1 Endosome mediated delivery of ceramide phosphoethanolamine (CPE) with
2 unique acyl chain anchors to the cleavage furrow is essential for male meiosis
3 cytokinesis.

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Abstract

Division of one cell into two daughter cells is fundamental in all living organisms. Cytokinesis, the final step of cell division, begins with the formation of an actomyosin contractile ring, positioned midway between the segregated chromosomes. Constriction of the ring with concomitant membrane deposition in a spatiotemporal precision generates a cleavage furrow that physically divides the cytoplasm. Unique lipids with specific biophysical properties have been shown to localize to midbodies however, their delivery mechanisms and biological roles were largely unknown. In this study, we show that Ceramide phosphoethanolamine (CPE), the structural analog of sphingomyelin, has unique acyl chain anchors in spermatocytes and is essential for meiosis cytokinesis. We found that disengagement of the central spindle from the contractile ring but not localization of phosphatidyl inositols (PIPs) at the plasma membrane was responsible for the male meiosis cytokinesis defect in CPE deficient animals. Further, we demonstrate that enrichment of CPE in Rab7 and Rab11 positive endosomes which in turn translocate to the cleavage furrows to promote cytokinesis. Our results implicate endosomal delivery of CPE to ingressing membranes is crucial for meiotic cytokinesis.

Introduction

Eukaryotic cells display high diversity in lipid species, that are chemically and structurally distinct. They are classified into eight major categories, each of which is further subdivided into classes and subclasses [1]. Lipids usually comprise of a polar head group that is linked by a structural backbone to hydrophobic tail groups composed of acyl chains. Lipid diversity that exists in cells arises from the large number of combinatorial chemical possibilities that the head and tail moieties could generate. Diversification of lipids to cope with evolving cellular complexity has resulted in thousands of lipid species each with purported unique function most of which still remain unknown [2].

The head group of each lipid class has been widely recognized for its biological function. For instance, the inositol head group of phosphatidylinositols and its modifications play critical roles in cellular signaling [3]. However, even within each lipid class, there are a number of molecularly distinct species that have identical head group but differ extensively in their acyl side chains such as number of carbon atoms, number of double bonds and the nature of the chemical linkage (e.g., ester or ether). An increasing number of studies are beginning to reveal the importance of acyl side chains by demonstrating that they selectively bind to target proteins and elicit biological function [4, 5]. For instance, a recent study has shown that acyl side chains of diacylglycerol significantly influence the recruitment of protein kinase C to the plasma membrane [6]. Similarly, Ceramide chain length was shown to be critical for selective protein cargo sorting at endoplasmic reticulum (ER) exit sites in yeast [7]. The COPI machinery protein p24 was shown to bind specifically to N-stearoyl sphingomyelin (SM C18) and acts as a cofactor to regulate COPI-dependent transport in the early secretory pathway. Further, this interaction depended on both the headgroup and the backbone of the sphingolipid [8].

Cell division is fundamental to all living organisms where one cell divides into two daughter cells. The last step of cell division, the cytokinesis, begins with the formation of an actomyosin contractile ring, positioned midway between the segregated chromosomes. Narrowing of this ring with the addition of membrane in a spatiotemporally defined mode produces a cleavage furrow that physically divides the cytosol [9]. During somatic cytokinesis, the two daughter cells are interconnected *via* midbodies prior to abscission. Although these structures are transient, they accumulate defined lipid species to mediate correct cell division [10-12]. Unlike somatic cytokinesis, in testis, the developing spermatogonial cells divide synchronously with an incomplete cytokinesis where all the daughter cells remain interconnected by cytoplasmic bridges. Thus, the spermatogonial cells develop as a syncytium and become separated from each other only at the end of spermatogenesis during spermatid individualization. The high degree of membrane curvature necessary for cleavage furrow constriction and stabilization of intercellular bridges (ICB)/cytoplasmic bridges necessitates lipid components with specific biophysical properties. While the presence of very long chain fatty acids (which are components of sphingolipids and glycosphingolipids) in cytokinesis has been shown, the relationship between membrane lipid composition, ring constriction and furrow ingression is still underexplored. Although sphingomyelins containing very long chain polyunsaturated fatty acids (VLC-PUFAs) were identified in various mammalian testis including humans, their direct role in spermatogenesis particularly in meiosis cytokinesis remains unknown [13, 14].

In this study we show that CPE, the structural analog of sphingomyelin (SM), has unique acyl chain anchors in spermatocytes and CPE is essential for meiosis cytokinesis, spermatid polarity and individualization. The head group of CPE is also important for spermatogenesis. Ceramide phosphoethanolamine synthase (CPES) expression in transit amplifying to spermatocyte stage is

essential for successful completion of spermatogenesis. We show aberrant central spindle behavior at the cleavage furrows is responsible for the meiosis cytokinesis defect. Further, we show endocytically retrieved CPE from plasma membrane is enriched in Rab7 containing endosomes and targeted to cleavage furrows during cytokinesis demonstrating the importance of CPE rich membrane addition at the cleavage furrows.

Results

Sphingosine with VLC-mono-unsaturated fatty acid and sphingadiene with VLC-saturated fatty acid containing sphingolipids are enriched in the testis

To investigate sphingolipid species diversity and their role in *Drosophila* tissues we performed super-critical fluid chromatography coupled to mass spectrometry (SFC/MS/MS) on extracted lipids from wild type fly head, dissected ovary and testis (Data S1). As shown in Fig.1A, CPE is a more abundant sphingolipid compared to ceramides (Fig.1B) and hexosylceramides (HexCer) (Fig.1C). The amount of CPE is significantly higher in testis compared to head and ovary (Fig.1A). In contrast hexosylceramides were significantly higher in the heads compared to ovary and testis (Fig.C).

Fig.1. Sphingolipid analysis in wild type *Drosophila* tissues. (A-C) Sphingolipids were extracted from wild type male and female heads and dissected ovary and testes using Methanol:Chloroform method and subjected to SFC/MS/MS. Wild type heads from male flies (WTHM), wild type heads from female (WTHF), wild type ovary (WTOY) and wild type testis (WTTS). Sphingolipids were quantitated by measuring large fragments in MRM and average amounts from 3 independent biological replicates were shown as picomols (pmol) in 100 micrograms of carbon. Each sphingolipid species (CPE (1A), Ceramide (1B) & HexCer (1C)) was further subdivided into 5 major subspecies. Each of the subspecies differed in acyl chain length but contained similar

chemical properties Dihydrosphingosine (DHSPH) base linked to Saturated fatty acid (SFA) (DHSPH_SFA), Sphingosine (SPH) base linked to SFA (SPH_SFA), Sphingosine (SPH) base linked to Monounsaturated fatty acid (MUFA) (SPH_MUFA), Sphingosine (SPH) base linked to Polyunsaturated fatty acid (2-6 double bonds, PUFA) (SPH_PUFA) and Sphingadiene (SPD) with SFA (SPD_SFA). (D) Cartoon showing the chemical structure of three major CPE subspecies including CPE_SPH_SFA, CPE_SPH_MUFA and CPE-SPD_SFA. (E) CPE subspecies within CPE_SPH_SFA that differed in number of carbon atoms in acyl chains of sphingosine base and fatty acid. (F) CPE subspecies within CPE_SPH_MUFA. (G) CPE subspecies within CPE_SPD_SFA. Statistical significance was calculated using mean, standard deviation (SD) and *N* in Prism 8. The 2way ANOVA multiple comparisons was used to calculate *P* values where *****P* ≤ 0.0001 ; ****P* ≤ 0.001 ; ***P* ≤ 0.01 ; **P* ≤ 0.05 and ns *P* > 0.05 . Three independent biological replicates were performed for each sample and lipids were extracted from 400 heads or 250 pairs of ovary or testis for each biological replicate.

