Mammalian Triacylglycerol Metabolism: Synthesis, Lipolysis and Signaling

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1.0. Introduction

Triacylglycerol (TAG) is the primary unit of energy storage in eukaryotic cells. TAG comprises more than 90% of the content of white adipocytes, and TAG synthesis is essential in enterocytes for the absorption of dietary lipids, in hepatocytes for the synthesis and transport of VLDL, and in mammary epithelial cells for the production of milk. TAG is critical for the formation of the water barrier of skin, it acts as a mechanical cushion within joints and around internal organs, and it also serves an important role in insulation. In virtually every type of cell, stored TAG sequesters essential fatty acids (FAs) and precursors of eicosanoids, as well as the DAG precursor of phospholipids. Storage of FAs in TAG protects cells from the potential detergent-like properties of FAs or their acyl-CoA derivatives which may injure cellular membranes. In addition, recent studies suggest that cellular stores of TAG in lipid droplets release FAs that are channeled selectively to β-oxidation and that act as signals to influence the transcriptional control of gene expression. Additional studies suggest that the process of synthesizing or degrading TAG produces lipid intermediates like lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG) that may serve as activators or inhibitors of signaling pathways controlled by peroxisome proliferator-activated receptor-γ (PPARγ), the mammalian target of rapamycin (mTOR), or protein kinase C (PKC) isoforms. These signaling pathways may link excess intracellular TAG storage with insulin resistance.

It is the goal of this review to provide an overview of TAG metabolism as a dynamic process that allows its lipid participants to play numerous inter-related roles within cells. Thus, we will focus on both the synthesis and degradation of TAG, the enzymes involved and their regulation, and the overall physiological regulation of these dynamic processes. In addition to the lipolytic enzymes, this review will also highlight co-regulatory proteins that affect the activity of specific lipases.

We have chosen to focus primarily on information reported during the past 10 years and recommend to readers several excellent reviews that present more detailed information from older studies: for the synthetic pathway as a whole,[1] for GPAT,[2] for GPAT, AGPAT and PAP,[3–4] for DGAT,[5] for the lipolytic pathway and ATGL,[6] for HSL,[7] and for MGL.[8]

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2.0. Enzymes of triacylglycerol metabolism

2.1. Enzymes of triacylglycerol synthesis

The three pathways of TAG synthesis begin with the acylation of glycerol-3-phosphate in mitochondria or the endoplasmic reticulum (ER), the acylation of dihydroxyacetone phosphate in peroxisomes, or the acylation of *sn*-2-monoacylglycerol in the ER[9–10] (Figures 1, 2). We will focus on the glycerol-3-phosphate pathway, the major pathway for TAG synthesis in most mammalian cells apart from enterocytes.

The biosynthesis of TAG begins with the sequential acylation of glycerol-3-phosphate by long-chain acyl-CoA thioesters. The initial step is catalyzed by *sn*-1-glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) to form LPA. A second acylation at the *sn*-2 position of LPA by acyl-CoA:1-acylglycerol-3-phosphate acyltransferase (AGPAT [also called LPA acyltransferase, LPAAT]) produces PA. As the precursor of CDP-diacylglycerol, PA lies at the branch-point of TAG synthesis and the synthesis of phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). PA is hydrolyzed by PA phosphatase (also called lipin) to form DAG. DAG also lies at a branch-point between TAG synthesis and the synthesis of the major phospholipids in mammalian cells, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The final esterification step by DAG acyltransferase (DGAT) produces TAG.

Kennedy and his colleagues identified the enzymes of the TAG pathway in the 1950s and 60s.[11–12] Pullman and his colleagues distinguished two GPATs with different subcellular locations and properties,[13–14] (summarized in [1,15]), and Brindley and his colleagues distinguished two different PAPs, one of which was important for TAG synthesis and the other for signaling.[16] Of all these steps in the pathway, only the mitochondrial GPAT was purified to homogeneity.[17] Only descriptive studies were performed until *Gpat1*, the first of the synthetic enzymes, was cloned.[18] Completion of the human genome project allowed related acyltransferase homologs to be identified, and it became apparent that multiple proteins were involved for each step. Although many genes involved in TAG synthesis have been identified, studies of knockout mice suggest that additional acyltransferase isoforms remain to be discovered.

For most synthetic pathways each step is catalyzed by a single enzyme unless specific regulation is required. Yet, in the glycerol-3-phosphate pathway of TAG synthesis, investigators have confirmed 4 independent GPAT isoforms, 3 AGPAT isoforms, 3 PAP isoforms, and 2 DGAT isoforms. In tissues that have a major function in synthesizing TAG, each of these isoforms is present, albeit in differing amounts. Studies in which deficiencies in a single isoform have been created show that the enzymes are non-redundant, and the ability to manipulate their expression independently has altered our conceptualization of the synthetic pathway. For example, the initial step catalyzed by GPAT was believed to be rate-limiting, but overexpression of one of the 4 isoforms, GPAT4, does not invariably increase FA incorporation into TAG.[19–20] Also, although the final step catalyzed by DGAT was thought to occur on the ER, it now appears that under some conditions, DGAT2 becomes closely associated with lipid droplets.[21] And finally, new information suggests that LPA, PA and DAG formed during TAG synthesis can initiate signaling pathways that were previously thought to be formed exclusively from the phospholipase-mediated hydrolysis of membrane phospholipids.

2.2. Enzymes of triacylglycerol lipolysis

Lipolytic activity was first measured in adipose tissue in the 1930's.[22] Although lipolytic activity was characterized in numerous other tissues, it was not until the 1960's when a lipase, now known as hormone-sensitive lipase (HSL), was identified that responded to β-

adrenergic signals (Figure 2).[23] Shortly thereafter, a second lipolytic enzyme, now known as monoglyceride lipase (MGL), which catalyzes the hydrolysis of monoacylglycerol (MAG), was discovered.[24] Until the early 2000s, most subsequent research on lipolysis focused on HSL, which was believed to catalyze the first two steps of TAG hydrolysis and produce a 2-MAG and 2 FAs. However, the discovery of ATGL in 2004 shifted our basic understanding of lipolysis. ATGL is now recognized as the principal TAG lipase in many tissues, whereas HSL catalyzes the hydrolysis of DAG in the second step of the lipolytic pathway. Although ATGL, HSL and MGL are the principal enzymes controlling lipolysis in most tissues, additional lipases also contribute to this pathway. These enzymes, which include PNPLA3 and TGH, are not as well characterized and may have tissue-specific roles in TAG hydrolysis.

3.0. Glycerol-3-phosphate acyltransferases

3.1. Overview and structure

The synthesis of TAG is initiated by the acylation of glycerol-3-phosphate by one of several independent GPAT isoforms (for details about older studies, see [1,25]). Historically, 2 GPAT isoforms had been postulated, based on differences in location, substrate preference, and sensitivity to sulfhydryl group modifiers like *N*-ethylmaleimide and iodoacetamide. By 2010, four GPAT isoforms had been identified, each the product of an independent gene, and at least one additional GPAT isoform seems likely to exist.[26]

GPAT1-4 are intrinsic membrane proteins that have two predicted transmembrane domains and active sites that face the cytosol.[27–28] From a detailed analysis of the GPAT from *E. coli* [29–30] four active site motifs were delineated. These motifs comprise the PlsC domain (In *Escherichia coli* AGPAT is encoded by the PlsC gene, or phospholipid synthesis gene C.), and identify a large super-family of phospholipid acyltransferases and a more distantly related family termed membrane bound O-acyltransferases (MBOAT).[31–33] The four known GPAT isoforms have similar active site regions (motifs I–IV), and GPAT3 and 4 have a fifth region (motif V) that was identified by protein homologies[34] and mutations in the acyltransferases that underlie the human disorders, Barth Syndrome[35] and AGPAT2-related congenital lipodystrophy.[36–37] (Table 1). The mechanism of action of the GPAT family members is believed to involve the histidine in motif I as a general base that deprotonates the hydroxyl moiety of the acyl acceptor. The arginine in motif II probably binds to the phosphate group of glycerol-3-phosphate or the comparable phosphate in a lysoglycerophospholipid.[29–30] Although GPAT1 was purified to homogeneity,[17] no mammalian GPAT has been crystallized. A related GPAT isoform from squash (*Cucurbita moschata*) has been crystallized,[38] and provides some information, but unlike the mammalian GPAT isoforms, this chloroplast GPAT is a soluble protein. Nevertheless, at 1.9 A resolution, the protein was determined to have two domains, one of which has a large cleft lined by hydrophobic residues and a cluster of positively charged residues flanked by motif I (H(X)(4)D). The authors predicted that the hydrophobic residues would bind the acyl-CoA chain, that the positively charged residues would bind the phosphate moiety of the glycerol 3-phosphate, and that motif I functions in catalysis.[39] Unlike mammalian GPATs which use an acyl-CoA, the squash plastidial GPAT uses an acyl-AMP; crystallization shows that acyl-AMP binds before glycerol-3-phosphate and that the enzyme can use short (4:0)- and medium-chain (6:0, 12:0) acyl-CoAs.[40] It is not known whether this catalytic mechanism bears any relevance to that of the mammalian enzymes.

3.2. Location and structure of *sn*-glycerol-3-phosphate acyltransferases

GPAT1 is located on the outer mitochondrial membrane and is enriched in ER-mitochondrial membrane contact sites.[41] In rat liver, most GPAT1 protein but little activity,

is present in vesicular membrane fractions associated with mitochondria, whereas the highest GPAT1 specific activity is present in purified mitochondria. Beyond their locations in the mitochondrial membrane (GPAT2) and ER membrane (GPAT3 and 4) little else is known about the location or topography of GPAT2, 3, or 4.

The topography of GPAT1 has been studied in some detail. Several in silico programs predicted that two transmembrane domains followed the active site motifs, and protease treatment of rat liver mitochondria together with immunolocalization of C and N termini epitope tags confirmed this configuration, with both N (aa 1–471) and C (aa 593-end) termini facing the cytosol and a single loop facing the intermembrane space.[28] Despite the fact that all the catalytic site motifs precede the first transmembrane domain, truncations at this domain are inactive. Further, GPAT1 with sequential truncations of the C-terminal domain are also inactive.[42] Chemical cross-linking and protein cleavage studies demonstrated that the N- and C-termini of GPAT1 interact. Although the 792 amino acid length of GPAT2 is similar to that of GPAT1 (828 amino acids), and GPAT2 also has a long C-terminal region, its predicted transmembrane domains flank the catalytic domains and would not allow interaction between the active site and the C-terminal domain (Figure 3). GPAT3 and 4 are only 432 and 451 amino acids in length, respectively, and both lack the extended C-terminal region (Figure 3). Thus, although the mechanism of catalysis is likely to be similar for all four GPAT isoforms, their structural dissimilarity predicts important differences in protein regulation.

3.3. sn-Glycerol-3-phosphate acyltransferase-1 (GPAT1)

GPAT1 was cloned from mouse[43] and rat[44] liver, and is the previously recognized "mitochondrial" isoform that is known to have a strong preference for saturated fatty acyl-CoAs and resistance to inactivation by sulfhydryl reagents.[1] GPAT1 contributes 30–50% of total GPAT activity in liver, but only 10% of GPAT activity in most other tissues examined, although the mRNA abundance and specific activities of GPAT1 are similar in liver and white adipose (A.A. Wendel and R.A. Coleman, unpublished). Despite its location on the outer mitochondrial membrane and the fact that the terminal enzymes of TAG synthesis are associated with the ER, GPAT1 expression and activity are positively regulated under conditions that increase TAG synthesis via SREBP1c-responsive sequences in the *Gpat1* promoter.[45]

Stable overexpression of GPAT1 in CHO cells results in increased TAG content and increased incorporation of labeled FA into TAG.[46] Adenovirus-mediated overexpression of GPAT1 in rat liver causes marked hepatic steatosis within 5 to 7 days.[47–48]

Studies of *Gpat1* knockout mice have confirmed the role of GPAT1 in TAG synthesis. Female *Gpat1*^{−/−} mice weigh less than controls and have smaller gonadal fat pads.[49] A high throughput phenotypic screen that used dual-energy X-ray absorptiometry technology to estimate body-fat stores confirmed the modestly lean phenotype.[50] An independent *Gpat1*^{−/−} model confirmed the reduction in female body weight-gain, adiposity, liver TAG content, plasma cholesterol and TAG levels and the increase in *ex vivo* liver FA oxidation and plasma ketone bodies compared with littermate wildtype controls.[51]

GPAT1 plays a major role in regulating hepatic TAG content, which is significantly lower in *Gpat1*^{−/−} mice than controls under a variety of conditions, including both low and high fat diets.[52] Even with the marked hepatic steatosis of leptin deficient *ob/ob* mice, adenovirus-mediated shRNA knockdown of GPAT1 largely normalizes the hepatic TAG and DAG content.[53] Although the effect on hepatic lipids appeared to be beneficial in this study, in mice with a double knockout of leptin and GPAT1 (*ob/ob-Gpat1*^{−/−}), near normalization of hepatic TAG and DAG did not improve insulin resistance.[54] The latter study, however,

confirmed the potent effect of GPAT1 on hepatic steatosis in this model. In $Gpat1^{-/-}$ mice, the decreased hepatic TAG content is also associated with a minor decrease in plasma TAG concentrations.[49] Lack of GPAT1 also protects the heart from TAG accumulation caused by high fat or high sucrose diets.[26]

Unlike other GPAT isoforms, GPAT1 has a preference for saturated FAs, particularly 16:0.[1] Thus, it is not surprising that the absence of GPAT1 decreases the amount of 16:0 in glycerolipids. Compared to controls, in $Gpat1^{-/-}$ liver, the 16:0 content is lower in PC and PE, particularly at the *sn*-1 position.[49] This alteration results in about 40% more 20:4ω6 at the *sn*-2 position of PC and PE. Similar changes are present in purified mitochondria from $Gpat1^{-/-}$ liver, compared to wildtype controls,[55] and in heart, $Gpat1^{-/-}$ mice contain 25–45% less 16:0 and more 20:4ω6 in PC, PE, and PS/PI.[26] Compared to wildtype mice, PC and PE from $Gpat1^{-/-}$ hearts also contain higher amounts of 18:0 and 18:1. Although increases in 24:4ω6 provide more potential substrate for eicosanoid synthesis, no studies have investigated the effect of these changes on liver or cardiac function.

Because GPAT1 is on the mitochondrial outer membrane, it was hypothesized that it might compete with carnitine palmitoyl transferase-1 (CPT1) for acyl-CoAs. In confirmation, mice with absent GPAT1 show increased partitioning of FA away from TAG synthesis and towards oxidation when fed a high-fat, high-sucrose diet for 4 months.[55] Despite developing obesity, livers from $Gpat1^{-/-}$ mice had a 60% lower TAG content and increased *HMG-CoA synthase-2* mRNA and acyl-CoA content regardless of being fed a chow or high fat diet. These mice also exhibited 2-fold increases in plasma β-hydroxybutyrate and acylcarnitines, consistent with elevated rates of FA oxidation.[55] It appeared that acyl-CoA content had increased because of an impaired ability to use long-chain acyl-CoAs derived from the diet, even when the dietary fat content was low. Adenovirus-mediated over-expression of GPAT1 in primary cultures of rat hepatocytes confirmed this hypothesis.[48] With a greater than 4-fold increase in GPAT activity, FA oxidation diminishes 80%, together with a 2-fold increase in hepatic diacylglycerol; when 300 μM labeled 16:0 or 18:1 is added, incorporation into phospholipid and TAG increases.

Compared to wildtype controls, liver mitochondria from $Gpat1^{-/-}$ mice also show a 20% increase in the rate of production of reactive oxygen species and markedly increased sensitivity to the induction of the mitochondrial permeability transition.[55] 4-Hydroxynonenal, a product of arachidonate peroxidation, is high in $Gpat1^{-/-}$ liver, and is associated with a 3- to 15-fold increase in TUNEL staining, a marker for apoptosis, compared to age-matched controls. Compared to controls, bromodeoxyuridine labeling is 50% and 7-fold higher in livers from young and old $Gpat1^{-/-}$ mice, respectively.[55] These alterations in hepatocyte apoptosis and proliferation suggest that $Gpat1^{-/-}$ mice might be resistant to hepatic carcinogenesis.

