Drosophila spermatid individualization is sensitive to temperature and fatty acid metabolism

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Fatty acids are precursors of potent lipid signaling molecules. They are stored in membrane phospholipids and released by phospholipase A$_2$ (PLA$_2$). Lysophospholipid acyltransferases (ATs) oppose PLA$_2$ by re-esterifying fatty acids into phospholipids, in a biochemical pathway known as the Lands Cycle. *Drosophila* Lands Cycle ATs *oys* and *nes,* as well as 7 predicted PLA$_2$ genes, are expressed in the male reproductive tract. Oys and Nes are required for spermatid individualization. Individualization, which occurs after terminal differentiation, invests each spermatid in its own plasma membrane and removes the bulk of the cytoplasmic contents. We developed a quantitative assay to measure individualization defects. We demonstrate that individualization is sensitive to temperature and age but not to diet. Mutation of the cyclooxygenase Pxt, which metabolizes fatty acids to prostaglandins, also leads to individualization defects. In contrast, modulating phospholipid levels by mutation of the phosphatidylcholine lipase Swiss cheese (Sws) or the ethanolamine kinase Easily shocked (Eas) does not perturb individualization, nor does Sws overexpression. Our results suggest that fatty acid derived signals such as prostaglandins, whose abundance is regulated by the Lands Cycle, are important regulators of spermatogenesis.

Introduction

In most species, spermatozoa contribute only DNA, centrioles, and select signaling molecules to the developing zygote.[1–3] In the testis, sperm are stripped of their cytoplasmic contents following terminal differentiation and acquire their characteristic streamlined shape.[4] The sperm of many species develop as a syncytium.[5] The mitotic and meiotic divisions of spermatogenesis end with incomplete cytokinesis, leaving intercellular bridges that permit cytoplasmic sharing among sister sperm, which are thought to synchronize differentiation and protect spermatids against the deleterious effects of haploidy.[6–8] In both mammals and *Drosophila*, the removal of excess cytoplasm and the dissolution of intercellular bridges occur concurrently in an actin mediated process.[4,9–12] This process is important for fertility, but the mechanisms that regulate it are not well understood.[13]

The *Drosophila* male gonad is organized such that the developing sperm cells are arranged chronologically within the testis tubule, permitting identification and observation of all spermatogenic stages simultaneously. The germline stem cells (GSCs) reside at the apical end of the testis, clustered around a stem cell niche called the hub.[14] Each GSC divides to produce another stem cell that remains associated with the hub and a gonialblast cell that is displaced from the hub and is destined to differentiate.[15] The gonialblast undergoes a series of 4 mitoses with incomplete cytokinesis to produce 16 interconnected spermatocytes that comprise the developing germ cell cyst. The spermatocytes increase their volume 25-fold and undergo 2 meiotic divisions to produce 64 spermatids.[85] As differentiation proceeds, each cyst is displaced basally down the testis by newly formed gonialblasts and cysts. After meiosis is complete, the spermatid nuclei condense, and the microtubule-based axonemes elongate. Finally, spermatid individualization occurs. Throughout the course of spermatogenesis, each cyst is enveloped by 2 non-mitotic somatic cells, called cyst cells, which originate from a population of somatic stem cells located at the hub.[16]

Cytoplasm removal and resolution of individual sperm are mediated by an actin-rich individualization complex (IC) following axoneme elongation.[17,85] Each IC is composed of 64 actin cones, which form around the 64 germ nuclei of the cyst.[9,11] Once the actin cones have formed, the IC moves away from the nuclei, which are located basally in the testis, and travels along

the axonemes back toward the apical end of the testis (**Fig. 1A, B**). As the IC progresses, the spermatids' cytoplasmic contents accumulate in a cystic bulge around the IC.[9] When the cystic bulge and associated IC reach the end of the axoneme, they form a waste bag and are degraded.[17] The ICs are readily visible with fluorophore-conjugated phalloidin.[11]

Many mutants that disrupt individualization have been identified.[18,19] However, different mutants have distinct effects, some disrupting both nuclei and actin cones, some disrupting the morphology of the actin cones, and others perturbing the movement of the ICs without affecting the nuclei or actin cones themselves.[11,20] We found previously that deletion of the lysophospholipid acyltransferases (ATs) Oysgedart (Oys) and Nessy (Nes) results in male sterility and failure of spermatid individualization.[21] In *oys nes* double mutants, actin cone formation and morphology appear normal, but synchronous translocation of the actin cones in the IC is disrupted.

Oys and Nes regulate the metabolism of membrane phospholipids via a biochemical pathway called the Lands Cycle (**Fig. 7A**).[21,22] Lands Cycle enzymes of the phospholipase A_2 (PLA_2) family catabolize phospholipids into lysophospholipids and fatty acids, which can be converted into potent short-range signaling molecules.[23,24] Oys and Nes catalyze the reverse reaction, re-esterifying lysophospholipids and fatty acids into phospholipids.[25,26] Both lysophospholipid and fatty acid derived signaling molecules bind G-protein coupled receptors (GPCRs) on the surface of target cells and activate a variety of downstream signal transduction cascades.[27,28] They can also bind and activate nuclear receptors, such as the peroxisome proliferator-activated receptor PPAR-γ.[29–31] Fatty acid derived mediators of the prostaglandin and leukotriene families are especially important in the mammalian immune and reproductive systems.[32,33] The lysophospholipid derived mediator lysophosphatidic acid is important for wound healing, vascular development, and neuronal development and function.[34] PLA_2s and ATs are critical for regulating lipid signal availability,[26] and disruptions in lipid signaling contribute to infertility, inflammation, atherosclerosis, metabolic syndrome, and cancer.[35,36] Lipid signaling molecules are thought to play important roles in invertebrates as well, but their target receptors and signal transduction pathways are uncharacterized.[37,38] In addition to regulating the availability of bioactive lipid mediators, the Lands Cycle modulates the molecular species composition of membrane phospholipids through acyl chain exchange.[22,39] The Lands Cycle is thought to function in all cells to maintain the membranes' physical properties (e.g. fluidity and curvature), but its regulation and its influence on cellular physiology and behavior are not well understood.[40,41]

Here we show that *oys* and *nes* are expressed in the *Drosophila* male germline and that 7 PLA_2 family members are expressed in the adult male reproductive tract. We introduce a quantitative method to measure defects in spermatid individualization and find that individualization is highly sensitive to temperature. Applying our methodology to other mutants reveals that individualization is sensitive to disruptions in prostaglandin metabolism but not other phospholipid metabolism pathways. Together, our results suggest that fatty acid derived signals in the testis are critical for successful completion of spermatogenesis.

Results

Characterization and quantification of individualization

To understand the individualization process in more detail, we characterized normal and abnormal ICs and waste bags. We conceptually divided the testis into 3 zones (**Fig. 1A, B**). In zone 1, the ICs formed around the spermatid nuclei, and nascent actin cones were thin and needle-shaped (**Fig. 1C**). Progressing actin cones in zone 2 accumulated actin and acquired their characteristic conical shape (**Fig. 1D**).[42] In normal progressing ICs, the actin cones of each IC remained tightly assembled (**Fig. 1D**). Waste bags were distinguishable from ICs by their disorganized appearance, degenerating actin cones, and the distinct cortical actin surrounding them (**Fig. 1E**). Under normal conditions, waste bags formed only in zone 3, upon reaching the ends of the axonemes (**Fig. 1A, B**).

Under environmental or genotypic conditions that disrupt individualization (see below), abnormal ICs were observed. Abnormal ICs had loosely associated actin cones (**Fig. 1F**) or formed waste bag-like structures that degenerated before reaching the apical region of the testis (**Fig. 1H**). Testes with aberrant individualization also had many stray actin cones not associated with a distinguishable IC (**Fig. 1G**). In some cases, abnormal ICs appeared basally oriented instead of apically oriented.

