Lis1, the Drosophila homolog of a human lissencephaly disease gene, is required for germline cell division and oocyte differentiation

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SUMMARY

Lissencephaly is a severe congenital brain malformation resulting from incomplete neuronal migration. One causal gene, LIS1, is homologous to nudF, a gene required for nuclear migration in A. nidulans. We have characterized the Drosophila homolog of LIS1 (Lis1) and show that Lis1 is essential for fly development. Analysis of ovarian Lis1 mutant clones demonstrates that Lis1 is required in the germline for synchronized germline cell division, fusome integrity and oocyte differentiation. Abnormal packaging of the cysts was observed in Lis1 mutant clones. Our results indicate that LIS1 is important for cell division and differentiation and the function of the membrane cytoskeleton. They support the notion that LIS1 functions with the dynein complex to regulate nuclear migration or cell migration.

Key words: LIS1, Germline, Cyst, Cell division, Fusome, Cytoskeleton, Drosophila, Lissencephaly

INTRODUCTION

Lissencephaly is a congenital brain malformation manifested by a smooth cerebral surface due to incomplete neuronal migration (Dobyns, 1989). Type I lissencephaly occurs either as an isolated abnormality or in association with dysmorphic facial appearance in Miller-Dieker syndrome (MDS; Miller, 1963; Dieker, 1967). Lissencephaly patients usually die young with profound mental retardation, muscle weakness and seizures. One causal gene for lissencephaly, lissencephaly 1 (LIS1), encodes a protein containing seven WD-40 repeats (Reiner et al., 1993). Heterozygous deletions or mutations in the LIS1 gene have been identified in patients with MDS and isolated lissencephaly (Reiner et al., 1993; Lo Nigro et al., 1997), indicating the haplo-insufficiency of LIS1. Mice homozygous for a null Lis1 mutation die early in embryogenesis soon after implantation. Heterozygous and compound heterozygous mice have dosage-dependent defects in neuronal migration and neurogenesis (Hirotsune et al., 1998). Lis1 is also caused by mutations in an X-linked gene encoding a potentially phosphorylated protein that may play a role in a signal transduction pathway overlapping with the function of LIS1 (des Portes et al., 1998; Gleeson et al., 1998).

LIS1 was also identified biochemically in bovine brain extracts as the regulatory subunit α of platelet-activating factor acetylhydrolase (PAFAH; Hattori et al., 1994). PAFAH contains two additional catalytic subunits β and γ, and the crystal structure of the β subunit closely resembles that of GTPases (Ho et al., 1997). PAFAH catalyzes the removal of the acetyl group at the sn-2 position and produces biologically inactive lyso-PAF. PAFAH thus is important for regulating levels of the active PAF, which has potent biological functions in diverse organs (Venable et al., 1993), including the central nervous system (Kornecki and Ehrlich, 1988; Bito et al., 1992; Marcheselli and Bazan, 1994).

A function for PAFAH in the brain is also suggested by the observation that LIS1 and the two catalytic subunits are highly expressed in the same developing brain tissues (Mizuguchi et al., 1995; Albrecht et al., 1996). Since PAF is membrane-localized (Vallari et al., 1990), it is possible that the role of LIS1 is to localize the two catalytic subunits (β and γ) of PAFAH to the plasma membrane. LIS1 has been shown to associate with tubulin (Sapir et al., 1997) and β-spectrin (Wang et al., 1995), to co-localize with microtubules in tissue culture cells, and to reduce microtubule catastrophe frequency in vitro (Sapir et al., 1997).

A LIS1 homolog, nudF (Xiang et al., 1995), was isolated in a screen for nuclear migration mutants in Aspergillus nidulans. NudF interacts genetically with nudC, another nuclear migration gene in A. nidulans (Osmani et al., 1990; Xiang et al., 1995). NUDC is required to maintain a normal concentration of NUDF protein (Xiang et al., 1995). The murine NUDC homolog and Lis1 are co-expressed in the ventricular zone of the forebrain and in the cortical plate, and they also interact in a two-hybrid system (Morris et al., 1998b), suggesting that nuclear migration may play an important role for neuronal or cell migration (Morris et al., 1998b).
Mutations in the genes encoding Hts or a adducin-like protein (Lin et al., 1994; de Cuevas et al., 1996). The largest polypeptide in the dynactin complex that stimulates vesicle movement by dynein, and centrin (RO-4; Plamann et al., 1994; Robb et al., 1995), the most abundant component in the dynactin complex. PAC1, sharing significant identity with LIS1, is one of the components of the cytoplasmic dynein pathway in Saccharomyces cerevisiae (Fig. 1A; Geiser et al., 1997). These results support the notion that LIS1 and the dynein complex function together to regulate nuclear movement in fungi.

Little is known about how LIS1 functions during development. To take advantage of the genetic and molecular technologies in Drosophila, we cloned the Drosophila homolog of LIS1 (Lis1) and found that Lis1 is expressed strongly during oogenesis, and so have utilized the Drosophila ova as a model system to investigate Lis1 function. With the development of the FLP/FRT technique (Golic and Lindquist, 1989; Chou et al., 1993), the Drosophila ova is used as a powerful system to study gene functions at the cellular level (reviewed by Spradling, 1993).