3.3.1. GPAT1 and immune function—GPAT1 appears to be involved in the inflammatory response, although the mechanism is unknown. Compared to control C57BL/6 mice, $Gpat1^{-/-}$ mice infected with coxsackievirus B3 have higher mortality, increased viral titers in the liver and heart, and a 50% increase in heart inflammatory infiltrate, as well as increased heart mRNA abundance of proinflammatory cytokines like tumor necrosis factor-α, interleukin (IL)-6, and IL-1β.[56] The response of $Gpat1^{-/-}$ splenic T cells to coxsackievirus B3 antigen is defective, and lack of GPAT1 activity appears to affect both innate and adaptive immune mechanisms. GPAT1 may also regulate T-lymphocyte proliferation. Reduced GPAT1 activity correlates with decreased splenic T-lymphocyte proliferation in aged rats[57], and reduced T-lymphocyte proliferation is observed in young $Gpat1^{-/-}$ mice[58]. Because the membrane lipid composition of the T-lymphocytes contains high amounts of 20:4ω6, similar to that measured in $Gpat1^{-/-}$ liver[49], the authors speculated

that changes in membrane composition underlie the T-lymphocyte proliferation rates. In addition, the authors observed 35% and 85% increases in prostaglandin E2 and leukotriene 4, respectively, in response to CD3/CD28 stimulation. [59] It has not yet been shown whether the changes in immune function and T-cell proliferation in *Gpat1* $^{-/-}$ mice are directly caused by lack of GPAT1 activity or, instead, are related to the altered membrane FA composition that results from the lack of GPAT1.

3.4. Regulation of GPAT1

Gpat1 mRNA decreases in adipose and liver with fasting and increases with refeeding, consistent with its regulation by SREBP-1c.[60] Comparison of *Gpat1* mRNA, GPAT1 protein and NEM-sensitive GPAT specific activity shows a general lack of coordination.[61] For example in rat tissues, the heart expresses the highest amount of mitochondrial GPAT protein, but low mitochondrial GPAT specific activity, whereas liver and adipose have the highest mitochondrial GPAT activity, but very low protein expression. These discrepancies suggest that GPAT1 is regulated post-transcriptionally. The fasting-induced decrease in liver and adipose GPAT1 activity and protein, and the overshoot of GPAT activity and protein after refeeding may result from both transcriptional and post-transcriptional regulation.[61]

Given its role in modulating acyl-CoA entry into the mitochondrial oxidation pathway, it is not surprising that GPAT1 also contributes to exercise-induced changes in energy metabolism. GPAT1 activity decreases 50% in rat muscle, adipose, and liver after 30 minutes of treadmill running.[62] The changes in liver and adipose were attributed to the effect of AMP-activated kinase (AMPK).[63] AMPK decreases GPAT1 specific activity in liver, although it has not been established that GPAT1 itself is directly phosphorylated.[64] GPAT1 is phosphorylated by casein kinase II,[58,65–66] and insulin stimulation of GPAT1 activity in rat adipocytes is accompanied by phosphorylation of the GPAT1 casein kinase sites, ser 632 and ser 639.[67] The physiological significance of these modifications is unknown.

To determine whether obesity increases hepatic GPAT1 activity, two models of rodent obesity were examined and shown to have a greater than 2-fold increase in liver GPAT activity.[48] Overall, these results support the concept that increased hepatic GPAT1 activity associated with obesity positively contributes to lipid disorders by reducing oxidative processes and promoting de novo glycerolipid synthesis.

3.5. *sn*-Glycerol-3-phosphate acyltransferase-2 (GPAT2)

The discovery in *Gpat1* $^{-/-}$ mice of a protein that was recognized by an antibody raised to full-length GPAT1, led to the discovery of GPAT2, a second mitochondrial isoform that is inhibited by NEM and by dihydroxyacetone phosphate.[68] Unlike GPAT1, GPAT2 has no substrate preference. GPAT2, cloned from mouse testis[69] and kidney,[70] is a 795 amino acid protein with 32% identity and 72% similarity to mouse GPAT1 and an estimated molecular mass of 89 kDa. Although expressed in many tissues, GPAT2 mRNA is 50-fold more abundant in testis.[69] Like GPAT1,[46] expression of GPAT2 in Cos-7 cells increases trace [1-^{14}C]oleate incorporation into [^{14}C]TAG, but not phospholipids, but in contrast to *Gpat1*, *Gpat2* mRNA abundance in liver is not altered by fasting or refeeding.[69] GPAT2 is likely to have a specialized function in testis that has yet to be discovered.

3.6. *sn*-Glycerol-3-phosphate acyltransferase-3 and -4 (GPAT3 and 4)

The first ER-associated GPAT to be cloned was GPAT3 (originally called AGPAT8).[71] This isoform is highly expressed in mouse adipose, small intestine, and heart, all tissues with high rates of TAG biosynthesis, and in human kidney, heart, thyroid and skeletal muscle.[71] Prediction programs suggest that GPAT3 has two transmembrane domains that precede the

active site motif region (Figure 3). *Gpat3* expressed in insect cells shows highest activity over background GPAT activity when assayed with 12:0-CoA. The activity is inhibited by NEM, and no activity is observed with other substrates, including lysophospholipids, monoacylglycerol and DAG.[71] Overexpressing *Gpat3* in HEK293 cells increases the formation of TAG, but not phospholipid.[71] GPAT3 is reported to have either no[71] or high[72] LPAAT activity, a discrepancy that requires further investigation. It is now apparent that most of the 70-fold increase in NEM-sensitive GPAT activity in differentiating 3T3-L1 adipocytes,[73] is due to the 60-fold induction of *Gpat3*.[31] *Gpat3* mRNA is also up-regulated in white adipose from *ob/ob* mice treated for 21 days with a PPARγ agonist. With a GPAT3 siRNA knockdown, total GPAT activity in 3T3-L1 adipocytes decreases 60%.[31]

A second ER GPAT, *Gpat4* (originally called AGPAT6) is highly expressed in brown and white adipose tissues, testes, liver and heart.[19,34,74] This enzyme does not have AGPAT activity.[19–20,34,74] GPAT4 is approximately 80% identical to GPAT3, with similar placement of its putative transmembrane domains and active site motifs (Figure 3). At least 50% of NEM-sensitive GPAT activity in mouse liver is due to GPAT4 activity.[19] The remaining activity might be due to GPAT3, even though the mRNA abundance in liver of *Gpat3* is extremely low.[74] Compared to *Gpat3, Gpat4* and *Gpat1* are more modestly induced (5-fold and 11-fold, respectively) in 3T3-L1 differentiating adipocytes,[43,73] and shRNA-mediated knockdown of *Gpat4* in adipocytes has little effect on enzyme activity or TAG synthesis.[74] Although these data suggest that GPAT3 provides most of the GPAT activity in fat cells,[19] *Gpat4* mRNA abundance is high in most adipose depots, and *Gpat4*^{−/−} mice have diminished subcutaneous fat depots (see section 3.6.1).[74] This discrepancy remains unexplained.

3.6.1 Mice deficient in GPAT4—*Gpat4*^{−/−} mice were derived from a gene-trap insertion technique.[74] The non-back-crossed mice resist weight gain on a high fat diet. Their subdermal fat depots are absent, and their gonadal and brown adipose fat pads and adipocytes are smaller. The differences in fat pad size are not solely due to absent GPAT4 activity within the tissue, because although NEM-sensitive GPAT activity in brown adipose is 50% lower than in wildtype brown adipose, gonadal NEM-sensitive GPAT activity is identical.[19] The diminished adipose stores occur despite normal food intake, respiratory quotient (RQ), physical activity, and body temperature, but energy expenditure is increased, perhaps because of the lack of insulating subdermal fat. Unlike other mouse and human models of lipodystrophy, *Gpat4*^{−/−} mice do not appear to have insulin resistance or hepatic steatosis. In fact, hepatic TAG content is 50% lower than in wildtype mice, suggesting that GPAT4, like GPAT1, is a significant contributor to hepatic TAG synthesis. When crossed with *ob/ob* mice, the absence of Gpat4 decreases female body weight gain about 20%.[19] Despite very strong expression of GPAT4 in brown adipose from wildtype mice, the *Gpat4*^{−/−} mice are able to maintain normal body temperature for 4 hours when kept at 4°C. Strikingly, lactation is abnormal in *Gpat4*^{−/−} dams, and they are unable to suckle their progeny.[34] *Gpat4* is normally expressed in mammary epithelium, and *Gpat4*^{−/−} dams have undeveloped mammary alveoli and ducts that produce milk containing little TAG or DAG. Therefore, it appears that lack of GPAT4 has its most significant impact on TAG metabolism in adipose tissue, especially subdermal depots, and in the mammary gland.

3.6.2. Regulation of GPAT3 and GPAT4—GPATs 1, 3, and 4 are variably expressed in different layers of the epidermis,[75] and the mRNA expression of both *Gpat1* and *3* increases during fetal rat epidermal development. Induction of keratinocyte differentiation by calcium increases *Gpat3* mRNA abundance together with a 2-fold increase in NEM-sensitive activity, whereas the mRNA for both *Gpat1* and *4* decrease. In differentiated keratinocytes only GPAT3 mRNA abundance is increased by PPARγ or PPARδ activators via increased transcription.[75] In 3T3-L1 adipocytes, both GPAT3 and GPAT4 are phosphorylated by

insulin at Ser and Thr residues, leading to increased GPAT activity that is sensitive to wortmannin.[31]

3.7. Evidence for additional GPATs and for TAG synthesis via dihydroxyacetone phosphate acyltransferase

Because mitochondria purified from *Gpat1* $^{-/-}$ hearts express a residual NEM-resistant GPAT activity, it is suspected that a fifth GPAT exists.[26] This potential GPAT activity could result from an AGPAT isoform that remains uncharacterized or incompletely characterized (Table 2) or from a novel protein. TAG synthesis may also, in theory, be initiated by the peroxisomal enzyme dihydroxyacetone phosphate (DHAP) acyltransferase (EC 2.3.1.42) (Figure 1). DHAP acyltransferase catalyzes the esterification of DHAP at the *sn*-1 position, and a peroxisomal acyl(alkyl)-DHAP oxidoreductase can convert the acyl-DHAP product to LPA which could then leave the peroxisome and enter the ER pathway of TAG synthesis. The specific activity of DHAP acyltransferase increases 9-fold as 3T3-L1 adipocytes differentiate and, in one study in glucose and serum-starved adipocytes, contributed 40–50% of total TAG synthesis.[76]

4.0 *sn*-1-Acyl-glycerol-3-phosphate acyltransferases

sn-1-Acyl-glycerol-3-phosphate acyltransferase (AGPAT; also known as LPAAT) (EC 2.3.1.51), catalyzes the synthesis of PA from acyl-CoA and LPA. AGPAT activity has been reported on both ER and mitochondrial membranes,[77] but it is not known which of the AGPAT isoforms is present in each location. In GenBank, multiple genes that encode proteins with similar structures and that contain PlsC domain motifs[34] have been named *Agpat1-13*, but only AGPAT1, 2, and 3 have been conclusively shown to catalyze the esterification of LPA to form PA. When *Agpat3, 4*, and *5* were cloned from mouse and expressed in Cos-1 cells, AGPAT activity increased only minimally over endogenous activity,[78] and AGPAT4 and 5 have not been further investigated. *Agpat8* and *Agpat6* have been renamed *Gpat3* and *Gpat4*, respectively, and several of the other *Agpat* genes encode proteins that esterify lysophospholipids (Table 2).[33]

4.1. *sn*-1-Acyl-glycerol-3-phosphate acyltransferase-1 (AGPAT1)

Originally termed LPAAT-α, the AGPAT1 gene was cloned by searching for human homologs to yeast and *E. coli* LPA acyltransferases.[79–80] Human *AGPAT1* encodes a 283 amino acid protein that is expressed in most tissues, with highest levels in liver, lung, heart, and pancreas.[79–80] Limited characterization shows that with an *sn*-1-16:0-LPA acceptor, 20:4-, 18:0-, and 14:0-CoAs are better acyl donors than 16:0-CoA.[79] Over-expression of AGPAT1 in 3T3-L1 adipocytes or in C2C12 myotubes enhances FA uptake and TAG storage.[81]

Because the predicted transmembrane domains of several of the AGPATs would place motifs I and motifs II and III (Table 1) on opposite sides of a membrane, and because only motifs I and II are highly conserved in plant GPATs, Yamashita and colleagues studied the topography of AGPAT1.[82] By mutating the protein to construct potential glycosylation sites, the authors confirmed that a site near motif III could be glycosylated, suggesting that it would lie near the luminal side of the ER. On the other hand, by mutating specific amino acids in each of the four motifs, they also confirmed that each one was essential for AGPAT activity. They concluded that motifs II and III might interact with the hydrophobic LPA substrate and lie within the membrane.[79–80] Although little is known regarding the regulation of AGPAT1, analysis of the *Agpat1* promoter shows binding sites for NOR1 and PPARα, which are both involved in muscle development.[83]

4.2. *sn*-1-Acyl-glycerol-3-phosphate acyltransferase-2 (AGPAT2)

Human AGPAT2 is a 278 amino acid protein with strong identify to AGPAT1 and has similar predicted transmembrane domains. The highest mRNA expression is in liver, heart, and adipocytes.[36,80] Analysis of the AGPAT2 promoter region shows consensus binding sites for C/EBPβ and PPARγ, as would be expected for a protein upregulated in adipocytes.

The mechanism by which AGPAT2 deficiency causes human lipodystrophy (see section 15.1) seems to be indirectly related to its role in the pathway of TAG biosynthesis, because AGPAT2 may have a second role in adipocyte differentiation.[84] When 3T3-L1 cells differentiate into adipocytes, *Agpat2* mRNA expression increases 30-fold. siRNA knockdown of *Agpat2* prevents the induction of C/EBPβ and PPARγ which slows the adipogenic program. Thus, AGPAT2 seems to be required for the appropriate temporal expression of these transcription factors. Surprisingly, these cells have 3-fold higher PA levels and although their TAG stores are low, phospholipid content is normal Therefore, either the PA formed is available for phospholipid, but not TAG synthesis, or lack of TAG synthesis results in a buildup of PA levels.[84]

4.3. *sn*-1-Acyl-glycerol-3-phosphate acyltransferase-3 (AGPAT3)

When AGPAT3 (LPAAT3), a 376 amino acid protein, is overexpressed in HeLa cells, AGPAT activity increases.[85] LysoPC, lysoPE, and lysoPS are not substrates,[86] but the mouse enzyme has a low activity using 20:4-CoA to acylate lysoPI.[85] *Agpat3* mRNA is expressed in brown and white adipose tissues, liver, and testis,[74,85] and expression increases in hearts that over-express PPARα.[87] Like AGPAT1, studies using digitonin permeabilization or protease protection indicated that a transmembrane domain separates motifs I and II.[88] The authors speculated that motif I faces the cytosol, motif II lies buried within the membrane, and motifs III and IV are located on the luminal side of the ER or Golgi; how catalysis would occur, however, remains unclear.

Over-expressed, tagged and endogenous AGPAT3 is located in ER/Golgi membranes and gain- and loss-of-function experiments show that the enzyme functions in the formation of Golgi membrane tubules and in protein trafficking. siRNA-mediated decrease of AGPAT3 fragments Golgi into mini-stacks.[86] It has been suggested that membrane fragmentation might occur via changes in membrane curvature resulting from the interconversion of LPA and PA or by the formation of signaling intermediates.[88–89]

shRNA knockdown of AGPAT3 in Sertoli cells decreases PC, PE, PS, and PG species that contain 20:4, 20:5, or 22:5 FAs.[90] Overexpression of AGPAT3 increases polyunsaturated phospholipid species, and the authors suggested that these species might supply polyunsaturated FAs to germ cells. Most phospholipids have an unsaturated acyl group in the *sn*-2 position, but the relative contributions of AGPAT isoforms and lysophospholipid acyltransferases to phospholipid composition remain unclear.