To quantify the individualization process, we counted the number of normal progressing ICs (those not affiliated with nuclei) and waste bags in 0–3 day old males from 3 control genotypes at room temperature (23–24°C): w^{1118}, Canton S, and the balancer stock *Sco/SM6;MKRS/TM6B* to control for non-specific effects from balancer chromosomes. The average number of normal progressing ICs and waste bags was similar among the 3 control genotypes (8–11 per testis, **Figure 2D**). This average was reproducible and consistent across many control genotypes (see below). We designated ICs having 4 or more separated actin cones as abnormal. Even with this stringent criterion, we did not observe more than 4 abnormal ICs per testis in either w^{1118} or the balancer stock at room temperature. Canton S showed slightly more variability, with an occasional testis displaying more than 4 abnormal ICs.

Aged males have smaller testes, because the GSCs divide less frequently.[43] We examined IC progression in testes of our 3 control genotypes in aged flies. Males were collected within 0–3 d of eclosion and allowed to age in the presence of females for 5 d, 10 d, 15 d, or 26 d. The average number of normal progressed ICs and waste bags per testis decreased dramatically with age. While the youngest males had an average of 8–11 normal individualization structures per testis, males aged for 5 d had 4–7 normal individualization structures per testis, and older males had fewer than 5 normal individualization structures per testis on average (**Fig. 2D**). The average number of abnormal ICs also declined with age, such that the proportion of abnormal ICs versus normal individualization structures stayed similar at all ages
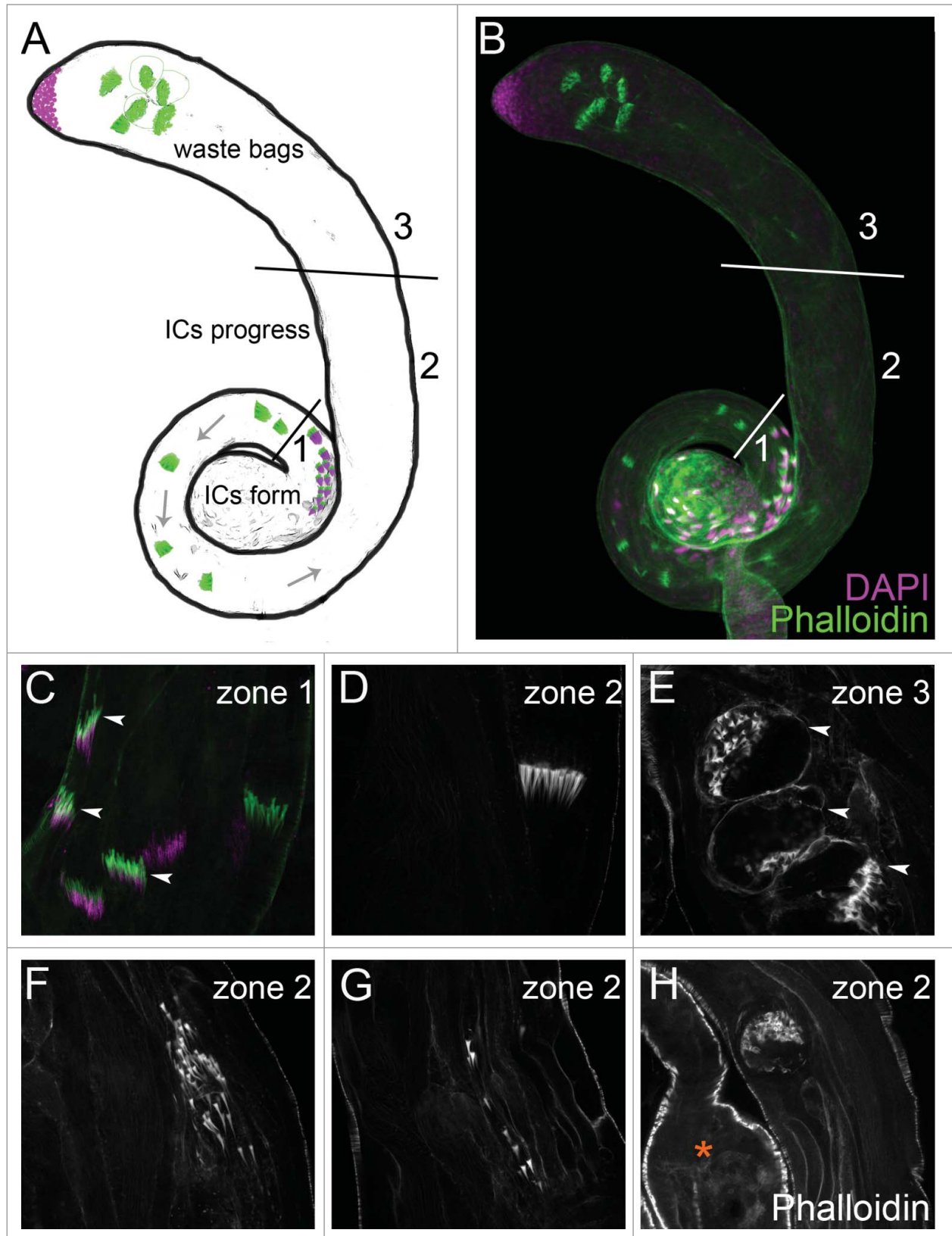
Figure 1. For Figure legend see Page 4.

(~1:10; **Figure S1**). This effect seemed to be due primarily to a smaller proportion of aged testes having any abnormal ICs. At least one abnormal IC was observed in over half of the testes from 0–3 day old males of all 3 genotypes. In contrast, after aging for 26 days, only 38% of testes from w^{1118}, 13% of testes from Canton S, and 16% of testes from *Sco/SM6;MKRS/TM6B* males had any abnormal ICs. Aged testes also had dramatically fewer nuclear bundles, suggesting that the decline in individualization structures was due to the production of fewer cysts (**Fig. 2A–C**, arrows).

Due to the decline in cyst number, we expected male fertility to decline with age. We performed single matings with 0–3 day old males or males aged for 5 days, 10 days, 15 days, or 30 d at room temperature. Suprisingly, progeny numbers did not decline significantly even after 30 d (**Fig. 2E, G**). This is consistent with the observation that aging did not disrupt the ICs, and it suggests that young males produce sperm in large excess, perhaps to foster sperm competition or to ensure reproductive success in the wild.[44] Additionally, older males may maintain their fertility by storing sperm.

Individualization is sensitive temperature

To determine whether spermatid individualization was sensitive to environmental conditions, we quantified individualization structures in flies reared at a range of temperatures. Flies raised at 27°C had a slight decrease in the average number of normal individualization structures per testis, whereas flies raised at 29°C showed severe individualization defects. At 29°C, w^{1118}, Canton S, and *Sco/SM6;MKRS/TM6B* showed averages of 5.5 or fewer normal progressed ICs and waste bags per testis (**Fig. 3A**). Low temperature also disrupted individualization. At 18°C, w^{1118} had an average of only 1.7 normal progressed ICs and wastebags per testis (**Fig. 3B**). Canton S males raised at 18°C showed an average of 8.2 normal individualization structures per testis, and *Sco/SM6;MKRS/TM6B* at 18°C displayed an average of 6.4 normal individualization structures per testis (**Fig. 3B**). More abnormal individualization structures were observed in all 3 genotypes at 18°C (**Fig. 3C-E**). This was in contrast to aged males, in which the presence of fewer cysts led to fewer individualization structures but the number of abnormal structures did not increase. Males also displayed reduced fertility at both 18°C and 29°C (**Fig. 3F**). Male fertility in all 3 genotypes was strongly reduced at 29°C, with Canton S showing the strongest effect. At 18°C, Canton S and balancer males had slightly reduced fertility, whereas the fertility of w^{1118} males was dramatically reduced. This is in accord with the effects on individualization, in which Canton S displayed the strongest effect at 29°C and w^{1118} showed the strongest effect at 18°C. Cytokinesis defects were occasionally observed in w^{1118} but not the other genotypes when males were reared at 18°C and in all 3 genotypes at 29°C (**Figure S2**). Thus, rearing flies at extreme temperatures disrupted spermatogenesis. Different genetic backgrounds rendered flies more or less susceptible to temperature-induced defects. Interestingly, the wild-type strain Canton S showed more variability in individualization at room temperature but was less sensitive to cooler temperatures.

