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Lipid metabolism and *Drosophila* sperm development

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Lipids are essential membrane structural components and important signal carriers. The major enzymatic metabolisms of various lipids (phospholipid, sphingolipid, cholesterol) are well studied. The developmental function of lipid metabolism has remained, for the most part, elusive. With the help of new techniques and model organisms, the important roles of lipid metabolism in development just start to emerge. *Drosophila* spermatogenesis is an ideal system for in vivo studies of cytokinesis and membrane remodeling during development. The metabolic regulators of many lipids, including phosphatidylinositol (PI) lipids, fatty acids and cholesterol, are reported to play critical roles in various steps during *Drosophila* spermatogenesis. In this mini-review, we summarized recent findings supporting a tight link between lipids metabolism and *Drosophila* sperm development.

***Drosophila* spermatogenesis, phosphatidylinositol lipids, fatty acids, cholesterol**

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1 Introduction

Lipids (phospholipid, sphingolipid, cholesterol, etc.) are essential components of cells. Besides the well-known membrane structural and storage roles, lipids also play critical roles in cellular signaling, e.g., PIPs and sphinogosines are key regulators in signaling transduction and developmental regulation [1]. In addition, lipid modifications of proteins, such as palmitoylation of Wnt and cholesterol modification of Hedgehog, have important roles during animal development and cancer [2].

Within the cells, lipid homeostasis is achieved through several lipid metabolic processes including lipid biosynthesis, lipid utilization, lipid storage, and lipid trafficking [3]. Under normal physiological conditions, lipid metabolism is tightly regulated and defects in lipid metabolism often result in various human diseases, including obesity, diabetes, and atherosclerosis. Nevertheless, compared to protein, DNA, and RNA, relative less is known about the lipid biology and the biological processes involved.

Brain and testis are two lipid-rich organs, where many membrane reorganizing processes happen. For instance, *Drosophila* spermatogenesis involves complex membrane remodeling processes, including cytokinesis and spermatid differentiation. Figure 1 depicts the *Drosophila* spermatogenesis. Briefly, two main types of cells build up the *Drosophila* testis: germ cell and cyst cell. During *Drosophila* spermatogenesis, a pair of cyst cells encloses a primary spermatogonia cell, and the whole structure is called a cyst. Within a cyst, a primary spermatogonia cell goes through four rounds of consecutive mitotic division and two rounds of meiotic division, to form 64 spermatid cells. The newborn spermatids are still interconnected by cytoplasmic bridges even after the cytokinesis is completed. After that, the spermatids differentiate synchronously from round spermatids to elongated spermatids to have the final shape

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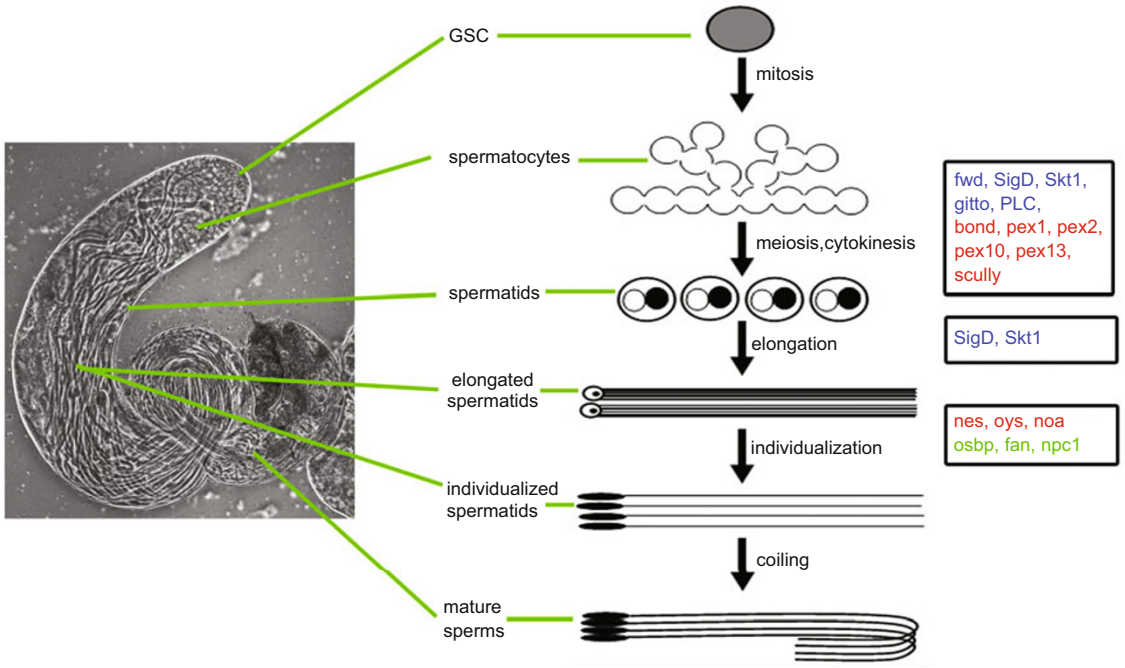

