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Diverse roles of phosphatidate phosphatases in insect development and metabolism

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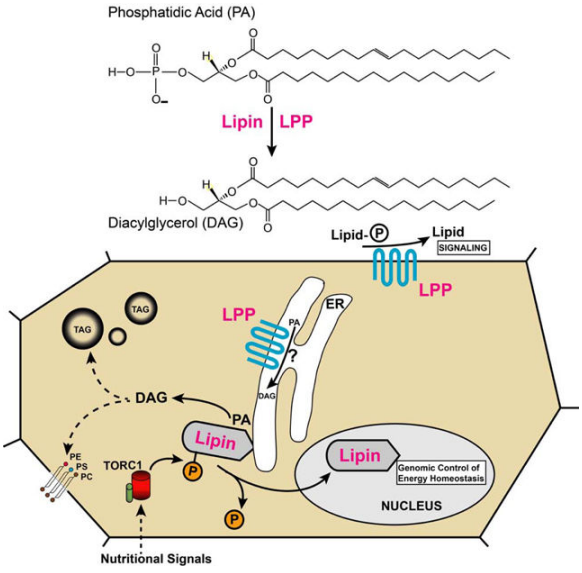
Abstract

The conversion of the glycerophospholipid phosphatidic acid (PA) into diacylglycerol (DAG) is essential for the biosynthesis of membrane phospholipids and storage fats. Importantly, both PA and DAG can also serve signaling functions in the cell. The dephosphorylation of PA that yields DAG can be executed by two different classes of enzymes, Mg^{2+}-dependent lipins and Mg^{2+}-independent lipid phosphate phosphatases. Here, I will discuss the current status of research directed at understanding the roles of these enzymes in insect development and metabolism. Special emphasis will be given to studies in the model organism *Drosophila melanogaster.*

Graphical Abstract



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Keywords

Phosphatidate phosphatases; lipins; nutrient signaling; lipid phosphate phosphatases; Wunen; Lazaro

1. Introduction

Lipids are essential structural and functional components of cells. A central intermediate in the synthesis of both membrane phospholipids and neutral fats is the glycerophospholipid phosphatidic acid (PA). PA can be produced in three different ways, (1) acylation of lysophosphatidic acid (LPA), (2) hydrolysis of phosphatidylcholine (PC), or (3) phosphorylation of diacylglycerol (DAG) (Fig. 1). The de novo synthesis of PA in the glycerol-3-phosphate pathway by acylation of LPA is an essential intermediate step in the synthesis of all glycerophospholipids. It is also the penultimate step in the synthesis of triacylglycerols (TAG) that are used as energy stores. Enzymes of the glycerol-3-phosphate pathway are located at the endoplasmatic reticulum. Some of them, including 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), the enzyme that produces PA by acylation of LPA, are also found at the surface of fat droplets (Jacquemyn et al., 2017; Wilfling et al., 2013).

Apart from its role as a biosynthetic precursor, PA can have structural and signaling functions. Compared to other phospholipids, PA is underrepresented in the biological membranes of eukaryotic cells, constituting only 1–4% of total cellular lipid in mammalian and yeast cells (Selvy et al., 2011; van Meer et al., 2008). PA content is equally low in membranes of insect cells (Carvalho et al., 2012). Local enrichment, however, can have profound effects. It can bring about changes in membrane properties that may, for instance, lead to increased membrane curvature. Such a structural role could be the mechanistic underpinning of PA's established function in intracellular vesicle trafficking in both mammals and *Drosophila* (Kooijman et al., 2003; LaLonde et al., 2006; Roth et al., 1999; Siddhanta and Shields, 1998). Local production of PA can also serve signaling purposes. PA derived from hydrolysis of PC by phospholipase D (PLD) is involved in a number of different signaling processes including nutrient signaling through the kinase target of rapamycin (TOR) and growth factor signaling mediated by receptor tyrosine kinases and Ras (Foster, 2013; Selvy et al., 2011; Zhao et al., 2007). Phosphorylation of DAG by diacylglycerol kinase (DGK) produces PA that plays a regulatory role in the vertebrate immune system and in photoreceptor function in *Drosophila* (Mérida et al., 2008) (Fig. 1). Conversion of DAG into PA by DGK may also serve structural functions as exemplified by the role of yeast DGK1 in nuclear envelope biogenesis (G.-S. Han et al., 2008).