We also quantified spermatid individualization in flies that were raised at room temperature and shifted to 29°C as adults. Males were collected 0–3 d after eclosion and allowed to age in the presence of females for 5 d either at room temperature or at 29°C. Temperature shifted males of all 3 control genotypes had significant individualization defects compared to siblings kept at room temperature, showing averages of fewer than 3.5 normal progressed ICs and waste bags per testis (**Fig. 3G**, peach bars). The temperature shifted males also had more abnormal ICs per testis than the corresponding room temperature controls (**Figure S3**). In the reverse experiment, males reared at 29°C and shifted to room temperature for 5 d showed rescue of individualization compared to age-matched males kept at 29°C (**Fig. 3H**). These experiments revealed that the individualization process itself was sensitive to temperature.

Individualization is not sensitive to diet

Because individualization was sensitive to environmental perturbations, we asked whether diet can affect the individualization process. We reared animals on regular medium and shifted them following eclosion to glucose medium for 4 d or 8 d at room temperature. This medium contained only glucose and cholesterol. Individualization appeared normal in these flies (**Fig. 4A, B**). Flies reared throughout their development on glucose medium did not survive to adulthood. However, some flies did survive to adulthood when reared on lipid depleted food supplemented with cholesterol.[45] These flies had normal individualization (data not shown). Thus, the individualization process was not sensitive to reduced dietary lipid intake.

Oys and Nes are expressed in the germline

Testes from 0–5 day old *oys nes* double mutant males raised at room temperature showed an average of ~2 normal individualization structures per testis (**Fig. 5A**). Many abnormal individualization structures were observed in the mutant (**Fig. 5C**). Actin cones from mutant ICs were almost always scattered (**Fig. 5B, D**), and waste bags appeared devoid of actin cones (**Fig. 5E**). Isogenic control males had 8.5 normal individualization structures per testis on average, like the control genotypes above. Males homozygous for either *oys* or *nes* and heterozygous for the other

Figure 1. (See previous page). Characterization of normal and abnormal individualization structures. (**A**, **B**) Testes can be divided schematically into 3 domains. In zone 1, the ICs (phalloidin, green) form in apposition to the nuclei (DAPI, purple). The ICs migrate away from the nuclei along the spermatid axonemes toward the apical testis (zone 2). In zone 3, the ICs are degraded within the waste bags. (**C–E**) Testes from *Sco/SM6;MKRS/TM6B*. (**C**) Newly formed actin cones in zone 1 are thin and needle-shaped (arrowheads). (**D**) As the IC progresses, actin cones in zone 2 become conical and migrate synchronously. (**E**) Waste bags in zone 3 are bounded by cortical actin and contain the degenerating actin cones (arrowheads). (**F-H**) Testes from Canton S males reared at 29°C. Abnormal ICs contain scattered actin cones (**F**), unaffiliated actin cones (**G**), or degenerate before reaching zone 3, as indicated in the photograph by the proximity to the seminal vesicle (asterisk, **H**).
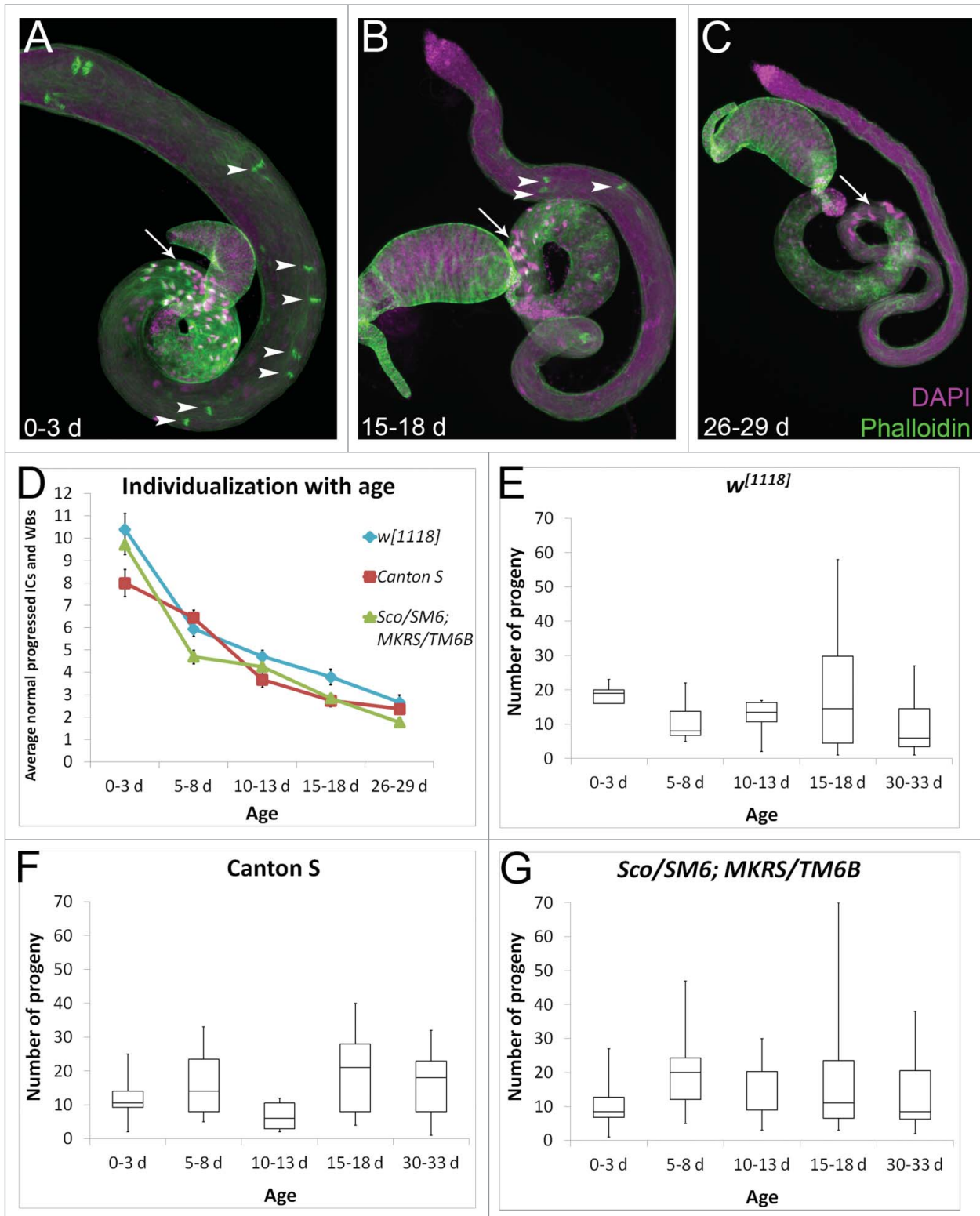
Figure 2. For Figure legend see Page 6.

also showed normal individualization (**Fig. 5A**). Thus, individualization was not sensitive to the dosage of *oys* and *nes*. Endogenous *oys* and *nes* transcripts were detectable in the germline using in situ hybridization (**Fig. 5F, G**). Cytokinesis was normal in *oys nes* homozygotes and heterozygotes (**Fig. 5H, I**).[21]

Because Oys and Nes esterify fatty acids into phospholipids,[21] thereby reducing free fatty acid concentrations, we expected fatty acid levels to increase in the *oys nes* double mutant. Therefore, we investigated whether reducing dietary intake of fatty acids could rescue the *oys nes* mutant phenotype. However, culturing *oys nes* mutant males on glucose food lacking fatty acids did not rescue the individualization defect (**Fig. 4C**).

