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Lipins, Lipinopathies, and the Modulation of Cellular Lipid Storage and Signaling

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Summary

Members of the lipin protein family are phosphatidate phosphatase (PAP) enzymes, which catalyze the dephosphorylation of phosphatidic acid to diacylglycerol, the penultimate step in TAG synthesis. Lipins are unique among the glycerolipid biosynthetic enzymes in that they also promote fatty acid oxidation through their activity as co-regulators of gene expression by DNA-bound transcription factors. Lipin function has been evolutionarily conserved from a single ortholog in yeast to the mammalian family of three lipin proteins—lipin-1, lipin-2, and lipin-3. In mice and humans, the levels of lipin activity are a determinant of TAG storage in diverse cell types, and humans with deficiency in lipin-1 or lipin-2 have severe metabolic diseases. Recent work has highlighted the complex physiological interactions between members of the lipin protein family, which exhibit both overlapping and unique functions in specific tissues. The analysis of “lipinopathies” in mouse models and in humans has revealed an important role for lipin activity in the regulation of lipid intermediates (phosphatidate and diacylglycerol), which influence fundamental cellular processes including adipocyte and nerve cell differentiation, adipocyte lipolysis, and hepatic insulin signaling. The elucidation of lipin molecular and physiological functions could lead to novel approaches to modulate cellular lipid storage and metabolic disease.

1. Introduction and Overview

Glycerolipids serve a variety of vital cellular functions. Phospholipids are a major component of cellular plasma membranes and intracellular membranes. Triacylglycerol (TAG) is critical for long-term energy storage in adipose tissue and provides a source of fatty acids for oxidation in skeletal muscle and cardiac muscle. TAG is also essential for the production of very low-density lipoproteins in the liver and chylomicrons in the intestine, as well as for the production of milk by mammary epithelial cells [1, 2]. Derangements in TAG synthesis and storage, including those occurring in the setting of obesity and lipodystrophy,

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are highly relevant to public health because they can increase susceptibility to cardiovascular disease, diabetes mellitus, and dyslipidemia [3-6].

In eukaryotes, TAG is synthesized by two pathways—the glycerol phosphate pathway and the monoacylglycerol pathway. The glycerol phosphate pathway is the primary pathway in most cell types [1, 2]. The acylation of glycerol 3-phosphate occurs in a stepwise fashion, with each acyl group added by a distinct class of acyltransferases. Lipins catalyze the penultimate step in the glycerol phosphate pathway—converting phosphatidic acid (PA) to diacylglycerol (DAG) (Fig. 1). By contrast, TAG synthesis in the small intestine is thought to occur predominantly by the monoacylglycerol pathway, which uses monoacylglycerol produced by the hydrolysis of dietary fats [7, 8].

The lipin gene family was first identified through studies of a spontaneous mutant mouse strain (i.e., the fatty liver dystrophy mouse) that exhibited dysregulated TAG storage in multiple tissues, leading to lipodystrophy and neonatal fatty liver [9, 10]. The cloning and sequencing of *Lpin1* (encoding lipin-1) also made it possible to identify two related mammalian genes, *Lpin2* and *Lpin3*, as well as orthologous genes in organisms as evolutionarily distant as yeast [11]. In the ensuing decade, numerous studies of lipin protein function have been performed in a wide range of experimental organisms. In addition, human diseases caused by mutation in *LPIN1* or *LPIN2* have been identified [12, 13]. Because of the critical roles that lipin proteins play in lipid homeostasis and metabolism, it is important to better define their molecular and physiological functions. Several excellent reviews on mammalian and yeast lipin protein function have appeared in the past few years [14-22]. Here, we will briefly highlight some aspects of the history of the lipin field and well-established molecular functions of the lipin proteins, and then will focus on recent studies of mouse and human lipin deficiencies that have provided insights into the physiological activities of, and relationships between, different members of the lipin family.

2. Lipin protein molecular functions

2.1. Lipin phosphatidate phosphatase (PAP) activity

The activities of the enzymes in the glycerol phosphate pathway [glycerol-3-phosphate acyltransferases (GPATs), acylglycerol-3-phosphate acyltransferases (AGPATs), acyl coenzyme-A:diacylglycerol acyltransferases (DGATs) and phosphatidate phosphatases (PAPs)] have been studied for decades [1, 2]. PAP activity, which converts PA to DAG, was first demonstrated by Eugene Kennedy in seminal work that established the connection between PA synthesized from glycerol and the downstream synthesis of phosphatidylcholine and TAG [23]. Subsequent attempts to purify PAP activity from mammalian liver were unsuccessful, likely thwarted by instability of the protein and the requirement at that time for large amounts of protein for sequence determination. Nevertheless, studies of PAP activity in rat liver revealed important features of the enzyme. Particularly noteworthy was the fact that PAP activity resides in the cytosol until stimulated with fatty acids, which causes translocation to internal membranes [24]. That characteristic distinguishes PAP from all of the acyltransferase enzymes in the glycerol phosphate pathway, which are integral membrane proteins.

The molecular cloning of members of the GPAT, AGPAT, and DGAT protein families in the 1990s provided insights into the physiology and biochemistry of these protein families [1, 2]. The identity of the PAP activity in the glycerol phosphate pathway was revealed only recently, and involved both classical biochemical purification studies in yeast and mouse genetics. In 2006, Carman and colleagues purified PAP activity from *Saccharomyces cerevisiae* and obtained protein sequence [15, 25]. The sequence revealed that the yeast PAP, Pah1p, corresponded to the yeast ortholog of the mammalian lipin family [11].

Whereas yeast and invertebrates have a single lipin ortholog, plants have two lipin genes, and mammals have three, encoding lipin-1, lipin-2, and lipin-3 (Fig. 2).