In the Drosophila ova, two or three germline stem cells are located at the tip of each ovariole, the germarium. Each stem cell divides asymetrically to produce a new stem cell and a cystoblast that undergoes four rounds of synchronized divisions with incomplete cytokinesis to form a 16-cell cyst. The cystocytes in one cyst are interconnected in a stereotypical pattern by stable intercellular bridges termed ring canals. Following cyst formation, oocyte-specific transcripts and proteins accumulate in one cell which contains four ring canals and then develops as the oocyte. The other fifteen cells become polyploid nurse cells supplying nutrients for oocyte maturation and subsequent embryonic development.

A germline-specific cytoplasmic vesicle, the fusome, grows from a small sphere in a cystoblast into a large branched structure that extends through the ring canals into every cell of a developing cyst (Lin et al., 1994; McKearin, 1997; de Cuevas and Spradling, 1998). During stem cell and cyst cell division, one pole of the mitotic spindle associates with the fusome (Lin and Spradling, 1995; Deng and Lin, 1997). Following mitosis, newly synthesized fusome plugs move toward and fuse with the pre-existing fusome. Their associated ring canals also move, changing the geometry of the cyst and resulting in the formation of a rosette. The fusome remains asymmetrically distributed within the cyst after the first division, so generating a polarity in the developing cyst (de Cuevas and Spradling, 1998).

The fusome is composed of membrane skeletal proteins α-spectrin, β-spectrin, ankyrin and Hu-li tai shao (Hts), an adducin-like protein (Lin et al., 1994; de Cuevas et al., 1996). Mutations in the genes encoding Hts or α-spectrin apparently eliminate the fusomes and cause the formation of cysts with fewer than 16 cells, usually without an oocyte (Yue and Spradling, 1992; de Cuevas et al., 1996).

Cytoplasmic dynein associates with the fusome in a cell-cycle-dependent manner. Mutations in the cytoplasmic dynein heavy chain gene, Dhc64C, cause disruption of spindle orientation in the dividing cyst and block oocyte differentiation (McGrai and Hays, 1997). The mutant cysts contain fewer than 16 cells, suggesting that dynein is also involved in cystocyte division. In Drosophila, dynein forms a protein complex with the p150 subunit of dynactin encoded by glued (McGrai et al., 1995). The gene bag-of-marbles (bam) encodes another component of the fusome required for cystoblast differentiation and is thought to be involved in vesicle recruitment for fusome assembly (McKearin and Ohlstein, 1995; McKearin, 1997). Taken together, these observations indicate that the fusome has a critical role in regulating the synchronous cystocyte division and establishing or maintaining cyst polarity.

We report the identification of the Lis1 gene and isolation of Lis1 mutants in Drosophila. Lis1 mutant larvae die at the second instar larval stage, indicating that Lis1 is an essential gene in Drosophila as it is in vertebrates. Analysis of Lis1 ovarian mutant clones demonstrates that Lis1 is required in the germline for synchronized cystocyte division and oocyte differentiation. Furthermore, fusomes are aberrantly formed in Lis1 mutant cysts similar to the phenotypes observed in Dhc64C mutants. These results suggest that Lis1 is involved in the formation and maintenance of fusomes, important for regulating cystocyte division and oocyte differentiation. Our study also supports the notion that LIS1 interacts with the dynem complex to regulate the function of membrane skeletons, necessary for nuclear and neuronal migration.

MATERIALS AND METHODS

**Drosophila strains and culture**

All flies were maintained at 25°C on standard medium. Flies carrying deficiencies in polytene regions 52F (Saxton et al., 1991), w$^{fa}$; Df[2R]Jp1/Y;CyO, (51C3; 52F5-9) and w$^{fa}$; Df[2R]Jp8, w+[CyO; (52F5-9); 52F10-53A1] were obtained from the Blooming Stock Center. w$^{a}$; Df[2R]Jp4/Y; CyO; (51F3; 52F8-9) and w; Df[2R]Jp6/CyO; (52E3-5; 52F) were provided by B. Saxton. Flies used for EMS mutagenesis, bt/tr/CyO DTS100 and P[tr+; his-neo; FRT]2R-43D (Xu and Rubin, 1993), were provided by T. Schupbach. Lines for germline clonal analysis, y w P[tr+; FLP]2R; CyO Sco, w; P[mini w+; FRT]2R-G13; w; P[mini w+; FRT]2R-G13 L/CyO, and P[mini w+; FRT]2R-G13 P[mini w+; ovoD]2R-J329] S Sp Ms(2)M bwD/CyO (Chou and Perrimon, 1996) were provided by T. Schupbach. y w P[tr+; FLP]2R; P[mini w+; FRT]2R-G13 P[arm-lacZ] was provided by N. Perrimon. Oregon R served as the wild-type strain for all experiments.

**Molecular cloning and analysis of Lis1**

To amplify DNA from the Drosophila homolog of LIS1/NUDF, we made primers corresponding to two conserved regions of the proteins. The sequences of the primers are as follows:

- primer 1. (A/C/G/T)/(C/T)/(A/C/G/T)GA(A/G)AA(A/G)AA-A/(G)TGGAC
- primer 2. (A/C/G/T)/(C/A/C/G/T)/(C/T)/(A/C/G/T)ACCCA-(A/G)TT/(A/G)TC/(A/G)TC/(A/C/G/T)/CC.