The focus of this review will be the conversion of PA into DAG by phosphatidate phosphatases (PAP; also referred to as phosphatidate phosphate hydrolases) (Fig. 2). Like PA, DAG can have signaling functions. Particularly well characterized are these functions for DAG produced by phospholipase C that binds to and activates protein kinases C and D (Mochly-Rosen et al., 2012; Roy et al., 2017). Similar to the production of PA, PA's conversion to DAG is compartmentalized and carried out by separate groups of PAP

enzymes that serve specific roles. Early biochemical characterization of PA phosphatases in both insects and mammals indicated that enzymatic activity could be stimulated by Mg^{2+} ions (Hirano and Gilbert, 1967; Jamdar and Fallon, 1973a). In 1973, Jamdar and Fallon reported the presence of at least two different types of PA phosphatases in rat adipose tissue that differed by their dependence on Mg^{2+}, one being Mg^{2+}-independent (Jamdar and Fallon, 1973b). Today, these groups are distinguished as lipins (formerly PAP1 enzymes; Mg^{2+}-dependent) and lipid phosphate phosphatases (LPPs; formerly PAP2 enzymes; Mg^{2+}-independent) (Brindley and Pilquil, 2009). Here, I will review studies that have identified roles of these types of PA phosphatases in insect development and metabolism.

2. Lipins

The first gene encoding an animal PAP1 enzyme was identified in 2001 in the mouse. However, it took six more years until the product was shown to catalyze the conversion of PA into DAG (Donkor et al., 2007; Peterfy et al., 2001). Mutations in the gene, originally referred to as the *fatty liver dystrophy* (*fld*) gene (Langner et al., 1989), cause fatty liver and a severe lipodystrophy in mice (Peterfy et al., 2001). Upon cloning of the gene, it was renamed as *lipin-1* (*Lpin1*) (Peterfy et al., 2001). The mouse and human genomes encode three lipin paralogs, whereas *Drosophila* and other insects have a single lipin gene (Alves-Bezerra and Gondim, 2012; Csaki and Reue, 2010; Kanost et al., 2016; Ugrankar et al., 2011; Valente et al., 2010). The basic structure of lipins, as outlined in figure 3 for *Drosophila* Lipin, as well as the basic functions of these enzymes, are conserved in all eukaryotes, from yeasts and plants to protozoans and vertebrates (Peterfy et al., 2001; Pillai et al., 2017).

Characterization of insect lipins has so far been limited to *Drosophila melanogaster* (Schmitt et al., 2015; Ugrankar et al., 2011; Valente et al., 2010). Similar to the phenotype caused by mutations in the mouse *Lpin1* gene, fruit fly larvae lacking Lipin display a severe lipodystrophy and increased hemolymph sugar levels indicating insulin resistance (Schmitt et al., 2015; Ugrankar et al., 2011). The Lipin protein is broadly expressed in *Drosophila* tissues including the fat body, the gut, the Malpighian tubules, the nervous system, the gonads, and the endocrine ring gland (Ugrankar et al., 2011; Valente et al., 2010). The *Lipin* gene encodes at least three protein isoforms that have been characterized to some extent, and the presence of additional isoforms is predicted (FlyBase version FB2020_03, released June 16, 2020). Some of the characterized isoforms show stage and tissue specificity in their expression (Valente et al., 2010). They differ in their C and N termini, but all contain intact catalytic and transcriptional co-regulator motifs. The conserved sequence motifs are part of the CLIP domain that is highly conserved between lipins from different species. A second highly conserved domain, the NLIP domain is located near the N terminus of the protein (Fig. 3). This domain contains an HVRF motif which constitutes a binding site for the catalytic subunit of protein phosphatase 1 (PP-1c). Interestingly, deletion of this motif completely abrogates PAP activity and nuclear translocation of lipin-1, indicating that the NLIP domain is required for both enzymatic and nuclear functions of lipin-1 (Kok et al., 2014). The *Drosophila* Lipin J isoform, which is specifically expressed in testis, lacks this conserved motif (Valente et al., 2010) (Fig. 3). Consistent with the predicted enzymatic inactivity of this isoform, TAG levels are low in testis, raising the question what the function

