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asunder is required for dynein localization and dorsal fate determination during *Drosophila* oogenesis

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SUMMARY

We previously showed that *asunder* (*asun*) is a critical regulator of dynein localization during *Drosophila* spermatogenesis. Because the expression of *asun* is much higher in *Drosophila* ovaries and early embryos than in testes, we herein sought to determine whether ASUN plays roles in oogenesis and/or embryogenesis. We characterized the female germline phenotypes of flies homozygous for a null allele of *asun* (*asun*^{d93}). We find that *asun*^{d93} females lay very few eggs and contain smaller ovaries with a highly disorganized arrangement of ovarioles in comparison to wild-type females. *asun*^{d93} ovaries also contain a significant number of egg chambers with structural defects. A majority of the eggs laid by *asun*^{d93} females are ventralized to varying degrees, from mild to severe; this ventralization phenotype may be secondary to defective localization of *gurken* transcripts, a dynein-regulated step, within *asun*^{d93} oocytes. We find that dynein localization is aberrant in *asun*^{d93} oocytes, indicating that ASUN is required for this process in both male and female germ cells. In addition to the loss of *gurken* mRNA localization, *asun*^{d93} ovaries exhibit defects in other dynein-mediated processes such as migration of nurse cell centrosomes into the oocyte during the early mitotic divisions, maintenance of the oocyte nucleus in the anterior-dorsal region of the oocyte in late-stage egg chambers, and coupling between the oocyte nucleus and centrosomes. Taken together, our data indicate that *asun* is a critical regulator of dynein localization and dynein-mediated processes during *Drosophila* oogenesis.

Keywords

Drosophila; oogenesis; dorsal fate determination; centrosomes; dynein

INTRODUCTION

Drosophila oogenesis is a powerful model system for studying various aspects of cell and developmental biology such as control of the cell cycle, axis formation, epithelial morphogenesis, cellular polarity, and cell fate determination. A wild-type *Drosophila* female has a pair of ovaries, each made up of 16 –18 independent “egg assembly lines” known as ovarioles (Bastock and St Johnston, 2008; Spradling, 1993). Each ovariole consists of a specialized anterior region (the germarium) where the progeny of germline and somatic stem cells are organized into distinct egg chambers. Each egg chamber consists of a cyst of 16

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germ cells (15 nurse cells and 1 oocyte) interconnected by cytoplasmic bridges called ring canals and surrounded by a single layer of somatic follicle cells. The development of the egg chambers into mature eggs has been divided into 14 stages based on egg chamber morphology (Spradling, 1993). The polarity of the mature egg, formed at the end of oogenesis, is characterized by certain prominent structures: an anteriorly positioned, cone-shaped micropyle that facilitates sperm entry prior to fertilization and, located above the micropyle, a pair of dorsal appendages that facilitate embryonic respiration.

Determination of eggshell polarity depends on key patterning events that occur during *Drosophila* oogenesis. Within the germarium, centrosomes migrate from the nurse cells into the future oocyte in a manner dependent on a branched cytoplasmic organelle called the fusome, which extends into all the germline cells within a cyst (Bolivar et al., 2001; Lin et al., 1994). A microtubule-organizing center (MTOC) forms in the oocyte posterior; microtubules originating from this MTOC pass through cytoplasmic bridges into adjacent nurse cells and are required for transport of maternal mRNAs and proteins from the nurse cells into the oocyte (Pokrywka and Stephenson, 1991; Theurkauf et al., 1992). Transport and asymmetric localization within the oocyte of *oskar* (*osk*), *nanos* (*nos*), *bicoid* (*bcd*), and *gurken* (*grk*) transcripts are critical for proper establishment of the embryonic body axes (Becalska and Gavis, 2009).

grk mRNA is localized to the posterior of the *Drosophila* oocyte prior to its translation to generate Gurken (Grk) protein, a TGFα-like ligand, which signals posterior follicle cells to adopt a posterior fate (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schupbach, 1993). The posterior follicle cells in turn trigger reorganization of the microtubule cytoskeleton of the oocyte that promotes localization of *bcd* transcript to the anterior pole and *osk* and *nos* transcripts to the posterior pole, thus establishing the anterior-posterior axis of the embryo. This microtubule reorganization also results in migration of the oocyte nucleus to the anterior-dorsal region of the oocyte (Zhao et al., 2012). *grk* mRNA, which associates with the oocyte nucleus, begins to accumulate in this region (Neuman-Silberberg and Schupbach, 1993). The resulting localized secretion of Grk protein, which signals to overlying dorsal-anterior follicle cells, initiates a signaling cascade that ultimately establishes the dorsal-ventral axis of the embryo (Peri and Roth, 2000; Sen et al., 1998; Van Buskirk and Schupbach, 1999; Wasserman and Freeman, 1998).

The microtubule motors, dynein and kinesin, are critical for the transport of various mRNAs to their specific sites during *Drosophila* oogenesis (Becalska and Gavis, 2009; Duncan and Warrior, 2002; Januschke et al., 2002). Localization of *grk* mRNA, which is required for the formation of both major axes, is dependent on the minus-end-directed motor, dynein (MacDougall et al., 2003; Rom et al., 2007; Swan et al., 1999). Dynein is a large complex composed of four types of subunits: heavy, intermediate, light intermediate, and light chains (Hook and Vallee, 2006; Susalka and Pfister, 2000). Dynein regulates multiple cellular processes such as organelle transport, chromosome movements, nucleus-centrosome coupling, nuclear positioning, and spindle assembly (Anderson et al., 2009; Gusnowski and Srayko, 2011; Hebbar et al., 2008; Huang et al., 2011; Jodoin et al., 2012; Salina et al., 2002; Sitaram et al., 2012; Splinter et al., 2010; Stuchell-Brereton et al., 2011; Wainman et al., 2009). During *Drosophila* oogenesis, dynein is required within the germ cells for maintenance of fusome integrity, centrosome migration, oocyte determination, migration of the oocyte nucleus, transport into the oocyte of various mRNAs and proteins that play critical roles in axis determination of the embryo, and localization of these mRNAs and proteins within the oocyte (Bolivar et al., 2001; Januschke et al., 2002; Lei and Warrior, 2000; McGrail and Hays, 1997; Schnorrer et al., 2000; Swan et al., 1999). Dynein is also important within the somatic follicle cells for maintenance of their apical-basal polarity as well as for the migration of the border cells from the anterior of the egg chamber to the

anterior of the oocyte (Horne-Badovinac and Bilder, 2008; Van de Bor et al., 2011; Yang et al., 2012).

We previously identified *asunder* (*asun*) as a critical regulator of dynein localization during *Drosophila* spermatogenesis (Anderson et al., 2009). Dynein enrichment on the nuclear surface of G2 spermatocytes and round spermatids is lost in *asun* testes; as a result, *asun* male germ cells exhibit defects in nucleus-centrosome and nucleus-basal body coupling. Northern blot analysis of *Drosophila* tissues revealed that *asun* transcripts, while detected in the testes, were present at much higher levels in ovaries and early embryos, suggesting that *asun* may play roles in oogenesis and/or embryogenesis (Stebbings et al., 1998). In this study, we investigate the role of *asun* during *Drosophila* oogenesis by characterizing the phenotypes of females homozygous for a null allele of *asun* ($asun^{d93}$). We provide evidence to show that, similar to its role in spermatogenesis, *asun* is required for regulating dynein localization and dynein-mediated processes within the germ cells such as nuclear positioning, centrosome migration, and dorsal-ventral patterning during *Drosophila* oogenesis.

