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Regulation of dynein localization and centrosome positioning by *Lis-1* and *asunder* during *Drosophila* spermatogenesis

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SUMMARY

Dynein, a microtubule motor complex, plays crucial roles in cell-cycle progression in many systems. The LIS1 accessory protein directly binds dynein, although its precise role in regulating dynein remains unclear. Mutation of human *LIS1* causes lissencephaly, a developmental brain disorder. To gain insight into the in vivo functions of LIS1, we characterized a male-sterile allele of the *Drosophila* homolog of human *LIS1*. We found that centrosomes do not properly detach from the cell cortex at the onset of meiosis in most *Lis-1* spermatocytes; centrosomes that do break cortical associations fail to attach to the nucleus. In *Lis-1* spermatids, we observed loss of attachments between the nucleus, basal body and mitochondria. The localization pattern of LIS-1 protein throughout *Drosophila* spermatogenesis mirrors that of dynein. We show that dynein recruitment to the nuclear surface and spindle poles is severely reduced in *Lis-1* male germ cells. We propose that *Lis-1* spermatogenesis phenotypes are due to loss of dynein regulation, as we observed similar phenotypes in flies null for Tctex-1, a dynein light chain. We have previously identified *asunder* (*asun*) as another regulator of dynein localization and centrosome positioning during *Drosophila* spermatogenesis. We now report that *Lis-1* is a strong dominant enhancer of *asun* and that localization of LIS-1 in male germ cells is ASUN dependent. We found that *Drosophila* LIS-1 and ASUN colocalize and coimmunoprecipitate from transfected cells, suggesting that they function within a common complex. We present a model in which *Lis-1* and *asun* cooperate to regulate dynein localization and centrosome positioning during *Drosophila* spermatogenesis.

KEY WORDS: *Drosophila*, Spermatogenesis, Meiosis, Centrosomes, Basal body, Dynein

INTRODUCTION

Dynein is a minus-end-directed microtubule motor that exists in two forms. Axonemal dynein promotes microtubule sliding for beating of cilia and flagella. Cytoplasmic dynein moves processively along microtubules and, in addition to organelle positioning and transport, plays key roles in cell cycle events, including nucleus-centrosome coupling, nuclear envelope breakdown, spindle assembly/positioning and chromosome segregation (Gusnowski and Srayko, 2011; Hebbar et al., 2008; Huang et al., 2011; Salina et al., 2002; Splinter et al., 2010; Stuchell-Brereton et al., 2011; Wainman et al., 2009). Dynein is a large complex composed of four subunit types: heavy (containing motor activity), light, intermediate and light intermediate chains (Höök and Vallee, 2006; Susalka and Pfister, 2000).

Dynactin and LIS1 are dynein accessory factors (King and Schroer, 2000; Mesngon et al., 2006). LIS1 directly binds several dynein and dynactin subunits through its C-terminal WD-repeat domain, and LIS1 binding enhances dynein motor activity (Faulkner et al., 2000; Mesngon et al., 2006; Sasaki et al., 2000; Smith et al., 2000; Tai et al., 2002). The importance of LIS1 for dynein function is evidenced by the fact that *LIS1* mutants have defects in many dynein-dependent processes (Faulkner et al., 2000; Hebbar et al., 2008; Li et al., 2005; Tai et al., 2002).

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Loss or mutation of one copy of human *LIS1* (*PAFAH1B1* – Human Gene Nomenclature Database) causes type I lissencephaly (‘smooth brain’), a brain malformation disorder associated with neuronal migration defects (Gambello et al., 2003; Hirotsune et al., 1998; Vallee and Tsai, 2006; Wynshaw-Boris, 2007). Neuronal migration requires proper migration and positioning of the nucleus (Malone et al., 2003; Tanaka et al., 2004; Tsai and Gleeson, 2005). Dynein plays a major role in regulating these processes by promoting interaction of the nucleus with microtubules and microtubule-organizing centers.

The *Drosophila* homolog of human *Lis1* plays key roles during neurogenesis and oogenesis, presumably via its regulation of dynein. *Drosophila Lis-1* neuroblasts have defects in centrosome migration, bipolar spindle assembly, centrosomal attachment to spindles and spindle checkpoint function (Siller and Doe, 2008; Siller et al., 2005). In *Drosophila* oocytes, *Lis-1* regulates nuclear migration and positioning (Lei and Warrior, 2000). A detailed characterization of the role of *Lis-1* in *Drosophila* spermatogenesis, however, has not been reported.

Drosophila spermatogenesis is an ideal system for studying cell division. Meiotic spindles of spermatocytes are large and, hence, convenient for cytological analysis, relaxed checkpoints facilitate the study of cell cycle mutants and alterations in the highly regular appearance of immature spermatids are diagnostic of meiotic division defects (Cenci et al., 1994; Rebollo and González, 2000). The stages of *Drosophila* spermatogenesis are well defined (Fuller, 1993). Germline stem cells give rise to spermatogonia, which undergo four synchronous mitotic divisions with incomplete cytokinesis to generate 16-cell cysts of primary spermatocytes. After premeiotic S phase, primary spermatocytes enter G2, a prolonged growth period. Meiosis I yields 32-cell cysts of

secondary spermatocytes and meiosis II generates 64-cell cysts of haploid spermatids. Immature, round spermatids differentiate into mature sperm. A unique feature of spermatids in *Drosophila* and other insects involves formation of a multi-layered mitochondrial aggregate, the Nebenkern, which provides energy for beating of the sperm flagella.

We have previously identified *asunder* (*asun*) as a regulator of dynein-dynactin localization during *Drosophila* spermatogenesis (Anderson et al., 2009). *asun* spermatocytes and spermatids show defects in nucleus-centrosome and nucleus-basal body coupling, respectively. Dynein mutation disrupts nucleus-centrosome attachments in *Drosophila* and *C. elegans* embryos (Gönczy et al., 1999; Robinson et al., 1999). A pool of dynein anchored at the nuclear surface is thought to promote stable interactions between the nucleus and centrosomes by mediating minus-end-directed movement of the nucleus along astral microtubules (Reinsch and Gönczy, 1998). We observed reduction of perinuclear dynein in *asun* male germ cells that we hypothesize causes loss of nucleus-centrosome and nucleus-basal body coupling (Anderson et al., 2009).

Drosophila Lis-1 was previously reported to be required for male fertility, although its role in the male germ line has not been further characterized (Lei and Warrior, 2000). In this study, we have analyzed the role of *Lis-1* during *Drosophila* spermatogenesis. We found that *Lis-1* regulates centrosome positioning in spermatocytes and promotes attachments between the nucleus, basal body and Nebenkern in spermatids. LIS-1 colocalizes with dynein-dynactin at the nuclear surface and spindle poles of male germ cells and is required for recruiting dynein-dynactin to these sites. We provide evidence to support our model that *Lis-1* and *asun* cooperate to regulate dynein localization and centrosome positioning during *Drosophila* spermatogenesis.

MATERIALS AND METHODS

Drosophila stocks

y w was used as 'wild-type' stock. Transgenic flies expressing β1-tubulin (product of *βTub56D* gene) fused at its C-terminal end to GFP and under control of the *Ubi-p63E* (ubiquitin) gene promoter were a gift from H. Oda and Y. Akiyama-Oda (JT Biohistory Research Hall, Osaka, Japan). Transgenic flies expressing GFP-PACT and DMN-GFP were gifts from J. Raff (University of Oxford, Oxford, UK) and T. Hays (University of Minnesota, Minneapolis, MN), respectively. Transgenic flies expressing GFP-ASUN were previously described (Anderson et al., 2009). *tctex-1*^{e155} was a gift from T. Hays. *piggyBac* insertion lines *asun*^{f02815} and *f01662* were from the Exelixis Collection (Harvard Medical School, Boston, MA). *Lis-1*^{k11702}, *Df(2R)JP5*, *Df(3R)Exel6178* and *piggyBac* transposase were from Bloomington Stock Center (Indiana University, IN).