of Lipin J is in this tissue. In general, it will be an important goal for future studies to identify functional differences between the Lipin isoforms. With today's availability of CRISPR/Cas9 mutagenesis, this should be an achievable task.

Lipins are highly phosphorylated proteins and the phosphorylation status controls intracellular localization of the protein. When nutrients are scarce, *Drosophila* Lipin and other lipins migrate into the cell nucleus. Nuclear translocation of mouse lipin-1 and *Drosophila* Lipin is under control of the nutrient-sensitive target of rapamycin (TOR) C1 pathway (Peterson et al., 2011; Schmitt et al., 2015). TOR is a serine/threonine kinase that has been shown to directly phosphorylate lipin-1, leading to retention of the protein in the cytoplasm (Peterson et al., 2011). Upon dephosphorylation, when TORC1 activity is low, dephosphorylated forms of lipins translocate to the ER and into the cell nucleus. Phosphatases involved in dephosphorylation of lipin-1 are CTDNEP1 and NEP1-R1, and potentially the above-mentioned pP-1c (S. Han et al., 2012; Kim et al., 2007; Wu et al., 2011). The *Drosophila* homolog of CTDNEP1, Dullard, genetically interacts with *Lipin* in wing vein formation (Liu et al., 2011). However, it yet has to be shown that Dullard directly contributes to the dephosphorylation of Lipin.

Flies expressing reduced amounts of Lipin exhibit increased sensitivity to starvation (Ugrankar et al., 2011). A similar increase is observed in flies generated by CRISPR/Cas9 mutagenesis that entirely rely on Lipin lacking the nuclear translocation signal (NLS) located between the conserved NLIP and CLIP domains (Fig. 3). The $Lipin^{\Delta NLS}$ flies have altered gene expression profiles in both the starved and fed state, which correlate with decreased survival during starvation and, notably, increased survival in the fed state (S.E. Hood, X.V. Kofler, Q. Chen, J. Scott, J. Ortega and M.L., manuscript in preparation). Genes showing changed expression in $Lipin^{\Delta NLS}$ flies are involved in energy metabolism, feeding behavior, and the immune response. Specifically, in the starved state, genes involved in lipolysis and carbohydrate breakdown are upregulated, suggesting that the flies exhaust energy reserves more rapidly than control flies. In the fed state, on the other hand, the changes in gene expression suggest a shift from fatty acid β-oxidation to lipogenesis, which would predict reduced energy production in $Lipin^{\Delta NLS}$ flies. Indeed, in support of this hypothesis, $Lipin^{\Delta NLS}$ flies exhibit a lowered metabolic rate. In the mouse, the genomic response to lipin-1 overexpression in the liver points to a similar involvement of the protein in the control of lipid and energy metabolism (Finck et al., 2006). Thus, the genomic responses to lipin are at least partially conserved between mammals and insects.

