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Short Communication

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A Comparison Between Canonical and Alternative Pathways in Triglyceride Synthesis

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Abstract

Background: Mammals have evolved the ability to store and transport energy as triglycerides (TGs). TG synthesis occurs primarily in the liver during fasting and in white adipose tissue (WAT) following feeding. In humans, the last step of the canonical pathway in TG synthesis is carried out by diacylglycerol O-acyltransferase 1 (DGAT1) and DGAT2, which catalyze the covalent addition of a fatty acyl chain to diacylglycerol in both the liver and WAT. Recently, an alternative TG synthesis pathway was discovered: transmembrane protein 68 (TMEM68) carries out cell-autonomous TG synthesis, with thioredoxin-related transmembrane protein 1 (TMX1) being its inhibitor.

Methods: Because TG synthesis in the body is tightly and accurately regulated, we compared and contrasted the expressions of genes involved in the two pathways during the fed-fast cycle in the liver and WAT of mice, in mice treated with a high-fat diet (HFD), and in young vs. old mice.

Results: We found that in the liver, fasting upregulated *Dgat1*, *Dgat2*, and *Tmx1*, while in WAT it reduced *Dgat2* but increased *Tmx1*. Moreover, HFD and aging suppressed the expression of *Dgat1* and *Dgat2* in WAT. Male *Tmem68* knockout mice generated by the International Mouse Phenotypic Consortium exhibited elevated plasma aspartate aminotransferase (AST) levels and reduced glucose levels, suggesting potential liver and glucose homeostasis abnormalities.

Conclusions: Our results are consistent with the notion that during fasting, the liver relies more on the canonical pathway and that in WAT, both pathways are upregulated following feeding.

Keywords: Dgat1; Dgat2; Tmem68; Tmx1; Triglyceride

Introduction

Because energy sources are not always available from the environment, mammals have evolved the ability to store and transport energy as triglycerides (TGs) [1-3]. TG are ideal for energy storage because they serve as stores of highly reduced carbon, high energy per unit mass, and do not require water for their storage, as opposed to glycogen, which is stored in hydrated form [4, 5].

Chemically, TG are neutral lipids composed of a glycerol backbone conjugated to three fatty acyl chains, hence the name "triglyceride". TG synthesis occurs in the endoplasmic reticulum (ER) membrane of cells by bonding three fatty acid molecules to a glycerol molecule. In humans, many types of cells have the capacity of TG synthesis, but the liver and white adipose tissue (WAT) are especially critical [6]. In the liver, TG synthesis primarily occurs in the fasting state, while in WAT, it happens predominantly in the fed state [1, 7].

In the liver, during fasting, TG are synthesized from fatty acids, which are from two sources: either the liver, which converts glucose into fatty acids, or WAT, which breaks down stored TG into fatty acids, which are then secreted into the circulation and, in turn, taken up by the liver. The newly synthesized TGs are then packaged by the liver into TG-rich lipoproteins called very low-density lipoprotein (VLDL). VLDL particles are secreted directly into blood, where TGs are delivered to peripheral tissues, such as the heart and muscle to generate energy during fasting through the process of β-oxidation [1].

In WAT, after food intake, dietary fats in the intestine are broken down into fatty acids, which are packaged into chylomicrons (CM). CM, high in TG, are then secreted into the bloodstream to meet lipoprotein lipase (LPL) in the capillaries of adipose tissues. LPL hydrolyzes TG into fatty acids, which are taken up by adipocytes to synthesize into TG for long-term energy storage. In adipocytes, TGs are stored in organelles called lipid droplets (LDs) [8, 9].

The last step of TG synthesis in humans is carried out by diacylglycerol O-acyltransferase 1 (DGAT1) and diacylglycerol O-acyltransferase 2 (DGAT2), which catalyze the covalent addition of a fatty acyl chain to diacylglycerol (DAG). DGAT1 and DGAT2 catalyze the same chemical reaction, but they are genetically unrelated. DGAT1 is part of the membrane bound O-acyl transferase (MBOAT) protein family, and DGAT2 is a member of the diacyl-glycerol acyltransferase (DAGAT) family [10-13]. Mouse gain- and loss-of-function studies showed that the two proteins have important, non-redundant roles in both the liver and WAT [13-20].