Figure 1 *Drosophila* spermatogenesis and lipid metabolic genes discussed in this review. Left panel is a *Drosophila* testis with different stages of spermatogenesis. Middle panel is a schematic drawing of key steps in spermatogenesis. Right panel lists the genes involved in cytokinesis, elongation and individualization. These genes are involved in the metabolism of PI (blue), fatty acids (red), and cholesterol (green).

of long-tail sperms. Individualization is an actin based, membrane reorganizing process that occurs after elongation. During individualization, the individualization complex (IC) composed of 64 actin cones, forms and progresses caudally along the cyst. The synchronic movement of actin cones remodels the syncytial membrane to remove excess cytosol and packs each spermatid into its own plasma membrane. The individualized sperms are then coiled and move into the seminal vesicles for storage [4].

The metabolic regulators of many lipids, including phosphatidylinositol (PI) lipids, fatty acids and cholesterol, are reported to play critical roles in the *Drosophila* spermatogenesis [5–9]. In addition, a single type of lipid may participate in several steps during sperm development. In this mini-review, we summarize recent findings supporting a tight link between lipids metabolism and *Drosophila* sperm development.

2 The levels of PIs are critical for cytokinesis in *Drosophila* sperm development

The metabolism process of PI lipids, also known as PI cycle, has been well studied. Briefly, PI can be phosphorylated by PI4K to form PI4P, and then PIP5-kinase (such as Skt1 in *Drosophila*) could phosphorylate PI4P to form PIP2. PIP2 could be further phosphorylated by PI3K to form PIP3 or be hydrolyzed by phosphorlipase C (PLC) to generate the second messengers, IP3 and DAG. PIP3 could be reversibly dephosphorylated sequentially by phosphatase, such as PTEN, SigD and Sac1, to generate PI back (Figure 2).

PIs have been involved in many different biological processes. Works from a variety of systems have suggested that PIs are important for cytokinesis. The first link between PIs and cell division is found in experiments showing that lithium (Li^+) blocks cytokinesis in sea urchin zygotes and could be rescued by addition of PI precursor, *myo*-inositol [10]. It has been known that Li^+ could be used as inhibitors for inositol biogenesis [10,11]. These results indicate that inositol, the precursor of PI, is important for cytokinesis process. Notably, block of PIP2 by microinjection PIP2 antibodies results in multinucleate cells [12]. Further studies show that block of PIP5-kinase or local depletion of PIP2 leads to cytokinesis failure [13,14].

Studies in *Drosophila* male sterile mutants provided strong *in vivo* genetic evidence that PIP2 is important for germ cell cytokinesis. In *Drosophila* testis, large amounts of germ cells during cytokinesis could be easily seen under the microscope. Therefore, it is an ideal system for *in vivo* cy-tokinesis studies. *four wheel drive* (*fwd*) is the first identified PI cycle related gene involved in *Drosophila* spermat-

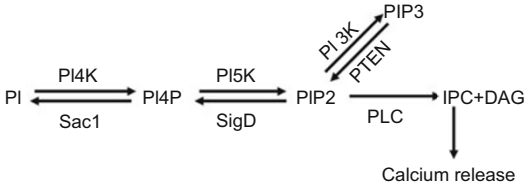

Figure 2 PI cycle pathway. PI cycle is regulated by multiple kinases and phosphatases.

ogenesis [5]. *fwd* mutants show cytokinesis defects during male meiosis. In particular, although the contractile rings could form, the cleavage furrows are unstable and fall apart. *fwd* encodes a homolog of phosphatidylinositol 4-kinase β (PI4Kβ). The kinase activity is required for Fwd function. Similarly, depletion of PIP2 by elevated phosphatase SigD expression, or knockout *gitto* (also known as *vibrator*), which encodes a PI trafficking protein, also leads to severe spermatogenesis defects [6,15]. PIP2 may function in regulating actin dynamics and membrane fusion during cytokinesis. These results indicate that a reduced level of PIP2 may lead to *Drosophila* cytokinesis defects.