Following the determination that yeast and human lipin-1 proteins exhibit PAP activity [25], it was shown that mouse lipin-1, lipin-2, and lipin-3 are all PAP enzymes, requiring Mg^{2+} for activity and exhibiting sensitivity to inhibition by N-ethylmaleimide [26]. Of the three family members, lipin-1 has substantially higher PAP specific activity than lipin-2 and lipin-3 [26]. It was also shown that the activity of all three lipin proteins is specific for PA as a substrate, with no activity against other lipid phosphates [26]. At present, it is unclear whether there are differences in substrate preferences among the mammalian lipin isoforms, and this is a fertile area for future research. It should be noted that a family of proteins that are structurally unrelated to lipin proteins, known as lipid phosphate phosphatases, also exhibit PAP activity. Importantly, however, lipid phosphate phosphatases are localized to the plasma membrane and do not contribute to cellular TAG or phospholipid biosynthesis [27, 28]. Biochemically, the two types of PAP enzymes are distinguishable by the fact that lipid phosphate phosphatases act on a wide variety of substrates in addition to PA, do not require Mg^{2+} for activity, and are resistant to N-ethylmaleimide inhibition.

2.2. Lipin-1 protein nuclear activity

The acyltransferase enzymes of the glycerol phosphate pathway are integral ER membrane proteins [1, 2]. By contrast, lipin proteins reside in the cytosol and transiently associate with the ER membrane during the PAP enzyme reaction. In addition, lipin proteins can localize to the nucleus [11, 29-33]. Exciting clues about lipin-1 nuclear function were first provided by Finck and colleagues, who demonstrated that lipin-1 interacts with the nuclear factor PPAR and the transcriptional coactivator PGC-1 to induce fatty acid oxidation genes in response to fasting [34]. Lipin-1 also activates PPAR and myocyte enhancer factor 2 (MEF2), and represses nuclear factor of activated T-cells c4 (NFATc4) [30, 35, 36]. Lipin-1 is present in complexes associated with the promoters of some target genes, but not others, and it appears that the mechanisms of lipin-1 co-regulatory activity may differ with individual transcription factors [34, 35, 37]. Most data are consistent with a model in which lipin-1 serves as a molecular scaffold for binding of histone modifiers [18]. In this model, the differential interaction of lipin-1-containing complexes with histone deacetylases or histone acetyltransferases may determine whether the interaction results in gene repression or activation.

There are important outstanding questions regarding the relationship between lipin-1 PAP and transcriptional activities. Studies in cultured cells indicate that lipin-1 co-activator/co-repressor activity is independent of PAP activity [34, 35]. However, since many of these studies were performed by overexpressing catalytically inactive lipin-1 protein in wild-type cells, it is possible that the endogenous wild-type lipin-1 protein is also recruited (directly or indirectly) to the transcriptional complexes, and could provide PAP activity required for the activator or repressor activity. In addition, lipin-1 may form homo-oligomers [38] (see section 2.3.3), raising the possibility that catalytically inactive and wild-type lipin-1 proteins could interact in co-regulatory complexes. Interestingly, lipin-1 regulates the nuclear abundance and promoter activity of the sterol response element binding protein 1 (SREBP1) lipogenic transcription factor, and the lipin-1 PAP activity is critical for this function [39]. Further elucidation of the lipin transcriptional regulatory mechanism is important because it likely influences important metabolic processes including the regulation of hepatic fatty acid oxidation genes [34, 37], adipocyte differentiation [36], and adipocyte inflammatory gene expression [35].

2.3 Lipin protein functional domains

Several structural features that are important for lipin protein function have been defined. All lipin family members contain extended stretches of highly conserved amino acid sequences known as the N-LIP and C-LIP domains, which are present at the amino- and carboxyl-terminal regions of the mammalian proteins, and occur at other sites in lipin proteins from other species (Fig. 2) [11]. The C-LIP domain contains the PAP enzyme active site motif, DXDXT, which also identifies these proteins as members of the haloacid dehalogenase superfamily [25, 40]. Mammalian and *Drosophila* lipin proteins also have an LXXIL motif in the C-LIP domain, which has been shown to mediate the interaction of lipin-1 with transcription factors and coactivators [34] (see section 2.2). Lipins from mammals and invertebrates, but not plants and yeast, contain a stretch of positively charged residues near the N-LIP domain. This motif is required for the translocation of lipin-1 to the nucleus [31, 33] and for lipin-1 binding to PA [33]. However, nuclear localization likely requires a second structural motif or partner molecular; only a fraction of lipin-1 localizes to the nucleus under most conditions [31], and lipin-2 appears to be excluded from the nucleus despite possessing the polybasic motif [38].

2.4. Lipin-1 isoforms, protein modifications, and subcellular localization

2.4.1. Properties of lipin-1 isoforms generated by alternative splicing—The genes encoding human and mouse lipin-1 undergo alternative splicing to generate multiple protein isoforms known as lipin-1_Δ, lipin-1_Δ, and lipin-1_Δ [32, 41, 42]. The 890-amino acid sequence of the human lipin-1_Δ isoform (891 amino acids in mouse) gains an additional in-frame exon of 36 amino acids (human) or 33 amino acids (mouse) to generate the lipin-1_Δ isoforms, or a different in-frame exon of 26 amino acids to generate the human and mouse lipin-1_Δ isoforms. The inclusion of the lipin-1_Δ or lipin-1_Δ-specific exons does not alter the PAP active site, the nuclear localization motif, coactivator motifs, or other known functional sites within the lipin-1 protein sequence. PAP activity studies on the three lipin-1 isoforms purified from an *E. coli* expression system indicated that the lipin-1_Δ and lipin-1_Δ isoforms have greater PAP specific activity by an order of magnitude than lipin-1_Δ, but that lipin-1_Δ has higher affinity for the PA substrate [42].