The CLIP domain harbors a transcriptional co-regulator motif and studies in mice and yeasts suggest that nuclear lipins indeed function as transcriptional co-regulators (Finck et al., 2006; Santos-Rosa et al., 2005). However, the corresponding mechanism of action has yet to be confirmed for *Drosophila* Lipin or the Lipin protein of another insect. Interestingly, mouse lipin-1 can alter nuclear localization of SREBP1, a key transcription factor in the activation of lipogenic genes (Kunte et al., 2006; Porstmann et al., 2008). This implies that a subset of lipin-1-responsive genes is controlled through this indirect mechanism (Peterson et al., 2011). However, it is unknown if *Drosophila* Lipin has a similar effect on SREBP1 localization.

In both mice and *Drosophila,* tissues that lack lipin exhibit insulin resistance, which leads to an increase in hemolymph sugar levels in fruit fly larvae and makes *Lpin1* mutant mice more susceptible to atherosclerosis (Reue et al., 2000; Schmitt et al., 2015). Genetic mosaic studies in *Drosophila* have shown that insulin pathway activity is cell autonomously downregulated in fat body cells that lack the PAP activity provided by Lipin. A nuclear function of Lipin does not seem to be required for generating insulin sensitivity, suggesting that it is the change in the intracellular concentration of a metabolite or metabolites in the wake of PAP reduction that causes insulin resistance (Schmitt et al., 2015). Candidates for this function are ceramides, which are elevated in cultured myotubes after *Lpin1* knockdown. The increase in insulin resistance that is observed in these cells after *Lpin1* knockdown is ameliorated by blocking ceramide synthesis (Huang et al., 2017). It will be important to determine in more detail in future studies the mechanism by which *Drosophila* Lipin influences insulin sensitivity and how it exerts its effects on gene expression.

Lipin is a broadly expressed gene in *Drosophila,* suggesting that it functions not only in fat body development and fat storage. A function in adult development is suggested by the occasional occurrence of notched wings in the few adult flies that the hypomorphic $Lipin^{e00680}$ mutant produces (Ugrankar, 2011). Indeed, recent studies have shown that expression of *Lipin* in the wing imaginal disc is required for normal wing development, apparently through a requirement for disc patterning by morphogenetic BMP signaling (Duy Binh et al., 2019; Liu et al., 2011; Raftery and Umulis, 2012). It will be interesting to further dissect the mechanism through which *Lipin* interacts with signaling pathways during wing development. More generally, it will be important to address in future studies whether lipins, similar to the PLDs, play roles in signaling processes and, if so, how. In a first step in this direction, it has been shown that mouse lipin-1 is required for activation of the protein kinase D (PKD)-Vps34 phosphatidylinositol 3-kinase signaling pathway that controls autolysosome formation during autophagy in mouse skeletal muscle (P. Zhang et al., 2014). This is the first example showing that a lipin can produce DAG that acts as a signaling molecule. While the source of the PA used in this pathway remains unknown, one obvious candidate is the breakdown of PC by PLD.

3. Lipid phosphate phosphatases (LPPs)

The cloning of the first cDNA encoding a vertebrate LPP from mouse kidney was reported in 1996. This was shortly followed by a report describing the *Drosophila* Wunen protein and its homology to the vertebrate LPP (Kai et al., 1996; N. Zhang et al., 1997). In addition to *wunen (wun),* the *Drosophila* genome harbors six genes that encode proteins with homology to vertebrate LPPs. All contain six putative transmembrane domains and three other domains that are characteristic of LPPs (Garcia-Murillas et al., 2006). While four of the LPP homologs are as yet uncharacterized, three of them, Wun, Wunen 2 (Wun2), and Lazaro (Laza), have undergone some functional characterization (Burnett and Howard, 2003; N. Zhang et al., 1997; 1996). In contrast, the mammalian genome encodes only three LPPs, LPP1, LPP2, and LPP3. Therefore, it appears that LPPs have diversified to a larger extent in the invertebrate lineage compared to vertebrates.

