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Yike Lu April 14, 2020

The Role of Leucine Carboxyl Methyltransferase-1 (LCMT-1) in Liver Lipid Homeostasis

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Protein phosphatase 2A (PP2A) family phosphatases (PP2A, PP4 and PP6) are an important family of phosphatases that regulate a variety of functions during cell growth, division, and development. LCMT-1 is a protein methyltransferase that methylates the catalytic C subunit of PP2A, PP4, and PP6. Loss of LCMT-1 leads to a reduction in methylation of PP2A family phosphatases, resulting in disruption of normal development. Previous studies indicate that LCMT-1 plays a key role in fetal liver development and its reduction is also associated with insulin resistance, a symptom linked to non-alcoholic fatty liver disease (NAFLD). NAFLD affects around 25% of people in the world and is especially common among middle-aged Americans. The long-term goal of our research is to determine whether modulation of PP2A family phosphatase methylation might be therapeutically beneficial. Preliminary data from experiments done in the Pallas lab using homozygous albumin-cre-directed LCMT-1 conditional knockout (cKO) mice suggest that LCMT-1 may play a vital part in liver lipid homeostasis. This research project studies a cKO mouse model in which LCMT-1 is only knocked out in liver cells in order to further understand the role of LCMT-1 in liver lipid accumulation and liver cell viability. Two feeding studies were performed with similar designs, the only difference being that the first used homozygous Alb-cre mice while the second used heterozygous Alb-cre mice. In each feeding study, cKO and corresponding sex-and-age-matched control mice were placed on a high-fat diet and a normal diet. After a 17-week feeding period, their livers and serum were harvested, analyzed, and compared to see whether the loss of LCMT-1 affects liver lipid accumulation and cell viability, both hallmarks of NAFLD. Alanine transferase (ALT) levels in serum served as an indicator of liver cell death, while western blot analysis provided insights into LCMT-1's mechanistic effects. Our data show that loss of LCMT-1 induced by both homozygous and heterozygous Alb-cre expression results in enlargement of livers and spleens and elevated alanine transferase (ALT) activity in blood in cKO mice placed on high-fat diet. In homozygous Alb-cre cKO mice, the loss of LCMT-1 also leads to faster weight gain. Overall, our data suggest that LCMT-1 plays a vital role in liver lipid homeostasis.

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Introduction

Protein phosphatase 2A (PP2A) is an essential threonine/serine protein phosphatase that regulates multiple cell functions.[1] PP2A carries out its functions by forming different hetero-trimeric holoenzymes using one each of different regulatory/targeting B subunits, catalytic C subunits (PP2Ac), and structural A subunits.[2] There are multiple different regulatory B subunits divided into four families (B, B', B'' and B''' families) according to structural differences. Genes that code for regulatory B subunits express in a tissue-specific manner, resulting in variable abundance of different B subunits in respective tissues/cells. These B subunits, combined with 2 isoforms of the C subunit and 2 isoforms of the A subunit, can constitute over 80 different PP2A holoenzymes.[1] The various holoenzymes regulate important cell functions such as the cell cycle transitions, multiple signaling pathways, DNA synthesis, to name a few.[3,4] A subset of PP2A holoenzymes is activated through carboxyl methylation of the catalytic C subunit by leucine carboxyl methyltransferase-1 (LCMT-1).[2] LCMT-1 methylates PP2Ac at its carboxyl-terminal Leu^{309}, which subsequently enables regulatory B subunits to assemble with the A/C heterodimer. LCMT-1 and protein phosphatase methylesterase-1 (PME-1), which demethylates the catalytic C subunit of PP2A holoenzyme, together regulate the activity of these select PP2A phosphatase heterotrimers, termed methylation-dependent PP2A heterotrimers.[5] More recently, Hwang and Pallas showed that LCMT-1 similarly regulates methylation-dependent heterotrimers of the related PP2A family phosphatase, PP4.[6] Thus, LCMT-1 is critical for the function of a subset of both PP2A and PP4 phosphatase complexes.

Data in a recent paper by Lee et.al (our lab) indicate that LCMT-1 plays a key role in fetal liver development in mouse models. Interestingly, both the blood system and liver cells co-develop in the fetal liver during embryogenesis until a point in late gestation when the hematopoietic cells leave the fetal liver and migrate to the bone marrow.[7] Lee et al.'s data

demonstrate that global loss of LCMT-1 leads to elevated apoptosis of blood cells and reduced hematopoietic stem and progenitor cells.[7] Furthermore, unpublished data from our lab indicate that in this same global LCMT-1 knockout mouse model, hepatoblasts, which ultimately generate the adult liver, are reduced 5- to 10-fold compared with wild-type controls. Thus, LCMT-1 appears to have a role in both normal hematopoietic and liver development.

Other research indicates that partial loss of LCMT-1 can lead to defects in insulin signaling in adult mice, resulting in an insulin-resistance phenotype.[8] Interestingly, insulin resistance is often associated with non-alcoholic fatty liver disease (NAFLD), though the mechanistic basis of this association is still largely unknown.[9] This association doesn't come as a surprise since liver is highly involved in several glucose metabolism processes such as glycogenesis, glycogenolysis, glycolysis, gluconeogenesis, and fatty acid synthesis.[10–12] NAFLD is usually characterized by triglyceride and free fatty acid accumulation in livers, obesity, metabolic derangement, and insulin resistance.[13] Consistent with PP2A being a possible relevant downstream target of LCMT-1 in liver glucose metabolism, liver specific deletion of the major PP2Ac gene (PP2Ac α) in mice enhances glucose metabolism and insulin sensitivity.[14] Additional studies have also implicated both PP2A and PP4 in glucose metabolism and/or lipid homeostasis.[15–20] Therefore, we postulated that LCMT-1 might regulate methylation-dependent PP2A and/or PP4 complexes that play a role in liver lipid homeostasis and that modulation of LCMT-1 may affect the development of NAFLD. Importantly, since methylation of PP2A and PP4 is reversible, a long-term goal of this project is to determine if modulation of LCMT-1 and/or PME-1 function might be a novel therapeutic target for NAFLD. Since PP2A family phosphatases have such broad functions in regulation of cell growth and development, it may be hard to manipulate them without

causing side effects. However, LCMT-1 and PME-1 have much more specific functions and therefore may serve as better therapeutic targets.[21]

In order to determine whether methylation-dependent PP2A heterotrimers and/or PP4 complexes are indeed involved in regulation of liver lipid homeostasis, we studied an Alb-cre-directed LCMT-1 conditional knockout mouse model so as to circumvent embryonic lethality and better explore the function of LCMT-1 in hepatic cells in adult mice. In this study, we investigated the effects of conditional loss of LCMT-1 in liver by comparing mouse weight changes over time, liver/mouse weight ratio, spleen sizes (indicator of systemic inflammation), and alanine transferase (ALT) levels (an indicator of liver cell death) between cKO and control mice that were either placed on high-fat diet or normal diet. Moreover, we examined signaling via the mTOR pathway and inflammatory signaling via NFkB.