Closer examination of acyl chain composition revealed that head and ovary derived CPEs are predominantly composed of sphingosine linked to saturated fatty acid (SPH-SFA) (Fig.1D and 1E). Interestingly, in addition to SPH-SFA, CPE in the testis is enriched in two distinct species with net two double bonds in their acyl chain composition including sphingosine (one double bond) linked to mono-unsaturated fatty acid (SPH- MUFA) (Fig.1D and 1F) and a sphingadiene (two double bonds) linked to saturated fatty acid (SPD-SFA) (Fig.1D and 1G). The fatty acid acyl chain lengths of these CPE species are predominantly composed of C20, C22, C24 and therefore could be classified as very long chain (VLC) sphingolipids. The sphingoid base acyl chain length composed of d14 and d16 (Fig.1F and 1G). The corresponding ceramide precursors are also evident in the wild type testis samples indicating acyl chain diversity is likely introduced during

ceramide synthesis but not after CPE synthesis (Fig.1B and Data S1). Remarkably, SPH_MUFA and SPD_SFA were also present in HexCer but at significantly lower levels in the testis compared to heads (Fig.1C vs Fig.1A). The fatty acid acyl chain length of HexCer SPH_MUFA and HexCer_SPD_SFA showed that they are predominantly composed of C18 and C20 while the sphingoid base is composed of primarily d14 and d16 (Fig.S1B and S1D). HexCer with d14 sphingosine base and C22 PUFA (SPH-PUFA) is enriched in heads compared to testis (Fig.1C and Fig.S1C). Taken together these results suggest that CPE in the testis and the HexCer in the central nervous system have unique acyl chain composition.

Fig.S1. Comparison of hexosylceramides and its subspecies in *Drosophila* tissues. (A) Hexosylceramides composed of sphingosine base linked to saturated fatty acid (HexCer SPH-SFA) and its subspecies were shown as picomols (pmol) in 100 micrograms of carbon. Wild type heads from male flies (WTHM), wild type heads from female (WTHF), wild type ovary (WTOY) and wild type testis (WTTS). (B) Hexosylceramides and its subspecies composed of sphingosine base linked to Monounsaturated fatty acid (HexCer-SPH-MUFA). (C) Hexosylceramides and its subspecies composed of sphingosine base linked to Polyunsaturated fatty acid (HexCer-SPH-PUFA). (D) Hexosylceramides and its subspecies composed of Sphingadiene base linked to Saturated fatty acid (HexCer SPD-SFA). Statistical significance was calculated using mean, standard deviation (SD) and N in Prism 8. The 2way ANOVA multiple comparisons was used to calculate P values where **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$ and ns $P > 0.05$. Three independent biological replicates were taken for each sample and lipids were extracted from 400 heads or 250 pairs of ovary or testis for each biological replicate.

CPE deficient males are sterile and show defects in male meiotic cytokinesis, spermatid polarity and individualization

To understand the biological significance of the unique CPE species in the testis, we reasoned that the *cpes* null mutant generated by us would serve as a suitable tool since *cpes* mutant males are sterile [15]. These mutants show accumulation of germ cells at the tip of the testis and absence of mature sperms in seminal vesicles (Fig.2B). To investigate cytological defects in *cpes* mutant testis, we have performed live testis squash preparation followed by phase contrast microscopy. This method allows for easy identification of almost all of the major stages of spermatogenesis [16]. During meiotic divisions, chromosomes and mitochondria are equally partitioned to each of the four daughter cells. Immediately following meiosis, in the round spermatid stage, mitochondria in each daughter cell aggregate to form a large phase dark structure known as the nebenkern. Round spermatids (onion stage spermatids) are characterized by phase dark nebenkern and phase light nucleus at roughly equal size and at a ratio of 1:1 per daughter cell. The nuclear size of round spermatid is directly proportional to the chromatin content, hence any errors in chromosome segregation during meiosis can lead to variability in nuclear size [17, 18]. Similarly defects in meiotic cytokinesis following normal chromosome segregation results in cells with two or four nuclei and an abnormally large nebenkern. These changes can be easily visualized by phase contrast microscopy and is often used as a diagnostic to detect male meiotic defects [18-24]. As shown in Fig.2D, wild type round spermatids contained an equal sized phase light nucleus and a phase dark nebenkern at a ratio of 1:1 per cell within the cyst of 64 cells. In contrast *cpes* mutant round spermatids showed four regularly sized nuclei (tetranucleate) and one abnormally large nebenkern, indicating a failure in cytokinesis in both meiosis I and II divisions (Fig.2E).

Fig.2. Cytological, immunofluorescence and sphingolipid analysis in cpes mutants.

(a-c) whole mount of Drosophila testis immunostained for germ cell specific Vasa protein primary antibody and Alexa fluor 488 secondary antibody to visualize various stages of germ cells

including stem cells, spermatogonia and spermatocytes (A) w^{1118} , (B) *cpes* mutant and (C) *Tub-Gal4>UAS CPES* rescue. (D-F) Phase contrast microscopy of live testis squash preparations to visualize round/onion stage spermatids (D) w^{1118} , (E) *cpes* mutant and (F) *Tub-Gal4>UAS CPES* rescue. (G-I) Testis squash preparation followed by fixation and immunostaining to visualize early elongating spermatids. The beta tubulin primary antibody and Alexa fluor 568 conjugated secondary antibody were used for immunostaining, w^{1118} (G), *cpes* mutant (H) and *Tub-Gal4>UAS CPES* rescue (I). (J-K) whole mount *Drosophila* testis immunostained with cleaved caspase specific DCP-1 primary antibody and Alexa fluor 568 secondary antibody to visualize individualizing spermatids (J) w^{1118} (K) *cpes* mutant and (L) *Tub-Gal4>UAS CPES* rescue. (M-O) Sphingolipid analysis in lipid extracts of dissected testis. (M) Comparison of CPE and its subspecies between w^{1118} , *cpes* mutant and *bam-Gal4>UAS CPES* rescue. (N) Comparison of ceramide and its subspecies between w^{1118} , *cpes* mutant and *bam-Gal4>UAS CPES* rescue. (O) Comparison of hexosylceramide and its subspecies between w^{1118} , *cpes* mutant and *bam-Gal4>UAS CPES* rescue. Statistical significance was calculated using mean, standard deviation (SD) and N in Prism 8. The 2way ANOVA multiple comparisons was used to calculate P values where **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$ and ns $P > 0.05$. Three independent biological replicates were taken for each sample and 250 pairs of testis were used for lipid extraction from each biological replicate.

Following the round/onion stage, the 64 interconnected haploid cells in the cyst become highly polarized with the nucleus localizing to one end and the sperm tail (axoneme) growing in the opposite side. These elongating spermatid nuclei face towards the seminal vesicles and the tails grow towards the germline stem cell hub/tip of the testis. As shown in Fig.2G, wild type early elongating spermatids had all the nuclei facing one side and their axoneme growing to the opposite

side indicating that they are highly polarized. In contrast, elongating spermatids in *cpes* mutant cysts appeared as sickle cell shaped with the nuclei present in the middle-bulged area (occasionally throughout the cyst) and their tails growing on both sides of the cyst (Fig.2H). This data indicates that spermatid polarity is compromised in *cpes* mutants.