Cherry-LIS-1 transgenic fly lines

cDNA encoding *Drosophila* LIS-1 (clone LD11219, *Drosophila* Gene Collection) with an N-terminal Cherry tag was subcloned into vector tv3 (a gift from J. Brill, The Hospital for Sick Children, Toronto, Canada) for expression of Cherry-LIS-1 under control of the testes-specific *β2-Tubulin* promoter (Wong et al., 2005). Transgenic lines were generated by *P*-element-mediated transformation via embryo injection (Rubin and Spradling, 1982).

Generation of a null allele of *asun*

piggyBac insertion lines *asun*^{f02815} and *f01662* were used to generate a two-gene [*belphegor* (*bor*) and *asun*] deletion line via FLP-mediated recombination of FRT sites in the transposons as previously described (Parks et al., 2004). A 4 kb genomic fragment containing *bor* and flanking regions (supplementary material Fig. S9) was PCR-amplified from BAC clone BACR05P04 (*Drosophila* Genomics Resource Center, Indiana University, IN) and subcloned into pCaSpeR4. A stop codon was added to 5′ *asun*-coding region, and a transgenic line was made using this construct. *asun*^{d93} flies are homozygous for the *bor asun* two-gene deletion and *bor* transgene.

Male fertility assay

Individual males (2 days old) were placed in vials with five wild-type females (2 days old) and allowed to mate for 5 days. The mean number of adult progeny eclosed per vial was determined (25 males tested per genotype).

Cytological analysis of live and fixed testes

Live and fixed testes cells were prepared for phase-contrast or fluorescent microscopy, as described previously (Anderson et al., 2009). Acetylated tubulin antibodies (6-11B-1, 1:50, Sigma-Aldrich) were also used herein. Wild-type and mutant testes were isolated and prepared for microscopy in parallel and under identical conditions for all experiments. Our designation of 'late G2' and 'prophase' primary spermatocytes corresponds to S5/S6 and M1a spermatocytes, respectively, according to the staging system of Cenci et al. (Cenci et al., 1994). We used four criteria to score primary spermatocytes as being in prophase: (1) well-separated centrosomes; (2) initiation of chromatin condensation (as evidenced by DAPI staining); (3) the presence of robust arrays of microtubules surrounding centrosomes (visualized by using the beta1-tubulin-GFP transgene); and (4) lack of appreciable nuclear envelope breakdown (as evidenced by clear demarcation between nucleus and cytoplasm when viewing beta1-tubulin-GFP in the cytoplasm) (Cenci et al., 1994; Fuller, 1993; Rebollo et al., 2004; Tates, 1971). Confocal images were obtained with a Leica TCS SP5 confocal microscope and Leica Application Suite Advanced Fluorescence (LAS-AF) software using maximum-intensity projections of *z*-stacks collected at 0.75 μm/step with a 63× objective lens.

Immunoblotting

Homogenized testes extracts from newly eclosed flies were analyzed by SDS-PAGE (four testes pairs/lane) and immunoblotting using standard techniques. Primary antibodies were used as follows: dynein heavy chain (P1H4, 1:2000), dynein intermediate chain 1 (74.1, 1:1000, Santa Cruz), Dynamitin (1:250, BD Biosciences or ab56687, 1:1000, Abcam), mCherry (1:500, Clontech), β-tubulin (E7, 1:1000, Developmental Studies Hybridoma Bank), Cdk1 (PSTAIR, 1:1000, Upstate) and GAPDH (14C10, 1:1000, Cell Signaling). HRP-conjugated secondary antibodies and chemiluminescence were used to detect primary antibodies.

Mammalian cell experiments

HeLa cells were maintained and transfected as described previously (Anderson et al., 2009). Plasmids for expression of N-terminally tagged versions of *Drosophila* ASUN and/or LIS-1 in cultured human cells were generated by subcloning into pCS2. For colocalization, HeLa cells were transfected with Cherry-LIS-1 and GFP-ASUN constructs using Lipofectamine 2000 (Invitrogen), treated with nocodazole (5 μg/ml) at 24 hours, fixed for 5 minutes at –20°C with methanol and mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). Images were obtained using an Eclipse 80i microscope (Nikon) with Plano-Apo 100× objective. For co-immunoprecipitation, lysates of transfected HEK293 cells co-expressing HA-ASUN with c-Myc tag or c-Myc-tagged *Drosophila* LIS-1 were made in non-denaturing lysis buffer [50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 1% Triton X-100]. Lysates (500 μg) were incubated with anti-c-Myc agarose beads (40 μl; Sigma) for 3 hours with shaking at 4°C. Beads were washed three times in lysis buffer and boiled in 6× sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting with c-Myc (9E10, 1:1000) and HA (CAS 12, 1:1000) antibodies.

RESULTS

Lis-1 is required for spermatogenesis

To analyze the role of *Lis-1* in *Drosophila* spermatogenesis, we obtained a male-sterile allele, *Lis-1*^{k11702}, with a *P*-element insertion in the 5′-UTR of *Lis-1* (supplementary material Fig. S1A) (Lei and Warrior, 2000). We found that homozygous and hemizygous *Lis-*

1^{k11702} males uniformly failed to produce any progeny (supplementary material Fig. S1B). Fertility of *Lis-1*^{k11702} males was fully restored via transgenic expression of Cherry-tagged LIS-1 in male germ cells or by precise *P*-element excision; the former fully rescued all other *Lis-1*^{k11702} phenotypes presented herein (supplementary material Fig. S1B; data not shown). To assess mature sperm production, we examined *Lis-1*^{k11702} seminal vesicles (supplementary material Fig. S1C). Although the size and shape of *Lis-1*^{k11702} testes appeared normal, seminal vesicles were empty, suggesting that *Lis-1*^{k11702} male sterility results from disruption of spermatogenesis.

Lis-1 spermatocytes have abnormal centrosome positioning and meiotic spindle formation

We sought to determine the earliest stage at which spermatogenesis is disrupted in *Lis-1*^{k11702} testes. As in wild type, we observed 16-cell cysts of primary spermatocytes in *Lis-1*^{k11702} testes (26/26 cysts scored), indicating successful completion of four rounds of spermatogonial divisions. *Lis-1*^{k11702} spermatocytes, however, exhibited profound defects in centrosome positioning and meiotic spindle structure.

During the G2 growth phase of wild-type primary spermatocytes, centrosomes are anchored at the cell cortex; at G2/M, centrosomes migrate back towards the nucleus and begin to separate from each other (Fuller, 1993; Rebollo et al., 2004; Tates, 1971). Once reattached to the nuclear surface, centrosomes move to opposite poles during prophase. Approximately 90% of *Lis-1*^{k11702} prophase spermatocytes had centrosomes positioned at the cortex rather than the nuclear surface, presumably owing to failure to break their cortical associations; wild-type cells rarely (<0.5%) showed this configuration (Fig. 1A-M, supplementary material Fig. S2). Cortical centrosomes of *Lis-1*^{k11702} prophase spermatocytes appeared to separate normally and undergo migration to opposite poles. >10% of *Lis-1*^{k11702} prophase spermatocytes had free centrosomes (unattached to the cortex or nuclear surface) similar to those of *asun* mutants, a phenotype observed in ~3% of wild-type cells (Anderson et al., 2009). Approximately 60% of prophase spermatocytes heterozygous for *Lis-1*^{k11702} had either cortical or free centrosomes.

