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Interactions Among Lipin Protein Family Members are Critical for Lipid Homeostasis

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Interactions Among Lipin Protein Family Members are Critical for Lipid Homeostasis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Jennifer Richardson Dwyer

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ABSTRACT OF THE DISSERTATION

Interactions Among Lipin Protein Family Members

are Critical for Lipid Homeostasis

by

Jennifer Richardson Dwyer

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2012

Professor Karen Reue, Chair

Members of the lipin protein family — lipin-1, lipin-2 and lipin-3 — catalyze the penultimate step in triacylglycerol (TAG) synthesis, the dephosphorylation of phosphatidic acid (PA) to form diacylglycerol. The founding member of the family, lipin-1, has been studied most extensively and has been implicated in metabolic diseases that include lipodystrophy, obesity and insulin resistance. The three lipin proteins exhibit distinct tissue distributions and therefore likely have unique physiological roles, which at present are not well understood. The focus of this dissertation has been to augment our understanding of the physiological and molecular roles of lipin family members through the use of various genetic and molecular tools. We generated lipin-2–deficient mice and determined that loss of lipin-2 causes hepatic and neurological

defects in mice. These symptoms were associated with impaired TAG homeostasis in liver, and the accumulation of the lipin enzyme substrate, PA, in cerebellum. Interestingly, in both tissues, we uncovered relationships between lipin-1 and lipin-2 activities, which were particularly important in maintaining hepatic lipid homeostasis under conditions of metabolic stress such as a high fat diet, and in cerebellar function during aging. In addition to the functional interactions between lipin-1 and lipin-2, we determined that the two proteins interact in a protein complex in liver. Furthermore, lipin-1 and lipin-2 influence the protein levels of one another, and this requires a sequence motif in lipin-1 that has been shown to promote nuclear localization and PA binding. Analysis of lipin-2 KO mice also led to the discovery of a role for lipin proteins in bone homeostasis, with lipin-2 deficiency causing altered trabecular bone architecture, which was exacerbated on a high fat diet. Finally, an initial characterization of lipin-3 deficiency in mice is presented. Overall, these studies show that lipin protein family members cooperate to maintain normal glycerolipid homeostasis *in vivo*. The further elucidation of the regulation and function of lipin protein interactions will contribute to our understanding of lipid homeostasis in normal and diseased states.

The dissertation of Jennifer Richardson Dwyer is approved.

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DEDICATION

This manuscript is dedicated in loving memory of my grandparents, Walter Allen Richardson, Jr. and Jane Personius Richardson. They instilled in me a curiosity about the way things work from a very young age and encouraged me to never stop learning (or I might get *old*).

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CHAPTER ONE

INTRODUCTION

THE OBESITY EPIDEMIC

The latest survey data from the Centers for Disease Control indicate that 35.7% of adults and 16.9% of children in the United States are obese (1, 2) and childhood obesity is on the rise in developing countries (3, 4). The factors that contribute to the development of obesity include poor nutritional knowledge, bad eating habits, lack of physical activity, genetics, socioeconomic status and sociocultural beliefs (1-3). The health concerns associated with obesity are serious and include increased risk for developing insulin resistance, dyslipidemia, metabolic syndrome, skeletal problems, certain cancers, and even depression (5–10). Clinical intervention largely focuses on strategies for weight loss and includes nutritional education, lifestyle changes, medication and sometimes surgery (11, 12). Medications aim to enhance weight loss by increasing metabolic rate, suppressing appetite or inhibiting intestinal fat absorption, but have undesirable side effects and limited effectiveness (11, 13). Despite these efforts, the road to a healthy weight for obese individuals is physically and psychologically arduous and often unsuccessful, creating a large population of patients suffering the secondary effects of obesity.

An additional approach to treatment involves seeking to understand how obesity damages the body in an effort to design interventions that will mitigate its complications. As the molecular pathways that link obesity to its side effects are mapped out, a recurrent theme is that dysregulated fat storage in adipose tissue is associated with the release of excess fatty acids, which are absorbed and deposited as triacylglycerol (TAG) in tissues that do not normally store fat (14, 15). Thus, a better understanding of the molecular pathways that regulate TAG synthesis and storage may contribute to the development of useful therapeutic interventions for obesity.

THE IMPORTANCE OF LIPID HOMEOSTASIS

There are three ways that cells in our bodies are supplied with the lipids they need in order to function. First, triglycerides consumed in the diet are absorbed in the small intestine. These

lipids are broken down, re-synthesized and packaged into chylomicrons that are released into the lymph system and subsequently, into the circulation. Chylomicrons in the blood travel throughout the body to the various tissues where they are metabolized by tissue-specific lipases that deliver lipids into the cells. Second, during the fasting state, adipose tissue triglyceride lipase and hormone-sensitive lipase in adipose tissue become activated, triggering the breakdown of stored triglyceride into fatty acids. These non-esterified fatty acids are released into the circulation, where they are bound to albumin and are carried into tissues via various transporters on the cell membrane. The final source of lipids is available only to liver and adipose tissues. Under conditions of high carbohydrate availability, these tissues have the ability to synthesize lipids *de novo* from glucose.

Once fatty acids enter the cell, they are routed towards specialized metabolic pathways in order to meet the needs of the specific cell type. Cells utilize lipids to accomplish a variety of vital functions. Fatty acids can serve as precursors for the synthesis of phospholipids, a major component of all cellular plasma and intracellular membranes. Cells also oxidize fatty acids to release energy to fuel cellular processes. Fatty acid oxidation is particularly important in skeletal and cardiac muscle, but also significant in liver and other tissues. In tissues such as adipose, liver, and muscle, fatty acids are incorporated into TAG or cholesteryl esters and stored as cytosolic lipid droplets for later use.

The ability of cells to synthesize and store TAG allows organisms to subsist during the fasting period of the diurnal cycle. However, as with most biological processes, homeostasis between TAG storage and fatty acid oxidation must be achieved to promote normal cellular function. Chronic fatty acid excess and chronic TAG storage in tissues other than adipose tissue is associated with the development of insulin resistance. This occurs in conditions such as obesity, where the release of fatty acids from adipose tissue TAG becomes excessive. Interestingly, it also occurs in lipodystrophies, where fat storage is impaired, forcing other

tissues in the body to handle the metabolic responsibilities of the dysfunctional or absent adipose tissue.

THE GLYCEROL-3-PHOSPHATE PATHWAY, LIPID SYNTHESIS, AND LIPIN PROTEINS

Most cells in the body utilize the glycerol-3-phosphate pathway to synthesize triglycerides (Figure 1.1)(16). This occurs on the membrane of the endoplasmic reticulum and begins with the esterification of a fatty acid onto a glycerol-3-phosphate backbone by members of the glycerol phosphate acyltransferase (GPAT) family to form lysophosphatidic acid (LPA). LPA is then further esterified by members of the 1-acylglycerol 3-phosphate acyltransferase (AGPAT) family to form phosphatidic acid (PA). PA is then dephosphorylated to form diacylglycerol (DAG) by PA phosphatase (PAP) enzymes. Finally, a third fatty acid is esterified onto DAG by the action of diacylglycerol acyltransferase (DGAT) enzymes to form TAG.

The GPAT, AGPAT, DGAT and PAP enzyme activities have been studied for decades. The molecular cloning of members of the GPAT, AGPAT and DGAT protein families the 1990's led to new insights into the physiology and biochemistry of these protein families. However, at the start of this dissertation project, the identity of the proteins that catalyze the dephosphorylation of PA remained a mystery. Shortly thereafter, findings from our laboratory on the lipin proteins (17) and those from a laboratory studying lipid synthesis in yeast converged (18), revealing that lipin proteins are responsible for the PAP enzyme activity in the glycerol-3-phosphate pathway.

In 2001, our laboratory used positional cloning to identify a null mutation in the Lpin1 (lipin-1) gene as responsible for the severe metabolic phenotype seen in *fld* (fatty liver dystrophy) mice (17). These mice display a fatty liver and hypertriglyceridemia in the neonatal period, a near absence of adipose tissue (lipodystrophy), insulin resistance, and peripheral neuropathy(19). Two additional mouse and human lipin gene family members, *Lpin2* and *Lpin3*, were identified based on sequence similarity of their predicted protein sequence to lipin-1 (17). While mammals and most other vertebrates possess three lipin genes, invertebrates and yeast

possess a single lipin ortholog. The extensive evolutionary conservation of lipin proteins down to single-celled eukaryotes indicates that lipin proteins function in a fundamental cellular process.

The lipodystrophy observed in lipin-1–deficient *fld* mice indicated that lipin-1 plays a critical role in adipose tissue biology, but the molecular function of the lipin-1 protein remained a mystery until it was shown that the yeast lipin ortholog, Pah1, possesses PAP enzyme activity (18). PAP activity is conferred by the DxDxT amino acid motif, which is conserved in lipin orthologs in all species. Subsequent studies demonstrated that mammalian lipin-1, lipin-2 and lipin-3 all have PAP activity, and that the specific activity appears to be highest for lipin-1 (20). Thus, lipin proteins catalyze the penultimate step in the glycerol-3-phosphate TAG synthesis pathway, converting PA into DAG. Since DAG is also a direct substrate for synthesis of the phospholipids phosphatidylcholine and phosphatidylethanolamine, lipin proteins occupy a pivotal position in determining the fate of lipid intermediates as TAG or phospholipids(16). Additionally, both PA and DAG are bioactive lipids, and regulate signaling cascades that have roles in numerous cellular processes, including growth and energy metabolism, and response to external stimuli (21).

The GPAT, AGPAT and DGAT enzyme components of the glycerol-3-phosphate pathway reside in the endoplasmic reticulum membrane, where lipid synthesis occurs (16). By contrast, lipin proteins reside primarily in the cytosol, and translocate to the ER membrane to catalyze the PAP reaction. Lipin-1 also translocates to the nucleus under some conditions (22–24). Although lipin-1 is not itself a transcription factor, the nuclear function of lipin-1 may be related to a role for lipin-1 as a modulator of gene expression in combination with transcriptional activators such as peroxisome-proliferator-activated receptor α (PPARα), PPARγ coactivator 1α (PGC-1α), and NFATc4 (25, 26). However, the functions of lipin-1 in regulating fat storage in adipose tissue have been attributed exclusively to the PAP enzyme activity (27).

The three lipin family members are found in a diverse array of tissues and have distinct, but slightly overlapping, tissue expression patterns (20). Lipin-1 is expressed at highest levels in adipose tissue and skeletal muscle, lipin-2 is primarily expressed in liver and brain, and lipin-3 is expressed at low levels in several metabolic tissues including intestine, kidney and liver. Several of the lipin-expressing tissues are not commonly associated with TAG storage, and the primary role of lipins in these tissues may be to support phospholipid homeostasis. The distinct tissue expression patterns for each lipin family member suggest unique physiological roles for each lipin, which remain to be elucidated.

Over the past several years, dozens of studies focused on elucidating the cellular and physiological roles of lipin-1 have been reported (28–31). These have been facilitated by the availability of the lipin-1–deficient *fld* mouse model. At present, only a handful of studies investigating the function of lipin-2 and lipin-3 have been published, which are summarized below.

LPIN2 MUTATIONS ARE ASSOCIATED WITH HUMAN DISEASE

Genome wide association and linkage studies conducted on a genetically-isolated population from the Southwest of the Netherlands detected an association between type 2 diabetes and chromosome 18p11, where *LPIN2* resides (32). Fine-mapping of this genetic locus in a group of 78 type 2 diabetics and 101 controls subjects from the genetically–isolated group revealed an association between a single nucleotide polymorphism in the 3' untranslated region of *LPIN2* (three nucleotides downstream of the stop codon) and type 2 diabetes. The initial studies failed to detect a direct effect of this polymorphism on the levels of lipin-2 mRNA in white blood cells or in microRNA binding to lipin-2 mRNA. It is likely that the identified polymorphism is in linkage disequilibrium with another functional variant that is responsible for the association with diabetes, and this warrants further investigation.

Mendelian mutations in *LPIN2* have been identified as the causative agent of Majeed Syndrome, a rare inflammatory disorder that arises in childhood, characterized by recurrent osteomyelitis, dyserythropoietic anemia, cutaneous inflammation and recurrent fever (33–35). Three different mutations in unrelated families in the Middle East have been identified. One mutation is a two-nucleotide deletion leading to a frame shift mutation within exon 4 leading to a premature stop codon (33) and the second mutation is missense mutation leading to the conversion of a highly conserved arginine to a serine (R776S) within the 5' spice site of exon 17 which is predicted to cause read-through into intron 17 and a truncated protein (34). The final mutation is the most studied and is a missense mutation that changes a highly conserved serine residue to a leucine (S734L) within exon 17 of *LPIN2* (33). This serine is conserved in vertebrate, invertebrates and plants and is also present in *LPIN1* (23). Mutation of the comparable serine within the murine coding sequences for *Lpin2* (S731L/D) or *Lpin1* (S721L/D) causes total loss of enzyme activity (23). Interestingly lipin-1 and lipin-2 have been shown to bind to PPARα via a nearby LXXIL motif within the C-LIP domain, which mediates the transcriptional-co-activation activities of lipin-1 and lipin-2 (23, 26). However, the S731L mutation had no effect on the ability of lipin-2 to co-activate PPARα response-elements or on the ability of lipin-2 to translocate to membranes in response to oleate treatment, indicating that it is likely the loss of PAP activity that is the cause of Majeed syndrome.

Given that both mouse and human lipin-2 is prominently expressed in liver and brain, with lower levels seen in kidney and intestine (20, 23), the spectrum of tissues affected in Majeed syndrome is somewhat unexpected. At present, it is unclear if the symptoms of Majeed syndrome result indirectly from the effects of dysregulated lipid metabolism in a tissue where lipin-2 is prominently expressed, or directly from the loss of the lipin-2 protein from specific cell populations in bone or other tissues. Interestingly, lipin-2 has recently been shown to play a protective role against inflammation in cultured macrophages by attenuating the inflammatory

effects of saturated fatty acids on macrophage activation through enhanced neutral lipid synthesis (36).

LIPIN-2 AND HEPATIC LIPID METABOLISM

Lipin-2 is most highly expressed in liver and it is the most abundant lipin in liver (20, 23). Hepatic lipin-2 protein levels increase with fasting and obesity (37), suggesting that lipin-2 is functional when the liver is actively metabolizing lipids. Lipin-2 protein levels are also upregulated in the lipid-loaded livers of *Lpin1*$^{fld/fld}$ mice during the neonatal period and remain elevated in young adult *fld* mice (37) presumably to help maintain lipid homeostasis is the absence of lipin-1. Over-expression of lipin-2 in the HepG2 hepatocyte cell line increased PAP activity, while knockdown of lipin-2 in isolated hepatocytes from wild-type or *Lpin1*$^{fld/fld}$ mice caused a decrease in PAP activity (37). These findings suggest that although all three of the lipin family members are expressed in liver, lipin-2 is likely the major source of PAP activity in the liver.

Lipins catalyze the penultimate step in TAG synthesis and the link between hepatic TAG accumulation and insulin resistance has been well documented (15). Therefore a natural question is whether altered hepatic lipin-2 levels can influence TAG accumulation and insulin resistance. Increasing PAP activity, as seen with adenoviral-mediated overexpression of lipin-2 in hepatocytes, does not result in increased DAG or TAG accumulation (37, 38). Conversely, knockdown of lipin-2 in cultured hepatocytes does not decrease TAG levels (37). However, TAG synthesis is attenuated under some circumstances when both lipin-1 and lipin-2 levels are reduced simultaneously. Thus, TAG synthesis was impaired when hepatocytes from lipin-1–deficient *fld* mice were subjected to shRNA-mediated lipin-2 knockdown in combination with the lipid stress of either a young age (when fatty liver is present due to lipin-1 deficiency) or oleate treatment (37). These results suggest that the activity of both lipin-1 and lipin-2 contribute to TAG homeostasis in liver under conditions of excess hepatic lipid influx.

Lipins may also have a role in glucose metabolism. Increased cellular content of lipid intermediates including PA and DAG, the substrate and product of lipin PAP activity, has been linked to changes in insulin signaling (38, 39). Elevated levels intracellular PA, caused by either overexpression of the lipid synthesis enzymes GPAT-1 or AGPAT-1 or by direct treatment of with PA, results in reduced AKT phosphorylation and disrupts the assembly of the TORC2 complex that functions downstream of the insulin receptor in cultured hepatocytes (38). In mice, adenoviral-mediated over-expression of lipin-2 caused increased hepatic DAG and TAG levels, as well as increased fasting blood glucose and plasma insulin levels when mice were stressed with a high fat diet (40), implying that excess lipin-2, enhances insulin resistance in the setting of obesity. Another possibly is that an imbalance between lipin-2 and lipin-1 levels causes improper regulation of PA and DAG pools which triggers insulin resistance. Lipin-1 levels, nor phosphatidic acid levels, were quantified in this study, so it is unclear what the mechanism of insulin resistance is in this situation. In obese humans, DAG accumulation in liver is associated with insulin resistance (39). Analysis of liver samples isolated from a cohort of obese non-diabetics with a wide range of insulin sensitivities showed that elevated levels of DAG and high levels of DAG localized to lipid droplets were the strongest predictors of insulin resistance (39). Higher levels of activated protein kinase Cε, which inhibits the phosphorylation of insulin receptor substrates 1 and 2 by the insulin receptor, also correlated with the elevated DAG levels in this cohort (39, 41). These results imply that the enzymatic function of lipins, the catalysis of PA into DAG, may have a role in glucose homeostasis that is yet to be elucidated.

LIPIN-3, THE UNKNOWN

Little is known about the physiological function of lipin-3. Lipin-3 is expressed at relatively low levels in metabolic tissues compared with lipin-1 and lipin-2, but significant mRNA levels are detectable in small intestine and kidney (20). The protein structure is similar to the other lipin protein family members, including known functional motifs, suggesting that it has similar

molecular activity to other lipin proteins. Indeed, lipin-3 is able to catalyze the conversion of PA into DAG, however its PAP specific activity is much less than that of lipin-1 (75% less) (20). Despite its lower relative PAP activity, lipin-3 can substitute for lipin-1 during adipocyte differentiation *in vitro* by promoting the expression of the adipocyte differentiation program and TAG accumulation (27). Lipin-3 mRNA expression is also induced in the liver of lipin-1–deficient mice, suggesting that it may also partially compensate for the function of hepatic lipin-1 in these mice (20).

To date, no homozygous mutations in *LPIN3* have been associated with human disease. Three unique *LPIN3* heterozygous missense mutations have been reported in individuals with severe myolysis and muscle pain (42). One of these (*LPIN3*-P24L) was also shown to have impaired ability to complement a Pah1-deficient yeast strain, suggesting impaired protein function. A mouse model of lipin-3 deficiency will be a valuable tool to investigate the physiological role of lipin-3.

DISSERTATION GOALS AND STRATEGY

The focus of this dissertation project has been to further elucidate the molecular and physiological functions of members of the lipin protein family using genetic and molecular approaches. Highlights include: the first report of a lipin-2–deficient mouse model, which revealed a critical role for lipin-2 in the cerebellum; the identification of physiological and molecular interactions between lipin-1 and lipin-2; the identification of a role for lipin proteins in maintenance of bone health; and the generation and initial characterization of a lipin-3–deficient mouse model.

Chapter 1 provides an introduction to the lipin protein family and its significance in metabolic disorders such as obesity and insulin resistance. Chapter 2 is a published review article on the lipin family of proteins that provides the state of knowledge prior to the subsequent studies presented in the thesis. Chapter 3, "Mouse Lipin-1 and Lipin-2 Cooperate to Maintain Glycerolipid Homeostasis in Liver and Aging Cerebellum*",* contains our recently accepted manuscript (Proceedings of the National Academy of Sciences USA) characterizing the lipin-2 knockout mouse phenotype. Chapter 4, "Lipin-1/Lipin-2 Interactions Regulate Lipin Protein Levels", contains a preliminary manuscript describing our findings regarding the molecular interaction between lipin-1 and lipin-2, which has affects on the levels of these proteins in liver. Chapter 5 provides a synthesis of the novel findings that have resulted from these studies, and describe additional preliminary data on the roles of lipin proteins in the maintenance of bone structure and the initial characterization of lipin-3–deficient mice. These data are presented in the context of Future Studies that will expand our knowledge of the physiological roles of the lipin protein family.

ADDITIONAL PROJECT

The mild phenotype of the lipin-3 knockout mice has led to a collaborative effort with Lauren S. Csaki, a Human Genetics graduate student in our lab who has bred lipin-1/lipin-3 and lipin-2/lipin-3 double knockout mice in an effort to elucidate the molecular function of lipin-3. An abstract regarding these projects is below:

Lipin protein partnerships uncovered in double knockout mouse models

Lauren S Csaki[1], Jennifer R Dwyer[2], Peixiang Zhang[1], Michael HK Nguyen[3], David N Brindley[4], Elisa Atti[5], Sotirios Tetradis[5], Loren Fong[5], Stephen G Young[1,2,5], and Karen Reue[1,2,5]

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Lipins are an evolutionarily conserved family of three proteins (lipin-1, lipin-2, and lipin-3) that catalyze a critical step in glycerolipid biosynthesis, namely the removal of the phosphate group from phosphatidic acid to form diacylglycerol. Mutations in lipin-1 and lipin-2 cause serious disease in humans and mice: Lipin-1 deficiency causes lipodystrophy in the mouse, a consequence of its vital role in adipogenesis and TAG storage in adipose tissue. Lipin-2 deficiency affects lipid homeostasis in non-adipose tissues, including liver and brain. However, little is known about the physiological function of lipin-3, expressed prominently in bone and at low levels in intestine, adipose, kidney, and liver. To investigate the elusive function of lipin-3, we developed a knockout mouse, but detected no apparent phenotype. To circumvent possible compensation from the other lipins in these mice, we generated lipin-1/lipin-3 and lipin-2/lipin-3 double knockout (DKO) models. We observed distinct phenotypes that reveal cooperative relationships between lipin-3 and lipin-1 in adipose tissue and between lipin-3 and lipin-2 in intestine and bone.

Lipin-1/3 DKO mice were even more profoundly lipodystrophic than mice lacking only lipin-1, with further reductions in body weight and fat pad mass. Interestingly, absence of either lipin-1 or lipin-3 dramatically reduced PAP activity in adipose tissue, indicating that the presence of both lipins is required for optimal PAP activity there. The activities of lipin-1 and lipin-3 appear to be cooperative, as adipose tissue TAG levels are nearly normal in lipin-3 KO mice, reduced by half in lipin-1-deficient mice, and almost completely ablated in DKO mice. We showed that lipin-3, like lipin-1, is induced during adipogenesis, and we hypothesize that the two lipins work together to regulate lipid intermediates that modulate adipogenic gene expression.

Lipin-2/3 DKO mice exhibit reduced viability, reduced body size, hunched posture and abnormal gait. These gross physical traits may be outward manifestations of abnormalities found in the bones of these mice, including abnormal skeletal traits and impaired trabecular and cortical bone density. Dramatic abnormalities in the small intestine include hyperplastic villi and lipid accumulation within enterocytes. The presence of lipid droplets suggests that triglyceride synthesis is intact, but chylomicron maturation and/or secretion are likely impaired. Impaired

intestinal nutrient assimilation could contribute to the small size and bone defects observed. However, we also hypothesize that lipin-3 has a local role in bone, due to strong mRNA expression levels there and the presence of lipin-3 protein within bone marrow stromal cells. We propose that lipin-2 and lipin-3 function together to maintain intestinal and bone lipid homeostasis.

