cell 15, 600–610.

Peifer, M., Orsulic, S., Sweeton, D., Wieschaus, E., 1993. A role for the *Drosophila* segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. Development 118, 1191–1207.

Roberts, D.B., 1998. *Drosophila*: A Practical Approach. IRL Press at Oxford Univ. Press, Oxford.

Robinson, D.N., Cooley, L., 1997. Genetic analysis of the actin cytoskeleton in the *Drosophila* ovary. Annu. Rev. Cell Dev. Biol. 13, 147–170.

Robinson, D.N., Cant, K., Cooley, L., 1994. Morphogenesis of *Drosophila* ovarian ring canals. Development 120, 2015–2025.

Rorth, P., 1998. Gal4 in the *Drosophila* female germline. Mech. Dev. 78, 113–118.

Rosenberg-Hasson, Y., Renert-Pasca, M., Volk, T., 1996. A *Drosophila* dystrophin-related protein, MSP-300, is required for embryonic muscle morphogenesis. Mech. Dev. 60, 83–94.

Spradling, A.C., 1993. Developmental genetics of oogenesis. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila meanogaser*. Cold Spring Harbor Lab., NY, pp. 1–70.

Starr, D.A., Han, M., 2002. Role of ANC-1 in tethering nuclei to the actin cytoskeleton. Science 298, 406–409.

Starr, D.A., Han, M., 2003. ANChors away: an actin based mechanism of nuclear positioning. J. Cell Sci. 116, 211–216.

Tran, P.T., Marsh, L., Doye, V., Inoue, S., Chang, F., 2001. A mechanism for nuclear positioning in fission yeast based on microtubule pushing. J. Cell Biol. 153, 397–411.

Van Doren, M., Williamson, A.L., Lehmann, R., 1998. Regulation of zygotic gene expression in *Drosophila* primordial germ cells. Curr. Biol. 8, 243–246.

Volk, T., 1992. A new member of the spectrin superfamily may participate in the formation of embryonic muscle attachments in *Drosophila*. Development 116, 721–730.

Wheatley, S., Kulkarni, S., Karess, R., 1995. *Drosophila* nonmuscle myosin II is required for rapid cytoplasmic transport during oogenesis and for axial nuclear migration in early embryos. Development 121, 1937–1946.

White, P., Aberle, H., Vincent, J.P., 1998. Signaling and adhesion activities of mammalian beta-catenin and plakoglobin in *Drosophila*. J. Cell Biol. 140, 183–195.

Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.

Zhang, Q., Skepper, J.N., Yang, F., Davies, J.D., Hegyi, L., Roberts, R.G., Weissberg, P.L., Ellis, J.A., Shanahan, C.M., 2001. Nesprins: a novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. J. Cell Sci. 114, 4485–4498.

Zhang, Q., Ragnauth, C., Greener, M.J., Shanahan, C.M., Roberts, R.G., 2002. The nesprins are giant actin-binding proteins, orthologous to *Drosophila melanogaster* muscle protein MSP-300. Genomics 80, 473–481.

Zhen, Y.Y., Libotte, T., Munck, M., Noegel, A.A., Korenbaum, E., 2002. NUANCE, a giant protein connecting the nucleus and actin cytoskeleton. J. Cell Sci. 115, 3207–3222.