In dividing *Lis-1*^{k11702} spermatocytes, meiotic spindles were typically associated with cortically positioned centrosomes (~95% vs. <2% for wild-type during metaphase; Fig. 1N-R). These observations suggest that cortical centrosomes present in *Lis-1* prophase I spermatocytes assemble meiotic spindles, although we have not excluded the possibility that spindles form normally in the mutants followed by pushing of centrosomes to the cortex during spindle elongation. *Lis-1*^{k11702} spindles were relatively long and wavy with occasional detachment of cortical centrosomes from spindle poles; centrosomal detachment from mitotic spindle poles has similarly been reported for *Lis-1* neuroblasts and early embryos (Robinson et al., 1999; Siller et al., 2005; Wojcik et al., 2001). Despite defects in centrosome positioning and meiotic spindle structure, cytokinesis and chromosome segregation surprisingly did not appear to be grossly affected in *Lis-1*^{k11702} spermatocytes, as most round spermatids contained a single nucleus of uniform size (99%; 612/619 spermatids) (Fuller, 1993).

We have previously reported that *asun* is required for centrosome positioning in *Drosophila* spermatocytes (Anderson et al., 2009). Most *asun*^{f02815} spermatocytes arrest in prophase I with free centrosomes. We did not, however, find an increased fraction of *Lis-1*^{k11702} spermatocytes in prophase (supplementary material Fig. S3). Microtubules on the nuclear surface have been implicated

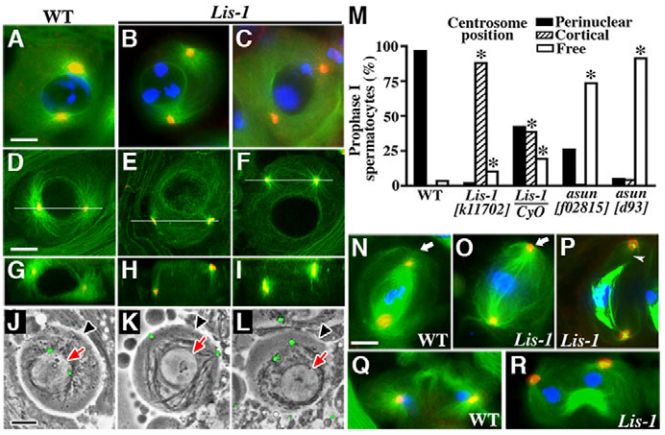

Fig. 1. Defective centrosome positioning in *Lis-1* spermatocytes. (A-L) Centrosomes normally at the nuclear surface (A,D,G,J) are cortical (B,E,H,K) or free (C,F,I,L) in *Lis-1*^{k11702} prophase I spermatocytes. (A-I) Spermatocytes expressing β-tubulin-GFP (green) stained for γ-tubulin (red; centrosome marker). (**A-C**) Epifluorescent micrographs. DNA in blue. (**D-I**) XY projections (D-F) and corresponding *xz* optical sections (G-I). White bars mark positions of corresponding *xz* optical sections. (**J-L**) Phase/fluorescence overlay images of spermatocytes expressing GFP-PACT (green; centriole marker). Red arrows and black arrowheads mark the surface of the nucleus (phase-light) and plasma membrane, respectively, of each cell. (**M**) Quantification of centrosome positioning defects in *Lis-1* and *asun* prophase I spermatocytes expressing β-tubulin-GFP and stained for γ-tubulin (over 100 cells scored per genotype). **P*<0.0001 (Fisher's exact test). (**N-R**) Centrosomes of metaphase (N-P) and telophase (Q,R) spindles are at the cortex (arrows) and often detached (arrowhead) in *Lis-1*^{k11702} but not wild-type spermatocytes. Scale bars: 10 μm.

in nuclear envelope breakdown at prophase exit (Beaudouin et al., 2002; Salina et al., 2002). We found an accumulation of microtubules (both total and acetylated) surrounding the nucleus of wild-type and *Lis-1*^{k11702} prophase spermatocytes that was absent in *asun*^{f02815} mutants (supplementary material Fig. S4); this difference may explain the prophase arrest observed in *asun*^{f02815}, but not *Lis-1*^{k11702}, mutants.

Lis-1 spermatids lack nucleus-Nebenkern-basal body attachments and have abnormal Nebenkern morphology

Wild-type round spermatids contain a phase-light nucleus and a phase-dark mitochondrial aggregate (Nebenkern) of roughly equal size; both organelles associate with a centriole-derived basal body at the site of nucleus-Nebenkern linkage (Fig. 2A) (Fuller, 1993). Given the lack of nucleus-centrosome attachments in *Lis-1* spermatocytes (Fig. 1), we assessed nucleus-basal body attachments in *Lis-1* spermatids using male germline expression of GFP-tagged Pericentrin/AKAP450 centrosomal targeting (PACT) domain to label basal bodies (Martinez-Campos et al., 2004). We observed nucleus-basal body uncoupling in most *Lis-1*^{k11702} hemizygous spermatids (Fig. 2B-D). Furthermore, we frequently observed loss of Nebenkern-basal body and nucleus-Nebenkern attachments in *Lis-1*^{k11702} hemizygous spermatids (Fig. 2B-D). These results suggest that LIS-1 is required to maintain normal linkages between the nucleus, Nebenkern and basal body during spermatogenesis. Nebenkerne of wild-type spermatids typically have a round uniform shape (Fig. 2E). In *Lis-1*^{k11702} spermatids,

however, we occasionally observed Nebenkerne with abnormal morphology (Fig. 2F-H). These findings suggest that LIS-1 plays a role in Nebenkern formation and/or maintenance.

During spermatid elongation, the Nebenkern unfurls and elongates with the growing axoneme (Fuller, 1993). Because the basal body nucleates the axoneme, we asked whether Nebenkern-basal body uncoupling in *Lis-1*^{k11702} spermatids would affect this process. We found that the Nebenkern properly associated with the axoneme in early elongating *Lis-1*^{k11702} spermatids, suggesting that Nebenkern-basal body coupling is not essential for this process (supplementary material Fig. S5) (Anderson et al., 2009; Inoue et al., 2004).

Defects in late spermatogenesis in *Lis-1* testes

Spermatids must undergo elongation and individualization to form functional sperm (Fuller, 1993). During elongation, nuclei and associated basal bodies are positioned at the proximal tips of growing spermatid bundles, and round nuclei acquire a needle-like shape (Fig. 2I). During individualization, actin investment cones move in unison along the axoneme length, resolving cytoplasmic bridges between spermatids formed in a common cyst (Fig. 2K). In *Lis-1*^{k11702} testes, however, we observed unattached round nuclei and basal bodies dispersed throughout the length of elongating spermatid bundles as well as sparse, disorganized investment cones (Fig. 2J,L). These results suggest that LIS-1 is required for positioning of spermatid nuclei within growing bundles. The random distribution of investment cones within elongating *Lis-1*^{k11702} spermatid bundles may reflect loss of nuclear positioning, as investment cones are thought to originate at the nuclear surface (Texada et al., 2008).

LIS-1 localization during spermatogenesis mirrors dynein-dynactin

Our results suggested roles for *Lis-1* in the regulation of centrosome positioning, meiotic spindle assembly, nucleus-Nebenkern-basal body associations, Nebenkern morphogenesis and nuclear positioning during *Drosophila* spermatogenesis. To gain insight into how *Lis-1* affects these processes, we examined the subcellular localization of LIS-1 during spermatogenesis using transgenic flies co-expressing Cherry-LIS-1 and β-tubulin-GFP. LIS-1 is dispersed in the cytoplasm during early G2 with enrichment around the nucleus by late G2 (Fig. 3A; data not shown). Perinuclear LIS-1 becomes focused at centrosomes during prophase I and II (Fig. 3B,E). Throughout both meiotic divisions, LIS-1 concentrates at spindle poles (Fig. 3C,D; data not shown). In early spermatids, LIS-1 forms a hemispherical cap on the nuclear surface (Fig. 3F). Similar localizations have been reported for LIS-1 during mitosis; in contrast to these studies, however, we did not detect LIS-1 at the cortex during *Drosophila* male meiosis (Cockell et al., 2004; Coquelle et al., 2002; Faulkner et al., 2000; Li et al., 2005; Tai et al., 2002).