Figure 1.1

The Glycerol-3-phosphate Pathway

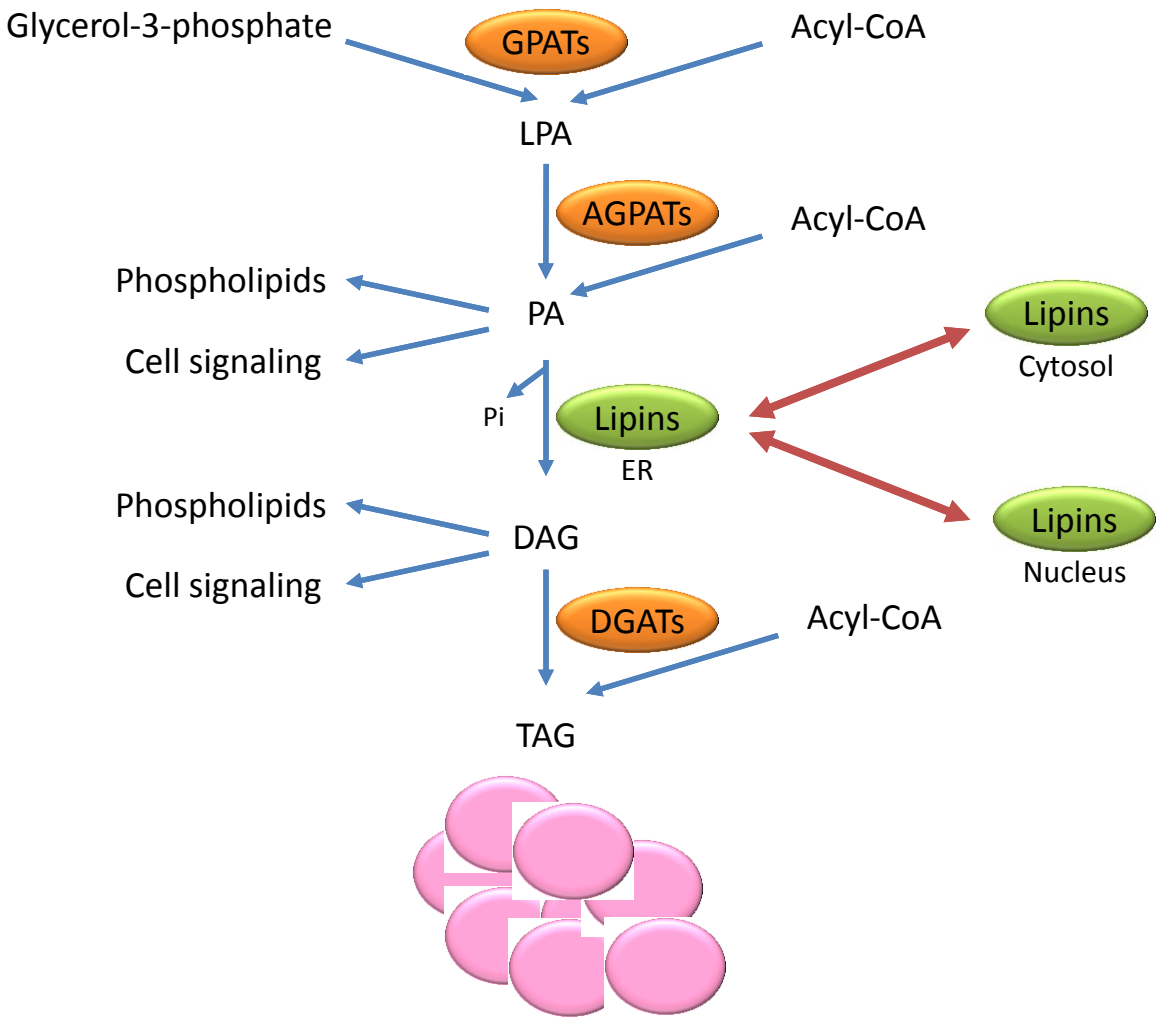

Figure 1.1 – The glycerol-3-phosphate pathway.
Triacylglycerol (TAG) is formed in the glycerol-3-phosphate pathway via the sequential addition of fatty acids to a glycerol-3-phosphate backbone. Fatty acid moieties are added by members of the glycerol phosphate acyltransferase (GPAT), 1-acylglycerol 3-phosphate acyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT) enzyme families. Lipins catalyze the penultimate step, the dephosphorylation of phophtatidic acid (PA) to form diacylglycerol (DAG). GPAT, AGPAT and DGAT are all membrane-anchored proteins, whereas lipins reside in the cytosol and can translocate to the endoplasmic reticulm (ER) membrane or to the nucleus. PA and DAG can also be metabolized into phospholipids and can function in cell signaling. LPA = lysophosphatidic acid.

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CHAPTER TWO

LIPIN PROTEINS AND METABOLIC HOMEOSTASIS

PREFACE

This chapter contains a reprint of a review that Dr. Karen Reue and I worked on together entitled “Lipin Proteins and Metabolic Homeostasis” from a special 50th Anniversary edition of *The Journal of Lipid Research* (2009 Apr;50 Suppl:S109-14). Permission not needed for reprint. My involvement in this review article was largely editorial, helped me to become familiar with the existing literature on lipin proteins, and exposed me to the steps needed to prepare a manuscript for publication.

JOURNAL OF LIPID RESEARCH

Lipin proteins and metabolic homeostasis

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Abstract **The lipin protein family, consisting of three members, was first identified early this century. In the last few years, the lipin proteins have been shown to have important roles in glycerolipid biosynthesis and gene regulation, and mutations in the corresponding genes cause lipodystrophy, myoglobinuria, and inflammatory disorders. Here, we review some of the progress toward elucidating the molecular and physiological functions of the lipin proteins.**—Reue, K., and J. R. Dwyer. **Lipin proteins and metabolic homeostasis.** ***J. Lipid Res.*** **2009.** 50: **S109–S114.**

Supplementary key words lipodystrophy • obesity • triglyceride • phosphatidate phosphatase • insulin resistance

THE LIPIN PROTEIN FAMILY

The lipin protein family consists of three members, lipin-1, lipin-2, and lipin-3. The founding member of the family, lipin-1, was identified via positional cloning as the mutated gene product in the fatty liver dystrophy (*fld*) mouse (1). The name of the mutation refers to the occurrence of fatty liver and hypertriglyceridemia during the neonatal period, and peripheral neuropathy, which progresses throughout adulthood (2, 3). In addition, these mice are lipodystrophic, develop insulin resistance, and have increased susceptibility to atherosclerosis (4). Lipin-1 is expressed in a variety of tissues, with the most prominent expression in adipose tissue, skeletal muscle, and testis (1). Two lipin-1 protein isoforms are generated by alternative mRNA splicing, giving rise to proteins with predicted sizes of ~98 and 102 kDa (5, 6). Interestingly, lipin-1 protein can localize to either the cytoplasm or the nucleus (6), which may be related to the dual roles of the protein (described below).

The genes for lipin-2 and lipin-3 were identified by similarity of their predicted protein sequence to lipin-1 (1). In addition, single lipin orthologs were identified in invertebrates, plasmodia, and yeast, and two lipin orthologs are present in plants. All lipin proteins exhibit two regions of especially high sequence conservation located in the N- and C-terminal protein regions, known as the N-LIP and C-LIP domains, respectively. This review will principally focus on lipin-1, about which the most is known, with information about the other two lipin family members provided where available.

MOLECULAR FUNCTION OF LIPIN PROTEINS

Lipin-1 has two recently discovered molecular functions. First, lipin-1 acts as a phosphatidate phosphatase (PAP) enzyme, which converts phosphatidate to diacylglycerol during triglyceride, phosphatidylcholine, and phosphatidylethanolamine biosynthesis (reviewed in Refs. 7, 8 and Brindley, this issue). Although PAP enzyme activity had been studied for more than 50 years, the molecular identity was a mystery until Han, Wu, and Carman (9) purified the enzyme from the yeast *Saccharomyces cerevisiae* and obtained peptide sequence that identified it as the yeast lipin ortholog. The PAP enzyme activity requires a DxDxT motif located in the C-LIP domain (see **Fig. 1**).

After identification of the yeast PAP enzyme, PAP activity was subsequently demonstrated for all three mammalian lipin proteins, with lipin-1 having the highest specific activity (10, 11). In the presence of elevated fatty acid levels within the cell, lipin proteins translocate from the cytosol to the endoplasmic reticulum membrane, where they encounter phosphatidic acid and catalyze its conversion to diacylglycerol (8). The three lipin genes have a distinct, but overlapping, tissue distribution (10), suggesting that each may be responsible for the PAP activity in a specific set of tissues. As determined in tissues from the *fld* mouse, lipin-1 accounts for all of the PAP activity in adipose tissue and skeletal muscle, but only part of the activity in liver, heart, kidney, and brain (10, 11), and lipin-2 and/or lipin-3 may be active in these tissues.

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Abbreviations: *fld*, fatty liver dystrophy; PAP, phosphatidate phosphatase; PPARα, peroxisome proliferator-activated receptor α.

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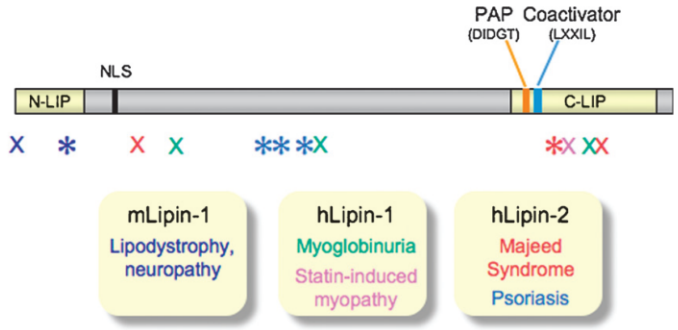

Fig. 1. Lipin protein domains and disease-related mutations. At top is shown a schematic diagram of lipin protein domain structure, with N-LIP and C-LIP domains indicated with yellow shading and other functional motifs labeled. NLS, nuclear localization signal; PAP, PAP active site; coactivator, nuclear receptor interaction motif. Below is shown the position relative to protein domains of nonsense (X) and missense (*) mutations related to disease. mlipin-1, mouse lipin-1; hLipin-1, human lipin-1; hLipin-2, human lipin-2. See text for primary references.

In addition to cytosolic localization, all of the mammalian lipin proteins possess a putative nuclear localization signal. It has been demonstrated that lipin-1 can localize to the nucleus in adipocytes (6) and hepatocytes (12), and subcellular localization may be influenced by protein phosphorylation (11). The role of nuclear lipin-1 may be related to its function as a transcriptional coactivator. Finck et al. (13) have shown that lipin-1 is required for the activation of hepatic fatty acid oxidation genes during fasting conditions. Lipin-1 directly interacts with the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) and PPARγ coactivator 1α (PGC-1α) in a complex that modulates fatty acid oxidation gene expression. Lipin-1 coactivator activity requires an LxxIL sequence motif within the C-LIP domain (Fig. 1). These studies established a key role for lipin-1 in hepatic gene expression during fasting, but it is not clear whether lipin-1 coactivator activity has a physiological role in other tissues nor whether the other lipin family members exhibit similar activity.

The recent elucidation of lipin-1 PAP and coactivator activities has provided new insight into the biological functions of lipin-1. One approach to understanding lipin-1 physiological function has been to study the effects of lipin-1 deficiency or enhanced lipin-1 expression using mouse models. As summarized below and in **Fig. 2**, lipin-1 has a unique physiological role in lipid homeostasis in tissues, including adipose tissue, skeletal muscle, liver, and peripheral nerve.

LIPIN PROTEINS AND ADIPOGENESIS

The lipodystrophy in lipin-1-deficient mice is characterized by the presence of immature adipocytes that fail to store lipid or express mature adipocyte markers (4). The lack of triglyceride storage in adipocytes from these mice can be attributed in large part to the deficiency in PAP activity, which is responsible for lipid synthesis in mature adipocytes. However, lipin-1-deficient cells exhibit additional defects in adipocyte differentiation. Lipin-1-deficient cells and adipose tissue fail to induce the key adipogenic transcription factor, PPARγ, and its target genes and instead express high levels of preadipocyte factor-1, an inhibitor of adipogenesis (14). Complementation of lipin-1-deficient preadipocytes with a retroviral vector expressing PPARγ partially rescues differentiation, suggesting that there is a requirement for lipin-1 at early stages of adipogenesis to facilitate PPARγ expression.

Lipin-2 may also have a role in adipogenesis. Whereas lipin-1 is the predominant lipin in adipose tissue and its expression increases during differentiation of the 3T3-L1 adipocyte cell line, lipin-2 protein can be detected in preadipocytes but diminishes during differentiation (15). The fact that lipin-2 cannot compensate for lipin-1 function in adipose tissue of *fld* mice indicates that the two proteins serve different roles and may suggest that lipin-2 provides a regulatory function during adipogenesis.

LIPIN-1, OBESITY, AND INSULIN SENSITIVITY

While lipin-1 deficiency produces lipodystrophy in the mouse, enhanced lipin-1 expression in either adipose tissue or skeletal muscle promotes obesity (16). On a high-fat diet, both adipose tissue and muscle-specific lipin-1 transgene expression induce more rapid weight gain than in nontransgenic mice by 2-fold (adipose transgenic) or 4-fold (muscle transgenic), despite equivalent food intake. However, the mechanism for the obesity and the resulting effects on glucose homeostasis differ depending on whether lipin-1 expression is enhanced in adipose tissue or in muscle.

The muscle-specific lipin-1 transgenic mice exhibit reduced energy expenditure and develop insulin resistance

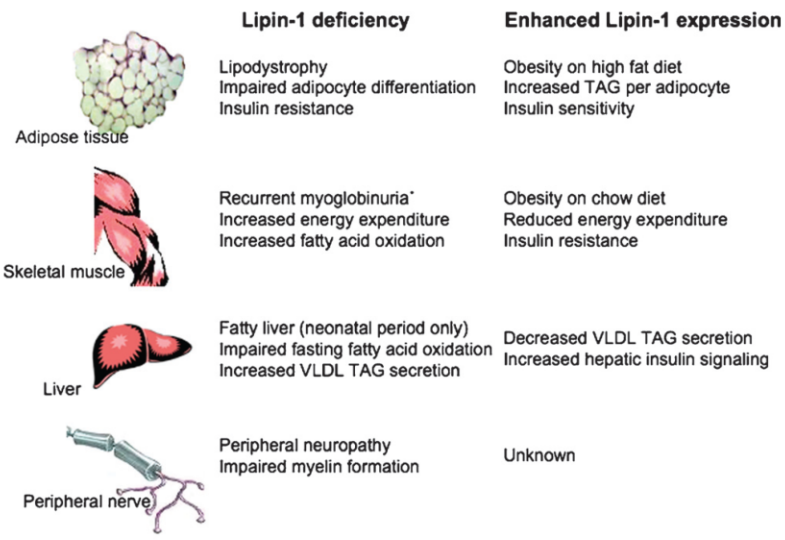

Fig. 2. Physiological effects of lipin-1 deficiency or enhanced expression. Summary of lipin-1 effects on metabolism in tissues shown. Characteristics shown in the left column are derived from studies in the lipin-1-deficient *fld* mouse or lipin-1-deficient human subjects (denoted by *). Characteristics shown in the right column are derived from studies of adipose-specific lipin-1 transgenic mice (adipose), muscle-specific lipin-1 transgenic mice (muscle), or from lipin-1 overexpression in liver via adenovirus (liver). See text for primary references.

(16). The insulin resistance in these mice is presumably secondary to the obesity and/or increased PAP activity leading to triglyceride accumulation and altered metabolism in muscle. In contrast, enhanced lipin-1 levels in adipose tissue lead to increased adipocyte triglyceride content but lower glucose and insulin levels than wild-type mice on both chow and high-fat diets (16). Importantly, studies in humans have revealed a similar positive correlation between lipin-1 expression levels in adipose tissue and insulin sensitivity. This is true in both obese subjects with normal or impaired glucose tolerance (17, 18) and in healthy young men (19). It is possible that lipin-1 may promote efficient incorporation of fatty acids into adipocyte triglycerides, thereby preventing lipid deposition in other tissues where they could compromise insulin action (20). Additionally, in human adipocytes, lipin-1 expression levels are correlated with glucose transporter 4 expression, which may increase glucose uptake (21), and with expression of PPARα and fatty acid oxidation genes, which may prevent fatty acid accumulation (19). Interestingly, lipin-1 expression is induced in adipocytes by insulin-sensitizing drugs such as thiazolidinediones and harmine (18, 22).

In addition to the connection between lipin-1 and insulin sensitivity discussed above, insulin induces phosphorylation of lipin-1 at multiple sites (5, 11). Phosphorylation does not appear to alter the PAP-specific activity but rather to shift the localization toward the cytosolic rather than microsomal compartment (11), potentially altering the proportion of lipin-1 that is available to catalyze the PAP reaction at the microsomal membrane. Studies in yeast have identified a specific phosphatase that acts on yeast lipin, and the mammalian counterpart of this phosphatase, known as Dullard, acts on mammalian lipin-1 (23, 24). Although the physiological significance of lipin protein phosphorylation is not yet fully understood, it is likely to represent an important mechanism for rapid modulation of lipin-1 compartmentalization and/or activity in response to insulin and other metabolic stimuli.

LIPIN-1 AND PERIPHERAL NERVE FUNCTION

The lipodystrophy in lipin-1-deficient mice is accompanied by severe peripheral neuropathy (3). It is now clear that lipin-1 deficiency causes a lack of PAP activity in the fat pads that constitute the bulk of the epineurium in adult nerve, as well as in Schwann cells themselves (25). Elegant studies of a Schwann cell-specific, lipin-1-deficient mouse demonstrated that lack of PAP activity in these cells is sufficient to cause peripheral neuropathy associated with myelin degradation and attenuated nerve conduction velocity (26). Furthermore, the accumulation of phosphatidate, the substrate for PAP, is responsible for eliciting aberrant signaling through the MEK/Extracellular signal-related kinase pathway, leading to dedifferentiation and proliferation of Schwann cells. These results raise the intriguing possibility that other symptoms of lipin deficiency are related not only to the loss of PAP enzyme function, but also to the

deleterious effects of phosphatidate accumulation within the cell (reviewed in Ref. 8).

LIPIN-1 AND HEPATIC LIPOPROTEIN SECRETION

It has been known for decades that PAP activity in the liver is regulated in response to changing metabolic conditions. For example, hepatic PAP activity is diminished in diabetes and starvation conditions and is increased with glucocorticoids (reviewed in Ref. (8). All three lipin family members are expressed in hepatocytes, but their expression is regulated independently. Glucocorticoids specifically increase mRNA and protein levels of lipin-1, but not lipin-2 or lipin-3 (27, 28). The effect of glucocorticoids on lipin-1 is enhanced by glucagon or cAMP and antagonized by insulin (27).

Recent studies have implicated lipin-1 in the synthesis and secretion of VLDL in liver. This function of lipin-1 has been investigated by modulating lipin-1 levels in cultured hepatocytes and in the mouse. Enhanced expression of lipin-1 in a rat hepatocyte cell line led to stimulation of triglyceride synthesis and secretion, while knockdown of endogenous lipin-1 expression decreased the secretion of newly synthesized triglycerides (12). Interestingly, deletion of the lipin-1 nuclear localization signal led to impaired association with the microsomal membranes and less effective induction of triglyceride synthesis. These results suggest that lipin-1 compartmentalization within hepatocytes may be a determinant of triglyceride synthesis and VLDL secretion.

However, studies performed in vivo indicate that the relationship between lipin-1 and VLDL secretion is more complex than observed in a hepatocyte cell line. Hepatocytes isolated from adult *fld* mice were shown to secrete VLDL at increased rates compared with wild-type hepatocytes (29), suggesting that lipin-2 and/or lipin-3 are capable of promoting VLDL synthesis and secretion. On the other hand, mice treated with an adenovirus to increase lipin-1 expression in the liver exhibited reduced rates of VLDL secretion (29), in direct contrast to results obtained in vitro (12). Through the use of mutant lipin-1 molecules, this effect was shown to require lipin-1 transcriptional coactivator but not PAP enzyme function. Consistent with the results of lipin-1 adenovirus overexpression in the mouse, hepatic lipin-1 expression levels and VLDL secretion both increased in obese individuals following gastric bypass surgery (30). Taken together, it appears that lipin proteins influence hepatic triglyceride synthesis and VLDL secretion, but at present, it is difficult to attribute effects solely to specific lipin family members and to distinguish the contributions of PAP versus coactivator activities.

LIPIN-1 GENE MUTATIONS AND POLYMORPHISMS ASSOCIATED WITH DISEASE

Mutations affecting lipin-1 and lipin-2 cause human disease. While two distinct mutations in mouse *Lpin1* cause lipodystrophy (1) (Fig. 1), analysis of human lipodystrophic subjects has failed to detect causative mutations in the *LPIN1* gene (31, 32). However, mutations in *LPIN1* have been identified in patients with recurrent acute myoglobinuria in childhood (33). Distinct inactivating mutations were detected in patients from several ethnic backgrounds and occur at dispersed locations throughout the lipin-1 protein structure (see Fig. 1). Unlike mice with lipin-1 deficiency, the patients were reported to have normal fat distribution, although no quantification of fat was provided, and all subjects were under 10 years of age (33). The authors suggest that lipin-2 expression in adipose tissue may compensate for lack of lipin-1, as lipin-2 expression has been detected in human adipose tissue (10). At present, it is unclear what the effect of lipin-1 deficiency will be in human adults and whether they will develop peripheral neuropathy as is also observed in adult lipin-1-deficient mice.

In addition to the mutations in human lipin-1 described above, *LPIN1* polymorphisms have been associated with numerous metabolic traits. Among these are insulin and/or glucose levels (17, 34, 35), resting metabolic rate (34), and systolic blood pressure (36, 37). *LPIN1* polymorphisms that are associated with response of type 2 diabetic patients to rosiglitazone have also been reported (36). One particularly notable *LPIN1* polymorphism causes an amino acid substitution within the C-LIP domain and is associated with statin-induced myopathy (33), an unfortunate side effect experienced by some of individuals who take statin drugs to treat hypercholesterolemia [reviewed in (38)]. This *LPIN1* variant merits further study in larger numbers of individuals.

LIPIN-2 GENE MUTATIONS AND DISEASE

LPIN2 mutations cause Majeed syndrome, an inflammatory disorder characterized by recurrent sterile osteomyelitis, cutaneous inflammation, and dyserythropoietic anemia (39, 40). Three independent *LPIN2* mutations have been described, two of which result in truncated protein or decayed mRNA, and a third point change altering a highly conserved amino acid residue downstream of the PAP active site in the C-LIP domain (see Fig. 1). It is unclear what the normal physiological role of lipin-2 is and whether the inflammatory symptoms observed in Majeed syndrome are direct effects of lipin-2 deficiency in tissues such as bone, skin, and erythrocytes, or perhaps secondary effects resulting from altered lipid homeostasis in other tissues. Finally, a polymorphism in the 3′ untranslated region of *LPIN2* has been linked to diabetes risk (41).

SUMMARY AND FUTURE DIRECTIONS

The lipin proteins represent relatively new identities for an "old" function, the PAP enzyme activity that is critical in glycerolipid biosynthesis. Lipin-1 (and most likely lipin-2 and lipin-3) leads a double life as a transcriptional

coactivator that promotes fatty acid oxidation, a function that, paradoxically, has the opposite effect on lipid storage as PAP enzyme activity. The dual functions of lipin proteins may allow them to adapt their activity to cellular lipid storage requirements. Many questions remain, particularly regarding the specific roles of lipin-2 and lipin-3, and the interrelationships among the family members in tissues where multiple lipins are expressed. These questions will be addressed in the coming years by identifying additional informative genetic variants in the human population and by the generation of genetically modified mouse models.

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CHAPTER THREE

MOUSE LIPIN-1 AND LIPIN-2 COOPERATE TO MAINTAIN GLYCEROLIPID HOMEOSTASIS IN LIVER AND AGING CEREBELLUM

PREFACE

This chapter is contains the first report of a lipin-2–deficient mouse model. This manuscript was accepted for publication in *The Proceedings of the National Academy of Sciences USA* on July 26, 2012. Permission is not needed for reprint.

At the onset of this project, little was known about lipin-2. Lipin-2 was discovered by sequence similarity to lipin-1 and mRNA analysis showed high levels of lipin-2 mRNA in liver and brain. *In vitro* studies had demonstrated that lipin-2 has phosphatidic acid phosphatase activity, but the *in vivo* function of lipin-2 was not well studied. To generate an experimental model to study lipin-2 physiology, we collaborated with Drs. Stephen G. Young and Loren G. Fong to generate lipin-2 knockout (KO) mice. We used a targeted trapping strategy to insert a β-galactosidase-neomycin (β-geo) resistance cassette within the *Lpin2* gene to disrupt its function and to generate a lipin-2–β-geo fusion transcript under the control of the endogenous *Lpin2* promoter. This was useful for comparing the expression pattern of lipin-2 protein with that of the *Lpin2* RNA, as an informative lipin-2 antibody did not exist when we began this work.

The characterization of the lipin-2 KO mouse revealed a previously unknown functional relationship between lipin-1 and lipin-2 in liver, and demonstrated that the presence of both lipin-1 and lipin-2 are optimal for handling the stress of high fat diet feeding. It also revealed that both lipin-1 and lipin-2 act in the cerebellum, and that lipin-2 is critical in the aging cerebellum, as lipin-1 expression is extinguished with ageing. Lipin-2–deficient mice develop a late onset ataxia related to loss of cerebellar phosphatidic acid phosphatase activity and accumulation of the lipid intermediate, phosphatidate. Lipin-2 staining was detected in the Purkinje cell layer of the cerebellum, a cell population that is known for coordinating movement. Further work will hopefully reveal the molecular mechanism by which loss of lipin-2 causes ataxia.

This was a highly collaborative project performed under the direction of Dr. Karen Reue. All of the authors participated to various degrees to the intellectual progress of this project, in addition to technical assistance. Drs. Pieter de Jong and Yuko Yoshinaga worked with Drs. Young and Fong to generate the *Lpin2* targeted trapping construct. Dr. Jimmy Donkor bred the initial lipin-2 KO mice and performed preliminary metabolic studies on the first few cohorts that became available. Dr. David N. Brindley and Mr. Jay Dewald performed PAP enzyme assays on our mouse tissue samples.

I took over maintenance of the lipin-2 KO mouse colony from Dr. Donkor when he left the laboratory. I bred mice for the studies, performed the β-galactosidase assays on the gross organs and tissue sections, and photographed them to show the expression pattern of lipin-2 protein. I photographed the excess lipid accumulation in the lipin-2 KO liver and performed TAG assays on hepatic extracts. I performed the majority of the quantitative real-time PCR (qPCR), all of the western blotting, subcellular fractionation and immunohistochemistry in this manuscript. I characterized the ataxia in lipin-2 KO mice using techniques that I learned at the UCLA Behavioral Testing Core. I coordinated most of the mouse dissections, including collecting bones for microCT analyses, and performed high fat diet feeding studies.

Dr. Peixiang Zhang performed tissue lipid extraction and was responsible for their analysis by mass spectrometry. Dr. Sotirios Tetradis and Mrs. Elisa Atti performed microcomputed tomography to assess changes in lipin-2–deficient bone. Mrs. Lauren S. Csaki was actively involved in many of the dissection procedures and assisted me in the SHIRPA behavioral analysis. Dr. Laurent Vergnes aided in the lipin-1 and lipin-2 double knockout embryology studies and Ms. Jessica M. Lee provided technical assistance with microscopy.