Like lipins, LPPs can dephosphorylate PA to generate DAG. However, LPPs have a broader substrate specificity that includes other lipid phosphates such as LPA, sphingosine 1-phosphate (S1P), ceramide 1-phosphate, and diacylglycerol pyrophosphate (Brindley and Pilquil, 2009). Therefore, identification of the specific substrate(s) and product(s) involved is a crucial, but challenging task when studying the biological roles of LPPs. In contrast to lipins, which are soluble proteins, LPPs are integral membrane proteins that are anchored in membranes through six transmembrane domains. When located in the plasma membrane of the cell, they exhibit extracellular catalytic activity that contributes to cell-cell signaling. LPPs are also found in the membranes of the ER and Golgi apparatus where catalytic activity is presumably directed toward the lumen (Tang et al., 2015). While preferred extracellular substrates of mammalian LPPs are LPA and S1P, PA is being discussed as a major substrate of intracellular activities of LPPs (Tang et al., 2015). For instance, LPP3 present in the Golgi apparatus of HeLa cells dephosphorylates de novo synthesized PA to generate DAG that is required for retrograde vesicle trafficking from the Golgi to the ER. Reduction of LPP3 disrupts this process (Gutiérrez-Martínez et al., 2013).

The Wun and Wun2 proteins of *Drosophila* are most similar to mammalian LPP3. That LPP3 represents the Wun/Wun2 ortholog in mammals is supported by the observation that LPP3, but not LPP1, shows the same biological activity in *Drosophila* as Wun (Burnett and Howard, 2003). Both Wun and Wun2 play an essential role in germ cell migration during *Drosophila* embryogenesis. Expression in the cells of somatic tissues repels migrating primordial germ cells that are homing in on the developing gonads during embryogenesis. Loss of *wun* and *wun2* results in a scattered distribution of the germ cells, which fail to reach their gonadal target tissue (Renault and Lehmann, 2006). It is believed that the catalytic activity of Wun/Wun2 degrades an extracellular phospholipid and, thus, contributes to formation of a chemotactic gradient that guides germ cell migration (LeBlanc and Lehmann, 2017). Biochemical assays show that, in vitro, a preferred substrate of Wun is LPA, whereas activity toward PA is negligible. However, although human LPP3 exhibits the same biological activity in germ cell repulsion as Wun when expressed in *Drosophila,* it metabolizes LPA only very poorly. Moreover, mouse LPP1, which dephosphorylates LPA very efficiently, is ineffective in *Drosophila.* These data strongly suggest that LPA may not be the in vivo target of Wun, but another, yet to be identified substrate (Burnett and Howard, 2003). This conclusion is further supported by the apparent absence of homologs of the vertebrate LPA and S1P receptors in *Drosophila* (Ile et al., 2012).

Interestingly, *wun2* is not only required in somatic tissues but also expressed in the germ cells themselves and required for their survival. Since overexpression of either *wun* or *wun2* in somatic tissues causes germ cell death, it is believed that germ cell survival requires uptake by germ cells of a lipid that Wun2 generates by breakdown of the phospholipid that serves as the chemotactic agent (Hanyu-Nakamura et al., 2004; Renault et al., 2004; Slaidina and Lehmann, 2017). It will be interesting to see if it is possible to visualize gradient formation and to elucidate the molecular nature of the gradient-forming phospholipid. In this context, it is interesting to note that, in a *Drosophila* model of muscular dystrophy, increased levels of S1P can suppress the dystrophic muscle phenotype. Reducing expression of *wun* has the same effect, suggesting that S1P is the biologically active target of Wun, at least in this system (Pantoja et al., 2013). During normal muscle development, Wun is required for