Intriguing observations have been reported regarding functional differences among lipin-1 isoforms, and the regulation of *LPIN1/Lpin1* alternative splicing. Using an assay that allows the direct comparison of lipin-1_Δ and lipin-1_Δ mRNA levels across a panel of mouse tissues, it was determined that lipin-1_Δ is more prominent in brain, spleen, and preadipocytes, whereas lipin-1_Δ is more prevalent in liver, heart, kidney, and mature adipocytes; levels of the two isoforms were similar in several other tissues (skeletal muscle, small intestine, lung, adrenal, ovary, and thymus) [32]. Thus far, the expression of the lipin-1_Δ isoform has been assessed in a limited number of tissues. It was shown to be expressed in human brain, but not liver, small intestine, or skeletal muscle [43]. There is some evidence that the three lipin-1 isoforms also exhibit differential subcellular localization. In adipocytes, lipin-1_Δ tends to have predominantly nuclear localization, whereas lipin-1_Δ is more often cytosolic [32]. The lipin-1_Δ isoform has been localized to lipid droplets, and expression of recombinant lipin-1_Δ in COS-7 cells led to the appearance of small, clustered lipid droplets compared to larger, dispersed lipid droplets in the absence of this protein [43]. However, some caution is warranted in interpreting studies of lipin-1 isoform localization because antibodies to discriminate between different lipin-1 isoforms are not available and all work has been performed by protein overexpression in cultured cells.

Lipin-1_Δ and -1_Δ isoforms may have distinct functions in adipocyte lipid metabolism. When lipin-1_Δ-deficient adipocytes were complemented with these isoforms individually, lipin-1_Δ promoted the expression of adipogenic genes (PPAR_γ, C/EBP_β, aP2), whereas lipin-1_Δ

promoted the expression of lipogenic genes (fatty acid synthase, acetyl CoA carboxylase, stearoyl CoA desaturase, diacylglycerol acyltransferase) [32]. Evidence for distinct effects of lipin-1 and lipin-1 isoforms has also emerged from human studies. For example, increased expression of lipin-1, but not lipin-1, in adipose tissue was associated with protection from lipodystrophy in patients undergoing antiretroviral therapy for human immunodeficiency virus [44]. Lipin-1, but not lipin-1, was induced in adipose tissue of insulin-resistant subjects in response to treatment with the insulin-sensitizing drug pioglitazone, which also promotes increased adiposity [45]. Lipin-1, but not lipin-1, levels in the liver were increased in obese patients following gastric bypass surgery [46]. It is interesting to note that alternative lipin-1 splice variants are conserved in species beyond human and mouse, including pig and fruit fly. In pigs, the ratio of porcine lipin-1 to lipin-1 in skeletal muscle differs between animals with high and low intramuscular fat content [47]. In *Drosophila*, the single lipin-1 ortholog undergoes alternative splicing to produce at least three protein isoforms, which exhibit distinct tissue expression and subcellular localization patterns [48].

The regulation of lipin-1 mRNA splicing is also intriguing. During adipocyte differentiation, lipin-1 is induced preferentially over lipin-1 by glucocorticoids, suggesting that hormones regulate *Lpin1* mRNA splicing [49]. Recently, the SFRS10 splicing factor has been implicated in the inclusion of the lipin-1 exon. SFRS10 was identified as showing differential expression levels in liver and muscle of lean and obese individuals [50]. SFRS10 was subsequently shown to regulate *LPIN1* splicing in an *in vitro* minigene assay, and SFRS10 knockdown in HepG2 cells led to increased lipin-1 expression relative to lipin-1 [50]. The authors suggested that the down-regulation of SFRS10 in the liver of obese individuals may lead to increased lipin-1 expression and contribute to hepatic steatosis in the setting of obesity [50]. However, in a subsequent study, SFRS10 or lipin-1 expression levels in human liver biopsies did not appear to correlate with obesity or liver histopathology [51]. Technical and biological details differed between the two studies of SFRS10 levels [52], and more studies will be required to fully establish the roles of SFRS10 and lipin-1 in obesity.

2.4.2. Lipin-1 protein phosphorylation and sumoylation—The subcellular localization of lipin proteins is dynamically regulated and is likely a determinant of their activity. It has been known for many years that fatty acid availability promotes the migration of rat liver PAP activity from the cytosol to microsomal membrane fractions [27]. PAP enzyme subcellular localization appears to be regulated, at least in part, by posttranslational protein modifications of lipin proteins. Phosphorylation and sumoylation are the primary lipin-1 protein modifications that have been characterized to date.

Mouse lipin-1 is phosphorylated at multiple serine and threonine residues upon treatment of adipocytes with insulin or amino acids [31, 41, 53]. Insulin-stimulated lipin-1 phosphorylation is regulated, at least in part, by the mammalian target of rapamycin (mTOR) pathway and causes phosphorylation of Ser-106 on lipin-1 [53]. Lipin-1 and lipin-2 are both phosphorylated during mitosis at consensus sites for cyclin-dependent kinase 1 [54]. The yeast lipin ortholog, Pah1p, is phosphorylated by several kinases, including Cdc28p [55-57], and is dephosphorylated by a dual protein complex composed of Nem1p (a phosphatase) and Spo7p (a regulatory unit) [55]. The mammalian counterpart of this phosphatase complex has now been identified as CTDNEP1 (C-terminal domain nuclear envelope phosphatase 1, formerly Dullard) [58] and NEP1-R1 (nuclear envelope phosphatase 1-regulatory subunit 1) [59]. The CTDNEP1-NEP1-R1 complex can dephosphorylate lipin-1, lipin-1, and lipin-2 in cultured cell lines [59]. As a consequence of this dephosphorylation, lipin-1 localization to the nucleus increases. The tissue expression patterns of CTDNEP1 and NEP1-R1 in mice and humans mirror that of lipin-1

[59], suggesting a potential relationship between this complex and lipin-1 activity. Further studies are required to establish the role of the phosphatase complex in the function of lipin proteins (and potentially other proteins) *in vivo*. Lipin-1 is also modified by sumoylation in neuronal cells [30]. Sumoylation promotes the nuclear localization of lipin-1, and intact sumoylation sites are required for lipin-1 coactivator activity [30].