Furthermore, studies in crane fly also support a pivotal role of PIP2 in meiotic cytokinesis. Many drugs (such as wortmannin and LY294002) could be used to interfere with the enzymes' activity specifically in PI cycle. Inhibiting of different steps in PI cycle with these drugs supplied a systemically view of PI function in spermatogenesis in crane fly. Reduction of PIP2 or its precursors' levels slows or even arrests the spermatocyte cytokinesis, indicating a requirement for PIs in the male meiosis cytokinesis [16], suggesting the PI dependent cytokinesis regulation is conserved in different species.

Interestingly, inhibiting the activity of PLC, which hydrolyzes PIP2 to form IP_3 and DAG, also leads to cytokinesis defect [17,18]. In this scenario, the PIP2 levels may be increased, which is opposite to the reduced PIP2 levels in *fwd* mutants or *SigD* overexpression conditions. How could both increased and decreased levels of PIP2 lead to the same cytokinesis defects in *Drosophila*? It is possible that instead of elevated PIP2 levels, reduced IP3 levels account for the phenotype observed. IP3 could be used as second messenger to stimulate calcium release. Indeed, supply of calcium to the cells could bypass the requirement of PIP2 hydrolysis [17,18]. These data highlight the important function of IP3. IP3 could activate the myosin via calcium and then regulate the actin dynamics in spermatogenesis [17,18]. Together, these results suggest that proper balance of PIP2 and IP3 is important for cytokinesis.

In addition to cytokinesis, PIP2 is also required for other steps in spermatogenesis. High level of *SigD* overexpression in testis leads to total loss of elongated spermatids and shows defects in the basal body docking to the nuclear envelope and the integrity of developing flagellar axoneme [19]. These results indicated a functional role of PIP2 in cooperating the membrane fusion events with microtubule organization to form the flagella [19]. Interestingly, a low level expression of *SigD* results in cell polarity defect in the elongation process and elongated spermatids could still be found in the testis [20]. The elongation defect is believed to be caused by the membrane addition and exocytosis defect, similar with what was found in *Sec8* mutants [20]. The phenotype could be suppressed by co-overexpress *SigD* with *Skt1*, which could promote PIP2 production [19,20]. These data indicated that the proper amount of PIP2 on plasma membrane is required both for the spermatocyte cytokinesis process and for the spermatid differentiation.

3 Fatty acid compositions are important for spermatogenesis

Fatty acids are the building block of phospholipids and glycerolipids. The first connection between fatty acid metabolism and *Drosophila* spermatogenesis originated from a functional study of fatty acid elongase [21]. Just as the name infers, the function of fatty acid elongase is to increase the length of acyl chain during fatty acid synthesis. NOA is a *Drosophila* homolog of mammalian elongase Elovl6 and it is essential for viability. Cyst cell-specific *noa* RNAi results in male sterility and defects in the spermatogenesis including individualization, indicating that *noa* plays an important role in cyst cells to cooperate germ cell development [21]. The communications between cyst cells and germ cells are important for the spermatogenesis. The cyst cell-specific requirement of NOA suggests that fatty acids or derivatives may be important for generating messengers, or they themselves could be messages, between germ cell and cyst cell.

On the germ cell side, from an EMS genetic screen aimed for male sterility mutations, *bond* was identified as an important regulator of cytokinesis during meiosis. Round spermatids from *bond* mutant males commonly display two or four nuclei associated with an abnormally large mitochondrial derivative [22]. Later, *bond* was found encoding a member of the Elovl family of enzymes involved in elongation of very-long-chain fatty acids (VLCFA) [7]. Therefore, elongases (NOA and Bond) are required in cyst cells and germ cells for proper spermatogenesis. Further study revealed a specialized VLCFA requirement for successful cleavage furrow ingression during germ cell division. In *bond* mutants, VLCFA levels may be reduced. VLCFA is proposed to stabilize highly curved membranes, which may be essential for the completion of cytokinesis [7,23].