PLA$_2$ genes are expressed in the male reproductive tract

PLA$_2$ enzymes act in opposition to ATs, hydrolyzing the *sn-2* ester bond in phospholipids and releasing lysophospholipids and fatty acids (**Fig. 7A**).[23] Mammals possess more than 20 PLA$_2$ homologs, which can be broadly grouped into 4 categories: cytoplasmic PLA$_2$s (cPLA$_2$s), secreted PLA$_2$s (sPLA$_2$s), calcium independent PLA$_2$s (iPLA$_2$s), and platelet-activating factor acetylhydrolases (PAFAHs) (**Fig. 6A**).[23,24] cPLA$_2$s reside in the cytosol and play key roles in fatty acid signaling.[24] sPLA$_2$s are secreted and also have been implicated in fatty acid signaling.[46,47] The iPLA$_2$s do not show strong specificity for the *sn-2* position, and they degrade phospholipids by deacylating at both *sn-1* and *sn-2* positions.[24] PAFAHs hydrolyze lipids like platelet-activating factor, which carry an acetyl group at the *sn-2* position.[24,48] We and others have identified 10 putative PLA$_2$ genes in the *Drosophila* genome.[49] *Drosophila* does not possess members of the cPLA$_2$ or PAFAH subfamilies (**Fig. 6A**).[24] Using RT-PCR, we found that the iPLA$_2$s encoded by *sws*, *CG6718*, and *CG10133* were expressed endogenously in the male reproductive tract, as were the sPLA$_2$s encoded by *CG14507*, *CG17035*, and *CG11124* (**Fig. 6B**). The sPLA$_2$ *CG1583* was weakly expressed as well (**Fig. 6B**), consistent with ModENCODE and FlyAtlas expression data (flybase.org). Thus, like Oys and Nes, PLA$_2$s may be important for *Drosophila* male fertility.

Perturbation of prostaglandin synthesis affects individualization

Phosphatidylcholine (PC) molecular species composition is abnormal in *oys nes* mutants compared to controls.[21] However, altering PC levels in the testis by overexpression or mutation of the iPLA$_2$/PC lipase Swiss cheese (Sws) did not affect individualization (**Fig. 7B**).[50] Inhibiting de novo phosphatidylethanolamine

(PE) synthesis by mutation of the ethanolamine kinase Easily shocked (Eas) also had no effect on individualization (**Fig. 7B**).[51] Thus, altered phospholipid composition did not inhibit individualization.

In mammals, fatty acids liberated from membrane phospholipids by the Lands Cycle are oxygenated and further metabolized into eicosanoid signaling molecules, such as prostaglandins.[27] The most upstream enzymes in the prostaglandin production cascade are the cyclooxygenases.[36] There is evidence for prostaglandin activity in invertebrates,[37,38,52] and the peroxidase Pxt was identified recently as having cyclooxygenase activity.[53] *pxt* mutant females have reduced fertility and actin-based oogenesis defects, and the severity of the defects increases with age. Similarly, males homozygous for the strong *pxt*[f0100] mutation showed individualization defects at all ages (**Fig. 7C, D**). Despite the individualization defects, Myosin VI localized normally to *pxt* mutant actin cones (**Fig. 7E, F**). Cytokinesis appeared normal in *pxt* mutants (**Fig. 7G**).

Discussion

Fatty acid mediators as regulators of spermatogenesis

Oys and Nes are ATs that esterify fatty acids to lysophospholipids to make phospholipids. They prefer long (C16-C20) unsaturated fatty acid substrates. Although Oys can act on a range of lysophospholipid substrates, Nes shows a strong preference for lysophosphatidylcholine.[21] Because Oys and Nes are genetically redundant, we expect that together they regulate levels of long chain fatty acids and lysophosphatidylcholine, as well as the molecular species composition of PC. Indeed, we have shown previously that PC acyl chain composition is altered in the *oys nes* mutant.[21] Which lipid is responsible for the mutant phenotype? Our data suggest that individualization is not sensitive to alterations in PC, since genetic perturbation of the PC lipase Sws does not affect individualization. Likewise, individualization is not disrupted by mutations in Eas, the ethanolamine kinase that initiates de novo PE synthesis.[54] Thus, we infer that phospholipid composition is not critical for individualization. Instead, our data suggest that altered levels of fatty acids and/or their metabolites disrupt individualization, as mutation of the cyclooxygenase Pxt leads to individualization defects.

In the absence of Oys and Nes, we expect fatty acid levels to be elevated. In the presence of wild-type Pxt, these excess fatty acids may be converted into prostaglandins. Prostaglandins regulate

Figure 2. (See previous page). The effects of aging on spermatogenesis. (**A**) Young testes are large and have many late stage spermatogenic cysts with clustered nuclei in the basal testis (arrow, DAPI, purple) and many progressing ICs (arrowheads, phalloidin, green). (**B, C**) Older testes are thin and have fewer cysts with basal nuclear clusters (arrows) and fewer ICs (arrowheads). Testes are from *w*[1118] males. (**D**) Young (0–3 d old) males at room temperature from 3 control genotypes have 8–11 normal progressed ICs and waste bags (WBs) per testis on average. Older males show a decline in the average number of individualization structures per testis, with 5–8 d old males having 4–7 normal individualization structures, 10–13 d old males having 3–5 normal individualization structures, 15–18 d old males having 2–4 normal individualization structures, and 26–29 d old males having fewer than 3 normal individualization structures per testis on average. N ≥ 18 testes each data point. In this an all subsequent figures, error bars represent standard error mean. (**E–G**) Fertility in 0–3 d old, 5–8 d old, 10–13 d old, 15–18 d old, or 30–33 d old males assessed in single matings (one male and one female each) for each control genotype (*w*[1118], Canton S, or the balancer stock *Sco/SM6;MKRS/TM6B*). F$_1$ progeny from 10 individual crosses were counted. Box plots show the distribution of progeny numbers for each age group, including maximum, minimum, interquartile range, and median numbers of progeny. ANOVA analysis comparing the mean number of progeny in each age group within each genotype reveals p > 0.1 for all 3 genotypes.

the actin cytoskeleton via various molecular pathways.[55] *pxt* mutant females show premature actin bundling in the female germline during stage 9 of oogenesis, as well as a failure of normal actin bundling during later stages of oogenesis.[55] Therefore, precise temporal regulation of prostaglandin signals organizes the actin cytoskeleton during oogenesis. A similar process may be occurring in spermatogenesis. Excess prostaglandins, formed from excess fatty acids in the *oys nes* mutant, may prevent timely completion of spermatid individualization. However, loss of prostaglandins in the *pxt* mutant also disrupts individualization. Thus, we hypothesize that individualization requires a precisely calibrated prostaglandin pulse. In the *Drosophila* oocyte, the actin regulatory proteins Fascin (Singed) and Enabled are downstream of prostaglandin signaling.[55,56] Interestingly, Singed localizes to the ICs, suggesting that molecular players downstream of prostaglandin signals may be conserved in gametogenesis.[20] Singed still localizes to *pxt* mutant ICs (data not shown), but its regulation might be altered. Endogenous Oys and Nes are expressed in the male germline. Similarly, Pxt acts in the germline during oogenesis.[53]

In mammals, altered dietary fatty acid intake affects phospholipid composition, lipid mediator availability, and fertility.[57,58] A recent study found that *Drosophila* phospholipid composition also is sensitive to dietary lipid alterations.[59] We were surprised to find that removal of fatty acids from the adult diet does not disrupt individualization in wild-type flies. This

Figure 3. For Figure legend see page 8.