MATERIALS AND METHODS

Drosophila stocks

y w was used as "wild-type" stock. The $asun^{d93}$ allele was previously described (Sitaram et al., 2012). png^{50} and png^{1058} were gifts from T. Orr-Weaver (Whitehead Institute, Cambridge, MA). $TrolGFP^{ZCL1700}$, which was used for observing the localization of *Drosophila* Perlecan, was a gift from A. Page-McCaw (Vanderbilt University School of Medicine, Nashville, TN).

Transgenesis

A 3.6-kb genomic fragment containing *asun* and its flanking regions (Fig. 1A) was PCR-amplified from BAC clone BAC37I18 (*Drosophila* Genomics Resource Center, Indiana University, IN) and subcloned into modified pCaSpeR4 for expression of full-length ASUN under control of its endogenous promoter (*asun* rescue construct). The following primers were used: 5′-GCA TGG CCG GCC ACT GCA CAA GAT T-3′ and 5′-GAC TGG CGC GCC CCG AAG AAA AGT T-3′. Transgenic lines carrying $P[asun^{FL}]$ were generated by *P*-element-mediated transformation via embryo injection (Rubin and Spradling, 1982).

Egg-laying assay

Females (2–4 days old) of each genotype tested were placed in a bottle with wild-type males, fattened with wet yeast for two days, and transferred to egg-collection chambers (five females and five wild-type males per chamber; two chambers per genotype) at 25°C. The number of eggs laid by females in each collection chamber was counted each day up to five days. Statistical analysis of the average number of eggs laid per day by females of the indicated genotypes was performed using an unpaired Student's *t*-test.

Cytological analysis of fixed ovaries

Ovaries were dissected and teased apart in Schneider's *Drosophila* medium (Life Technologies, Carlsbad, CA), fixed for 18 minutes in phosphate-buffered saline (PBS) plus 4% formaldehyde, washed for 2 hours in PBS plus 0.1% Triton X-100 (PBT), incubated for 3 hours in PBT plus 5% normal goat serum (PBT-NGT), and incubated overnight at 4°C in PBT-NGT containing primary antibodies. Ovaries were then washed for 2 hours in PBT, incubated for 4 hours in PBT-NGT containing fluorophore-conjugated secondary antibodies, incubated in PBT plus 0.5 μg/ml DAPI for 6 minutes, washed for 2 hours in PBT, rinsed

once in PBS, and mounted in Prolong Gold Antifade Reagent (Life Technologies). All steps were done at room temperature unless otherwise noted. For all experiments, ovaries from all genotypes tested were dissected in parallel and fixed/stained under identical conditions. For staining with anti-PLP antibody, the same protocol was followed except that ovaries were fixed with 100% methanol at −20°C for 10 minutes and rehydrated with decreasing concentrations of methanol in PBT before the first PBT wash.

Primary antibodies directed against the following proteins were used: dynein heavy chain (P1H4, 1:120; gift from T. Hays, University of Minnesota, Minneapolis, MN), PLP (1:500; gift from J. Raff, University of Oxford, Oxford, UK), lamin (ADL67.10, 1:500, Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), α-spectrin (3A9, 1:20, DSHB), Orb (4H8+6H4, 1:20, DSHB), DE-Cadherin (DCAD2, 1:20, DSHB), aPKC (sc216, 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX), Fasciclin III (7G10, 1:3, DSHB), and Gurken (1D12, 1:100, DSHB). Alexa Fluor 488 Phalloidin (A12379, 1:250, Life Technologies) was used to label F-Actin. Wide-field fluorescent images were obtained using an Eclipse 80i microscope (Nikon, Melville, NY) with Plan-Fluor 40X objective (all micrographs presented unless otherwise indicated). 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 63X objective. Fisher's exact test was used for statistical analyses of data.

Egg-chamber area analysis

Ovaries were fixed in 4% formaldehyde in PBS and stained with Alexa Fluor phalloidin (Life Technologies) to mark actin at the cell membranes. A Leica TCS SP5 confocal microscope was used to obtain images of optical sections of individual stage 10B egg chambers such that the largest areas were obtained. Areas of total egg chambers (oocyte + nurse cells) and oocytes alone were calculated using ImageJ software (National Institutes of Health, Bethesda, MD). 20 egg chambers were imaged per genotype for the calculation of egg chamber and oocyte area. Stage 10B egg chambers were identified by their follicle cell morphology.

Cytological analysis of fixed embryos

Embryos (0–2 hours) were collected on grape plates, dechorionated in 50% bleach, and devitellinized by shaking in a solution of methanol and heptane (1:1). Embryos were then stained with 1 μg/ml propidium iodide plus 1 mg/ml RNase A in PBT for 20 minutes, washed thrice with PBT, once with 50% methanol in PBT, and thrice with 100% methanol. Embryos were cleared and mounted in clearing solution (2:1 benzyl benzoate:benzyl alcohol). Wide-field fluorescent images were obtained using an Eclipse 80i microscope (Nikon) with Plan-Apo 20X objective.

Whole-mount RNA in situ hybridization

Whole-mount enzymatic in situ hybridization was performed as previously described (Suter and Steward, 1991). Fluorescent in situ hybridization was performed following the same protocol with the following modifications: Cy3-conjugated digoxigenin antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) replaced alkaline phosphatase-conjugated digoxigenin antibody, and the development step was omitted. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using a digoxigenin RNA labeling kit (Roche Applied Science, Indianapolis, IN). Antisense probes were prepared using the following full-length cDNA clones: *grk* (gift from A. Page-McCaw, Vanderbilt University School of Medicine, Nashville TN), *bcd*, and *osk* (*Drosophila* Genomics Resource Center). No significant signal was observed in control experiments using sense probes. Fluorescent in situ hybridization images were obtained using a Zeiss Apotome mounted on an Axio

ImagerM2 with a 20X/0.8 Plan-Apochromat objective, and images were acquired with an AxioCam MRm camera (Zeiss, Thornwood, NY). Enzymatic in situ hybridization images were obtained using a Zeiss LumarV12 fluorescence stereomicroscope with a 1.5X Neolumar objective (zoomed to 80X), and images were acquired with an AxioCam MRc camera (Zeiss).

Immunoblotting

Ovaries from newly eclosed females or embryos (0–2 hour) were homogenized in nondenaturing lysis buffer (50 mM Tris-Cl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100) and analyzed by SDS-PAGE (25 μg protein/lane) and immunoblotting using standard techniques. Primary antibodies were used as follows: anti-dynein heavy chain (P1H4, 1:2000), anti-dynein intermediate chain (74.1, 1:250, Santa Cruz), anti-Cyclin B (F2F4, 1:100, DSHB), and anti-beta-tubulin (E7, 1:1000, DSHB). HRP-conjugated secondary antibodies were used to detect primary antibodies by chemiluminescence.