DNA from embryo, ovary and disc fusion cDNA libraries (RFLY1, RFLY3 and RFLY5, gifts from R. Finley) was amplified by PCR and cloned into pBluescript T vector (Novagen). Conditions for PCR were as follows: 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 45°C for 2 minutes, 72°C for 3 minutes, followed by 72°C for 10 minutes. Random white colonies were sequenced and analyzed by computer. The amplified DNA fragment sharing sequence homology with LIS1/NUDF was used as a probe to screen a 4-8 hour embryonic cDNA library (Brown and Kafatos, 1988), according to Sambrook et al. (1989). One 1.8 kb Lis1 cDNA clone was partially digested and subcloned into the
The EcoRI site of pBluescript KS (+) (Strategene). The resulting construct is called pZL701. Double-strand sequencing was performed on this clone and showed that it contained a full-length open reading frame. A 1.8 kb BamHI and XhoI fragment containing the full-length Lis1 cDNA was used as a probe for northern blot analysis, screening of a genomic library (provided by Y. Hiromi) in λFIX vector (Strategene), and screening of an ovarian cDNA library (Stroumbakis et al., 1994) according to Sambrook et al. (1989). Four cDNA clones of 1.8 kb, 2.0 kb, 2.8 kb and 3.0 kb were isolated from the ovarian cDNA library. To map intron-exon boundaries, coding regions of the Lis1 genomic DNA was sequenced. This sequence was then compared to the genomic sequence published by the genome project and our Lis1 cDNAs.

**Whole-mount tissue in situ hybridization**

In situ hybridization to whole-mount ovaries and embryos was performed essentially as described by Tautz and Pfeifle (1989) with DIG-labeled (Boehringer Mannheim) full-length Lis1 antisense DNA as a probe. The control with the sense probe did not show any significant signal.

**Antibody induction and western blot analysis**

Anti-LIS1 antiserum was induced in mice against the N-terminal part of LIS1 (amino acid 1-164) overexpressed in bacteria and was used at a 1:1,500 dilution in western blot analysis that was performed according to Sambrook et al. (1989).

**Chromosome mapping and mutant isolation**

Chromosomal in situ hybridization was done as described by Ashburner (1989) with DIG-labeled (Boehringer Mannheim) full-length Lis1 cDNA from pZL701 as a probe. The hybridization was performed on larval polytene chromosomes isolated from wild type and heterozygous larvae carrying deficiencies Df(2R)Jp1, Df(2R)Jp4 and Df(2R)Jp8. To create point mutations in the Lis1 gene, a standard EMS screen for recessive lethals uncovered by Df(2R)Jp8 was performed essentially as described by Lewis and Bacher (1968). Homozygous males isogenic for the P[rY]; hs-neo; FRT201 chromosome were exposed to 50 mM ethyl methanesulfonate in 5% sucrose for 15 hours then mass mated with virgins of genotype b Tfl/CyO DFS100. Individual F1 progeny P[rY]; hs-neo; FRT201.

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**Fig. 1.** Alignment of the predicted *Drosophila* LIS1 protein sequence with its homologs and domain structure of the *Drosophila* LIS1 protein. (A) Sequence alignment of the deduced *Drosophila* LIS1 protein (DLIS1) with its homologs in human (LIS1), *A. nidulans* (NUDF) and *S. cerevisiae* (PAC1). The alignment was produced with CLUSTALW software. Identical residues are highlighted. Regions used to generate degenerate primers are underlined. (B) Alignment of the seven WD-40 repeats of *Drosophila* LIS1. Numbers on the left indicate the number of the repeat unit. Numbers on the right indicate the amino acid position. Repeats were identified based on Neer et al. (1993). Identical residues are highlighted. (C) Schematic representation of domains of *Drosophila* LIS1 and positions of LIS1 point mutations. The coiled-coil domain is denoted by a black box and WD-40 repeats are indicated by the hatched boxes. The GenBank accession number for the sequence reported in this paper is AF152419.
Df(2R)Jp4 was mutagenized second chromosomes. Each was tested for complementation with Df(2R)Jp4 and the other lethals, to establish the different complementation groups.

Transgenic rescue and mapping of Lis1 mutations
A 16 kb genomic insert from phage λδ7 contains the full-length Lis1 transcript and 8 kb of upstream region. This fragment was cut from λδ7 with XbaI restriction enzyme and subcloned into the pCaSpeR transformation vector (pZL1050), which contains a w' marker (Pirrotta, 1988). P-element-mediated transformation (Spradling and Rubin, 1982) was done with Turbo DNA as the transposase source. Transformed lines carrying the w' insert were crossed to w; Df(2R)Jp6/CyO and w; Lis1/CyO flies and tested for rescue of zygotic lethality of possible hemizygous or homozygous Lis1 mutants. Genomic DNA was isolated from heterozygous Lis1 mutants and the Lis1 coding region was sequenced to identify point mutations in the gene.

Genetic clonal analysis
Lis1 mutations were recombined onto P[mini w'; FRT]/2R-G13 chromosome by genetic crosses for induction of germline clones. Lis1 germline clones were generated with the FLP/DFS techniques as described by Chou and Perrimon (1996), y w P[hsFLP]12; FRT2R-G13 P[ovoD1]12; CyO males were crossed to w; FRT2R-G13 Lis1/CyO virgin females to produce y w P[hsFLP]12; FRT2R-G13 P[ovoD1]12; FRT2R-G13 Lis1 progeny. The same experiment was repeated with y w P[ery]; FLP]12; FRT2R-G13 P[arm-lacZ] males. 0-24 hour eggs were collected from the above crosses and heat shocked in a 37°C water bath for 2 hours over 2 consecutive days starting from the first day or the fifth day. The ovaries were dissected 1-3 days after eclosion and examined by immunofluorescence microscopy.