Our data demonstrate that cKO mice placed on high-fat diet have a higher liver to body weight ratio, possibly due to abnormal regulation of lipid homeostasis induced by reduced levels of LCMT-1. They also indicate increased inflammation in mice placed on a high-fat diet. Finally, they reveal that LCMT-1 loss in liver cells has unique effects on the mTOR pathway in mice fed a high-fat diet. Overall, the results of the study show some potential for the cKO mouse model as a novel model for NAFLD, which could potentially be beneficial for future studies related to NAFLD.

Methods

a. Albumin-cre-directed LCMT-1 cKO mouse model

Cre recombinase is an enzyme that deletes genes between 2 *loxP* sites. The gene (or portion of a gene) encoded between the *loxP* sites is called a floxed gene. Floxing marks a specific gene to be cut out by cre recombinase in the genome. The *Alb-cre* gene expresses cre recombinase under the expression pattern of the albumin promoter in liver. A mouse with a +/+ (no floxing) *LCMT-1* gene and a +/alb-cre or alb-cre/alb-cre *Alb-Cre* gene is considered a control mouse. A mouse with a flox/flox (floxed) *LCMT-1* gene and a +/alb-cre or alb-cre/alb-cre *Alb-Cre* gene is considered a cKO mouse. C57BL/6 mice of desired genotypes for the feeding study, controls and cKO, were bred and genotyped from an existing colony of mice that have both the *Alb-cre* gene and the floxed *LCMT-1* gene (Exon 3 flanked by loxP sites). Mice were genotyped through isolation of genomic DNA from tail biopsies, PCR with primers flanking one of the loxP sites, and gel electrophoresis to look for two bands, the shorter of which represents non-floxed *LCMT-1*, and the longer of which represents floxed *LCMT-1* (presence of loxP increases the PCR product size; see Figure 1B). Figure 1 shows representative PCR results for both genotypes of the *Alb-cre* gene and the floxed *LCMT-1* gene.

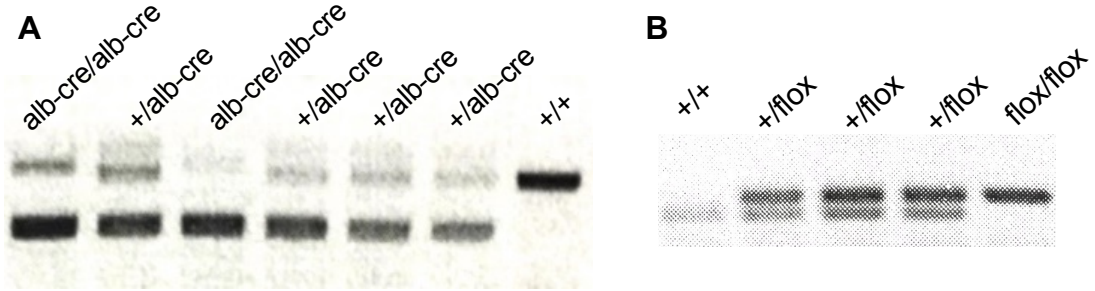

FIGURE 1. Genotyping PCR Results from DNA (A) *Alb-cre* genotypes **(B)** floxed *LCMT-1* genotypes

For the first feeding study, 5 cKO mice were placed on a high-fat diet (HF-cKO) while another 5 cKO mice were placed on a normal diet (N-cKO). Similarly, 5 control mice were on a high-fat diet (HF-C) while another 5 control mice were on a normal diet (N-C). In the second feeding study, 7 cKO mice were on a high-fat diet (HF-cKO) while another 2 cKO mice were

on a normal diet (N-cKO). 7 control mice were on a high-fat diet (HF-C) while another 3 control mice were on a normal diet (N-C).

b. Feeding Period and Diets

In both feeding studies, we use a high-fat diet produced by ENVIGO that contained 40 % fat while the normal diet contained less than 4% fat for respective experimental groups. The feeding period lasted for 17 weeks in both studies. Because high-fat diet is softer, mice on this diet are at higher risk of malocclusion (overgrowth of teeth that affects their ability to eat). During the feeding period, mice were weighed and checked for health and malocclusion, and high-fat food was replenished on a weekly basis. One control mouse on high-fat diet developed malocclusion and its teeth were clipped regularly for the remainder of the study. However, due to initial weight loss due to malocclusion, the malocclusion may have affected results so analyses on data obtained after that point did not include data from that mouse. Weights were recorded for future analysis.

c. Subsequent Analysis

At the end of the feeding period, mice were sacrificed, and their livers and blood were harvested. Blood was processed to generate serum by allowing it to clot for 30 minutes at room temperature and then centrifuging at 13,000 x g for 15 minutes and taking the supernatant (serum). If there was any red blood cell contamination, the serum was spun again for 5 minutes to further improve the serum quality, and ALT levels in blood serum were measured by our collaborator's lab (Czaja lab) along with a standard curve. Liver dimensions and weights were also measured. qPCR was performed on liver tissue to determine percent of deletion of the *LCMT-1* gene in livers. Western blots were performed on liver lysates to analyze phosphorylation levels of ribosomal protein S6, a downstream target of mTOR, and NFkB, an inflammatory transcription factor. Further planned analyses, immunohistochemistry to look for

NFkB subcellular localization and lipid content, and western blotting to look for evidence of caspase-3 cleavage (apoptosis) were prevented by the COVID-19 situation.

d. Colony Maintenance

A breeding cage and backup female and male mice were carried along during the feeding study to ensure a small colony of mice of desired *Alb-cre* genotype and floxed *LCMT-1* genotype for future study of the mouse model.

e. Data Analysis

Data were analyzed at the end of the 17-week feeding period. Weights and sizes of livers, ALT levels, and other values obtained were compared. Microsoft Excel was used to perform t-tests on data and Prism was used to make graphs. Western blot bands were quantified using a BioRad Fluor-S Max Chemiluimager and quantified using Quantity One software (BioRad).