RESULTS

asun is required for oogenesis

To address whether *asun* plays a role in *Drosophila* oogenesis, we first tested the fertility of females homozygous for a null allele of *asun* (*asun*^{d93}) that we previously generated (Fig. 1A) (Sitaram et al., 2012). We found that *asun*^{d93} females had a severely reduced egg-laying rate (average of <1 egg/day/female compared to 55 eggs/day/female for a control stock; Fig. 1B). Heterozygous *asun*^{d93} females, however, exhibited egg-laying rates comparable to that of control females, indicating that *asun* is a haplosufficient locus (Fig. 1B). To confirm that the egg-laying defect of *asun*^{d93} females was a direct consequence of loss of *asun* function, we generated transgenic *Drosophila* lines (*P[asun*^{FL}*]*) expressing full-length ASUN under control of its endogenous promoter (Fig. 1A). The egg-laying rate of *asun*^{d93} females was restored nearly to control levels by introduction of the *P[asun*^{FL}*]* transgene (Fig. 1B).

We then sought to determine if *asun*^{d93} females had gross defects in oogenesis that could account for their reduced egg-laying rate. We dissected whole ovaries from two-day old female flies fattened by addition of wet yeast paste to their food. Ovaries isolated from a majority of *asun*^{d93} females were considerably smaller in size than those isolated from wild-type females or *asun*^{d93} females carrying the *P[asun*^{FL}*]* transgene (herein referred to as "rescued *asun*^{d93}" line) (Fig. 1C–F). To test if the reduced size of *asun*^{d93} ovaries was due to a decrease in egg chamber and/or oocyte size, we measured the area of stage 10B egg chambers and oocytes (as a representative stage) isolated from wild-type or *asun*^{d93} females. We found no striking difference in stage 10B egg chamber or oocyte area between these genotypes (Fig. 1G), suggesting that the reduction in *asun*^{d93} ovary size might be due to defects in proper progression to later developmental stages of oogenesis.

Whereas individual ovarioles isolated from wild-type or rescued *asun*^{d93} ovaries were clearly ordered by increasing stages of development (Fig. S1A,C), the arrangement of a majority of ovarioles isolated from *asun*^{d93} ovaries was highly disorganized (Figs S1B, 1E). Ovarioles from the larger *asun*^{d93} ovaries typically contained early- and late-stage egg chambers with a paucity of intermediate stages (for example, Fig. S1B shows an *asun*^{d93} ovariole with two mature oocytes to the right immediately adjacent to a stage 5 egg chamber). Thus, the mature oocytes that are occasionally produced by *asun*^{d93} females tend to accumulate within the ovaries. These findings suggest that, in addition to abnormal oogenesis, *asun*^{d93} females have defects in related processes downstream of oogenesis such as ovulation, mating, sperm storage, fertilization, and/or egg laying (Sun and Spradling, 2013). We did not observe any overt differences, however, in the morphological appearance

of the reproductive glands (parovaria, spermathecae, or seminal receptacles) of *asun*^{d93} females compared to wild-type controls (Fig. S1D,E).

asun^{d93} egg chambers exhibit structural defects

We occasionally observed abnormal numbers of oocytes and nurse cells within *asun*^{d93} egg chambers. Whereas wild-type egg chambers normally contain 15 nurse cells and one oocyte, we found that 20% of *asun*^{d93} egg chambers (compared to <1% and <4% of wild-type and rescued *asun*^{d93} egg chambers, respectively) contained an increased number of germ cells (nurse cells and oocyte), possibly as a consequence of fusion of two or more egg chambers (Fig. 2A–C). Furthermore, we occasionally observed disruption of the follicle cell border that clearly demarcates nurse cells and the oocyte in wild-type egg chambers at or beyond stage 10 (Fig. 2D,D′); in 10% of *asun*^{d93} egg chambers at or beyond stage 10 (compared to 1% and 2% of wild-type and rescued *asun*^{d93} egg chambers, respectively), nurse cells appeared to protrude across this border and into the oocyte (Fig. 2E,E′,F).

asun-derived embryos do not phenocopy *png* mutants

ASUN was identified in an *in vitro* screen for substrates of the serine/threonine protein kinase encoded by *pan gu* (*png*), a critical regulator of the S-M cell cycles of early embryogenesis in *Drosophila* (Fenger et al., 2000; Lee et al., 2005; Shamanski and Orr-Weaver, 1991). Based on this association, we assessed *asun*^{d93}-derived embryos for the presence of *png*-like phenotypes. We found that *asun*^{d93}-derived embryos did not exhibit the giant nuclei phenotype that is characteristic of *png*-derived embryos (Fig. S2A–D). Furthermore, we did not observe genetic interaction between *png* and *asun*: introduction of a single copy of *asun*^{d93} into the *png*^{50} background failed to modify the *png*^{50} giant nuclei phenotype (Fig. S2E). PNG kinase mediates derepression of translation during early embryogenesis, thereby ensuring that Cyclin B levels are sufficiently high to promote mitotic entry (Fenger et al., 2000; Lee et al., 2001; Vardy and Orr-Weaver, 2007). In contrast, immunoblotting revealed normal levels of Cyclin B in *asun*^{d93}-derived embryos, suggesting that ASUN is not required for this function (Fig. S2F).

asun^{d93}-derived embryos have dorsal-ventral patterning defects

While performing experiments with *asun*^{d93}-derived embryos, we noticed abnormalities in the appearance of the dorsal appendages, a pair of paddle-shaped eggshell structures located on the anterior-dorsal surface of the embryo that form as a result of normal dorsal-ventral patterning events (Schupbach, 1987; Spradling, 1993). We used a previously reported scheme for classifying dorsal appendage defects to characterize this phenotype in *asun*^{d93}-derived embryos (Fig. 3A) (Lei and Warrior, 2000). Class I embryos have a pair of distinct dorsal appendages (wild-type appearance) that are positioned much closer to each other in class II embryos, fused at the base in class III embryos, and fused along their entire lengths in class IV embryos; class V embryos lack visible dorsal appendages. We found that a majority (54%) of *asun*^{d93}-derived embryos had dorsal appendage defects (compared to 1% and 3% for wild-type and rescued *asun*^{d93} embryos, respectively), including 23% in class V (Fig. 3B). These data suggest that *asun* is required for proper dorsal-ventral patterning of the *Drosophila* embryo. We also examined *asun*^{d93}-derived embryos for the presence of the micropyle, another eggshell structure located at the anterior end of the embryo that is required for sperm entry (Spradling, 1993). We found that a small fraction of *asun*^{d93}-derived embryos lacked a micropyle (6% compared to 1% in wild-type and *asun*^{d93}-derived embryos; Fig. S3).

grk mRNA localization is abnormal in *asun*^{d93} oocytes

The dorsal appendage defects of *asun*^{d93}-derived embryos resemble those reported for embryos produced by females homozygous for a hypomorphic allele of the dynein accessory factor, *Lis-1* (Lei and Warrior, 2000). The defect in dorsal-ventral patterning in *Lis-1*-derived embryos was attributed to loss of anterior-dorsal anchoring of *grk* mRNA in the oocyte. This asymmetric anchoring of *gurken* transcripts allows Grk, a TGFα-like protein that acts as a ligand, to asymmetrically activate the EGF receptor homolog, Torpedo/DER, specifically within the dorsal-anterior follicle cells (Neuman-Silberberg and Schupbach, 1993). Dynein light chain, a cargo-binding subunit of dynein, directly binds to *grk* mRNA and is required for its tight localization to the anterior-dorsal region of the oocyte (Rom et al., 2007).