Immunostaining and fluorescence microscopy
Ovaries were dissected in PBS and fixed for 20 minutes in 4% paraformaldehyde dissolved in PBS. They were washed for 40 minutes in PBT (1% BSA, 0.1% Triton X-100 in PBS), incubated for 30 minutes in PBT-DS (PBT plus 5% donkey serum; Jackson ImmunoResearch Laboratories), and then incubated at 4°C overnight in PBT-DS containing primary antibodies at the appropriate dilution. Ovaries were then washed for 1 hour in PBT, incubated for 30 minutes in PBT-DS and then for 1-2 hour in PBT-DS containing fluorescence-conjugated secondary antibodies. The stained ovaries were washed for 1 hour in PBS and mounted in PBS plus 50% glycerol and 1 mg/ml p-phenylene diamine (Sigma) for microscopy. The following antisera were used: rabbit anti-β-galactosidase antibody (1:2000) (Cappel) and mouse monoclonal anti-Hts antibody B1 (1:1) (Zaccari and Lipshitz, 1996). All the fluorescence-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory) were used at 1:200 dilution. Immunofluorescently labeled samples were also counterstained with the nuclei-philic stain Hoechst 33257 (Molecular Probes) at 1 µg/ml for 5 minutes before the final wash. Actin was labeled with FITC-conjugated phalloidin (Molecular Probes) at 0.4 U/ml in PBS for 20 minutes. All steps were performed at room temperature unless otherwise noted. Stained ovaries were examined by epifluorescence and confocal microscopy on a Leica TCS NT microscope. DNA staining was photographed with a Princeton Instruments cooled CCD camera.

RESULTS

Molecular cloning and characterization of Drosophila LIS1 (Lis1)
To clone the Drosophila homolog of the human LIS1 gene, degenerate primers were designed based on the amino acid similarity between human LIS1 and A. nidulans NUDF (Fig. 1A). A single fragment amplified by PCR reactions from Drosophila fusion cDNA libraries shared significant sequence similarity with human LIS1. It was used as a probe to screen a Drosophila embryonic cDNA library. The longest cDNA clone

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**Fig. 2.** Molecular structure of the Lis1 locus and Lis1 expression. (A) Restriction map of ~16 kb Lis1 genomic region (λδ7) that was used for the transgenic rescue experiment. The 1.8 kb Lis1 transcription unit is shown beneath the genomic map. Exons are represented by black boxes and thin lines represent introns. (S) Salt; (H) HindIII. (S) polymorphism between genomic clones isolated in this study and the genomic sequence deposited by the Berkeley genome project. (B) Northern blot of poly(A) + RNA from wild-type ovaries hybridized with the Lis1 cDNA probe. About 10 ng poly(A) + RNA was loaded. Note the four different-sized transcripts. (C) Developmental western blot of extracts from staged embryos, larvae, pupae, adult males, adult females and ovaries probed with a mouse anti-LIS1 antibody. Approximately 30 µg protein was loaded in each lane.
obtained was a 1756 bp fragment that contained an open reading frame of 1233 bp, encoding a conceptual protein of 411 amino acids. The amino acid sequence of this protein is 70% identical to LIS1, 41% to NUDF and 26% to PAC1 (Fig. 1A). The similarities extend along the whole proteins. They share an N-terminal coiled-coil motif and seven WD-40 repeats and are likely to form similar secondary structures (Fig. 1B,C). The high level of conservation suggests that the new gene is...
an ortholog of human \textit{LIS1}. We name the \textit{Drosophila} gene \textit{Lis1}.

\textbf{\textit{Lis1} is highly expressed in the gerarium and the developing oocyte}

To study at which developmental stages \textit{Lis1} is expressed, we performed developmental northern analysis and found that \textit{Lis1} mRNA is present throughout the \textit{Drosophila} life cycle with high levels in the ovary (Fig. 2B). Four major transcripts of \textit{Lis1} are detected in all developmental stages. From an ovarian cDNA library, we isolated cDNAs corresponding to each of the mRNAs and determined by sequence analysis that they share the same coding region and 5’ untranslated region. Thus the difference in size is likely to arise from the usage of alternative polyadenylation sites or alternative splicing in the 3’ untranslated region. Mouse antisera raised against the N-terminal 164 amino acids of LIS1 recognizes a single band at 45 kDa in extracts from every developmental stage of flies, as indicated by western analysis (Fig. 2C). Unfortunately, the antisera fails to recognize the native protein in whole-mount ovaries or embryos.

To further define which cell types express \textit{Lis1} in the ovary, we performed whole-mount mRNA in situ hybridization using a \textit{Lis1} antisense probe. \textit{Lis1} transcripts were detected in all germline and somatic cells in the gerarium except the terminal filament cells and in the egg chambers (Fig. 3A,B). From stage 4 to stage 7 egg chambers, \textit{Lis1} RNA accumulated in the oocytes (see arrows in Fig. 3A). Only low levels of \textit{Lis1} RNA in stage 8 and 9 egg chambers were detected. The RNA was strongly expressed in nurse cells of stage 10 egg chambers, indicating that \textit{Lis1} may function in early embryogenesis. Indeed, \textit{Lis1} RNA was uniformly distributed in early embryos before the onset of zygotic gene expression (Fig. 3C). The results from northern analysis and mRNA in situ hybridization experiments suggest that \textit{Lis1} may well be required throughout \textit{Drosophila} development.