Initial studies indicated that lipin-1 phosphorylation status primarily affects lipin-1 subcellular localization: highly phosphorylated lipin-1 is enriched in the cytosol and dephosphorylated lipin-1 is enriched on ER/nuclear membranes [31, 53]. Somewhat surprisingly, the phosphorylation status in these cases did not directly affect PAP activity as assessed in an *in vitro* assay performed with micelles containing the PA substrate. Very recent work by Harris, Brindley and colleagues has provided important insight into the relationship between lipin-1 phosphorylation and PAP enzyme activity. By manipulating the presentation of PA substrate in liposomes containing various amounts of phosphatidylcholine and phosphatidylethanolamine, these investigators demonstrated that lipin-1 enzymatic activity is influenced by the phospholipid composition of membranes containing PA, as well as by pH [60]. The proposed mechanism is that neighboring membrane phospholipids and cellular pH both influence the ionization status of PA, which in turn affects lipin-1 binding and PAP activity. The phosphorylation status of lipin-1 influences its ability to recognize di-anionic PA [60], and this interaction is mediated through the polybasic domain on lipin-1 (previously shown to influence both nuclear localization and PA binding [31, 33]). Thus, current knowledge paints a complex picture in which the regulation of lipin-1 PAP activity is influenced by multiple metabolic variables, including intracellular pH, the phospholipid composition of the ER membrane, and hormones or specific kinases and phosphatases that influence lipin-1 protein phosphorylation.

2.4.3. Lipin protein oligomer formation—Harris and colleagues demonstrated that lipin proteins form homo- and hetero-oligomeric structures [38]. Endogenous lipin-1 isolated from 3T3-L1 adipocytes—as well as recombinant lipin-1 purified from an insect cell expression system—self-associates as dimers and tetramers. Interestingly, they appear to associate in a head-to-head/tail-to-tail manner through N-LIP/N-LIP and C-LIP/C-LIP interactions on lipin-1 proteins. Lipin-1 can also form hetero-oligomers in combination with lipin-2 or lipin-3. Curiously, however, the formation of lipin-1 oligomers is not required for, nor does it enhance, lipin-1 PAP activity [38]. The purpose of lipin oligomer formation remains unknown. One suggestion is that oligomerization influences lipin protein subcellular localization. This is supported by the demonstration that lipin-1 protein that was targeted to the plasma membrane with an isoprenyl lipid anchor attracted wild-type lipin-1 molecules (i.e., proteins lacking lipid anchors) to the plasma membrane, presumably through lipin–lipin protein interactions [38]. It will be important to further investigate the physiological relevance of lipin homo- and hetero-oligomer formation.

3. The physiological role of mammalian lipin proteins

3.1. Lipin tissue expression profiles

The demonstration that mammals express three lipin proteins, each of which catalyzes the same biochemical reaction, raises questions about the physiological role of each family member. Gene-expression studies in both mice and humans have demonstrated that each lipin has a unique tissue expression pattern, but that overlapping expression of more than one lipin gene occurs in many tissues [26] (Fig. 3). *Lpin1* and *LPIN1* are expressed at high levels in skeletal muscle and adipose tissue depots, and at lower levels in liver, kidney, heart, brain, and intestine. *Lpin2* and *LPIN2* are expressed at highest levels in liver and brain, with lower levels in the gastrointestinal tract [61]. *LPIN2* was detected in a sample of

pooled human adipose tissue (but not appreciably in mouse adipose tissue) [26], raising the possibility that lipin-2 has a role in human adipose tissue under metabolic conditions (e.g., obesity, diabetes) that may have been present in some of the human subjects contributing to the pool. *Lpin1* and *Lpin2* are also expressed in mouse bone and/or bone marrow [61], a tissue that has not yet been assessed in humans. *Lpin3* and *LPIN3* expression levels are generally much lower than those of the other two lipins, with the highest levels of expression in the small intestine and in bone.

The common enzyme activity and overlapping expression patterns of lipin family members raises questions about the physiological roles of each family member. For example, *Lpin1* and *Lpin2* are both expressed in liver and brain, but what are the relative levels and roles of each in these tissues? With regard to the liver, *Lpin1* and *Lpin2* were assessed for their relative hepatic expression levels with an assay that relied on PCR amplification with a single primer set followed by discrimination of the products by restriction endonuclease digestion [61]. This analysis showed that the levels of *Lpin2* transcripts in the liver are several-fold higher than those of *Lpin1*. *Lpin1* and *Lpin2* expression are both regulated by fasting, induced by 25-fold and 5-fold, respectively [34, 61, 62]. *Lpin3* is also expressed in liver, and expression levels are increased in lipin-1-deficient mice, suggesting cross-talk and/or compensation between lipin family members [26, 63]. Recent *in vivo* studies in experimental mouse models have begun to shed light on the relationships between lipin family members that are co-expressed within the same tissue (see section 3.2).

3.2. Lipin deficiencies in mice and humans

The study of lipin deficiencies has provided new insights regarding the physiological function of lipins. Even before the identification of the *Lpin1* gene, studies of the phenotype of *fld* mice implicated lipin-1 in hepatic lipid metabolism, peripheral nerve function, and adipose tissue development [9, 10, 64, 65]. In recent years, null mutations in *LPIN1* and *LPIN2* have been identified in humans and found to cause serious disease. The rarity of these deficiency states, along with logistic and ethical issues surrounding human studies, have made it essential to investigate lipin pathophysiology in mouse models. Some phenotypic differences have been noted between lipin-deficient mice and humans, but at least some of the perceived differences are probably related to incomplete characterization of the mouse models and the human disease state. In any case, it is highly likely that the basic biology of lipin functions is conserved between mouse and human, even if the phenotypic presentation of loss-of-function mutations may differ in some respects. In the following sections, we describe aspects of lipin biology that have been gleaned from studies of lipin deficiencies in mice and humans.

3.2.1. Lipin-1 physiological functions revealed in mouse models—The *fld* mouse carries a complex gene rearrangement resulting in a null mutation and a variety of metabolic defects. A point mutation in the *Lpin1* gene leading to a single amino acid substitution (Gly84Arg) was identified in the *fld^{2J}* mouse strain and confers the same phenotypes observed in *fld* mice [11]. The *fld* mutation was named “fatty liver dystrophy” based on the presence of a severe fatty liver that is evident during the neonatal period until about 2 weeks of age, and a progressive peripheral neuropathy that develops in young adult mice [9, 64]. The *fld* mice have lipodystrophy characterized by impaired adipocyte differentiation and a near-complete absence of TAGs in white adipose tissue depots [10, 11, 66-68]. Lipin-1-deficient mice have dramatically altered diurnal metabolism and are insulin resistant, likely related to dysregulated lipid metabolism in adipose tissue, liver, and muscle [69, 70]. Finally, male *fld* mice are infertile, despite producing motile spermatozoa [9].