Individualization is the final stage of spermatid differentiation wherein structures called individualization complex (IC) containing actin cones assemble at the head/rostral end of elongated spermatids and start moving away from the nuclei towards the tail/caudal end of the cyst. As the IC travels it removes the cytoplasmic contents of the cyst and will individualize each spermatozoon with its own plasma membrane. The extruded cytoplasmic contents are collected into a sac like structure called the ‘cystic bulge’ that forms around the IC. As this process proceeds and the IC and cystic bulge reach the end of the flagella, the actin cones and cytoplasmic contents are extruded in a waste bag, the contents of which are degraded later [25]. Activated caspases have a non-apoptotic role in cystic bulge and the waste bag of individualizing spermatids and are essential for successful spermatid individualization [26]. Immunostaining of wild type testis with *Drosophila* Caspase 1 (DCP-1) antibody showed elongated spermatids undergoing individualization that contained cystic bulges and waste bags (Fig.2J). In contrast, *cpes* mutant testis showed defectively elongated spermatids and were devoid of cystic bulges and waste bags indicating failure in spermatid individualization (Fig.2K).

Ubiquitous expression of wild type cDNA copy of CPES completely rescued male sterility. As shown in Figs.2C, f, i and l, phase contrast imaging of live testis squash preparations showed 1:1 nebenkern to nucleus ratio indicating rescue of meiotic cytokinesis defect (Fig.2F). Further immunostaining of testis squash preparations with tubulin antibody and DAPI showed that spermatid polarity was completely restored wherein all the nuclei face one side and their tails face

the other side of the cyst (Fig.2I). DCP-1 staining showed individuating spermatids with cystic bulges and waste bags indicating normal individualization (Fig.2L).

To investigate the sphingolipid content in *cpes* mutant we dissected wild type, *cpes* mutant and germ cell specific rescue (Bam Gal4> UAS CPES) testes were dissected, sphingolipids were extracted and analyzed by SFC/MS/MS as described in the methods (Data S1). There was complete loss of CPE in *cpes* mutant testis (Fig.2M) and expression of CPES only in the germs cells was sufficient to restore CPE to near wild type level. The mutant and rescue samples were processed at the same time as the wild type for mass spectrometry, hence the wild type testis sample is the same as in Fig.1 (Data S1). Loss of CPE synthesis in *cpes* mutants caused concurrent accumulation of ceramides particularly SPH-MUFA and SPD-SFA species (Fig.2N) and expression of CPES in germ cells reduced ceramide levels. HexCer levels were also slightly increased in *cpes* mutant testis (Fig.2O), interestingly expression of CPES in germ cells did not reduce the HexCer levels indicating their levels did not correlate with the *cpes* mutant phenotypes in the testis.

CPES enzymatic activity is required for spermatogenesis and accumulation of ceramide is not the cause for spermatogenesis defects

In order to determine whether enzymatic activity of CPES is required for spermatogenesis we have generated active site mutants of CPES. The CPES has conserved amino acid motif D(X)2DG(X)2 (A/Y)R(X)8-16G(X)3D(X)3D in the CDP-alcohol phosphotransferase domain (CAPT) [27]. The final aspartates of this motif were shown to be essential for catalysis of human choline/ethanolamine phosphotransferase CEPT1 [28, 29]. We substituted the last two active site aspartates of CPES with alanine residues and subcloned into pUAST vector for generating transgenic flies. The UAS_CPES (DX₃D to AX₃A) mutant transgene was expressed in *cpes* mutant background using Bam Gal4 to test rescue of spermatogenesis phenotypes. As shown in Fig.S2A

and B, the active site mutant did not rescue spermatogenesis phenotypes suggesting a crucial role for CPES enzymatic activity in spermatogenesis.

Fig.S2. CPES enzymatic activity is required for spermatogenesis and accumulation of ceramide is not responsible for *cpes* mutant phenotypes. (A-C) Live testis squash preparation followed by phase contrast microscopy of round spermatids (A) The UAS CPES active site mutant (*GX₃DX₃D* to *GX₃AX₃A*) was expressed using *Bam-Gal4* in *cpes* mutant background. (B) *cpes*; *dcert* double mutants (C) The UAS CDase (neutral ceramidase) was expressed using *Bam-Gal4* driver in *cpes* mutant background.

We next investigated whether accumulation of ceramide at the Golgi is responsible for the observed phenotypes. Ceramide is generated in the membranes of endoplasmic reticulum (ER) via the *de novo* biosynthetic pathway catalyzed by the rate limiting enzyme serine palmitoyltransferase (SPT). Subsequently ceramide is actively transported from ER to Golgi via ceramide transfer protein (DCERT) and thus absence of DCERT could prevent ceramide accumulation at the Golgi [30]. To investigate whether blocking ceramide transport from ER to Golgi could restore spermatogenesis, we have generated *cpes* and *dcert^l* double mutants and their testis was analyzed for meiotic cytokinesis and spermatid polarity phenotypes. However, as shown in Fig.S2C and D. *cpes*; *dcert^l* double mutants did not rescue meiotic cytokinesis and spermatid polarity indicating ceramide accumulation may not be responsible for the phenotypes. In *Drosophila*, transgenic expression of neutral ceramidase was shown to reduces ceramide levels *in vivo* [31]. To further confirm, we have overexpressed ceramidase (UAS CDase) in germs cells using *Bam-Gal4* in *cpes* mutant background and the progeny testis was analyzed for the rescue of mutant phenotypes. However, again expression of CDase did not rescue meiosis cytokinesis and spermatid polarity (Fig.S2E and F) suggesting that absence of CPE but not accumulation of ceramide is responsible

for the phenotypes. Also, ceramide levels in testis do not correlate with sterility as will be discussed in figure 4J and K.

Genetic dissection of spatiotemporal role of CPES in spermatogenesis

To determine the stage of germ cell differentiation at which CPES expression has an important role, we have performed cell type/stage specific rescue experiments using UAS/Gal4 system in the *cpes* mutant background (Fig.3A). We first expressed UAS CPES in cyst cells using C587-Gal4 to investigate a possible non-cell autonomous role. However, as shown in Fig.3, expression of wild type CPES in cyst cells did not rescue meiotic cytokinesis, spermatid polarity and individualization (Fig.3 B, F and J respectively). We next expressed wild type UAS CPES in germ cells using three different Gal4 drivers that have been previously shown to be specifically expressed in stem cells (nos-Gal4), transit amplifying spermatogonial cells (bam-Gal4) and late spermatocytes (chif-Gal4) (Fig.3A). Interestingly, we observed varying degree of rescue, depending on the germ cell differentiation stage at which UAS CPES was expressed. The nos-Gal4 dependent expression of UAS CPES partially rescued both meiosis cytokinesis and spermatid polarity (Fig.3C, and G) however, individualizing spermatids lacked cystic bulges and waste bags indicating individualization was not fully rescued (Fig.3K). The bam-Gal4 dependent expression of UAS CPES in transit-amplifying cells completely rescued meiosis cytokinesis, spermatid polarity and individualizing spermatids contained cystic bulges and waste bags (Fig.3D, H and L). In contrast, only partial rescue of meiosis cytokinesis was observed when UAS CPES was expressed in spermatocytes using chif-Gal4 (Fig.3E). Although, the spermatid polarity was completely rescued, the individualizing spermatids largely lacked cystic bulges and waste bags indicating that individualization was defective (Fig.3I and M).