\textbf{\textit{Lis1} is essential for \textit{Drosophila} development}

To reveal its function, we generated mutations in the \textit{Lis1} gene. First, \textit{Lis1} was mapped to the polytene chromosome region 52F-53A by in situ hybridization experiments. We further defined its location by in situ hybridization to different deficiency chromosomes. Deletions, \textit{Df(2R)Jp1} to \textit{Df(2R)Jp8}, were isolated in this region in an effort to generate mutations in the kinesin heavy chain gene (Saxton et al., 1991). We found that the \textit{Lis1} gene was not removed by deficiency \textit{Df(2R)Jp1} (51C3-52F5-9), but that it was removed by two deficiencies, \textit{Df(2R)Jp8} (52F5-9;52F10-53A1) and \textit{Df(2R)Jp4} (51F3;52F8-9), indicating that \textit{Lis1} is located in chromosome region 52F5-9.

Based on the observation that \textit{Lis1} is expressed throughout development and that a null \textit{Lis1} mutation in the mouse is lethal, we expected that \textit{Lis1} should be an essential gene also in \textit{Drosophila}. However, no lethal mutants were known to map to this deficiency interval so we carried out a standard EMS screen to identify recessive lethal mutations uncovered by the smaller deficiency \textit{Df(2R)Jp8}. A total of 10,000 single chromosomes were screened and 72 lethal mutations were isolated and assigned to 19 complementation groups, referred to as \textit{l(2R)W1} to \textit{l(2R)W19}. Eleven of the complementation groups also failed to complement deficiency \textit{Df(2R)Jp4}, indicating that they are candidate \textit{Lis1} mutants.

To identify mutations in the \textit{Lis1} gene, a 16 kb genomic fragment from one \textit{λ} clone (\textit{λGb7}) containing the entire \textit{Lis1} coding region and about 8 kb of upstream region (Fig. 2A) was used as a transgene for genetic rescue experiments. This construct rescued the lethality of flies hemizygous for each of the four alleles of \textit{l(2R)W8}. Consistent with these rescue results, sequencing of the \textit{Lis1} coding region identified a single point mutation in each \textit{l(2R)W8} mutant allele. They are a dG-to-dA transition at nucleotide 570 (\textit{Lis1}^23F2), resulting in an E128K amino acid substitution; a dC-to-dT transition at nucleotide 903 (\textit{Lis1}^{G10.14}), resulting in an R239X premature translation termination; a dG-to-dA transition at nucleotide 1160 (\textit{Lis1}^D), resulting in a W324X premature translation termination; and a dG-to-dA transition at nucleotide 1286 (\textit{Lis1}^{21IC14}), resulting in a W366X premature translation termination (Fig. 1C). These data thus confirmed that the \textit{l(2R)W8} alleles are \textit{Lis1} mutants.

Western analysis of \textit{Lis1} mutant larvae showed the protein encoded by \textit{Lis1}^23F2 to be of normal size and as abundant as wild-type protein, and that \textit{Lis1}^{G10.14} encodes a stable but truncated protein. No proteins can be detected in either \textit{Lis1}^{G10.14} or \textit{Lis1}^D, indicating that they are likely to be LIS1 protein null alleles (data not shown).

\textit{Lis1} homozygous embryos hatched normally and the first instar larvae looked indistinguishable from their heterozygous siblings. At the L2 stage, the development of the homozygotes was clearly retarded and they died 5-6 days later without phenotypically entering the L3 stage. The survival of \textit{Lis1} mutants through embryonic and early larval development is probably due to the contribution of maternal wild-type \textit{Lis1} protein, as directly demonstrated by western analysis. When homozygous larvae were picked at L1 stage or early L2 stage, wild-type \textit{Lis1} protein was clearly detectable in the extract. But LIS1 protein was reduced or not detectable when the extracts were made from homozygotes close to death (data not shown).

\textbf{\textit{Lis1} is required in the gerarium for germline cell division and oocyte differentiation}

The expression pattern of \textit{Lis1} during oogenesis and early embryogenesis (Fig. 3) suggested that \textit{Lis1} may have important functions in the ovary and embryo. To investigate such a possibility, mosaic egg chambers whose germline lacked wild-type \textit{Lis1} were produced using the FLP/DFS technique (Chou and Perrimon, 1996). The germline phenotype in clones of all four \textit{Lis1} mutants were similar and failed to produce any eggs. Staining of the dissected ovaries with Hoechst 33258 revealed a phenotype indistinguishable from those of control \textit{ovo}^D females, and no vitellogenic oocytes were observed. The control experiment with the \textit{FRT2R-GI3} chromosome not carrying a \textit{Lis1} mutation produced wild-type clones containing many developing oocytes and mature eggs. These results demonstrate that \textit{Lis1} is required in the germline for early oogenesis.

To identify mutant germline and follicle clones, we repeated the experiment in the absence of \textit{ovo}^D and used \textit{arm-lacZ} as a marker. Mosaic ovaries were dissected and stained with anti-\textit{β}-galactosidase antibodies, identifying mutant germline and follicle clones by the absence of \textit{β}-gal protein expression. The
ovaries were counter-stained with Hoechst 33258 to determine the number and size of nuclei. The Lis1G10.14 allele was used to perform mutant clonal analysis. It does not produce mutant LIS1 protein and has no additional lethal mutations on the second chromosome.