4.4. Function of AGPAT in glycerolipid synthesis

Apart from increased incorporation of labeled FA into TAG with overexpressed AGPAT1[91] and AGPAT3,[81] surprisingly few studies have been performed to elucidate the function of the three confirmed AGPATs, AGPAT1, 2, and 3 in glycerolipid synthesis. Although *Agpat1* and *2* mRNAs are present in mouse epidermis, the most widely expressed forms are *Agpat3, 4*, and *5*.[92] mRNA for *Agpat1, 2*, and *3* and higher AGPAT activity both increase rapidly after disruption of the permeability barrier, suggesting roles in reparative membrane phospholipid synthesis.

4.5. Additional AGPATs and proteins with LPAAT activity

Because of their homology to *E. coli* GPAT and AGPAT, many proteins containing the PlsC domain have been listed as AGPATs in GenBank (Table 2). Several members of this family are either unstudied or have been shown to acylate one or more lysophospholipids. The nomenclature is confusing, because different investigators have persisted in using both the AGPAT and GPAT names and numbers. Further, members of both this family and that of some of the MBOAT family members have similar or overlapping lysophospholipid acyltransferase activities.[33]

In addition, other proteins that lack homology to either the AGPAT or MBOAT families, also exhibit LPAAT activity. Of these, the multifunctional protein endophilin-1 has been best studied and may play a role in modulating vesicle curvature via its ability to convert LPA into PA.[93]. However, the intrinsic acyltransferase function of a related protein, CtBP/BARS (carboxy-terminal binding protein/brefeldin A-ribosylated substrate), has been questioned.[94] AGPAT activity has also been reported for comparative gene identification-58 (CGI-58), a lipid droplet protein and activator of ATGL (see section 14.1).[95–97] CGI-58 may also help to partition hydrolyzed FA from TAG to phospholipid synthesis[97–98] and VLDL assembly.[99] The presence of this LPA acyltransferase on lipid droplets suggests that other proteins involved in TAG synthesis might also be located on this organelle.

5.0. Phosphatidic acid phosphatase/Lipin

Mammalian lipid PA phosphatases exist in two well-defined groups. One family (PAP2; LPP) is comprised of plasma membrane enzymes that remain active in the absence of Mg^{2+} or other divalent cations and in the presence of NEM.[100] These enzymes hydrolyze circulating lipid phosphates and signaling PA after its release from membrane phospholipids by phospholipase D. Unrelated structurally or functionally to this PAP signaling-related family is the PA phosphatase (PAP; lipin; LPIN) (EC 3.1.3.4) that translocates from the cytosol to the ER to hydrolyze the PA formed by AGPAT. This PA phosphatase produces the DAG that is the precursor for the synthesis of PC, PE, PS, and TAG.[101] The protein remained unknown until the yeast Pah1p was shown to be a homolog of Lipin1,[102–103] a protein that had been positionally cloned in a search for the underlying mutation in *fld* mice (see section 5.1).[104]

Three mammalian PAP (Lipin) isoforms exist. Structurally, they each contain a nuclear localization sequence, a DIDGT motif that identifies the catalytic site,[102] and an LXXIL motif believed to be required for activity as a coactivator of transcription.[105] The structure of the mammalian Lipins has not been extensively studied, and readers are referred to several excellent papers on the yeast homolog regarding mechanisms involved in translocation to membranes,[106] regulation by phosphorylation, and role in the nucleus.[107]

5.1. Lipin1

Lipin1 (gene: LPIN1) is a 890 amino acid protein whose encoding mRNA is most highly expressed in adipose tissue, skeletal muscle and testis.[104] Lipin1 is covalently modified by sumoylation which facilitates nuclear localization,[108] and by interaction with 14-3-3 scaffolding proteins in the cytosol which inhibit the nuclear localization of the protein.[109] Lipin1 is also regulated by phosphorylation which occurs in response to mTOR signaling[110] and during mitosis when Lipin1 phosphorylation decreases PAP activity.[111]

Lipin1 was first identified[104] as the deficient protein underlying the spontaneously mutated fatty liver dystrophy (*fld*) mouse.[112] During the suckling period, these mice have markedly fatty livers and are hypertriglyceridemic. After weaning they become lipodystrophic, with loss of both brown and white adipocytes. The lipodystrophy does not result from lack of

TAG synthesis due to absent PAP activity; instead, it is caused by lack of a required effect of Lipin1 on the adipogenesis transcription factors, PPARγ and C/EBPα (see section 5.2).[113] However, it remains unknown if Lipin1 directly alters transcriptional activity or if changes in its PA substrate or its DAG product mediate the effects. Male *fld* mice are infertile, perhaps related to the role of Lipin1 as a transcriptional activator. With time, the mice additionally develop a peripheral neuropathy characterized by Schwann cell dedifferentiation, myelin degradation and reduced nerve conduction velocity.[112,114–115] These neurological features may result from elevated PA levels and the consequent activation of MEK/Erk signaling.[115] In marked contrast to the phenotype of *fld* mice, inactivating human mutations in *LPIN1* cause episodes of recurrent myoglobinuria (see section 15.2).[4] Children with LPIN1 mutations do not have lipodystrophy, fatty liver, peripheral neuropathy, or manifestations of abnormal TAG metabolism.

Human *LPIN1* has 3 splice variants (α, β, γ) of undetermined individual functions that were expressed with His_6 tags in *E. coli* and studied after purification to near homogeneity on Ni^{2+} columns.[116] The PAP activities require divalent cations for activity and are inhibited by sphingosine and sphinganine. Kinetic studies show cooperativity in mixed micellar assays, and maximum activity occurs when the PA substrate has at least one unsaturated fatty acyl moiety.

In McA-RH7777 cells, overexpressed *Lpin1*α or β splice variants increase glycerolipid synthesis and VLDL-TAG secretion, and reduce the intracellular degradation of apolipoprotein B- 100.[117–118] Conversely, shRNA knockdown of *Lpin1* decreases glycerolipid secretion, despite the normal presence of *Lpin2* and *3*. When the nuclear localization signal was mutated, the stimulated TAG synthesis and secretion were diminished, indicating that both enzymatic and nuclear signaling functions of Lipin1 are responsible. In contrast to this report, the rate of TAG synthesis is normal in primary hepatocytes from adult *fld* mice, and the rate of VLDL-TAG secretion is markedly increased.[119] Further, both in mouse hepatocytes and in liver from obese, insulin-resistant mice, overexpression of *Lpin1* diminishes VLDL-TAG secretion. These discrepant results underline the potential problems of trying to relate results from studies in hepatoma cells to those performed in mice.

5.2. Lipin1 as a Transcriptional Coactivator

In addition to its roles as a PA phosphatase, Lipin1 is also a transcriptional coactivator. In the nucleus Lipin1 physically associates with PGC-1α and PPAR-α to promote their transcriptional activities.[105] Lipin1 also coactivates other PPAR isoforms, PPAR-γ and -δ, and HNF-4α. Truncation studies show that its role as a transcriptional coactivator is independent of its PAP activity.[105] Because of the importance of the PPAR family in controlling energy metabolism, Lipin1 is predicted to modulate the expression of many genes involved in FA and glucose metabolism. Lipin1 also interacts with and represses nuclear factor of activated T cells c4 (NFATc4) in adipose tissue.[120] This transcription factor increases the expression of fatty acid binding protein 4 (FABP4) and numerous inflammatory proteins such as TNFα and resistin. Taken together with the anti-inflammatory role of the PPAR family, these data suggest that Lipin1, through changes in transcription, may also reduce inflammation.

5.3. Lipin2 and lipin3

Highest *Lpin2* expression is in liver, brain, and kidney.[121] The 896 amino acid protein has a lower PAP specific activity than Lipin1,[121] but has similar transcriptional coactivator activity.[4] In contrast to *Lpin1*, however, *Lpin2* is not regulated by PGC-1α,[105] and it is repressed during adipogenesis,[111] indicating that the two isoforms have non-redundant

functions. Inactivating human mutations in *LPIN2* cause Majeed syndrome (see section 15.3).[122] *Lpin3* is highly expressed in liver and the gastrointestinal tract and encodes an 851 amino acid protein.[121] It has not yet been characterized.

5.4 Regulation of Lipins

PGC1α and glucocorticoids increase the expression of hepatic *Lpin1*, but not *Lpin2* or *3*. [123,105,124] *Lpin1* also has two splice variants, α and β, the latter of which is more highly upregulated by glucocorticoids and is further enhanced by β-adrenergic signaling.[123–124] Fasting increases hepatic *Lpin1* expression, possibly because of the elevated glucocorticoids that coincide with fasting.[105] Transcription of *Lpin1* is increased by SREBP1 and NF-Y,[125], although others have suggested that insulin, an activator of SREBP1, attenuates *Lpin1* expression.[123] Consistent with its role in PPARγ regulation, the expression of *Lpin1* mRNA increases during adipogenesis and precedes that of PPARγ. Inflammatory signals like lipopolysaccharide and TNFα reduce *Lpin1* expression.[126–128]. Postranslational modifications of LPIN1 are key determinants of the protein's cellular localization, and therefore, its function as either an enzyme or a transcriptional co-regulator. In addition to its role in enhancing *Lpin1* expression, insulin, through mTOR signaling, increases LPIN1 phosphorylation at Ser 106, which increases the interaction of LPIN1 with 14-3-3 proteins, and results in more cytosolic and less nuclear and membrane-associated LPIN1 protein.[109–110,129] Oleate supplementation decreases LPIN1 phosphorylation, increases hepatic LPIN1 association with membranes, and facilitates microsomal PAP activity, consistent with the known effects of oleate in promoting TAG synthesis and VLDL secretion.[129–131] In contrast to phosphorylation, sumoylation of LPIN1 facilitates nuclear localization and transcriptional coregulator activity.[108] *Lpin2*, unlike *Lpin1*, is repressed during adipogenesis.[111] Hepatic LPIN2 protein is also increased by fasting and obesity, independent of mRNA abundance,[132] however, additional information on the regulation of *Lpin2* and *3* is lacking.

6.0. *sn*-1,2-Diacylglycerol acyltransferases

Diacylglycerol acyltransferases (DGAT)-1 and -2 (EC 2.3.1.20) catalyze the final esterification step in the pathway of TAG synthesis. Both proteins are located in the ER, although when FAs are added to cell cultures, some DGAT2 partitions to mitochondrial-associated membranes near lipid droplets.[21] This interaction requires a positively charged, targeting signal between amino acids 61 and 66. Human *DGAT1* and *DGAT2* mRNAs are high in tissues that specialize in TAG synthesis. Expression of either DGAT1 or DGAT2 in cells increases TAG synthesis, and knockdown or absence of either protein decreases TAG synthesis,[133–135] However, when overexpressed in cells, DGAT2 appears to cause a larger accumulation of TAG.[134] Conclusive studies of substrate preference have not been performed. Neither DGAT isoform appears to have a preference for specific acyl-CoAs of a particular chain length or degree of saturation. One study suggested that DGAT2 was more active at lower acyl-CoA substrate concentrations and more sensitive to Mg^{2+} concentrations.[136] Preferences for different DAG species is unknown, but only DGAT1 can esterify retinol to form retinyl esters.[137] The importance of this function can be observed in intestinal cells from *Dgat1*$^{-/-}$ mice, suggesting that DGAT1 contributes to the absorption of dietary vitamin A.[137] Retinol metabolism is also disturbed in *Dgat1*$^{-/-}$ liver.[138] DGAT1 also has high MGAT activity and can synthesize wax esters from fatty acyl alcohols.[137]

6.1. *sn*-1,2-Diacylglycerol acyltransferases-1 (DGAT1)

DGAT1 is a member of the acyl-CoA: cholesterol acyltransferase (ACAT) family, and has retinol acyltransferase, MGAT, and monoester wax synthase activities.[5,137] *Dgat1* is expressed strongly in small intestine, adipose tissue, mammary gland, testis, thymus,

skeletal muscle, spleen, heart, and skin.[5] The DGAT/ACAT (acyl-CoA:cholesterol acyltransferase) family members are members of the MBOAT family that includes both lipid and protein acyltransferases that have a distant resemblance to the GPAT/AGPAT PlsC family. Although initially thought to have 6 to 8 transmembrane domains,[5] similar to the multi-transmembrane topographies of ACAT1 and 2,[139–140] studies of over-expressed, tagged mouse DGAT1 that used protease protection and indirect immunofluorescence reveal data consistent with the protein having only three transmembrane domains.[141] Mutagenesis of a highly conserved histidine residue (H426A) present in a region within the ER lumen inactivates the synthesis of TAG, retinyl esters, and wax esters. The N-terminal region faces the cytosol and is not required for activity, but could facilitate the formation of tetramers.[142] DGAT2 and (sterol-CoA desaturase-1 (SCD1) colocalize and may interact in HeLa cells.[143] Although a previous study had shown that long-chain acyl-CoAs bind to an N-terminal fragment,[144] the topography study places the active site of DGAT1 in the ER lumen and requires the transport of acyl-CoAs into the lumen.[141] An ER carnitine transport system for shuttling FAs has been reported,[145] and several papers have found mildly latent DGAT activity,[145–147] but data from an older study showed that acyl-CoAs cannot permeate ER membranes from rat liver.[148] Thus, the question remains open as to where each DGAT isoform releases its TAG product.

6.2. *sn*-1,2-Diacylglycerol acyltransferases-2 (DGAT2)

DGAT2 is a member of a seven-member family that bears little resemblance to DGAT1 and includes MGAT1, 2, and 3, and wax monoester synthases.[5] Human DGAT2, which was cloned based on the sequence of a fungal DGAT,[149] is a 388 amino acid protein believed to span the ER membrane twice, with both N- and C-terminal domains facing the cytosol.[136] Highest expression of human *Dgat2* mRNA is in liver, adipose, mammary gland, testis, peripheral leukocytes, and heart.[5] Unlike DGAT1, DGAT2 does not catalyze the synthesis of retinyl esters or wax esters.

6.3. Regulation of DGAT1 and DGAT2

Dgat1 and *2* are both regulated transcriptionally and probably post-translationally, but extensive studies have not been performed.[5] mRNA abundance of both *Dgat1* and *Dgat 2* is markedly upregulated during 3T3-L1 differentiation to adipocytes and the mRNA increases in response to 25 mM glucose in mature adipocytes.[150] In a similar study, *Dgat1* mRNA and activity increase in carbohydrate-starved 3T3-L1 adipocytes incubated with D-glucose, but not in response to L-glucose or 2-deoxyglucose, whereas *Dgat2* mRNA increases in response to insulin.[151] *Dgat1* in muscle may also be upregulated by exercise.[152] In people with impaired glucose tolerance, treatment with pioglitazone increases the expression of *Dgat1* in adipose.[153] *Dgat2*, in differentiating 3T3-L1 and mouse pre-adipocytes, is initially upregulated by C/EBP-β, then by C/EBP-α via promoter elements in the *Dgat2* gene.[154]

6.4. DGAT1 and 2: VLDL secretion vs. cytosolic TAG

It remains unclear, especially in liver, as to whether DGAT1 and DGAT2 catalyze the synthesis of different pools of TAG. One current model for TAG synthesis and lipid droplet formation places the newly synthesized TAG in a growing lens between the ER monolayers from which it might partition in either direction. Another view is that DGAT1 might synthesize the TAG destined for assembly into VLDL particles, whereas DGAT2 might synthesize TAG that is incorporated into lipid droplets. The small amount of latent activity in liver microsomes was interpreted as indicating that the active site of DGAT1 faced the ER lumen, and the topography is consistent with this view.[141] However, studies of mice deficient in DGAT1 do not support this idea of exclusivity, because both overt and latent DGAT activities are diminished in *Dgat1*$^{-/-}$ mice[5] and serum TAG is normal.[135] DGAT1 appears to be important for the HNF4α-mediated increase in the secretion of TAG-rich

lipoproteins from human hepatoma Huh7 and HepG2 cells,[155] and for the fatty liver that results from the exogenous FAs that enter the liver during high fat feeding or prolonged fasting.[156] Further, overexpression of either isoform increases VLDL production in some cells and increases cytosolic lipid droplets in both hepatoma cells and liver,[143] whereas an antisense oligonucleotide knockdown of *Dgat2* diminishes both VLDL and cytosolic droplets.[157]

Although overexpression studies are potentially problematic because downstream pathways may not be able to accommodate excess substrate, overexpressed DGAT1 in McA-RH7777 rat hepatoma cells results in increases in both the secretion of apoB-containing lipoproteins and in cellular TAG content.[158] In contrast, adenovirus-mediated overexpression of either DGAT1 or DGAT2 in mice increases hepatic cytosolic TAG, but not VLDL secretion.[159] Thus, the increase in the amount of cytosolic TAG does not appear to affect the production of VLDL-TAG.