Dr. Reue and I worked closely together to interpret and assemble all of the data into figures and tables. I prepared all of the figures shown in the manuscript. I also assisted in revising this manuscript and submitting it for publication.

Mouse lipin-1 and lipin-2 cooperate to maintain glycerolipid homeostasis in liver and aging cerebellum

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AUTHOR SUMMARY

Lipid homeostasis requires a continual balance among cellular lipid species to coordinate the metabolic and structural needs of the cell. The synthesis of glycerolipids, phospholipids, and triacylglycerol serves to transform reactive lipid intermediates into stable lipid components that play key roles in membrane structure (phospholipids) and cellular lipid storage (triacylglycerol). Members of the lipin protein family perform the penultimate step in glycerolipid synthesis, dephosphorylation of phosphatidic acid (PA) to diacylglycerol (reviewed in ref. 1). Three lipin proteins are found in mammals (lipin-1, lipin-2, and lipin-3), and each exhibits PA phosphatase activity, but the specific physiological function of each family member is not well understood. Here, we determined that lipin-2 is prominently expressed within hepatocytes in the liver and is localized to the cerebellum, cerebral cortex, and hippocampus within the brain. These findings suggest a role for lipin-2 in the liver and brain.

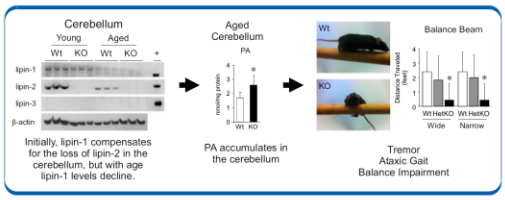

Fig. P1. Lipin-2–deficient mice develop age-dependent cerebellar defects. *Left*, In young mice (2 mo of age), lipin-1 can compensate for the lack of lipin-2 in the cerebellum of lipin-2–deficient mice. However, cerebellar lipin-1 levels diminish in aging mice (10 mo of age), leaving no detectable lipin proteins in the cerebellum of lipin-2–deficient mice. *Center*, Lack of functional lipin in the cerebellum of aged mice leads to the accumulation of the lipin substrate phosphatidate (PA). *Right*, Aging lipin-2–deficient mice show a tremor, ataxic gait, and severely impaired balance.

Naturally occurring mutations in mice and humans reveal that lipin-1 plays a critical role in regulating triglyceride storage and levels of the bioactive lipid phosphatidate in fat tissue, muscle, and the peripheral nervous system (reviewed in ref. 2). *LPIN2* mutations cause the rare disease Majeed syndrome, characterized by anemia and recurrent multifocal osteomyelitis (3). As a result of limitations imposed for conducting human studies, patients with Majeed syndrome have not been examined for abnormalities in tissues in which lipin-2 is most prominently expressed, such as the liver and brain. We describe here the generation of a knockout (KO) mouse model of lipin-2 deficiency to investigate the role of lipin-2 in normo- and pathophysiology. We uncovered a functional relationship between lipin-1 and lipin-2, which operates in a tissue-specific and age-dependent manner to maintain lipid homeostasis in the liver and brain.

We used a targeted trapping technique to replace mouse *Lpin2* with a gene encoding a lipin-2–β-gal fusion protein that is expressed under the control of the endogenous *Lpin2* promoter but does not possess PA phosphatase activity. Lipin-2 mRNA and protein were undetectable in tissues of the lipin-2–KO mice. By visualizing the β-gal fusion protein, we determined that lipin-2 is prominently expressed within hepatocytes in the liver and is localized to the cerebellum, cerebral cortex, and hippocampus within the brain. Because lipin-2 is the most prominent lipin species in the liver, we expected that KO mice would exhibit a dramatic reduction in hepatic PA phosphatase activity. Surprisingly, PA phosphatase activity was instead increased in liver homogenates from heterozygous and homozygous KO compared with WT mice and was associated with a compensatory increase in lipin-1 protein levels. Under basal metabolic conditions, this compensatory change was adequate to maintain nearly normal hepatic triglyceride levels and phospholipid profiles. However, when subjected to metabolic stress by feeding a high-fat diet, lipin-2–KO mice accumulated excess hepatic triglyceride, suggesting that the combined action of lipin-1 and lipin-2 in the liver serves to maintain homeostasis under dietary stress.

Evidence of a role for lipin-2 in brain lipid homeostasis became apparent as lipin-2–deficient mice aged (Fig. P1). Beginning at the age of 5 mo, the KO mice developed motor defects, first noticeable as a tremor and ataxic gait. The ataxic gait was reminiscent of human diseases affecting the cerebellum, which is crucial for controlling balance and gait. We therefore analyzed lipin protein levels and the phospholipid content of the cerebellum before and after onset of ataxia (Fig. P1). In young (2 mo old) WT mice, lipin-1 and lipin-2 were evident in the cerebellum, whereas lipin-3 was not detectable. Lipin-1 levels diminished dramatically as mice aged, and lipin-2 levels decreased somewhat, leaving lipin-2 as the primary lipin present in the cerebellum of aged WT mice (Fig. P1, *Left*). Because of this, lipin-2–deficient mice had virtually no cerebellar lipin proteins at advanced age. This was reflected by a 50% increase in the levels of the lipin substrate, PA (Fig. P1, *Center*).

Author contributions: J.R.D., S.G.Y., and K.R. designed research; J.R.D., J. Donkor, P.Z., L.S.C., L.V., J.M.L., J. Dewald, E.A., and L.G.F. performed research; Y.Y., P.J.D.J., and L.G.F. contributed new reagents/analytic tools; J.R.D., J. Donkor, P.Z., L.S.C., L.V., J. Dewald, D.N.B., S.T., and K.R. analyzed data; and J.R.D., S.G.Y., and K.R. wrote the paper.

The authors declare no conflict of interest.

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Mouse lipin-1 and lipin-2 cooperate to maintain glycerolipid homeostasis in liver and aging cerebellum

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The three lipin phosphatidate phosphatase (PAP) enzymes catalyze a step in glycerolipid biosynthesis, the conversion of phosphatidate to diacylglycerol. Lipin-1 is critical for lipid synthesis and homeostasis in adipose tissue, liver, muscle, and peripheral nerves. Little is known about the physiological role of lipin-2, the predominant lipin protein present in liver and the deficient gene product in the rare disorder Majeed syndrome. By using lipin-2–deficient mice, we uncovered a functional relationship between lipin-1 and lipin-2 that operates in a tissue-specific and age-dependent manner. In liver, lipin-2 deficiency led to a compensatory increase in hepatic lipin-1 protein and elevated PAP activity, which maintained lipid homeostasis under basal conditions, but led to diet-induced hepatic triglyceride accumulation. As lipin-2–deficient mice aged, they developed ataxia and impaired balance. This was associated with the combination of lipin-2 deficiency and an age-dependent reduction in cerebellar lipin-1 levels, resulting in altered cerebellar phospholipid composition. Similar to patients with Majeed syndrome, lipin-2–deficient mice developed anemia, but did not show evidence of osteomyelitis, suggesting that additional environmental or genetic components contribute to the bone abnormalities observed in patients. Combined lipin-1 and lipin-2 deficiency caused embryonic lethality. Our results reveal functional interactions between members of the lipin family in vivo, and a unique role for lipin-2 in central nervous system biology that may be particularly important with advancing age. Additionally, as has been observed in mice and humans with lipin-1 deficiency, the pathophysiology in lipin-2 deficiency is associated with dysregulation of lipid intermediates.

Lipid homeostasis requires a precise balance among cellular lipid species to coordinate the metabolic and structural needs of the cell. The synthesis of glycerolipids serves to transform reactive lipid intermediates into stable lipid components that play key roles in membrane structure (i.e., phospholipids) and cellular lipid storage (i.e., triacylglycerol) (1, 2). Members of the lipin protein family perform the penultimate step in glycerolipid synthesis, dephosphorylation of phosphatidic acid (PA) to diacylglycerol (DAG) (reviewed in refs. 3–5). Three lipin proteins are found in mammals (lipin-1, lipin-2, and lipin-3), and each exhibits phosphatidate phosphatase (PAP) activity, but the specific physiological function of each family member is not well understood. The elucidation of mechanisms that regulate triglyceride storage in various tissues is of interest because of an association between obesity and metabolic dysregulation including insulin resistance, type 2 diabetes, hyperlipidemia, and atherosclerosis (6).

To date, the majority of in vivo studies on lipin protein physiology have involved lipin-1 function. A naturally occurring mutation in the mouse lipin-1 gene causes generalized lipodystrophy (7, 8), and lipin-1–deficient humans have severe myopathy and rhabdomyolysis in childhood (9, 10). These phenotypes correlate with the requirement for lipin-1 to provide PAP activity in adipose tissue and skeletal muscle (11, 12). Growing evidence suggests that in addition to the synthesis of glycerolipids, an important role of lipin-1 is to prevent the accumulation of lipid intermediates in the cell. Lipin-1–deficient tissues in mouse, and lipin-1–deficient muscle in humans, accumulate PA, the substrate of lipin PAP activity (10, 13, 14). PA accumulation in lipin-1–deficient adipose tissue and Schwann cells leads to inappropriate activation of the MAPK/ERK signaling pathway and inhibition of cellular differentiation, contributing to the lipodystrophy and peripheral neuropathy occurring in these mice (13, 14). Lipin-1 can also function as a transcriptional coactivator (15) or corepressor (16). The physiological roles of these activities include the activation of hepatic fatty acid oxidation gene expression in fasting liver (15), and the repression of inflammatory and adipogenic genes in adipocytes (16).

Relatively little is known about the functions of lipin-2 and lipin-3 in vivo. Homozygous mutations in human *LPIN2* lead to the rare disorder known as Majeed syndrome (17–19). Majeed syndrome has been reported thus far in only three families, all residing in the Middle East (20). The hallmarks of the disease are congenital dyserythropoietic anemia and recurrent episodes of osteomyelitis; transient dermatosis may also be present (19, 21). Thus far, three mutant alleles have been identified: a nonsense mutation, a splice site mutation, and a missense mutation involving a highly conserved serine residue (17, 18, 21). The first two mutations are predicted null alleles in which functional lipin-2 protein is not present. The missense mutant protein is stable and exhibits coactivator function similar to lipin-1 protein but lacks PAP activity, indicating that the loss of lipin-2 PAP activity is a defining feature of the disease (22). No human diseases caused by lipin-3 deficiency have been described.

In the mouse, lipin-2 mRNA is expressed most prominently in liver, with substantial levels in other tissues including brain (22), raising the possibility that lipin-2 deficiency may alter glycerolipid metabolism in these tissues. Consistent with a role for lipin-2 PAP activity in liver, knockdown of lipin-2 in isolated hepatocytes leads to reduced PAP activity and triglyceride synthesis (23). Data from in vivo studies are limited, but indicate that lipin-2 levels are regulated by various metabolic perturbations. Mouse lipin-2 protein levels are increased by fasting, obesity,

Author contributions: J.R.D., S.G.Y., and K.R. designed research; J.R.D., J. Donkor, P.Z., L.S.C., L.V., J.M.L., J. Dewald, E.A., and L.G.F. performed research; Y.Y., P.J.D.J., and L.G.F. contributed new reagents/analytic tools; J.R.D., J. Donkor, P.Z., L.S.C., L.V., J. Dewald, D.N.B., S.T., and K.R. analyzed data; and J.R.D., S.G.Y., and K.R. wrote the paper.

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consuming a high-fat diet, and treatment with inducers of endoplasmic reticulum stress (23, 24), suggesting a potential role in metabolism of excess fatty acids released from adipose tissue stores or diet.

To gain better insight into the physiological role of lipin-2, we developed and characterized a mouse model of lipin-2 deficiency. We identified in vivo functional interactions between lipin-1 and lipin-2 that contribute to lipid homeostasis in liver and cerebellum, and uncovered a critical role for lipins in the aging brain. These results illustrate how members of a protein family can cooperate to maintain tissue homeostasis, and reveal that the nature of the cooperation may occur in tissue- and age-specific manners.

Results

Generation of Mice with Lipin-2 Deficiency and in Vivo Reporter for Lipin-2 Expression. Lipin-2–deficient mice were developed by homologous recombination in ES cells. The *Lpin2* gene, which comprises 20 exons and spans 67 kb, was inactivated by insertion of a promoterless β-geo gene-trapping cassette into intron 3 (Fig. 1*A*). Transcription of this allele produces a fusion transcript consisting of exons 1 through 3 of *Lpin2* followed by the β-geo cassette, and a corresponding protein with β-gal activity that is expressed under the control of *Lpin2* gene-regulatory elements. Mice that are heterozygous or homozygous for the mutant allele exhibit the expected reductions in *Lpin2* mRNA levels (Fig. 1*B*), and lipin-2 protein is virtually undetectable in the liver of homozygotes (Fig. 1*C*). β-Gal activity was evident in tissues in which we previously detected prominent lipin-2 mRNA expression (22). Thus, there was robust staining in liver within hepatocytes, and prominent staining in the brain in the cerebellum, cerebrum, and hippocampus (Fig. 1*D*). We did not detect staining in several tissues in which lipin-1 accounts for nearly all PAP activity (e.g., white adipose tissue, brown adipose tissue, skeletal muscle) (11, 12).

Hepatic Compensation of Lipin-2 Deficiency by Increased Lipin-1 Protein Levels. As the most prominent site of lipin-2 expression is liver (22, 23) (Fig. 1*D*), we expected that lipin-2–KO mice would have reduced hepatic PAP activity. Instead, we found that PAP activity in liver of the lipin-2–KO homozygous and heterozygous mice was higher than in WT mice (Fig. 2*A*). By contrast, PAP activity in white adipose tissue and skeletal muscle was normal in lipin-2–deficient mice (Fig. 2*A*), consistent with the previous demonstration that lipin-1 is responsible for PAP activity in these tissues (7, 11, 12). Consistent with the lack of impairment in PAP activity in lipin-2–deficient liver, levels of PA and other phospholipid species were normal (Fig. 2*B*).

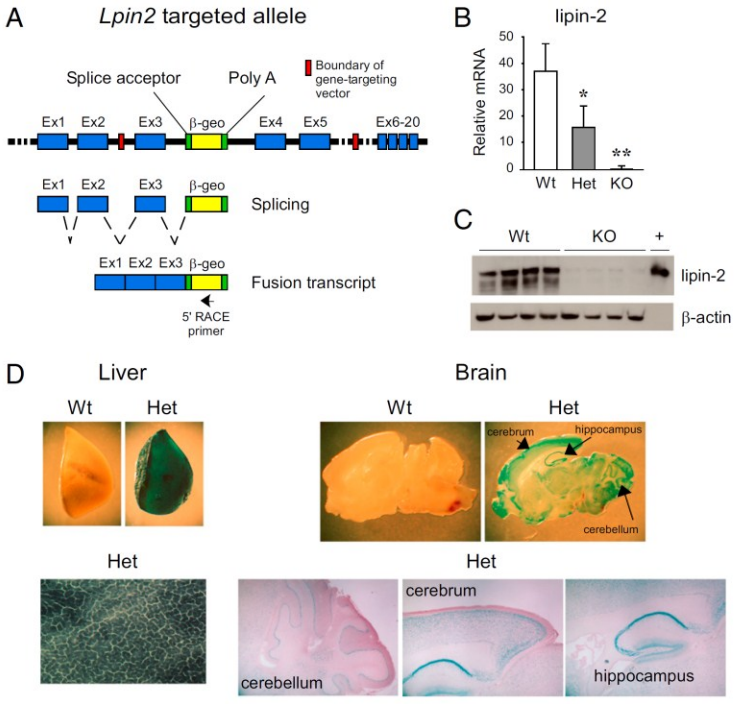

Fig. 1. Inactivation of *Lpin2* by targeted trapping. (*A*) *Lpin2* was inactivated by insertion of the β-geo (fusion of β-gal and neomycin resistance genes) trapping cassette into intron 3 of the gene. Normal gene transcription leads to the production of a fusion mRNA transcript consisting of exons 1 to 3 of *Lpin2* and the β-geo cassette (omits exons 4–20 of *Lpin2*). Targeted cells were identified by 5′ RACE with a β-geo primer (arrow) and by DNA sequencing to confirm expression of a fusion transcript that includes the β-geo gene and extends beyond the 5′ boundary of the targeting vector, into exon 2 of the endogenous *Lpin2* gene. (*B*) Lipin-2 mRNA was quantified in liver of WT (*Lpin2*$^{+/+}$), heterozygous (Het, *Lpin2*$^{+/-}$), and KO (*Lpin2*$^{-/-}$) mice by qPCR ($n = 5$ for each genotype). Values represent mean ± SD (*$P < 0.05$ and **$P < 0.01$ vs. WT). (*C*) Lipin-2 protein was detected in liver of WT or KO mice by using lipin-2–specific antibody. β-Actin was detected as a loading control, and recombinant lipin-2 protein expressed in HEK293 cells was used as a positive control (+). (*D*) Lipin-2–β-geo fusion protein was assessed in tissues of WT and heterozygous mice by staining for β-gal activity. In heterozygous mice, blue staining was most abundant within hepatocytes and the indicated brain regions. Gross sections were magnified 20× to 50×; liver sections were magnified 200×; brain sections were magnified 40×.

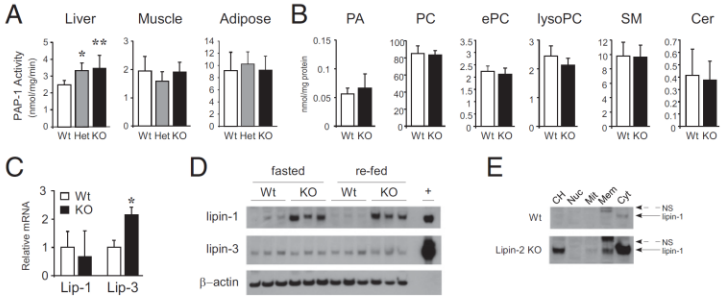

Fig. 2. Enhanced PAP activity and lipin-1 protein expression in lipin-2–KO mice. (*A*) PAP enzyme activity in liver, muscle, and adipose tissue from WT, heterozygous, and KO mice fed a chow diet. Enzyme activity is normalized to cellular protein. (*B*) Total phospholipids were quantified in lipid extracts made from WT and KO liver by ESI-MS and normalized to cellular protein. PC, phosphatidylcholine; ePC, ether-linked PC; SM, sphingomyelin; Cer, ceramides. (*C*) Relative mRNA levels for lipin-1 and lipin-3 were quantified in livers of WT and KO mice by qPCR. In *A*–*C*, $n = 5$ or 6 for each genotype; values represent mean ± SD (*$P < 0.05$ and **$P \leq 0.005$ vs. WT). (*D*) Western blot analysis of lipin-1 and lipin-3 protein levels in liver from fasted and fasted/refed WT and lipin-2–KO mice. β-Actin was used as a loading control, and recombinant lipin proteins were expressed in HEK 293 cells and used as positive controls (+). Note the large increases in lipin-1 protein in lipin-2–deficient samples. (*E*) Subcellular distribution of lipin-1 protein in liver of WT and lipin-2–deficient mice. Hepatic protein lysates were separated into total cell homogenate (CH), nuclear (Nuc), mitochondrial (Mit), membrane (Mem), and cytosolic (Cyt) fractions. Lipin-1 in lipin-2–deficient liver resides primarily in cytosolic and membrane fractions. NS, nonspecific band.

The elevated PAP activity in the liver of lipin-2–deficient mice suggested that that there may be compensatory increases in lipin-1 or lipin-3. Lipin-1 mRNA levels were unchanged, whereas lipin-3 mRNA levels were increased (Fig. 2*C*); however, it should be noted that the absolute levels of lipin-3 mRNA are very low. At the protein level, lipin-1 levels in the liver were far higher in lipin-2–deficient mice than in WT mice in fasted and refed states, whereas similar low levels of hepatic lipin-3 were present in lipin-2 KO and WT liver (Fig. 2*D*). Subcellular fractionation studies showed that the lipin-1 in the liver of lipin-2 KO mice exhibits a similar subcellular distribution to that in WT liver. In both cases, the majority of lipin-1 was present in the cytosol, and a much smaller proportion was associated with the membrane fraction (Fig. 2*E*). The latter includes endoplasmic reticulum (ER) membranes, to which lipin-1 translocates to catalyze the PAP reaction (4). Nuclear lipin-1 levels were very low in WT and lipin-2–KO liver. Thus, the maintenance of PAP activity and phospholipid levels in lipin-2–KO liver are likely a result of compensation by lipin-1 protein.

We assessed whether the increased PAP activity in liver of lipin-2–KO mice correlates with increased hepatic triacylglycerol or circulating lipid levels. On a chow diet, hepatic triacylglycerol and fasting plasma lipid levels (total cholesterol, HDL cholesterol, triglycerides, free fatty acids) in lipin-2–KO mice were similar to those in WT mice (Fig. 3*A* and Table S1). However, when stressed with a high-fat diet (60% fat calories), lipin-2–KO mice accumulated significantly more hepatic triacylglycerol stores than WT mice (Fig. 3*A* and *B*), likely related to higher hepatic PAP activity levels. In contrast, plasma triglyceride and free fatty acid levels were 20% lower in lipin-2–KO mice (Table S1). There were no substantial differences in lipin-2 heterozygous or homozygous null mice in body weight or proportional liver weight on chow or high-fat diets; the gonadal fat pad mass was modestly increased in heterozygous lipin-2–KO compared with WT mice fed a high-fat diet ($P < 0.05$; Fig. S1 *A* and *B*). Thus, the compensation for lipin-2 loss by hepatic lipin-1 largely maintains hepatic and plasma lipid homeostasis in chow fed animals. However, the compensation is imperfect in mice fed a high-fat diet and highlights a requirement for normal levels of lipin-1 and lipin-2 to maintain lipid homeostasis under dietary stress.

In addition to its enzymatic activity, lipin-1 acts as a transcriptional coactivator for expression of fatty acid oxidation genes in the liver (15). To evaluate whether the increased lipin-1 protein present in lipin-2 KO liver had coactivator activity, we quantitated expression levels for known lipin-1 coactivator target genes (15). We found no difference between chow-fed lipin-2–KO and WT mice in hepatic expression of peroxisome proliferator-activated receptor α (*Ppara*), carnitine palmitoyltransferase 1a (*Cpt1a*), and medium chain acyl-CoA dehydrogenase (*Acadm*; Fig. 3*C*). In mice fed a high-fat diet, *Ppara* and *Acadm* expression levels were modestly but significantly reduced in lipin-2–KO liver (Fig. 3*C*). Thus, the elevated levels of lipin-1 protein in lipin-2–KO liver appear not to promote fatty acid oxidation, but likely contribute to enhanced triacylglycerol storage in mice fed a high-fat diet.

Previous work suggests that hepatic lipin-2 has a role in the response to ER stress, particularly in mice fed a high-fat diet (24). We therefore assessed whether the absence of lipin-2 would alter glucose homeostasis or the expression of ER stress markers in mice maintained on chow and high-fat diets. The expression levels for unfolded protein response genes *Chop* (C-EBP homologous protein), *Grp78* (glucose-regulated protein, 78 kDa), and *Xbp1* (X-box binding protein 1, unspliced and spliced forms) were similar in livers of WT and lipin-2–KO mice on chow and high-fat diets, except for a reduction in *Grp78* expression on a chow diet (Fig. 3*D*). Fasting plasma glucose levels were slightly reduced in homozygous KO mice on a chow diet, but this difference was not present in mice fed a high-fat diet, and the KO mice did not appear to have altered glucose tolerance (Fig. S1*C*). These results suggest that a chronic deficiency of lipin-2 does not directly alter hepatic ER stress, potentially because of compensatory changes by other lipin protein family members.

Lipin-2–Deficient Mice Develop Ataxia Associated with Age-Dependent Loss of Lipin-1 in Cerebellum. Throughout development and young adulthood, lipin-2–KO mice were grossly indistinguishable from WT mice. However, beginning at 5 to 6 mo of age, the lipin-2–deficient mice developed a tremor (Fig. 4*A*). The lipin-2–KO mice had an ataxic gait and flicked their tails in a rapid side-to-side movement, most likely to aid in maintaining balance. They were able to rear on their hind legs but typically toppled over after a few seconds. Lipin-2–KO mice exhibited normal grip strength and righting reflex (Fig. S2), but were unable to walk along a balance beam (Fig. 4*B*). WT and heterozygous mice traversed the entire length of wide (2.2 cm) and narrow (1.6 cm) beams nearly 100% of the time without prior training. In contrast, lipin-2–KO mice immediately swung perpendicular to the beam, hung by the

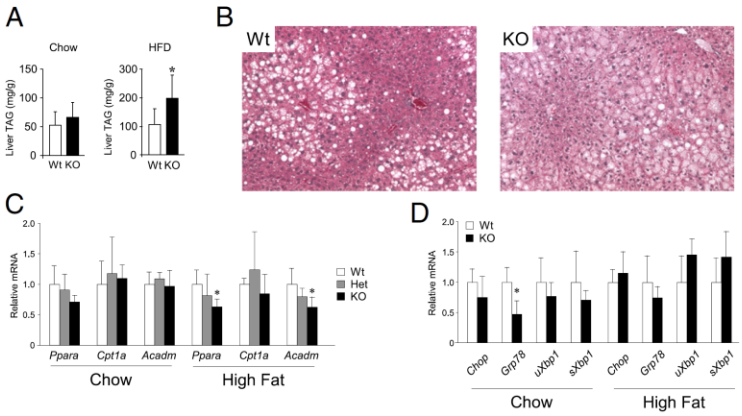

Fig. 3. Lipin-2–KO mice accrue excess hepatic triglyceride on a high-fat diet. (*A*) Hepatic triglyceride (TAG) content in WT and lipin-2–KO mice fed a chow or high-fat diet for 7 wk. Lipid values are normalized to cellular protein (n = 5–6 for each genotype; values represent mean ± SD; *P < 0.05 vs. WT). (*B*) Photomicrographs (magnification of 200×) show representative liver sections from mice fed the high-fat diet and stained with H&E. Lipid accumulation is visible as unstained droplets. (*C*) Expression levels of known lipin-1 coactivator target genes were determined by qPCR in liver samples from WT, heterozygous, and lipin-2–KO mice. *Acadm*, medium chain acyl CoA dehydrogenase; *Cpt1a*, carnitine palmitoyltransferase-1α; *Ppara*, peroxisome proliferator-activated receptor α (n = 5 per genotype; *P < 0.05 vs. WT). (*D*) Expression levels of ER stress genes were determined by qPCR in liver of WT and lipin-2–KO liver. *Chop*, C/EBP homologous protein (also known as DNA-damage-inducible transcript 3); *Grp78*, glucose-regulated protein, 78 kDa (also known as heat shock protein 5); *Xbp1*, X-box binding protein 1, unspliced (uXbp1) and spliced (sXbp1) forms (n = 6–9 per genotype).

forepaws, and fell off within a few seconds (Fig. 4*B*, *Lower*). As the mutant mice did not have impaired grip strength or righting reflex (Fig. S2), the most likely cause for this phenotype was impaired balance.