may indicate that sufficient fatty acids are stored during development, which may be facilitated by the relatively short life span of *Drosophila*.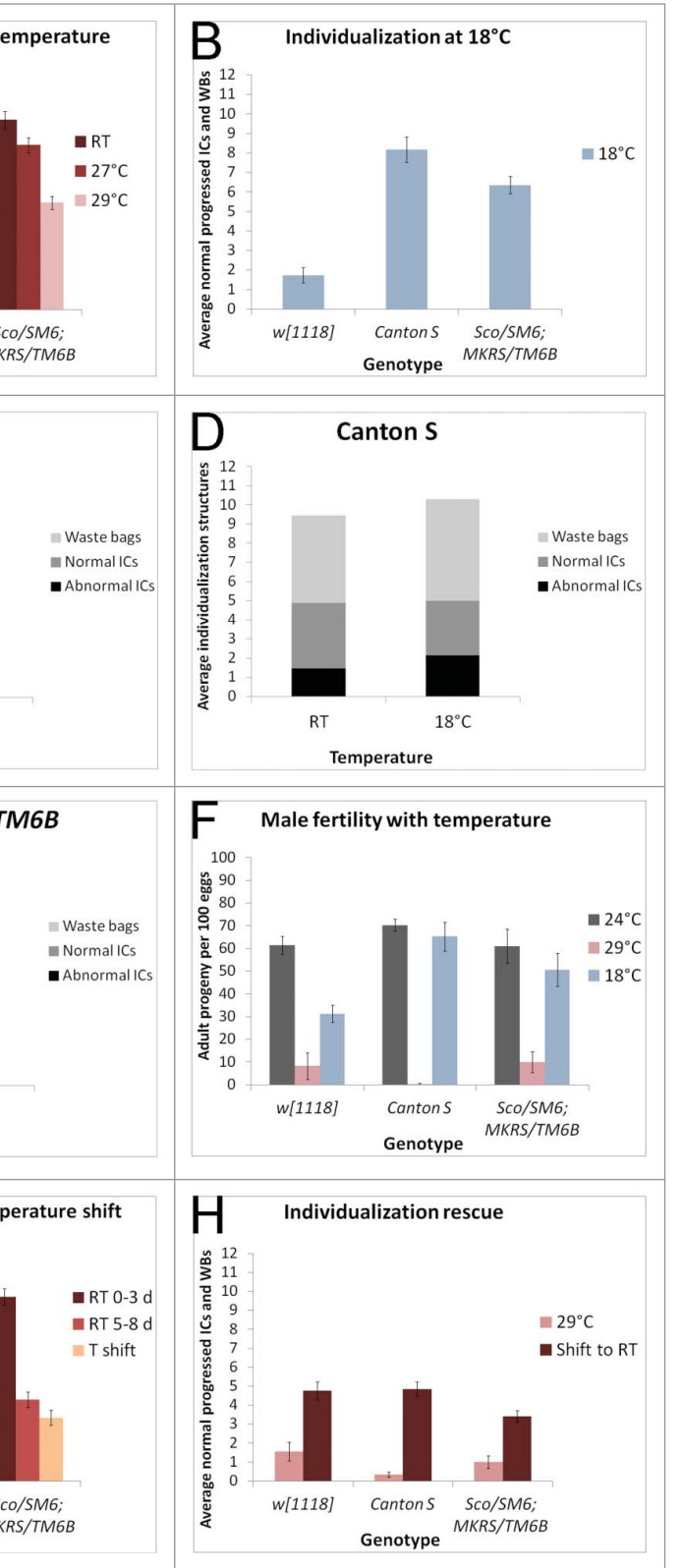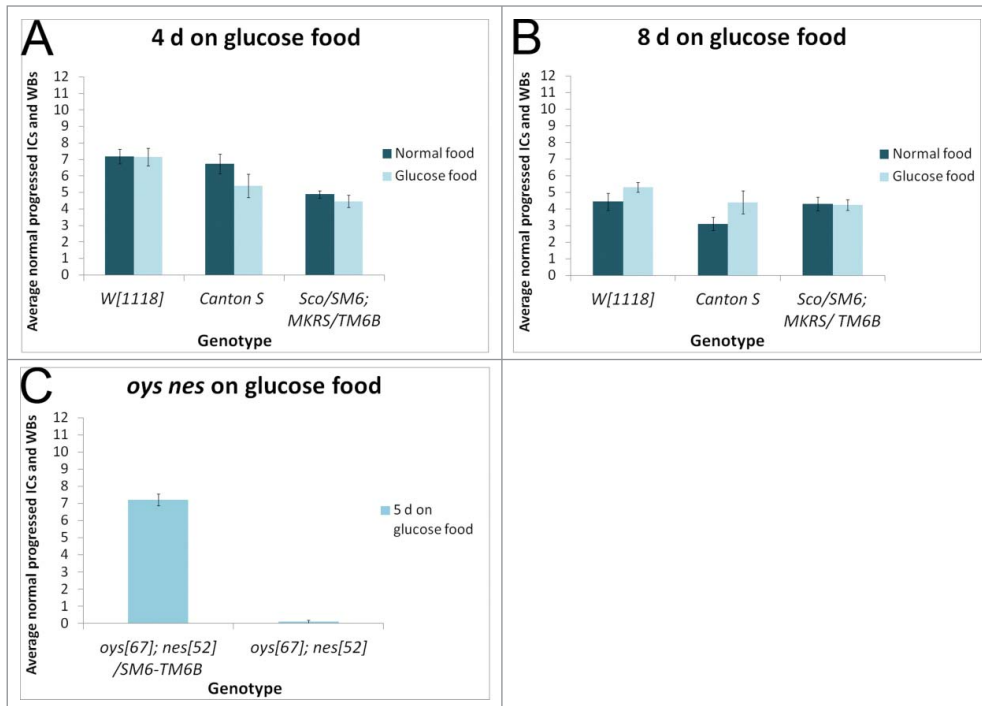 Alternatively, flies may be capable of synthesising necessary fatty acids from carbohydrate sources.[37,60,61] Dietary fatty acid depletion also does not rescue the individualization defect of *oys nes* mutant flies. Thus,

Figure 4. Individualization is not sensitive to diet. Males reared on normal food and shifted within 3 d of eclosion to glucose food (light bars) for either 4 d (**A**) or 8 d (**B**) show no individualization defects compared to age matched siblings kept on normal food (dark bars). N ≥ 10 testes each condition. (**C**) $oys^{\Delta67}$ $nes^{\Delta52}$ double mutant males collected within 5 d of eclosion and cultured on glucose food for 5 d show highly abnormal individualization, with an average of fewer than one normal IC per testis. Controls are balancer siblings cultured in parallel. N ≥ 17 testes each condition.

Sperm cell development before individualization is normal in *oys nes* mutants,[21] again implicating a specific regulatory pathway, as opposed to a general perturbation of cell function, in the mutant phenotype. Furthermore, other unrelated lipid metabolic pathways affect different aspects of spermatogenesis. Phosphatidylinositol lipds are required for spermatocyte cytokinesis, cyst polarity, and axoneme growth.[63–69] A fatty acid elongase, encoded by the *bond* gene, is required for cytokinesis as well,[60,70] while Oys, Nes, and Pxt are dispensable for cytokinesis. These observations indicate that different lipids play distinct roles during sperm development.

The Lands Cycle has been found to influence Golgi dynamics,[71] and alterations in membrane phospholipid composition have been seen to induce ER stress and apoptosis.[72,73] It is unlikely that these effects underlie the *oys nes* phenotype, because mutations that perturb trafficking through the Golgi apparatus, such as *4 way stop* and *syntaxin 5*, disrupt spermatocyte cytokinesis and axoneme elongation, and such defects are not observed in the *oys nes* mutant.[74,75] Additionally, immunofluorescent staining of the Golgi appears normal in *oys nes* mutant cells, and we do not observe ectopic germ cell apoptosis in the *oys nes* mutant.[21]

individualization is not sensitive to global changes in fatty acid levels. Consistently, Sws overexpression is expected to increase fatty acid levels as a byproduct of PC catabolism,[62] yet it has no effect on individualization. Together, our results support the idea that spermatid individualization relies on a specific fatty acid signaling molecule.