6.5. Mice deficient in DGAT1 or DGAT2

Mice deficient in *Dgat1* have reduced adiposity compared to wildtype mice and are resistant to diet-induced obesity. [133,135] Their energy expenditure and activity are increased, and their lactation is defective.[133,135] Dry fur and hair loss result from atrophic sebaceous glands and abnormal fur lipids, such that the fur does not repel water.[160] $Dgat1^{-/-}$ mice do not develop hepatic steatosis and obesity when fed a high fat diet, despite full absorption of the dietary fat. This phenotype appears to be due to a defect in either the timing or location of TAG absorption,[161] because *when Dgat1* is replaced in enterocytes, the $Dgat1^{-/-}$ mice are no longer resistant to obesity when fed a high fat diet.[162] $Mgat2^{-/-}$ mice have a similar phenotype that appears to be caused by a delay in the timing of intestinal uptake of TAG or the relative lack of TAG uptake by the duodenum.[163] Abnormalities in TAG-enhanced secretion of gut peptides like GLP-1 and PYY may be important for the phenotype.[164]

The lack of DGAT2 in mice is lethal shortly after birth.[134] At birth the $Dgat2^{-/-}$ mice weigh 20% less than their wildtype littermates, suggesting that intrauterine growth is defective. The permeability barrier function of $Dgat2^{-/-}$ skin is impaired because of abnormalities in the lamellar body secretory system, and the mice rapidly become dehydrated.[134] The combination of restrictive skin, lack of energy substrates, and hypothermia contribute to their difficulty in suckling; the mice became hypoglycemic and lipopenic and die within the first day of life.

7.0. TAG Catabolism

Lipolysis is the process by which FAs are hydrolyzed from the glycerol backbone of TAG (Figure 2). Complete lipolysis yields three FAs plus glycerol. Although lipolysis occurs in all tissues, it has primarily been studied in white adipose because of the abundance of TAG in this tissue and its contribution to serum FA levels. In heart, skeletal muscle, and brown adipose tissue, the primary function of lipolysis is to generate FAs that can be used as substrates for β-oxidation and energy production. Lipolyzed FAs may also be reesterified to form TAG or be activated for use in the synthesis of phospholipids, cholesteryl esters, or other FA-containing lipids. Recent insights into lipid droplet biology have highlighted novel roles for lipolysis in contributing to cell signaling. As a result, a growing body of literature has focused on the numerous intracellular TAG lipases involved in lipolysis and the subsequent channeling and signaling effects of their released products. Although many putative TAG lipases have been identified, we will focus on those that have been shown to hydrolyze TAG, DAG and MAG and that have been adequately characterized.

8.0. Adipose triglyceride lipase (ATGL)

For many years, hormone-sensitive lipase (HSL) was believed to catalyze multiple steps of TAG catabolism, including the initial reaction that produces DAG from TAG.[23,165] However, the discovery that HSL knockout mice accumulate DAG in numerous tissues and have unaltered body fat content suggested that HSL hydrolyzes DAG rather than TAG and that an additional TAG hydrolase must exist.[166–167] In 2004 three independent groups discovered and characterized the major TAG hydrolase in adipose tissue, adipose TAG lipase (ATGL) (EC 3.1.1.3) (also called PNPLA2, desnutrin, and calcium-independent phospholipase A2ζ).[167–169]

ATGL shares homology with a family of patatin-like phospholipase domain-containing proteins (PNPLA).[167] These proteins include patatin, a protein in tubers, that possesses acyl hydrolase activity.[170] Human ATGL is a 486 amino acid protein with a predicted molecular mass of 54 kDa. Conserved regions within the N-terminal patatin-like domain include a GXGXXG nucleotide binding motif, a GXSXG serine hydrolase motif common to all PNPLA members, and a DG(G/A) motif. The conserved Asp 166 and Ser 47 residues form a catalytic dyad that is essential for lipase activity.[171] In addition, the C-terminus domain contains a hydrophobic region that is required for ATGL binding to lipid droplets.[172] ATGL shows the highest substrate reactivity towards TAG, but it can also hydrolyze DAG, albeit with at least an order of magnitude lower activity.[167] Despite its TAG hydrolase activity, it is not known whether ATGL preferentially hydrolyzes FAs based on their position (i.e. sn-1/3 or sn-2) or their structure (i.e. chain length and saturation) although ATGL knockdown in the liver results in accumulation of TAG enriched in C18:1 and with decreased C16:0, C18:0 and C18:3.[173] In vitro studies show that ATGL also possesses acylglycerol acyltransferase and phospholipase activities, although the contribution of these activities to the observed physiological effects of ATGL remains unknown.[168,174–175] ATGL is highly expressed in brown and white adipose tissues; expression levels are lower in heart and skeletal muscle.[167,169,176–177]

8.1. *Atgl*$^{-/-}$ mice

The physiological role of ATGL has been largely inferred from studies of *Atgl* knockout mice. These mice have increased body fat content, resulting from elevated TAG content in adipose tissue, as well as ectopic TAG accumulation in nearly all tissues, suggesting the importance of the lipase activity in non-adipose tissue.[167] Severe TAG accumulation in the heart causes cardiac dysfunction and premature death, occurring as early as 12 weeks of age.[166,178] In agreement with increased white adipose in ATGL null mice, silencing ATGL in human adipocytes robustly decreases lipolysis.[179–180] In contrast to these findings, humans lacking functional ATGL do not have increased adiposity. The discrepancy between the phenotypes in mice and humans (see section 15.6) has yet to be clarified, although it is likely that changes in energy metabolism resulting from ATGL ablation play a role. Possible changes include reduced lipogenesis, increased fatty acid oxidation, and compensatory expression of additional lipases.

8.2. Role of ATGL in FA oxidation and partitioning

Mice that lack ATGL have an increased RQ, indicating that carbohydrate oxidation is elevated at the expense of FA oxidation.[166] Consistent with increased glucose use, muscle and liver glycogen stores are depleted.[181] These shifts in substrate use may occur because lower rates of adipose TAG lipolysis result in decreased serum FA levels and an inadequate supply of FA to tissues for β-oxidation.[166,181] However, ATGL plays an important role in FA oxidation, independent of changes in circulating FA, because it contributes hydrolyzed FA to β-oxidation within the same cell. Most FAs taken up by myocytes are not directly

oxidized, but instead, are initially esterified to form TAG from which they must be subsequently hydrolyzed before being oxidized.[182] Thus, the rate of TAG hydrolysis within muscle may be a regulatory step that controls FA β-oxidation. Studies using stable FA isotopes in humans show enrichment in muscle acylcarnitines, which are indicators of FA flux through the β-oxidation pathway. The high acyl-carnitine levels are more closely associated with intramyocellular TAG enrichment than with plasma FA enrichment.[183] These data support the hypothesis that FAs that undergo β-oxidation in muscle are primarily derived from the hydrolysis of intracellular TAG stores rather than from exogenous FA taken up from the blood. Consistent with the findings in muscle, adenoviral-mediated overexpression of *Atgl* in liver increases FA oxidation whereas hepatic *Atgl* knockdown decreases FA oxidation.[173,184] In agreement with these data, ATGL null mice exhibit a markedly reduced running velocity and diminished endurance compared to wildtype mice.[181] Taken together, these data highlight a critical role of ATGL in hydrolyzing TAG to supply mitochondria with FAs for β-oxidation in muscle and liver. Data derived from studies in other tissues also support a link between ATGL and FA oxidation. In mice with adipocyte-specific overexpression of ATGL, FA oxidation in adipocytes increases without altering the release of FAs from the cells.[185] Thus, it is possible that the FAs hydrolyzed by ATGL are either reesterified to TAG or used in β-oxidation, whereas the FAs hydrolyzed by HSL, which is much more responsive to lipolytic stimuli, are the ones that are preferentially released into the blood. ATGL may play a similar role in brown adipose. Because of its thermogenic capacity, brown adipose is essential for body temperature homeostasis in infants and hibernating animals. Recent renewed interest in brown adipose results from studies showing that brown adipose is present in adults and is negatively correlated with obesity.[186–189] Brown adipose relies primarily on the oxidation of FA in order to fuel thermogenesis. However, the role of intracellular TAG hydrolysis in supplying FA to the mitochondria for oxidation has not been critically considered. The importance of TAG hydrolysis in fueling oxidation is evidenced by *Atgl* null mice, which are unable to perform thermogenesis and, as a result, die within 6 h after they are exposed to 4 °C.[190] In contrast, and in support of differential FA partitioning between HSL and ATGL thermogenesis is normal in mice lacking HSL.[191]

9.0. PNPLA3

PNPLA3 (also called adiponutrin[192] or calcium-independent phospholipase A2η[168]) shares the highest homology to ATGL (PNPLA2) in the PNPLA family. Human PNPLA3 is a 413 amino acid protein with a predicted MW of 53 kDa. *Pnpla3* is highly expressed in adipose tissue and expression in other tissues is relatively low.[176,192] PNPLA3 is located on both lipid droplets and on other, as yet undefined, intracellular membranes.[193] PNPLA3 is predicted to have 4 transmembrane domains, 3 of which are located within the N-terminus region.[192] In addition to TAG hydrolase activity, PNPLA3 also possesses transacylase and phospholipase A_2 activities.[168] Despite robust TAG lipase activity in vitro, the physiological contribution of PNPLA3 to TAG hydrolysis remains unclear. Gain- and loss-of-function studies reveal that PNPLA3 has no effect on TAG accumulation or hydrolysis in cultured cells.[176–177,193] In *Pnpla3* $^{-/-}$ mice, liver TAG content is normal, and the phenotype does not differ from that of wildtype mice.[194] These data are especially surprising given the extensive literature showing that sequence variations, especially I148M, in human *PNPLA3* are associated with NAFLD (see section 15.7). In agreement with the cell culture studies, adenoviral-mediated overexpression of *Pnpla3* in mouse liver does not influence hepatic TAG content.[193] However, when *Pnpla3* with the I148M mutation is overexpressed, hepatic TAG content increases markedly, and TAG hydrolysis is inhibited.[193] Pulse-chase experiments with 18:1 in primary hepatocytes also show that overexpressing the mutant *Pnpla3* increases the incorporation of radiolabeled oleate into TAG during the chase period, suggesting that the mutated PNPLA3, in addition to inhibiting TAG hydrolysis, may be

involved in transferring acyl groups from other cellular lipids to TAG.[193] Additional studies are needed to define the normal function of PNPLA3.

10.0. Triacylglycerol hydrolase (TGH)

TGH (also called Ces3 in mice and CES1 in humans) belongs to the carboxylesterase family of enzymes. Because carboxylesterases can hydrolyze ester, amide and thioester bonds, they are involved in metabolism of xenobiotics as well as lipids.[195] Human TGH is a 568 amino acid protein with a predicted MW of 62 kDa. TGH has little homology to other known lipases. In human TGH, Ser 221, Glu 354 and His 468 are highly conserved residues that form the catalytic triad critical for lipolytic activity.[196] Additionally, amino acids 414–429 form a hydrophobic domain that is thought to bind lipid substrates.

TGH is expressed predominantly in liver and is located in the ER and on lipid droplets within the lumen of the ER.[197–199] In humans, a HIEL sequence at the C-terminus is responsible for retention of TGH in the ER.[200] The catalytic site of TGH faces the lumen of the ER, suggesting that TGH plays a role in VLDL metabolism.[201] Overexpression of TGH in hepatoma cells or mouse liver results in increases in TAG hydrolysis, hepatic TAG secretion, and plasma concentrations of TAG and apoB.[202–203] In contrast, suppression of TGH in 3T3-L1 adipocytes decreases basal FA efflux without affecting isoproterenol-stimulated lipolysis and increases cellular TAG and CE content.[204]

10.1. *Tgh*$^{-/-}$ mice

Consistent with the overexpression studies, mice lacking TGH have reduced serum TAG and apoB in both fed and fasted states.[205] Despite the reduction in hepatic TAG output, ablation of TGH does not increase hepatic TAG content, which may be due to lower serum FA levels in the knockout mice. Indeed, in primary hepatocytes isolated from wildtype and *Tgh*$^{-/-}$ mice, lack of TGH causes nearly a 3-fold increase in cellular TAG accumulation.[198] Hepatocytes lacking TGH contain smaller lipid droplets, possibly by decreasing the rate at which TAG in newly formed lipid droplets on the ER are transferred to existing cytosolic lipid droplets.[198] Additionally, TGH ablation causes phospholipid and DAG to accumulate in the ER, suggesting that TGH may preferentially hydrolyze DAG rather than TAG.[206] Thus, although TGH clearly plays a role in TAG metabolism and VLDL synthesis, much remains to be discovered regarding the mechanisms by which it mediates these processes.

11.0. Hormone sensitive lipase (HSL)

For the past half-century, HSL (EC 3.1.1.79) has been the primary focus of research involving lipolytic regulation. Human HSL is a 775 amino acid protein with a calculated mass of 84 kDa. Expression is highest in adipose and adrenals, but is also present in gonads, placenta and muscle.[207] Nine exons are expressed in all HSL variants, however, three different exons (A, B and T) upstream of exon 1 are differentially spliced into exon 1 to form three HSL isoforms. Exon B, which is non-coding and is the predominant isoform expressed in adipocytes, results in an 84 kDa protein.[208] Exon A encodes an additional 43 amino acids, resulting in an 88 kDa protein, and is primarily expressed in ovaries, adrenal gland, β-cells, and at a lower level in adipose.[209] The T exon, which is expressed exclusively in testes, encodes an additional 300 amino acids and results in an isoform with a molecular mass of 120 kDa.[210–211] Despite the significant differences in the N-terminus of HSL, the physiological importance of these splice variants remains unknown. The C-terminal domain of human HSL contains the catalytic triad of Ser 424, Asp 693 and His 723 located within the α/β hydrolase fold.[212–213] Additionally, the C-terminal domain also contains the regulatory region (amino acids 522–699 in human HSL) that contains all phosphorylation sites known to mediate hormonal regulation of enzyme activity.[214] Apart

from this catalytic domain, HSL shares no sequence homology to other proteins. Although the HSL monomer has some enzymatic activity, HSL is thought to act primarily as a homodimer, which forms through interactions within the N-terminal domains.[214]

HSL was originally named for its activation in response to hormonal signals such as norepinephrine and epinephrine.[165] Early investigators concluded that HSL catalyzed the hydrolysis of both TAG and DAG, but subsequent studies showed that HSL has broad substrate specificity towards tri-, di-, and monoacylglycerols as well as cholesterol and retinyl esters (reviewed in[7]). However, the rates of hydrolysis of DAG are significantly higher than for other substrates and at least 10-fold higher than for TAG.[167] In mice lacking HSL, DAG levels are elevated in numerous tissues, including adipose, thus supporting the idea that HSL preferentially hydrolyzes DAG.[166] At present, HSL is recognized as being largely responsible for the second step of lipolysis, the hydrolysis of DAG to MAG, although roles in hydrolysis of other lipid esters may still be important. HSL prefers *sn*-1,2-DAG compared to 1,3-DAG and preferentially hydrolyzes unsaturated acyl chains.[7]

11.1. *Hsl*$^{-/-}$ mice

The physiological role of HSL has been primarily inferred from studies of HSL knockout mice. Mice with a whole-body HSL deletion have normal body weight, despite reduced white adipose mass, and are resistant to high fat diet-induced obesity.[166] Several mechanisms may explain the decreased adiposity. First, basal lipolysis is increased in *Hsl*$^{-/-}$ mice, whereas fasting lipolysis is normal, although loss of HSL markedly blunts β-adrenergic stimulated lipolysis.[215] Second, adipogenesis is decreased in white adipose, as evidenced by decreased expression of adipocyte differentiation markers (PPARγ, C/EBP-α, FABP4, lipoprotein lipase) and lipogenic genes (GPAT1, DGAT1 and 2, fatty acid synthase, ATP citrate ligase).[216] Finally, white adipose from *Hsl*$^{-/-}$ mice takes on characteristics of brown adipose, including elevated mRNA expression of uncoupling protein-1 and CPT1, an increase in mitochondria, and a nearly 3-fold increase in oxygen consumption. [217] Thus, increased basal lipolysis, decreased adipogenesis and increased oxidation all contribute to the diminished white adipose in *Hsl*$^{-/-}$ mice. Although the mechanism linking HSL to these effects is not fully understood, the data suggest that the retinyl ester hydrolase activity of HSL may contribute. Retinyl ester hydrolase activity is lower in white adipose from HSL null mice, and is accompanied by higher levels of retinyl esters and lower levels of retinol, retinaldehyde and *all-trans* retinoic acid.[218] Genes positively regulated by retinoic acid are down-regulated in HSL null mice, including those involved in lineage differentiation to white adipose [retinoblastoma protein (pRb) and receptor interaction protein 140 (RIP140)], and lipogenesis (S14, SREBP1c, RXR-α). Moreover, supplementing the diets of *Hsl*$^{-/-}$ mice with retinoic acid partially restores the gene expression profiles and the white adipose phenotype. These data support the idea that the retinyl ester hydrolase activity of HSL, rather than the DAG hydrolase activity, may account for much of its signaling effects.