Cherry-LIS-1 localization during *Drosophila* spermatogenesis is strikingly similar to that of dynein-dynactin, suggesting that LIS-1 and dynein-dynactin may colocalize at these sites (Anderson et al., 2009). We examined male germ cells co-expressing Cherry-LIS-1 and GFP-tagged Dynamitin (DMN), the p50 subunit of dynactin, which colocalizes with dynein throughout spermatogenesis (McGrail and Hays, 1997; Wojcik et al., 2001). LIS-1 colocalized with dynactin at the nuclear surface of G2 spermatocytes and spermatids and at meiotic spindle poles (Fig. 3G-L). In prometaphase spermatocytes, LIS-1 colocalized with dynactin at kinetochores (Fig. 3I). These results are consistent with tight association between LIS-1 and dynein-dynactin complexes during *Drosophila* spermatogenesis, as has been reported in other systems (Faulkner et al., 2000; Mesngon et al., 2006; Sasaki et al., 2000; Smith et al., 2000; Tai et al., 2002).

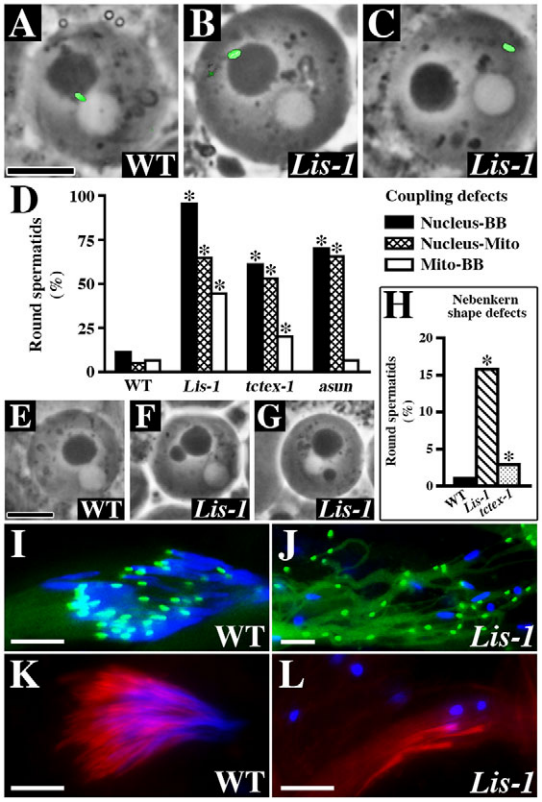

Fig. 2. *Lis-1* spermatid defects. (**A**-**C**) Phase/fluorescence overlay images of round spermatids expressing GFP-PACT (green). Normal associations between the nucleus (phase-light), Nebenkern (phase-dark) and basal body (green) are lost in *Lis-1*^{k11702} spermatids. (**D**) Quantification of coupling defects in *Lis-1*, *tctex-1* and *asun* round spermatids observed in phase/fluorescence overlay micrographs. A given spermatid may have been scored as defective in more than one category (loss of nucleus-basal body (BB), nucleus-Nebenkern (Mito) and/or Nebenkern-basal body coupling). (**E-G**) Phase-contrast images reveal abnormal Nebenkern morphology in *Lis-1*^{k11702} round spermatids. (**H**) Quantification of Nebenkern morphology defects in *Lis-1* and *tctex-1* spermatids. (**I-L**) Elongating bundles of spermatids expressing GFP-PACT (green; I,J) or stained with phalloidin (red; marks individualization cones; K,L). DNA in blue. *Lis-1*^{k11702} bundles are disorganized compared with wild type. Scale bars: 10 μm. Genotypes used for graphs: *Lis-1*^{k11702}*/Df(2R)JP5*, *tctex-1*^{e155}*/Df(3R)Exel6178*, *asun*^{f02815}*/asun*^{d93} (over 500 spermatids scored per genotype). **P*<0.0001 (Fisher's exact test).

Lis-1 male germ cells show loss of dynein-dynactin localization

Although *Lis-1* is an established regulator of dynein-dynactin, its mechanism of action is unclear. Localizations of LIS-1 and dynein-dynactin within cells have been shown in several cases to be dependent on each other, although their interdependency varies with the model system and subcellular sites (Cockell et al., 2004; Coquelle et al., 2002; Lam et al., 2010; Lee et al., 2003). We examined localization of dynein-dynactin complexes in *Lis-1*^{k11702} male germ cells using antibodies against dynein heavy chain and transgenic expression of DMN-GFP (McGrail and Hays, 1997). Dynein-dynactin is normally enriched at the nuclear surface of G2 spermatocytes and round spermatids, and at spindle poles of meiotic spermatocytes (Anderson et al., 2009; Li et al., 2004). We found a significant reduction in dynein-dynactin localization to

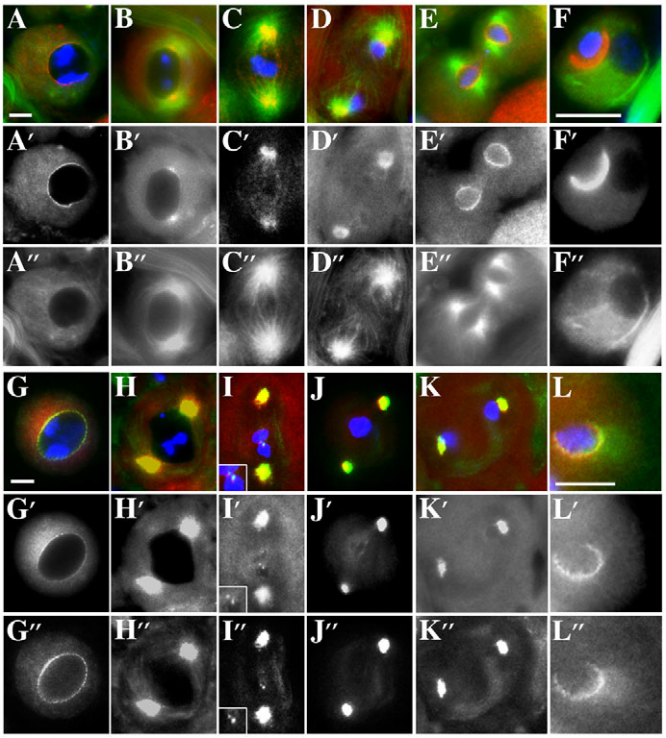

Fig. 3. Colocalization of LIS-1 and dynactin during male meiosis. (**A-F″**) Wild-type male germline cells co-expressing Cherry-LIS-1 (red, A-F; grayscale, A′-F′) and β-tubulin-GFP (green, A-F; grayscale, A″-F″). (A-A″) Late G2 spermatocyte. (B-B″) Late prophase I. (C-C″) Metaphase I. (D-D″) Telophase I. (E-E″) Early prophase II. (F-F″) Round spermatid. (**G-L″**) Wild-type male germline cells show colocalization of Cherry-LIS-1 (red, G-L; grayscale, G′-L′) and DMN-GFP (green, G-L; grayscale, G″-L″). (G-G″) Late G2 spermatocyte. (H-H″) Prophase I. (I-I″) Prometaphase I. Inset shows colocalization of Cherry-LIS-1 and DMN-GFP at kinetochores. (J-J″) Metaphase I. (K-K″) Telophase I. (L-L″) Round spermatid. DNA in blue. Scale bars: 10 μm.