Results

a. Conditional loss of LCMT-1 in liver results in greater liver to the body weight ratio when either on normal diet or high-fat diet

In both feeding studies, cKO mice show significantly higher liver to body weight ratio on high-fat diet (Figure 2A, 2B). In the first feeding study using homozygous Alb-cre mice, cKO mice on high-fat diet average a 15.2% liver to body weight ratio, 2-fold higher that of control mice on high-fat diet and 2.4-fold higher than cKO mice on control diet (Figure 2A). In the second feeding study using heterozygous Alb-cre mice, the liver to body weight ratio in HF-cKO mice was 24% greater than for HF-C mice and 66% greater than for N-cKO. The differences are smaller compared to that of the first feeding study, but they are still statistically significant. Interestingly, in both feeding studies, there are significant differences in liver to body weight ratio between cKO mice and control mice even when both were placed on normal diet, with a 21% and a 38% increase, respectively (Figure 2A, 2B).

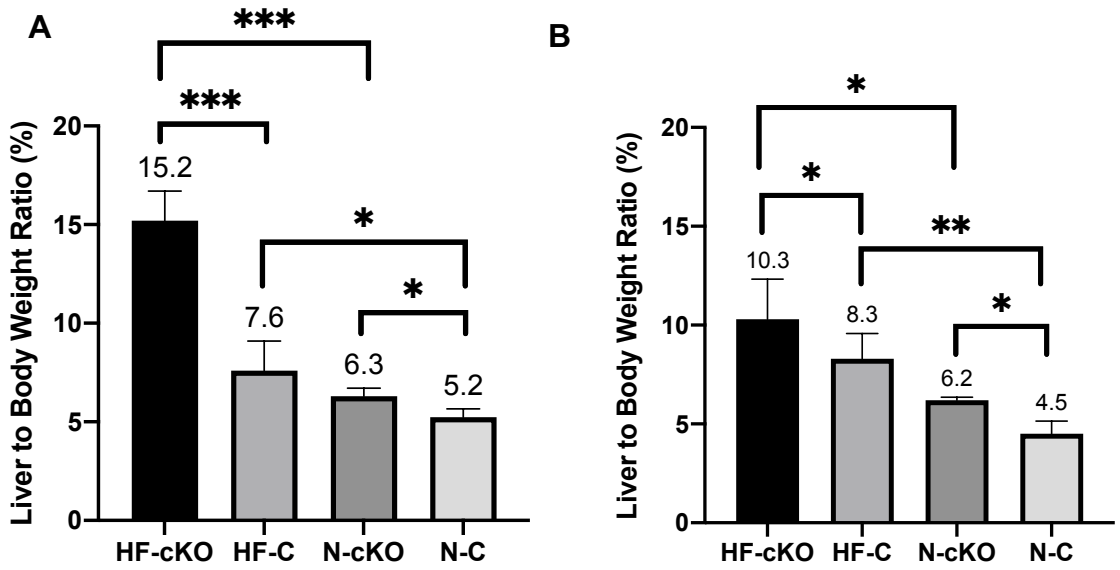

FIGURE 2. Liver to Body Weight Ratio Data in the figure were collected at the end of the feeding period. (Feed and genotype abbreviations: HF-cKO: cKO mice on high-fat diet; HF-C: control mice on high-fat diet; N-cKO: cKO mice on normal diet; N-C: control mice on normal diet.) **A)** liver to body weight ratio in respective groups in the first feeding study. **(B)** liver to body weight ratios in respective experimental groups in the second feeding study. For both studies, the liver to body weight ratios in each group were compared by two-tailed t-tests as indicated by brackets in the figure. Data are reported as average ± standard deviation as

represented by columns and error bars in the figure. Significance of p-values are indicated by * above the error bar (*$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$).

b. Conditional loss of LCMT-1 in liver results in greater systemic inflammation when on a high-fat diet

In both feeding studies, cKO mice show a higher spleen to body weight ratio when placed on a high-fat diet compared to control mice on a high-fat diet (Figure 3A, 3B). In the first feeding study, homozygous Alb-cre cKO mice on high-fat diet have a 0.35% spleen to body weight ratio on average, which is 75% higher than that in control mice on high-fat diet (Figure 3A). In the second feeding study, heterozygous Alb-cre cKO mice on high-fat diet averages a 0.40% spleen to body weight ratio, 74% higher than control mice on high-fat diet (Figure 3B). In both studies, control mice on high-fat diet have the lowest spleen to body weight ratio (Figure 3A, 3B). cKO mice and control mice on normal diet in both studies all have similar spleen to body weight ratio.