Figure 3. (See previous page). Individualization is sensitive to temperature. (**A**) Young (0–3 d old) males raised at 27°C have a slight decrease in the average number of normal individualization structures per testis (red bars, n ≥ 18 each genotype) compared to room temperature (RT) males of the same genotypes (maroon bars, n ≥ 18 each genotype). At 29°C, testes average fewer than 6 normal individualization structures each (pink bars, n > 10 each genotype). (**B**) At 18°C, young w^{1118} males (0–6 d old) have fewer than 2 normal individualization structures per testis on average (n = 57). In contrast, Canton S males at 18°C have more than 8 normal individualization structures per testis (n = 37), and balancer males at 18°C have an average of more than 6 normal individualization structures per testis (n = 34). (**C**) Young w^{1118} males raised at room temperature have an average of 1.15 abnormal ICs per testis, compared to young w^{1118} males raised at 18°C, which have an average of 3.42 abnormal ICs per testis. p < 0.001. (**D**) Young Canton S males raised at room temperature have an average of 1.44 abnormal ICs per testis, compared to young Canton S males raised at 18°C, which have an average of 2.14 abnormal ICs per testis. (**E**) Young Sco/SM6;MKRS/TM6B males raised at room temperature have an average of 0.875 abnormal ICs per testis, compared to young Sco/SM6;MKRS/TM6B males raised at 18°C, which have 1.94 abnormal ICs per testis. p < 0.001. (**F**) Young males raised and mated at 29°C or 18°C have reduced fertility compared to young males raised at 24°C. Number of adult progeny resulting from 100 eggs collected from each cross was counted and averaged between 3 trials. Error bars represent SEM. (**G**) Allowing males to develop at room temperature and shifting them to 29°C within 0–3 d of eclosion significantly reduces the average number of normal individualization structures per testis (peach bars, T shift, n ≥ 15 each genotype). Temperature shifted males were kept at 29°C for 5 d. Control males were 0–3 d old room temperature males (maroon bars, n ≥ 18 each genotype) or 5–8 d old room temperature males (light red bars, n ≥ 18 each genotype). (**H**) Rearing males at 29°C and shifting them to room temperature within 0–3 d of eclosion (maroon bars, n ≥ 16 each genotype) rescues the average number of normal individualization structures per testis. Temperature shifted males were kept at room temperature for 5 d. In contrast, males kept at 29°C for 5 d have fewer than 2 normal individualization structures per testis on average (pink bars, n ≥ 11 each genotype).
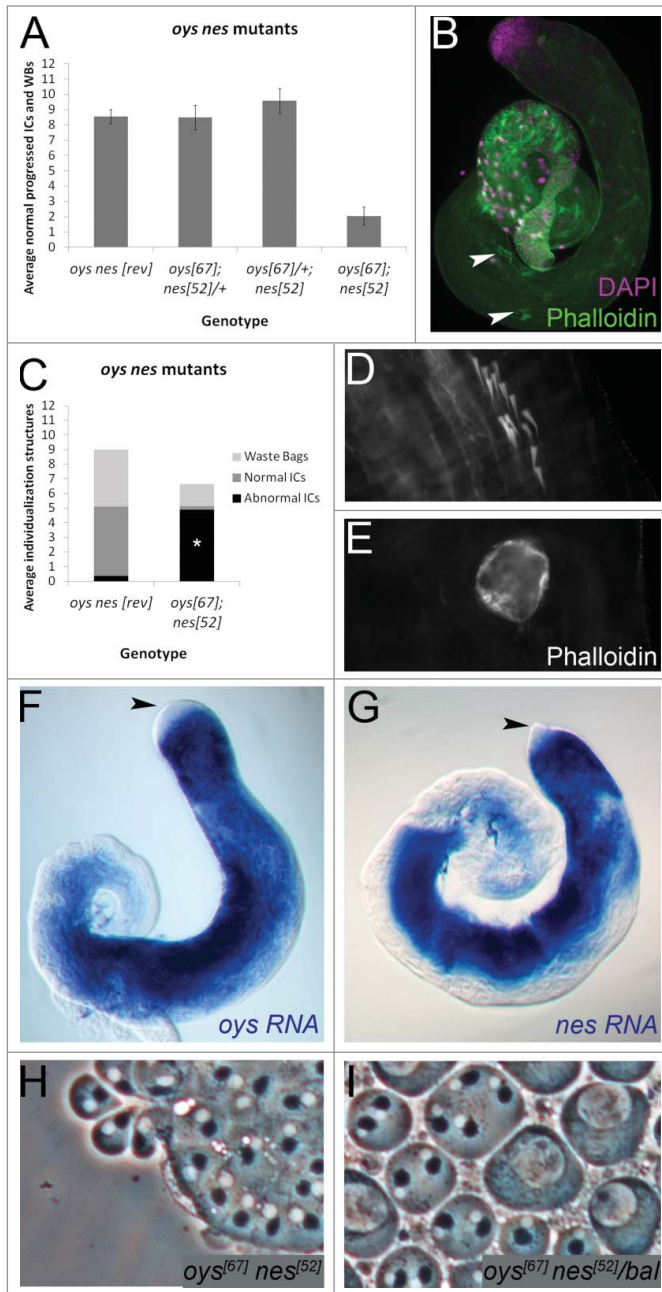
Figure 5. Oys and Nes are required for individualization and are expressed in the germline. (**A**) $oys^{\Delta 6}$ $nes^{\Delta 52}$ mutant males (0–5 d old) have an average of 2 normal individualization structures per testis (n = 20), while precise excision controls (revertant, "rev") have more than 8 (n = 31). Males homozygous for either $oys^{\Delta 67}$ or $nes^{\Delta 52}$ and heterozygous for the other also have more than 8 normal individualization structures per testis on average (n ≥ 24 each). (**B**–**E**) oys nes mutant testes have scattered ICs (**B**, arrowheads, phalloidin, green, close-up in **D**) and waste bags devoid of actin cones (**E**). The average number of abnormal ICs in the oys nes mutant (4.9, n = 22) is significantly increased compared to the revertant control (0.36, n = 11), p <0.001 (**C**). (**F**–**G**) RNA in situ hybridization to endogenous oys (**F**) or nes (**G**) in w^{1118} testes shows expression in the germline beginning in premeiotic spermatocytes (blue). Endogenous transcripts are not detected in the germinal proliferation center (black arrowheads). (**H**-**I**) Phase contrast microscopy of testis squashes reveals no cytokinesis defects in either $oys^{\Delta 67}nes^{\Delta 52}$ homozygotes (**H**) or sibling heterozygotes (**I**, "bal" = SM6-TM6B balancer). Each phase dark Nebenkern has one associated phase light nucleus.

Temperature sensitivity of individualization

Individualization in *Drosophila melanogaster* is extremely temperature sensitive. Our temperature shift experiments demonstrate that individualization itself is the sensitive process, as opposed to gonad formation during larval development. Cytokinesis defects also are apparent at extreme temperatures, but at lower penetrance than the individualization defects. It has been shown previously that spermatogenesis is sensitive to temperatures above 28°C.[76] Here we show that low temperature, i.e. 18°C, also disrupts spermatogenesis. Individualization defects may underlie the observed effects on male fertility, because these 2 parameters are well correlated at both high and low temperatures. A practical implication of this observation relates to the temperature at which *GAL4* experiments are conducted in lab strains. Although *GAL4* activity is maximized at 29°C,[77] we recommend using 27°C when assessing spermatogenesis phenotypes, as few defects are observed at this temperature. A conceptual implication relates to how different populations and species cope with seasonal or daily climatic variations.[76,78]

In conclusion, we have developed a quantitative assay to assess defects in *Drosophila* spermatid individualization, using fluorescent detection of the actin-rich ICs. We have characterized the individualization process as it occurs over a range of environmental parameters. Many mutations have been discovered that perturb individualization, and we hope that our system will be of use in analyzing these mutations in more detail. Individualization is sensitive to genetic disruption of the Lands Cycle and prostaglandin metabolism, and AT and PLA$_2$ homologs are expressed in the *Drosophila* male reproductive tract. Fatty acid mediators are important for male fertility in mammals as well as *Drosophila*.[32,36,58] PLA$_2$ knockout mice exhibit spermatogenesis defects[24] and mammalian ATs are highly expressed in testis,[79] suggesting that the roles of the Lands Cycle in spermatogenesis are evolutionarily conserved throughout metazoa.

Materials and Methods

Fly stocks

Stocks used were w^{1118}, Canton S, Sco/SM6;MKRS/TM6B, tj-GAL4, sws^4, eas^{alaE1} (from Bloomington Stock Center), UAS-Sws (gift from D. Kretzschmar),[50] bam-GAL4-VP16 (gift from Y. Yamashita), pxt^{EY0350}, pxt^{f0100}, and $pxt^{revTT274}$ (gifts from T. Tootle).[53] The $oys^{\Delta 6}$ $nes^{\Delta 52}$ mutant stock and $oys^{\Delta 8}$ $nes^{\Delta 19}$ precise excision revertant control were as described.[21]

Aging and temperature shift individualization experiments

For aging experiments, males were collected within 3 d of eclosion and aged for 5 days, 10 days, 15 days, or 26 d at room temperature in the presence of sibling females. Flies were transferred to new vials every few days. For aging of *pxt* mutants, males were collected within 3 d of eclosion and aged for 5 d or 13 d at room temperature in the presence of sibling females.