To determine if the dorsal-ventral patterning defects observed in *asun*^{d93} egg chambers could be due to a loss of dynein-mediated regulation of *grk* transcripts, we assessed the localization of *grk* mRNA using both enzymatic and fluorescent in situ hybridization methods. We consistently observed a loss of the tight anterior-dorsal localization of *grk* transcripts (in 42% and 45% of *asun*^{d93} egg chambers by enzymatic and fluorescent in situ hybridization, respectively (compared to 4% and 6% of wild-type and rescued *asun*^{d93} egg chambers, respectively), suggesting that the ventralization of *asun*^{d93}-derived embryos could be a consequence of loss of dynein regulation by ASUN (Figs 4A–C, S4). In contrast, Grk protein appeared to localize normally to the anterior-dorsal region of the oocyte in *asun*^{d93} egg chambers. In ~21% of *asun*^{d93} egg chambers (31/147 egg chambers), however, the Grk protein localized in a more diffuse manner and with decreased signal intensity compared to 1.3% (3/225 egg chambers) of wild-type egg chambers and 1.7% (5/287 egg chambers) of rescued *asun*^{d93} egg chambers (Fig. 4D,E). We did not observe any defects, however, in the localization of *osk* or *bcd* transcripts, which encode anterior-posterior patterning factors, in *asun*^{d93} egg chambers (Fig. S5).

Dynein localization is disrupted in *asun*^{d93} oocytes

We previously identified *asun* as a critical regulator of dynein localization in *Drosophila* spermatogenesis and in cultured mammalian cells (Anderson et al., 2009; Jodoin et al., 2012; Sitaram et al., 2012). To determine if *asun* performs the same function during *Drosophila* oogenesis, we examined the localization of dynein in *asun*^{d93} oocytes using antibodies against the dynein heavy chain. Dynein accumulates within the oocyte in region 2b of the germarium and remains there throughout oogenesis (Li et al., 1994). In early egg chambers of wild-type females, dynein is enriched around the oocyte nucleus, and it localizes to the posterior pole of the oocyte in stage 9 chambers (Fig. 5A,D). We observed a significant loss of dynein localization to these sites in >35% of *asun*^{d93} egg chambers (compared to <1% and <3% of wild-type and rescued *asun*^{d93} egg chambers, respectively; Fig. 5A–G). Immunoblotting revealed normal levels of dynein heavy and intermediate chains in *asun*^{d93} ovaries, suggesting that the loss of dynein localization was not due to instability of core components of the complex (Fig. 5H).

Nucleus-centrosome coupling and nuclear positioning are abnormal in *asun*^{d93} oocytes

The dynein motor is required at multiple steps during *Drosophila* oogenesis, and its role in this system has been well characterized (Januschke et al., 2002; Lei and Warrior, 2000; McGrail and Hays, 1997; Schnorrer et al., 2000; Swan et al., 1999). Because we observed a loss of dynein localization in *asun*^{d93} ovaries, we sought to determine if dynein-mediated processes (in addition to *grk* transcript localization) were disrupted. We observed oocyte nucleus-centrosome coupling defects in 18% of *asun*^{d93} egg chambers (compared to 5% and 6% in wild-type and rescued *asun*^{d93} egg chambers, respectively), suggesting that, similar to

its role in male germ cells, *Drosophila* ASUN promotes dynein-mediated association between the nucleus and centrosomes during oogenesis (Fig. 5I–K). Our criterion for categorizing an egg chamber as having uncoupling of the oocyte nucleus and centrosomes was that the egg chamber contained one or more centrosomes completely detached and at a visible distance from the outer membrane of the oocyte nuclear envelope when observed as a two-dimensional image using fluorescence microscopy.

We next assessed the positioning of the oocyte nucleus in *asun*^{d93} egg chambers. The oocyte nucleus, which normally migrates from the posterior of the oocyte to the future anterior-dorsal region in stage 7 egg chambers, is incorrectly positioned in the absence of dynein or its accessory factors (Januschke et al., 2002; Lei and Warrior, 2000; Swan et al., 1999). This phenotype has been attributed to a failure in the dynein-dependent anchoring of the nucleus at the anterior-dorsal of the oocyte (Zhao et al., 2012). We found that oocyte nuclei in stage 9 egg chambers from wild-type and *asun*^{d93} females were similarly positioned, suggesting that nuclear migration takes place normally in *asun*^{d93} females (Fig. 6A–C,I). By stage 10, however, only 67% of *asun*^{d93} egg chambers (compared to 94% of wild-type egg chambers) exhibited normal anterior-dorsal anchoring of the oocyte nucleus, suggesting that the attachment of the oocyte nucleus at that position is not maintained in the absence of ASUN; introduction of the genomic *asun* transgene into the *asun*^{d93} background partially rescued this defect with 81% of these egg chambers containing a properly positioned oocyte nucleus (Fig. 6D–I).

asun^{d93} egg chambers exhibit defects in centrosome migration

At the end of the four mitotic germ cell divisions, centrosomes migrate from the nurse cells into the pro-oocyte in a manner dependent on a large cytoplasmic organelle called the fusome, which extends into all 16 cells within a cyst through the ring canals (Bolivar et al., 2001; Grieder et al., 2000). Mutation of dynein heavy chain has been reported to result in loss of fusome integrity and centrosome migration (Bolivar et al., 2001; McGrail and Hays, 1997).

We assessed *asun*^{d93} germaria for fusome integrity and centrosome migration. We observed no obvious differences in fusome structure in wild-type and *asun*^{d93} germaria (Fig. 7A,A′,B,B′). *asun*^{d93} germaria, however, exhibited defects in centrosome migration. Most nurse cell centrosomes have migrated to the oocyte (located at the posterior of the egg chamber) by stage 2B in wild-type germaria (Fig. 7A,A′,D). Centrosome migration was disrupted in 67% of *asun*^{d93} stage 3 germaria (compared to <2% in wild-type stage 3 germaria) with centrosomes still distributed throughout the cyst (Fig. 7B,B′,D). Centrosome migration was significantly restored in rescued *asun*^{d93} ovaries (with only 15% of their stage 3 germaria showing disruption of centrosome migration) (Fig. 7C,C′,D). The timing of this process, however, appeared to be delayed: in contrast to wild-type ovaries, most centrosomes were found scattered throughout stage 2B germaria of rescued *asun*^{d93} ovaries (Fig. 7C′). Possibly as a result of the loss of centrosome migration in *asun*^{d93} germaria, we observed a decreased number of centrosomes (fewer than five) associated with the oocyte nucleus in 42% of *asun*^{d93} stage 5 egg chambers (compared to 8% of wild-type egg chambers; Fig. S6).

Dynein-mediated processes within follicle cells are not disrupted in *asun*^{d93} egg chambers

In addition to the functions reported for dynein within the germ cells, two major functions have been identified for dynein within the somatic follicle cells: dynein is required for the normal polarization of the epithelial follicle cells as well as for the normal migration of the border cells (Horne-Badovinac and Bilder, 2008; Van de Bor et al., 2011; Yang et al., 2012). To determine if ASUN is required for dynein functions within the follicle cells, we assessed whether *asun*^{d93} egg chambers exhibited defects in these processes. We observed normal

localizations of aPKC, which marks the apical surface of follicle cells, and of Perlecan, which marks the basal surface of follicle cells, in *asun*^{d93} egg chambers compared to wild-type egg chambers (Fig. S7A–D′; 161/163 *asun*^{d93} egg chambers and 142/142 wild-type egg chambers with normal aPKC localization; 149/149 *asun*^{d93} egg chambers and 141/141 wild-type egg chambers with normal Perlecan localization). We additionally determined that the border cells migrated normally to the border between the nurse cells and the oocyte in stage 10 *asun*^{d93} egg chambers compared to wild-type egg chambers (Fig. S7E–F′; 132/132 *asun*^{d93} egg chambers and 134/134 wild-type egg chambers).