cell migration that leads to proper formation of longitudinal muscles that ensheathe the gut (Stepanik et al., 2016). Wunens are not only required for muscle development and germ cell migration, but also for normal development of the tracheae and the heart. Analysis of tracheal defects in wunen mutants indicated a requirement of *wun,* but not *wun2,* for proper functioning of septate junctions between tracheal cells leading to defects in the integrity of the tracheal lumen (Ile et al., 2012). The mechanistic basis for heart defects in *wun* and *wun2* mutants is less well understood. *Wun/wun2* mutant embryos exhibit a 'broken-hearted' phenotype in which cardioblasts fail to properly associate with pericardial cells and bilaterally positioned cardioblasts fail to move medially leading to breaks in the cardiac lumen. Wunens are expressed in both pericardial cells and the overlying ectoderm and mutant phenotypes can only be fully rescued if *wun* function is restored in both tissues (Haack et al., 2014). The implication of wunens with not only germ cell migration, but also tracheal, muscle, and heart development indicates that these lipid phosphatases have broad functions in *Drosophila.* This conclusion is also supported by the broad expression patterns of wunens in larval and adult tissues (Flybase, version FB2020_03, released June 16, 2020). It will be interesting to further dissect these functions and to explore whether wunens also function intracellularly and in PA dephosphorylation as their mammalian LPP3 counterpart.

A third *Drosophila* LPP that has been functionally characterized is Laza (Garcia-Murillas et al., 2006; Kwon and Montell, 2006; Kwon et al., 2008). Laza is required for phototransduction in the *Drosophila* complex eye and the regulation of thermotactic behavior. In the eye, overexpression of *laza* enhances the retinal degeneration phenotype of mutations in the DGK-encoding *rdgA* gene and loss of *laza* suppresses the phenotype (Garcia-Murillas et al., 2006). Before degeneration sets in, mutations in *laza* also attenuate and shorten the light response (Kwon and Montell, 2006). Retinal PA levels are reduced in $rdgA^{3}$ flies and this reduction is strongly enhanced when LPP activity is increased. Reduction of *laza* also suppresses retinal degeneration induced by overexpression of PLD, supporting a model in which PLD-produced PA feeds into DAG production that causes degeneration (Kwon and Montell, 2006). Together, the data support a model in which Laza specifically metabolizes PA and cooperates with the *rdgA*-encoded DGK and PLD to adjust PA levels during phototransduction (Garcia-Murillas et al., 2006). It is currently unclear how PA influences photoreceptor function mechanistically. Phototransduction is accomplished by the opening of TRP Ca^{2+} channels that is regulated by a phospholipase C (PLC)-controlled pathway (Hardie and Juusola, 2015). PA does not seem to directly alter activity of the TRP Ca^{2+} channels. It has been proposed that, instead, altered PA levels act through an effect on the production of phosphatidylinositol, which is needed for feeding substrates into the PLC-controlled pathway that controls the TRP channels (Garcia-Murillas et al., 2006). TRP Ca^{2+}channels acting downstream of a PLC pathway also control thermotactic behavior that responds to mutations in *laza.* Thus, Laza seems to have a more general role in controlling TRP channel activity (Kwon et al., 2008).

4. Conclusion and outlook

PA phosphatases have been primarily studied in mammalian systems and much remains to be learned about the functions of these enzymes in insects, in particular in non-drosophilids. There are still many questions that can be addressed using *Drosophila* by taking advantage

of the genetic toolbox available for this model organism. To what extent are Lipin and the wunens integrated into signaling pathways in which PA or DAG function as ligands of downstream effectors? What are the tissue-specific functions of these PA phosphatases in the larval and adult stages? *Lipin* is, for instance, strongly expressed in the endocrine ring gland and in Malpighian tubules. Are there specific requirements for the presence of Lipin in hormone production or in excretion? Similarly, *wun* is strongly expressed in adult salivary glands and the larval and adult digestive tract. *Wun2* shows relatively high expression in the ovary compared to other tissues. What are the roles that these two LPPs have in these tissues? A long-standing open question is the molecular nature of the phospholipid or -lipids that are the substrates of the exo-enzymatic activity of Wun and Wun2. Experimental evidence is needed to support the model in which uptake of a Wun2 product by primordial germ cells supports germ cell survival. What is this product and how does it prevent germ cell death? There are major open questions regarding the mechanism by which nuclear Lipin exerts its effects on gene expression. So far, it has not been shown that Lipin can bind to specific loci on fat body polytene chromosomes or that it can be located on chromosomal loci by ChIP assays. Which role, if any, does the PAP activity play in nuclear functions of Lipin? Finally, what are the functions of the four LPPs encoded in the *Drosophila* genome that have so far remained uncharacterized? It will be exciting to see these, and other questions answered in the coming months and years.