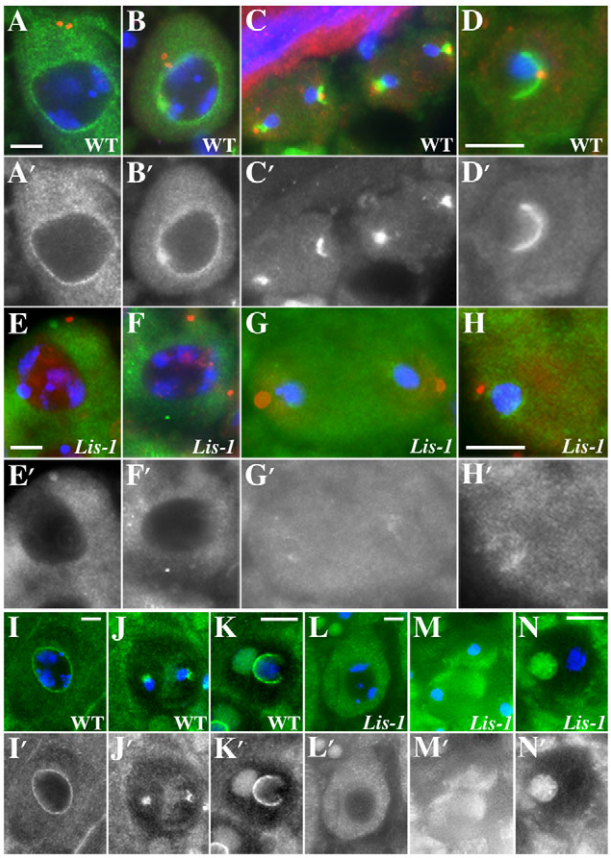

Fig. 4. Loss of dynein-dynactin localization in *Lis-1* male germline cells. (**A-H′**) Male germline cells stained for dynein heavy chain (green, A-H; grayscale, A′-H′) and PLP (red; centriole marker). (**I-N′**) Male germline cells expressing DMN-GFP (green, I-N; grayscale, I′-N′). *Lis-1*^{k11702} cells have reduced dynein-dynactin localization relative to wild type. Late G2 (A,A′,E,E′,I,I′,L.L′), prophase I (B,B′,F,F′) and telophase (C,C′,G,G′,J,J′,M,M′) spermatocytes and round spermatids (D,D′,H,H′,K,K′,N,N′) shown. DNA in blue. Scale bars: 10 μm.

these sites in *Lis-1*^{k11702} testes (over 95% reduction in G2 spermatocytes and over 80% reduction in spermatids, over 200 cells scored each; Fig. 4, supplementary material Fig. S6). Immunoblotting revealed normal levels of core dynein-dynactin proteins in *Lis-1*^{k11702} testes, suggesting that decreased enrichment at these sites was not due to decreased stability of complex components (supplementary material Fig. S7).

tctex-1 male germ cells have *Lis-1*-like phenotypes

We hypothesized that the defects we observed in *Lis-1*^{k11702} male germ cells are a consequence of decreased dynein function. To test this hypothesis, we sought to assess spermatogenesis in dynein-dynactin mutants. Most null mutations in *Drosophila* dynein-dynactin subunits are lethal, however, thus complicating an analysis of their roles during spermatogenesis. We used flies null for the *Drosophila* ortholog of Tctex-1 (Dlc90F – FlyBase), the 14 kDa dynein light chain, because they are viable but male sterile (Li et al., 2004).

In *tctex-1* male germ cells [*tctex-1*^{e155}/*Df(3R)Exel6178* used in this study], we identified all *Lis-1*^{k11702} phenotypes described above, albeit to a lesser degree. Both cortical and free centrosomes were observed at higher rates in *tctex-1* prophase spermatocytes (~14% and ~17%, respectively) compared with wild-type cells (<0.5% and ~3%, respectively; $P<0.001$) (Fig. 5A-I). *tctex-1* spermatids exhibited loss of wild-type nucleus-basal body, nucleus-Nebenkern and Nebenkern-basal body attachments and occasionally had Nebenkern shape defects (Fig. 2D,H, Fig. 5J-L). We observed significant loss of dynein localization to the nuclear surface of *tctex-1* G2 spermatocytes and spermatids (87% and 64% of cells, respectively; over 200 cells scored each) and spindle poles during meiotic divisions (Fig. 5M-R, supplementary material Fig. S6). Another group previously reported nucleus-basal body coupling defects and reduced perinuclear dynein in *tctex-1* spermatids (Li et al., 2004). We observed a less severe degree of reduction of perinuclear dynein in *tctex-1* male germ cells compared to *Lis-1*^{k11702}; this difference might explain the lower percentage of spermatocytes with centrosome positioning defects in *tctex-1* versus *Lis-1*^{k11702} testes (supplementary material Fig. S6). Wild-type levels of core dynein-dynactin subunits were detected in *tctex-1* testes by immunoblotting, suggesting that loss of dynein localization is not secondary to decreased levels of complex components (supplementary material Fig. S7).

Our phenotypic characterization of *Lis-1*^{k11702} male germ cells suggested that LIS-1 is required for proper subcellular localization of dynein. To test for a reciprocal requirement, we assessed Cherry-LIS-1 localization in *tctex-1* male germ cells. We found that *tctex-1*

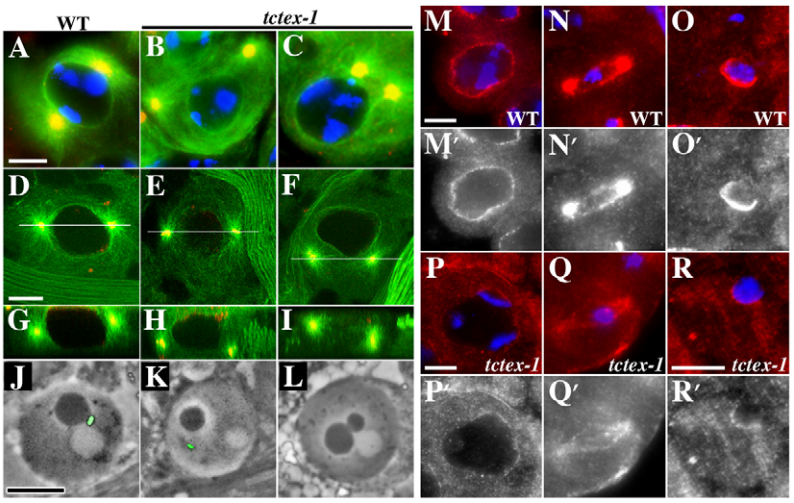

Fig. 5. Dynein light chain mutant male germline cells exhibit *Lis-1* phenotypes. (A-I) Prophase I spermatocytes expressing β-tubulin-GFP (green) stained for γ-tubulin (red). Roughly one-third of *tctex-1* spermatocytes have cortical (B,E,H) or free (C,F,I) centrosomes (normally at the nuclear surface; A,D,G). (**A-C**) Epifluorescent micrographs. DNA in blue. (**D-I**) *xy* projections (D-F) and corresponding *xz* optical sections (G-I). White bars mark positions of corresponding *xz* optical sections. (**J**,**K**) Phase/fluorescence overlay images of round spermatids expressing GFP-PACT (green) show wild-type nucleus-Nebenkern-basal body interactions that are lost in *tctex-1* mutants. (**L**) Phase-contrast image of *tctex-1* spermatid with defective Nebenkern morphology. (**M-R′**) Male germline cells stained for dynein heavy chain (red, M-R; grayscale, M′-R′) and DNA (blue). *tctex-1* cells have reduced dynein localization relative to wild type. Late G2 (M,M′,P,P′) and metaphase II (N,N′,Q,Q′) spermatocytes and round spermatids (O,O′,R,R′) shown. Scale bars: 10 μm.

spermatocytes and spermatids had wild-type levels of Cherry-LIS-1 on the nuclear surface (>200 of each cell type scored), suggesting that LIS-1 can be recruited to this site independent of dynein complexes (supplementary material Figs S6, S8).