DISCUSSION

We report herein that *asun* is a critical regulator of *Drosophila* oogenesis. *Drosophila* females that are homozygous for a null allele of *asun* (*asun*^{d93}) have highly reduced egg-laying rates as a result of defects in oogenesis as well as in processes downstream of oogenesis. We have focused in this study on characterizing the oogenesis defects in *asun*^{d93} females and have found that eggs laid by these females are ventralized. This phenotype may be secondary to the improper localization of mRNA transcripts encoding the dorsal fate determinant, Grk, in *asun*^{d93} oocytes. The dynein motor, which is required for transport of *grk* mRNA during *Drosophila* oogenesis, is also mislocalized in a significant fraction of *asun*^{d93} oocytes. We have also determined that other reported dynein-mediated processes such as nuclear positioning, nucleus-centrosome coupling, and centrosome migration are also defective in *asun*^{d93} egg chambers.

We previously identified ASUN as a regulator of dynein localization during *Drosophila* spermatogenesis and in cultured human cells (Anderson et al., 2009; Jodoin et al., 2012). Loss of ASUN in *Drosophila* spermatocytes results in the loss of perinuclear localization of dynein at the G2-M transition, leading to defects in coupling between the nucleus and centrosomes, spindle assembly, chromosome segregation, and cytokinesis during the meiotic divisions (Anderson et al., 2009). Similar defects were observed in mitotically dividing cultured human cells following siRNA-mediated down-regulation of the human homologue of ASUN (Jodoin et al., 2012).

The role of dynein during *Drosophila* oogenesis has been well characterized. Within the germ cells, the dynein motor has been implicated in maintaining fusome integrity, centrosome migration, and oocyte determination within the germarium (Bolivar et al., 2001; McGrail and Hays, 1997; Mische et al., 2008; Swan et al., 1999). Additionally, dynein is critical for the transport and normal localizations of various patterning factors throughout oogenesis as well as maintenance of the anterior-dorsal positioning of the oocyte nucleus in late-stage egg chambers (Clark et al., 2007; Duncan and Warrior, 2002; Januschke et al., 2002; Lan et al., 2010; Lei and Warrior, 2000; Rom et al., 2007; Swan et al., 1999). Within the somatic follicle cells, dynein plays a role in maintaining apical-basal polarity as well as in the migration of border cells (Horne-Badovinac and Bilder, 2008; Van de Bor et al., 2011; Yang et al., 2012).

We have observed disruption of several of these dynein-regulated processes in *asun*^{d93} ovaries, likely as a result of the loss of dynein localization that occurs in the absence of ASUN. *asun*^{d93} ovaries exhibit defects in centrosome migration, *grk* mRNA localization, and nuclear positioning. *asun*^{d93} ovaries exhibit additional defects in the structure of the egg chamber and in the coupling between the oocyte nucleus and centrosomes. Dynein has been shown to facilitate nucleus-centrosome coupling in other systems, fused egg chambers have been observed in *Drosophila* dynein light chain mutants, and the displacement of nurse cells toward the oocyte has been reported in ovaries from flies mutant for *Bicaudal-D*, a known regulator of dynein; thus, these defects of *asun*^{d93} egg chambers could potentially also be

due to loss of dynein function (Anderson et al., 2009; Bolhy et al., 2011; Dick et al., 1996; Jodoin et al., 2012; Malone et al., 2003; Robinson et al., 1999; Sitaram et al., 2012; Splinter et al., 2010; Swan and Suter, 1996).

In spite of the complete loss of ASUN in *asun*^{d93} egg chambers, we found that certain dynein-mediated processes that have been reported to occur during *Drosophila* oogenesis were not affected in *asun*^{d93} ovaries. The fusome, a cytoplasmic organelle, is highly disorganized in ovaries that lack dynein or its accessory factor, LIS-1 (Bolivar et al., 2001; McGrail and Hays, 1997). The normal asymmetric distribution of the fusome within the different cells of a female germline cyst plays an important role in the determination of the future oocyte (de Cuevas and Spradling, 1998; Lin and Spradling, 1995; McKearin, 1997). Not surprisingly, *Drosophila* lines mutant for dynein or LIS-1 exhibit defects in oocyte determination (McGrail and Hays, 1997; Mische et al., 2008; Swan et al., 1999). We observed, however, that fusome structure within *asun*^{d93} germaria was indistinguishable from that of wild-type females, and oocyte determination appeared to occur normally in *asun*^{d93} ovaries (P.S. and L.A.L., unpublished observations). The migration of centrosomes from the nurse cells into the oocyte within the germaria is considered to be a fusome-dependent process (Bolivar et al., 2001; Grieder et al., 2000). Despite our observations of wild-type fusome structure in *asun*^{d93} germaria, they exhibit defects in centrosome migration. This discrepancy suggests that either an additional factor required for centrosome migration is affected in *asun*^{d93} mutants, the function (but not structure) of the fusome is compromised in *asun*^{d93} germaria, or that the fusomes of *asun*^{d93} germaria have subtle structural defects that we were unable to detect. In addition to being required only for a subset of dynein-dependent processes within germ cells during *Drosophila* oogenesis, ASUN appears to be dispensable for the functions of dynein within follicle cells, as *asun*^{d93} egg chambers do not exhibit any defects within these cells.

asun^{d93}-derived embryos exhibit defects in dorsal-ventral patterning. We found that the localization of *grk* mRNA to the anterior-dorsal region of the oocyte is lost in late-stage *asun*^{d93} egg chambers, suggesting that the ventralization of *asun*^{d93}-derived eggs could be a consequence of this defect. Additionally we observed that 21% of *asun*^{d93} egg chambers exhibited mild defects in the localization of the Grk protein. This result corresponds well with the percent of *asun*^{d93}-derived embryos exhibiting complete ventralization (23% of *asun*^{d93}-derived embryos). Our results suggest that even a mild disruption of Grk protein localization might lead to severe consequences in the dorsal-ventral patterning of the embryo. We speculate that the partial ventralization that we observed in 31% of *asun*^{d93}-derived embryos could be a result of milder, undetected defects in Grk protein localization. Alternatively, the function of the Grk protein could be compromised due to its dissociation from the oocyte nucleus as a result of nuclear mislocalization in late-stage *asun*^{d93} egg chambers. Similar defects in the localization of *grk* mRNA as well as the dissociation between Grk protein and the oocyte nucleus have been reported in *Drosophila* females mutant for a dynein light chain (Rom et al., 2007). Furthermore, additional unknown defects within the signaling cascade activated by the Grk ligand might exist within the dorsal-anterior follicle cells in *asun*^{d93} egg chambers that could contribute to the ventralization phenotype.

The oogenesis defects reported herein for *asun*^{d93} females appear to be only partially penetrant. Centrosome migration, which was the most penetrant ovarian phenotype that we observed (with 65% of germaria showing defects), occurs at a relatively early stage of oogenesis. The remaining defects reported in *asun*^{d93} egg chambers and *asun*^{d93}-derived embryos had a maximum penetrance of ~50%, with some, such as the structural defects of egg chambers, occurring at a frequency of less than 20%. In contrast, the defects observed in *asun*^{d93} testes are generally present at a higher frequency (Sitaram et al., 2012).