The canonical TG synthesis pathway from fatty acyl-CoA and DAGTs has been clearly defined. Recently a new alternative TG synthesis pathway has been discovered [21, 22], and

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it is reported that transmembrane protein 68 (TMEM68, also named DIESL) and thioredoxin-related transmembrane protein 1 (TMX1), within the ER, carry out cell-autonomous alternative TG synthesis. TMEM68 functions as a 1,2-diacylglycerol O-acyltransferase, and TMX1 potently inhibits TMEM68. It is proposed that TMEM68 converts endogenous acyl chains into TG, a process that requires regulations of TMX1, and that TMX1-TMEM68 may enable the supply of fatty acids to mitochondria during times of lipid or nutrient scarcity [21].

TG synthesis in the body is tightly and accurately regulated during the fed-fast cycle. We then asked the following questions: 1) During the fed-fast cycle, how are the two pathways regulated? 2) Does the body use the two pathways similarly or differently in tissues including the liver and WAT? To answer the questions, we examined mRNA levels of these genes in the liver and fat during the fed-fast cycle, and in other conditions, including aging and high-fat diet (HFD) treatment.

Materials and Methods

Mice

Mice were housed at 22 - 24 °C with a 14-h light, 10-h dark cycle and provided with *ad libitum* water and a chow diet (6% calories from fat, 8664; Harlan Teklad, Indianapolis, IN) unless otherwise indicated. Eight-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were treated with 20-h fasting with fed mice as controls. Young (3-month-old) and old (20-month-old) C57BL/6J mice were purchased from the Jackson Laboratory. To examine nutritional stimulation, 10 4-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were placed on either a chow diet or a high-fat, high-sucrose diet (58% kcal from fat, 26% kcal from sucrose, D-12331; Research Diets, New Brunswick, NJ) for 3 months. At International Mouse Phenotyping Consortium (IMPC), the mouse strain is C57BL/6NTac. All animal protocols were approved by the Animal Care and Use Committee of Wayne State University.

RNA extraction, quantitative real-time polymerase chain reaction (PCR)

Dissected tissues were immediately placed into RNAlater solution (Ambion, Austin, TX) for subsequent RNA extraction. Total RNA was isolated from tissues with RNeasy Tissue Mini Kit with deoxyribonuclease treatment (Qiagen, Valencia, CA). One microgram of RNA was reverse transcribed to cDNA using random hexamers (Superscript; Ambion). Relative expression levels were calculated, and β-actin was used as an internal control. Primer sequences for mouse *Dgat1* were forward: 5′-TCCGTCCAGGGTGGTAGTG-3′; reverse: 5′-TGAACAAAGAATCTTGCAGACGA-3′. Primer sequences for mouse *Dgat2* were forward: 5′-GCGCTACTTCCGAGACTACTT-3′; reverse: 5′-GGGCCTTATGCCAGGAAACT-3′. Primer sequences for mouse *Tmem68* were forward: TGTGGGATGGTGCAAGGAAAA-3′; reverse: 5′-CAGCTACTACTCGGCAAGTTC-3′. Primer sequences for mouse *Tmx1* were forward: 5′-CACTTGGGGCGTCTTATGGTT-3′; reverse: 5′-CCAGTTCTCATCGGTGAGGAC-3′. Primer sequences for mouse β-actin were forward: 5′-GTGACGTTGACATCCGTAAAGA-3′; reverse: 5′-GCCGGACTCATCGTACTCC-3. All methods were carried out in accordance with relevant guidelines and regulations.

Statistical analysis

Data are expressed as the mean ± SEM (standard error of the mean). Statistical significance was tested with unpaired two-tailed Student's *t*-test unless otherwise indicated. One-way analysis of variance (ANOVA) was used to compare the means of three groups, e.g., hepatic expression levels of the genes in fed, fasting, re-fed mice. Prism Graphpad was used to draw figures. The differences were considered statistically significant if $P < 0.05$.