Notably, the study of peroxisome function in *Drosophila* revealed that accumulation of VLCFA could also result in cytokinesis defect [8]. In animals, β-oxidation of VLCFA is exclusively performed in peroxisomes. Disruption of peroxisomes in *Drosophila* peroxisome biogenesis defective mutants, such as *pex1*, *pex2, pex10* and *pex13*, leads to abnormal accumulation of VLCFA and profound defects in spermatogenesis including cytokinesis [8]. Interestingly, *pex* mutations could suppress the cytokinesis defects in *bond* mutants in a dosage dependent fashion, suggesting a balance of VLCFA levels is required in spermatogenesis. This study also suggests that the accumulation of VLCFA may change the membrane dynamics. However, it is not certain whether the mildly changed membrane dynamics is the cause of the cytokinesis failure. In addition, VLCFA may also participate in other processes during spermatogenesis,

such as elongation. Instead of long bundles, elongated spermatid in *pex* mutants displays a "cotton-ball" like shape, which has been reported before in the *syntaxin* (*syx1A*) mutants [24]. Moreover, it remains to be determined that whether the total levels of VLCFA or a specific species of VLCFA are responsible for the phenotype observed.

Besides VLCFA, other fatty acids may also be involved in the spermatogenesis in *Drosophila*. Actually, the first piece of evidence comes from the characterization of *scully*, which encodes a protein with high structural homology to type II 3-hydroxy-acyl-CoA dehydrogenase (HADH) [25]. Type II HADHs catalyze β-oxidation of short chain fatty acids in mitochondria. *scully* mutants show a dramatic reduction of the testis size [25]. The ultrastructural analysis of *scully* mutant testis revealed abnormal accumulations of lipids containing vesicles in spermatocytes [25]. Therefore, normal β-oxidation of short chain fatty acids is required for sperm development, though the mechanisms are still unclear.

Lysophospholipid acyltransferase (LPLAT) is another lipid metabolic enzyme which is involved in the individualization process [26]. LPLATs catalyze the reacylation step in membrane phospholipids remodeling [27]. There are three LPLAT coding genes in *Drosophila*, *nessy* (*nes*), *farjavit* (*frj*) and *oysgedart* (*oys*), and they often exhibit redundant functions. *oys;nes* double mutant males are sterile with individualization defects [26]. It is possible that LPLATs may execute their function by affecting the PI levels, just like other PI cycle related genes. Alternatively, LPLATs may function in spermatogenesis by affecting the distribution of fatty acids.

Based on the reports on fatty acids and sperm development, both excess accumulation and reduction of fatty acids all result in sperm development failure. Since the length of fatty acid carbon chain is important for the membrane dynamics, it is reasonable that fatty acids with different carbon chain length may have distinguishable functions in sperm development. Similar to PIs, fatty acids may function in multiple biological processes in spermatogenesis.

4 Cholesterol plays key roles in the individualization process

Recently, cholesterol is found important during spermatogenesis based on our studies of *npc1* and *Osbp* [9,28]. *npc1* encoded a cholesterol binding transmembrane protein, which is known as one of the two key genes responsible for the Niemman-Pick type C (NPC) disease. *NPC* mutant cells are defective in intracellular trafficking of endocytic-originated cholesterol [29]. *Drosophila npc1* mutants are first instar larvae lethal with cholesterol accumulation in multi-lamellar body like structures [30,31]. The lethality is due to a shortage of ecdysone, which is synthesized from cholesterol. Interestingly, ecdysone shortage-rescued *npc1* mutants show a male sterile phenotype, indicating that *npc1* is also involved in process(es) other than ecdysone biosynthesis [30,31]. Further studies show that *npc1* deficiency leads to individualization failure in *Drosophila*. In addition, we found that *npc1* function is required cell-autonomously in germ cells [28].

Oxysterol binding protein (OSBP), which is involved in non-vesicular mediated intracellular sterols trafficking and distribution, is also required for spermatogenesis [9]. Similar with *npc1*, *Osbp* mutants show individualization defects. During individualization, OSBP protein is localized as punctated structure at the leading edge of IC. Cholesterol, stained by a vital dye filipin, displays a similar punctated pattern in the same place. It is not known whether OSBP and cholesterol are localized to the same puncta, which appears to be Golgi-like structure. In *Osbp* mutants, the sterol-rich puncta are missing. FAN, a VAMP-associated ER protein, modulates OSBP-mediated sterol trafficking and interacts with OSBP both genetically and physically [9]. *fan* mutants show similar individualization defects with *Osbp* [9]. Notably, both of *npc1* and *Osbp* mutants could be rescued by adding 7-dehydrocholesterol (7-dC) in food [9,28]. Additional cholesterol feeding also could rescue the fertility of *Osbp* mutant males, though it does not work well in *npc1* mutants [9,28]. These results highlight an important role of cholesterol in spermatogenesis in *Drosophila*, especially in the individualization process.