Lipin-2 is prominently expressed in the cerebellum (Fig. 1*D*), and the ataxic gait in lipin-2–KO mice is reminiscent of human diseases affecting the cerebellum, which is crucial for controlling balance and gait (25, 26). It has been previously demonstrated that PAP activity in the rat cerebellum decreases with age (27–29), but we are aware of no studies that have investigated the role of lipin proteins in cerebellum. Western blot analysis of the three lipin family members was performed in young mice at an age before onset of ataxia in lipin-2–KO mice (2 mo of age), and in old mice at a point when ataxia was severe (10–12 mo of age). In young mice, lipin-1 and lipin-2 proteins were both present in cerebellum, whereas lipin-3 was not detectable (Fig. 4*C*, *Left*). Interestingly, lipin-1 protein levels diminished with age, and nearly undetectable levels of lipin-1 remained in cerebellum of WT and lipin-2–KO mice at 10 to 12 mo of age (Fig. 4*C*, *Right*). Lipin-2 protein levels also decreased in cerebellum of aged WT mice, but remained clearly detectable (Fig. 4*C*). By contrast, lipin-2–KO mice had virtually no remaining lipin proteins at advanced age.

In lipin-1–deficient mice, the accumulation of the PAP enzyme substrate, PA, in Schwann cells and adipose tissue has been implicated in the pathogenesis of peripheral neuropathy and lipodystrophy, respectively (11, 30). This raised the possibility that PA accumulation in lipin-2–KO cerebellum as the mice age contributes to the ataxia. It is also possible that a diminished supply of the PAP product, DAG, could influence intracellular signaling and cerebellar function. We performed electrospray ionization (ESI)-MS on lipid extracts from presymptomatic (2 mo of age) and ataxic (10–12 mo of age) mice. We detected normal PA levels in the young mice (Fig. 4*D*, *Left*), but ataxic lipin-2–KO mice exhibited a 50% increase in total PA levels affecting all of the most abundant PA species (Fig. 4*E*). The PA accumulation was specific for cerebellum and not detected in cerebral cortex (Fig. S3). Several other phospholipid classes, including sphingomyelin and ceramides, were normal in lipin-2–KO cerebellum (Fig. 4 *D* and *E*), aside from modest changes in specific phosphatidylcholine (PC) species (Fig. S4). Total DAG levels were normal in lipin-2–KO mice at both ages assessed, although some individual DAG species exhibited 20% higher or lower levels in aged lipin-2–KO cerebellum (Fig. 4 *D* and *E*). Thus, it appears that lipin-2 activity is not required for the maintenance of DAG levels in the cerebellum, and suggests that insufficient DAG is unlikely to be a major contributor to the phenotype. Together, our results indicate that lipin-2 plays a critical role in maintaining lipid homeostasis in cerebellum as mice age and the levels of lipin-1 protein diminish.

To further define the role of lipin-2 in cerebellum, we localized the protein within this tissue. β-Gal activity staining (Fig. 4*F*) and immunocytochemistry with lipin-2 antibody (Fig. 4*G*) revealed that lipin-2 localizes to the cell layer between the granular and molecular layers of the cerebellum, where Purkinje cells reside. As expected, β-gal staining was not detectable in WT mice, but was robust in the Purkinje cell layer of mice heterozygous for the lipin-2–β-gal allele (Fig. 4*F*, *Center*). Interestingly, the stained cell layer in 10 mo-old lipin-2–KO mice consistently exhibited an irregular appearance, suggesting partial cell loss (Fig. 4*F*, *Right*). Lipin-2 antibody revealed diffuse staining throughout the cytoplasm of cells at the border of the granular and molecular layers, and staining was absent in these cells of lipin-2–KO mice (Fig. 4*G*). Lipin-2 localization to Purkinje cells together with the late-onset ataxia and cerebellar PA accumulation in lipin-2–KO mice suggest a critical function for lipin-2 in maintenance of Purkinje cell lipid homeostasis.

Anemia but No Osteomyelitis in Lipin-2–Deficient Mice. The primary symptoms of Majeed syndrome in humans are dyserythropoietic anemia and recurrent osteomyelitis (20). Reminiscent of humans with Majeed syndrome, the lipin-2–KO mice had mild anemia, with reduced RBC volume and hemoglobin levels, and increased RBC distribution width, indicating the presence of immature erythrocytes (Table 1). However, lipin-2–KO mice studied through 18 mo of age failed to exhibit features of osteomyelitis, such as tail kinks, foot deformities, joint swelling, or thickened ears. We examined tibias from numerous mice of advanced age (n = 13 WT and n = 11 KO mice) for evidence of osteomyelitis by radiography and low-resolution micro-computed tomography (microCT),

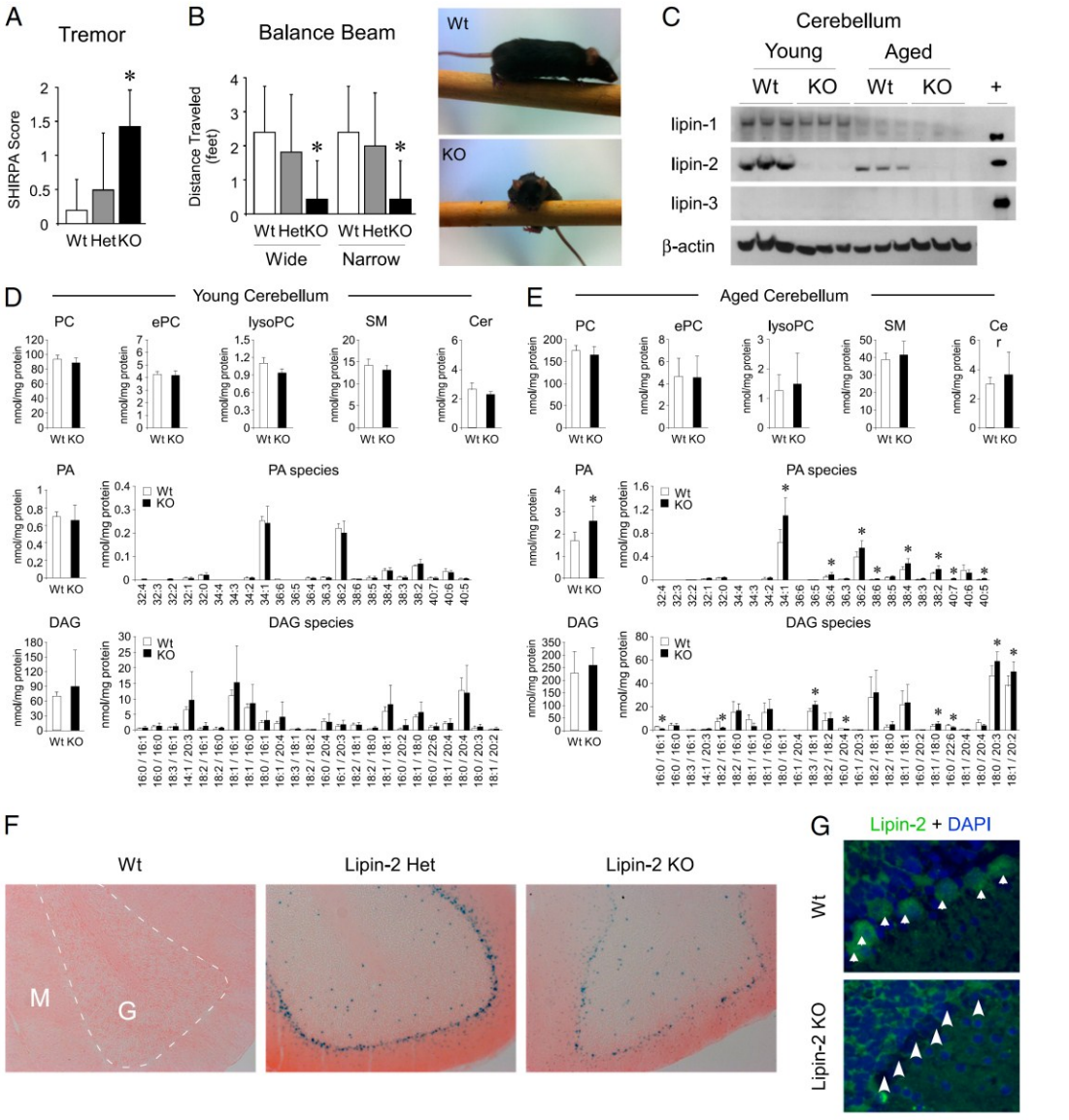

Fig. 4. Lipin-2–KO mice exhibit age-dependent balance impairment associated with phosphatidate accumulation in the cerebellum. (*A*) WT, heterozygous, and lipin-2–KO mice were observed in a viewing jar by an observer blind to genotype, and scored for abnormalities according to SHIRPA guidelines (*Methods*). Lipin-2–deficient mice exhibited a marked tremor that was significantly more apparent compared with WT controls. (*B*) Balance was assessed as the distance traveled on a 3-ft-long beam of wide (2.2 cm) or narrow (1.6 cm) width. WT mice were able to traverse the beam of either width nearly perfectly, but lipin-2–KO mice typically took only a few steps before they turned sideways and dropped from the beam. For *A* and *B*, $n = 5$ to 7 per genotype. Values represent mean ± SD (*$P < 0.05$ vs. relevant WT). (*C*) Western blot analysis of lipin-1, lipin-2, and lipin-3 protein levels in the cerebellum of young (2 mo) and aged (10–12 mo) mice. In young mice, lipin-1 levels were similar in cerebellum of WT and lipin-2–KO, and lipin-2 levels were robust in WT mice. In older mice, lipin-1 protein was reduced in WT and lipin-2–KO mice, but WT mice maintained detectable lipin-2. Lipin-3 protein was undetectable. β-Actin was used as a loading control and recombinant lipin proteins were expressed in HEK293 and used as positive controls (+). (*D*) Lipid analysis of young (2 mo old) WT and lipin-2–KO cerebellums by ESI-MS. *Top*, Total levels of PC, ether-linked PC (ePC), lysoPC, sphingomyelin (SM), and ceramides (Cer). Lipid values are expressed relative to cellular protein. *Middle*, Total phosphatidate (PA; *Left*) and individual PA species (*Right*) are shown normalized to cellular protein. The molecular species of PA are indicated as total number of carbons:number of double bonds. *Lower*, Total DAG and DAG species ($n = 5$ per genotype). Values represent mean ± SD (*$P <$ 0.05 vs. WT). (*E*) Lipid analysis of aged (10–12 mo old) WT and lipin-2 KO cerebellums by ESI-MS. Samples are as described in *D* ($n = 6$–7 per genotype; values represent mean ± SD; *$P < 0.05$ vs. WT). (*F*) Localization of lipin-2 in cerebellar sections of 10-mo-old mice by detection of β-gal activity. Regions of lipin-2–β-gal fusion protein activity are indicated by deep blue color; pink represents eosin counterstain (magnification of 100×). G, granular layer; M, molecular layer. (*G*) Cellular localization of lipin-2 in cerebellum by immunofluorescence using lipin-2 antibody (green). Nuclei are stained with DAPI (blue). Lipin-2 is visible as cytoplasmic staining in large cells located between the molecular and granular layers (arrows, *Top*). The similar regions in lipin-2–KO mice are unstained because of a lack of lipin-2 protein (arrowheads). Diffuse staining of molecular and granular areas is nonspecific (magnification of 882×).

Table 1. Complete blood count analysis

Variable	WT	Het	KO
RBC count, $\times 10^6/\mu L$	10.50 ± 0.58	10.70 ± 0.78	9.82 ± 0.20*
RBC distribution width, %	15.9 ± 0.72	16.8 ± 0.76	17.8 ± 1.18*
Hemoglobin, g/dL	16.4 ± 0.67	16.0 ± 1.27	15.1 ± 0.48*
Platelet count, $\times 10^3/\mu L$	597 ± 16.6	510 ± 83.1	710 ± 82.7*
Platelet volume, fL	4.5 ± 0.08	4.5 ± 0.13	5.0 ± 0.26*

Complete blood cell count was performed on whole blood from WT, heterozygous (Het), and lipin-2 KO mice (n = 5 per genotype). All values represent mean ± SD.
*$P < 0.05$ vs. WT.

but detected no lesions. The lack of osteomyelitis in lipin-2–KO mice suggests that additional genetic or environmental factors in addition to lipin-2 deficiency contribute to osteomyelitis in humans with Majeed syndrome, or that species differences exist.

Deficiency of both Lipin-1 and Lipin-2 Is Lethal. The availability of lipin-1– and lipin-2–deficient mouse models allowed us to ask whether a combined deficiency of both lipin-1 and lipin-2 is tolerated during development. To address this question, *Lpin1*$^{+/-}$ *Lpin2*$^{+/-}$ mice were intercrossed and the offspring were screened for viable double-KO mice (*Lpin1*$^{-/-}$*Lpin2*$^{-/-}$). *Lpin1*$^{-/-}$ and *Lpin2*$^{-/-}$ pups were born at the expected Mendelian ratios. We expected to find *Lpin1*$^{-/-}$*Lpin2*$^{-/-}$ pups at a frequency of one in 16, but we never observed any double-KO pups in 282 births, suggesting embryonic lethality (χ^2 test, $P = 0.008$).

To determine when *Lpin1*$^{-/-}$*Lpin2*$^{-/-}$ mice die during embryonic development, we examined embryos at 8.5, 10.5, 11.5, and 12.5 d post coitum (dpc). Double-KO embryos were indistinguishable from other genotypes at 8.5 dpc, but, by 10.5 dpc, they were small, and there was no evidence of a circulatory system (Fig. 5). At 11.5 dpc, all double-KO embryos were dead. Heterozygosity for lipin-1 deficiency with a complete lipin-2–KO background was associated with retarded growth in approximately half the embryos (n = 5 of 11) at 10.5 dpc. Because a deficiency in lipin-1 alone or lipin-2 alone is compatible with normal development, it appears that lipin-1 and lipin-2 can compensate for one another, at least to some degree. However, the embryonic lethality of the double-KO mice shows that lipin-3 cannot compensate for the absence of both lipin-1 and lipin-2.

Discussion

Lipin-1, -2, and -3 PA phosphatase enzymes likely provide the majority of PAP activity for the Kennedy pathway, required for the synthesis of triglycerides as well as PC and phosphatidylethanolamine. To date, in vivo studies have focused on the functions of lipin-1, largely because of the availability of a lipin-1–deficient mouse strain that arose by spontaneous mutation (reviewed in refs. 4, 5, 31). Here, we generated a mouse model to investigate the role of lipin-2 in lipid homeostasis. Characterization of these animals revealed an intricate in vivo relationship between lipin-1 and lipin-2 that acts in a tissue-specific and age-dependent manner (summarized in Fig. 6). In liver, lipin-2 deficiency led to a compensatory increase in hepatic lipin-1 protein and elevated PAP activity, which maintained lipid homeostasis under basal conditions, but led to diet-induced hepatic triglyceride accumulation. As lipin-2–deficient mice aged, they developed ataxia and impaired balance. This was associated with the combination of lipin-2 deficiency and an age-dependent reduction in cerebellar lipin-1 levels, resulting in altered cerebellar phospholipid composition and ataxia. These results highlight a unique role for lipin-2 in cerebellar glycerolipid homeostasis. We also learned that a deficiency of either lipin-1 or lipin-2 is tolerated, but the absence of both enzymes is lethal.

Based on the extremely high levels of lipin-2 protein in liver (22) and the demonstration that knockdown of lipin-2 in isolated hepatocytes reduces PAP activity (23), we expected that lipin-2 KO mice would exhibit a dramatic reduction in hepatic PAP activity and triglyceride content. Surprisingly, PAP activity was instead increased in KO compared with WT liver. This was associated with a compensatory increase in lipin-1 protein levels. This finding highlights a critical difference between the response to acute lowering of lipin-2 expression levels in cultured cells and the chronic loss of endogenous lipin-2 expression in the whole animal. The in vivo response to lack of lipin-2 was an adaptive response that is presumably designed to retain sufficient PAP activity to meet the needs of hepatocytes. Under basal conditions (i.e., chow diet), the compensation by lipin-1 appeared to be adequate to maintain lipid homeostasis in the hepatocytes. However, when subjected to metabolic stress by feeding a high-fat diet, lipin-2–KO mice accumulated excess triglycerides, suggesting that the combined action of lipin-1 and lipin-2 is important for fine-tuned regulation of glycerolipid synthesis under such conditions.

There are many known examples in which genetic deficiency in a given protein is compensated by the activity of a related protein, as occurs in some respects between lipin-1 and lipin-2. However, three aspects of the relationship between lipin-1 and lipin-2 observed here are noteworthy. First, the compensatory increase in lipin-1 levels in lipin-2–deficient mice was tissue-specific, occurring in liver but not in brain. Second, the compensatory increase in lipin-1 occurred through modulation of lipin-1 protein levels without a change in lipin-1 mRNA levels. This suggests a posttranscriptional mechanism that allows rapid changes in protein levels to maintain lipid homeostasis. This is somewhat different from the effects of manipulation of lipin-2 levels in HeLa M cells, which led to changes in lipin-1 mRNA levels (32). Differences may be related to cell/tissue type, the acute or chronic nature of the modulation, and the difference between in vitro and in vivo systems. Third, the increased lipin-1 levels in the liver led to enhanced PAP activity, but did not increase expression levels of genes that are coactivated by lipin-1 via its interaction with PGC-1α (15). This raises the possibility that lipin-2 is required to potentiate the effects of lipin-1 on gene expression. Along these lines, it is interesting that recombinant

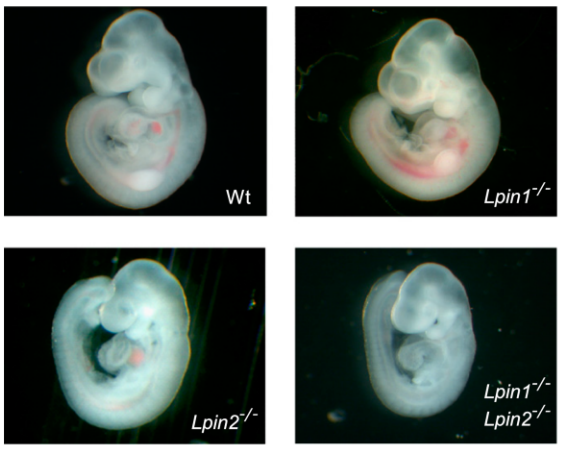

Fig. 5. Combined lipin-1/lipin-2 deficiency is embryonic-lethal. Representative embryos isolated at 10.5 dpc are shown for WT, *Lpin1*$^{-/-}$, *Lpin2*$^{-/-}$, *and Lpin1*$^{-/-}$*Lpin2*$^{-/-}$. *Lpin1*$^{-/-}$*Lpin2*$^{-/-}$ embryos were reduced in size beginning at 9.5 dpc, and could not be recovered much beyond 10.5 dpc.

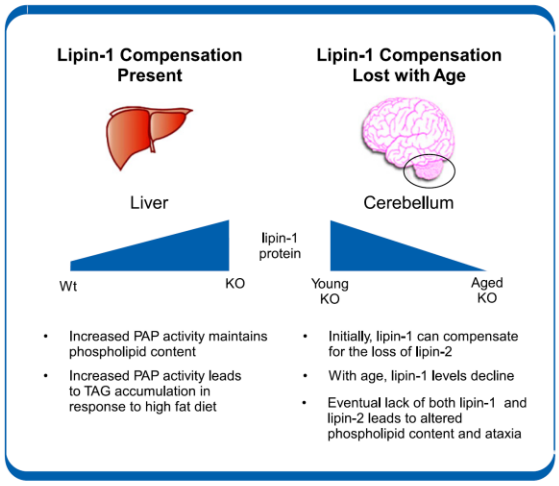

Fig. 6. Tissue pathologic findings seen in lipin-2–deficient mice are governed by the presence or absence of compensation by lipin-1. The effects of lipin-2 deficiency differ among tissues depending on the tissue-specific and age-dependent compensation by lipin-1. In liver of lipin-2–KO mice, lipin-1 protein levels are elevated, leading to maintenance of relatively normal phospholipid levels. However, with a high-fat diet, the increased PAP activity promotes enhanced triacylglycerol storage. In cerebellum of lipin-2 KO mice, lipin-1 levels are similar to those in WT, and decrease with age. This eventual loss of lipin-1 in cerebellum of aged mice on a background of lipin-2 deficiency leads to altered phospholipid content and ataxia.

lipin-1 and lipin-2 proteins appear to form heterodimers (33). The potential functional and physical interaction between lipin-1 and lipin-2, and its role in lipin-1 transcriptional coactivator activity, merits further investigation.

Lipin-2 deficiency resulted in an unexpected central nervous system abnormality: cerebellar disease associated with late-onset ataxia and tremor. This phenotype was 100% penetrant by 6 mo of age, making it possible to identify KO mice simply by observing their gait. Unlike the case with liver, lipin-1 protein levels were not increased to compensate for loss of lipin-2, and analysis of the basis for the late-onset nature of the phenotype revealed an important temporal component to the regulation of lipin proteins in the cerebellum. In young WT mice, lipin-1 and lipin-2 proteins are both expressed in the cerebellum. In young, asymptomatic lipin-2 KO mice, lipin-1 levels in cerebellum are similar to those in WT. Upon aging, the levels of both lipin-1 and lipin-2 diminished in WT cerebellum, but lipin-2 levels remained sufficient to maintain lipid homeostasis and normal cerebellar function. These results are consistent with previous reports of decreasing PAP activity levels within the cerebellum of aging rats (27–29). In contrast to WT mice, aged lipin-2–KO mice experienced an absence of lipin-1 and lipin-2; this was associated with a 50% increase in total PA levels but only minor changes in specific species of other phospholipid classes and DAG. PA has previously been shown to accumulate in tissues of lipin-1–deficient mice and contribute to impaired adipose tissue development and peripheral neuropathy in those animals (13, 14). Recent studies have implicated tissue PA content in several aspects of metabolic homeostasis, including reductions in the expression and activation of peroxisome proliferator-activated receptor-γ (14, 34), activation of ERK signaling (13), inhibition of mammalian target of rapamycin complex 2 (35), inflammatory signaling (36), and others (reviewed in ref. 37). Our studies demonstrate that lipin-2 has a critical role in maintaining PA levels in cerebellum, particularly after loss of lipin-1 during aging.

The lipin-2–KO mice have revealed fundamental information about the role of lipin-2 in glycerolipid homeostasis and its relationship to lipin-1 function in liver and cerebellum. These features of lipin-2 protein function would be very difficult to assess in human patients. Our studies also provided information about the role of lipin-2 deficiency as a determinant of key features of the Majeed syndrome, anemia, and osteomyelitis. As observed in patients with Majeed syndrome, we found that lipin-2 deficiency is sufficient to cause anemia, characterized by reduced blood cell volume and immature erythrocytes (19, 21). However, in contrast to patients with Majeed syndrome, we did not observe swollen joints in our mice, nor did we find osteolytic lesions in bones, as judged by radiography and microCT scans, although we cannot assess whether the mice felt any bone or joint pain. There are various possible explanations for the lack of obvious osteomyelitis in lipin-2–deficient mice. One possibility is that asymptomatic bone lesions might be present but require techniques such as isotope uptake to detect (18, 20). The osteomyelitis in patients with Majeed syndrome is considered to be sterile based on failure to respond to i.v. antibiotic treatment and the inability to culture microorganisms from bone biopsies (21). Nevertheless, the lack of obvious osteomyelitic lesions in lipin-2–KO mice raises the possibility that additional genetic or environmental factors not encountered in our mouse colony (e.g., a fastidious microorganism or virus, or dietary component) act in conjunction with lipin-2 deficiency to promote osteomyelitis in patients with Majeed syndrome. Alternatively, the lack of osteomyelitis in the mice may reflect a species difference in lipin family compensation in bone.

The detection of late-onset ataxia in the lipin-2–deficient mice raises a question about whether patients with Majeed syndrome may be susceptible to similar symptoms. Most patients with Majeed syndrome reported thus far have been studied during the neonatal or early childhood period, with the oldest individual studied at 21 y of age (17, 18, 21). Hence, it is currently unknown to us whether patients with Majeed syndrome may develop neurological syndromes at later stages of life.