Results

In the liver, fasting upregulates the canonical-pathway genes

Because TG synthesis is tightly and accurately orchestrated by the fed-fasting cycle, we hypothesized that expression of the TG synthesis genes is altered by treatments of fasting and refeeding. The liver is a central organ that is involved in TG synthesis, and thus we first checked the expression of these genes in the liver. The mice were treated with *ad libitum* feeding, 20-h fasting, and 4-h refeeding following the fasting. qPCR analysis was then performed to examine mRNA expressions. Fasting increased liver *Dgat1* expression at least two-fold, and 4 h after refeeding the expression returned to the basal level (Fig. 1), consistent with previous results [14]. In other words, expression of liver *Dgat1* was significantly reduced 4 h after refeeding, compared to fasted mice. *Dgat2* showed a similar pattern to that of *Dgat1*, that is, fasting upregulated its expression, and re-feeding significantly reduced its expression. These results suggested that both *Dgat1* and *Dgat2* were upregulated during fasting in the liver, while in a fed state, their expressions were reduced.

Both fasting and refeeding did not significantly alter the expression of *Tmem68*. However, fasting significantly increased the expression of *Tmx1*, and 4 h following the refeeding, its expression returned to the basal level. Thus, the hepatic expression of *Tmx1* was increased by fasting, but repressed by feeding (Fig. 1). Because Tmx1 is a potent inhibitor of Tmem68, the enzyme for TG synthesis, these results are consistent with a notion that fasting suppressed the alternative TG synthesis pathway but upregulated the canonical pathway.

In WAT, fasting suppresses *Dgat2* but upregulates *Tmx1*

WAT is a critical organ for postprandial TG synthesis. Following a meal, TG synthesis is increased in WAT, while fasting