We compared egg chambers specifically lacking Lis1 in germline cells and control egg chambers retaining Lis1 in both germline and follicle cells. Control egg chambers invariably contained 15 nurse cells and one oocyte (Fig. 4A,C). Lis1 egg chambers often contained fewer than 16 cells and usually lacked an oocyte (Fig. 4B,D). All the cells in the mutant Lis1 cysts appeared to develop as nurse cells, as indicated by their polyploid nuclei. We examined 498 egg chambers and among 5340 polyploid nurse cells found only 6 oocytes. Because the normal ratio between nurse cells and oocytes is 15:1, our observations indicate a 59-fold reduction in the frequency of oocyte formation. As a result of synchronized cystocyte division, there are either 1, 2, 4, 8 or 16 cells in developing wild-type cysts. However, in Lis1 mutant cysts, the cell numbers range consecutively from 1 to 16 (Fig. 4G), indicating that the synchrony of germline cell divisions was disrupted in the absence of Lis1 germline function.

Quantitative differences in the number of cells in the Lis1 mutant cysts were observed dependent on when the germline clones were induced. The earlier the induction, the fewer cells in the mutant cysts that were observed (Fig. 4G). When clones were induced at embryonic or first instar larval stages, the average nurse cell number in Lis1 egg chambers was 7.2 (n=125 egg chambers). The average cell number increased to 11.9 (n=373 egg chambers) with egg chambers containing mostly even numbers of nurse cells, when the induction was done at the third instar larval stage. The frequency of mutant clone formation was also increased significantly with late induction. However, the frequency of oocyte differentiation was similar (2 in 125 egg chambers versus 4 in 373 egg chambers) in early and late induction. These results suggest that oocyte differentiation is more sensitive to the perturbation of Lis1 function than is cystocyte division. The weaker phenotype observed with later induction may be a reflection of the perdurance of wild-type LIS1 protein in germline stem cells. The relatively low frequency of clones resulting from early induction may be due to the death and growth arrest of germline stem cells or cystoblasts. LIS1 protein level in these cells is expected to be lower in early induced clones than in those induced later.

Mosaic egg chambers that contain wild-type germline cells surrounded by only mutant follicle cells produce normal germline cysts of 15 nurse cells and one oocyte (Fig. 4E,F). Thus, LIS1 function is not required in the follicle cells for normal cyst formation. Our results clearly demonstrate that LIS1 is required in the germline but not in somatic follicle cells to regulate cystocyte division and oocyte differentiation.

**Fusomes are defective in Lis1 cysts**

The cyst defects observed in Lis1 mutants are similar to those of hts and α-spectrin mutants. The hts and α-spectrin genes encode membrane skeletal proteins that are components of the fusome (Lin et al., 1994), and mutations in either gene abolish fusome formation (Lin et al., 1994; de Cuevas et al., 1996). The absence of a fusome is thought to block the rapid and synchronous cystocyte divisions in both mutants. We examined the morphology of fusomes in cysts mutant for Lis1 by double-staining the egg chambers with anti-β-galactosidase antibody and monoclonal anti-Hts antibody 1B1 (Zaccai and Lipshitz, 1996).

In a wild-type germarium (Fig. 5A), a spherical fusome, also called spectrosome (ss), is found in germline stem cells (Lin et al., 1994). The fusome (f) grows from a spherical fusome in the cystoblast into a polarized branched structure in 2-, 3-, 8- and 16-cell cysts (de Cuevas and Spradling, 1998). Several major defects in Lis1 mutant cysts were observed (Fig. 5B-D). First, some mutant cystoblasts fail to divide, while their fusomes continue to grow to a larger sphere and their nuclei sometimes become polyploid (see long arrows in Fig. 5B), suggesting that LIS1 is required for cystoblast division. Second, when all germline cells in a germarium were mutant for Lis1, the number of developing cysts was dramatically reduced compared with a wild-type germarium (Fig. 5B,C), suggesting that the division of germline stem cells is also retarded in Lis1 mutants. Third, branched fusomes in the Lis1 mutant cysts were often thinner and fragmented (see short arrows in Fig. 5C,D) as compared to the intact, smoothly branched fusomes in wild-type cysts. The DNA staining of these mutant cysts indicated that the nuclear appearance of most cystocytes was normal, although some cystocytes appeared arrested in cell division and the nuclei became prematurely polyploid (data not shown). Finally, fusomes in more posterior regions of the germarium were sometimes absent (see arrowhead in Fig. 5C) and, in all these cysts, the nuclei were often polyploid or apoptotic as determined by DNA staining. Most likely, these cysts were growth-arrested based on their location in the more posterior region of the germarium, eventually degenerating the fusomes.

Sometimes, cysts with apparently normal fusomes were also detected. However, they probably did not function normally, since virtually no mutant egg chambers with a normal complement of 15 nurse cells and one oocyte were observed. We conclude that the fusome is defective in the absence of LIS1 protein. The wide spectrum of fusome phenotypes may result from the different levels of perdurance of LIS1 protein in germline stem cells after the clone is induced.