For temperature experiments, flies were reared at room temperature (23–24°C), 29°C, 27°C, or 18°C. Males at room temperature, 29°C, or 27°C were collected and dissected within 3 d
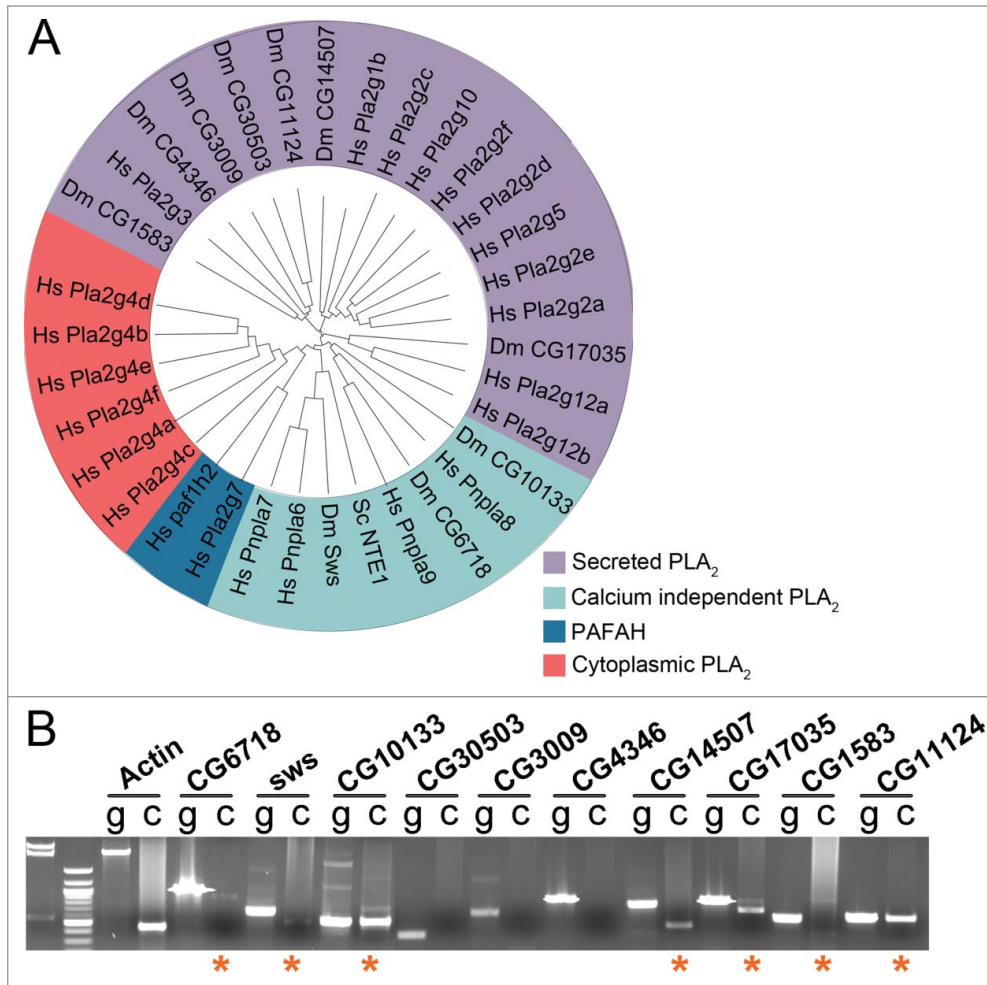
Figure 6. *Drosophila* PLA₂ homologs are expressed in the male reproductive tract. (A) Phylogenetic tree of PLA₂ family members from *Saccharomyces cerevisiae (Sc), Drosophila melanogaster (Dm),* and *Homo sapiens (Hs).* Tree was compiled using software provided by Interactive Tree of Life (itol.embl.de)[82] and following classifications in ref. 24 (**B**) RT-PCR on cDNA ("c") prepared from *w^1118* reproductive tracts showing expression of *CG6718, sws, CG10133, CG14507, CG17035, CG1583* and *CG11124* (asterisks). Control PCR was run for each set of primers on genomic DNA ("g"). *Actin-5c* PCR was used as a control for cDNA integrity.

was adapted from ref. 45 and contained 10% glucose, 0.5% agar (MoorAgar Inc., 41004), 0.11% methyl paraben, 0.38% propionic acid, and 6.2 μg/mL cholesterol.[45] Cholesterol was dissolved in 100% ethanol before addition to food. Males from control genotypes were collected within 3 d of eclosion and kept on glucose food for 4 d or 8 d at room temperature in the presence of sibling females. Control sibling males were collected within 3 d of eclosion and kept on normal food. *oys^Δ67 nes^Δ52* mutant males or balancer sibling males were collected within 5 d of eclosion and kept on glucose food for 5 d at room temperature in the presence of sibling females.

Lipid depleted food recipe was adapted from ref. 45 and contained 10% chloroform-extracted yeast autolysate (Sigma-Aldrich, 73145–500G-F), 10% glucose, 0.5% agar, 0.11% methyl paraben, 0.38% propionic acid, and 6.2 μg/mL cholesterol.[45]

Fertility tests

For aging fertility tests, males of each genotype reared at room temperature were collected within 3 d of eclosion and mating singly to one virgin *w^1118* female each or allowed to age for 5 days, 10 days, 15 days, or

of eclosion. Males at 18°C were collected and dissected within 6 d of eclosion.

For the temperature upshift experiment, stocks were reared at room temperature. Males were collected within 3 d of eclosion and shifted to 29°C for 5 d in the presence of sibling females. Control flies were collected within 3 d of eclosion and kept at room temperature for 5 d. For the temperature downshift experiment, stocks were reared at 29°C. Males were collected within 3 d of eclosion and shifted to room temperature for 5 d in the presence of sibling females. Control flies were collected within 3 d of eclosion and kept at 29°C for 5 d.

Drosophila food

Normal food contained 3.83% molasses, 1.58% yeast, and 3.83% corn meal supplemented with 0.11% methyl paraben and 0.38% propionic acid as mold inhibitors. Glucose food recipe

30 d in the presence of sibling females at room temperature and thereafter mated singly to one virgin *w^1118* female each (virgin females were also reared at room temperature). Matings were performed in standard food vials supplemented with granular dry active yeast (Lab Scientific, FLY-8040–10F). Each mating was allowed to proceed for 3 d at room temperature. After 3 days, P₀ adults were discarded, and larvae were allowed to develop at room temperature. F₁ adults were scored every 3–4 d until the 20th day after establishment of the vial. Ten matings were performed for each age group. Crosses that yielded no progeny were discarded.