The role for lipin-1 as the primary PAP activity in adipose tissue [26, 53] provides a partial explanation for the dramatically reduced TAG stores in adipose tissue of *flid* mice. The requirement for lipin-1 in adipose tissue has been further elucidated by studies of adipose tissue-specific lipin-1 knockout mice, which also manifest lipodystrophy, confirming the cell autonomous function of lipin-1 in white and brown adipocytes [67]. In addition, the adipose tissue-specific expression of a truncated lipin-1 that retains co-regulator function but lacks PAP activity confirmed the requirement for lipin-1 PAP activity in adipocyte maturation and TAG storage [66]. It is possible that the loss of lipin-1 coactivator activity and dysregulation of hepatic fatty acid oxidation genes [34] contributes to the neonatal fatty liver phenotype in *flid* mice. However, the recent demonstration that neonatal hepatic steatosis occurs in mice with lipin deficiency exclusively in adipose tissue [66] suggests that the fatty liver may result from metabolic dysfunction in adipocytes and/or macrophages.

The peripheral neuropathy in *flid* mice is characterized by demyelination and lipotrophy in peripheral nerves [64, 71]. Inactivation of lipin-1 function specifically in Schwann cells revealed that it is the loss of lipin-1 function in that cell type, rather than in the adipocytes of the epineurium, that is responsible for the neuropathy [72]. Furthermore, Chrast and colleagues determined that the lack of lipin-1 PAP activity in Schwann cells leads to an accumulation of the PAP enzyme substrate, PA, and activation of MEK-Erk signaling pathway, which inhibits Schwann cell differentiation [72]. PA accumulation also occurs in other cell types of lipin-1-deficient mice, including heart [73] and adipose tissue [66, 67, 74]. In adipocytes, PA accumulation and Erk activation inhibits the expression of PPAR α , a key transcriptional regulator of adipogenesis, providing an explanation for the impaired adipocyte differentiation in *flid* mice [74]. The accumulation of PA in adipose tissue also causes impaired adipocyte lipolysis through allosteric effects on phosphodiesterase 4 activity [66].

The cause of male infertility in *flid* mice has not been investigated extensively, but this phenotype may be a behavioral change related to the peripheral neuropathy. However, recent findings implicate lipin-1 as a determinant of nuage structure in gonads [75], which could potentially affect testis function. Nuage is a structure that occurs between aggregated mitochondria and is implicated in the generation of small non-coding Piwi-interacting RNAs (piRNAs) that are expressed in animal gonads and are thought to influence retrotransposase-induced DNA damage, mitochondrial fission/fusion dynamics, and other processes [76]. Interestingly, the testes from *flid* mice exhibit abnormal nuage density and length [75].

3.2.2. The role of lipin-1 in muscle is highlighted in human lipin-1 deficiency—

The first human lipin-1-deficient patients were identified in 2008 [13]. *LPIN1* mutations were in children from consanguineous families who had experienced episodes of rhabdomyolysis with muscle pain, myocyte degradation, and fever. Homozygosity mapping directed attention to a region on chromosome 2, and sequencing revealed unique pathogenic *LPIN1* mutations in multiple families [13]. Michot and colleagues have subsequently cataloged *LPIN1* mutations in patients with childhood rhabdomyolysis from around the world [77, 78]. To date, at least 19 distinct *LPIN1* mutations have been identified, most of which are point changes or small insertion/deletions that result in premature termination codons. In addition, an intragenic deletion of 73 amino acids has been reported in numerous unrelated patients [77, 78]. The occurrence of this mutation in multiple families suggests a founder effect within an isolated population.

LPIN1 mutations account for a significant proportion of childhood rhabdomyolysis in cases where fatty acid oxidation defects have been excluded. Michot and colleagues sequenced *LPIN1* in 141 cases of severe or moderate childhood rhabdomyolysis [78]. Biallelic *LPIN1* mutations were present in two-thirds of the individuals presenting with severe

rhabdomyolysis with onset before 7 years of age, but were infrequently observed in individuals with later onset or less severe rhabdomyolysis (Fig. 4). Interestingly, heterozygous *LPIN2* and *LPIN3* mutations were identified in a handful of patients with moderate rhabdomyolysis or with severe rhabdomyolysis with late onset (Fig. 4). Given that these mutations were observed only in the heterozygous state and that *LPIN2* and *LPIN3* are expressed at much lower levels in muscle than *LPIN1* [26], it is unclear whether these mutations are truly related to the development of rhabdomyolysis.

Lipin-1 deficiency can produce dramatic, life-threatening illness in toddlers, with severe cardiac muscle damage leading to cardiac arrest [79]. In addition to the evidence for rhabdomyolysis, analysis of muscle lipids in two affected individuals revealed increased levels of lipid intermediates in the glycerol phosphate pathway [13]. An in-depth analysis of muscle metabolism in lipin-1-deficient mouse models could prove valuable in elucidating the basis for rhabdomyolysis in lipin-1-deficient individuals. In mouse skeletal muscle, lipin-1 provides virtually all of the PAP activity [26, 53], and muscle lipin-1 levels affect energy metabolism [80] and mitochondrial content [81]. Although rhabdomyolysis and muscle pathology have not been noted in earlier descriptions of *flid* mice, this may be related to difficulties recognizing episodic muscle pain in mice and a lack of stimuli that normally trigger those episodes in affected humans (e.g., infection/fever, strenuous exercise, fasting). Indeed, preliminary studies in our laboratory indicate that, under metabolic stress, lipin-1-deficient mice exhibit histopathologic changes in muscle and elevated creatine kinase levels (Zhang and Reue, unpublished). Thus, lipin-1-deficient mice will likely be useful in better understanding muscle disease in lipin-1-deficient humans.