In summary, our analysis of lipin-2–deficient mice uncovered unexpected aspects of lipin-2 biology that would not have been predicted based on analysis of lipin-2 tissue expression, by in vitro studies with transfected cells, or by in vivo studies of lipin-2 overexpression with adenoviral vectors (22–24, 32). Our results revealed an intricate in vivo relationship between lipin-1 and lipin-2, with tissue- and age-specific components. In the liver, lipin-2 function can be adequately compensated by an adaptive increase in lipin-1 protein levels under basal conditions, but both proteins are required to maintain lipid homeostasis under the increased lipid load encountered with a high-fat diet. In the central nervous system, lipin-2 has a unique role in maintaining lipid homeostasis in the cerebellum, particularly with advancing age. These studies raise the possibility that lipin-2 levels may be relevant in idiopathic late-onset cerebellar ataxias (38).

Our studies reveal the presence of an intricate relationship between two members of a protein family, and hint that several mechanisms may act in concert to ensure that homeostasis is maintained. Protein families are a common feature of many biological systems, including other enzymes that function in the glycerol-3-phosphate pathway (1, 2). Family members typically have similar molecular activities, raising questions about the physiological significance of the individual members. Future studies will be aimed at further characterizing distinctions and interactions between the members of the lipin protein family that support lipid homeostasis in a tissue-specific and age-dependent manner.

Methods

Mice. *Lpin2*-deficient mice were generated by targeted trapping (39) in ES cells. ES cells were electroporated with the trapping construct consisting of a promoterless β-geo gene (fusion of a β-gal reporter and a neomycin-

resistance gene) with an upstream splice acceptor site (40) inserted into intron 3 of the *Lpin2* gene. ES cells were selected under G418 and targeted cells identified by 5′ RACE with a β-geo primer and DNA sequencing to confirm expression of a fusion transcript that includes the β-geo gene and extends beyond the 5′ boundary of the targeting vector, into exon 2 of *Lpin2*. Targeted ES cells were injected into blastocysts, and resulting chimeras were crossed to C57BL/6J mice to establish germline transmission and backcrossed for four to six generations. WT and mutant alleles were detected by PCR by using primer sets that spanned the β-geo coding sequence within intron 3 or amplified a portion of it, respectively [cctgtctaatgtgcctctcct, agcaggtttagagccatgtga (WT); ttatcgatgagcgtggtggtt, gcgcgtacatcgggcaaataa (mutant)].

Mice were maintained in 12-h light/dark conditions and fed a laboratory chow diet that consisted of 4.5% fat, 50% carbohydrate (wt/wt) (LabDiet; Purina). WT and KO littermates were used for all studies. For specified studies, mice were fed a high-fat diet (35% fat, 33% carbohydrate; Diet F3282, BioServe). Animal studies were approved by the UCLA Animal Research Committee and performed according to guidelines established in the Guide for Care and Use of Laboratory Animals.

Gene Expression Analysis. RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized and analyzed by quantitative PCR (qPCR) as previously described (41). The qPCR primers sequences were from previous publications (22, 24, 42, 43) and were as follows: *Lpin1* (ccttctatgctgcttttgggaacc; gtgatcgaccacttcgcagagc); *Lpin2* (agttgaccccatcaccgtag, cccaaagcatcagacttggt); *Lpin3* (tggaattgggatgacaaggt, cactgcaagtacccctttggt); peroxisome proliferator-activated receptor α, *Ppara* (aatgcaattcgctttggaag, ggccttgaccttgttcatgt); medium chain acyl-CoA dehydrogenase, *Acadm* (aggtttcaagatcgcaatgg, ctccttggtgctccactagc); β-2-microglobulin (cagcatggctcgctcggtgac, cgtagcagttcagtatgttcg); TATA box binding protein, *Tbp* (acccttcaccaatgactcctatg, atgatgactgcagcaaatcgc); 18S ribosomal RNA (accgcagctaggaataatgga, gcctcagttccgaaaacca), carnitine palmitoyltransferase-1α, *Cpt1a* (aaacccaccaggctacagtg, tccttgtaatgtgcgagctg); C/EBP homologous protein, *Chop* (cagtcatggcagctgagtcc, taggtgcccccaatttcatc); glucose-regulated protein, 78 kDa, *Grp78* (tgcagcaggacatcaagttc, tttcttctggggcaaatgtc); unspliced X-box binding protein 1, u*Xbp1* (tatccttttgggcattctgg, aaagggaggctggtaaggaa); and spliced XBP-1, s*Xbp1* (ctgagtccgaatcaggtgcag, gggagtggagtaaggctggt).

Western Blot Analysis of Lipin Proteins. Protein lysates were generated by homogenization of tissues in lysis buffer [250 mM sucrose, 20 mM Tris, 1 mM EDTA, 1.4% Triton X-100, 1× Complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics) and 1× protein phosphatase inhibitor mixtures 1 and 2 (Sigma)]. Protein lysates were electrophoresed on acrylamide gels, transferred to nitrocellulose membrane, blocked, and incubated with primary antibody. Lipin-1 antibody (used at 1:10,000) (44) was a gift of Maroun Bou Kahlil (University of Ottawa, Ottawa, Canada). Lipin-2 antibody (used at 1:1,000) (23) was a gift of Brian Finck (Washington University, St. Louis, MO). β-Actin antibody (used at 1:5,000) was from Sigma (A1978). Lipin-3 antibody (used at 1:10,000) was from Lifespan Biosciences (C37207). Species-appropriate HRP-conjugated secondary antibodies were used to detect the primary antibody binding by enhanced chemiluminescence (GE Healthcare).

Tissue Histology and Detection of Lipin-2/β-Gal Fusion Protein. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks, and sections were stained with H&E to reveal tissue morphology. β-Gal staining of tissues was performed as previously described (45). Briefly, fresh tissue pieces from WT and lipin-2–deficient mice were placed in prewarmed Solution A (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS solution, pH 7.5) containing 1.33 mg/mL X-gal and incubated at 37 °C to allow accumulation of product. Samples were then fixed in 10% buffered formalin overnight at 4 °C, rinsed with PBS solution, and digitally imaged under an M8 dissecting microscope (Wild Heerbrugg). For β-gal staining of tissue cryosections, fresh tissues were placed in optimal cutting temperature embedding medium (Tissue-Tek; Sakura Finetek) and frozen on dry ice. Following cryosectioning, samples were fixed in 2% formaldehyde with 0.2% glutaraldehyde for 5 min. Sections were then incubated with Solution A containing X-gal, and counterstained with eosin Y. Images were obtained by using an Eclipse E600 microscope (Nikon) and SPOT imaging software (Diagnostic Instruments).

PAP Activity Assay. PAP activity was measured on tissue extracts essentially as described previously (11, 30). Briefly, fresh mouse tissues were directly homogenized in lysis buffer [250 mM sucrose, 2 mM DTT, protein phosphatase inhibitor mixtures 1 and 2 (Sigma), 1× Complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics), and 0.15% Tween-20]. PAP-1 activity was measured in 100 mM Tris/HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 2 mg/mL fatty acid-poor BSA with 0.6 mM tritiated PA (approximately 6×10^4 dpm labeled with [^{3}H]palmitate) mixed with PA derived from egg PC, 0.4 mM PC, 1 mM EDTA, 1 mM EGTA and 200 μM tetrahydrolipstatin to block the degradation of DAG. Tween-20 was adjusted to a final concentration of 0.05%, and the reactions were incubated at 37 °C. Chloroform containing 0.08% olive oil was added to stop the reaction and basic alumina was added to remove any PA or [^{3}H]palmitate formed by phospholipase A activity. The [^{3}H]DAG product was then isolated and quantified by scintillation counting. Lysate protein amounts and incubation times were optimized to ensure <15% of PA was consumed during incubation. Three different protein concentrations were analyzed for each sample to ensure the proportionality of the assay. Parallel analyses were done in the presence of excess NEM (5 mM) to assess the contribution of lipid phosphate phosphatase activity. This latter activity was subtracted from the total activity to yield true PAP activity values. These assay conditions were chosen to maximize the PAP activity relative to that of lipid phosphate phosphatases.

Metabolic Characterization. Mice were fasted for 5 h (0800 h to 1300 h) before blood and tissue collection. Blood glucose levels were determined with a One Touch Ultra Blood Glucose monitor (Lifescan). Plasma lipids, including triglycerides, free fatty acids, total cholesterol, and HDL cholesterol were quantified as previously described (46). Glucose tolerance tests were performed after a 4.5-h fast by injecting mice i.p. with glucose (2 mg/g body weight) and determining blood glucose levels at 0, 15, 60, and 120 min after injection (47).

Blood Analysis. Complete blood counts were performed on fresh blood samples containing EDTA by using a Hemavet 950 Hematology System (Drew Scientific) at the University of California, Los Angeles, Department of Laboratory and Animal Medicine Diagnostic Laboratory. The following major blood cell groups were quantified: total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, total RBCs, and platelets. Mean RBC volume, mean RBC distribution width, hemoglobin concentration, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean platelet volume, and platelet distribution width were also measured.

Bone Imaging and Analysis. Femurs and tibias from WT and lipin-2–KO mice were dissected, cleaned of soft tissue, and stored in 70% ethanol before radiography and microCT scanning. Femurs and tibias were imaged by using a DX50 Core Specimen Core Radiography system (Faxitron Biooptics/Siemens) with an exposure of 16 s and 35 kV. MicroCT scanning was performed on tibias with a Scanco-40 scanner (Scanco) or a Skyscan 1172 scanner (Skyscan) with the X-ray energy equaling 55 KVp and 72 μA or 55 KVp and 167 μA, respectively. A 0.5-mm aluminum filter and a voxel isotropic resolution of 12 μm were used with both scanners. To evaluate the secondary spongiosa trabecular pattern, 200 slices were measured for each sample covering 2.4 mm of the distal metaphysis starting 0.048 mm below the curvature of the growth plate and moving along the shaft. Contours were drawn in the medullar cavity at a fixed distance from the endosteum, avoiding the cortical bone to define tissue volume. Bone volume, bone volume fraction, connectivity density, and structure model index values were calculated according to the method described by Hildebrand and Rüegsegger (48). A hydroxyapatite phantom was used for density calibration and calculation of apparent density.

Behavioral Analyses. The SHIRPA primary screen is a broad panel of simple behavioral tests that screen for neurological deficits (49). We performed a subset of 14 of these tests that focus on characterizing defects in movement, attention, balance and coordination. We observed WT, heterozygous, and lipin-2–deficient mice, and recorded differences in body position, spontaneous activity, tremor presence, transfer arousal, locomotor activity, gait, pelvic elevation, tail elevation, touch escape, startle response, wire maneuver, righting reflex, contact righting reflex, and negative geotaxis. Each test was scored with a system that categorizes normal and deviant responses to each situation.

Forelimb grip strength was assessed by using a spring scale (8262-M; Ohaus) rigged with a trapeze (50). Each mouse was allowed to grasp the trapeze by its forelimbs and was then gently pulled downward by the tail until it let go. The weight pulled as the mouse released was recorded. A total of five trials were conducted, body weight was subtracted from the weights pulled, and the best three trials were used.

Balance was assessed as the ability of a mouse to traverse a marked 4-ft-long, round balance beam at a height of ~60 cm from the floor. Balance beams with wide (2.2 cm or 5/8″) and narrow (1.6 cm or 3/8″) widths were used. Mice were placed at the 1-ft mark and the distance traveled (as much as 3 ft) was recorded; no time limit was imposed. Mice that fell from the beam were caught in the investigator's hands or on padded material placed below the beam. Mice were given four trials on the wide beam, and the best score was used. Mice were then given two trials on the narrow beam, and the greater distance of these two was recorded.

Quantification of Tissue Lipids. For quantification of hepatic triglycerides, lipids were extracted by a modification of the Bligh and Dyer method (51). Triglyceride concentration was determined using a colorimetric biochemical assay (L-type triglyceride M; Wako). For quantification of phospholipid and DAG species, lipids were prepared from indicated tissues and dried under a gentle stream of argon. An automated ESI-MS approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (52, 53) with modifications. The lipid samples were dissolved in 1 mL chloroform. An aliquot of 15 to 50 μL of extract in chloroform was used. Precise amounts of internal standards were added, and unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole mass spectrometer (API 4000; Applied Biosystems). The data were corrected for the fraction of the sample analyzed and normalized to milligrams of cellular protein analyzed.

Immunohistochemistry. Immediately after euthanasia, brains were dissected and placed in 4% paraformaldehyde overnight at 4 °C. The samples were then washed with PBS solution and kept in 70% alcohol until embedding in paraffin. Sections (4 μm) were deparaffinized by using xylenes and graded alcohol washes. Pepsin-mediated antigen retrieval was performed using pepsin (00-3009; Invitrogen) diluted in an equal volume of 0.2 N HCl at 37 °C for 10 min. The sections were blocked with 5% donkey serum in PBS solution for 90 min, and then incubated with Lipin-2 antibody (1:200) (23) at 4 °C overnight in a humidified chamber. Secondary antibody (A21206; 1:1,000; Molecular Probes) was applied to the sections for 1 h in the dark. Nuclear staining was performed with DAPI (0.3 ng/mL) for 30 min, and then the sections were mounted in fluorescent mounting medium (Vectashield). Images were acquired using a Zeiss Observer.D1 microscope outfitted with an AxioCam MRc camera and AxioVision software.

Statistical Analyses. Statistical significance for data involving WT, heterozygous, and KO groups was determined by one-way ANOVA (STATAversion 11) followed by pair-wise comparisons by Student *t* test. Analysis of lipid profile data for PA and PC species was performed by two-way ANOVA followed by pairwise comparisons with Bonferroni correction for multiple comparisons. Analysis of embryonic lethality of *Lpin1*$^{-/-}$*Lpin2*$^{-/-}$ mice was performed by χ^2 test.

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Supporting Information

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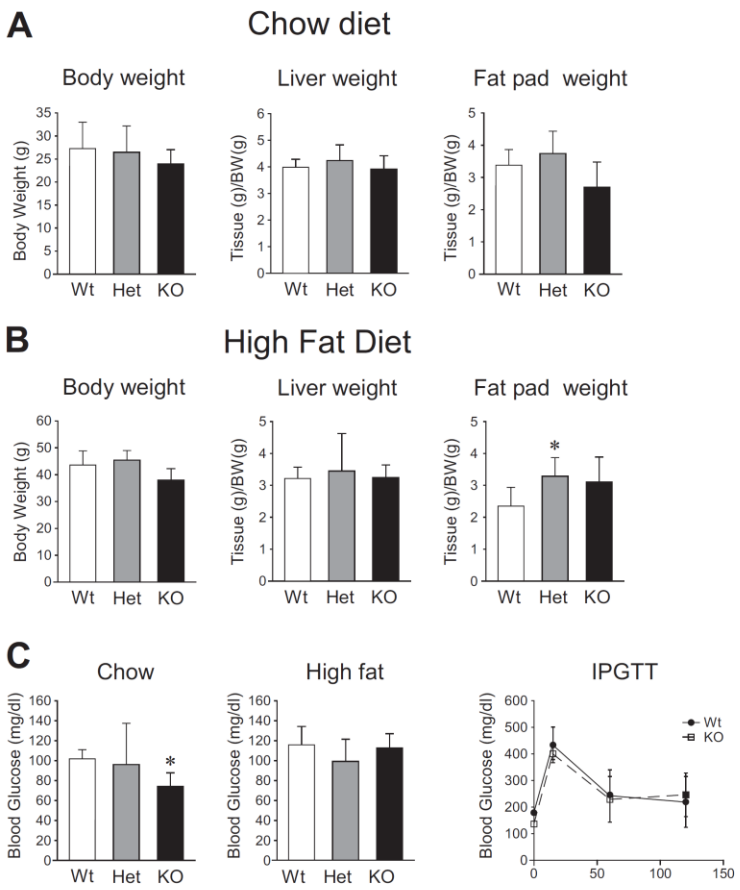

Fig. S1. Metabolic studies of lipin-2–deficient mice. (*A*) WT, *Lpin2*$^{+/-}$ [i.e., heterozygous (Het)] and *Lpin2*$^{-/-}$ (KO) mice were fed a chow diet until 12 wk of age. Liver and gonadal fat pad weights were expressed in proportion to body weight. (*B*) Mice were fed a high-fat diet for 4 wk beginning at 8 wk of age, and organ weights were determined upon dissection. Liver and gonadal fat pad weights were expressed in proportion to body weight. (*C*) Fasting blood glucose was measured before dissection for the cohorts described in *A* and *B* fed chow or high-fat diet, respectively. *Right*, Glucose tolerance test was performed on chow-fed WT and lipin-2–KO mice by i.p. injection of 2 mg glucose per gram body weight, and blood glucose levels measured before injection and thereafter at 15, 60, and 120 min (n = 4 per genotype).

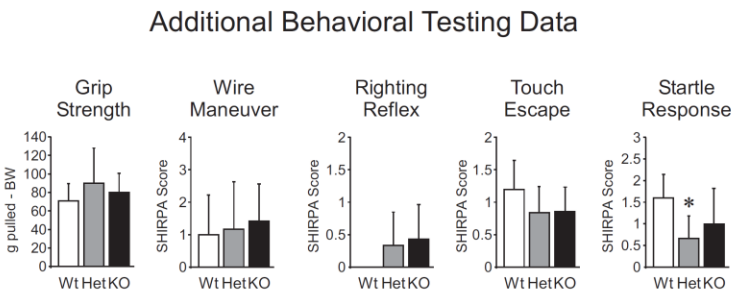

Fig. S2. Additional behavioral tests in lipin-2–KO mice. The indicated tests were performed on WT, heterozygous (Het), and lipin-2–KO mice ($n \geq 5$ individuals per genotype; values represent mean ± SD; $*P < 0.05$ vs. WT).

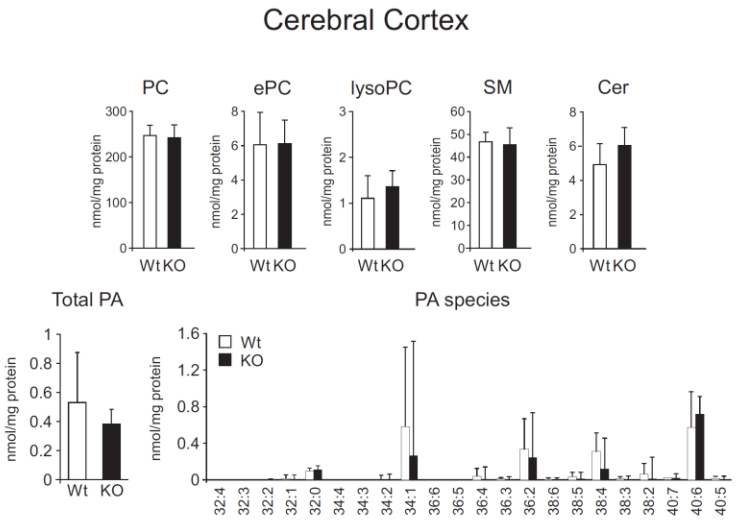

Fig. S3. Cerebral cortex phospholipid levels of WT and lipin-2–KO mice. Electrospray ionization MS analysis of phospholipid species extracted from cerebral cortex ($n = 6$ to 7 per genotype). *Upper*, Total levels of phosphatidylcholine (PC), ether-linked PC (ePC), lysoPC, SM (sphingomyelin), and Cer (ceramides). *Lower*, Levels of total PA and the various molecular species of PA as total number of carbons:number of double bonds. Lipid values were normalized to cellular protein. Values represent mean ± SD.

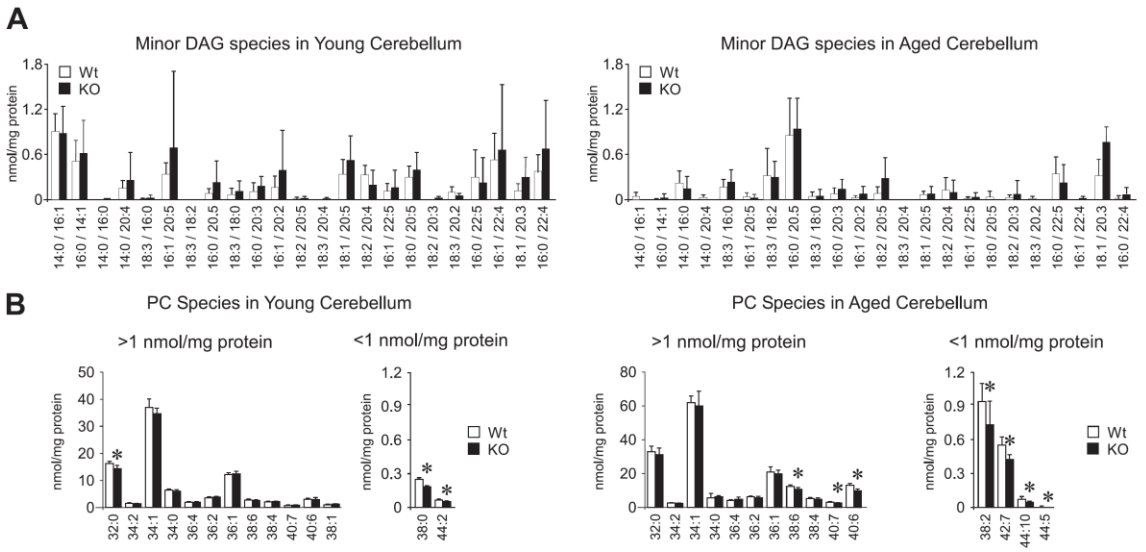

Fig. S4. Diacylglycerol (DAG) and PC species in cerebellum of young and aged WT and lipin-2–KO mice. Electrospray ionization MS analysis of DAG and PC species from young (2 mo of age, $n = 5$ per genotype) and aged (10–12 mo of age, $n = 6$ to 7 per genotype) mice. The molecular species are indicated as total number of carbons:number of double bonds. Lipid values were normalized to cellular protein. Values represent mean ± SD (*$P < 0.05$ vs. WT). Minor DAG species (mean, <1 nmol/mg protein) are shown. The following DAG species were completely undetectable: 18:1/22:6, 18:2/22:5, 18:3/22:4, 20:2/20:5, 20:3/20:4, 18:0/22:6, 18:1/22:5, 18:2/22:4, 20:2/20:4, 20:3/20:3, 18:0/22:5, 18:1/22:4, and 20:2/20:3.

Table S1. Plasma lipid levels of WT and lipin-2–KO mice maintained on chow or high-fat diet

Lipid	Chow		High-fat diet	
	WT	Lipin-2 KO	WT	Lipin-2 KO
Total cholesterol	144.4 ± 39.5	175.4 ± 32.1	193.0 ± 37.9	209.3 ± 61.2
HDL cholesterol	127.4 ± 32.6	160.0 ± 27.1	145.0 ± 29.7	154.0 ± 45.1
Triglycerides	69.2 ± 59.3	34.8 ± 12.7	66.0 ± 7.3	52.0 ± 7.2 *
Free fatty acids	36.0 ± 3.5	32.4 ± 6.9	30.2 ± 2.6	23.0 ± 1.8 *

Plasma lipids indicated were measured in mice maintained on a chow or high-fat diet for 4 wk and fasted 5 h before blood collection ($n = 5$ per genotype). All values represent mean ± SD.
*$P < 0.05$ vs. WT.

CHAPTER FOUR

LIPIN-1/LIPIN-2 INTERACTIONS REGULATE LIPIN PROTEIN LEVELS

PREFACE

Studies of the lipin-2 knockout mouse indicated that lipin-1 and lipin-2 cooperate to regulate phosphatidate phosphatase (PAP) activity in the liver (1). This chapter, "Lipin-1/Lipin-2 Interactions Regulate Lipin Protein Levels", contains a preliminary manuscript providing evidence for a physical interaction between endogenous hepatic lipin-1 and lipin-2, and is a work in progress.

This work builds from previous observations in our laboratory and a study by Liu *et al.* (2) indicating that lipin-1 and lipin-2 are present in a protein complex in cultured cells transfected with expression constructs for lipin-1 and lipin-2. Here we demonstrate that endogenous hepatic lipin-1 and lipin-2 can occur in a complex and identify a novel domain within lipin-1, the lipin-interacting domain (LID), which is required for lipin-1/lipin-2 interaction. We further demonstrate that lipin-1 and lipin-2 have distinct regulatory effects on one another. Lipin-1 enhances the proteasomal degradation of lipin-2, whereas lipin-2 causes increased lipin-1 protein levels. We find that the formation of the lipin-1/lipin-2 complex is required for the effect of lipin-1 on lipin-2 degradation and that the nuclear localization sequence within lipin-1 has a critical role in mediating both lipin-1-induced lipin-2 degradation and lipin-2–mediated lipin-1 stabilization. These findings provide a model for how lipins regulate one another and our future work will aim to define the function of the lipin-1/lipin-2 complex and identify the physiological conditions that influence its formation.

I have generated the majority of the data for this paper, but several individuals have made notable contributions. Dr. Jimmy Donkor cloned many of the lipin-1 and lipin-2 mutants described herein and performed initial co-immunoprecipitation (co-IP) experiments using transfected cells to demonstrate that lipin-1 and lipin-2 interact. Under my supervision, an undergraduate student, Mr. Andrew Park, cloned deletion constructs spanning the lipin-1 LID domain and also performed co-IPs using lysates from transfected cells. Dr. Jasmine Li

performed the co-IPs using lipin-2 functional mutants in combination with lipin-1 that are shown in Fig. 4.2C.