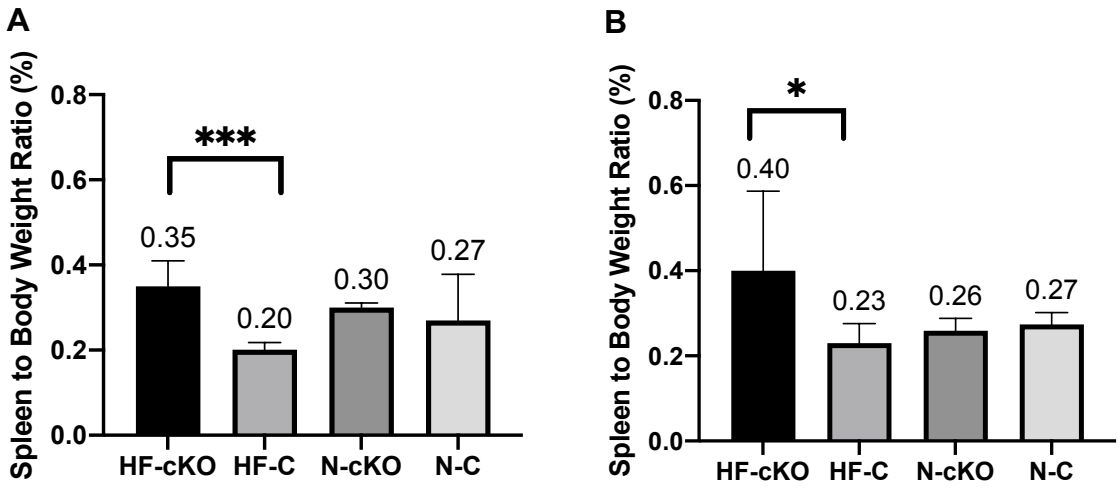

FIGURE 3. Spleen to Body Weight Ratio Data in the figure were collected at the end of the feeding period. (Feed and genotype abbreviations are the same as in Figure 1.) **(A)** spleen to body weight ratio in respective experimental groups in the first feeding study. Mice in the HF-cKO group showed significantly higher spleen to mouse ratio compared to that of the HF-C group as demonstrated by two-tailed t-test. **(B)** spleen to body weight ratio in respective experimental groups in the second feeding study. Mice in the HF-cKO group showed significantly higher liver to body weight ratio as compared to that of the HF-C group as demonstrated by two-tailed t-test. For both A and B, data are reported as average ± standard deviation as represented by columns and error bars in the figure. Significance of p-values are indicated by * above the error bar (*$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$).

c. Conditional loss of LCMT-1 in livers results in elevated ALT activity when on a high-fat diet

In the first feeding study, homozygous cKO mice have the highest average ALT activity in blood serum with 396 IU/L (Figure 4A). However, the HF-C group in the first feeding study also has elevated ALT levels, averaging to 289 IU/L (Figure 4A). The results from the second feeding study using heterozygous mice have a different pattern. In the second feeding study, although both HF-cKO and HF-C have elevated ALT activity, control mice on high-fat diet has higher average ALT levels than cKO mice on high-fat diet, unlike in the first feeding study (Figure 4B). It is worth noting that in both studies, cKO mice and control mice on normal diet all show normal ALT levels, which are significantly different from their experimental counterparts, cKO mice and control mice placed on high-fat diet (Figure 4A, 4B).

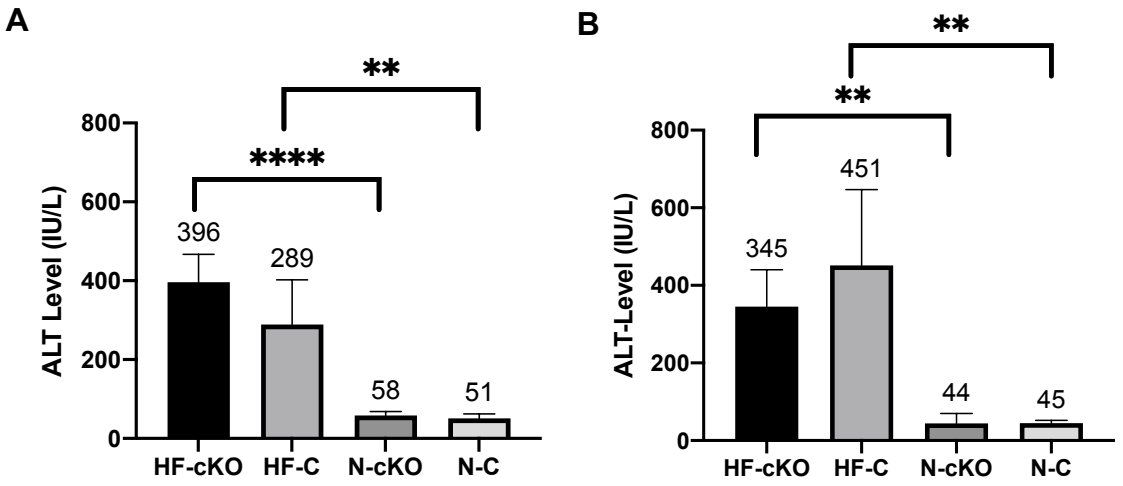

FIGURE 4. Alanine Transferase (ALT) Activity in Blood Serum This figure presents the ALT levels in serum obtained at the end of the 17-week feeding period. (Feed and genotype abbreviations are as in Figure 1.) **(A)** ALT activity of mice in the first feeding study. HF-cKO mice and N-cKO mice have significantly elevated ALT-activity. **(B)** ALT activity of mice in the second feeding study. HF-cKO mice and N-cKO mice have significantly elevated ALT-activity. For both A and B, data are reported as average ± standard deviation as represented by columns and error bars in the figure. Significance of p-values are indicated by * above the error bar (*$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$).

d. Conditional loss of LCMT-1 in liver results in faster weight gain in homozygous Alb-cre mice on high-fat diet

In the first feeding study, the homozygous Alb-cre cKO mice show a faster weight gain at the beginning of the feeding period (Figure 5A). At the 12-week mark, there is a significant difference between the average weights of HF-cKO mice and that of HF-C mice. However, the gap narrows after 12 weeks and by the end of 17 weeks, HF-cKO mice and HF-C mice have similar weights (Figure 5A). In the second feeding study, heterozygous control mice maintain a faster weight gain than all other experimental groups, unlike in the first feeding study (Figure 5B).