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Abbreviations:

AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DGk	diacylglycerol kinase
GPAT	glycerol-3-phosphate acyltransferase
Laza	Lazaro
LPA	lysophosphatidic acid
LPP	lipid phosphate phosphatase
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PLC	phospholipase C
PLD	phospholipase D
PI	phosphatidylinositol
PS	phosphatidylserine
S1P	sphingosine 1-phosphate
TAG	triacylglycerol
Wun	Wunen
Wun2	Wunen 2

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Highlights

- Phosphatidic acid phosphatases convert phosphatidic acid into diacylglycerol
- Two classes: Mg^{2+}-dependent lipins and Mg^{2+}-independent lipid phosphate phosphatases (LPPs)
- Lipins have essential cytoplasmic and nuclear functions in energy homeostasis
- LPPs are transmembrane proteins with broader substrate specificity
- Insect LPPs function in development, germ cell migration, and phototransduction

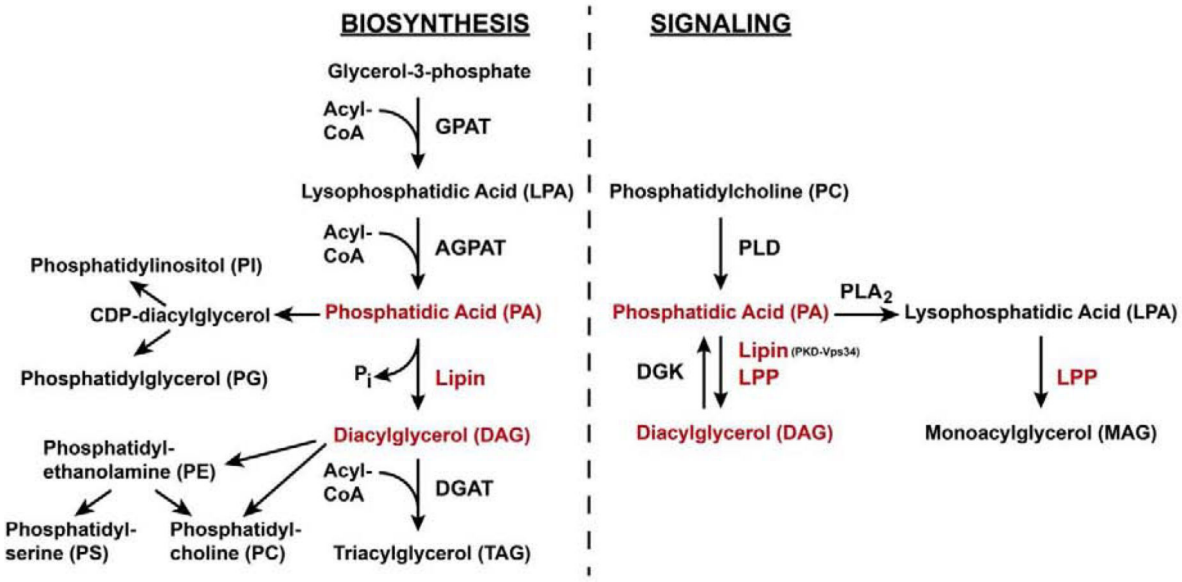

Figure 1.

Integration of phosphatidic acid and phosphatidic acid phosphatases (Lipin and LPP) into biosynthetic and signaling pathways. Substrates and products of PA phosphatases and the enzymes themselves are highlighted in red.