Fig.3. Genetic dissection of spatiotemporal role of CPES in spermatogenesis. (A) Schematic illustration of early germ cell differentiation and the stage specific Gal4 expression patterns. The C587-Gal4 expresses in somatic cyst cells, nos-Gal4-VP16 expresses in GSCs and spermatogonia, bam-Gal4-VP16 expresses in 4-16 stages of spermatogonia to spermatocytes, and Chif-Gal4 expresses in early spermatocytes. (B-E) Phase contrast microscopy of round spermatids in live testis squash preparations. (F-I) Early elongating spermatids immunostained for beta tubulin primary antibody (Alexa 568) and DAPI for DNA (J-M) Individualizing spermatids immunostained for cleaved caspase DCP-1 antibody and DAPI for DNA. (B,F and J) C587-Gal4 drives the expression of UAS CPES in cyst cells of cpes mutants. (C, G and K) Nanos-Gal4 drives the expression of UAS CPES in GSCs and spermatogonia. (D, H & I) bam-Gal4 drives the expression of UAS CPES in spermatogonia and spermatocytes (E, I & M) chif-Gal4 drives the expression of UAS CPES in early spermatocytes. (N) Male fertility test where individual males from each of the UAS CPES rescue experiment was crossed with 3 wild type females. The vials which produced at least 5 pupae or progeny were counted as fertile and plotted as percent fertility compared to wild type males. (O-R) Whole mount immunostaining followed by confocal imaging of genomic CPES flies where CPES is tagged with V5 at the C-terminus (red). The GM130 was a cis-Golgi marker (green) and DAPI (blue) stains the DNA.

To determine the extent to which male fertility was rescued by cell type specific CPES expression compared to wild type, we performed the male fertility test as described in the methods section. As shown in Fig.3N, expression of UAS CPE in transit amplifying spermatogonial cells and spermatocytes (bam-Gal4) rescued male fertility to wild type levels. In contrast only partial rescue was observed when UAS CPES was expressed only in stem cells (nos-Gal4) or in spermatocytes

(chif-Gal4). Expression of UAS CPES in cyst cells (C587-Gal4) did not rescue male sterility and as all of the males were sterile (Fig.3N).

To determine *in vivo* CPES expression more precisely at the protein level, we have expressed genomic CPES tagged with V5 at the C-terminus. Immunostaining followed by confocal imaging showed that CPES_V5 was highly expressed in the cells that are present just below the tip of the testis (Fig.3O and R). This area of the testis is known to be enriched in spermatogonia and early spermatocytes [16].

Taken together these results show that testis specific CPE generation by CPES from transit amplification stage to spermatocyte stage is crucial for successful meiosis cytokinesis, spermatid polarity and spermatid individualization.

Insect CPE Synthases but not sphingomyelin Synthase rescue male sterility defects

Previously we have shown that CPE has an important structural role in cortex glial plasma membranes [15]. The cortex glial plasma membranes are severely defective in *cpes* mutants leading to loss of cortex glia neuronal cell body interactions during development and increased susceptibility to light inducible epilepsy in adults. Further, we have shown that overexpression of human sphingomyelin synthase 1 (hSMS1) that produces SM instead of CPE was sufficient to rescue cortex glia plasma membrane defects via establishment of detergent resistant membranes. We wondered whether overexpression of hSMS1 could also rescue the spermatogenesis phenotypes of *cpes* mutant. To this end, UAS hSMS1 was overexpressed using Tubulin-Gal4 in *cpes*, the adult male testes were dissected, and live testis squash preparations were observed using phase contrast microscopy. However, as shown in Fig.4A, hSMS1 overexpression did not rescue male meiosis cytokinesis. Further, immunostaining analysis showed that spermatid polarity was also not rescued (Fig.4E) suggesting an important role for the CPE head group in spermatogenesis.

We next investigated whether overexpression of other insect derived CPES could rescue *cpes* mutant phenotypes. To test this, we have overexpressed *Aedes aegypti* (Yellow fever mosquito, 60% identity and 77% similarity), *Bombyx mori* (domestic silk moth, 43% identity and 58% similarity) and *Tetranychus urticae* (two-spotted spider mite, 33% identity and 51% similarity) derived UAS CPES homologs in *cpes* mutant background using Tubulin-Gal4. As shown in Fig.4 mosquito and silk moth CPES were able to completely rescue meiosis cytokinesis (Fig.4B and C) and spermatid polarity (Fig.4F and G). However, spider mite CPES was not able to rescue any of the *cpes* phenotypes (Fig.4D and H).

Fig.4. Insect CPE synthases but not sphingomyelin synthases rescue spermatogenesis defects.

(A-D) Phase contrast microscopy of round spermatids where UAS *hSMS1* (A), UAS *Aedes* CPES (B), UAS *Bombyx* CPES (C), and UAS *T.Urticae* CPES (D) were ubiquitously expressed in *cpes* mutant background using Tubulin Gal4. The scale bar is equivalent to 10 μ m. (E-H) Confocal images of elongating spermatids immunostained with beta tubulin primary antibody and Alexa fluor 568 secondary antibody and DAPI for DNA. The UAS *hSMS1* (E), UAS *Aedes* CPES (F), UAS *Bombyx* CPES (G), and UAS *T.Urticae* CPES (H). (I-L) Sphingolipid analysis of lipids extracted from testis of wild type, *cpes* mutant, and various rescues expressed in germ cells using *Vasa-Gal4*. In contrast to Figs.1 and 2, here we used whole male organ (testis, accessory glands, collecting ducts and ejaculatory bulb) for lipid extraction. (I) The amounts of CPE and its subspecies in wild type, *cpes* mutant and various rescues were shown. (J) Ceramide and its subspecies were shown in picomoles per 100 μ g of carbon. (K) Hexosylceramides and its major subspecies amounts were shown in bar diagram. (L) Amount of sphingomyelin and its subspecies were shown as picomoles per 100 μ g of carbon. Dihydrosphingosine (DHSPH), Sphingosine (SPH), Sphingadiene (SPD) Saturated fatty acid (SFA), Monounsaturated fatty acid (MUFA) and

polyunsaturated fatty acid (PUFA). Statistical significance was calculated using mean, standard deviation (SD) and N in Prism 8. The 2way ANOVA multiple comparisons was used to calculate P values where **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$ and ns $P > 0.05$. Three independent biological replicates were taken for each sample and lipids were extracted from 250 male organs for each biological replicate.

To investigate the nature of sphingolipid species synthesized by hSMS1, *Aedes aegypti*, *Bombyx mori* and *Tetranychus urticae* derived CPES, we have specifically expressed these transgenes in germ cells using vasa-Gal4, their male reproductive system was dissected, lipids were extracted and subjected to sphingolipid analysis using SFC/MS/MS as described in the methods section (Data S2). As shown in Fig.4I germ cell specific expression of *Drosophila* CPES, *Bombyx mori* CPES completely restored CPE to the wild type amounts. *Aedes aegypti* CPES also synthesized significant amount of CPE but to a lower amount than *Drosophila* CPES. However, all three distinct CPE species are synthesized by *Aedes aegypti* CPES and *Bombyx* CPES (Fig.4I). As anticipated, hSMS1 did not synthesize CPE (Fig.4I) but produced significant amount of SM (Fig.4L). However, the amount of SM is relatively low compared to CPE, perhaps due to limited expression/stability of hSMS1 in germ cells (vasa-Gal4). Indeed, ubiquitous expression of hSMS1 using actin-Gal4 driver significantly increases the SM amounts (Fig.4M), yet at this level of expression we did not see rescue of *cpes* mutant phenotypes (Fig.4A and E) suggesting the importance of the CPE head group. We next analyzed the ceramide content in these samples and found that ceramide levels were significantly high in *cpes* mutants and hSMS1 rescue compared to *Drosophila* CPES rescue. Interestingly, even though *Tetranychus urticae* CPES was not able to synthesize CPE, it significantly reduced the ceramide and HexCer amounts of *cpes* mutants (Fig.4 J, and K). However, despite the reduction in ceramide and HexCer levels, *Tetranychus urticae*

CPES failed to rescue *cpes* mutant phenotypes suggesting lack of correlation between ceramide accumulation and rescue of *cpes* mutant phenotypes (Fig.4J). Similarly, higher amounts of HexCer in *Aedes aegypti* CPES rescue and *Bombyx mori* CPES rescue did not correlate with the spermatogenesis phenotype rescue (Fig.4K).