**Lis1 mutant egg chambers have packaging defects**

About 5% of the mutant cysts were compound egg chambers that contained a wild-type cyst and some mutant cells. These hybrid cysts were almost always found in association with a mutant cyst (Fig. 6). This condition occurred even in egg chambers that were surrounded by wild-type follicle cells. There were usually fewer than 16 combined Lis1 cells in the two adjacent mutant cysts. The mutant cells in the compound egg chamber were not transient clones generated during cystocyte division because the size of the mutant nuclei were the same in the two neighboring mutant egg chambers and were different in size from the wild-type nurse cells in the same egg chamber. Also, the wild-type cyst in the hybrid egg chamber contained a full complement of 15 nurse cells and one oocyte. The likely explanation for this packaging defect is that the Lis1 cysts were unstable and broke into smaller groups of interconnected cells during or shortly after the cystocyte divisions. It is possible that LIS1 is required for normal functions of the cyst cytoskeleton, important for maintaining...
the integrity of a cyst. Alternatively, the formation of compound egg chambers is due to mis-signaling between the Lis1 germline cells and the wild-type follicle or wild-type germline cells.

**Ring canals are morphologically normal in Lis1 egg chambers**

Ring canals are formed by dynamic assembly of cytoskeletal proteins (Robinson et al., 1994). In wild-type ovaries, they are composed of two layers: an outer rim, which is laid down upon arrest of the cleavage furrow and is adjacent to the plasma membrane, and an actin-rich inner rim, which accumulates after cysts are surrounded by follicle cells. The hts gene encodes a component of the inner rim and the hts cysts have defective ring canals which fail to accumulate actin (Yue and Spradling, 1992; Robinson et al., 1994). Since the defective cysts produced by Lis1 germline clones are similar to those observed in hts mutants, we examined ring canal formation in Lis1 clones.

Ovaries were double-stained with anti-β-galactosidase antibodies to mark the clones; FITC-conjugated phalloidin was used to visualize the filamentous actin component of ring canals and Hoechst 33258 to determine the number and size of nuclei. In all Lis1 egg chambers (Fig. 7B,D,F), the staining intensity and pattern of ring canals were indistinguishable from the control egg chambers (Fig. 7A,C,E), even though the polarized organization of ring canals seen in the wild-type (Fig. 7C) was disrupted. We conclude from these results that ring canal formation is apparently normal in the absence of LIS1.

**Fig. 5.** Fusomes are defective in Lis1 cysts. Germaria are stained with anti-β-galactosidase antibodies (green) to identify Lis1 cysts and a monoclonal anti-Hts antibody 1B1 (red) to visualize the fusomes. All images are stacks of confocal sections. Anterior is to the left. (A) Control gerarium containing spectosomes (ss.) in germline stem cells and fusomes (f.) in dividing cysts. (Only the most anterior fusome has been labeled.) (B,C) Germaria lacking Lis1 in most germline cells. The number of developing cysts is reduced. Fusomes in Lis1 cysts are unbranched (arrows in B), aberrantly fused (long arrow in C), fragmented (short arrow in C), or absent (arrowhead in C). (D) A gerarium containing a Lis1 cyst that has a fragmented fusome (arrow). All panels are shown at the same scale; bar in D represents 10 μm.

**Fig. 6.** Lis1 egg chambers have packaging defects. Egg chambers are stained with anti-β-galactosidase antibody to identify Lis1 clones. (A) Control egg chambers. (B,C) A Lis1 mutant egg chamber followed by a compound egg chamber that contains a wild-type cyst and some mutant cells (arrows). Note the Lis1 mutant egg chamber in B is aberrantly enclosed by follicle cells in conjunction with the compound egg chamber (arrowhead). All panels are shown at the same scale; bar in A represents 10 μm.
DISCUSSION

Despite increasing experimental insight into the possible function of LIS1, the mechanisms controlling neuronal migration and nuclear migration and the contribution of LIS1 to these mechanisms remain elusive. We have taken a reverse genetic approach to our study of the function of Lis1 in Drosophila and find that, as in vertebrates, Lis1 is an essential gene. Homozygous mutant larvae do not develop further than the second instar and die several days after growth-arrest. In vertebrates, the brain phenotype is due to haplo-insufficiency (Reiner et al., 1993; Lo Nigro et al., 1997) while, in Drosophila, one wild-type copy of the gene is apparently sufficient for normal function. However, the wide spectrum of phenotypes related to the perdurance of maternal LIS1 protein is consistent with the dosage-sensitivity observed in gene knock-out experiments in mice (Hirotune et al., 1998). Lis1 is strongly expressed in the central nervous system in Drosophila. It will be interesting to investigate whether LIS1 is required for embryonic development and for the function of the CNS. The stringent requirement of LIS1 during oogenesis prevents us from directly testing its function in embryonic development.

LIS1 is required for germline cell division, fusome integrity and oocyte differentiation

The rapid, synchronous divisions of the cystoblast are the basis for the formation of a normal germline cyst and the ultimate differentiation of the oocyte and 15 nurse cells. We found that, in Lis1 mutant germline clones, these critical cell divisions are disrupted, resulting in cysts with significantly reduced number of cells that almost never contain an oocyte. In these mutant cysts, the fusomes were aberrantly formed and did not branch normally, they were either fragmented or grew to larger than normal spheres that eventually disintegrated.

What is the connection between the cystoblast divisions and the fusome formation and branching, and how does LIS1 function impact these processes? At least two hypotheses can be advanced to explain the phenotypes that we observed. First, fusome formation may be more sensitive to the levels of LIS1 than are cystoblast divisions. If this is the case, the disruption of the rapid and synchronized germline cell divisions would result from the defects in the fusome. This hypothesis is supported by the similarity of the Lis1 cyst phenotypes to those observed in hts and α-spectrin mutants. In such mutants, the abnormal cysts are apparently caused by the disruption of the fusome. Both these genes encode membrane skeletal proteins that are integral parts of the fusome and, in mutants, the fusome is not formed (Yue and Spradling, 1992; Lin et al., 1994; de Cuevas et al., 1996).