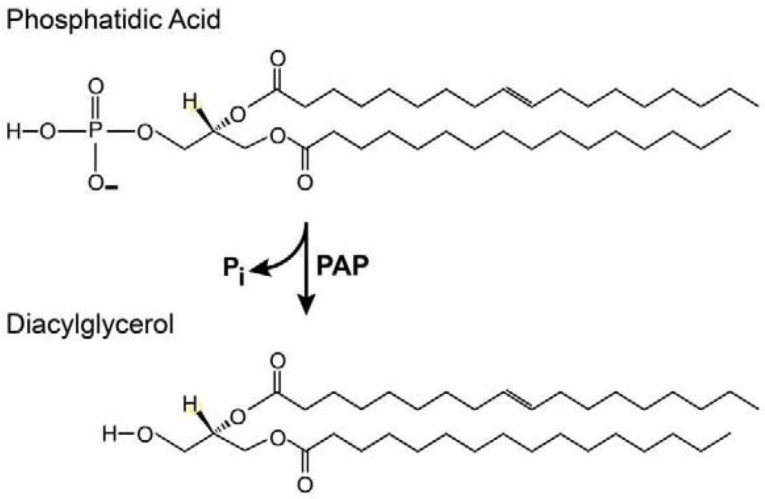

Figure 2.

Reaction catalyzed by PAP enzymes. The general structure of phosphatidic acids and diacylglycerols is shown. Different species of these glycerolipids exist in cells that are specified by their fatty acid side chains.

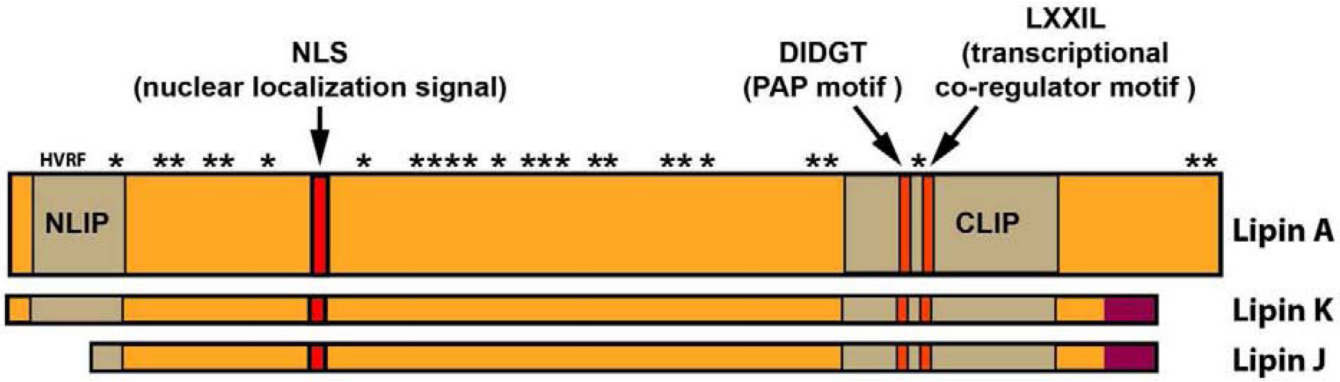

Figure 3.

Structure of the *Drosophila* Lipin protein. Lipin A is the most widely expressed isoform, whereas Lipin K dominates in the central nervous system and Lipin J is specific to the male gonads (Valente et al., 2010). The N-terminal and C-terminal NLIP and CLIP domains are highly conserved between lipins from yeasts, plants, and animals (Peterfy et al., 2001). CLIP harbors two conserved sequence motifs characteristic of PA phosphatases and transcriptional co-regulators. The asterisks indicate serine and threonine phosphorylation sites identified by mass spectrometry (Bodenmiller et al., 2008; Bridon et al., 2012). The HVRF motif is a recognition site of protein phosphatase PP-1c (Kok et al., 2014).