Aberrant central spindle behavior but not localization of PIPs at the plasma membrane is responsible for defective meiosis cytokinesis in *cpes* mutants

To determine the mechanism by which CPES regulates meiosis cytokinesis we have performed RNAseq with total RNA isolated from dissected testis from *w¹¹¹⁸*, *cpes* mutant and bam-Gal4>UAS CPES rescue. Gene set enrichment analysis (GSEA) comparison between *w¹¹¹⁸* (WT), *cpes* (KO) and rescue (RES) samples indicated that several pathways involved in spindle organization, metabolism, endocytosis, signaling, male meiosis cytokinesis, spermatid differentiation, sperm individualization etc., are significantly altered in *cpes* mutants (Fig.5A). Red bars in the Fig.5A indicates gene sets enriched in *cpes* mutants and blue bars indicate gene sets enriched WT & RES. GSEA showed several genes involved in spindle organization and spindle elongation positively correlated with the mutant phenotype (Fig.5B and C). The heat map comparison for genes involved in spindle organization and elongation are shown in Data S3 and S4. To study spindle behavior during male meiosis cytokinesis *in vivo*, we performed live imaging on isolated cysts. We first dissected the testis in M3 insect cell culture media and muscle sheath was cut open to release intact spermatocyte cysts into the media. Subsequently spermatocyte cysts were transferred to poly-D-lysine coated cover glass dishes and live imaging was performed using Andor spinning disk confocal microscopy as described in the methods section. We chose mCherry tagged microtubule cross linking protein Feo (Ubi-p63E-feo-mCherry) as a marker for central spindle. Feo-mCherry was shown to accumulate at the anaphase B and telophase central spindle

[32]. We have also used spaghetti squash (sqh) gene encoding myosin II regulatory light chain (RLC) tagged to GFP (Sqh-GFP-RLC) as a marker for actin-myosin based structure known as contractile ring that assembles during late anaphase [33]. The actin and myosin ring positioned midway between two spindle poles is thought to drive the formation of the cleavage furrow during telophase. As shown in Movie S1, Fig.5D, the actomyosin ring (Sqh-GFP-RLC) quickly assembles around the central spindle during late anaphase and both simultaneously constrict through early to late telophase. At the end of late telophase Feo-mCherry disassembles however, Sqh-GFP-RLC remains and becomes part of ring canal/cytoplasmic bridges between daughter cells (Movie S1). Interestingly, in *cpes* mutants, the initial assembly of central spindle and actomyosin ring occurred normally (Movie S2) however, central spindle and actomyosin ring constriction became uneven during cleavage furrow ingression leading to substantial disengagement between them (Fig.5D). As a result, central spindle disassembled prematurely leading to the cleavage furrow regression and disassembly of actomyosin ring (Movie S2). Expression of UAS CPE in germ cells was sufficient to rescue this phenotype (Movie S3, Fig.5D).

Fig.5. Aberrant central spindle behavior but not PIPs localization at the plasma membrane is responsible for meiosis cytokinesis defect in *cpes* mutants. (A) GSEA pathway analysis of RNA seq data from *w¹¹¹⁸*, *cpes* mutant and *bam-Gal4>UAS CPES* rescue testis. Three independent biological replicates were taken for each sample and RNA was extracted from 200 pairs of testis for each biological replicate. (B) Enrichment plot showing genes involved in spindle organization are positively correlated in *cpes* mutants compared to *w¹¹¹⁸* and rescue. (C) Enrichment plot showing genes involved in spindle elongation are positively correlated in *cpes* mutants compared to *w¹¹¹⁸* and *bam-Gal4>UAS CPES* rescue. (D-F) Snapshots of live spermatocytes undergoing meiosis I cytokinesis. Horizontal view(left), one slice of a z-stack was shown and cross-sectional

view across cleavage furrow, maximum intensity projection image was shown (right). Control spermatocytes (top row), *cpe*s mutant spermatocytes (middle row) and *Tub-Gal4>UAS CPES* rescue (bottom row). (D) Spermatocytes expressing myosin II regulatory light chain tagged to GFP under its own promoter (*sqh*) that localizes to contractile ring and mCherry tagged fascetto (*feo*) expressing under the control of *Ubi-p63E* promoter that binds to central spindle microtubules. (E) Spermatocytes expressing GFP tagged pleckstrin homology (PH) domain of PLCD (*UAS-PLCδ-PH-EGFP*) under the control of *Tub-Gal4* that localizes to plasma membrane in the presence of phosphatidylinositol-4,5-bisphosphate (PIP2) and *Ubi-p63E-Feo-mCherry*. (F) Spermatocytes expressing GFP tagged PH domain of *Grp1* under the control of alpha *Tub84B* promoter (*tGPH*) that localizes to plasma membrane in the presence of phosphatidylinositol-3,4,5-triphosphate (PIP3) and *Ubi-p63E-Feo-mCherry*.

Previous studies have shown that phosphatidyl inositols (PIPs) play critical roles in somatic and meiotic cytokinesis [34]. Further, in somatic cells undergoing mitosis, sphingomyelin-rich lipid domains in the outer leaflet of cleavage furrow were shown to be required for accumulation of PIP (2) to the cleavage furrow which in turn is required for proper translocation of RhoA and progression of cytokinesis. We wondered whether the distribution of PIPs at the plasma membrane or at the cleavage furrow is altered in *cpe*s mutants during meiosis. To investigate PIPs distribution in *cpe*s mutant membranes we used PIP2 reporter *UAS PLCδ-PH-EGFP* (*UAS* regulatory sequence drives expression of EGFP tagged with Tag:PH(hPLCD1)) and PIP3 reporter *GFP-PH-Steppke*, also known as *tGPH* (an alpha*Tub84B* promoter drives expression of EGFP fused to Tag:PH(step) [35, 36]. The PIP2 reporter *PLCδ-PH-EGFP* was shown to uniformly localize to entire membrane including the cleavage furrow in *Drosophila* spermatocytes [37]. Indeed, we have seen similar uniform distribution in both control and *cpe*s mutant spermatocyte plasma membranes

indicating normal distribution of PIP2 (Movie S4 and S5, Fig.5E). Live imaging analysis on *cpes* mutant spermatocytes expressing PLC δ -PH-EGFP revealed normal cleavage furrow initiation but progression of furrow failed due to disconnect between central spindle and the furrow (Movie S5, Fig.5E). Expression of wild type CPES restored this phenotype (Movie S6, Fig.5E). Of note, PLC δ -PH-EGFP localization to spermatocyte plasma membrane was significantly reduced upon loss of cyst cells while, this acute reduction did not prevent spermatocytes from completing meiosis I cytokinesis. We next investigated the PIP3 distribution in control meiotic spermatocytes using GFP-PH-Steppke (tGPH), interestingly, we found that tGPH localized to plasma membranes and became significantly enriched at cleavage furrows (Movie S7, Fig.5F). Of note, even here, we have observed that plasma membrane localization and cleavage furrow enrichment of tGPH strictly depended on the intact cyst. While it is currently unknown if PIP3 localization to the spermatocyte cleavage furrows has any impact on cytokinesis, a recent study has shown that cytohesin Steppke reduces tissue tension by inhibiting the actomyosin activity at adherens junctions and actomyosin network assembly is necessary and sufficient for local Steppke accumulation in *Drosophila* embryos [38]. We next wondered cleavage furrow enrichment of PIP3 is altered in *cpes* mutants. However, as shown in Movie S8, Fig5F, PIP3 localization to the cleavage furrows was not altered but again there was a disconnect between cleavage furrow and central spindle during cleavage furrow ingression. Expression of wild type CPES restored this phenotype (Movie S9; Fig.5F). Overall, these observations suggest that aberrant spindle behavior but not the localization of PIPs to the plasma membrane or cleavage furrow, is responsible for meiotic cytokinesis defect in *cpes* mutants.