For temperature fertility tests, 10 males of each genotype reared at the experimental temperature were mated to 15–20 virgin *w^1118* females and transferred to egg collecting cages. Both mating and egg laying were allowed to proceed at the experimental temperature. Within a 24 hour egg laying period, 100 eggs
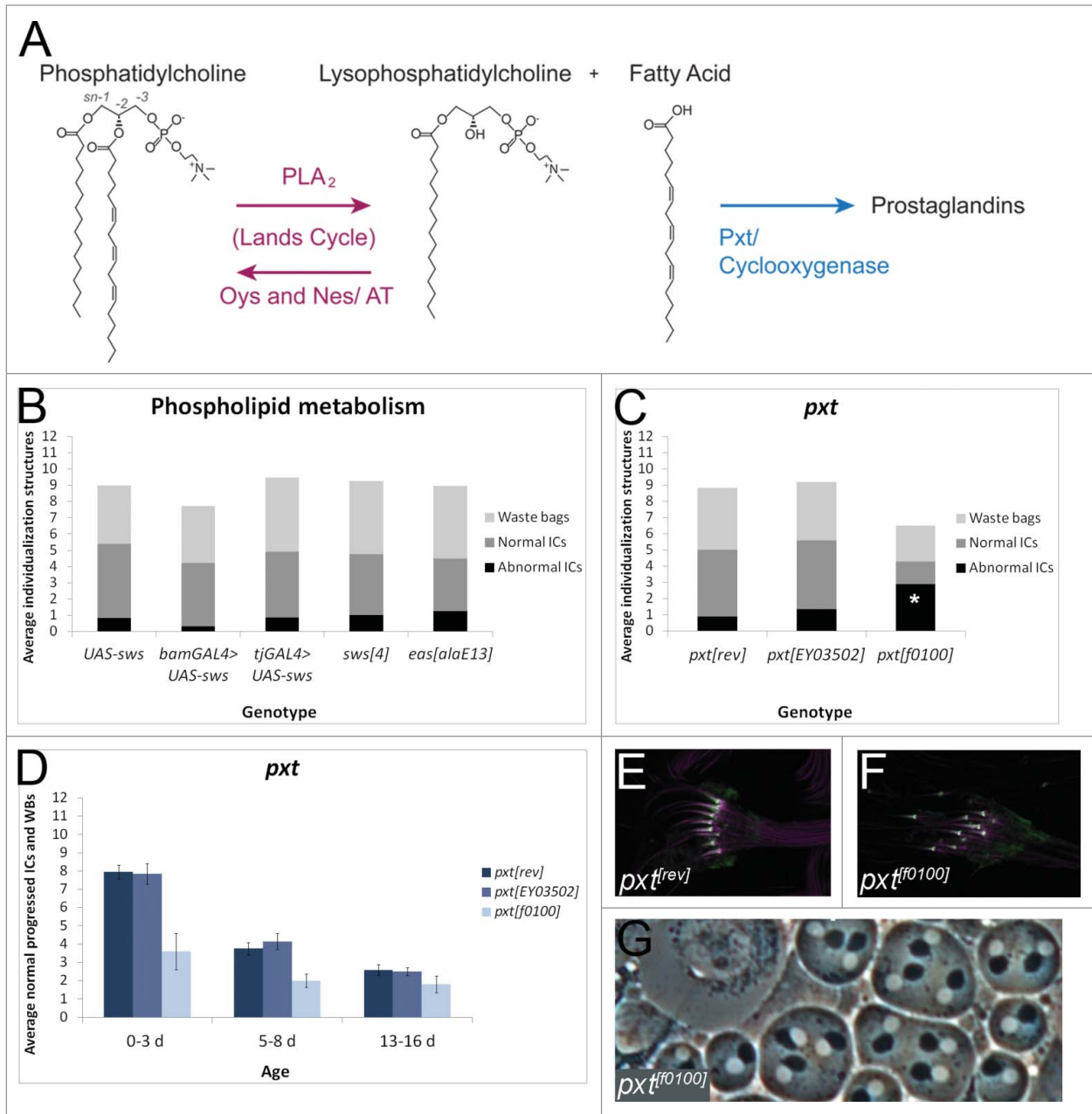
Figure 7. A strong pxt mutant disrupts individualization. (**A**) Schematic of the Lands Cycle. Long chain unsaturated fatty acids are esterified to single chain lysophospholipids, like lysophosphatidylcholine, by ATs Oys and Nes to form PC.[21] When stimulated, PLA_2 enzymes catabolize phospholipids to release fatty acids and lysophospholipids.[23] Cyclooxygenases such as Pxt metabolize fatty acids to prostaglandin signaling molecules.[83] (**B**) Males overexpressing *UAS-Sws* in the germline with *bam-GAL4-VP16* (n = 18), males overexpressing *UAS-Sws* in the somatic cyst cells with *tj-GAL4* (n = 21), males hemizygous for the strong hypomorphic *sws*[4] allele[50] (n = 36), or males hemizygous for the strong hypomorphic *eas*[alaE13] allele[84] (n = 25) show no individualization defects compared to control males bearing *UAS-Sws* alone (n = 13). p > 0.1 for each experimental condition. (**C**) 0–3 d old homozygous *pxt*[f0100] males have significantly more abnormal ICs per testis (average 2.9, n = 10) than revertant males (*rev*, average 0.9, n = 20), p <0.05. *pxt*[EY03502] homozygotes do not show a significant increase in abnormal ICs (average 1.35, n = 20), p = 0.32. (**D**) Males homozygous for the strong hypomorphic *pxt*[f0100] mutation[55] (light blue bars) have a reduced number of normal individualization structures at 0–3 d old, 5–8 d old, and 13–16 d old, when compared to precise excision controls (*rev*, dark blue bars). Males homozygous for the weak *pxt*[EY03502] mutation do not show individualization defects (medium blue bars). N ≥ 10 testes each condition. (**E, F**) Actin cones (purple, phalloidin) from *pxt*[rev] (**E**) and *pxt*[f0100] (**F**) males both show normal Myosin VI localization to the front of the cones (green). (**G**) *pxt*[f0100] mutants do not show cytokinesis defects in phase contrast testis squashes. p values were obtained using 2-tailed students' t-test with unequal variance. Experiments were repeated at least twice; data shown are from a representative trial.

from each cross were collected and placed into food vials, in order to control for reduced egg laying at extreme temperatures. The progeny were allowed to develop in food vials at 24°C, in order to control for larval developmental defects at extreme temperatures. Males reared at 24°C or 29°C were collected within 3 d of eclosion. Males reared at 18°C were collected within 6 d of eclosion. Adult progeny were counted until the 19th day after establishment of the vial.

Fluorescence staining

Adult testes were dissected in phosphate-buffered saline (PBS) and fixed in 5% formaldehyde for 20 min at room temperature, washed in PBX (PBS + 0.1% Triton X-100) for 15–20 min, and stained with rhodamine-phalloidin (1:200, Sigma-Aldrich, P1951) and DAPI (1:4000, Roche, 10236276001) for 20 min at room temperature. Following three washes in PBX, testes were mounted in Fluoromount G (Southern Biotechnology, 0100–01). For antibody staining, the apical ends of the testes were removed before fixation to expose the spermatids. Following fixation in 5% formaldehyde for 20 min, testes were blocked in PBS + 5% normal donkey serum + 1% Triton X-100. Testes were incubated in primary antibodies overnight (+ 5% normal donkey serum) at 4°C, washed 3 times in PBX, and incubated with secondary antibodies (Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody from Molecular Probes, 1:1000, A-21202), DAPI, and rhodamine-phalloidin for 2 hours at room temperature. Mouse anti-myosin VI was kindly provided by K. Miller and used at 1:20.[80] Mouse anti-Singed (Fascin) was used undiluted (DSHB, sn 7C). Images were captured using a Zeiss LSM510 Confocal or an Olympus IX-81 motorized inverted microscope with XM-10 monochrome camera. ICs were scored using a 20x objective.

RT-PCR

Testis cDNA was prepared following ref. 81 using SuperScript III first-strand synthesis kit (Life Technologies, 18080051).[81] PCR primer pairs were as follows:

Gene	Forward primer	Reverse primer
CG1583	5′-CGAACGCCATCGGAAAGCTC	5′-CATAGACGTGGTGTCTCTGG
CG3009	5′-CATCTGGTTCGGCGGTGC	5′-ACCTCTGGTGTGCCGAGC
CG11124	5′-CGACGAGGCTATCTTTGAGG	5′-ACGCTGGGATTGTGAAGAAC
CG30503	5′-GCAGCCAACTACGATGACCTG	5′-CACTCGGTAGCTGAGACATCG
CG4346	5′-CTTCAGCTGCTGGCCTCG	5′-GGAGTCGTTGCTCACACGC
CG14507	5′-GTGGCGACGACTTCGAGCTG	5′-CATGGAGTAACCGAAGGGATC
CG6718	5′-CGACCCACCTCGGATTCC	5′-GACCACCAGACATTGGACG
CG10133	5′-GAAGCGGAGGAGCTGTGC	5′-GTGCGGCGGGATGAGAAC
CG17035	5′-CTAACCTACGCCTATTCAGG	5′-CGTTGCGATCCTTCCAGC
Sws	5′-CTAAGCGGTTGGTGGCTGC	5′-CAGGTCAAGCTCCACGTCC

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