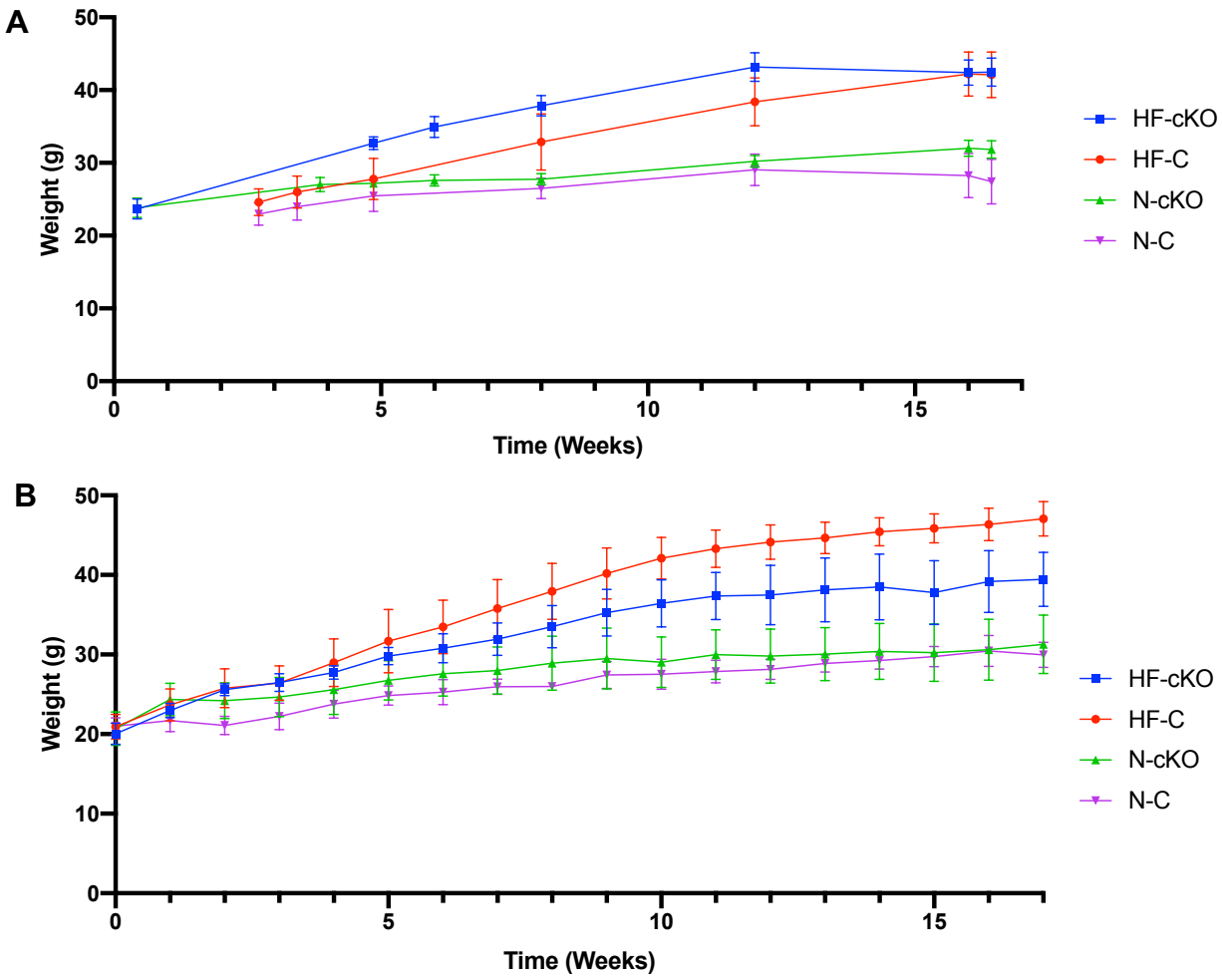

FIGURE 5. Weights Over the 17-Week Feeding Period This line plot shows weights of each experimental groups as measured throughout the feeding period. Points represent averages of all weights in each experimental group for the same time point as indicated by weeks. (Feed

and genotype abbreviations are as in the legend in Figure 1.) Blue, red, green, and purple lines indicate average weights of HF-cKO mice, HF-C mice, N-cKO mice, and N-C mice respectively. **(A)** weights over time in the first feeding study. **(B)** weights over time in the second feeding study. Data are reported as average ± standard deviation as represented by columns and error bars in the figure.

e. Conditional loss of LCMT-1 in livers results in reduced ribosomal S6 phosphorylation in homozygous Alb-cre mice when on a high-fat diet

Western blots were performed on mice liver lysates from the first feeding study, which used homozygous Alb-cre cKO and control mice. Figure 6A shows a representative western blot, in which cKO mice on high-fat diet show a significant downregulation of phosphorylated ribosomal protein S6 levels. This observation is supported by quantification as shown in Figure 6B. HF-cKO mice exhibit the lowest relative ribosomal S6 phosphorylation activity of all groups and is significantly different from that of HF-C mice. In addition, ribosomal S6 phosphorylation level of N-cKO mice is also visibly higher than that of HF-cKO (Figure 6B). Although statistical analysis does not indicate a significant difference between these two, the p-value yielded by the two-tailed t-test is 0.07 which is very close to significance.

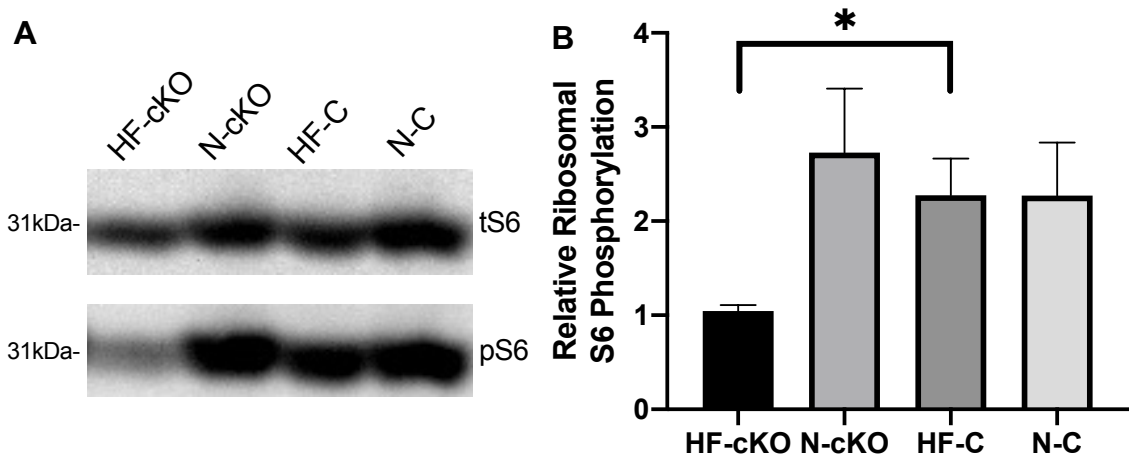

FIGURE 6. Western Blot Analysis of Ribosomal S6 Phosphorylation in Hepatic Cells This figure shows ribosomal S6 protein quantification in liver lysates of mice. **(A)** lysates from mice liver cells were probed with both total ribosomal S6 and phosphorylated ribosomal S6 antibodies. As expected, both proteins migrate at ~31kDa. **(B)** this bar graph shows relative ribosomal S6 phosphorylation levels in each experimental group as indicated by western blot quantification. Relative ribosomal S6 phosphorylation is calculated by dividing the phosphorylated ribosomal S6 by the amount of total ribosomal S6, and then normalizing to the values of HF-cKO group. The relative ribosomal S6 phosphorylation of HF-cKO mice is significantly less than that of the HF-C group. Data are reported as average ± range of two experiments as represented by columns and error bars in the figure. Significance of p-values are indicated by * above the error bar (*$p<0.05$).

f. High-fat diet results in elevated p57 NFkB phosphorylation in homozygous Alb-cre cKO mice and control mice

Figure 7 shows result of probing liver lysates for both total p65 NFkB protein and phosphorylated p65 NFkB. Figure 7A shows a representative western blot, while Figure 7B and 7C show quantification result of respectively the ~65kDa band and the ~57kDa band. Phosphorylated p65 NFkB protein migrates primarily at two different positions, ~65kDa and ~57kDa. Relative p65 NFkB phosphorylation levels are similar across all experimental groups (Figure 7B). However, relative p57 NFkB phosphorylation activity is visibly higher in HF-cKO and HF-C mice compared to that in N-cKO and N-C (Figure 7A).