As described earlier, the importance of lipin-1 in adipose tissue in rodents is well established. It is therefore surprising that lipin-1-deficient children do not exhibit overt lipodystrophy [13]. Nevertheless, there is strong evidence that lipin-1 is a determinant of adiposity and related metabolic traits in humans. Both lipin-1 expression levels in human adipose tissue and *LPIN1* genetic polymorphisms are associated with body mass index and insulin sensitivity in multiple human cohorts [17, 82-87]. Furthermore, most of the reports of human lipin-1 deficiency have been in children, and it remains possible that some affected patients will develop adipose tissue phenotypes as they age. It is also possible that metabolic differences between mice and humans may protect lipin-1-deficient humans from overt lipodystrophy. These include a greater reliance on de novo lipogenesis in mouse adipose tissue than in human adipose tissue [88], and/or potential compensation for the loss of lipin-1 in humans by other lipin family members. A better characterization of adiposity in lipin-1-deficient patients, and of the role of lipin-1 in the differentiation of human adipocytes, would be useful.

Whether lipin-1-deficient humans develop fatty liver during the first few weeks of life is unknown. However, it is interesting that a genetic variation in *LPIN1* has been linked to the presence and the severity of fatty liver disease in children [89].

3.3. Lipin-2 and lipin-3 physiological function

3.3.1. Lipin-2 and lipin-3 molecular function and regulation—Since the identification of the three lipin genes in mammals, lipins have been the subject of >180 publications, but fewer than 10% of those have dealt with lipin-2 or lipin-3. Both lipin-2 and lipin-3 are PAP enzymes, albeit with lower specific activities than lipin-1 [26]. Lipin-2 and lipin-3 carry the coactivator motif found in lipin-1 [34], and lipin-2 exhibits coactivator activity in a cell-based assay [61]. Lipin-2 is prominently expressed in liver [61], and knockdown studies have shown that lipin-2 contributes substantially to PAP activity and TAG synthesis in hepatocytes [62]. Lipin-2 expression levels in mouse liver are induced by fasting, diet-induced obesity, and ER stress [61, 62, 90], suggesting that modulating lipin-2

levels may be important for hepatic lipid homeostasis. Lipin-2 is also expressed in macrophages and protects against saturated fatty acid-induced inflammation in these cells *in vitro* [91]. Lipin-2 appears to differ from lipin-1 in its subcellular localization. While data on the localization of lipin-2 in tissues is not available, lipin-2 localizes primarily to the cytosol in HeLa cells, whereas lipin-1 is found in both the cytosol and an ER/nuclear membrane-associated pool [54].

Virtually no studies have addressed the physiological function of lipin-3. However, genome-wide association studies in humans have identified genetic variation in *LPIN3* (as well as *LPIN2*) as determinants of metabolic traits. A variant near *LPIN3* has been associated with fasting serum glucose levels in healthy individuals [92]. A polymorphism in the 3' UTR of *LPIN2* has been linked to fat distribution, glucose, and insulin levels in type 2 diabetics [93]. Furthermore, *LPIN2* null mutations cause the rare disease, Majeed syndrome. Studies of lipin-2-deficient humans and mice have begun to define some of the physiological roles of lipin-2 (see sections 3.3.2. and 3.3.3).

3.3.2. Majeed syndrome—In 2005, Ferguson and colleagues identified the first mutations in *LPIN2* in children from two families in Jordan [12]. The initial symptoms are usually fever and pain in bones and joints, typically beginning in the first year of life [94]. Radiography reveals osteomyelitic lesions near the ends of long bones and other locations, but these lesions are sterile [94-96]. In addition, subjects develop congenital dyserythropoietic anemia of varying severity [12, 96]. Some patients develop transient inflammatory dermatosis, but this is not a consistent disease phenotype.

Majeed syndrome is rare, having been observed in only four families, all from the Middle East [97-99]. Four *LPIN2* mutations are known, three frameshift or splice site mutations that result in premature stop codons, and one missense mutation that changes a conserved serine to a leucine (Ser734Leu). The latter mutation occurs downstream of the PAP active site and coactivator motif and is conserved in mammalian lipin-1, -2, and -3 [12]. The Ser734Leu mutation abolishes PAP enzyme activity but does not impair the ability of lipin-2 to associate with microsomal membranes or adversely affect lipin-2's transcriptional coactivator function [61]. Of note, lipins in all multicellular organisms have a serine at the corresponding position, whereas single-celled organisms have a threonine. Thus, a hydroxyl group at this position may be critical for their phosphatase activity. In any case, it is clear that the loss of lipin-2 catalytic activity is sufficient to cause Majeed syndrome.

Due to the rarity of Majeed syndrome and the limitations on performing studies in human subjects, mysteries remain regarding the pathogenesis of the disease and how it relates to lipin-2 function and glycerolipid metabolism. A recent report implicated interleukin-1 (IL-1), an inflammatory cytokine, in the bone abnormalities in Majeed syndrome. Two Majeed patients showed clinical improvement when treated with drugs that block IL-1 action (Anakinra, an IL-1 receptor antagonist, and Canakinumab, an IL-1 antibody) [99]. Therefore, it seems possible that the bone abnormalities in Majeed syndrome are due to cytokine elaboration, either systemically or locally in bone. Cytokine production does occur within joints in disorders such as osteoarthritis [100, 101]. Lipin-2 mRNA expression is detectable in mouse bone and bone marrow [61], so further studies in mice could prove to be useful for defining the pathogenesis of Majeed syndrome (see section 3.3.3).

3.3.3. Lipin-2-deficient mice reveal cooperative and unique functions of lipin-1 and lipin-2—To investigate the functions of lipin-2, a Lipin-2-deficient mouse model was generated by targeted trapping [102]. Based on the prominent lipin-2 expression in liver [26, 62] and the substantial reduction in PAP activity in isolated hepatocytes with lipin-2 knockdown [62], it was expected that these mice would exhibit dramatically reduced liver

PAP activity and impaired hepatic lipid metabolism. Unexpectedly, however, lipin-2 knockout (KO) mice have slightly elevated hepatic PAP activity and had a compensatory increase in lipin-1 protein levels [102]. As a result, hepatic and plasma lipid levels in chow-fed lipin-2 KO mice are nearly normal. However, when mice are challenged with a high-fat diet, Lipin-2-deficient mice accumulate excess hepatic TAG. Adenoviral knockdown of lipin-2 in wild-type mouse liver led to increased expression of ER stress genes [90], but no evidence for ER stress was detected in the liver of Lipin-2-deficient mice [102].