Additionally, we observed that females that are heterozygous for the null allele of *asun* as well as females that are homozygous for a weak allele of *asun* (*asun*^{f02815}) do not exhibit any obvious defects in oogenesis, whereas spermatogenesis is severely compromised in *asun*^{f02815} males (P.S. and L.A.L., unpublished observations) (Anderson et al., 2009). Taken together, these data suggest that either ASUN plays a less critical role during *Drosophila* oogenesis in comparison to spermatogenesis and/or that other factors can better compensate for the loss of ASUN in this system.

ASUN has also been identified as a functional component of an evolutionarily conserved nuclear complex known as the Integrator in cultured mammalian cells (Chen et al., 2012; Malovannaya et al., 2010). Integrator, which is composed of at least 14 distinct subunits (including ASUN), mediates 3′-end processing of small nuclear RNAs (Baillat et al., 2005; Chen and Wagner, 2010). We have recently determined that several Integrator subunits, like ASUN, are required in cultured human cells for recruitment of dynein motors to the nuclear envelope during mitosis (J.N. Jodoin and L.A.L., unpublished observations). Our current model for the role of ASUN in controlling dynein localization is that ASUN, in conjunction with other subunits of the Integrator complex, mediates the proper processing of a specific mRNA target encoding a critical regulator of dynein recruitment to the nuclear envelope in cultured human cells. The high degree of conservation between *Drosophila* ASUN and its human homologue, and our data showing that *Drosophila* ASUN can be used to rescue loss of mammalian ASUN and vice versa, makes it likely that *Drosophila* ASUN functions in a similar manner to regulate the localization of dynein during oogenesis and spermatogenesis (Anderson et al., 2009; Jodoin et al., 2012).

In addition to the defects in oogenesis that we report herein, the capacity of *asun*^{d93} females to produce mature eggs that accumulate within the ovary suggests that these females also have defects downstream of oogenesis. Given that we have been able to ascribe a majority of the defects in *asun*^{d93} mutants to disruption of dynein-mediated processes, it is possible that these downstream phenotypes of *asun*^{d93} females may represent novel functions of dynein. Alternatively, these processes may be directly regulated by ASUN or by a different target of the ASUN/Integrator complex. It would therefore be of importance in future studies to determine if ASUN functions as a component of the Integrator complex in this system, and if so, to identify the targets of this complex required for normal progression through *Drosophila* oogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Females null for *asun* (*asun*^{d93}) have a severe egg-laying defect.
- *asun*^{d93}-derived embryos are ventralized due to mis-localization of *grk* transcripts.
- Dynein, required for *grk* localization, is mis-localizaed in *asun*^{d93} egg chambers.
- *asun*^{d93} ovaries exhibit defects in known dynein-mediated processes.
- ASUN regulates dynein and dynein-mediated processes during *Drosophila* oogenesis.

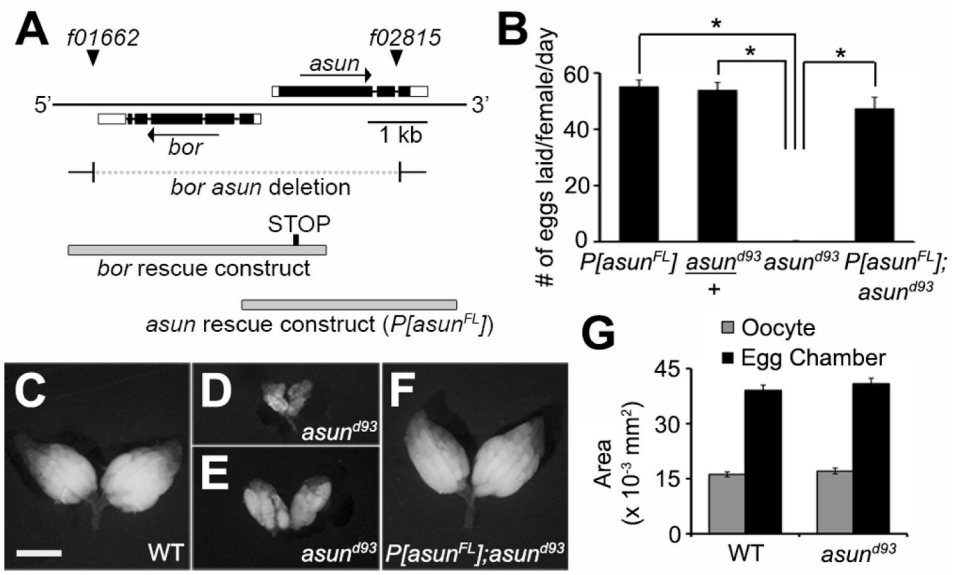


Figure 1. Reduced egg-laying rates and ovary size of *asun*^{d93} females

(A) Schematic diagram of the *asun* gene region. Coding regions and UTRs are represented as filled and unfilled boxes, respectively, introns as thin lines, and *piggyBac* transposons *f01662* and *f02815* as triangles. Breakpoints of a *bor asun* two-gene deletion (generated through FLP-mediated recombination of FRT sites within the transposons) and design of a *bor* transgene are shown; as previously described, *asun*^{d93} flies are homozygous for the *bor asun* two-gene deletion and *bor* transgene (Sitaram et al., 2012). Design of the full-length *asun* transgene (*P[asun*^{FL}*]*) generated for this study is also shown. (B) Quantification of egg-laying rates for females of the indicated genotypes. Asterisks, $p<0.0001$. (C–F) Whole ovaries dissected from 2-day old fattened females of the indicated genotypes. Ovaries from *asun*^{d93} females (D,E) are highly reduced in size compared to those from wild-type (C) or *P[asun*^{FL}*];asun*^{d93} rescue (F) females. Scale bar, 1 mm. (G) Quantification of the average area of stage 10B egg chambers and oocytes isolated from females of the indicated genotypes.

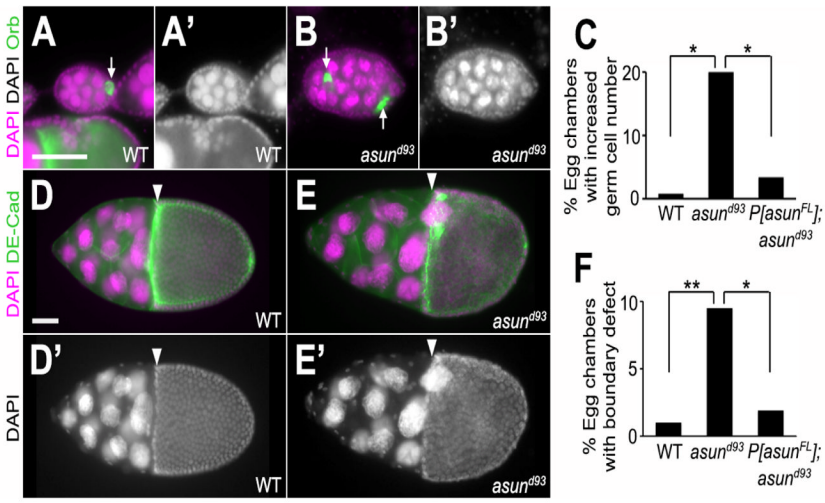
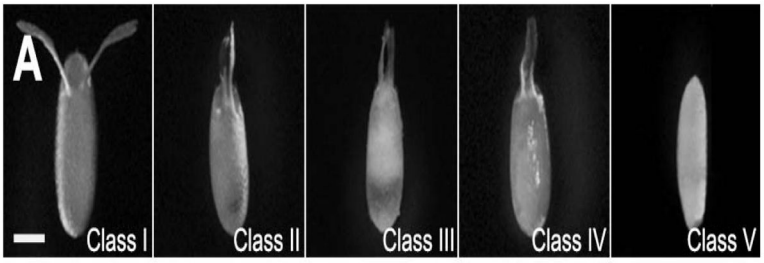