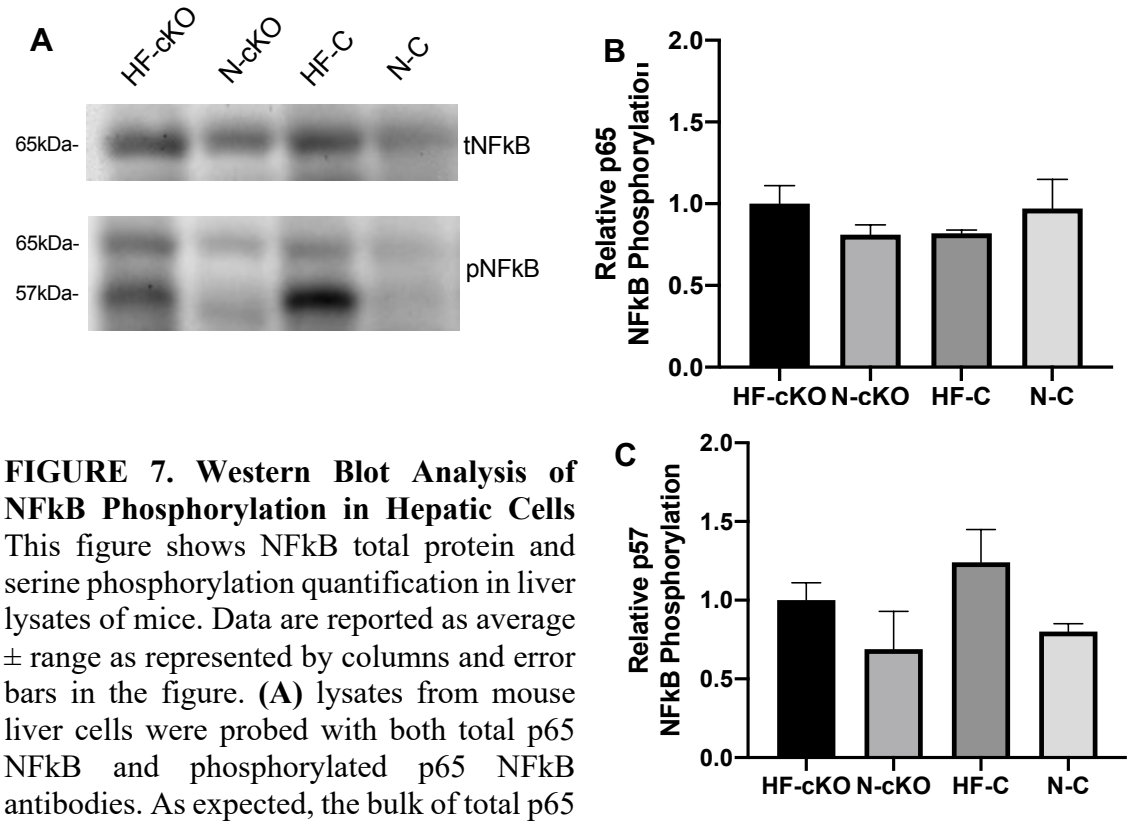

FIGURE 7. Western Blot Analysis of NFkB Phosphorylation in Hepatic Cells This figure shows NFkB total protein and serine phosphorylation quantification in liver lysates of mice. Data are reported as average ± range as represented by columns and error bars in the figure. **(A)** lysates from mouse liver cells were probed with both total p65 NFkB and phosphorylated p65 NFkB antibodies. As expected, the bulk of total p65 NFkB migrates at ~ 65kDa. However, phosphorylated p65 NFkB migrates at both ~65kDa and ~57kDa. **(B) and (C)** bar graphs show the relative NFkB phosphorylation levels in each experimental group as obtained from western blot quantification. Relative NFkB phosphorylation is calculated by dividing the quantity of phosphorylated NFkB over that of total NFkB, then normalizing to the values of HF-cKO group. B and C show relative NFkB phosphorylation levels at the p65 band and the p57 band, respectively, both normalized to the 65kDa p65 band in the total NFkB blot.

Conclusions & Future Work

In this study we have begun to probe for a role of LCMT-1 in adult liver function. By performing high-fat vs control diet feeding studies with LCMT-1 cKO and control mice, we have the opportunity to look for LCMT-1 functions that are revealed either independent of, or dependent on, a particular diet. Of greatest interest is the question as to whether methylation of PP2A family phosphatases by LCMT-1 is important for preventing liver damage in animals on a high-fat diet. Indeed, in some cases the loss of LCMT-1 does seem to show additive or more than additive effects with high-fat diet and the results suggest LCMT-1 function is important for minimizing inflammation induced by a high-fat diet.

One of the most striking results we obtained was that in both feeding studies, loss of LCMT-1 corresponds with enlargement of livers independent of the diet the mice consumed (Figure 2). Possible explanations include effects on cell division and/or lipid homeostasis. In a small preliminary test of lipid content in one adult male cKO mouse liver and one age-matched male control mouse liver, we found greatly elevated triacylglycerol levels in the cKO mouse liver (data not shown). Thus, the increased liver size may indeed reflect in part altered lipid homeostasis. Alternatively, the increase in cKO liver size might be due to altered numbers of liver cells. These possibilities will need to be researched further. This result is also relevant in that we did not expect increased cell numbers based on the fact that the Pallas lab has found that fetal livers of a LCMT-1 global knockout mouse model have 5- to 10-fold reduced hepatoblasts when compared to fetal livers of control mice (unpublished data). Drastically reduced hepatoblasts levels would be presumed to have a negative effect on development of livers and liver size, but that is not observed here.[22] Our current result of larger livers in LCMT-1 cKO mice is in strong contrast to the fetal hepatoblasts reduction. Two major explanations are possible. The first explanation is that LCMT-1 may have a crucial positive role in early hepatoblast development. Since cKO mice only start losing LCMT-1 expression after albumin

protein starts expressing (mid to late gestation), the conditional loss of LCMT-1 may have a limited effect in the early development of hepatic cells. In order to test for this hypothesis in the future, we would need to examine fetal livers of cKO and control mice, but that is beyond the scope of my thesis. The second possible explanation for the difference LCMT-1 loss has on fetal vs adult livers is that loss of LCMT-1 in tissues other than liver in the global knockout mouse model exert extrinsic effects on the development of fetal liver cells. In cKO mice, the loss of LCMT-1 is specific to albumin-expressing liver tissue so there would be no extrinsic effects like are possible in global LCMT-1 knockout mice.