Lis-1 dominantly enhances *asun*

We have previously identified *asun* as a regulator of dynein during *Drosophila* spermatogenesis (Anderson et al., 2009). Both $asun^{f02815}$ (hypomorphic allele) and $Lis\text{-}1^{k11702}$ male germ cells show loss of nucleus-centrosome and nucleus-basal body attachments, probably owing to reduction of perinuclear dynein. Given these shared phenotypes, we questioned whether ASUN and LIS-1 might cooperate in regulating spermatogenesis. We tested for genetic interactions between *asun* and *Lis-1* and found that the phenotype of $asun^{f02815}$ males carrying a single copy of $Lis\text{-}1^{k11702}$ was strongly enhanced; similar results were obtained using a deficiency that uncovers *Lis-1* (Fig. 6; data not shown). The testes of these $Lis\text{-}1^{k11702}/+$; $asun^{f02815}$ males were small compared with $Lis\text{-}1^{k11702}/+$ or $asun^{f02815}$ males (Fig. 6A-C). The reduction in size ranged from mild to severe; an example of the latter is shown in Fig. 6C. Conversely, we did not detect dominant enhancement of *Lis-1* by *asun* (data not shown).

We found an extreme paucity of sperm bundles in $Lis\text{-}1^{k11702}/+$; $asun^{f02815}$ testes compared with $asun^{f02815}$ testes, suggesting a block in spermatogenesis that would account for the reduced size of $Lis\text{-}1^{k11702}/+$; $asun^{f02815}$ testes (Fig. 6D,E). Although $asun^{f02815}$ testes contain an increased fraction of prophase I spermatocytes, cells at all stages of spermatogenesis can be readily identified (Anderson et al., 2009) (Fig. 6F). We observed a preponderance of late G2 primary spermatocytes in $Lis\text{-}1^{k11702}/+$; $asun^{f02815}$ testes with very few cells beyond this stage of spermatogenesis, indicative of a severe G2 block (Fig. 6G). This phenotype was more severe than meiotic phenotypes observed in male flies homozygous for $Lis\text{-}1^{k11702}$ (no block), $asun^{f02815}$ (prophase block) or a null allele of asun (prophase block, described below); thus, it does not appear to represent merely an additive effect of the two alleles. These findings suggest that *Lis-1* and *asun* cooperate in the regulation of *Drosophila* spermatogenesis.

asun-null phenotype

In contrast to $Lis\text{-}1^{k11702}$, we rarely observed cortical centrosomes in $asun^{f02815}$ prophase spermatocytes (Fig. 1M). Our previous studies suggested that $asun^{f02815}$ is a hypomorphic allele (Anderson et al., 2009). We questioned whether lack of the cortical centrosome phenotype in $asun^{f02815}$ spermatocytes might be due to low allele strength. To obtain a null allele of *asun*, we generated a two-gene deletion that removed most of the *asun* coding region and its entire neighboring gene, *belphegor* (*bor*) (supplementary material Fig. S9A). *bor* is predicted to encode an ATPase of unknown function. Homozygous lethality of this deletion was rescued by a *bor* transgene, thus demonstrating that *bor*, but not *asun*, is essential for viability. Males homozygous for the two-gene deletion and carrying the *bor* transgene (referred to hereafter as $asun^{d93}$) were completely sterile. All $asun^{d93}$ phenotypes reported herein were fully rescued via male germline-specific expression of GFP-ASUN, confirming that they were due to loss of *asun* (data not shown).

Nucleus-centrosome uncoupling was more severe in $asun^{d93}$ than $asun^{f02815}$ prophase spermatocytes (Fig. 1M, supplementary material Fig. S9B-D). As for $asun^{f02815}$, cortical centrosomes were rare in $asun^{d93}$ prophase spermatocytes, suggesting that centrosome detachment from the cortex during late G2 requires LIS-1 but not ASUN (Fig. 1M). As expected, based on our study of $asun^{f02815}$, perinuclear dynein-dynactin enrichment was greatly diminished in $asun^{d93}$ spermatocytes and spermatids (supplementary material Figs S6, S9E,F; data not shown) (Anderson et al., 2009). $asun^{d93}$ round spermatids, which were scarce due to strong prophase I arrest, contained multiple nuclei and four basal bodies, indicative of severe cytokinesis defects (99%; 99/100 cells) (supplementary material Fig. S9G,H). $asun^{d93}$ spermatids exhibited nucleus-basal body and nucleus-Nebenkern coupling defects; in contrast to $Lis\text{-}1^{k11702}$ spermatids, however, Nebenkern-basal body coupling appeared normal (20/20 cells; supplementary material Fig. S9G,H). Most transheterozygous $asun^{d93}/asun^{f02815}$ spermatids exhibited the same constellation of coupling defects as the null mutants (Fig. 2D).

LIS-1 localization is ASUN dependent

Given shared spermatogenesis phenotypes and genetic interaction between *Lis-1* and *asun*, we questioned whether LIS-1 and ASUN might regulate the localization of one another. We expressed Cherry-LIS-1 in $asun^{f02815}$ testes to assess the effects of decreased ASUN function on LIS-1 localization. We observed severe reduction of Cherry-LIS-1 on the nuclear surface of spermatocytes and spermatids and at spindle poles of dividing spermatocytes in $asun^{f02815}$ testes (over 97% of G2 spermatocytes and over 80% of spermatids, over 200 of each cell type scored; Fig. 7, supplementary material Fig. S6). Cherry-LIS-1 accumulation at these sites remains normal in males with mutation of a testes-specific β-tubulin subunit, suggesting that

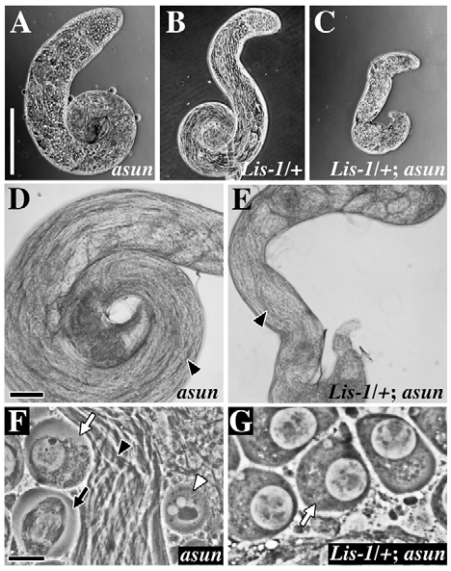

Fig. 6. *Lis-1* dominantly enhances *asun*. (**A-C**) Phase-contrast images of whole testes show reduced size of *Lis-1*^{k11702}*/+;asun*^{f02815} compared with *asun*^{f02815} and *Lis-1*^{k11702}/+. Scale bar: 250 μm. (**D**,**E**) Higher magnification images show paucity of sperm bundles (arrowheads) in *Lis-1*^{k11702}*/+;asun*^{f02815} testes compared with *asun*^{f02815}. Scale bar: 50 μm. (**F**,**G**) Phase-contrast image shows *asun*^{f02815} male germ cells at various stages of spermatogenesis: G2 spermatocytes (white arrow), dividing spermatocytes (black arrow), round spermatids (white arrowhead) and sperm bundles (black arrowhead); most cells from *Lis-1*^{k11702}*/+;asun*^{f02815} testes are G2 spermatocytes (white arrow). Scale bar: 10 μm.

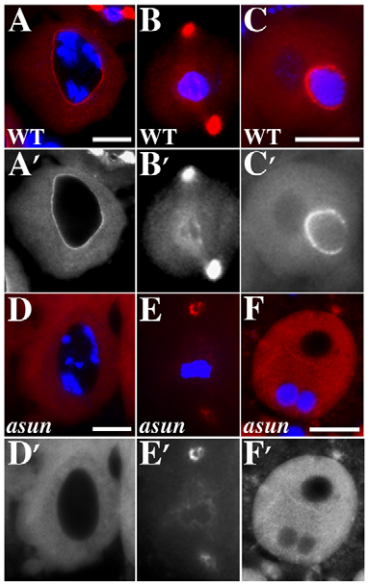

Fig. 7. Loss of LIS-1 localization in *asun* male germline cells. (**A-F′**) Male germline cells expressing Cherry-LIS-1 (red, A-F; grayscale, A′-F′) and DNA-stained (blue). *asun* cells have reduced Cherry-LIS-1 localization relative to wild type. Late G2 (A,A′,D,D′) and metaphase I (B,B′,E,E′) spermatocytes and round spermatids (C,C′,F,F′) shown. Scale bars: 10 μm.

its recruitment is not microtubule dependent (data not shown) (Kemphues et al., 1982). We detected wild-type Cherry-LIS-1 levels in *asun*^{f02815} testes; thus, LIS-1 stability does not appear to require ASUN (supplementary material Fig. S10). GFP-ASUN shows a wild-type localization pattern when expressed in *Lis-1*^{k11702} testes (intranuclear during early G2, appearing in cytoplasm during late G2), suggesting that LIS-1 is not reciprocally required for ASUN localization (supplementary material Fig. S11) (Anderson et al., 2009). We infer that ASUN regulates localization of LIS-1 and dynein-dynactin, whereas LIS-1 regulates localization of dynein-dynactin but not ASUN.