Figure 2. Defects in the cellular composition and arrangement of *asun*^{d93} egg chambers

(AB′) Stage 5 egg chambers stained for DNA (magenta; grayscale in A′ and B′) and Orb (green; oocyte marker) from wild-type and *asun*^{d93} ovaries (dorsal, top; anterior, left). Wild-type egg chambers (A,A′) contain 15 polyploid nurse cells and 1 haploid oocyte. *asun*^{d93} ovaries occasionally contain compound/fused egg chambers (B,B′). White arrows point to oocytes. (C) Quantification of fused egg chamber defect in ovaries of indicated genotypes (>250 chambers scored per genotype). Asterisks, $p<0.0001$. (D–E′) Stage 10 egg chambers stained for DNA (magenta; grayscale in D′ and E′) and DE-cadherin (green; cell membrane marker) from wild-type and *asun*^{d93} ovaries (dorsal, top; anterior, left). There is a clear boundary (white arrowhead) between the nurse cells and the oocyte in stage 10 wild-type egg chambers (D,D′). Nurse cells occasionally extend past this boundary in *asun*^{d93} stage 10 egg chambers (E,E′). (F) Quantification of nurse cell-oocyte boundary defect in ovaries of indicated genotypes (>200 chambers scored per genotype). Single asterisk, $p=0.0006$; double asterisk, $p<0.0001$. Scale bars, 50 μm.



B

Maternal genotype	Dorsal appendage phenotype				
	Class I	Class II	Class III	Class IV	Class V
yw	98.8%	0.9%	0.1%	0.1%	0.1%
$asun^{d93}$	46.4%	12.7%	7.3%	10.7%	22.9%
$P[asun^{FL}];asun^{d93}$	96.9%	0.7%	0.6%	0.5%	1.3%

Figure 3. Ventralization of $asun^{d93}$-derived eggs

(A) Eggs laid by $asun^{d93}$ females. Anterior, top; dorsal side facing outward. Classification scheme for ventralized eggs is adapted from (Lei and Warrior, 2000). Class I eggs appear wild type with a pair of dorsal appendages in the anterior-dorsal region. Dorsal appendages in class II eggs are positioned abnormally close to each other. Class III and class IV eggs contain dorsal appendages that are partially fused at the base and completely fused along the length, respectively. Class V eggs lack dorsal appendages. Scale bar, 250 μm. (B) Quantification of dorsal appendage phenotypes in embryos from wild-type, $asun^{d93}$, and rescued $asun^{d93}$ females (>200 embryos scored per genotype).

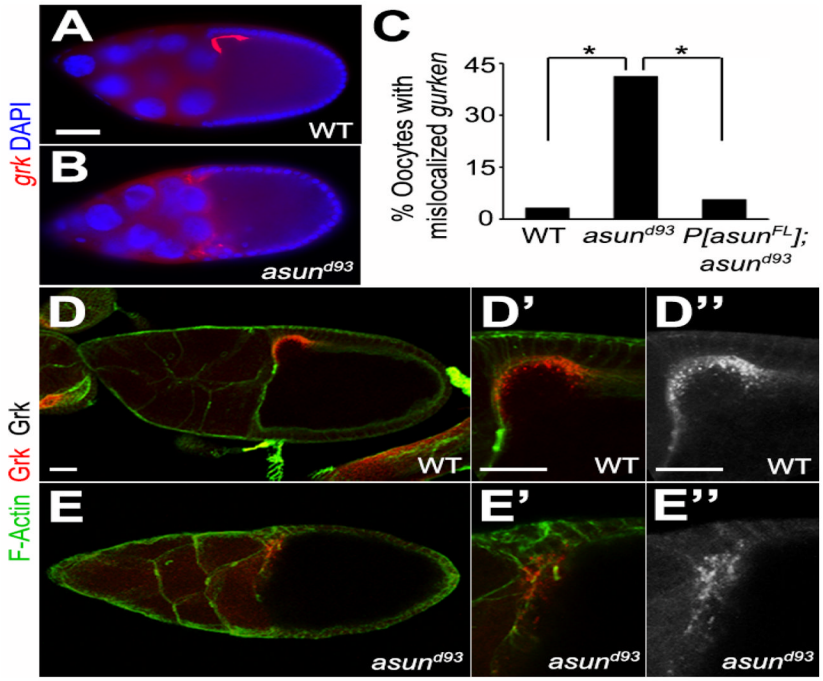


Figure 4. Localization of *grk* transcripts and Grk protein in *asun*^{d93} oocytes

(A–B) Fluorescent in situ hybridization of stage 9 egg chambers using *grk* probe (red). Dorsal, top; anterior, left. *grk* mRNA localization is tightly restricted to the anterior-dorsal region of the oocyte in wild-type egg chambers (A). In *asun*^{d93} egg chambers, *grk* transcripts are more diffusely localized throughout the anterior oocyte (B). Scale bars, 50 μm. (C) Quantification of abnormal *gurken* mRNA localization in wild-type, *asun*^{d93}, and rescued *asun*^{d93} egg chambers (>100 chambers scored per genotype) by fluorescent in situ hybridization. Asterisks, $p<0.0001$. (D–E″) Maximum projection confocal images of stage 10 wild-type and *asun*^{d93} egg chambers stained with Gurken (red; grayscale in D″ and E″) and F-Actin (green; cell membrane marker). Anterior, left; dorsal, top. Gurken protein localizes normally to the anterior-dorsal region of *asun*^{d93} oocytes (E, E′, E″), but occasionally with a lower intensity and more diffuse pattern than that observed in wild-type oocytes (D, D′, D″). Scale bars, 20 μm.

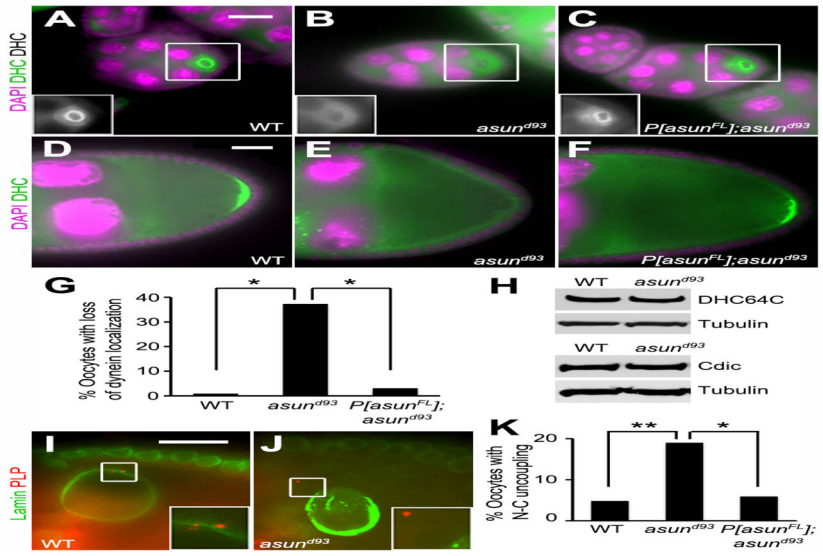


Figure 5. Loss of dynein localization and nucleus-centrosome coupling in *asun*^{d93} oocytes