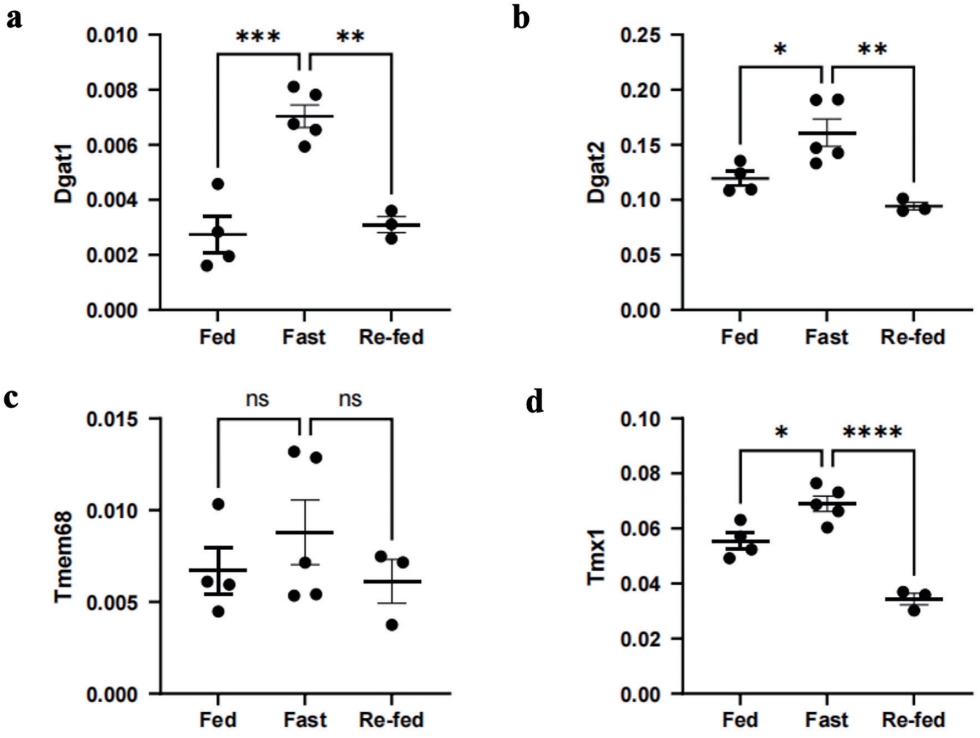

Figure 1. Liver expression of *Dgat1*, *Dgat2*, *Tmem68*, and *Tmx1*. Hepatic mRNA levels of (a) *Dgat1*, (b) *Dgat2*, (c) *Tmem68*, and (d) *Tmx1* in mice with ad libitum feeding, 20-h fasting, and 4-h refeeding following the fasting (n = 4, 5, 3, for the fed, fasting, and re-fed groups, respectively). Data are represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns: non-significant; SEM: standard error of the mean.

induces lipolysis leading to release of fatty acids into the circulation. We, therefore, examined the expression of TG synthesis pathways in WAT.

Fasting dramatically suppressed the expression of *Dgat2* (more than 80%), while feeding dramatically increased its expression (Fig. 2). This is consistent with the role of *Dgat2* in WAT. Both fasting and re-feeding did not alter the expression of *Dgat1*, consistently with the notion that *Dgat2*, but not *Dgat1*, is physiologically relevant in WAT.

Both fasting and refeeding did not significantly alter the expression of *Tmem68*. However, fasting significantly increased the expression of *Tmx1* (Fig. 2). Thus, this result is consistent with a notion that fasting downregulates, and feeding upregulates the alternative TG synthesis pathway. In other words, it is likely that in WAT, both canonical and alternative pathways are induced by feeding and suppressed by fasting, consistently with their physiological roles.

A high-fat diet downregulates the canonical-pathway genes in WAT

Excess TG synthesis contributes to obesity and metabolic disease. To study this, we subjected mice to HFD for 3 months and analyzed gene expression in both the liver and WAT. In the liver, HFD treatment did not significantly alter the expression of any of these genes (data not shown). In WAT, HFD dramatically reduced the expression of *Dgat1* (85% reduced) and *Dgat2* (60%) (Fig. 3). The expression levels of *Tmem68* and *Tmx1* in WAT remained unaffected by the HFD.

The canonical-pathway genes are downregulated by aging

Aging and TG metabolism are closely related [23], and therefore we examined the expression of these genes in young vs. aged mice. It is commonly believed that 3-month-old mice are considered young, and 20-month is considered old mice [24]. Thus, we compared the expression of these genes in 3-month vs. 20-month-old mice. In the liver, there were no significant differences between young and old mice for all these genes (data not shown). In WAT, aging significantly reduced the expression of *Dgat1* and *Dgat2*, while expression of *Tmem68* and *Tmx1* was not changed (Fig. 4).

Tmem68 knockout (KO) mice exhibit liver and glucose phenotypes

Gene deletion has become a powerful way to examine gene functions. Brummelkamp's group reported that *Tmem68* KO mice exhibited increased respiratory exchange ratio in response to fasting, indicating an attenuated switch from carbohydrate to lipid oxidation [21]. The International Mouse Phenotypic Consortium (IMPC) produces whole body KO mouse lines for community use and delivers broad comprehensive