LIS-1 and ASUN colocalize and coimmunoprecipitate

We hypothesized that LIS-1 and ASUN interact at the nuclear surface of late G2 spermatocytes to recruit dynein-dynactin. Our efforts to demonstrate colocalization of LIS-1 and ASUN at the nuclear surface of spermatocytes, however, were complicated by the low frequency and weak accumulation of GFP-ASUN that we have observed at this site. We previously reported colocalization of endogenous dynein and GFP-tagged *Drosophila* ASUN at the nuclear surface of transfected, nocodazole-treated, cultured mammalian cells (Anderson et al., 2009). Taking a similar approach, we found that 74% of co-transfected cells with perinuclear localization of GFP-tagged *Drosophila* ASUN exhibited colocalization of Cherry-tagged *Drosophila* LIS-1 at this site (Fig. 8A; 68/92 cells scored). Furthermore, we demonstrated co-immunoprecipitation of tagged versions of *Drosophila* LIS-1 and ASUN from cultured mammalian cells, suggesting LIS-1 and ASUN can exist within a common complex (Fig. 8B).

DISCUSSION

Our analysis of a hypomorphic, male-sterile allele of *Lis-1* revealed that *Lis-1* plays essential roles during *Drosophila* spermatogenesis. Our data suggest that loss of dynein function is the root cause of the defects that we observe in *Lis-1*^{k11702} testes, as mutation of the dynein light chain gene *tctex-1* phenocopies mutation of *Lis-1*. Based on their overlapping phenotypes in male germ cells, genetic interaction, colocalization and co-immunoprecipitation, we present a model in which *Lis-1* and *asun* cooperate to regulate dynein localization during spermatogenesis.

Our observations suggest that centrosomes of *Lis-1* spermatocytes remain attached to the cell cortex and fail to migrate to the nuclear surface at entry into meiotic prophase. The phenotype of persistent cortical centrosomes during meiotic divisions has been characterized in *abnormal spindles* and *nudE* testes; Wainman et al. also noted the presence of cortical centrosomes in *Lis-1*^{k11702} metaphase spermatocytes in their study of *nudE* mutants (Rebollo et al., 2004; Wainman et al., 2009). Dynein-dynactin and LIS-1 localize to the cell periphery in lower eukaryotes and cultured mammalian cells, as well as to the posterior cortex of *Drosophila* oocytes (Busson et al., 1998; Dujardin and Vallee, 2002; Faulkner et al., 2000). We have not, however, detected enrichment of dynein-dynactin or LIS-1 at the cortex of *Drosophila* spermatocytes. Cortical dynein has been implicated in regulation of mitotic spindle orientation in several systems, although the mechanism is not clear (Gusnowski and Srayko, 2011; Markus et al., 2009; Woodard et al., 2010). Our data suggest that dynein and LIS-1 are required in spermatocytes to release centrosomes from the cortex prior to meiotic entry.

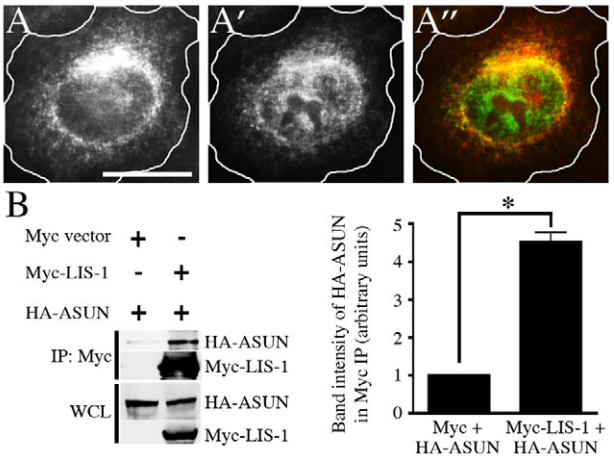

Fig. 8. LIS-1 and ASUN colocalize and coimmunoprecipitate. (**A**) Colocalization of Cherry-tagged *Drosophila* LIS-1 (A; red in A″) and GFP-tagged *Drosophila* ASUN (A′ green in A″) in transfected HeLa cells. Scale bar: 10 μm. (**B**) HEK293 cells were transfected with tagged *Drosophila* LIS-1 and ASUN expression plasmids as indicated. Myc (control) or Myc-LIS-1 was immunoprecipitated from lysates. Immunoblots of whole cell lysates (WCL) and Myc immunoprecipitates were probed using HA and Myc antibodies. Representative blot is shown on left; quantification on right (*P<0.001, paired Student's t-test, n=4 experiments, ImageJ analysis of band intensities).

We have shown that *Lis-1* spermatocytes exhibit free centrosomes, albeit at a much lower frequency than the phenotype of cortical centrosomes. Detachment of centrosomes from the cortex of primary spermatocytes is an earlier step in male meiosis than reassociation of the centrosomes with the nuclear surface at G2/M; hence, a failure of centrosomes to detach from the cortex is likely to mask a subsequent failure of nucleus-centrosome coupling. We found that LIS-1 colocalizes with dynein-dynactin at the nuclear surface, and localization of dynein-dynactin to this site is severely impaired in *Lis-1* spermatocytes and spermatids. Dynein-dynactin anchored at the nuclear surface has previously been implicated in mediating interactions between the nucleus and centrosomes during both mitotic and meiotic cell cycles (Anderson et al., 2009; Gönczy et al., 1999; Li et al., 2004; Malone et al., 2003; Robinson et al., 1999; Salina et al., 2002). We propose that defects in nucleus-centrosome coupling in *Lis-1* spermatocytes stem from disruption in localization of dynein-dynactin to the nuclear surface.

Previous studies in other systems concerning the role of LIS1 in dynein-dynactin recruitment to the nuclear surface have yielded conflicting results. In *C. elegans* embryos, dynein-dynactin was reported to localize normally to this site in the absence of *Lis-1* (Cockell et al., 2004). In mammalian neural stem cells, however, *Lis1* was shown to be required for recruitment of dynein to the nuclear surface at prophase entry (Hebbar et al., 2008). Similarly, we observed severe reduction of perinuclear dynein-dynactin in *Drosophila Lis-1* spermatocytes at meiotic onset, suggesting that *Lis-1* is required for this process. Conversely, we found normal levels of *Drosophila* LIS-1 at the nuclear surface of *tctex-1* spermatocytes; thus, dynein-dynactin does not appear to be reciprocally required for LIS-1 recruitment to this site. Our finding of reduced levels of dynein heavy chain on the nuclear surface of *tctex-1* spermatocytes suggest that Tctex-1 light chain plays a specific role in localizing dynein complexes to the nuclear surface; alternatively, complex integrity may be compromised in *tctex-1* mutants.