CPE endocytosis and targeting to cleavage furrows occurs during meiosis cytokinesis

468 To determine how CPE is promoting the synergy between central spindle constriction and cleavage
 469 furrow ingression during cytokinesis we have utilized recombinant CPE binding protein tagged
 470 with mCherry as a reporter for visualization of CPE on spermatocyte plasma membranes. The
 471 mushroom-derived protein of the aegerolysin family pleurotolysin A2 (PlyA2) was shown to
 472 specifically bind to CPE and was demonstrated to be a versatile tool for visualizing CPE in live
 473 *Drosophila* tissues [39]. We have reevaluated the efficacy of recombinant PlyA2-mCherry
 474 proteins in detecting endogenous CPE on live spermatocyte membranes. The wild type and *cpe*
 475 mutant spermatocytes were treated with 10 µg/ml of PlyA2-mCherry in M3 insect media for 1 hour
 476 at room temperature, washed with fresh M3 media, and imaged on an Andor spinning disk confocal
 477 microscope. As shown in Fig.6A-D, PlyA2-mCherry specifically bound to the plasma membranes
 478 of wild type spermatocytes but not to the *cpe* mutant spermatocytes showing specificity of this
 479 protein binding to CPE. Interestingly, PlyA2-mCherry was endocytosed in wild type
 480 spermatocytes (Fig.6). To determine the nature of these endosomes where CPE is enriched, we
 481 have performed live imaging on spermatocytes expressing endogenous EYFP-MYC tagged Rab
 482 proteins [40]. The small GTPases Rab4, Rab7 and Rab11 were shown to mark early, late and
 483 recycling endosomes respectively [41-43] while Rab6 was shown to be associated with the Golgi
 484 membranes [44]. The spermatocytes expressing individual EYFP-Rab proteins were treated with
 485 recombinant PlyA2-mCherry and imaged. As shown in Fig.6G, M and P, PlyA2-mCherry
 486 colocalizes with Rab4, Rab7 and some of the Rab11 endosomes respectively. However, PlyA2-
 487 mCherry containing structures did not colocalize with Rab 6 positive Golgi membranes (Fig.6J).
 488 These results suggested that CPE on spermatocyte plasma membranes is actively targeted to the
 489 endocytic pathway. We next wondered whether endosomes carrying CPE migrate to cleavage
 490 furrows during meiosis cytokinesis. EYFP-Rab 7 and EYFP-Rab11 were strongly expressed in

spermatocytes compared to Rab4 and therefore we followed their dynamics during meiosis cytokinesis. Strikingly, as shown in Fig.7A-F, & Movie.S10, PlyA2-mCherry containing Rab7 positive endosomes were actively targetted to cleavage furrows. Similar EYFP-Rab7 localization was seen in intact cysts (Fig.7G-I). The PlyA2-mCherry also colocalized to some of the EYFP-Rab11 recycling endosomes which in turn localized to ingressing membranes at the cleavage furrow (Fig.7J-O and Movie S11). Overall, the endocytosis of CPE and targeting to the ingressing cleavage furrows via Rab7 and Rab11 endosomes indicates their importance in delivery of lipids to the ingressing membranes of the furrow and coordination of this event with proper spindle behaviour and meiosis cytokinesis.

Fig.6.Ceramide phosphoethanolamine (CPE) localizes to plasma membrane and endosomes.

(A-D) Live spermatocytes were incubated with purified PlyA2-mCherry (10 µg/ml) for 30 min followed by washes with M3 insect media (2x) and imaged using spinning disk confocal microscopy. (A and B) w¹¹¹⁸ spermatocytes, (C and D) cpe^s spermatocytes. (E-P) Live spermatocytes expressing endogenous EYFP tagged Rab proteins were incubated with PlyA2-mCherry for one hour followed by washes with M3 insect media (2x) and imaged using spinning disk confocal microscopy. (E-G) EYFP-Rab4 spermatocytes, (H-J) EYFP-Rab6 spermatocytes, (K-M) EYFP-Rab7 spermatocytes, (N-P) EYFP-Rab11 spermatocytes. Arrow heads indicates colocalizing structures.

Fig.7. CPE containing endosomes translocate to cleavage furrows during male meiosis

cytokinesis. *(A-F) The EYFP-Rab7 expressing spermatocytes were treated with PlyA2-mCherry and snapshots of spermatocytes undergoing meiosis I cytokinesis were shown. (A-C) Horizontal view of one z slice was shown (D-F) Cross sectional view of a spermatocyte undergoing cytokinesis, maximum intensity projection image was shown. (G-I) Localization of EYFP-Rab7 in*

intact cysts where spermatocytes are encapsulated by cyst cells. Horizontal view of a single z slice was shown. (J-O) EYFP-Rab11 expressing spermatocytes were treated with PlyA2-mCherry and meiosis I cytokinesis was imaged (J-L) Horizontal view of single z slice was shown (M-O) Cross sectional view, maximum intensity projection image was shown. White arrows indicate colocalization and white arrow heads indicates lack of colocalization.

Discussion

Plasma membrane expansion and ingression of the cytokinetic furrow still remains one of the least understood horizons in the field of cell division [45]. We are only beginning to appreciate the involvement of lipids in structural integrity, membrane expansion, and transmission of signals during cytokinesis. PIP2 is the most studied lipid in cytokinesis and has been shown to localize to the cleavage furrow during ingression. In addition to PIP2, cholesterol, very long chain fatty acids and sphingolipids have also been associated with cytokinesis [46] [10, 12]. In mitotic cells, PIP2 accumulation at the inner leaflet and subsequent recruitment of RhoGTPase (RhoA) was shown to be coordinated with sphingomyelin rich domains on the outerleaflet [10]. However, it is unknown if a similar mechanism exists in germ cells which have different characteristics and different lipid composition. While mitotic cells were shown to accumulate classical sphingolipids with long to very long acyl chains at midbodies [12], male germ cells accumulated complex sphingolipids with verylong/ultralong chain polyunsaturated fatty acids (VLC-PUFAs) [13]. These differences in sphingolipid composition with distinct biophysical properties between mitotic cells and male meiotic cells point towards existence of distinct underlying mechanisms.

In this study, we show the sphingolipid composition of the major species in *Drosophila* testis for the first time and demonstrate significant enrichment of unique CPE species containing SPH_MUFA and SPD_SFA. Unlike mammalian testis, the *Drosophila* testis lacks significant

amounts of VLC-PUFA containing sphingolipids. However, due to conserved unsaturation in acyl chains, it is likely that SPH_MUFA and SPD_SFA containing CPE could complement for VLC-PUFA functions in *Drosophila* male germ cells. Glycosphingolipids with VLC/ULC PUFAs have been shown to be required for mammalian male meiosis cytokinesis but their actual role has not been delineated [13, 47, 48]. It is interesting to note that CPE mimics certain physical properties of galactosylceramide (GalCer) a major sphingolipid in myelin sheath [49]. Previous studies have shown that CPE has an essential role in axonal ensheathment and cortex glial plasma membrane expansion in *Drosophila* [15, 50]. It was hypothesized that the axonal ensheathment in mammals and *Drosophila* is based on similar physical process with different lipids [49]. We have previously shown that sphingomyelin, a structural analogue of CPE, could rescue cortex glial membrane defects in *cpe* mutants suggesting the head group is not as important as the tail group in glial membranes. However, here we show that sphingomyelin could not rescue spermatogenesis defects in *cpe* mutants alluding to the importance of the ethanolamine head group in spermatocytes. Taken together, we could suggest that *Drosophila* CPE would have evolved to have both the properties of mammalian glycosphingolipids and sphingomyelin to promote spermatogenesis and glial ensheathment of neurons.