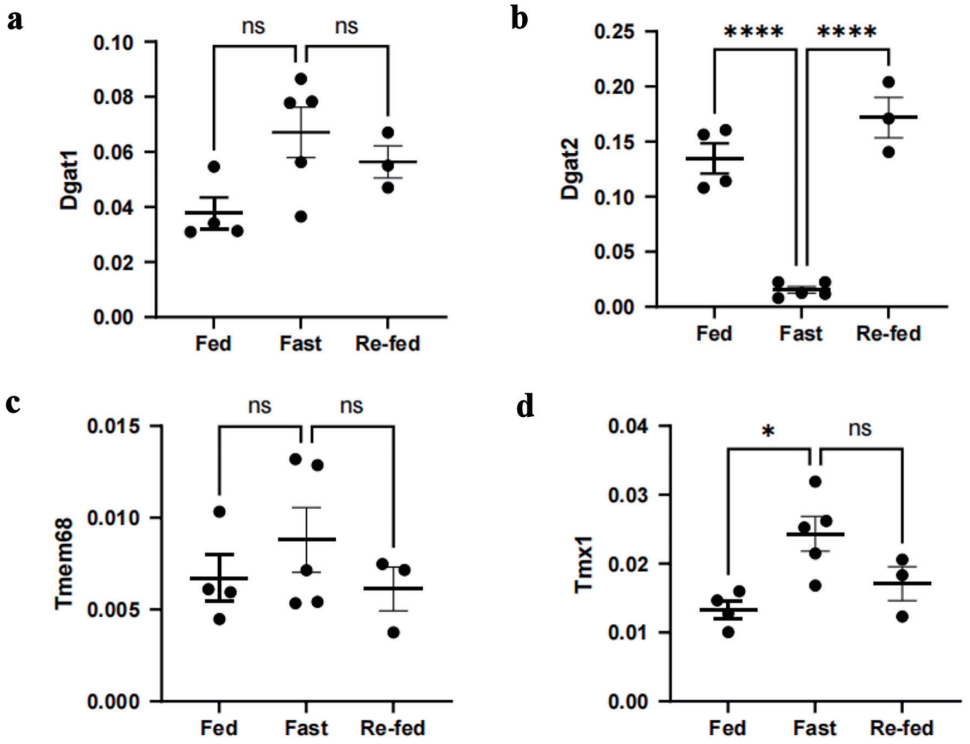

Figure 2. Expression of *Dgat1*, *Dgat2*, *Tmem68*, and *Tmx1* in white adipose tissue (WAT). WAT mRNA levels of (a) *Dgat1*, (b) *Dgat2*, (c) *Tmem68*, and (d) *Tmx1* in mice with *ad libitum* feeding, 20-h fasting, and 4-h refeeding following the fasting (n = 4, 5, 3, for the fed, fasting, and re-fed groups, respectively). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as mean ± SEM. ns: non-significant; SEM: standard error of the mean.