The second hypothesis is that cystoblast division is directly affected by the absence of Lis1 function, resulting in arrest of division in early stages. The abnormality in cell division would result in the formation of the large spherical fusomes and their ultimate disintegration. Four observations support this second hypothesis. (1) In a germinarium that contained only Lis1 mutant cysts, the number of developing cysts was dramatically reduced. Lin and Spradling (1997) showed that fusomes are not essential for the division of germline stem cells and cystoblasts. Thus, the reduction in division rates of the stem cells or cystoblasts appears to be due to a lack of LIS1. (2) In some mutant cysts cell division was arrested and the nuclei apparently underwent endoreplication and became prematurely polyploid. The fusomes in these cysts often grew in size without branching and some fusomes later degenerated. (3) When we investigated the function of Lis1 by clonal analysis in the
eye, we never observed a mutant clone even though \( w^+ \) twin clones were present (Z. L. and R. S., unpublished data), a result consistent with \( Lis1 \) also functioning in the zygote to control cell division and differentiation. (4) In agreement with a function of \( Lis1 \) in cell division and differentiation, homozygous \( Lis1 \) knock-out mice die early in embryogenesis (Hirotsune et al., 1998).

Although it is as yet impossible to pinpoint the precise role of LIS1 in early oogenesis, it is tempting to speculate that LIS1 is involved in the movement of the ring canals and the newly formed fusome plugs to the original fusomes to form continuous fusomes and the rosette structure. LIS1 may also be involved in the constant transport of vesicles into fusomes to maintain the dynamic fusome structure.

The failure of \( Lis1 \) mutant cysts to differentiate an oocyte is not surprising. The disruption in germline cell division and fusome formation leads to the disruption of polarity of the cyst and consequently the differentiation of the oocyte. Our results show that, even when the fusome and cysts appear normal, oocyte differentiation is affected, suggesting a more stringent and constant requirement for LIS1 function in oocyte differentiation.

**LIS1, the dynein motor and the membrane cytoskeleton**

In \( A. nidulans \), the \( LIS1 \) homolog \( nudF \) interacts genetically with the cytoplasmic dynein heavy chain gene \( nudA \) (Willins et al., 1997). Other components of the dynein motor complex, cytoplasmic dynein light chain, p150, and centrin subunits of dynein receptor dynactin have also been implicated in the same pathway in filamentous fungi (Morrisset al., 1998a). Our results show that \( Lis1 \) and \( Dhc64C \) mutants have overlapping phenotypes in cyst formation, fusome integrity and oocyte differentiation, suggesting that the two genes also function in \( Drosophila \) in the same processes. An overlapping function is also suggested by our biochemical experiments.

LIS1 is present in microtubule-associated proteins (MAPs) prepared from \( Drosophila \) embryo extracts. However, unlike dynein, LIS1 is not enriched in MAP preparations and a substantial pool of LIS1 remains in the supernatant. Moreover, a significant amount of LIS1 is released when the MT pellet is washed in buffer and MTs are repelled. The significance of the association of LIS1 with MTs in vitro remains unclear but may reflect a low affinity interaction (Z. L., R. S. and T. Hays, unpublished data).

Several observations suggest that LIS1 not only functions in maintaining the integrity of fusomes, but also in maintaining membrane skeletons in general. First, vertebrate \( Lis1 \) was shown to associate in vitro with the pleckstrin homology domain of \( \beta \)-spectrin (Wang et al., 1995). Second, platelet-activating factor, the substrate of PAFAH, is localized to the plasma membrane (Vallari et al., 1990), suggesting a role for LIS1 in the localization of the enzyme complex to the plasma membrane. Third, in the heterotrimeric G protein, \( G \beta \) serves to anchor \( G \alpha \) to the cytoplasmic face of the plasma membrane. PAFAH is a unique G-protein-like trimer (Ho et al., 1997). By analogy, LIS1 could have a similar function as \( G \beta \).

The finding that centractin, a component of the dynactin complex, is associated with spectrin (Holleran et al., 1996) provides a link of the dynein motor complex with membrane skeletal proteins, thus dynein and LIS1 may interact at the plasma membrane.

In support of the idea that LIS1 functions in maintaining the integrity of membrane skeletons in general, we observed that mutant \( Lis1 \) cysts were mispackaged, a phenotype that has not been described for mutants in any of the genes that function in cyst formation in early gerarium. This phenotype may be due to a loss of organization and integrity of cyst cytoskeletons, such that migrating follicle cells separate one cyst into two parts.

The function of microtubules in germline cell division and in fusome formation remains to be elucidated. A prominent MT organization center is established only after the formation of the 16-cell cyst (Theurkauf et al., 1993). LIS1 and dynein are likely to interact, and to function throughout oogenesis to affect the transport of determinants to the oocyte and the migration of the oocyte nucleus. Indeed, both processes were affected in \( Lis1 \) hypomorphic mutations (A. Swan, T. Nguyen and B. Suter, personal communication).

The integrity of the membrane skeleton is essential for the organization of cells and the establishment of the MT network. Their disruption would affect both nuclear and cellular migration. \( Lis1 \) is likely to function in these two processes through the interactions with the dynein motor complex and membrane skeletons.

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**REFERENCES**


Deng, W. and Lin, H. (1997). Spectrosomes and fusomes anchor mitotic...
spindle during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. Dev. Biol. 247, 246-2476.