Phosphoinositides play critical role in cytokinesis particularly PIP2 was shown to be enriched at the furrows and directly binds to several proteins such as anillin, septins and MgcRacGAP to mediate furrow stability [51, 52]. Unlike mitotic cells, PIP2 in male meiotic cells do not show selective accumulation at cleavage furrows instead it is uniformly distributed throughout the plasma membrane including at furrows ([37] and Fig.5E and Movie S4, S5 and S6). Interestingly PIP2 hydrolysis and calcium release were shown to be required for cytokinesis in *Drosophila* spermatocytes [37]. These observations suggest that PIP2 in meiotic cell has more broader role at

the plasma membrane and at furrows to promote cytokinesis. Interestingly, we show that the PIP3 binding protein Steppke (tGPH) localizes to cleavage furrows indicating a potential role for PIP3 during male meiosis cytokinesis. However, the accumulation of PIP3 to the cleavage furrows occurs only in the intact cysts i.e, 16 interconnected spermatocytes wrapped up by two somatic cyst cells suggesting the need for certain mechanical force. The role of PIP3 accumulation in the male meiotic cleavage furrows is an open question and warrants future investigation. Interestingly, recently it was shown that cytohesin Steppke reduces tissue tension by inhibiting the actomyosin activity at adherens junctions in embryos [38]. Although broad accumulation of PIP2 to the plasma membranes and PIP3 accumulation at the cleavage furrows was not altered in *cpe*s mutants their specific localization to membrane microdomains and their dynamics on functional alterations cannot be ruled out. Sphingadienes with Δ 4.6 conjugated double bonds were previously identified in *Drosophila*, *Manduca sexta*, and *Bombyx mori* [53-55], although their enrichment in the testis was not known until now. However, normal sphingolipid synthesis and their degradation are shown to be required for spermatogenesis [56, 57]. The sphingadiene sphingolipids were shown to have inhibitory effects on AKT-dependent signaling [58] and downregulate Wnt signaling via a PP2A/Akt/GSK3 β pathway [59]. Accumulation of sphingadiene with saturated fatty acid containing sphingolipids in the testis suggests that they could have a role in regulation of PIP2/PIP3 mediated signaling *in vivo*. Consistent with this suggestion, total RNAseq results show an upregulation of ERBB signaling in male germ cells. Apart from CPE_SPD_SFA the *Drosophila* testis is also enriched in CPE_SPH_MUFA suggesting they could mediate distinct functions. Lipids with unsaturated fatty acids mediate a number of biological functions [60]. It is widely known that phospholipids with unsaturated fatty acids promote membrane fluidity and elasticity

[61, 62]. Sphingolipids with unsaturated fatty acids could thus provide unique biophysical properties necessary for the membrane curvature requirements during meiosis cytokinesis. Human genome encodes more than 60 Rab proteins and several of them including Rab1, Rab8, Rab10, Rab14, Rab21, Rab24 and Rab35 were shown to localize to intercellular bridges. However, most of the studies primarily focused on the recycling role of Rab11 and Rab35 endosomes and their effector proteins in cytokinesis [52]. Currently it is unknown if these endosomes are also involved in delivery of specific plasma membrane derived lipids to the cleavage furrows. Our study shows that there is an increased localization of CPE to the endosomal compartments in spermatocytes. The CPE containing Rab7 and Rab11 endosomes are selectively targeted to ingressing cleavage furrows suggesting possible addition of CPE rich membranes to the growing furrows. Further investigations are needed to determine whether CPE enriched endosomes actively participate in male meiosis cytokinesis, spermatid polarity and individualization by providing CPE enriched membranes and or act as a signaling center to mediate spermatogenesis.

Materials and Methods

Fly stocks

tubulin-Gal4 (BDSC#5138), UAS-PLCdelta-PH-EGFP (BDSC#39693), tGPH (BDSC#8163), Sqh-GFP.RLC (BDSC#57145), Sqh-mCherry.M (BDSC#59024), p(Ubi-p63E-Feo-mCherry)3 (BDSC#59277), bam-Gal4 [63], nos-Gal4 (BDSC#4937), chif-Gal4 (BDSC#13134), C587-Gal4 (BDSC#67747), EYFP-Rab4 (BDSC#62542), EYFP-Rab6 (BDSC#62544), EYFP-Rab7 (BDSC#62545), EYFP-Rab11(BDSC#62549), genomic CPES_V5tag [15], *cpes* mutants [15], UAS-hSMS1 [15], dcert1[30], UAS CDase [31], UAS CPES active site mutants, UAS Aedes CPES, UAS-Bombyx CPES, UAS-T. Urticae CPES (this study).

Fly husbandry

All *Drosophila* stocks were raised on standard fly food and maintained 25°C unless otherwise mentioned. Due to increased lethality *cpes* mutants were raised at 18-21°C. Certain genetic backgrounds increased pupal death in *cpes* mutants such as overexpression of UAS CDase, UAS-PLCδ-PH-EGFP, *cpes;dcert^l* double mutants. In such cases we have separated 3rd instar larvae of *cpes* mutants and allowed them to develop into the pupae. The testis were dissected before the death of pupae and analyzed.

Cloning of UAS-*Aedes aegypti* CPES, UAS-*Bombyx Mori* CPES, UAS *Tetranechos Urticae* CPES

The coding sequence for *Aedes aegypti* CPES ([XM_021851518.1](#)), *Bombyx mori* CPES ([XM_004923146.3](#)) and *Tetranychus urticae* CPES ([XM_015926572.2](#)) were codon optimized and synthesized by GenScript® and subcloned into pUAST vector. The clones were sent to BestGene Inc for embryo injection service. The transgenic flies were balanced and crossed with Gal4 drivers to conduct rescue experiments.

Cloning of CPES active site mutant

Two conserved aspartates in CPES active site were mutated to alanine using PCR based site directed mutagenesis method as described by [64]. PCR was performed using pBluescript SK+ CPES_V5 clone as template with appropriate sense and antisense primers (Table S1) and Phusion polymerase (NEB). The PCR product was digested with DpnI to remove templates followed by transformation into DH5α competent cells. Plasmids were isolated from colonies grown in LB media and screened by restriction digestion with appropriate restriction enzymes (Table S1) and sequencing. The pBluescript SK+ CPES DD-AA clone was used as template to PCR amplify the insert using appropriate primers (Table S1), restriction digested and cloned into pUAST vector. The clones were confirmed with restriction digestion followed by DNA sequencing.

Cloning, expression, and purification PlyA2mCherry

Mushroom-derived aegerolysin PlyA2 was cloned, expressed and purified as described by [65]. Briefly, the coding sequence of PlyA2 (accession number AB777517) fused to mCherry at the C-terminus was synthesized by GenScript®. The linker amino acid sequence between PlyA2 and mCherry was VDGTAGPGSIAT. This clone was used as template to PCR amplify using appropriate forward and reverse primers (Table S1) and cloned into p24 a vector at appropriate restriction sites (Table S1). The clones were transformed into Escherichia coli strain BL-21 (DE3) and single colony was inoculated in to 5ml of LB broth containing 50µg/ml kanamycin and grown overnight in orbital shaker (200rpm) at 37°C. 2ml of overnight culture was inoculated into 250ml of fresh LB broth containing kanamycin and grown at 37°C until the optical density reached to 0.6. Subsequently, the culture was incubated in cold (4°C) for 2 hours, followed by induction with 0.3mM isopropyl-B-D-thiogalactopyranoside and grown at 25°C in orbital shaker (200rpm) overnight. The cells were harvested by centrifugation at 5,000g for 10min at 4°C and the pellet was resuspended in binding buffer (20mM sodium phosphate buffer (pH 7.4), 500mM NaCl, 20mM imidazole) containing EDTA free protease inhibitor cocktail (Sigma, P8340). The cell suspension (50 ml) was sonicated at 4°C (Cole-Parmer ultrasonic processor model CP130,) with microprobe at an amplitude of 30 and on and off cycles of 10sec each for about 30 min to 1 hour. The lysate was centrifuged at 10,000g for 10min to remove insoluble proteins and debris. To the supernatant 1ml of 50% Ni-NTA agarose (Thermo Scientific) was added and incubated on end-to-end rotor for 3 hours and the beads were collected on a column and washed with 30ml of binding buffer and eluted with binding buffer containing 200mM imidazole. The fractions were dialysed in 20mM sodium phosphate buffer 3 times (1L each) and the final protein was concentrated with Amicon centriprep filter devices for volumes up to 15ml. The protein concentration was

determined using Bradford assay. The proteins were resuspended in 20% glycerol, aliquoted (100µl) and stored at -80°C for future use.