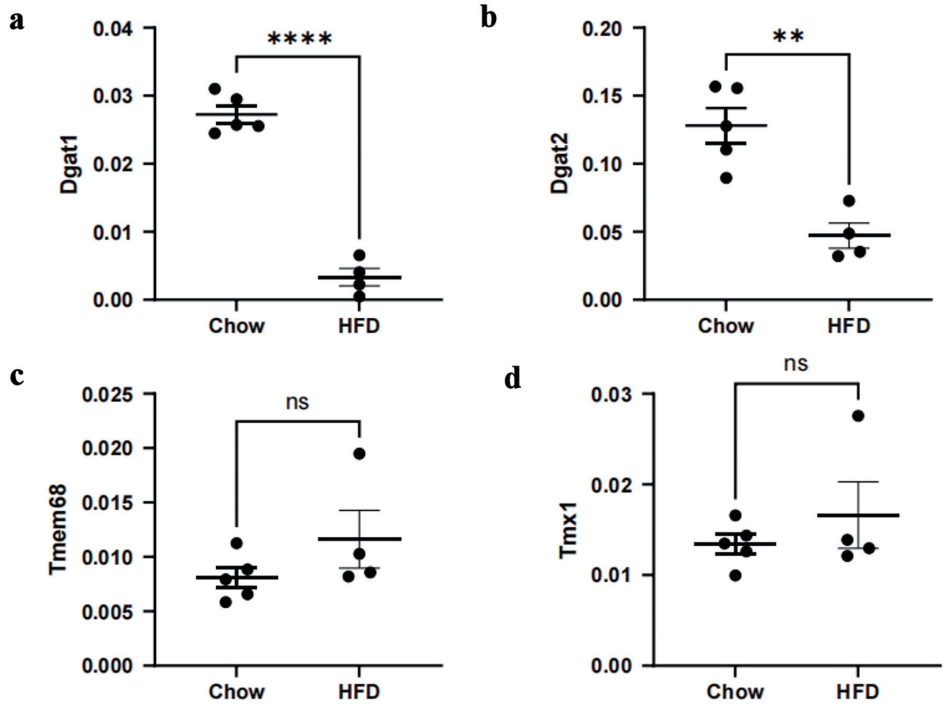

Figure 3. Expression of *Dgat1*, *Dgat2*, *Tmem68*, and *Tmx1* in white adipose tissue (WAT). WAT mRNA levels of (a) *Dgat1*, (b) *Dgat2*, (c) *Tmem68*, and (d) *Tmx1* in mice on chow and 3-month HFD (n = 5 per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as mean ± SEM. ns: non-significant; SEM: standard error of the mean; HFD: high-fat diet.

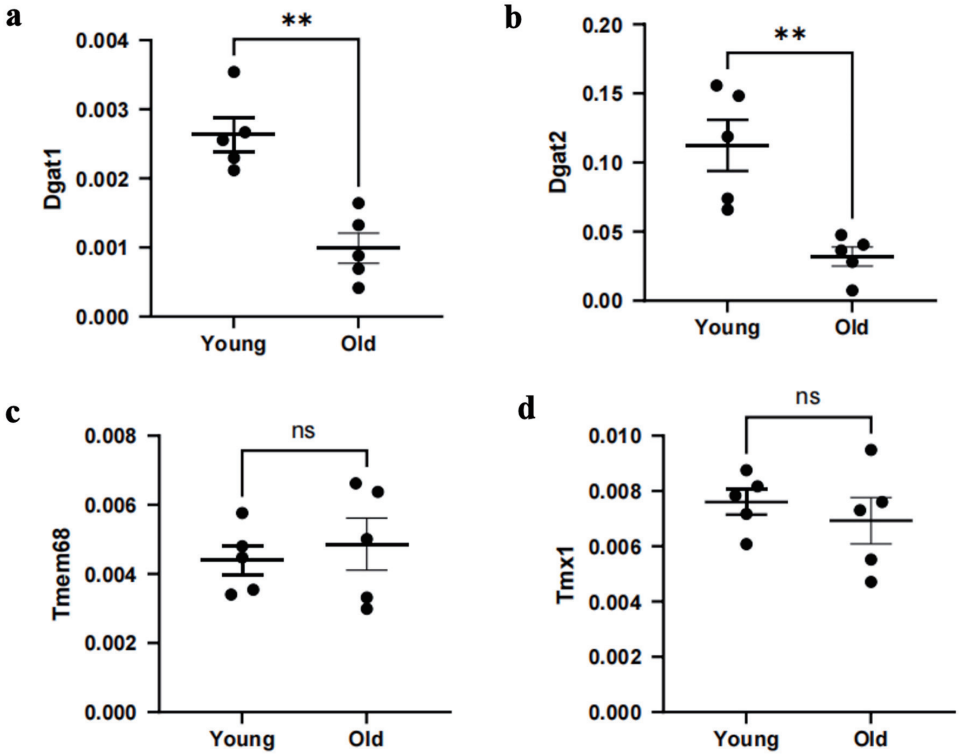

Figure 4. Expression of *Dgat1*, *Dgat2*, *Tmem68*, and *Tmx1* in white adipose tissue (WAT). WAT mRNA levels of (a) *Dgat1*, (b) *Dgat2*, (c) *Tmem68*, and (d) *Tmx1* in young and old mice. Young and old mice that are 3 and 20 months old, respectively (n = 5 per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are represented as mean ± SEM. ns: non-significant; SEM: standard error of the mean.

phenotyping data and the KO lines as an international resource enabling functional analyses with genome-wide coverage [25, 26]. *Tmem68* KO mice have been generated and phenotyped by IMPC (Tmem68$^{tm1a(EUCOMM)Wtsi}$). In male *Tmem68* KO mice generated by IMPC, plasma aspartate aminotransferase (AST) levels were significantly increased (Fig. 5), suggesting liver phenotypes. Plasma glucose levels were reduced (Fig. 5), suggesting that Tmem68 is involved in glucose homeostasis. Therefore, *Tmem68* KO mice generated by IMPC independently show consistent metabolism phenotypes.