I developed co-immunoprecipitation conditions used to isolate endogenous hepatic lipin complexes, as well as complexes with mutant lipin-1 proteins, based on the kind technical advice of Dr. Thurl Harris of the University of Virginia. I discovered the unique alterations in protein levels that occur upon co-expression of lipin-1 and lipin-2, and also performed the screening of panels of mutants to identify active functional motifs. I compiled the data and devised the figures and models presented in this chapter, and wrote the original draft of the work contained herein with Dr. Karen Reue's supervision.

LIPIN-1/LIPIN-2 INTERACTIONS REGULATE LIPIN PROTEIN LEVELS

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INTRODUCTION

Insulin resistance in obese humans is associated with increased levels of lipid synthesis intermediates and TAG in the liver that can disrupt insulin signaling (3, 4). Given that more than one third of the American public is now clinically obese (5), understanding the molecular mechanisms that regulate hepatic triacylglycerol (TAG) synthesis is of paramount importance to enable the development of useful therapeutics to effectively treat this growing patient population.

Members of the lipin protein family catalyze the penultimate step in the formation of TAG via the glycerol-3-phosphate pathway, leading to the removal of a phosphate group from phosphatidic acid (PA) to form diacylglycerol (DAG). All three lipin family members exhibit PAP activity in an *in vitro* assay (6). In this assay, lipin-1 has the highest specific activity and lipin-2 and lipin-3 have approximately one quarter the activity of lipin-1. Lipin-2 is the most abundantly expressed lipin in liver (7), although low levels of lipin-1 and lipin-3 are also present in this tissue.

Several lines of evidence suggest that lipins are important to the maintenance of hepatic lipid homeostasis and may have a role in glucose metabolism. Hepatic lipin-1 and lipin-2 protein levels are upregulated by fasting and down-regulated in response to feeding, in parallel with the utilization of fatty acids as metabolic fuel (8). Lipin-1 and lipin-2 are also highly upregulated in the livers of mouse models of obesity and diabetes, such as in *ob/ob* and *db/db* mice, where lipid storage becomes dysregulated (8, 9). To begin to understand the role of lipin proteins in hepatic lipid and glucose metabolism, studies have been performed to actively modulate lipin levels in liver. Since lipin-2 is the predominant lipin in liver, it is a good candidate for having a major role. For example, transient overexpression of lipin-2 in the liver of young mice fed a high-fat diet caused increased hepatic DAG and TAG levels, as well as increased fasting blood glucose and plasma insulin levels (10). Conversely, knockdown of hepatic lipin-2 improved these measures of hepatic lipid and glucose homeostasis (10), implying that excess

lipin-2 worsens insulin resistance in the setting of high fat diet feeding. Further, lipin-1 deficient *fld* mice develop a fatty liver during the neonatal period (11) and lipin-2 KO mice accrue excess hepatic TAG when fed a high fat diet (1), indicating that both lipin-1 and lipin-2 are needed for normal lipid balance in the liver.

The modulation of lipin levels using *in vitro* techniques has shown that DAG and TAG pools are tightly regulated to maintain homeostasis. Adenoviral-mediated overexpression of lipin-2 in hepatocytes or in a cultured cell line did not result in increased DAG or TAG accumulation, although it did substantially increase PAP activity (8, 12). Knockdown of lipin-2 in cultured hepatocytes did not decrease TAG levels (8). However, TAG synthesis can be attenuated under some circumstances when both lipin-1 and lipin-2 levels are reduced simultaneously. Thus, TAG synthesis was impaired when hepatocytes from lipin-1–deficient *fld* mice were subjected to shRNA-mediated lipin-2 knockdown in combination with the lipid stress of either a young age (when fatty liver is present due to lipin-1 deficiency) or oleate treatment (8). These results suggest that the activity of both lipin-1 and lipin-2 contribute to TAG homeostasis in liver under conditions of excess hepatic lipid influx.

Lipins may mediate their effects on glucose metabolism by balancing intracellular concentrations of PA and/or DAG, two lipid intermediates that have been linked to impaired insulin signaling (12, 13). The elevation of PA levels in cultured hepatocytes, either by direct treatment with PA or by upregulation of other enzymes in the glycerol-3-phosphate pathway, such as glycerol phosphate acyltransferase-1 (GPAT-1) or 1-acylglycerol 3-phosphate acyltransferase-2 (AGPAT-2), caused attenuated insulin signaling as evidenced by reduced pAKT levels (12). Elevated PA levels were also associated with dissociation of the mammalian target of rapamycin (mTOR) from rictor, disabling the mTOR Complex 2 (mTORC2), another downstream effect of insulin signaling (12). Elevated DAG levels have also been implicated in insulin resistance in humans (13). Fresh liver samples were isolated from a cohort of obese non-diabetics undergoing bariatric surgery that displayed a wide range of insulin sensitivities

and subjected to lipidomic analysis. Increased total hepatic DAG levels and high levels of DAG localized to lipid droplets in liver were the strongest predictors of insulin resistance in this cohort (13). Further, higher levels of activated protein kinase Cε also correlated with higher DAG levels in these patients. PKCε has been shown to inhibit kinase activity of the insulin receptor on insulin receptor substrates 1 and 2 (13, 14). These results imply that the enzymatic function of lipins, the catalysis of PA into DAG, likely has a role in lipid and glucose homeostasis.

We endeavored to elucidate how lipin-1 and lipin-2 might cooperate to manage hepatic nutrient homeostasis. We determined that lipin-1 and lipin-2 physically interact in a complex that can be isolated from mouse liver, and identified a novel lipin-1 protein domain that is required for formation of the lipin-1/lipin-2 complex. We further determined that lipin-1 and lipin-2 have distinct regulatory effects on one another. Lipin-1 enhances the degradation of lipin-2 and lipin-2 mediates increased lipin-1 protein levels. These effects required the presence of a lipin-1 motif that has been implicated in its nuclear localization and in its ability to bind PA. Our findings suggest a model for lipin-1/lipin-2 interaction that may contribute to the regulation of PA and DAG levels in liver.

RESULTS

Lipin-1 and Lipin-2 are regulated by fasting and feeding.

Previous work by our laboratory and others has shown that lipin-1 and lipin-2 mRNA and protein expression are regulated by fasting and feeding (7, 8). After an overnight fast, lipin-1 and lipin-2 are readily detectable by immunoblotting of liver extracts from C57BL/6J mice (Figure 4.1A). When the mice are subsequently re-fed for 3 hours, lipin-1 protein levels decline to nearly undetectable, and lipin-2 protein levels become greatly diminished. Hepatic lipin-3 protein levels are similar in the fasted and re-fed states, and do not appear to respond to fasting/feeding (Figure. 4.1A).

Previously, we reported that lipin-1 protein levels are elevated in the liver of lipin-2 KO mice (10, and Figure 4.1B). Interestingly, the elevated lipin-1 levels were not associated with increased lipin-1 mRNA levels, suggesting that lipin-1 mRNA or protein stability is altered or that lipin-1 mRNA translation is enhanced. We also showed that in the absence of lipin-2, lipin-1 levels are not modulated in response to fasting/feeding (Figure 4.1B). We next investigated whether lack of lipin-1 influences the regulation of lipin-2 in liver. We found that in the liver of lipin-1–deficient *Lpin1*$^{fld/fld}$ (*fld)* mice, the modulation of lipin-2 protein levels by fasting/feeding is attenuated compared to wild-type liver (Figure 4.1C). Additionally, hepatic lipin-2 in *fld* mice runs as a doublet on polyacrylamide gel electrophoresis, suggesting that this pool of lipin-2 may undergo protein modifications not occurring in wild-type liver. Lipin-3 protein levels appear not to be affected bylipin-1 or lipin-2 deficiency and are present at similar levels throughout the fasting/feeding cycle (Figure 4.1B, C).

Endogenous lipin-1 and lipin-2 are present in a complex.

The results above indicate that the modulation of hepatic lipin-1 and lipin-2 protein levels in the fasting/feeding cycle is dependent on the presence of both proteins. Recently, Liu et al. demonstrated that lipin-1 and lipin-2 overexpressed in cultured cells can form heterodimers (2).

To determine whether lipin-1/lipin-2 protein interactions may contribute to their functional relationship in liver, we tested whether the two proteins physically interact *in vivo*. We extracted protein from the livers of fasted and non-fasted wild-type mice and used anti-lipin-1 antibody to isolate lipin-1–containing protein complexes from the lysates. Anti-lipin-2 antibody was then used to detect the lipin-1-lipin-2 complex within the immunoprecipitates via western blot (Figure 4.2A). Lipin-2 was readily detectable in the lipin-1 immunoprecipitate under both fasted and non-fasted conditions, but not in the control IgG pulldowns. Although lipin-1 and lipin-2 proteins were more abundant in liver from fasted compared to fed mice, lipin-1/lipin-2 co-precipitating complexes were detected under both conditions.

Lipin-1 interacts with lipin-2 via a lipin-1-unique sequence, the lipin-interacting domain (LID).

All vertebrate lipin proteins contain highly conserved regions near the amino- and carboxy-terminal ends, denoted N-LIP and C-LIP domains, respectively (Peterfy 2001; Figure 4.2B). Key functional motifs on lipin proteins include the DXDXT catalytic motif required for PAP enzyme activity and a nuclear localization sequence (NLS). The NLS in lipin-1 has dual functions, promoting nuclear localization in response to physiological stimuli (15, 16), as well as serving as a binding site for phosphatidate, the substrate of the PAP enzyme reaction (17). Mammalian lipin proteins also contain two putative coactivator binding motifs, LXXIL and IXXII (9). The LXXIL motif in both lipin-1 and lipin-2 amplifies the ability of PPARα and PGC-1α to co-activate gene expression in a cell-based assay, but no activity has been associated with the IXXII motif (7, 9). Mutation of the DXDXT motif abolishes PAP activity but retains coactivator function, whereas mutation of the LXXIL motif abolishes both PAP and coactivator activities (9). Lipin-1 sequence diverges from the other lipin family members downstream of the NLS, where several specific domains of unknown function have been identified, including a region rich in acidic amino acids (AcR), and a serine-rich domain (SRD) (18 and Figure 4.2B).

To map the regions of lipin-1 and lipin-2 proteins that participate in the interaction detected in liver, we co-expressed wild-type lipin-1 and lipin-2 containing a C-terminal V5 epitope in HEK-293 cells, which have very low levels of endogenous lipin expression. As observed in liver, lipin-1 co-immunoprecipitated with lipin-2, but did not pull-down with control IgG antibody (Figure 4.2B). Deletion of the nuclear localization sequence (ΔNLS) in either lipin-1 or lipin-2 did not perturb the lipin-1/lipin-2 interaction (Figure 4.2C; left panel: lipin-1 mutants, right panel: lipin-2 mutants). This suggests that neither nuclear localization nor the binding of PA to lipin-1 is critical for the physical association of lipin-1 and lipin-2. Mutation of the co-activator binding motifs in the C-LIP domains of lipin-1 or lipin-2 likewise did not impair protein association. Since mutation of the coactivator motifs abolishes both PAP and coactivator functions, these results imply that the interaction does not require either of these activities. Consistent with this, mutation of the lipin-1 PAP active site (D679E), or mutation of a conserved serine that is necessary for lipin-2 PAP activity (S731L), did not interfere with the lipin-1/lipin-2 interaction (Figure 4.2C, right, and data not shown). Deletion of the lipin-1 N-LIP, AcR (aa 170-215), or SRD (aa219-265) domains did not prevent formation of the lipin-1/lipin-2 complex (data not shown). Deletion of the conserved C-LIP region from lipin-1 resulted in no detectable lipin-1 protein, indicating that this protein is likely unstable (data not shown).

Having determined that the known lipin functional motifs were not required for association of lipin-1 and lipin-2 proteins, we evaluated the effect of lipin-1 deletion mutations in regions that are unique to lipin-1 by producing large deletions encompassing amino acids 261-366 or amino acids 472-576. The lipin-1Δ472-576 mutant formed a complex with lipin-2 (data not shown), but deletion of aa261-366 prevented the interaction (Figure 4.2C, bottom left). As of yet, no other function has been reported for this region of lipin-1, so we named it the lipin-interacting domain (LID). Lipin-1 mutations containing sub-deletions within the LID sequence exhibited normal interaction with lipin-2, suggesting that interaction with any of multiple contact sites within this domain may be sufficient to promote lipin-1/lipin-2 interaction.

Lipin-1 mediates lipin-2 proteasomal degradation and lipin-2 increases lipin-1 protein levels

We were next interested to determine the function of the interaction between lipin-1 and lipin-2. To assess this, we transfected HEK-293 cells with plasmids expressing lipin-1, lipin-2, or the two in combination, and examined protein levels by immunoblotting (Figure 4.3A left). Expression of lipin-1 or lipin-2 independently produced the levels shown in Figure 4.3A (left and middle lanes). However, co-expression of the two proteins using the same amounts of plasmid as when expressed independently led to altered protein levels. Specifically, the presence of lipin-2 caused a substantial increase in lipin-1 protein levels, whereas lipin-1 diminished lipin-2 protein levels (Figure 4.3A, right lanes; Figure 4.3B). Similar results were obtained using Hepa 1-6 mouse hepatoma cells (Figure 4.3A, right), indicating that these effects are not specific to HEK-293 cells. Furthermore, the effect of lipin-2 on lipin-1 was dose-responsive (Figure 4.3C).

To determine if the effects of lipin-1 and lipin-2 co-expression on the protein levels of each requires formation of the lipin-1/lipin-2 protein complex, the non-interacting lipin-1ΔLID mutant was co-expressed with increasing amounts of lipin-2 plasmid (Figure 4.3D). As observed with wild-type lipin-1 (Figure 4.3C), lipin-1ΔLID protein levels were increased with increasing amounts of lipin-2, indicating that this effect does not require the physical association of lipin-1 and lipin-2. However, lipin-2 levels were not reduced in the presence of lipin-1ΔLID, indicating that the interaction is required for this effect. This suggests a model in which the reduction in lipin-2 levels by lipin-1 occurs in a direct manner, requiring interaction between the proteins, whereas the enhancement of lipin-1 protein levels by lipin-2 involves an indirect mechanism not requiring formation of the lipin-1/lipin-2 complex (Figure 4.3E).

A prominent mechanism for the modulation of cellular protein levels is the regulation of protein degradation by the proteasome. To determine whether the reduction in lipin-2 protein levels that occurs in the presence of lipin-1 involves proteasomal protein degradation, we

treated cells with the proteasomal inhibitor MG132 and assessed lipin levels when lipin-2 was co-expressed with either wild-type lipin-1 or lipin-1ΔLID. Co-expression of lipin-1 and lipin-2 resulted in the expected increase in lipin-1 protein levels and decrease in lipin-2 levels (Figure 4.3F, top, lanes 1 and 3 compared to lane 4). The presence of lipin-1ΔLID did not result in reduced levels of lipin-2 (compare lanes 2 and 3 with lanes 6 and 7). MG132 treatment resulted in increased levels of lipin-1, lipin-1ΔLID and lipin-2 under all conditions. Importantly, the lipin-1–mediated reduction in lipin-2 was blunted, indicating that the decrease in lipin-2 by lipin-1 occurs, at least in part, via proteasomal degradation (Figure 4.3F).

The lipin-1 NLS is imperative for the effects of the lipin-1/ lipin-2 interaction

To identify functional domains that are necessary for the observed effects of the lipin-1/lipin-2 interaction on lipin protein levels, we assessed the ability of mutant lipin proteins to promote the lipin-2–mediated stabilization of lipin-1 and lipin-1–mediated degradation of lipin-2. To enable comparison between all mutants, cell extracts from all mutants were immunoblotted to a single membrane and all panels for each antibody were scanned from a single exposure (Figure 4.4A). As seen previously, wild-type lipin-1 protein levels increased when co-expressed with lipin-2 (lanes 1 vs. 3), and wild-type lipin-2 protein levels decreased when co-expressed with lipin-1 (lanes 2 vs. 3). Lipin-1 lacking PAP activity (D679E mutant) had a higher level of protein expression than wild-type lipin-1 when expressed alone (lanes 1 v. 4). Lipin-1D679E levels were enhanced by lipin-2 co-expression (lane 5), and this mutant was slightly less potent than wild-type lipin-1 in promoting lipin-2 degradation (lanes 5 vs. 3). Lipin-1 lacking the conserved N-LIP region had much higher basal protein expression levels than wild-type lipin-1 (lanes 1 vs. 6). Lipin-1ΔN-LIP levels increased slightly when present in combination with wild-type lipin-2 (lane 7), and the degradation of lipin-2 was slightly impaired by deletion of the N-LIP domain from lipin-1 (lanes 7 vs. 3).

By contrast to the mutants described above, deletion of the lipin-1 NLS had a dramatic effect on the modulation of lipin protein levels by lipin-1/lipin-2 interaction. Lipin1∆NLS was expressed at higher baseline levels than wild-type lipin-1 protein. Lipin-1ΔNLS protein levels were not increased in the presence of lipin-2, and lipin-1ΔNLS was unable to mediate lipin-2 protein degradation (lanes 9 vs. 3). Thus, the 9 basic amino acid residues comprising the NLS are critical for the modulation of lipin-1/lipin-2 protein levels.

Deletion of the AcR from lipin-1 resulted in low baseline protein levels comparable to wild-type lipin-1. When co-expressed with lipin-2, lipin-1ΔAcR protein levels increased, and lipin-2 levels decreased, although to a lesser degree than with wild-type lipin-1 (lanes ll vs. 3). Mutation of the co-activator motif from lipin-1 (ΔLXXIL, lanes 12-13) or deletion of the lipin-1 SRD (lanes 14-15) resulted in very low protein levels that are far below expression of wild-type lipin-1, and were detectable only with long exposure of the blots. These proteins were not stabilized by lipin-2 co-expression, but interestingly, even these low amounts of protein were sufficient to mediate the degradation of lipin-2 when compared to wild-type lipin-1 (lanes 13 and 15 vs. 3).

Finally, lipin-2 PAP activity was not required for lipin-1–mediated lipin-2 degradation, as lipin-2 harboring the S731L point mutation was degraded to a similar degree as wild-type lipin-2 (S731L, lanes 16-17 and Wt, lanes 2-3). Furthermore, this enzymatically inert lipin-2 protein also enhanced lipin-1 protein levels above those of wild-type lipin-2 (lanes 17 vs. 3).

As summarized in Figure 4.4B, deletion of the lipin-1 NLS prevented both the lipin-2–mediated enhancement of lipin-1 protein levels and reduced the lipin-1–mediated degradation of lipin-2. This suggests that subcellular localization of the lipin-1 protein and/or its binding to phosphatidate are critical for this effect. We also determined that interference with the lipin-1/lipin-2 interaction by deletion of the lipin-1 LID also prevented the lipin-1–mediated degradation of lipin-2. On the other hand, the stabilization of lipin-1 by lipin-2 was impaired by deletion of the SRD or LXXIL sequences from lipin-1.

DISCUSSION

The prevalence of obesity in our society and the major health complications associated with it have fueled an interest in understanding the mechanisms that govern lipid homeostasis. The glycerol-3-phosphate pathway is the primary TAG synthesis pathway in most tissues in the body and the lipin protein family catalyzes the conversion of PA to DAG, the penultimate step in TAG formation. Lipin-1 and lipin-2 in liver are elevated during overnight fasting, reflecting the liver's reliance on fatty acids as a fuel source during this period. When fasting terminates with feeding, lipin-1 and lipin-2 protein levels are down-regulated. Several facets of hepatic lipin biology in mouse models of lipin deficiency have suggested that lipin-1 and lipin-2 may cooperate to maintain normal lipid homeostasis in this tissue. The data presented in this study demonstrate that lipin-1 and lipin-2 form a complex in the liver, define a novel lipin-interacting domain on lipin-1, and show that lipin-1 and lipin-2 influence the protein levels of one another. We propose that this intricate regulatory mechanism is in place to promote hepatic lipid homeostasis.

Lipin-2-deficiency in mice leads to increased hepatic TAG accumulation when animals are fed a high fat diet (1). The TAG accumulation was associated with elevated levels of lipin-1 protein in liver of lipin-2 KO mice compared to wild-type littermates. The pool of lipin-1 protein that accumulates in lipin-2 KO liver is unusual in that it results from a post-transcriptional mechanism and is not regulated by fasting and feeding. Here we demonstrated that lipin-2 is abnormally regulated in the livers of lipin-1-deficient, *Lpin1*$^{fld/fld}$ mice. Two features of this lipin-2 pool are distinct from lipin-2 in wild-type mice. First, it shows an impaired response to changes in fasting and feeding, and second, it runs as a doublet when resolved by electrophoresis, suggesting the presence of unusual lipin-2 post-translational modification. Because lipin-1 is altered in lipin-2 KO mice and lipin-2 is abnormally regulated in *fld* mice, we hypothesized that lipin-1 and lipin-2 might cooperate to achieve optimal hepatic lipid homeostasis.

Liu *et al.* recently reported that lipin-1 and lipin-2 can form a complex in an *in vitro* transfection system (2), raising the possibility that lipin-1 and lipin-2 may form a complex *in vivo*.

Here we show that endogenous lipin-1 and lipin-2 do indeed form a stable complex in mouse liver, suggesting a physiological role for this interaction. We screened lipin protein domains to characterize the sites of lipin-1/lipin-2 interaction. Interestingly, the deletion of known functional motifs within lipin-1, such as the PAP enzyme active site, nuclear localization sequence and co-activator binding site, were not critical to the formation of the lipin-1/lipin-2 interaction complex. However, lipin-1 harboring a deletion of a stretch of amino acids that are unique to lipin-1 located downstream of the N-LIP domain was unable to precipitate with lipin-2. As this domain has no other known function, we named it the lipin-interacting domain (LID). Comparison of the sequence within this domain across 7 mammalian species revealed 21 highly conserved residues. Of these, five were lysines, suggesting that the interaction between lipin-1 and lipin-2 may have a role in protein ubiquitination and degradation. However, we could detect no difference in ubiquitination of wild-type lipin-1 and the lipin-1ΔLID mutant. Lipin-2 nuclear localization, co-activator binding and PAP enzyme activity were also unnecessary for the formation of the lipin-1/lipin-2 interaction complex. Perhaps, mutation of amino acid sequences that are unique to lipin-2 will shed light on which segments of lipin-2 are needed for protein interaction.

Lipin-2–mediated lipin-1 stabilization

We next turned our attention to the possible function of the lipin-1/lipin-2 interaction complex. Co-expression of lipin-1 and lipin-2 resulted in a lowering of lipin-2 protein levels and an elevation of lipin-1 protein levels. Deletion of the lipin-interacting domain from lipin-1 did not alter lipin-2–mediated lipin-1 stabilization, indicating that this did not require lipin-1/lipin-2 complex formation. Lipin-1 protein stabilization in the presence of lipin-2 therefore apparently involves an indirect mechanism that merits further investigation.

The screening of lipin-1 mutants alone or in combination with lipin-2 to examine the effects of lipin-1 and lipin-2 on one another revealed several interesting aspects of lipin-1 biology. First,

several mutations in lipin-1 had large effects on lipin-1 stability in the absence of lipin-2. Two mutations that are known to influence the handling of PA (D679E and ΔNLS) resulted in very high levels of lipin-1 protein compared to wild-type lipin-1, implying that impairment of PA binding and catalysis slows the turnover of lipin-1 protein. Lipin-1 D679E was further stabilized by the presence of lipin-2, but lipin-1ΔNLS was not.

On the other hand, two lipin-1 mutants (ΔLXXIL and ΔSRD) resulted in nearly undetectable lipin-1 protein levels. This could be the result of mutant mRNA or protein instability and further experiments are necessary to delineate the cause of the low protein levels of these mutants. However, it is notable that both of these mutants participate in the lipin-1/lipin-2 complex formation and the low levels of these proteins were surprisingly efficient at facilitating the degradation of lipin-2. It is intriguing that the lipin-1 co-activator binding motif and SRD may have a role in modulating the turnover of lipin-1 protein. Lipin-1 has been shown to interact with PPARα via the LXXIL motif in lipin-1 (9) and this interaction has been associated with increased expression of fatty acid oxidation genes, but no direct function of the PPARα–lipin-1 interaction on lipin-1 protein stability has been shown. Co-expression of lipin-2 had no effect on these mutants, suggesting that the lipin-2–mediated stabilizing effect on wild-type lipin-1 acts via proteins that bind to these domains. PPARα would be a plausible candidate, and knockdown of PPARα in the presence of lipin-1 and lipin-2 could reveal whether lipin-2 utilizes PPARα to stabilize lipin-1.

Lipin-1–mediated lipin-2 degradation

Co-expression of lipin-1 with lipin-2 resulted in lower lipin-2 protein levels, and the presence of the lipin-1 LID sequence was required to mediate the degradation of lipin-2. This suggests that lipin-1 itself, or a protein that is present in the lipin-1/lipin-2 complex mediates lipin-2 degradation. Treatment with the proteasomal inhibitor MG132 slowed lipin-1–mediated degradation of lipin-2, occurring largely through a proteasomal mechanism.