Testis squash preparation, phase contrast microscopy

Drosophila testis squash preparations for phase-contrast microscopy and immunostaining were performed as described by [16]. Briefly, young male (0-2 days old) testis were dissected (5 pairs) in phosphate-buffered saline (PBS, 130 mM NaCl, 7mM Na₂HPO₄, 3mM H₂PO₄). The testis was torn open on a slide at appropriate location with a pair of Black Anodized Steel needles (tip diameter 0.0175mm) and a cover glass was gently placed without air bubbles. The slides were directly observed under phase-contrast microscopy or snap frozen in liquid nitrogen for immunostaining. After freezing the cover glass was removed from the slide with razor blade and immersed in cold 99.5% ethanol and incubated in -20°C for 10 min. The slides were then fixed with 4% paraformaldehyde (PFA) in PBS containing 0.1% Triton-X-100 (PBST) for 15 min. Subsequently, the slides were washed with PBS and the area of squashed tissue was circled with a hydrophobic barrier pen, permeabilized with PBST (30 min) and blocked with 5% normal goat serum in PBST (1 hour at RT). Squashed tissues were incubated with primary (1µg in 100µl) and secondary antibody (1:100 dilution) (8-12 hours each at 4°C in a moist chamber). Slides were washed with PBST (3x 10 min each) after each antibody treatment, finally incubated with DAPI solution (1:2000 dilution from 5mg/ml stock, Molecular Probes) and mounted with Vectashield H1000 mounting media.

Male fertility assay

Male fertility assay was performed by setting up individual crosses with 1 male and 3 wild type females. Each replicate involved 30 males and three independent replicates (total of 90 males) were performed for each experiment.

Immunostaining and confocal imaging

Drosophila tissues were dissected and immunostained as described previously [15]. Briefly, dissected testes were fixed with 4% PFA in 0.3% PBST for 1 hours at RT, washed (3x 10 min each), permeabilized in 0.3% PBST (1 hour), blocked with 5% NGS (1hour) and incubated with primary antibody (1:100 dilution or 1µg in 100µl of 5% NGS in PBST) overnight (8-12 hours). Testes were washed with 0.3% PBST (3x10min each) and incubated with secondary antibody (1:100 dilution in 5%NGS in PBST). Testes were washed with PBST (3x 10 min each), stained with DAPI (1:2000 dilution of 5mg/ml solution in PBST) for 15 min, and mounted with Vectashield H1000 mounting medium. The slides were imaged on ZEISS confocal laser scanning microscope LSM 780 or LSM880.

Live cell imaging of meiotic cysts

About 15-20 pairs of young (0-2days old) *Drosophila* testis were dissected in 600µl of insect cell culture media (M3 media supplemented with heat inactivated FBS, Pen/Strep) in a glass well plate. Testis were transferred to fresh a well with 400µl of media and tore open with a pair of Black Anodized Steel needles (tip diameter 0.01mm) to release the cysts into media. This was repeated until all the testes were torn opened, they were gently agitated so that all the cysts were released into the media followed by removal of empty testis or debris from the media. The cysts were gently washed with fresh 400µl media (2x), then transferred via pipetting into the middle of 35mm cover glass bottom dish (Poly D-Lysine coated) and fresh media (max. 200µl) was gently added to the cleft formed between cover glass and the dish. The cysts tend to move while handling but remains settled on the cover glass and ideally remain in position while imaging. The intact cysts and cysts that have lost their cyst cells could be distinguished in brightfield view. The cysts that are beginning to undergo meiosis could be identified in brightfield view by looking at cell morphology

and differences in nucleus and spindle appearance. Cells beginning to undergo meiosis could also be identified by Feo-mCherry which is localized to nucleus in interphase but translocates to cytosol during prophase to anaphase. Prophase to anaphase cysts were identified using 25x water objective and subsequently imaged using 63x water objective on a Leica Andor spinning disk confocal microscope. The time lapse imaging was done at 2 min intervals with the z stacks (about 80 slices, 0.5µm per slice) at each time point for 60 to 90 min for completion of meiosis I cytokinesis. Laser power and exposure times were preferably kept low to prevent bleaching and aberrant effects on cytokinesis due to generation of free radicals.

Sphingolipid estimation in fly tissues using Supercritical fluid chromatography coupled to mass spectrometry (SFC/MS/MS)

Sphingolipid estimation using supercritical fluid chromatography coupled to mass spectrometry (SFC/MS/MS) was performed as described previously [15]. Lipids were extracted from heads, ovary and testis samples as described [66, 67]. About 400 fly heads, about 250 pairs of ovaries and about 250 pairs of testes were used for each biological replicate. Three independent replicates were taken for each sample. For Figs.1 and 2 only testis alone samples were used whereas for Fig.4 whole male organ (testis, accessory glands, collecting ducts and ejaculation bulb) were used for lipid extraction. Internal standards (ISTD) added to the tissues before extraction included 500 pmol (20 µl) of Cer/Sph Mixture I (Avanti Polar Lipids LM-6002), and 1nmol of C12 Sphingosyl PE (C17:1/C12:0) (Avanti Polar Lipids). Lipids from all samples were normalized to carbon content (100µg/ml) and 100 µg/ml sample was diluted 10 times before injection for SFC. We have used large-fragment MRM method for quantitation of amount per 100µg of carbon.

Total RNA sequencing

Three independent biological replicates were performed for each sample and about 200 pairs of *w¹¹¹⁸* (WT 1-3), *cpes* mutant (KO 1-3) and Bam-Gal4>UAS CPES rescued (RES 1-3) fly testis were dissected for each replicate. The testes were dissected in S2 cell culture media, washed with PBS and RNA was extracted. Total RNA was extracted using Trizol method followed by DNAase I treatment and purification using RNA Clean and ConcentratorTM-5 kit (Zymoresearch). A total of 2µg of RNA was submitted to Novogene for total RNA seq. The RNA seq data was further analyzed for differential gene expression and pathway analysis using Gene Set Enrichment Analysis (GSEA). The gene sets specific for drosophilla were downloaded from <http://www.bioinformatics.org/go2msig/> and <http://ge-lab.org/gskb/>.

Acknowledgments

This study was funded by the intramural division of the National Cancer Institute, National Institutes of Health (Division of Health and Human Services). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

This work was partially supported by the Grant-in-Aid for Scientific Research on Innovative Areas (17H06304) [T.B.] and a Grant-in-Aid for Scientific Research (B) (18H01800) [T.B.] from Japan Society for the Promotion of Science (JSPS).

Conflict of Interest Statement

The authors declare no conflicts of interests.

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Spermatids

Round

Elongating

Individualizing

(A) GSEA Pathway Analysis

(B) Enrichment plot: Spindle Organization

(C) Enrichment plot: Spindle Elongation

Figure 7
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