12.0. Monoacylglycerol lipase (MGL)

MGL (EC 3.1.1.23) catalyzes the terminal step in TAG catabolism, the hydrolysis of monoacylglycerol to glycerol and one FA. Although MGL shares the conserved GXSXG motif within the α/β domain, it has little homology to other lipases. At the protein level, human, mouse and rat MGL share 85% homology. Human MGL is comprised of 303 amino acids with an estimated molecular mass of 33 kDa. The active site of mouse Mgl is comprised of Ser 111, Asp 239 and His 269.[219] *Mgl* is broadly expressed, with high mRNA abundance in testis, kidney and adipose tissue,[219] and is located within a variety of intracellular compartments.[8] Unlike many lipases that have a broad substrate specificity, MGL does not hydrolyze other glycerolipids, like DAG, TAG, cholesterol esters, or retinyl esters. MGL hydrolyzes both *sn*-1(3)-MAG and *sn*-2-MAG,[220] especially the MAG

endocannabinoid, *sn*-2-20:4-glycerol.[220–221] *Mgl* is expressed at high levels in specific regions of the brain (hippocampus, cortex, anterior thalamus and cerebellum) where cannabinoid receptors are also highly expressed, further supporting a role in endocannabinoid metabolism and, thereby, diminishing cannabinoid signaling.[220] *Mgl* is highly expressed in human cancer and appears to release FAs that are converted into downstream metabolites (LPA and PGE2) that support the malignancy.[222] The normal physiological role of MGL remains largely unknown (see section 16.3).

13.0. Regulation of TAG catabolism by gene expression and phosphorylation

In order to maintain cellular and whole-body energy homeostasis, TAG hydrolysis must be highly regulated. Both lipolytic and anti-lipolytic signals govern the activity of specific enzymes involved in TAG hydrolysis. Additionally, changes in gene expression and the amounts of several co-activators and inhibitors play key roles in regulating both basal and stimulated lipolysis.

13.1. Regulation of ATGL

Atgl expression increases markedly during adipocyte differentiation and parallels lipid droplet accumulation.[167,223–225] Consistent with a role in adipocyte differentiation, *Atgl* is a direct target of the adipogenic transcription factor PPARγ;[224,226] administration of antidiabetic thiazolidinedione drugs, which are PPARγ agonists, increases *Atgl* expression in mice.[227–229] *Atgl* mRNA abundance is higher in retroperitoneal adipose compared to mesenteric and inguinal,[230] and this variable expression of *Atgl* in different adipose depots, likely contributes to depot-specific differences in metabolism. In rats, *Atgl* mRNA increases with age in inguinal and retroperitoneal, but not epididymal and mesenteric adipose depots.[231] However, the physiological relevance of these differences or how the expression of ATGL co-regulatory proteins differs among adipose depots remains unknown.

Given the importance of hormonal control of lipolysis, there has been considerable interest in hormonal regulation of ATGL through both transcriptional and posttranslational (see section 13.1) mechanisms. *Atgl* mRNA abundance in adipose tissue and liver increases with fasting and decreases with refeeding.[176–177,232] In 3T3-L1 cells, insulin inhibits ATGL mRNA expression.[224,233] When targeted by insulin, the Forkhead box O1 (FoxO1) transcription factor translocates out of the nucleus and becomes inactive. Two FoxO1-binding sites are present on the ATGL promoter, and gain- and loss-of-function studies show that FoxO1 regulates ATGL in an insulin-dependent manner.[223] Insulin also signals through mTORC1 to control numerous physiological processes, including protein translation. Studies in 3T3-L1 cells and in C2C12 myotubes show that mTORC1 activation inhibits the expression of both *Atgl* and *Hsl* and decreases lipolysis, whereas inhibiting mTORC1 increases *Atgl* expression and lipolysis.[234] Although the mechanism by which mTORC1 regulates *Atgl* is unknown, it may be intertwined with that of FoxO1. FoxO1 physically interacts with TSC2, an inhibitor of mTORC1, thereby relieving the TSC2 inhibition of mTORC1.[235] Thus, insulin appears to regulate *Atgl* expression via both FoxO1 and mTORC1.

Atgl expression is also influenced by diet, obesity, and insulin resistance. In mice, ATGL protein increases in visceral and subcutaneous fat after 8 weeks of high fat feeding.[236] and *Atgl* mRNA is more highly expressed in large adipocytes.[237] *ATGL* mRNA abundance is higher in adipose tissue from obese humans, but protein and TAG lipase activity remain unchanged.[180,238] Although *Atgl* expression tracks with obesity, most studies in humans show that high adipose *ATGL* expression is associated with improved insulin sensitivity. In

obese subjects, those who are insulin resistant have lower mRNA and protein levels of ATGL compared with those who are insulin sensitive.[239–240] Similarly, *ob/ob* and *db/db* genetically obese rodents have reduced *Atgl* mRNA.[232] Consistent with this positive role in insulin sensitivity, *ATGL* mRNA expression in human omental adipose tissue correlates positively with insulin-stimulated glucose uptake.[239] Because inflammation is commonly linked to insulin resistance, it is not surprising that TNF-α decreases *Atgl* mRNA expression both in vitro and in vivo.[224,233,241] Additionally, exercise training, which is known to improve insulin sensitivity, increases ATGL protein content in human muscle.[242]

The regulation of ATGL through covalent modifications is unclear. Although human ATGL can be phosphorylated on Ser 404 and Ser 428, it is not modified by the classical lipolytic signal protein kinase A.[167] Nonetheless, covalent modification of other proteins may indirectly lead to changes in ATGL activity. These proteins, many of which are located on lipid droplets, influence either the enzymatic activity of ATGL or its access to the lipid droplet (see section 14.0)

13.2. Regulation of PNPLA3 and TGH

In contrast to ATGL, PNPLA3 expression decreases in adipose during fasting and increases in both adipose and liver after refeeding.[176,192] Consistent with an increase in the fed state, PNPLA3 mRNA increases in genetically obese *ob/ob* and *fa/fa* rodents,[176,192] and its activity is transcriptionally increased by insulin[177] through the insulin-responsive lipogenic transcription factor SREBP-1c.[243] No studies have evaluated posttranslational modifications of PNPLA3.

Few studies have evaluated the regulation of TGH expression. *Tgh* expression increases ~10- fold during 3T3-L1 differentiation, via mechanisms that are PPARγ-independent.[244] Isoproterenol does not alter *Tgh* expression in porcine adipocytes,[245] and hepatic *Tgh* expression does not respond to PPARα agonists or high fat diet feeding.[244]

In hepatoma cells, TGH phosphorylation and activity are not regulated by insulin or glucagon.[246] Although rat TGH can be phosphorylated on Ser 506, this residue is not conserved among species and, therefore, is unlikely to play a significant role in modifying TGH function.[247]

13.3. Regulation of HSL and MGL

Despite decades of active research on HSL, the regulation of *Hsl* mRNA expression has been minimally studied, perhaps because of the major role phosphorylation plays in controlling HSL activity. Like *Atgl, Hsl* is a PPARγ target and is induced during adipocyte differentiation.[248] *Hsl* expression is also controlled by a glucose-responsive element in its promoter.[249] When glucose is omitted from adipocyte culture medium, *Hsl* expression decreases, whereas increasing the glucose concentration enhances *Hsl* expression.[249–251] Several studies have suggested that altering insulin within physiological concentrations inhibits *Hsl* expression.[252–253] In agreement with the idea that insulin and a positive energy state inhibit *Hsl*, the abundance of adipose *HSL* mRNA is reduced in obese subjects.[254–257] Moreover, *HSL* mRNA is further reduced in obese, insulin-resistant subjects compared to those who are obese and insulin sensitive.[240] *HSL* mRNA and protein levels increase after a fast; thus, taken as a whole, it appears that feeding status plays a significant role in governing *HSL* expression.

The best studied regulation of HSL activity is by β-adrenergic stimulation which activates PKA. Activated PKA phosphorylates HSL on multiple serine residues, although Ser 659 and Ser 660 (rat sequence) are the two sites responsible for increasing HSL catalytic activity.[258] In contrast, insulin antagonizes lipolysis, in part through activating phosphodiesterase 3B,

which promotes cAMP degradation and thereby reduces β-adrenergic signaling.[259–262] Several phosphatases also affect HSL phosphorylation, although the physiological relevance of these effects is largely unknown.[263] In addition to PKA, numerous other protein kinases phosphorylate HSL, including extracellular signal regulated kinase (ERK)[258] and AMPK.[264] Unlike PKA and ERK, which promote HSL activity, AMPK phosphorylates Ser 565 and attenuates PKA-mediated phosphorylation of HSL,[264] thereby decreasing PKA-stimulated lipolysis.[265–266] Glycogen synthase kinase-4 also phosphorylates HSL, although the function of this modification is unknown, as it does not appear to influence HSL activity.[267]

Similar to *Atgl* and *Hsl*, PPARγ agonists increase *Mgl* mRNA expression,[227] but little more is known about its regulation.

14.0. Regulation by interacting proteins

14.1. CGI-58 and perilipin

The best studied of the ATGL-interacting proteins is CGI-58 (alias is ABHD5), which enhances ATGL activity 20-fold. [96] Knockdown or ablation of CGI-58 in cells or animals markedly reduces TAG hydrolysis.[96] C-terminus truncations of CGI-58, which maintain its LPA acyltransferase activity (see section 4.5), are unable to activate ATGL or bind to lipid droplets, suggesting that the co-activator and acyltransferase activities are independent.[268]

The function of CGI-58 as an ATGL co-activator depends on its location. Under basal conditions, CGI-58 lies primarily on the surface of lipid droplets. This location is facilitated by perilipin (PLIN1), a protein that acts as a scaffold on the droplet surface.[269] Fluorescence resonance energy transfer and biomolecular fluorescence complementation analysis show that PLIN1 interacts directly with CGI-58.[270] Moreover, under basal conditions, CGI-58 interacts with only ~25% of the ATGL; the remainder of the ATGL is cytosolic, but after lipolytic stimulation with forskolin, CGI-58 dissociates from PLIN1 and binds ATGL at the lipid droplet surface.[270–271] Thus, during lipolytic stimulation, there is a net movement of ATGL from the cytosol to lipid droplets.[270] PKA initiates this series of events by phosphorylating Ser 517 on PLIN1.[271–272] Although ATGL is not directly phosphorylated by PKA, PKA-stimulated lipolysis is mediated through its dynamic interaction with CGI-58 after phosphorylation of PLIN1.[272] In addition, PLIN1 phosphorylation on Ser 494 may also enhance lipolysis by promoting lipid droplet dispersion so that the surface area of the droplets is increased for lipase attack.[273–275] Both ATGL[167,270,276] and phosphorylated HSL[274] facilitate lipid droplet dispersion.

Similar to its role in regulating ATGL, PLIN1 also mediates HSL activity. β-adrenergic stimulation promotes the phosphorylation of PLIN1 allowing the physical interaction of PLIN1 and HSL.[270,277] This functional interaction promotes the translocation of HSL to lipid droplets and increases HSL activity and TAG lipolysis.[269–270,278] Consistent with this intracellular translocation, HSL phosphorylated on Ser 660 is found almost exclusively on lipid droplets.[279] Thus, through its direct interaction with HSL and CGI-58, and its promotion of ATGL activity, PLIN1 has emerged as a central mediator of stimulated lipolysis

14.2. Other perilipin family members

In addition to Plin1, the perilipin family also contains Plin2 (ADRP), Plin3 (TIP47), Plin4 (S3–12) and Plin5 (OXPAT, LSDP5 or MLDP). Although less well studied than Plin1, these proteins may also contribute to lipolytic regulation. For example, Plin2 overexpression in HEK293 cells reduces ATGL localization to lipid droplets and inhibits TAG turnover.[280] Conversely, treating hepatoma cells with siRNA against Plin2 and Plin3 reduces the amount of ATGL on lipid droplets.[281] In contrast, Plin5 promotes ATGL and CGI-58 interactions on

the lipid droplet surface and increases ATGL activity.[282] This area requires additional study to understand the individual roles of the Plin proteins and the mechanism through which they work.

14.3. Pigment epithelium-derived factor (PEDF)

PEDF, a secreted glycoprotein with a broad range of biological effects, is a high affinity binding partner of ATGL; ATGL has also been shown to localize to the ER and plasma membrane in addition to its aforementioned presence in the cytosol and on lipid droplets.[175] PEDF stimulates the phospholipase activity of ATGL, which facilitates PEDF signal transduction.[175] PEDF is highly expressed in liver, and whole-body ablation of *Pedf* results in hepatic steatosis.[283] Primary hepatocytes isolated from $Pedf^{-/-}$ mice also accumulate TAG. Overexpressing recombinant *Pedf* in $Pedf^{-/-}$ hepatocytes, partially reduces their TAG content, but has no effect on wildtype hepatocytes. Interestingly, serum levels and adipose mRNA expression of *Pedf* are elevated in rodent models of obesity and in obese humans,[284] whereas PEDF-neutralizing antibodies improve the insulin sensitivity of obese mice.[285] Consistent with these findings, acute or prolonged recombinant PEDF administration in vivo promotes insulin resistance in mice.[285] Although PEDF clearly has potent effects on energy metabolism, its specific role in regulating TAG hydrolysis through its interaction with ATGL remains unresolved.

14.4. G_0/G_1 switch gene 2 (G0S2)

The G_0/G_1 switch gene 2 (G0S2) was named because its mRNA abundance changes as cells progress from G_0 to G_1 of the cell cycle, although its role in cell proliferation remains unknown.[286] G0S2 is highly expressed in adipose tissue and liver, and is upregulated during 3T3-L1 adipocyte differentiation.[287–288] Overexpressing G0S2 causes lipid droplets to accumulate in 3T3-L1 adipocytes.[287] After lipolytic stimulation with isoproterenol, G0S2 migrates to the surface of lipid droplets together with ATGL, to which it binds independent of lipolytic stimulation.[287] CGI-58 overexpression is unable to overcome G0S2-mediated inhibition of ATGL suggesting that G0S2 and CGI-58 function through non-competing mechanisms.[289] Additional studies are needed to expand our understanding of G0S2 and its contribution to lipolysis and whole-body energy metabolism.