A second important result seen with both feeding studies is that loss of LCMT-1 results in enlargement of spleens in cKO mice on high-fat diets, suggesting that LCMT-1 function is important to minimize inflammation due to a fatty liver (Figure 3). Specifically, there was a ~75% increase in spleen size in the cKO mice on high-fat diet vs control mice on the same diet (Figure 3). Given that LCMT-1 is not knocked out in this mouse model in splenocytes, this result likely reflects an increase in general inflammation caused by the high-fat diet in the absence of LCMT-1 in most liver cells. It will be of great interest in the future to determine if increased methylation levels of PP2A family phosphatases can reduce inflammation due to fatty liver disease.

Homozygous Alb-cre cKO mice gained weight more quickly than control mice in the beginning of the first feeding study, which might reflect a defect in lipid homeostasis in cKO liver tissue (Figure 5). The difference in weights of cKO and control mice diminished after the 12-week time-point and by the end of the 17-week feeding study, cKO and control mice on high-fat diet had similar weights (Figure 5A). The change in weight-gaining pattern after 12 weeks can be clearly seen to be due to a great reduction in weight gain by the cKO mice while the rate of weight gain by the control mice remained constant. We hypothesize that this reduction in cKO weight gain might have resulted from increased inflammation and

accumulating liver damage in the cKO mice. However, we did not observe a similar phenomenon in the second feeding study. In the second feeding studying using heterozygous Alb-cre cKO and control mice, HF-C mice gained weight faster than all other groups throughout the feeding period (Figure 5B). More study will have to be done to resolve these discrepancies, but it might represent an effect caused by more efficient LCMT-1 knockout due to homozygous Cre expression.

Analysis of ALT levels in mice revealed that for both homozygous and heterozygous Alb-cre mice, a high-fat diet caused liver damage as indicated by elevated alanine transferase activity (Figure 4). Interestingly, weight-gaining patterns coincide with the ALT activity. In the first feeding study, cKO mice on high-fat diet gained weight more quickly (Figure 5A) and they also have the higher ALT activity (Figure 4A). In the second feeding study, control mice on high-fat diet gained weight more quickly (Figure 5B) and they also have the higher ALT activity in that study (Figure 4B). This correlation further supports our postulation that the rapid weight gain is possibly due to cKO mice's inability to process lipid and contributes to liver damage.

There are two potential explanations for the discrepancies between results of the first and second feeding study (e.g., cKO liver size, weight gain differences). The first possible explanation is that a heterozygous Alb-cre gene cannot achieve an as efficient knockdown of LCMT-1 in hepatic cells as a homozygous Alb-cre gene. We have conducted qPCR analysis on livers of the cKO mice to determine the percentage deletion of LCMT-1 in liver tissue and the results indicate that there were no significant differences between percentage deletions in homozygous and heterozygous Alb-cre mice (around 60-80% deletion of LCMT-1 in hepatic cells, data not shown). However, the variability (~±10%) in results from this assay and the fact that only certain liver cells express albumin makes it difficult to rule out more efficient LCMT-1 deletion in albumin-expressing liver cells of homozygous Alb-cre mice. This is because Alb-

cre-directed-LCMT-1 knockout targets hepatocytes and certain other albumin-expressing liver cells specifically and does not knockout LCMT-1 in all liver cells. Thus, 60-80% deletion efficiency in a piece of liver tissue could be 100% knockout of LCMT-1 in 60-80% of liver cells, or 60-80% deletion in 100% of liver cells or something in between. It has been estimated that approximately 60-70% of liver cells express albumin, so our results are consistent with >95% deletion of LCMT-1 in albumin-expressing liver cells, but we cannot rule out differences between the homozygous and heterozygous Alb-cre mice.

Another possibility is that when the Alb-cre 'driver' mice were created, the albumin-cre gene may have integrated in a gene important to liver lipid homeostasis regulation, thus inactivating its function. If this were the case, in a homozygous Alb-cre mouse the gene where Alb-cre integrated might be completely inactivated whereas in the heterozygous Alb-cre mouse, one copy would still be functional. The Alb-cre gene is known to be integrated into chromosome 13 and interrupts the *Speer6-ps1*(spermatogenesis associated glutamate (E)-rich protein 6, pseudogene 1) gene.[23] While currently there is no evidence suggesting that *Speer6-ps1* plays a role in liver lipid regulation, there is also no evidence suggesting that it does not. Therefore, it remains possible that differences in results between the two feeding studies result from homozygous inactivation of the *Alb-cre* gene.

To probe for effects on signaling pathways central to cell growth and proliferation or inflammation, western blots were performed to probe for S6 ribosomal protein and NFkB protein to determine the levels of phosphorylation at sites previously demonstrated to indicate activation of the relevant growth or inflammation pathways. Since PP2A regulates the mTOR growth-promoting pathway upstream of ribosomal S6 protein, S6 phosphorylation serves as an indication of effects on the mTOR pathway.[24] Also, if diet or conditional KO of LCMT-1 affect inflammation of the liver, activation of the proinflammatory NFkB pathway serve as one key measure of inflammatory signaling.[25] Phosphorylation of ribosomal S6 is associated with