Observation of the Lipin-2-deficient mice as they aged revealed an unexpected role for lipin-2 in lipid homeostasis in the cerebellum. Beginning at ~6 months of age, Lipin-2-deficient mice develop a tremor and ataxic gait [102]. An analysis of lipin protein levels in the cerebellum of wild-type mice revealed that both lipin-1 and lipin-2 (but not lipin-3) are abundant in the cerebellum of young animals, suggesting that lipin-1 compensates for the lack of lipin-2 in the cerebellum of young Lipin-2-deficient mice. However, as mice age, lipin-1 levels in the cerebellum fall to nearly undetectable levels. Thus, after 6 months of age, the cerebellum of Lipin-2-deficient mice have virtually no lipin protein. This leads to an accumulation of PA. In the cerebellum, lipin-2 is normally localized to Purkinje cells [102]. Aging Lipin-2-deficient mice exhibit a substantial reduction in Purkinje cell number, implying that lipin-2 and glycerolipid synthesis may play a role in maintenance of that cell type. Cerebellar disease has not been reported in Majeed syndrome, but all of the reported patients have been under 25 years of age. It seems possible that Majeed patients might develop cerebellar disease later in life, and it is also possible that lipin-2 levels in the cerebellum could prove to be relevant to the pathogenesis of idiopathic late-onset cerebellar ataxias.

Like Majeed patients, Lipin-2-deficient mice develop dyserythropoietic anemia [102]. It will be important to determine if erythrocytes and hematopoietic tissues from these mice have PA accumulation and/or abnormalities in phospholipid content. It is difficult to ascertain whether lipin-2 KO mice experience episodic bone pain, as in Majeed syndrome. No bony lesions were detected in Lipin-2-deficient mice by routine radiography or micro-computed tomography of tibiae [102]; however, conventional radiographs can be normal in Majeed patients early in the disease course [94, 97]. Detecting bone lesions in lipin-2 KO mice may require more sensitive techniques (*e.g.*, isotope uptake) [96] or studies of older mice. Monitoring mice for markers of inflammation or fever would also be informative.

The availability of both lipin-1-deficient and Lipin-2-deficient mouse strains made it possible to determine whether mice lacking both of these proteins were viable. It was determined that deficiency in both lipin-1 and lipin-2 is lethal during embryonic development, with affected embryos failing to develop a functional circulatory system [102]. This finding indicates that the presence of lipin-1 or lipin-2 is required during embryogenesis, and that lipin-3 cannot substitute for these functions.

The characterization of Lipin-2-deficient mice uncovered physiological functions of lipin-2 in the liver under dietary stress, in the cerebellum, in erythrocytes, and in embryonic development [102]. More generally, these studies have begun to illuminate physiological relationships between members of the lipin protein family, which operate in a tissue- and age-specific manner. In the case of lipin-1 and lipin-2, it is clear that each of these proteins has unique roles in specific tissues, while other physiological functions are shared by both lipin-1 or lipin-2 (Fig. 5). Further analysis of mice with global and tissue-specific deficiencies in the lipin proteins will likely enhance our understanding of the physiological relationships between lipin-1 and lipin-2.

4. Aberrant intracellular lipid signaling in lipinopathies

Data is beginning to mount suggesting that a hallmark of lipin deficiencies in vivo is the accumulation of the lipin enzyme substrate, PA. Thus, an imbalance in lipid intermediates may be a common theme in the tissue-specific pathologies observed in lipin deficiencies (Fig. 6). Thus far, it is known that PA accumulates in Schwann cells, heart, and adipose tissue of lipin-1-deficient mice (and likely in lipin-1-deficient skeletal muscle), and the cerebellum of aging Lipin-2-deficient mice [13, 66, 67, 72-74, 102]. PA has been implicated in many aspects of cellular lipid metabolism. It is a potent activator of MAP kinase/Erk and other signaling pathways, which contribute to the phenotypes observed in lipin-1-deficient mice, such as impaired Schwann cell and adipocyte differentiation [72, 74] (Fig. 6). PA synthesized by the glycerol phosphate pathway inhibits mTORC2 (mammalian target of rapamycin complex 2) activity, and impairs insulin signaling in mouse hepatocytes [103]. In yeast, PA accumulation impairs normal lipid droplet dynamics and leads to “super-sized” lipid droplets [104]. In addition, yeast PAP activity is required for membrane fusion events, such as vacuolar (lysosomal) fusion [105]. PA also regulates inflammatory signaling pathways [27, 106]. In addition to PA accumulation, it is possible that lipin deficiencies also lead to reduced DAG levels and reduced activation of protein kinase C, protein kinase D, and potentially other signaling cascades (Fig. 6). This aspect of lipin biology has not been explored in depth at present, but represents a promising avenue for future investigation. The characterization of lipid intermediate profiles and related signal transduction pathways may provide additional insights into the molecular basis for manifestations of lipin deficiencies, and may help to devise rational therapeutic approaches for treating these diseases.

5. Future Perspectives

In the dozen years since the identification of the lipin genes, lipin proteins have become established as critical players in TAG and phospholipid biosynthesis, and both rare mutations and common polymorphisms in lipin genes have been associated with human disease.