14.5. Fatty acid binding protein-4

FABP4 (aP2) is the fatty acid binding protein isoform most highly expressed in adipose tissue where it acts as a carrier for FA and retinoic acid.[290] FAs produced from HSL-mediated hydrolysis of DAG feedback to inhibit HSL activity. FABP4 directly interacts with HSL and facilitates the efflux of FA, thereby alleviating product inhibition and increasing HSL catalytic activity.[291] In agreement with this interpretation, mutating FABP4 to block its ability to bind FA abolishes its stimulatory effects on HSL activity.[292–293] Phosphorylation of HSL on Ser 659/660 is required for the physical association of FABP4 and HSL.[294] Consistent with these results, adipose tissue lipolysis and serum FFA are reduced in *Fabp4* null mice together with increased adipose tissue mass.[295–297]

14.6. Vimentin

Vimentin, a filament protein that forms part of the cytoskeleton, is present on lipid droplets,[298–299] and directly interacts with HSL to promote lipolysis.[300] Inhibiting vimentin through siRNA or a dominant negative construct attenuates β2 and β3 adrenergic activation of lipolysis.[300–301] Adipocytes isolated from vimentin knockout mice have normal maximal rates of isoproterenol-stimulated lipolysis, but require approximately 10-fold higher concentrations of isoproterenol to reach half maximal rates of lipolysis compared to wild type adipocytes.[300] Surprisingly, although adipocytes in vimentin knockout mice contain

smaller lipid droplets, the mice have no observable phenotype, and the amount of adipose tissue is normal.[300,302] In contrast, vimentin knockdown in 3T3-L1 cells attenuates lipid droplet accumulation during differentiation because TAG turnover is enhanced.[303] Thus, although vimentin appears to be involved in lipolysis, much remains to be discovered about its physiological role.

15.0. Genetic variation and disease in humans

15.1. Deficiency of AGPAT2

Mutations in *AGPAT2* cause a human congenital lipodystrophy characterized by liver steatosis and insulin resistance.[304] The lack of adipose tissue in subjects who lack functional AGPAT2 results in low serum leptin levels; providing exogenous leptin to these subjects suppresses appetite and improves hepatic steatosis and insulin sensitivity.[305] As in the human disorder, *Agpat2*$^{-/-}$ mice have a severe lipodystrophy of white and brown adipose depots and severe hepatic steatosis with upregulation of several lipogenic genes.[306]

15.2. Deficiency of LPIN1

LPIN1 deficiency may result from a variety of deleterious mutations and, unlike its lipodystrophic and neurological phenotypes in mice (see section 5.1), causes only myoglobinuria in children.[307] *LPIN1* polymorphisms are linked to increased body mass index, metabolic rate, blood pressure and hemoglobin A$_{1c}$ levels.[308–311] Variants of *LPIN1* also modulate the metabolic response to the antidiabetic drug pioglitazone. For example, the rs10192566 SNP in *LIPIN1* enhances the beneficial effects of pioglitazone on serum glucose concentrations and hemoglobin A$_{1c}$[312]

15.3. Deficiency of LPIN2

Mutations in *LPIN2* cause Majeed syndrome.[122] This autosomal recessive disorder is characterized by chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anemia that begin in childhood, as well as a transient inflammatory dermatosis with a neutrophilic skin infiltration. Three different mutations in *LPIN2*, a SNP (S734L), a frame shift mutation, and a splice mutation, have all been linked to Majeed syndrome.[122,313] Replication of the human mutation in the mouse *Lpin2* (S734L) cDNA abolishes PA phosphatase activity, but does not alter the ability of the protein to act as a transcriptional coactivator.[314] Thus, it appears that alterations in the PAP activity of LPIN2 contribute to disease etiology, although the mechanism remains unknown. In addition to its role in Majeed syndrome, several variants of *LPIN2* have been linked to psoriasis[315] and to type 2 diabetes and body fat distribution.[316]

15.4. *MGAT1* single-nucleotide polymorphism

To date, only one study has characterized mutations in *MGAT1*. A SNP (rs12517906) that is 45 kb downstream of the *MGAT1* gene is associated with body weight.[317]

15.5. *DGAT1* and *DGAT2* variants

Given their pivotal role in TAG synthesis, DGAT variants are logical targets for genetic variation. Analysis of C79T, a common variant in the promoter of *DGAT1*, revealed that the variant is associated with lower BMI and, after correction for BMI, is linked to increased HLD-cholesterol and lower diastolic blood pressure.[318] However, a subsequent study found no relationship between this or other *DGAT1* variants to obesity.[319] Interestingly, a SNP in *Dgat1* has also been linked to milk yield and composition in the bovine. The K232A mutation results in increased milk fat and protein composition, but decreased milk yields.[320–321] The K allele, which is characterized by a higher Vmax,[322] also increases the

proportion of saturated FAs in milk TAG.[323–324] These data support an important role for DGAT1 in lactation and are consistent with the inability of *Dgat1* knockout mice to produce milk.[135] The effects of *DGAT1* variants on lactation in humans have not been studied. DGAT2 variants have been linked to hepatic TAG accumulation, but not obesity or insulin sensitivity.[325–326] These findings are consistent with liver-specific *Dgat2* overexpression in mice, in which hepatic steatosis develops in the absence of insulin resistance.[327]

15.6. Deficiency of ATGL or CGI-58

The best documented disease caused by a mutation in a lipolytic enzyme or co-regulatory protein is neutral lipid storage disease (NLSD) (reviewed by[328]). This disorder is characterized by the accumulation of TAG in virtually all tissues. NLSD with myopathy (NLSDM) is caused by mutations in *ATGL*,[329] and NSLD with ichthyosis (NLSDI) is caused by mutations in *CGI-58*.[96] In 2006 Fischer et al. identified three mutations that produce a truncated ATGL protein with defects in its hydrophobic region.[329] The patatin domain, which contains the catalytic site, was unaltered in these mutations. Mutations have also been reported in the ATGL C-terminus, which is responsible for binding to lipid droplets.[330–332] Thus, even if a mutated ATGL has normal or elevated enzymatic activity towards synthetic lipid substrates, the inability to bind to lipid droplets prevents TAG hydrolysis in vivo.[172,332] Other mutations that affect the N-terminus, which contains the patatin domain, also result in NLSDM. For example, a retrotransposal insertion in exon 3[333] or a duplication mutation in exon 4[334] yield catalytically inactive enzymes. Therefore, both the inability to bind lipid droplets or the reduced catalytic activity result in NLSDM.

In the initial paper that identified *CGI-58* as the mutated gene in NLSDI,[335] eight distinct mutations were reported, and numerous additional mutations have been identified.[96,336–341] Because CGI-58 functions as both an ATGL co-activator and a LPA acyltransferase, studies have tried to determine which of these functions is involved in the pathogenesis of NLSDI. Acyltransferase activity is normal in two *CGI-58* mutations that cause NLSDI, Q130P and E260K,[95] but the proteins do not activate ATGL,[96] and they impair the ability of CGI-58 to bind PLIN1 and to lipid droplets.[342] Thus, it appears that altered activation of ATGL, rather than impaired CGI-58 acyltransferase activity, may be responsible for the development of NLSDI.

In addition to its role in NLSDM, genetic variations in *ATGL* are also linked to other metabolic measures and disease incidence. Several SNPs in *ATGL* are associated with higher serum FA, TAG, and glucose concentrations, and are linked to a higher risk of type 2 diabetes.[343] Two *ATGL* haplotypes are associated with lower risk for familial combined hyperlipidemia, lower serum TAG and FFA, and higher HDL-cholesterol.[344–345] Taken together, these studies support a potential link between *ATGL* genetic variation and metabolic disease risk.

15.7. *PNPLA3* mutations

In 2008, Romeo *et al.* used ^{1}H-MRS to measure hepatic TAG content and genome-wide association scans of participants in the Dallas Heart Study to identify the I148M variant of PNPLA3 that was tightly linked to non-alcoholic fatty liver disease (NAFLD).[346] Since that initial publication, numerous additional studies have supported a link between PNPLA3 and NAFLD.[347–351] The I148M polymorphism influences the severity of steatosis and fibrosis in subjects with NAFLD,[352–354] and is associated with increased serum levels of enzymes that are markers of liver damage.[347,351,355] NAFLD is tightly linked to insulin resistance, obesity, and the metabolic syndrome,[356–357] and several studies show a positive relationship between the presence of the I148M variant and obesity.[358–359] However, despite its relationship to NAFLD and obesity, the I148M variant is not associated with alterations in

insulin sensitivity.[348–351] Thus, it appears that variant PNPLA3 may couple NAFLD to the development of insulin resistance. Although less well documented, the I148M variant of PNPLA3 is also associated with alcoholic fatty liver disease.[360]

15.8. HSL polymorphisms

The most widely studied polymorphism in *HSL* is a common single base pair change (T/C) in intron 4 of the *HSL* promoter. Reporter gene assays reveal that the -60G construct has ~40% lower activity than the -60C construct, suggesting that the -60G SNP results in a lower *HSL* expression.[361] The -G60 variant is associated with lower fasting insulin levels and improved insulin sensitivity in several populations.[362–363] Reduced serum FFA levels and increased waist circumferences in subjects with the -G60 variant have also been reported.[363–364] Thus, in general, the -G60 variant appears to be associated with changes in insulin sensitivity and body mass, and may be involved in the interaction between these variables.

Several studies have linked a dinucleotide repeat polymorphism within intron 6 of *HSL* (HSLi6) with various metabolic outcomes. Women who are homozygous for HSLi6 have increased BMI, body fat mass and serum TAG, and male homozygotes have elevated fat mass and waist-to-hip ratios.[365] In agreement, HSLi6 is associated with obesity, type 2 diabetes and impaired catecholamine-induced lipolysis in abdominal adipocytes.[366–367] Other variants of HSL are also associated with elevated serum cholesterol levels[368–369] and abdominal obesity.[370]

16.0. TAG metabolism and intracellular signaling

Both the synthetic and the degradative pathways of TAG metabolism produce lipid molecules that are known to initiate signaling pathways when released from phospholipids: phospholipase A produces LPA, phospholipase C produces DAG, and phospholipase D produces PA. A major unanswered question is whether the LPA, PA, and DAG produced as intermediates in TAG synthesis, or the FA, DAG and MAG that result from TAG lipolysis are capable of initiating signaling pathways. Further, do changes in rates of TAG synthesis or hydrolysis alter cellular amounts of acyl-CoA precursors and products, and of related lipids like ceramide, all of which are potential activators of signaling pathways or ligands for transcription factors? In this section, we will summarize the current evidence for signaling effects in insulin resistance and other pathological events.

16.1. Insulin resistance

Increased TAG content in liver, pancreatic β-cells, and muscle is highly associated with impaired insulin-stimulated glucose metabolism, and both human and animal studies support the idea that this impairment results from actions of lipid intermediates on signaling pathways.[371] The major suspected candidates for these actions include fatty acyl-CoAs, DAG, and ceramide, as well as fatty acid oxidation products and oxidized lipids. Multiple studies have supported the idea that specific lipids, either synthesized de novo or released from intracellular lipid droplets, can inhibit insulin-stimulated glucose uptake or block insulin-stimulated down-regulation of hepatic glucose production. However, despite strong associations of insulin resistance with an increased tissue content of specific lipids, as well as a theoretical framework for the inhibited signaling pathway, definitive data are not available. In diabetic patients, inhibiting adipocyte lipolysis and decreasing FA delivery to tissues decreases muscle long-chain fatty acyl-CoA content and diminishes insulin resistance.[372] In contrast, increasing FA entry into either mouse liver or muscle by over-expressing lipoprotein lipase in the specific tissue results in tissue-specific insulin resistance

with increases in TAG, DAG acyl-CoA and ceramide.[373] Hypothesized mechanisms include inhibiting the PI3 kinase pathway via the DAG activation of protein kinase C isoforms.

One reason for the inconclusive nature of current studies is our lack of information about the location of the putative lipid signals. For example, the synthesis of acyl-CoA occurs on numerous intracellular membranes, but the resulting acyl-CoAs do not appear to be free to interact with all downstream pathways, implying that they exist in specific, poorly mixing pools.[374] Measuring the cellular content of DAG is problematic, because most DAG is probably sequestered within lipid droplets where it cannot interact with signaling protein kinase C. Finally, the potential for signaling by LPA and PA has not been well evaluated.

16.1.1. Relationship of cellular TAG and DAG content to insulin resistance—
Strong support for the effect of lipid intermediates on insulin signaling comes from studies of $Gpat1^{-/-}$ mice. In the absence of $Gpat1$, livers of mice fed a high fat safflower oil diet for 3 weeks retain insulin sensitivity, whereas livers from control mice become insulin resistant. [375–376] In this model, compared to wildtype controls, the insulin sensitive $Gpat1^{-/-}$ mice have lower hepatic content of DAG and lack of activation of protein kinase Cε (PKCε), as determined by the relative amount of membrane-associated PKCε. The hepatic content of GPAT's substrate, acyl-CoA, is 2-fold higher in the $Gpat1^{-/-}$ livers, thus eliminating this lipid intermediate as a contributor to the insulin resistance. Further, GPAT's LPA product is lower than in the control mice. Conversely, adenovirus-mediated over-expression of $Gpat1$ in rat liver causes hepatic insulin resistance within 5–7 days, associated with an increase in DAG and activation of PKCε.[47] In this study, the overexpressing $Gpat1$ rats had a 2.5-fold higher hepatic glucose output than controls during a hyperinsulinemic-euglycemic clamp. There was little evidence for hepatic inflammation playing a role in the development of insulin resistance, because hepatic NF-kappaB activity is 50% lower in adenovirus-$Gpat1$-treated rats, and the expression of TNFα and interleukin-β were unchanged. A similar study was performed in mice in which an adenoviral construct over-expressed either $Gpat1$ or a catalytically inactive variant D235G (see motif I in Table 1).[377] Hepatic TAG and DAG content increased dramatically in this model, but insulin resistance was not tested. Although these studies strongly implicate the pathway of TAG synthesis in producing lipid intermediates that alter insulin signaling, again however, the data are associative and indirect and do not consider potential changes in TAG hydrolysis.

Another association between DAG accumulation and insulin resistance occurred in mice fed a 60% fat diet for 10 weeks. Insulin-stimulated glucose oxidation in the mouse hearts decreased, and insulin resistance was attributed to elevated DAG because other potential lipid modulators (acyl-CoA, ceramide, TAG) did not accumulate.[378] In this model, GPAT activity increased, $Dgat2$ mRNA decreased, PKCα translocated to membranes, and p70S6K phosphorylation increased. Similarly, in skeletal muscle, overexpression of PPARγ coactivator (PGC-1α) increased FA uptake and incorporation into TAG, with increases in the expression of $Dgat1$ and $Gpat1$, as well as increased DAG and PKC activation that inhibited IRS-1 and diminished insulin-stimulated glucose uptake.[379]

Not all increases in TAG accumulation result in insulin resistance. Female $Gpat1^{-/-}$ mice fed a high fat and high sucrose diet for 4 months develop impaired glucose tolerance,[52] however, DAG was not measured, and the hepatic content of TAG increased in the $Gpat1^{-/-}$ mice fed the obesogenic diet, although TAG content remained considerably lower than in wildtype controls fed this diet. More strikingly, mice overexpressing $Dgat2$ either 1.5-fold or 3.5-fold in mouse liver had 5- or 17-fold increases, respectively, in hepatic TAG content, as well as in DAG, ceramide, and acyl-CoAs, but no changes occurred in insulin sensitivity or in measures of inflammation or ER stress.[327] Similar results are observed with hepatic overexpression of $Dgat1$,[327] and when DGAT1 is overexpressed in adipocytes and

macrophages, the mice become obese when fed a high fat diet and their macrophages contain more TAG, but macrophage inflammatory reaction is diminished, and the mice are protected from insulin resistance.[380] Further, when these DGAT1-expressing macrophages are transplanted into wildtype mice, protection from insulin resistance is maintained. In another study with antisense oligonucleotide-mediated knockdown of *Cgi-58*, the mice are more glucose tolerant and insulin sensitive despite elevated hepatic TAG, DAG and ceramide levels, although these lipids may be sequestered within the accumulating lipid droplets.[381] These disparate studies suggest the presence or absence of an as yet unidentified lipid mediator that is present when *Gpat1*, but not *Dgat*1, is overexpressed.