(A–F) Stage 5 (A–C) and stage 9 (D–F) egg chambers stained for dynein heavy chain (green; grayscale in inset) and DNA (magenta). Dorsal, top; anterior, left. *asun*^{d93} oocytes (B,E) have reduced dynein localization relative to wild-type (A,D) or rescued *asun*^{d93} (C,F) oocytes. (G) Quantification of loss of dynein localization in egg chambers of indicated genotypes (>200 chambers scored per genotype). Asterisks, $p<0.0001$. (H) Immunoblot showing wild-type levels of dynein heavy (DHC64C) and intermediate (Cdic) chains in extracts of *asun*^{d93} ovaries. Loading control, tubulin. (I,J) Stage 10 egg chambers stained for lamin (green; nuclear envelope marker) and PLP (red; centriole marker) (enlarged insets shown below). Dorsal, top; anterior, left. Unlike wild-type oocytes (I), centrosomes are not tightly coupled to the nuclear envelope in *asun*^{d93} oocytes (J). (K) Quantification of nucleus-centrosome coupling defect in ovaries of indicated genotypes (>100 chambers scored for wild-type and *asun*^{d93}; >50 chambers scored for rescue). Single asterisk, $p<0.05$; double asterisk, $p<0.0025$. Scale bars, 20 μm.

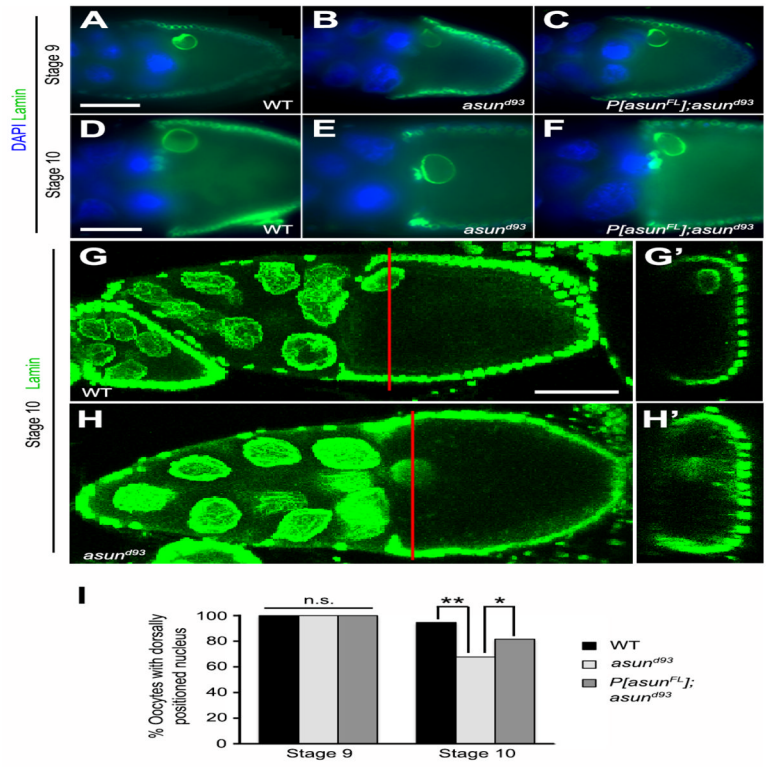


Figure 6. Loss of anterior-dorsal positioning of the oocyte nucleus in *asun*^{d93} egg chambers

(A–H′) Wild-type anterior-dorsal positioning of the oocyte nucleus (A,D,G) is observed in stage 9 *asun*^{d93} oocytes (B), but this positioning is not maintained in stage 10 *asun*^{d93} oocytes (E,H). This positioning defect is not observed in rescued *asun*^{d93} oocytes (C,F). (A–F) Epifluorescent micrographs of stage 9 (A–C) and stage 10 (D–F) egg chambers stained for lamin (green; nuclear envelope marker) and DNA (blue). (G–H′) Confocal micrographs of stage 10 egg chambers stained for lamin (green). XY projections (G,H) and corresponding XZ optical sections (G′,H′). Red bars mark positions of corresponding XZ optical sections. Dorsal, top; anterior, left. Scale bars, 50 μm. (I) Quantification of anterior-dorsal positioning of oocyte nucleus in wild-type, *asun*^{d93}, and rescued *asun*^{d93} stage 9 and 10 egg chambers (>100 chambers scored per genotype). Single asterisk, $p<0.006$; double asterisk, $p<0.0001$; n.s., not significant.

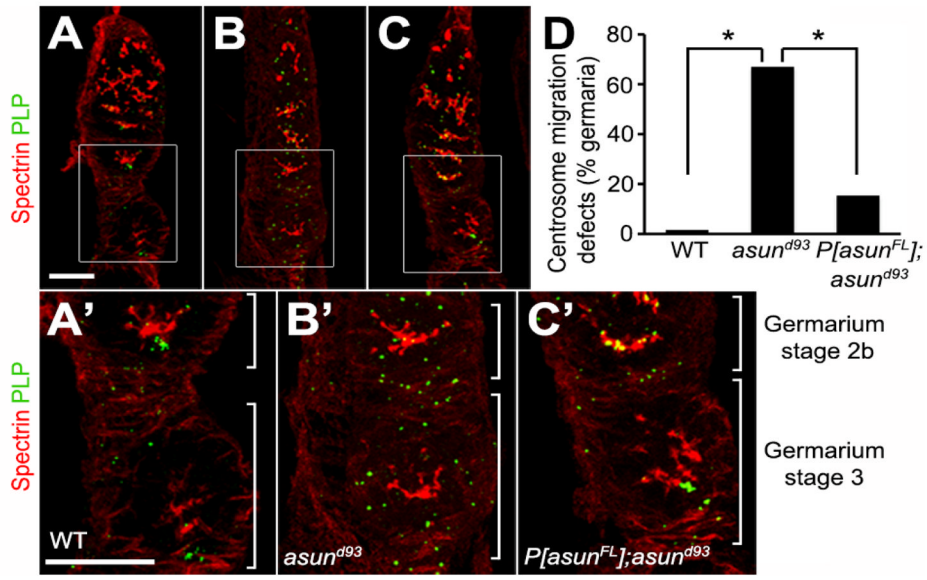


Figure 7. Centrosome migration defects of *asun*^{d93} germaria

(A–C) Projections of confocal sections of wild-type, *asun*^{d93}, and rescued *asun*^{d93} germaria stained for spectrin (red; fusome marker) and PLP (green; centriole marker). Enlarged insets shown below. Anterior, top; dorsal, left. Within wild-type cysts, most centrosomes have migrated from the nurse cells into the oocyte (located at posterior of egg chamber) by stage 2b of the germarium (A, A′). Centrosomes do not properly migrate into the oocyte in *asun*^{d93} germaria and are found distributed throughout the entire cyst (B, B′). Centrosome migration occurs in rescued germaria but is delayed (C, C′). Scale bars, 20 μm. (D) Quantification of centrosome migration defects in wild-type, *asun*^{d93}, and rescued *asun*^{d93} germaria (>100 germaria scored per genotype). Asterisks, $p<0.0001$.