Deletion of the NLS from lipin-1 was the sole mutation that abolished both of the effects of lipins on one another. This is interesting because not only does the NLS facilitate nuclear localization of lipin-1 (15, 16), but it also serves as the binding site for PA (17). The dual function of the NLS is consistent with several possible models of lipin-1–mediated lipin-2 degradation. One possibility is that the binding of PA may cause a conformational change in lipin-1, allowing lipin-1 or a protein that binds to lipin-1 (such as a ubiquitin ligase), to alter lipin-2 in such a manner as to accelerate its degradation. Another possibility is that the nuclear localizing function of this motif allows lipin-1 to physically chaperone lipin-2 from the cytosol, where it normally resides, to the nuclear-associated endoplasmic reticulum where the proteasomes are located, to bring about lipin-2 degradation. A third possibility is that both of these functions are vital to lipin-1–mediated lipin-2 degradation. Lipin-1 may chaperone lipin-2 to the ER where PA is present, and this localization facilitates the destruction of lipin-2. The fact that mutation of the lipin-1 PAP active site (D679E) attenuates lipin-1–mediated lipin-2 degradation provides additional evidence that the handling of PA by lipin-1 is critical for the observed effect.

Our results suggest further experiments that may refine our understanding of the mechanism of lipin-1–mediated lipin-2 degradation. First, it would be interesting to determine whether lipin-1 increases lipin-2 ubiquitination. It will also be useful to determine whether lipin-1 alters the subcellular localization of lipin-2. Finally, isolation of the lipin-1-lipin-2 interaction complex and analysis of its constituent proteins by mass spectrometry will reveal the composition of the complex and provide clues about its function and regulation.

Our studies of wild-type mouse liver have revealed that lipin-1 and lipin-2 interact in physical complex and that this interaction influences lipin protein levels. These data suggest that an intricate mechanism is in effect to ensure homeostatic balance of PA and DAG levels, which influence cellular signaling pathways involved in glucose and energy homeostasis. It will be interesting to further examine these interactions in an *in vivo* system and elucidate how it

operates in normal and lipid-overloaded disease states. However, our data also demonstrate that studies of lipid homeostasis in mice that completely lack lipin-1 or lipin-2 in the liver may not be instructive regarding homeostatic mechanisms that act under typical physiological circumstances. They further suggest that pathologies occurring in human lipin-1 and lipin-2 deficient individuals may be partly attributed to a loss of the functions that are conferred by the interaction of these two proteins. Thus, future experiments that employ more subtle modulation of lipin levels, such as partial knockdown or modest overexpression in liver, should be instructive. The study of heterozygous lipin-1– or lipin-2–deficient animals, or humans with functional polymorphisms in lipin-1 and lipin-2, may also be informative.

FIGURE LEGENDS

Figure 4.1 – Lipin-1– or lipin-2–deficiency causes altered hepatic lipin levels and impaired responses to feeding.

A. C57Bl/6 mice were fasted overnight or fasted then re-fed for 3 hours prior to dissection. Western blotting for lipin-1, lipin-2 and lipin-3 were performed using hepatic tissue lysates. Tubulin was used as a loading control. NS = non-specific band.

B. Western blots showing changes in to lipin-1 and lipin-3 in hepatic tissue extracts from wild-type (Wt) and lipin-2 knockout (KO) mice that were fasted overnight or fasted then re-fed. Tubulin was used as a loading control. Recombinant lipin-1 and lipin-3 were expressed in HEK-293 cells and used as a positive control.

C. Wt or lipin-1–deficient *Lpin1*$^{fld/fld}$ (*fld*) mice were fasted overnight or fasted and then re-fed. Lipin-2 and lipin-3 levels were assessed by immunoblotting. B-actin was used as a loading control. Recombinant lipin-2 and lipin-3 were expressed in HEK-293 cells and used as a positive control.

Figure 4.2 – Lipin-1 and lipin-2 form an interaction complex in hepatic lysates via a novel lipin-interacting domain.

A. Protein lysates from fasted and non-fasted livers from Wt mice were subjected to immunoprecipitation (IP) using anti-lipin-1 antibody or control IgG antibodies, then immunoblotted (IB) for lipin-1 and lipin-2. Lysate lane represents 10% of the amount of protein used for IP. FT = flow-through. Lipin-1 and lipin-2 were expressed in HEK-293 cells and used as a positive control.

B. Conserved and functional motifs within mouse lipin-1 and lipin-2. N-LIP= amino-terminal conserved region; NLS = nuclear localization sequence; AcR = acidic region; SRD = serine-rich domain; LID = lipin-interacting domain; C-LIP = carboxy-

terminal conserved region. S731L is a PAP inactivating point mutation found in Lipin-2. * = regions that are unique to lipin-1.

C. Co-immunoprecipitation studies utilizing Wt and mutant forms of lipin-1 and lipin-2. Various forms of lipin-1 and lipin-2-V5 were co-expressed in HEK-293 cells and immunoprecipitated using V5 antibody or IgG control antibody, then lipin-2-V5 and lipin-1 were detected by western blotting. Lipin-1-Wt and lipin-2-Wt IP is shown at top left. IP of lipin-1 mutants (ΔNLS, Δ LXXIF and ΔLID) with lipin-2-Wt are shown at left. IP of lipin-1-Wt with lipin-2-V5 mutants (ΔNLS, ΔLXXFF and S731L) are shown at right.

Figure 4.3 – Lipin-1 and lipin-2 have distinct effects on one another.

A. HEK-293 or Hepa 1-6 cells were transfected with lipin-1 or lipin-2-V5 alone or in combination with one another. Western blotting of was performed on equal amounts of protein lysates shows that co-expression results in distinct changes in lipin-1 and lipin-2 protein levels. B-actin was used as a loading control.

B. Schematic representation of effects on lipin-1 and lipin-2 protein levels when these lipins are co-expressed. Lipin-1 causes lower lipin-2 protein levels, while lipin-2 causes elevated lipin-1 levels.

C. Lipin-2 increases lipin-1 protein levels in a dose dependent manner. Lipin-1 or lipin-2 were expressed alone (left), then the same amount of lipin-1 was transfected with and equal amount, double (++) or triple (+++) the amount of lipin-2-V5 plasmid. pcDNA3.1 background vector was used to maintain equal DNA amounts across conditions.

D. Mutation of the LID domain in lipin-1 is unable to lower lipin-2 protein levels, but lipin-2 can cause increased levels of Lipin-1ΔLID mutant protein. Lipin-1ΔLID and Lipin-2-V5 were transfected using the same experimental design as in C.

E. The effects of lipin-1 on lipin-2 are direct as they require the formation of the lipin interaction complex. The same is not true of lipin-2–mediated lipin-1 stabilization; removal of the LID domain from lipin-1 does not alter the effect of lipin-2 on lipin-1 and is therefore an indirect effect.

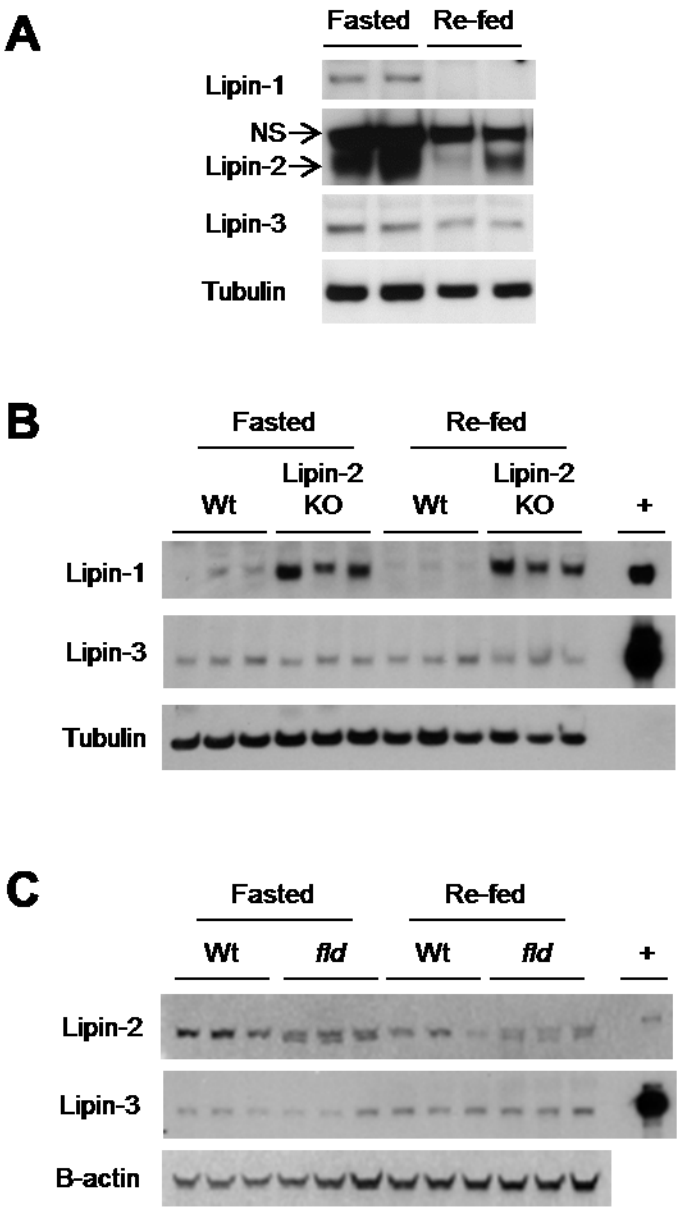
F. Lipin-1, Lipin-1ΔLID or lipin-2-V5 were transfected alone (lanes 1-3) or in combination (lanes 4-7) in the presence of control vehicle (top) or proteasomal inhibitor MG132 (bottom). ++ = double the amount of DNA in the (+) condition. pcDNA 3.1 background vector was used as to maintain equal amounts of DNA in each condition. The effect of lipin-1 on lipin-2 was blunted in the presence of MG132, showing that lipin-1 accelerates lipin-2 protein degradation to lower lipin-2 protein levels.

Figure 4.4 – The lipin-1 nuclear localization sequence is required for the effects of lipin-1 and lipin-2 on each other.

A. Wild-type lipin-1 (lane 1) and wild-type lipin-2 (lane 2) were transfected alone or in combination (lane 3). Then lipin-1 mutants were transfected alone or in combination with wild-type lipin-2. Lipin-1 mutants were as follows: D679E = lipin-1 PAP site mutant (lanes 4-5); N-LIP = conserved amino-terminal region (lanes 6-7); NLS = nuclear localization sequence (lanes 8-9); AcR = acidic amino acid region (lanes 10-11); LXXIL = co-activator binding site (lanes 12-13); SRD = serine rich domain (lanes 14-15). Finally, wild-type lipin-1 and lipin-2 carrying the S731L point mutation that lacks enzyme activity were used alone (lane 1, 16) or in combination (lane 17). Lysates were separated by electrophoresis and transferred to a single membrane to assess changes in lipin-1 and lipin-2 by western blotting. All panels were scanned from a single exposure to enable comparison across conditions. Tubulin was used as a loading control. * = longer exposure.

B. Diagram representing functional domains within lipin-1 and lipin-2 that are necessary for lipin-1–mediated lipin-2 degradation and lipin-2 dependent lipin-1 stabilization. The lipin-1 NLS motif, SRD and LXXIL motif were critical for the stabilization of lipin-1. The lipin-1 NLS motif and LID were indispensable for the degradation of lipin-2 via lipin-1.

Figure 4.1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Re-fed
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NS
Lipin-2
Lipin-3
Tubulin
B
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Re-fed
Wt
Lipin-2 KO
Wt
Lipin-2 KO
+
Lipin-1
Lipin-3
Tubulin
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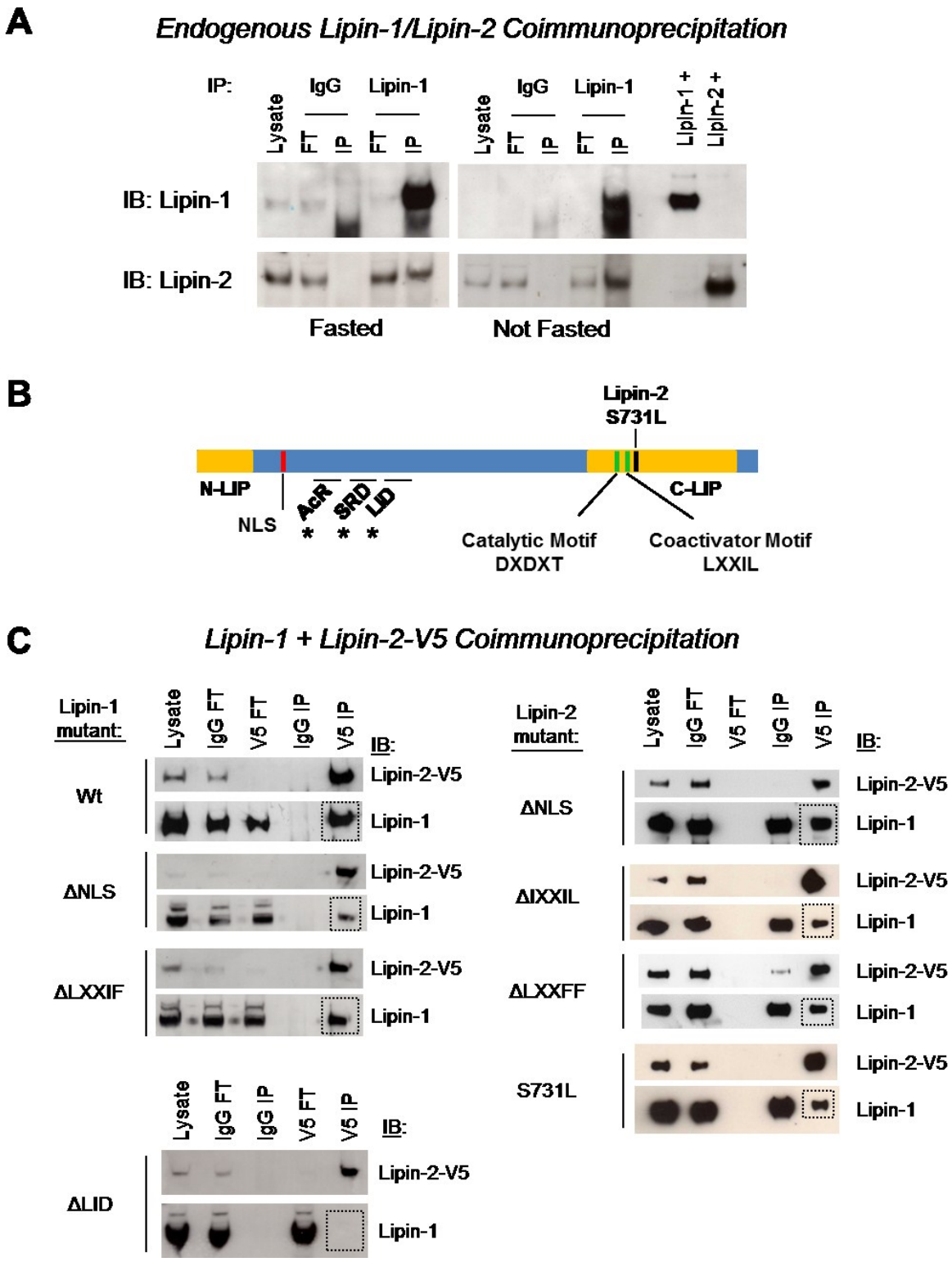
fld
+
Lipin-2
Lipin-3
B-actin

Figure 4.2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Lipin-1
Lysate
FT
IP
Lipin-1 +
Lipin-2 +
IB: Lipin-1
IB: Lipin-2
Fasted
Not Fasted
B
Lipin-2
S731L
N-LIP
C-LIP
NLS
AcR
SRD
LID
Catalytic Motif
DXDXT
Coactivator Motif
LXXIL
C
Lipin-1 + Lipin-2-V5 Coimmunoprecipitation
Lipin-1
mutant:
Lysate
IgG FT
V5 FT
IgG IP
V5 IP
IB:
Wt
ΔNLS
ΔLXXIF
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mutant:
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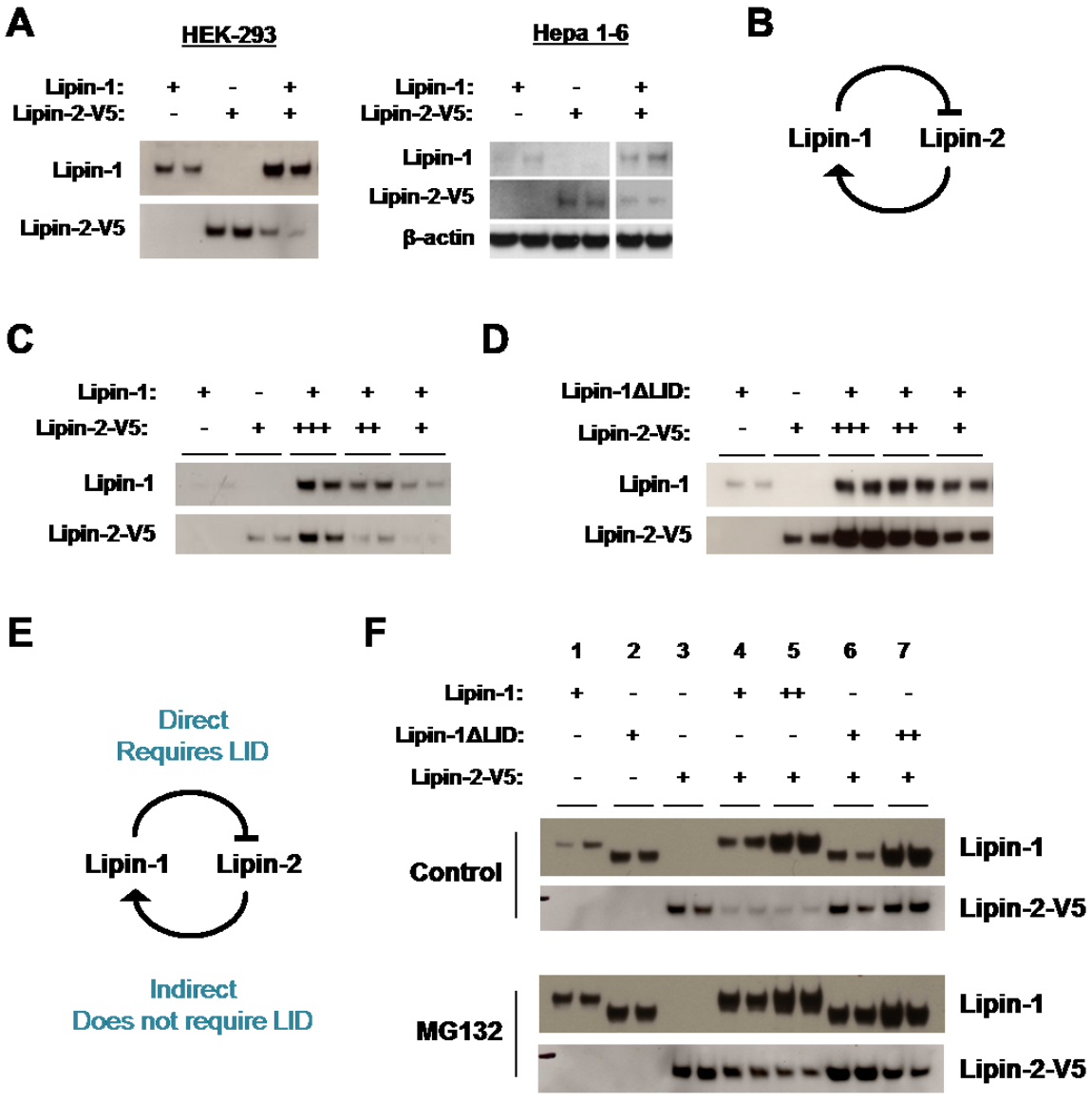
ΔLXXFF
S731L

Figure 4.3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Lipin-2-V5: - + +
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Lipin-2-V5
Hepa 1-6
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Lipin-2-V5: - + +
Lipin-1
Lipin-2-V5
β-actin
B
Lipin-1
Lipin-2
C
Lipin-1: + - + + +
Lipin-2-V5: - + +++ ++ +
Lipin-1
Lipin-2-V5
D
Lipin-1ΔLID: + - + + +
Lipin-2-V5: - + +++ ++ +
Lipin-1
Lipin-2-V5
E
Direct
Requires LID
Lipin-1
Lipin-2
Indirect
Does not require LID
F
1 2 3 4 5 6 7
Lipin-1: + - - + ++ - -
Lipin-1ΔLID: - + - - - + ++
Lipin-2-V5: - - + + + + +
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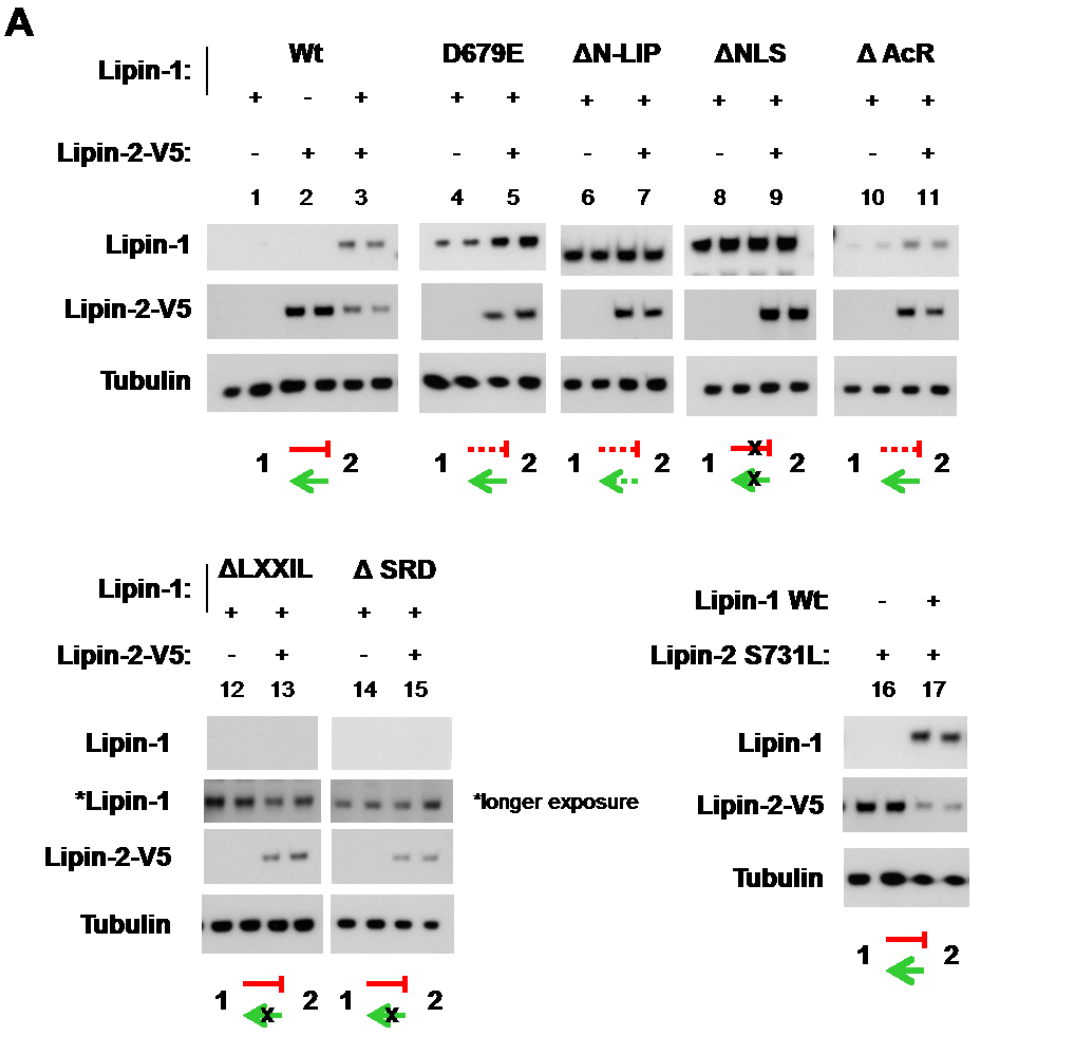
Lipin-2-V5
MG132
Lipin-1
Lipin-2-V5

Figure 4.4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Wt
D679E
ΔN-LIP
ΔNLS
Δ AcR
Lipin-2-V5:
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Lipin-1
Lipin-2-V5
Tubulin
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Δ SRD
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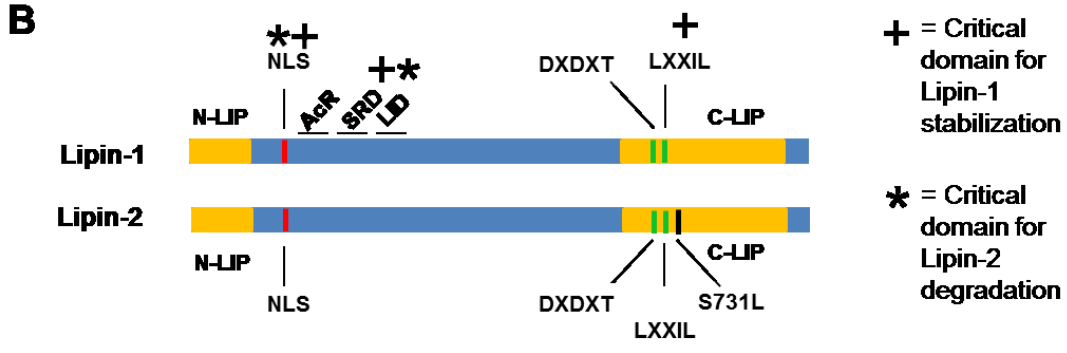
*Lipin-1
*longer exposure
Lipin-1 Wt:
Lipin-2 S731L:
16 17


B
NLS
N-LIP
AcR
SRD
LID
DXDXT
LXXIL
C-LIP
Lipin-1
Lipin-2
S731L
+ = Critical domain for Lipin-1 stabilization
* = Critical domain for Lipin-2 degradation

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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Summary

In 2001, our laboratory discovered the lipin protein family consisting of lipin-1, lipin-2 and lipin-3. All three lipin proteins have the ability to catalyze the conversion of phosphatidic acid (PA) into diacylglycerol (DAG) (1), the penultimate step in the glycerol-3-phosphate triacylglycerol (TAG) synthesis pathway, making it unclear what the physiological and cellular roles of the individual lipin family members might be. The focus of this dissertation project has been to begin to elucidate the physiological and molecular roles of lipin-2 and its relationship to the other lipin family members through the use of various genetic and molecular tools.