Increasing the sequestration of membrane-destructive FA and acyl-CoA can be protective. In mice that develop a severe cardiomyopathy after cardiac overexpression of acyl-CoA synthetase-1, overexpression of *Dgat1* (via the alpha-myosin heavy chain promoter) causes DGAT activity and TAG content to double, but diminishes the heart content of ceramide, DAG, and FA 20–35% and improves cardiac function.[382] A similar result is observed in skeletal muscle.[383] In mice overexpressing *Dgat1* in skeletal muscle (via a creatine kinase promoter) and fed a high fat diet for 2 months, skeletal muscle, but not liver, shows improved insulin sensitivity and more efficient FA oxidation.[383] This result was interpreted to mean that the improved insulin sensitivity occurred because enhanced DGAT1 activity in skeletal muscle reduces excess DAG and its activation of PKC and JNK, thereby reducing the inactivating serine phosphorylation of IRS-1 and enhancing GLUT4-mediated uptake of glucose. The opposite effect, however, is observed with overexpression of *Dgat2* in skeletal muscle (again via the alpha-myosin heavy chain promoter).[384] Compared to wildtype controls, these transgenic mice store more TAG and less DAG in glycolytic muscle, but have increases in ceramide and long-chain acyl-CoAs. In contrast to skeletal muscle *Dgat1* transgenic mice, transgenic *Dgat2* mice are insulin resistant and have inhibited IRS-1/PI3-K and PKC-λ activity. Further, the *Dgat2* mice also have whole-body glucose intolerance and insulin resistance. It is not clear why *Dgat1* and *Dgat2* overexpression should result in totally different responses unless they are altering different signaling pools.

On the lipolytic side, because ATGL enhances cellular TAG turnover and, therefore, the content of lipid intermediates, it is not surprising that manipulating ATGL significantly affects insulin sensitivity. Mice lacking *Atgl* have enhanced whole-body glucose tolerance with insulin sensitivity, despite ectopic lipid accumulation in muscle, heart and liver.[166] $Atgl^{-/-}$ mice also have increased 2-deoxyglucose uptake in muscle and liver and an elevated respiratory quotient, indicating that these tissues use glucose at the expense of FA oxidation,[166,385] $Atgl^{-/-}$ mice have reduced adipose lipolysis and serum free FAs; thus, a reduction in FA availability is likely to play a key role in the shift in substrate oxidation.[166] Despite improved whole-body insulin sensitivity, tissue-specific insulin signaling varies in *Atgl* null mice; insulin signaling is reduced in liver and brown adipose, but increases in muscle and white adipose.[385] However, ex vivo studies on insulin signaling could not recapitulate these findings, suggesting that systemic effects of *Atgl* absence, such as reduced serum RBP-4 or related adipokines, could play a significant role in the observed effects of *Atgl* in muscle.[385] *Atgl* null mice have increased DAG content in muscle, possibly resulting from reduced DGAT2 expression and, therefore, reduced DAG reacylation, together with unaltered ceramides and decreased acyl-CoAs.[385] Clearly, additional studies are needed to determine the role of ATGL or other TAG lipases in the formation of lipid intermediates and the etiology of insulin resistance.

16.2. Contribution of LPA and PA to cellular signaling

LPA is a ligand for at least 8 different G-protein coupled cell-surface receptors,[386] and may also activate PPARγ.[387] Intracellular LPA may also activate LPA1 receptors on nuclear membranes to stimulate proinflammatory gene expression.[388–389] PA, predominantly

derived as a product of phospholipase D action on the plasma and mitochondrial membranes,[390] also initiates intracellular signaling pathways, including those of mTOR, Raf-1, and others,[390–393] but it has not been established whether the PA synthesized by AGPAT can do this. GPAT3 (LPAAT-Θ) overexpression in HEK293T cells increases phosphorylation of the mTOR target S6 kinase on Thr 389 and eukaryotic translation initiation factor 4E-binding protein 1 on Ser 65, suggesting that LPA or PA produced by the TAG synthetic pathway might activate mTOR.[394]

The most convincing evidence that de novo lipogenesis produces signaling PA comes from studies of the peripheral neuropathy that develops in *fld* mice that are deficient in *Lpin1*.[112,114–115] The neuropathy results from elevated PA levels which activate MEK/Erk signaling.[115] PA has not been measured in Majeed Syndrome resulting from defective *LPIN2*.

It has not been shown that LPA synthesized by GPAT can be secreted, and GPAT is not believed to contribute to external LPA signaling pathways. However, internal signaling resulting from GPAT activity remains a possibility because of the relationship between GPAT and cell proliferation. For example, GPAT4 (called TSARG7) is strongly expressed in spermatocytes during meiosis,[395] and, when GPAT4 is transfected into the mouse spermatogonial cell line, GC-1spg, the rate of cellular proliferation increases, together with a decrease in the percentage of cells in the G0/G1 phase and an increase in cells in the S phase.[395] In addition, one might reinterpret the loss- and gain-of-function studies with *Gpat1* light of their manipulation of LPA levels in cells. Thus, livers from *Gpat1*$^{-/-}$ mice might have remained insulin sensitive because LPA content was low,[375] whereas overexpression of *Gpat1* increased LPA content and resulted in insulin resistance.[47]

A knockout of *Agpat* should similarly increase the levels of its substrate, LPA. The siRNA-mediated knockdown of *Agpat1*, but not *Agpat2*, disrupts the myogenic program of C2C12 cells with a 50% reduction in the transcriptional activator myogenin and a 70% down-regulation of myosin heavy chain expression.[83] The effect is rescued by replacing *Agpat1*. Transfection with *Agpat1* shows localization to areas of active actin polymerization. Although PA levels do not increase, the method used to measure them was relatively insensitive; it is possible that the changes observed are, in fact, due to PA-mediated signaling.

Stable overexpression of *Agpat1* or *2* in A549 or ECV304 cells synergistically enhances production of cytokines like IL-6 and TNFα by as much as 10-fold after the cells are stimulated.[80] Increases in *Agpat2* mRNA during fetal epidermal development also suggest a possible signaling role.[92] Further, it was suggested that over-expression of *Agpat2* might increase certain cytokines.[396] Because *Agpat2* mRNA is elevated in some human tumors (e.g.[397–398]), it has been proposed that PA synthesized by AGPAT could mediate oncogenesis. In fact, targeted small molecule inhibition of AGPAT2 diminishes cell proliferation and induces tumor cell apoptosis,[399] in conjunction with decreasing signaling via Ras/Raf/Erk and PI3K/Akt pathways.[400] Thus, small molecule inhibitors that cause apoptosis have been tested for a possible therapeutic role.[399–402]

16.3. MGL and endocannabinoid signaling

Unlike other enzymes involved in TAG catabolism, MGL has been studied more for its role in cell signaling that its function as a lipolytic enzyme. This emphasis has arisen because MGL catalyzes the breakdown of *sn*-2-20:4-glycerol (2-AG), a member of the endocannabinoid family. Endocannabinoids signal through receptors to affect a wide range of physiological processes including energy metabolism, inflammation, food intake and

behavior. By promoting the degradation of 2-AG and subsequently attenuating receptor binding, MGL may play a critical role in endocannabinoid signaling.[403]

16.4. Signaling by FA and acyl-CoA originating from TAG hydrolysis

Because acyl-CoAs and FAs are the substrates and products of TAG anabolism and catabolism, respectively, TAG metabolism has a large influence on the content and composition of their cellular pools. The relevance of this regulation becomes apparent when the bioactive properties of FAs and acyl-CoAs are considered. These two lipid classes alter metabolic and signaling pathways through a wide range of allosteric, covalent, and transcriptional mechanisms. Additionally, bioactive effects are highly dependent on FA or acyl-CoA chain length and saturation. However, few studies have attempted to dissociate the intracellular sources (i.e. consumed or produced by TAG metabolic reactions) of FAs or acyl-CoA that might contribute to their signaling roles. Our lack of understanding in this area is likely due to the fact that each anabolic or catabolic reaction in TAG metabolism also results in the formation or degradation of glycerolipid intermediates. Because many of these glycerolipid intermediates may also act as signaling molecules, it is often difficult to attribute an observed signaling effect to a specific molecule.

Recent studies have focused on signaling as a consequence of TAG hydrolysis. DNA microarrays of tissues from *Atgl* and *Hsl* knockout mice reveal divergent effects of these two enzymes on gene expression patterns.[404] Although deficiency of either lipase decreases the expression of oxidative genes in brown adipose, only ATGL deficiency suppresses oxidative gene expression in other tissues like heart and skeletal muscle. In *Atgl* and *Hsl* null mice, numerous other biological processes, such as cell growth and death, lipid synthesis, and signal transduction, are also differentially modulated in specific tissues. These results suggest that lipolysis, particularly by ATGL, may play a role in transcriptional control of FA catabolism. In support of this hypothesis, *Atgl* overexpression in adipocytes results in the expression of genes involved in FA oxidation and oxidative metabolism, including PPARα and –δ and results in enhanced fatty oxidation.[185] Hepatocytes that overexpress *Atgl* have higher PPARα activity and target gene expression, whereas knockdown of hepatic *Atgl in vivo* suppresses the expression of PPARα target genes,[173,405] as does the knockdown of CGI-58, the putative activator of ATGL.[381] Because FAs are known ligands for PPARα, these data suggest that ATGL-mediated TAG hydrolysis provides an important source of PPARα ligands. However, administering the PPARα agonist fenofibrate to mice with suppressed hepatic ATGL does not increase the expression of PPARα target genes to levels in control mice treated with the same drug.[173] Thus, the exact mechanism through which ATGL or its lipid products regulate oxidative gene expression remains unknown. In addition to the PPAR family, FA and/or acyl-CoA are known to regulate a host of transcription factors that govern energy metabolism. These transcription factors include SREBP, ChREBP, RXR, CREBh and NF-κB (reviewed by[406–407]). Despite this broad regulation of transcription, it is not known how FA or downstream metabolites that originate from TAG hydrolysis contribute to this process

In addition to regulating gene expression, FA and acyl-CoA may indirectly regulate energy metabolism via effects on AMPK. Once hydrolyzed, FAs must be activated to form acyl-CoAs before they can enter reesterification or oxidation pathways. In the activation reaction catalyzed by acyl-CoA synthetases (ACSL), ATP is hydrolyzed to AMP plus orthophosphate. Thus, during times of enhanced lipolysis, ACSL activity could modulate cellular levels of ATP and AMP. One example is that of 3T3-L1 adipocytes, in which isoproterenol-stimulated lipolysis decreases cellular ATP levels while increasing AMP concentration and AMPK activity.[408] Inhibiting acyl-CoA synthetase activity with triacsin C attenuates the changes in nucleotide levels and blocks the activation of AMPK. This study demonstrates a novel mechanism that links FA released from lipolysis to the regulation of

cellular energy metabolism mediated by phosphorylation. Somewhat problematic, however, is the fact that the same activation of FA by ACSL occurs when large amounts of FA enter adipocytes after a meal. The major fate of these FAs is their incorporation into TAG, a pathway that should be down-regulated by AMPK.[409–410] However, as stated above, TAG hydrolysis may release a distinct pool of FA and may produce AMP that specifically activates AMPK. FA and acyl-CoA also regulate PKC[411] and protein phosphatase 2a,[412] although the role of hydrolyzed products in their regulation has not been studied.

As noted above, FA released from the hydrolysis of DAG can feedback to inhibit HSL, thereby providing an example of how FA alters metabolism through allosteric regulation. In addition, many other enzymes are regulated by FA and acyl-CoA including hexokinase,[413] pyruvate dehydrogenase,[414] acetyl-CoA carboxylase,[415] and phosphofructokinase.[416] Acyl-CoAs also modify ATP-sensitive K^+ channels to affect biological processes, like the neurological control of food intake and insulin secretion from β-cells.[417–419] Despite the absence of studies testing the importance of TAG hydrolysis on these enzymes and pathways, hydrolyzed FA could play key roles when they are produced at high rates, as occurs during stimulated lipolysis.

17.0. Perspectives

Although the past 10 years have seen major advances, research in the area of TAG metabolism remains hampered by several technical difficulties. None of the mammalian enzymes of TAG synthesis have been studied in a purified state without epitope tags. Because the enzymes have not been crystallized, we do not understand their mechanisms of action, how they recognize and interact with their substrates and release their products, how they are oriented within membranes, or how they interact with other proteins. It is clear that the mRNA of individual isoforms is varyingly regulated physiologically by nutrients, exercise, and fasting and refeeding, as well as during transitional stages, such as adipocyte differentiation and the nutrient and maturational changes that occur between fetal and postnatal life. In addition, although studies have shown multiple post-translational variations in phosphorylation and acetylation, for the most part, the functional significance of any of these modifications remains poorly understood.

Positive steps currently in progress include the development of specific inhibitors that would allow one to differentiate between the diverse isoforms without requiring knockouts or knockdowns that often result in unforeseen compensatory effects. Although several DGAT1 inhibitors appear to reduce postprandial hyperlipidemia or fat depots in rodents,[420–422] the paucity of information currently available about the functions of the independent DGAT isoforms suggests that inhibiting drugs may have surprising, and perhaps unwelcome, effects.[423] For example, although antisense oligonucleotide knockdown of DGAT2 in obese, diabetic (*db/db*) mice improved hepatic steatosis, the mice developed worsening hepatic fibrosis.[424]

The consequences of deficient MGL or tissue-specific deficiencies of ATGL on triacylglycerol metabolism are unknown. Because ATGL knockout mice have such a striking phenotype, it is difficult to separate the contribution of ATGL in individual tissues from the indirect effects of altered concentrations of serum FA, adipokines, and other metabolites. Moreover, the substrate preferences and transcription control of ATGL and the lipases is largely unknown.

Future studies that should be undertaken include investigating the different white adipose depots in mice and people, as it has become clear that each fat depot is differently constituted in its effect on insulin resistance, the amount of its TAG stores, the accessibility of its TAG stores to lipases, and its enzyme constituents.[425–426] Information is also needed

on human variations of the enzymes of TAG metabolism and SNPS that may alter risks for obesity, hepatic steatosis, atherosclerosis, and cancer. Nonetheless, it is clear that TAG metabolism affects a multitude of biological processes, well beyond simply storing energy. It is likely that future studies will identify novel crosstalk between TAG metabolism and additional processes or pathways, as well as further defining its relationship to metabolic diseases such as obesity, diabetes and cardiovascular disease.

Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
AGPAT	acyl-CoA:1-acylglycerol-3-phosphate acyltransferase (LPAAT)
AMPK	AMP-activated kinase
ARAT	acyl-CoA:retinol acyltransferase
ATGL	adipose TAG lipase
CGI-58	comparative gene identification-58
DAG	diacylglycerol
DGAT	acyl-CoA:diacylglycerol acyltransferase
ER	endoplasmic reticulum
FABP4	fatty acid binding protein 4
G0S2	G_0/G_1 switch gene 2
GPAT	glycerol-3-phosphate acyltransferase
HSL	hormone sensitive lipase
LPA	lysophosphatidic acid
MAG	monoacylglycerol
MGAT	acyl-CoA:monoacylglycerol acyltransferase
MGL	MAG lipase
mTOR	mammalian target of rapamycin
PLIN	perilipin
NAFLD	non-alcoholic fatty liver disease
NEM	*N*-ethylmaleimide
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase (lipin)
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKC	protein kinase C
PS	phosphatidylserine
PPAR	peroxisomal proliferator-activated receptor

RQ	respiratory quotient
SREBP	sterol-regulatory element binding protein
TAG	triacylglycerol (triglyceride)
TGH	TAG hydrolase
VLDL	very low density lipoprotein

Acknowledgments

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Biographies

Rosalind Coleman received a BA *cum laude* from Harvard University and an MD from Case Western Reserve University. After her pediatric residency at New York Hospital-Cornell Medical Center and Duke University, she completed a clinical fellowship in Endocrinology and Metabolism and a postdoctoral fellowship in Biochemistry at Duke University with Robert M. Bell. As a Duke University faculty member, she diagnosed and treated children with inborn error of metabolism and continued to study enzymes of triacylglycerol metabolism. Currently in the Department of Nutrition at the University of North Carolina, she has expanded her research to encompass the roles of enzymes of triacylglycerol synthesis in whole animal studies designed to understand the development of insulin resistance and diabetes.
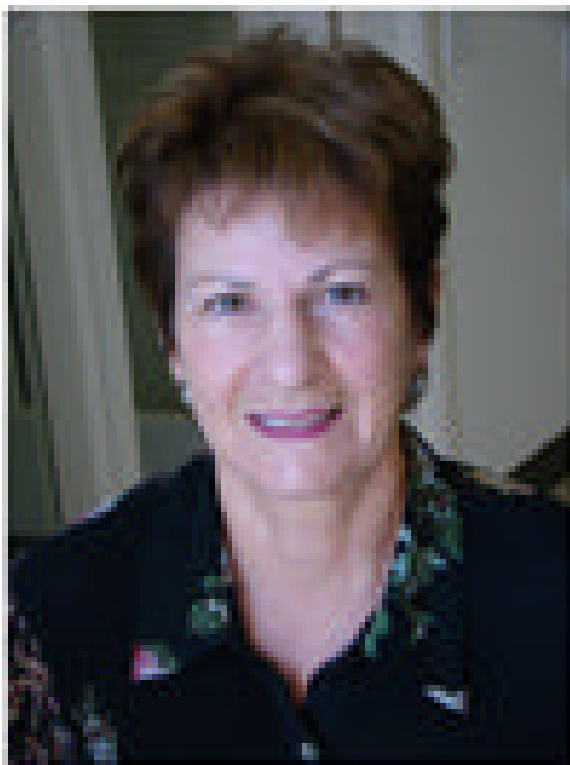
Douglas G. Mashek graduated with a B.S. in dairy science from Iowa State University in 1996. Subsequently, he received M.S. and Ph.D. degrees in animal/dairy science from Michigan State University and the University of Wisconsin-Madison, respectively. Throughout this time, his research largely focused on hepatic glucose and fatty acid metabolism and the etiology of hepatic steatosis. After a postdoctoral fellowship studying acyl-CoA synthetases in the laboratory of Dr. Rosalind Coleman (co-author) at the University of North Carolina-Chapel Hill, he was appointed in 2006 as an Assistant

Professor in the Department of Food Science and Nutrition at the University of Minnesota. The main emphasis of his research is the enzymatic regulation of intracellular fatty acid trafficking and signaling, especially as it relates to metabolic diseases such as type 2 diabetes and non-alcoholic fatty liver disease.
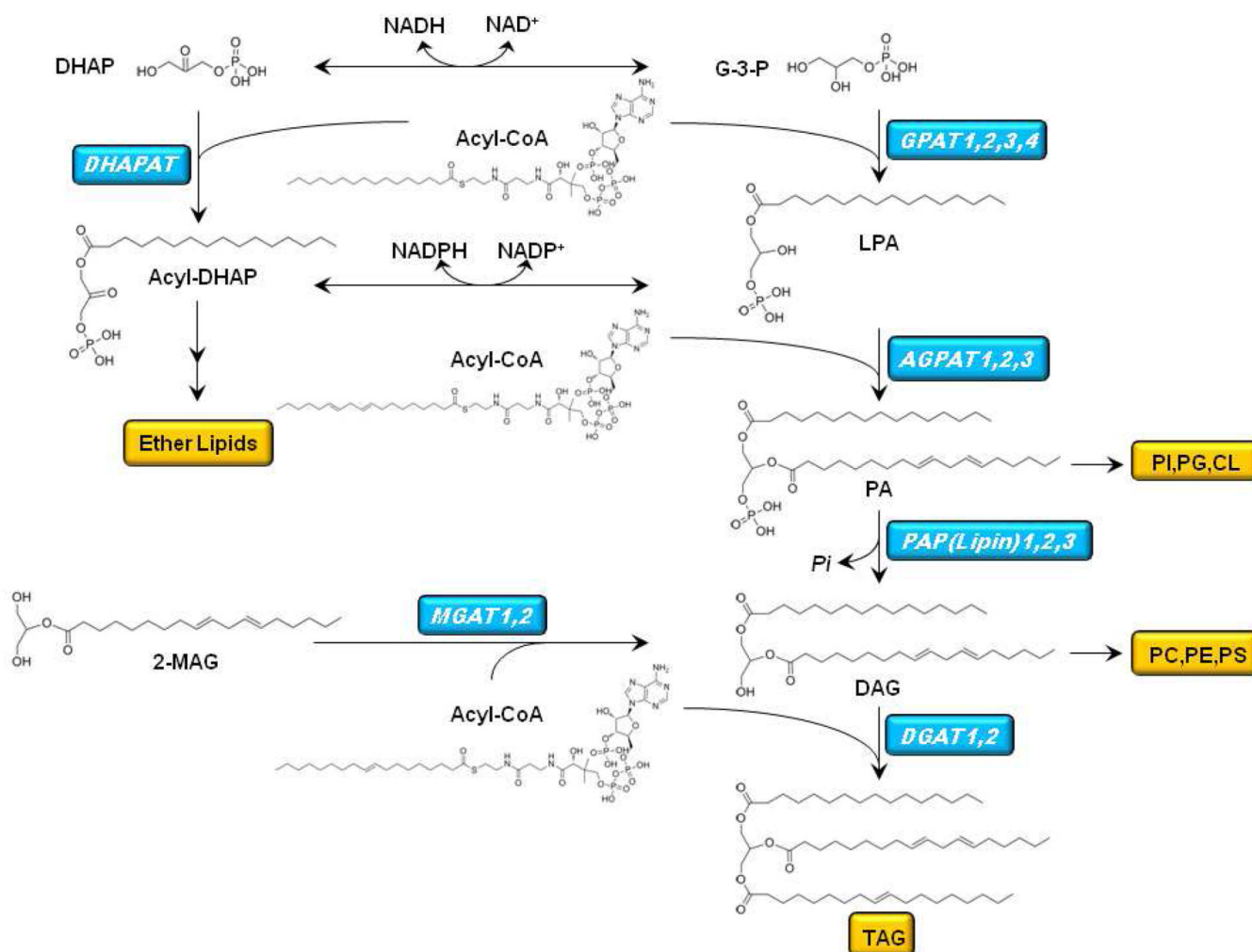
Figure 1. Pathway of glycerolipid synthesis
The biosynthesis of TAG begins with the sequential acylation of glycerol-3-phosphate (G-3-P) by *sn*-1-glycerol-3-phosphate acyltransferase (GPAT) to produce lysophosphatidic acid (LPA) and by acyl-CoA:1-acylglycerol-3-phosphate acyltransferase (AGPAT) to produce phosphatidic acid (PA). G-3-P can also be produced by the oxidation of dihydroxyacetone phosphate (DHAP), and the oxidation of acyl-DHAP produces LPA. PA is hydrolyzed by PA phosphatase (also called lipin) to form diacylglycerol (DAG). A final esterification step by DAG acyltransferase (DGAT) produces triacylglycerol (TAG). PA is also a precursor of CDP-diacylglycerol and the anionic phospholipids phosphatidylglycerol (PG), phosphatidylinositol (PI), and cardiolipin (CL). DAG is a precursor of the phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Acyl-DHAP is the precursor of the ether lipids.
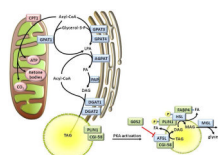
Figure 2. Integrated model of adipose TAG synthesis and lipolysis
Acyl-CoAs are oxidized in mitochondria after they have been transported into the matrix as acyl-carnitines via carnitine palmitoyltransferase-1 (CPT1). Alternatively, acyl-CoAs may be esterified to glycerol-3-phosphate by a glycerol-3-phosphate acyltransferase (GPAT) isoform, resulting in the production of lysophosphatidic acid (LPA). One or more acyl-CoA:acylglycerol-3-phosphate acyltransferase (AGPAT) isoforms uses a second acyl-CoA to esterify the *sn*-2 position of LPA to form phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) dephosphorylates PA to form *sn*-1,2-diacylglycerol (DAG). Diacylglycerol acyltransferase (DGAT) isoenzymes use a final acyl-CoA to synthesize triacylglycerol (TAG) from DAG. Upon lipolytic stimulation and protein kinase A (PKA) activation, perilipin (PLIN1) is phosphorylated, thereby promoting the release of comparative gene identification-58 (CGI-58) which recruits hormone sensitive lipase (HSL) to the lipid droplet. CGI-58 binds adipose triglyceride lipase (ATGL), facilitates the translocation of ATGL to the lipid droplet, and promotes ATGL's TAG hydrolytic activity. G0S2 inhibits ATGL, although its interaction with ATGL is not dependent upon PKA activation, as illustrated. PKA also phosphorylates HSL, thereby allowing it to move to the surface of the lipid droplet, and increasing its activity towards DAG. The interaction of HSL with fatty acid binding protein-4 (FABP4) promotes FA efflux from the lipid droplet and alleviates its product inhibition of HSL. Finally, monoacylglycerol lipase (MGL) hydrolyzes monoacylglycerol (MAG), releasing glycerol and a fatty acid (FA).
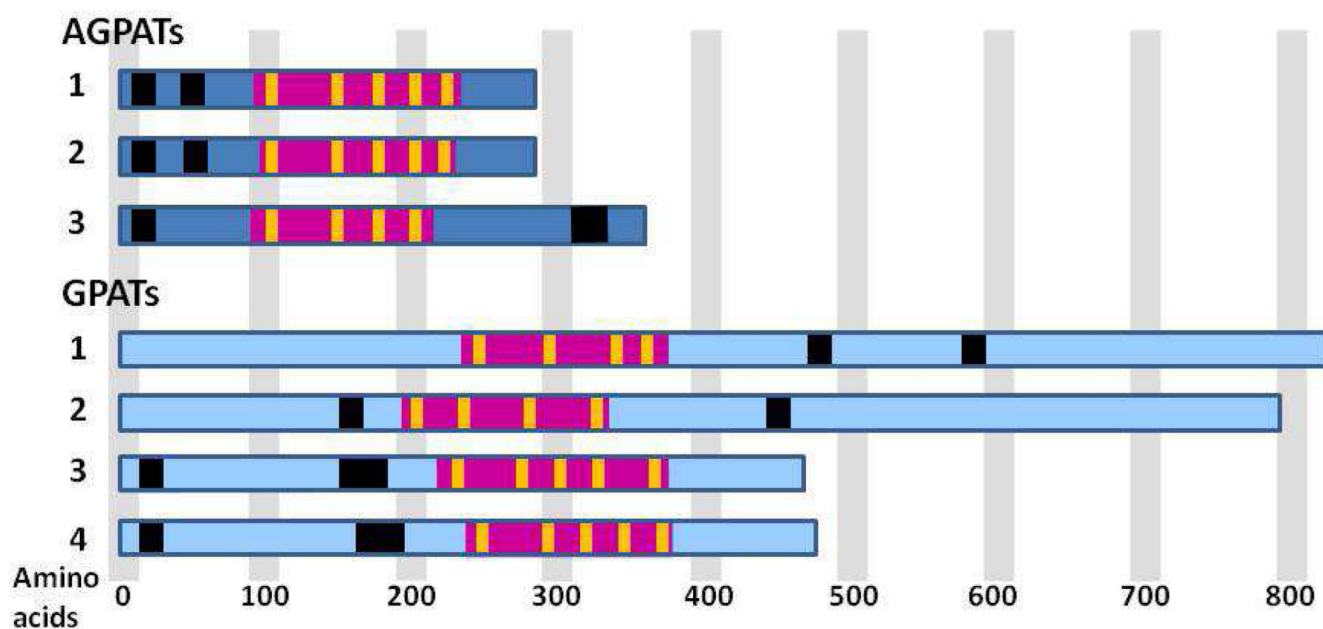
Figure 3. Predicted structures of confirmed mammalian AGPAT and GPAT isoforms
Black regions represent predicted transmembrane domains (PredictProtein; TMHMM; TMpred). Additional transmembrane domains are predicted, but are not shown because they would place sections of the active site region on opposite membrane sides. These predicted domains could interact closely with the membrane. The large rose regions represent the active site region intersected by yellow stripes to indicate the 4 or 5 conserved motifs. Only the topography of GPAT1 has been confirmed experimentally.[28] All active site regions face the cytosol.[27]

Table 1

Active site motifs in human members of the PlsC superfamily that have GPAT or AGPAT activity

	Motif I catalysis /acyl-CoA binding	Motif II G-3-P/LPA binding	Motif III G-3-P binding	Motif IV acyl-CoA binding	Motif V	Accession Number	Alias
GPAT1	PVHRSHIDY	LGGFFIRR	FLEIFLEGTRSRSGKTSCARAG	ILIIPVGISY		Q9HCL2	Gpam, mtGPAT
GPAT2	STHKTLLDS	RACSPALR	LEPPGALGPRLSALGQAWVGFV	ALLVPVAVTY		Q6NUI2.2	xGPAT[70]
GPAT3	ANHTSPIDV	CPHVWFER	PILIFPEGTCINNTSVMMFKKG	GTIHPVAIKY	VWYMPPMTREEGED	Q53EU6.2	See Table 2
GPAT4	ANHTSPIDV	CPHVWFER	PILIFPEGTCINNTSVMMFKKG	ATVYPVAIKY	VWYLPPMTREADED	Q86UL3.1	See Table 2
GNPAT	PSHRSYIDF	SGAFFMRR	PVEFFLEGTRSRSAKTLTPKFG	TYLVPISISY		O15228.1	DHAPAT
AGPAT1	SNHQSSLDL	AGVIFIDR	RVWVFPEGTRNHNGSMLPFKRG	QAQVPIVPIV	VLPPVPTEGLTPDD	Q99943.2	LPAATα
AGPAT2	SNHQSILDM	GGVFFINR	KVWIYPEGTRNDNGDLLPFKKG	QAQVPIVPVV	VLEAIPTSGLTAAD	O15120.1	LPAATβ
AGPAT3	LNHNFEIDF	TVVEGLRR	WFLLYCEGTRFTETKHRVSMEV	YHLLPRTKGF		Q9NRZ7.1	

Bolded and underlined amino acids are believed to be critical for function, based on site-directed mutagenesis and/or mutational analysis in *E. coli*,[29–30] and mouse GPAT[427] or human AGPAT1,[82] and by analysis of human disorders caused by deficiencies of AGPAT2,[36–37] dihydroxyacetone phosphate acyltransferase (GNPAT),[428] and tafazzin, which is a 1-palmitoyl-2-[14C]linoleoyl-phosphatidylcholine:monolysocardiolipin linoleoyltransferase.[35,429]

Table 2

Putative AGPAT isoforms

This Table lists all the so-called AGPAT proteins because several have multiple aliases and only a few have been confirmed to have LPA acyltransferase activity.

	Confirmed activity	Other names	GenBank No. (human)	Amino acids (human)	Comments
AGPAT1	LPAAT[79–80]	LPAATα	Q99943.2	283	See text
AGPAT2	LPAAT[80]	LPAATβ	O15120.1	278	Mutations cause congenital lipodystrophy[36]
AGPAT3	LPAAT		Q9NRZ7.1	376	See text
AGPAT4			Q9NRZ5.1	378	
AGPAT5			Q9NUQ2.3	364	
AGPAT6	GPAT4[19–20]	LPAAT-zeta TSARG7	Q86UL3.1	456	See text
AGPAT7	LPE acyltransferase> LPSAT, LPCAT; no GPAT or AGPAT activity[430]	LPEAT2, AYTL3, LPCAT, LPAAT	Q643R3.1	524	ER location[431]
AGPAT8	monolysoCL-AT[432] LPAAT[433]	LYCAT, LCLAT1, ALCAT1	Q6UWP7.1	414	
AGPAT9	GPAT3[71]	GPAT3, AGPAT8, LPAAT-theta AGPAT10[72]	Q53EU6.2	434	See text
AGPAT9	LPCAT1[434–435] LPAAT[436]	LPCAT1	Q1HAQ0.2	534	Increased in colon cancer[437]
AGPAT11	LPCAT2 & lyso platelet-activating factor acetyltransferase[438] LPAAT[439]	LPCAT2	Q7L5N7.1	544	Increased in breast, cervical, and colorectal cancer tissues[439]