Cholesterol is an essential component of membrane structure and the amounts of cholesterol in membranes could regulate the membrane dynamics [32]. Since both NPC1 and OSBP are required for the normal cholesterol trafficking, it is possible that the sterol components in the membrane structure are altered in *npc1* and *Osbp* mutants and subsequently affecting the membrane dynamics. Consistent with this hypothesis, the individualization defects in *npc1* mutants are temperature sensitive [28]. It is not clear that whether *npc1* and *Osbp* functions in the same or parallel pathway. NPC1 function is likely not dependent on OSBP, since both the OSBP-rich and sterol-rich speckles are still present in *npc1* mutants [28]. Moreover, over expression of *npc1* in germ cells could not rescue the male infertility of *Osbp* mutants, suggesting that *npc1* unlikely acts downstream of *Osbp* [28]. More work is expected before the exact functional mechanisms of sterols in individualization are found.

Cholesterol is transported into cells by the endocytic pathway. So it is possible that endocytosis-related mutants may show cholesterol deficiency in germ cells and then cause individualization defects. For example, clathrin heavy chain (Chc) and dynein light chain 1 are reported to be involved in individualization process [33,34]. Another possible candidate may be ER-Golgi retrograde trafficking regulator *vps54*, which is also known as *scattered* (*scat*). *scat* is essential for sperm development both in mouse and *Drosophila* [33,35]. A possible explanation for the requirement

of *scat* in sperm development is that it is involved in subcellular cholesterol trafficking or metabolism, since *scat* mutants show development delay in larva stages and could be rescued by additional sterols in food (our unpublished data). However, the functions of these genes may be more complicated because the infertility of *vps54* and *Chc* mutants could not be rescued by cholesterol or 7-dC (our unpublished data).

5 Perspectives and future challenges

There is no doubt that lipids (PIs, fatty acids and cholesterol) are essential for normal sperm development in *Drosophila.* More attention on lipid metabolism will help us to elucidate the role of proper lipid metabolism in spermatogenesis and to better understand the spermatogenesis process.

The main challenge now is to depict the exact role of lipids in spermatogenesis. Does a particular lipid species or the total amount of one type of lipids matter? Lipid metabolism is a complex network process and disturbing the metabolism of a single step could potentially interfere with the whole network. Identifying the exact species of lipids will be difficult. Utilizing the mass-spec technology may be a feasible way to retrieve a clue. Regulating the expression of a specific enzyme or culturing with a particular lipid may be useful to elucidate contributions from different lipids.

On the other hand, as essential membrane components, various lipids may affect different membrane properties. It has been reported that most of the sperm developmental defects happen in the cytokinesis and individualization process [36], which contain large amounts of membrane biogenesis and remodeling events. FRAP has been widely used to examine the membrane dynamics in spermatogenesis. However, the result of this technology is not enough to secure a link. More tries in the development of new technologies in this field will accelerate the studies of lipid's function in spermatogenesis. With GFP fusion to PH domains specifically binding different PI derivates, the PIs levels could be visualized and measured easily [17]. These markers highly facilitated and promoted the studies of different PIs function in spermatogenesis. However, it is difficult to label other kinds of lipids in such a specific manner. Specific markers, dyes, and examination technologies for different lipids are required, especially in the studies of fatty acid and cholesterol.

Current findings about lipid metabolism and spermatogenesis raise more questions than answers. For example, what is the role of different PIs in different sperm developmental stages? What are the downstream effectors of calcium release stimulated by IP3 signaling? How could both reduced and increased levels of VLCFA affect cytokinesis? What is the exact role of cholesterol in spermatogenesis? Is any lipid related signaling pathway involved in spermatogenesis? Characterization and functional analysis of more lipid metabolism mutants and/or disturbing the metabolic processes by gene overexpressing will be greatly helpful for answering these questions. Moreover, further studies of lipid metabolism may reveal basic concepts in spermatogenesis. The new findings in *Drosophila* spermatogenesis will lead to better understanding of the same process in human, since the underlying mechanisms are highly conserved.

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