Lipin-2 deficiency in humans causes a rare Mendelian disease, Majeed syndrome (2, 3). Only a handful of patients have been characterized and these studies have been necessarily superficial. This has precluded significant insights into the effects of lipin-2 deficiency and the normal physiological role of lipin-2. We have generated lipin-2 knockout (KO) mice in order to study the physiological function of lipin-2. Our initial characterization of lipin-2 deficiency in the mouse revealed a physiological relationship between lipin-1 and lipin-2 and follow-up studies have shown a physical interaction and an intricate regulatory loop between lipin-1 and lipin-2.

Lipin-2—deficiency causes hepatic defects in lipid metabolism

The highest levels of lipin-2 mRNA expression are found in liver and lipin-2 is the most abundant lipin in liver (1, 4). Therefore we expected that lipin-2 deficiency would result in altered lipid homeostasis in the liver and reveal a critical role for lipin-2 in regulating hepatic TAG and phospholipid levels. Thus, our finding that loss of lipin-2 caused increased, rather than decreased hepatic phosphatidate phosphatase (PAP) activity (5), was somewhat surprising. We determined that the liver has a safeguard, lipin-1 protein upregulation, which allows for glycerolipid synthesis and is satisfactory to maintain normal hepatic lipid homeostasis

under basal conditions. However, under the metabolic challenge of a high fat diet, lipid homeostasis becomes unbalanced and excess TAG accumulates in the lipin-2 KO liver (Dwyer, 2012). Analogously, lipin-1 deficiency in mouse liver leads to disrupted lipid homeostasis, particularly during the neonatal period when the liver is stressed by the high fat content of mouse mother's milk (6). These observations provided a clue that lipin-1 and lipin-2 may cooperate to maintain normal lipid homeostasis. This hypothesis is further supported by our demonstration that endogenous lipin-1 and lipin-2 physically associate in a protein complex in liver (Chapter 4). The function of this complex is not yet understood, but was found to influence levels of both lipin-1 and lipin-2 protein, suggesting that it serves as a means to maintain balance between the two family members and presumably to promote an optimal rate of glycerolipid synthesis.

Lipin-1 has been shown to bind peroxisome proliferator-activated receptor (PPAR) α and PPARγ coactivator-1α (PGC-1α) and to amplify their coactivation of fatty acid oxidation target gene expression (7). However, despite the elevated hepatic lipin-1 levels in lipin-2 KO mice, PPARα/PGC-1α target gene expression was not enhanced, and was even downregulated on a high fat diet. Therefore, excess lipin-1 in the setting of lipin-2 deficiency and metabolic stress may cause decreased fatty acid oxidation, contributing to enhanced TAG storage that was observed in these mice. These results also raise the possibility that lipin-2 is required to potentiate the full effects of lipin-1 on fatty acid oxidation gene activation. Thus, compensation by lipin-1 in lipin-2–deficient liver is imperfect, and optimal utilization of excess fatty acid influx that occurs with high fat diet feeding appears to require both lipin-1 and lipin-2.

The role of lipins in lipid homeostasis in brain

Lipin-1 and lipin-2 also have a role in maintaining lipid homeostasis in the brain (5). Lipin-1 is expressed at low levels throughout the brain (1), whereas lipin-2 appears to be localized to specific brain regions, including cerebellum, hippocampus and cerebral cortex (5). An

unexpected consequence of lipin-2–deficiency was the development of an age-dependent movement disorder. Evaluation of lipin levels in the cerebellum, which has a role in coordinating movement, showed that both lipin-1 and lipin-2 are present in young wild-type cerebellum, but lipin-1 levels decline with age, making lipin-2 the sole lipin present in aged cerebellum. The age-related reduction in lipin levels in this tissue may reflect a reduced requirement for PAP activity in phospholipid synthesis in the ageing brain. Perhaps related to reduced lipin levels, the PA concentration in wild-type cerebellum increases approximately 4-fold between young and aged mice. In lipin-2–deficient mice, PA levels increase substantially more than in wild-type brain and are linked to the neurological dysfunction seen in lipin-2 KO mice. It will be interesting to determine whether lipin-1 and lipin-2 protein complexes form in the cerebellum as we observed in liver and, if so, what regulates lipin-1/lipin-2 complex formation in this tissue. Because the neuropathy in the lipin-2 KO mouse is age-dependent and Majeed syndrome presents in childhood — the oldest reported patient was 21 years old — it is unknown if human lipin-2–deficient patients might develop neurolosgical dysfunction later in life. A greater understanding of the role of lipins in the context of brain lipid homeostasis may not only benefit Majeed Syndrome patients, but may also prove informative in the study of other neurological diseases associated with abnormal lipid levels (8).

FUTURE DIRECTIONS

Lipin Proteins and Bone Health

Mutations in *LPIN2* have been shown to be the cause of Majeed syndrome, a rare disorder consisting of chronic recurrent osteomyelitis, recurrent fever, anemia and skin inflammation (REFs). Our *a priori* expectation was that lipin-2–deficient mice would closely resemble humans with Majeed Syndrome. We detected anemia in lipin-2 KO mice, and observed isolated occurrences of skin inflammation, but were surprised that osteomyelitic bone lesions were not detected upon analysis of tibias and femurs by X-ray and low-resolution microcomputed tomography (microCT) (5). We previously detected lipin-2 mRNA in bone and bone marrow (4), and therefore further assessed whether lipin-2 deficiency affected bone by careful analysis with high-resolution microCT. MicroCT analysis of tibias from aged (14–16 month) lipin-2 KO mice revealed 36% decreased tissue volume (TV; marrow cavity plus trabecular bone) compared to wild type controls, but lipin-2 KO bones were otherwise normal (Figure 5.1A). This suggested that an additional factor not present in our mouse colony — such as an infectious agent, dietary or genetic component — may contribute to the symptoms of human Majeed syndrome.

Previous studies in mice have shown that increased dietary fat content is associated with decreased bone density (9–11), including decreases in trabecular volume (9, 11). We wondered whether the effects of lipin-2 deficiency on bone would be exacerbated by a high fat diet, and performed high-resolution microCT analysis of bones from mice fed a high-fat diet for 7 weeks beginning at 8 months of age. Lipin-2 KO mice fed the high fat diet sustained significant damage to the trabecular microarchitecture indicated by reductions in trabecular bone volume (BV; absolute bone volume in a defined region), bone volume fraction (BV/TV: proportion of total tissue volume composed of bone), connectivity density (a quantification of the connections between trabeculae), and apparent density (density of the total tissue region scanned) (Figure 5.1B, C). Further, the structure model index was increased, indicating a shift in the trabecular

structure from plate-like trabeculae to weaker rod-like trabeculae (Figure 5.1B). These differences in bone parameters were visible in microCT images as increased space within the trabecular structures of lipin-2 KO bone (Figure 5.1C). These findings suggest that lipin-2 deficiency sensitizes mice to the negative effects of high fat diet on bone structure and density.

It is worthwhile to note that bone biopsies from three children with Majeed syndrome exhibited “excessive resorption of bony trabeculae” (12), and loss of trabecular bone is one of the hallmark symptoms of osteoporosis. Interestingly, genome-wide association studies in humans have found associations between chromosome 18p11, which harbors *LPIN2*, and lumbar spine bone mineral density (13) and stature (14). Thus, lipin-2 KO mice may provide a useful model in which to explore the mechanisms involved in trabecular bone maintenance.

The detection of lipin-2 effects on bone density raised a question about whether the other two lipin protein family members may also have roles in bone.

We have previously detected low-level expression of lipin-1 mRNA in bone and bone marrow (1). Using specific antibodies, we assessed whether lipin-1 and lipin-3 proteins are present in bone marrow sections, and found that both proteins are detectable within bone marrow adipocytes and stromal cells (Figure 5.2). To examine the role of lipin-1 in bone maintenance, tibias from lipin-1–deficient *fld* mice were subjected to microCT analysis. Significant reductions in bone volume and tissue volume in both trabecular and cortical bones were detected in *fld* bones compared to wild-type controls, indicating smaller bones (Figure 5.3). Interestingly, indices related to the structure of the trabeculae (bone patterning factor and structure model index) were altered in *fld* mice towards a more rod-like structure, indicating weaker trabeculae and cortical bone mineral density was increased in *fld* mice compared to controls. Experiments are under way to determine if lipin-1–deficiency also sensitizes bone to the stress of a high fat diet. These findings raise the possibility of a local role for the lipin family in bone formation or remodeling.

The deleterious effects on lipin-2–deficient bone structure during dietary stress could be due solely to the loss of lipin-2 or could be due to imperfect compensation by another lipin family member in the absence of lipin-2, as was seen in the liver of high fat fed lipin-2 KO mice. Because bone marrow is an extremely heterogeneous organ, composed of osteoblasts, osteoclasts, adipocytes and numerous cell-types of the hematopoietic lineage, it will be necessary to identify the cell populations within bone that express lipins in order to gain insight into the specific function of lipins in bone. In the future, cell-type–specific knockout models could be useful in determining the molecular mechanisms underlying the trabecular abnormalities associated with lipin-2 deficiency.

The physiological role of the lipin-1/lipin-2 interaction complex

The interaction between lipin-1 and lipin-2 in a physical protein complex has revealed a previously unexpected mechanism for cooperative function of lipin protein family members. Our most pressing questions concerning the lipin-1/lipin-2 interaction complex is to determine what physiological cues govern its formation, and what are the consequences on lipin protein activities. We hypothesize that the lipin-1/lipin-2 interaction may be of significance in liver during the transition from the fasted to the fed state. Some observations consistent with this are that lipin-1 levels and subcellular localization are regulated by insulin (15), and that lipin-2 levels decline dramatically with feeding (16). In the short term, we plan to analyze several more lipin-2 mutants (including ΔNLS and ΔN-LIP) in combination with wild-type lipin-1 to map the protein domains in lipin-2 that participate in the interaction, reduce lipin-1 protein stability, and play a role in lipin-1–mediated lipin-2 degradation. We are currently investigating whether alterations in the balance between lipin-1 and lipin-2 *in vivo* lead to dysregulated lipid and glucose homeostasis through partial knockdown using anti-sense oligonucleotides. We will also investigate whether lipin-1/lipin-2 protein dynamics are responsive to various hormone treatments (such as insulin), knockdown of PPARα, and various fasting and feeding regimes.

Additionally, isolation of the lipin-1/lipin-2 complex and identification of other proteins that are present by mass spectrometry may aid in defining a physiological function of the interaction. A knock-in transgenic mouse that is missing the LID sequence of lipin-1 would also be particularly useful for determining the physiological role for the lipin-1/lipin-2 interaction *in vivo*.

The elucidation of lipin-3 function

Work in our laboratory has determined that lipin-3 is expressed at low levels in many tissues (liver, intestine, kidney and fat) (1) and at high levels in bone. However, lipin-3 cannot compensate for the loss of lipin-1 and lipin-2 since lipin-1/lipin-2 double KO (DKO) mice died as embryos (5). This may be related to a lack of lipin-3 expression during development, the reduced PAP specific activity of lipin-3, or perhaps lipin-3 requires the presence of at least one other family member for its function.

Lipin-3–deficiency in the mouse resulted in no obvious phenotype under basal conditions in several metabolic parameters (body weight, body composition, glucose tolerance, bone density, plasma lipid levels, etc.; data not shown). Unlike lipin-2 KO mice, when lipin-3 KO mice were stressed with high fat diet feeding, no abnormalities in bone microarchitecture were detected by microCT analysis, and we observed only a modest increase in body weight compared to wild-type littermates (data not shown). Due to the presence of compensatory mechanisms seen in the lipin-2 KO mouse, we presume that other lipin family members may be compensating for lack of lipin-3 in these mice. A better strategy to eliminate the effects of compensation and reveal the elusive the role of lipin-3 has been to cross lipin-3–deficient mice with lipin-1– and lipin-2–deficient heterozygous mice to generate lipin-1/lipin-3 and lipin2-/lipin-3 double knockout animals. I have been involved in the characterization of these animals with Lauren S. Csaki.

Lipin-1/3 doubly-deficient mice are even more profoundly lipodystrophic than lipin-1–deficient animals, with nearly non-detectable fat pads and no PAP activity. Interestingly, lipin-1–

deficient and lipin-3–deficient mice each exhibit reduced adipose tissue PAP activity, indicating that both lipin-1 and lipin-3 are needed for optimal PAP activity in this tissue (data not shown). We hypothesize that lipin-1 and lipin-3 cooperate to provide optimal PAP activity in adipose tissue.

Lipin-2/3 doubly-deficient mice have a reduced viability, small body size, and hunched posture (data not shown). MicroCT analysis of bones from these mice show reductions in trabecular and cortical bone density compared to wild-type and single knockout controls (data not shown). In addition, lipin-2/3 KO mice display severe lipid accumulation within the intestinal enterocytes and hyperplastic villi. It is unknown at this point if the bone abnormalities in these mice are secondary to impaired intestinal absorption, and is an area of active investigation. These results suggest that both lipin-2 and lipin-3 may be the pertinent lipin family members in intestine and bone.

Analogous to the lipin-1/lipin-2 protein complex, it will be interesting to explore whether lipin-1 and lipin-3 proteins form an interaction complex in adipose tissue, and if lipin-2 and lipin-3 form a similar complex in bone and intestine. Finally, it will be of great interest to elucidate the physiological functions of these complexes.

FUTURE PERSPECTIVES

The existence of protein families in mammals is a common theme in biology. Why are several, highly similar proteins designed to carry out the same molecular function? The studies performed in in this dissertation project suggest that it is not a simple matter of individual family members providing a particular function in distinct tissues. Rather, we have found that members of the lipin protein family act both independently and in combination, which may allow fine-tuning of biological processes under stressful situations. Thus, lipin-1 and lipin-2 were found to be necessary to mediate optimal liver function when presented with the metabolic

stress of high fat intake. We also found that lipin-1 and lipin-2 can each influence the protein levels of the other, suggesting that physical interactions among protein family members may provide an additional level of regulation for key cellular processes.

The further elucidation of the function of the individual lipin proteins and of the interactions between lipin family members may shed light on mechanisms that allow our bodies respond to the physiological stresses of lipid dysregulation, and illuminate a path towards better therapies for alleviating the systemic complications of obesity.

FIGURE LEGENDS

FIGURE 5.1 – Lipin-2–deficient mice exhibit reduced trabecular bone density.

(A) μCT scanning of proximal tibias from 16-month old male mice on chow diet revealed reduced tissue volume and tissue surface. *n* = 5 Wt and 7 KO.

(B) Trabecular bone parameter measurements determined by μCT of tibias from 8-month-old male mice fed a high-fat diet for 7 weeks. Lipin-2 KO mice exhibit reduced tissue volume, trabecular number and connectivity density, and increased trabecular spacing. *n* = 3 Wt and 4 KO.

(C) Top panels show representative tibia cross-sections from μCT scanning performed in C. Middle and lower panels display representative three-dimensional reconstructions of μCT scans of trabecular bone structure. Middle panels show the bone from the side, while lower panels display the axial view from the growth plate.

Figure 5.2 – Lipin-1 and Lipin-3 are expressed in bone marrow adipocytes and stromal cells.

Immunohistochemical staining of bone marrow sections for lipin-1 (top) and lipin-3 (bottom). Lipins are visible as brown staining. Nuclei were stained green. IgG from rabbit and goat were used as controls for the lipin-1 and lipin-3 antibodies, respectively.

Figure 5.3 – Lipin-1–deficiency results in altered bone microarchitecture.

MicroCT analyses in lipin-1-deficient (fld/fld) bone. Trabecular bone (top) exhibits reduced bone volume and tissue volume and increased bone patterning factor and structure model index. Cortical bone (bottom) shows reduced bone volume, tissue volume, medullary cavity size and increased bone mineral density. Images are representative of each group scanned.

Figure 5.1

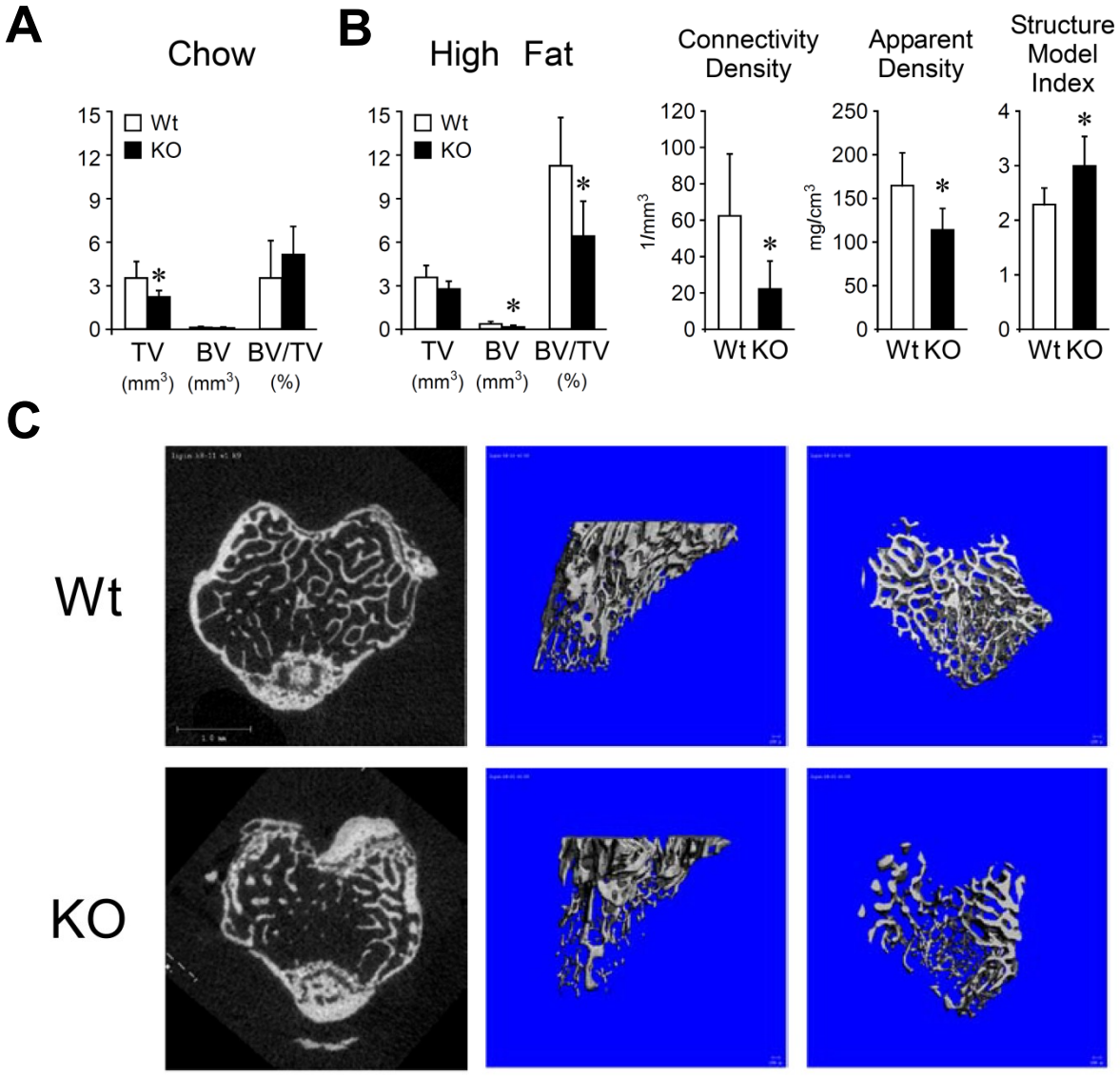
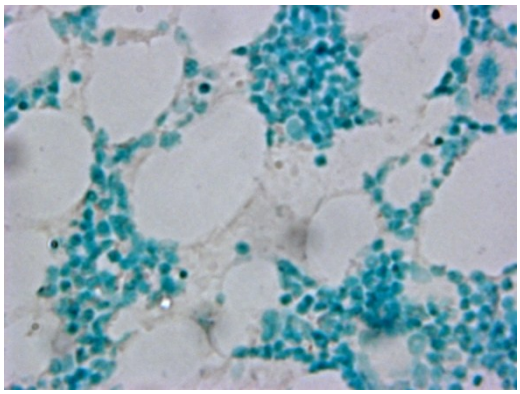
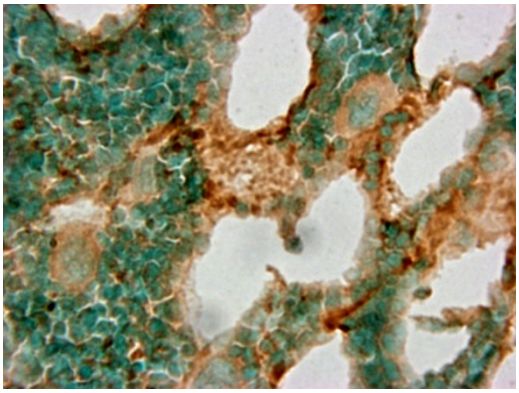

A
Chow
Wt
KO
15
12
9
6
3
0
TV (mm3)
BV (mm3)
BV/TV (%)
B
High Fat
Wt
KO
TV (mm3)
BV (mm3)
BV/TV (%)
Connectivity Density
1/mm3
120
100
80
60
40
20
0
Wt KO
Apparent Density
mg/cm3
250
200
150
100
50
0
Wt KO
Structure Model Index
4
3
2
1
0
Wt KO
C
Wt
KO

Figure 5.2

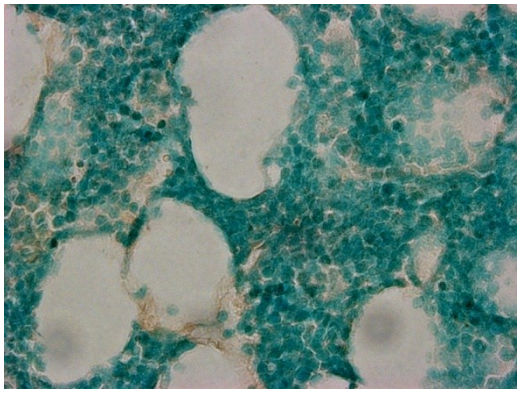
Rabbit IgG



Lipin-1



Goat IgG



Lipin-3

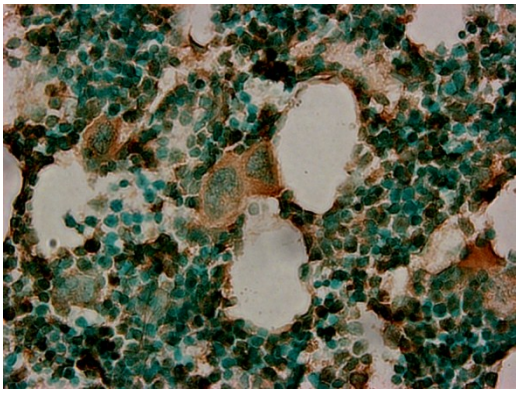
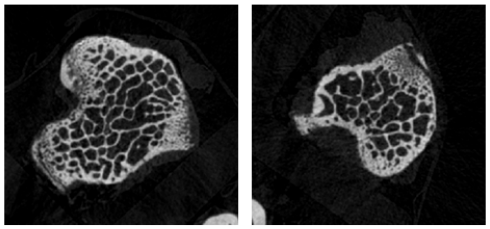


Figure 5.3

Trabecular Bone

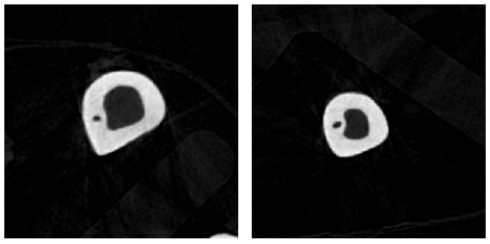
Wt *fld*



	Wild Type		*fld/fld*		
Bone Parameter	Mean	SD	Mean	SD	p-value
Tissue Volume (mm^3)	2.38	0.15	1.50	0.11	0.01 *
Bone Volume (mm^3)	0.30	0.02	0.18	0.04	0.01*
BV/TV (%)	12.79	0.61	11.72	1.76	0.49
Trabecular Patterning Factor (mm^{-1})	14.35	0.76	18.26	1.75	0.04*
Structure Model Index	1.66	0.05	1.89	0.06	0.03*

Cortical Bone

Wt *fld*



	Wild Type		*fld/fld*		
Bone Parameter	Mean	SD	Mean	SD	p-value
Tissue Volume (mm^3)	0.22	0.02	0.14	0.01	0.01*
Bone Volume (mm^3)	0.15	0.01	0.11	0.00	0.01*
BV/TV (%)	70.97	1.41	75.21	1.00	0.09
Cortical thickness (mm)	0.21	0.00	0.20	0.00	0.02*
Bone Mineral Density (g/cm^3)	1.59	0.01	1.64	0.01	0.01*
Medullary Cavity Size (mm^3) (TV-BV)	0.06	0.01	0.04	0.00	0.03*

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