The lipins, as well as other enzymes in the glycerol phosphate pathway (GPATs, AGPATs, DGATs), exist in a family of proteins with the same enzymatic activities [1, 2]. The physiological distinctions between the isoforms of these enzymes are incompletely understood. Do multiple lipin family members exist to guarantee adequate PAP activity through redundancy? Or do individual lipins have a unique physiological purpose? Do lipin family members function together, either by coupled regulation or through physical interactions? Studies in lipin-1 and Lipin-2-deficient mice have yielded some insights into these issues, but lipin physiology is complex. Different lipins within the same tissue can compensate for each other in some settings but not others (Fig. 5). For example, the induction of lipin-1 in the liver of Lipin-2-deficient mice is sufficient to maintain hepatic lipid homeostasis under basal conditions but not during a high-fat diet. In the cerebellum, lipin-1 expression compensates for lipin-2 in young mice, but this compensation wanes with age. The elucidation of interrelationships between lipin family members will require knockout mouse studies and in most cases would not be uncovered with overexpression studies in cultured cells or by modulating lipin expression levels with adenoviral vectors. In the future, additional mouse models—including lipin-3-deficient mice, tissue-specific lipin knockout mice, and mice with deficiencies in two of the three lipin proteins—will likely provide new insights into the function of the lipin proteins in health and disease.

Elucidating molecular functions of lipin proteins is fertile territory for new discovery. The lipins occupy a critical regulatory point in the balance between lipid storage (through PAP activity) and lipid oxidation (through modulation of gene expression regulation). A better

understanding of the relationship between lipin catalytic and co-regulatory activities is necessary to understand the effects of lipin proteins on lipid homeostasis. Emerging data from both mice and humans have revealed roles for lipins in modulating the balance between PA and DAG lipid intermediates, which act as signaling molecules as well as building blocks for glycerolipids. Disrupting the balance between PA and DAG in lipin-deficient states leads to impaired cell adipocyte differentiation and pathology in both peripheral nerves and cerebellum, and it seems likely that these observations constitute only the tip of the iceberg. The levels of PA and DAG lipid intermediates are known to influence adipocyte lipolysis [66] and hepatic insulin signaling [103]. Thus, a better understanding of both the molecular and physiological functions of lipin proteins may lead to novel approaches for modulating the levels of lipid storage as well as levels of lipid intermediates that regulate cell signaling.

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Fig. 1. The glycerol phosphate pathway for glycerolipid synthesis

Lipin proteins catalyze the conversion of phosphatidic acid (PA) to diacylglycerol (DAG), which is the penultimate step in triacylglycerol (TAG) and zwitterionic phospholipid synthesis via the glycerol phosphate pathway. The acyltransferase enzymes in this pathway reside within the ER membrane, but lipin proteins can transit from the cytosol to the ER membrane to catalyze their enzymatic reaction; lipin proteins may also localize to the nucleus. GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerol-3-phosphate acyltransferase; DGAT, acyl coenzyme-A:diacylglycerol acyltransferase; LPA, lysophosphatidic acid.

Fig. 2. Domain structure and evolutionary conservation of lipin proteins

Lipin proteins are evolutionarily conserved from yeasts to humans. Mammalian genomes encode three lipin proteins, some of which have multiple splice variants (not shown). Lower organisms possess two (*Arabidopsis*) or one lipin ortholog (invertebrates, single-celled eukaryotes). Lipin proteins possess two highly conserved domains, N-LIP and C-LIP (yellow bars); the PAP enzyme active site motif (DXDXT, green bar) is present within the C-LIP domain. Most lipin orthologs contain an identifiable basic motif that is required for nuclear localization and/or PA binding (blue bar), and mammalian and *Drosophila* lipins contain a motif that is required for lipin interaction with transcriptional activators and coactivators (orange bar).

Fig. 3. Mouse lipin tissue expression patterns

The diagram indicates the relative levels of lipin-1, lipin-2 and lipin-3 mRNA expression in mouse tissues. Lipin-1 is expressed prominently in adipose tissue and muscle, and at lower levels in several other tissues. Lipin-2 is the predominantly expressed lipin in liver and cerebellum, and is also found in intestine, bone, and kidney. Lipin-3 is expressed prominently in small intestine and bone, and at low levels in other tissues indicated.

Fig. 4. Summary of LPIN1 gene mutations detected in rhabdomyolysis patients without fatty acid oxidation disorders

Of 141 rhabdomyolysis patients investigated by Michot et al. [78], cases were described as either severe or moderate. The severe cases were further categorized by age of onset: childhood (< 7 years), adolescent (7-16 years), or adult (> 16 years). Of the 49 severe childhood onset cases, 67% were found to carry mutations in both LPIN1 alleles. Although biallelic or heterozygous LPIN1 mutations were also detected in a few cases of later onset, LPIN1 mutations are predominantly associated with severe rhabdomyolysis with early onset. Interestingly, a few heterozygous LPIN2 or LPIN3 mutations were documented in patients with moderate rhabdomyolysis.

Fig. 5. Unique and overlapping lipin-1 and lipin-2 physiological functions gleaned from studies in lipin-deficient mouse models

Lipin-1 plays a dominant role in glycerolipid synthesis in adipose tissue and Schwann cells, and as a regulator of hepatic fatty acid oxidation. Lipin-2 has a unique role in the maintenance of erythrocytes. The two proteins have overlapping roles in embryonic development, such that loss of either lipin-1 or lipin-2 is surmountable, but loss of both causes embryonic lethality. Lipin-1 and lipin-2 cooperate in the maintenance of lipid homeostasis in liver and cerebellum of young mice. However, lipin-2 provides a function that cannot be provided by lipin-1 in maintaining hepatic lipid homeostasis on a high fat diet, and in promoting normal cerebellar function in aging mice.

Fig. 6. Model for aberrant lipid signaling in lipin deficiencies

By catalyzing the transformation of PA to DAG, lipin PAP enzyme activity modulates the balance of these two important lipid intermediate signaling molecules. In a lipin-deficient state, PA accumulates and DAG levels may be reduced. An abundance of PA may activate mTOR, PLC, PI-4K, and ERK signaling, while low DAG levels may lead to reduced PKC or PKD activation. Tissue-specific macroconsequences of lipin-deficiency may reflect the downstream manifestations of lipid intermediate imbalance in distinct tissue environments. DAG, diacylglycerol; DGK, diacylglycerol kinase; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; PC, phosphatidylcholine; PLD, phospholipase D; PI-4K, phosphatidylinositol 4-kinase; PKC, protein kinase C; PKD, protein kinase D.