We have previously reported that *asun* regulates dynein localization during *Drosophila* spermatogenesis (Anderson et al., 2009). Our characterization of the hypomorphic *Lis-1*^{k11702} allele and the null *asun*^{d93} allele during *Drosophila* male meiosis reveals overlapping but distinct phenotypes. *Lis-1*^{k11702} spermatocytes exhibit two classes of centrosome positioning defects: cortical (major phenotype) and free centrosomes (minor phenotype). By contrast, although most *asun*^{d93} spermatocytes have free centrosomes, they do not share with *Lis-1*^{k11702} spermatocytes the phenotype of cortical centrosomes. These observations suggest that the role of *asun* in spermatocytes is limited to events at the nuclear surface, whereas *Lis-1* additionally regulates cortical events. *asun*^{d93} spermatocytes undergo severe prophase arrest, possibly owing to failure of astral microtubules of free centrosomes to promote nuclear envelope breakdown. In *Lis-1*^{k11702} spermatocytes, however, meiosis apparently progresses on schedule despite cortical positioning of centrosomes. The high percentage of *asun*^{d93} spermatids with increased numbers of variably sized nuclei, probably a consequence of cytokinesis and chromosome segregation defects, are also absent in *Lis-1*^{k11702} testes. These observations suggest that spindle formation and normal progression through male meiosis require centrosomes to be anchored, either to the nuclear surface or the cortex.

Hypomorphic *Lis-1*^{k11702} and null *asun*^{d93} round spermatids also show similarities and differences in their phenotypes. Both genes are required for recruitment of dynein-dynactin to the nuclear surface; this pool of dynein probably mediates nucleus-basal body and nucleus-Nebenkern attachments, which are defective in both mutants. Genes encoding Spag4 (a SUN protein), Yuri Gagarin (a coiled-coil protein) and GLD2 [a poly(A) polymerase] are required for nucleus-basal body coupling in spermatids, although it is not known whether they interact with ASUN or LIS-1 in this process (Kracklauer et al., 2010; Sartain et al., 2011; Texada et al., 2008). Our studies suggest that *Lis-1*, but not *asun*, is required for proper Nebenkern shaping and Nebenkern-basal body association; these functions might be mediated by dynein/microtubules acting at the Nebenkern surface. Nebenkerne are generated through fusion of mitochondria following *Drosophila* male meiosis (Fuller, 1993). Two Nebenkerne bodies are occasionally present in *Lis-1* and *tctex-1* spermatids, implicating dynein in regulation of mitochondrial aggregation at this stage. Together, these observations suggest that the role of *asun* in spermatids is limited to events at the nuclear surface, whereas *Lis-1* plays additional roles in regulating Nebenkerne.

Based on our studies of hypomorphic *Lis-1*^{k11702} and null *asun*^{d93} mutant testes, we propose a model in which LIS-1 is required for several dynein-mediated processes during *Drosophila* spermatogenesis, and ASUN is required for the subset of these processes that involve the nuclear surface (Fig. 9). Both LIS-1 and ASUN promote recruitment of dynein-dynactin to the nuclear surface of spermatocytes and spermatids. The strong genetic interaction that we observe between *Lis-1* and *asun* suggests that they cooperate in regulating dynein localization during spermatogenesis; our finding that LIS-1 accumulation on the nuclear surface is lost in *asun* male germ cells provides further support for this notion. The observed colocalization and co-immunoprecipitation of LIS-1 and ASUN suggest that they function within a shared complex to promote dynein-dynactin recruitment to the nuclear surface. We did not detect interaction

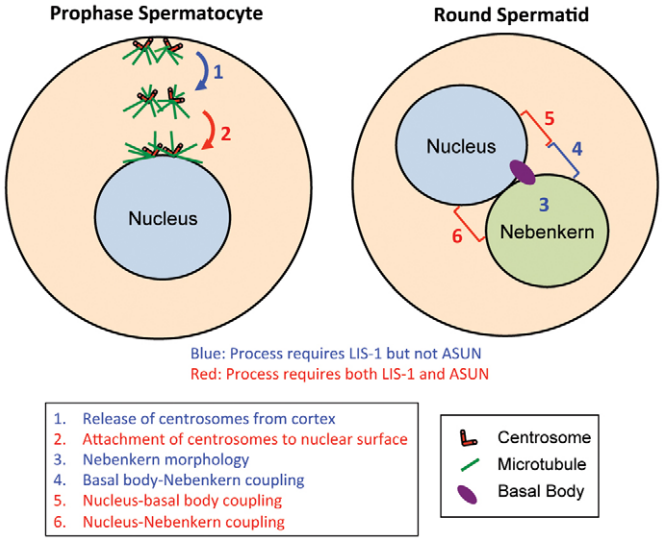

Fig. 9. Cytoplasmic dynein-mediated processes in *Drosophila* spermatogenesis: differential requirements for LIS-1 and ASUN. LIS-1 is required during spermatogenesis for cytoplasmic dynein-mediated processes, whereas ASUN is required for the subset of these processes that occur at the nuclear surface.

between *Drosophila* LIS-1 and ASUN proteins by in vitro binding or yeast two-hybrid assays, suggesting that their association may be mediated by another protein(s) rather than being direct (P.S. and L.A.L., unpublished). Future studies on the nature of the ASUN-LIS-1 interaction should help elucidate the mechanism by which dynein-dynactin localizes to the nuclear surface during spermatogenesis.

Several proteins that promote dynein recruitment and centrosomal tethering to the nuclear surface have been identified. In *C. elegans* embryos, the KASH-domain protein ZYG-12, which localizes to the outer nuclear membrane and binds the inner nuclear membrane protein SUN-1, is required for these events (Malone et al., 2003). Another KASH-domain protein, Syne/Nesprin-1/2, works in concert with SUN-1/2 to mediate nucleus-centrosome interactions during mammalian neuronal migration (Zhang et al., 2009). Two additional pathways required for dynein recruitment to the nuclear surface at prophase have recently been identified in cultured mammalian cells. BicD2 binds dynein and anchors it to the nuclear envelope via its interaction with a nuclear pore complex protein, RanBP2 (Splinter et al., 2010). Similarly, CENP-F and NudE/EL act as a bridge between dynein and Nup133 (Bolhy et al., 2011). It has not yet been determined whether mammalian LIS1 and ASUN function within these pathways or whether they act via a parallel mechanism to promote dynein recruitment to the nuclear surface.

Our finding that a single copy of *Lis-1*^{k11702} can drastically decrease the size of *asun*^{f02815} testes suggests potential roles for *Lis-1* and *asun* in regulating division of male germline stem cells of *Drosophila*, as loss of cell proliferation can lead to reduction of testes size (Castrillon et al., 1993). Interestingly, *Lis-1* has been reported to regulate germline stem cell renewal in *Drosophila* ovaries (Chen et al., 2010). Orientation of the cleavage plane during male germline stem cell division requires proper migration of centrosomes along the nuclear surface, and misorientation of the plane can lead to stem cell loss (Cheng et al., 2008; Yamashita et al., 2003; Yamashita et al., 2007). Given the importance of *Lis-1* and *asun* in mediating nucleus-centrosome coupling in *Drosophila* spermatocytes, it is possible that these genes also cooperate to regulate centrosomes during stem cell divisions in testes.

In humans, the *LIS1* gene is dose sensitive during brain development, as the disorder lissencephaly results from deletion or mutation of a single copy (Wynshaw-Boris, 2007). *Lis-1* spermatogenesis phenotypes reported herein were observed in flies homozygous for a hypomorphic *Lis-1* allele; flies carrying one copy of this allele displayed many of the same phenotypes but to a lesser degree. These findings suggest that precise regulation of LIS-1 protein levels is essential for normal development in *Drosophila*.

A requirement for *Lis1* during spermatogenesis is conserved in mammals. Deletion of a testis-specific splicing variant of *Lis1* in mice blocks spermiogenesis and prevents spermatid differentiation (Nayernia et al., 2003). LIS1 and dynein were shown to partially colocalize around wild-type spermatid nuclei, but dynein localization in *Lis1* testes was not assessed. It remains to be determined if the functions of LIS1 in mammalian spermatogenesis are mediated through dynein and if the ASUN homolog regulates LIS1 localization in this system.

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Competing interests statement
The authors declare no competing financial interests.

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