defective glucose homeostasis and insulin secretion.[26] Since PP2A negatively regulates Akt signaling pathway, loss of LCMT-1 was expected to increase mTOR activation as well as other downstream pathways (Figure 8A).[27] Unpublished data in the Pallas lab show that phosphorylation levels of ribosomal S6 is significantly increased in bone marrow and spleen cells (data not shown). However, contrary to expectation, cKO mice on high-fat diet show a significantly decreased level in ribosomal S6 phosphorylation (Figure 6). Interestingly, cKO mice on normal diet do not show a similar decrease (Figure 6). The results suggest that conditional loss of LCMT-1 and high-fat diet must work together synergistically to lead to reduced phosphorylation level of ribosomal protein S6. LCMT-1 may have an unknown role in the Akt/mTOR/S6K pathway that positively regulates it. Previous research in yeast indicates that the yeast homolog of LCMT-1, PPM-1, is involved in the regulation of a SAM-dependent sensor mechanism that positively regulates the TOR wpathway.[28] If a similar pathway operates in the mammalian systems as well, then this kind of regulation is a possible candidate for explaining our result. Moreover, B regulatory subunits of PP2A can be highly tissue-specific so that PP2A may have different functions in different cells. Different regulatory B subunits in PP2A holoenzymes enable PP2A to target different pathways and exhibit substrate specificity when modulating signaling pathways.[1] Further research on the abundance and effects of respective B subunit families in hepatic cells are necessary to achieve a more comprehensive conclusion.

Probing of NFkB also showed effects of LCMT-1 loss. NFkB is a transcription factor that is activated as a response to inflammation and is a key inflammatory mediator. NFkB consists of two subunits, the p65 subunit and the p50 subunit. Upon entering the nucleus, p65 is phosphorylated, resulting in transcription of additional inflammation mediators (Figure 8B).[29] Therefore, probing of phosphorylated p65 NFkB should give an accurate estimation of inflammatory responses within hepatic cells. Previous research has shown that PP2A

negatively regulates the NFkB pathway by terminating IKK activity.[30] In this project, we used a phosphorylated p65 NFkB antibody that detects phosphorylation at Ser^{536}, which is a phosphorylation site associated with importation of phosphorylated NFkB into nucleus.[31] However, relative phosphorylation of p65 NFkB does not differ greatly across all experimental groups (Figure 7B), but interestingly, phosphorylated p65 NFkB antibody also recognized another band that migrates at ~57kDa (Figure 7A). The unknown ~57kDa protein is upregulated in both HF-cKO mice and HF-C mice (Figure 7B). This ~57kDa protein is likely a degradation product of p65 NFkB subunit. Degradation of p65 NFkB subunit is a way to regulate the NFkB pathway and actively promotes transcription termination.[32] Therefore, our data suggests that cKO and control mice on high-fat diet have more activation of p65 NFkB in hepatic cells, possibly contributing to liver damage caused by high-fat diet, but that much of the phosphorylated p65 NFkB subunit is being degraded. It might be useful to perform an immunohistochemistry analysis on our experimental liver samples to visualize the subcellular localization of p65 NFkB as activation of NFkB should correspond to increased nuclear localization of this inflammatory transcription factor. This should help to give a clearer view on the effects of a conditional loss LCMT-1 on the regulation of NFkB pathway.

In summary, this study aimed to investigate the role LCMT-1 plays in adult liver function and liver lipid homeostasis. Our results suggest that LCMT-1 function in liver cells is important to prevent inflammation and liver damage, and therefore may be relevant to the development of NAFLD. Recently, two classes of inhibitors of the PME-1 methylesterase enzyme have been reported, and it will be very interesting to determine if treatment of mice on a high-fat diet with these inhibitors might have a therapeutic benefit.[33]

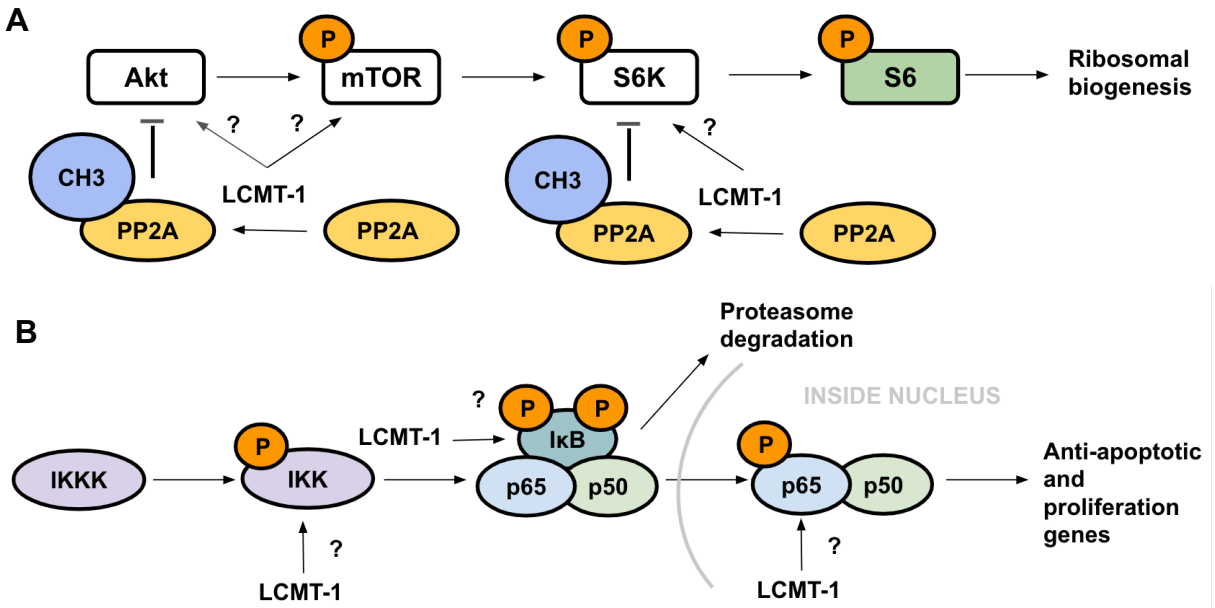

FIGURE 7. Simplified Diagrams of mTOR and NFkB Pathway Both diagrams show potential roles LCMT-1 may play in the regulation of both pathways. **(A)** ribosomal S6 protein is a downstream target of the mTOR pathway.[34] LCMT-1 is possibly involved in regulation of Akt, mTOR, and S6K signaling. **(B)** NFkB is phosphorylated inside the nucleus and subsequently becomes activated to start transcription of anti-apoptotic and proliferation genes.[30] LCMT-1 may be directly or indirectly involved in the regulation of phosphorylation of the p65 subunit of NFkB.

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