Discussion

TG metabolism has profound implications for human health and disease [27, 28]. As a standard component of the vascu-

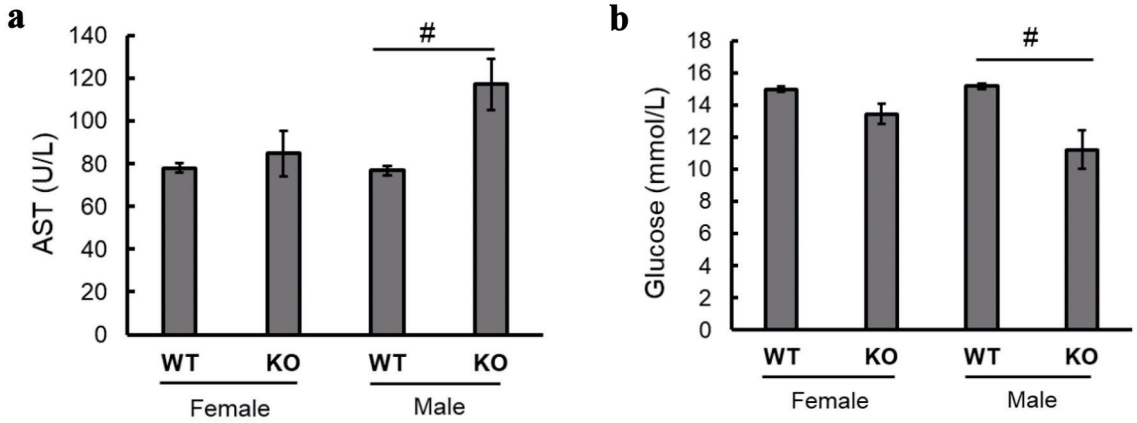

Figure 5. Plasma AST and glucose levels in *Tmem68* KO mice. (a) plasma AST and (b) glucose levels in WT and *Tmem68* KO mice. Data were obtained from the International Mouse Phenotyping Consortium (IMPC). #P < 0.01. Data are represented as mean ± SEM. SEM: standard error of the mean; AST: aspartate aminotransferase; WT: wild-type; KO: knockout.

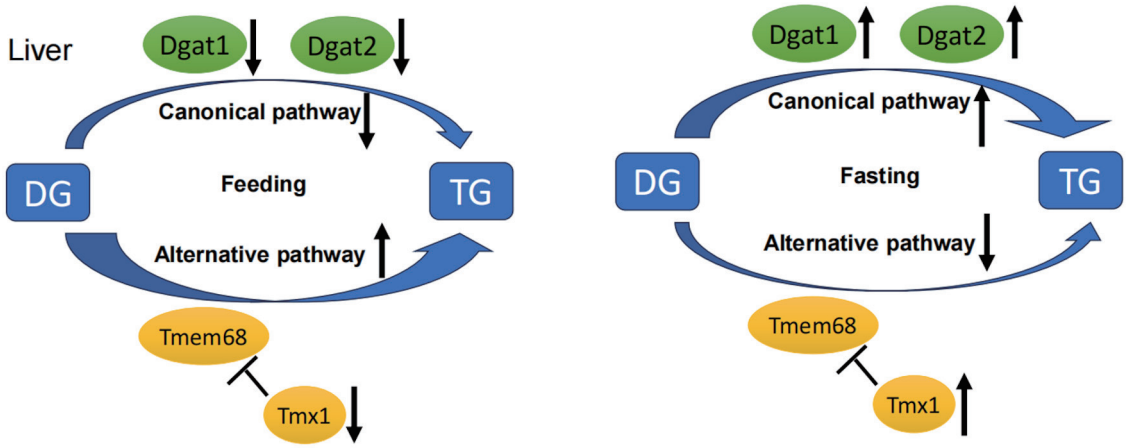

Figure 6. A model of how canonical and alternative pathways work during the fed-fasting cycle in the liver. DG: diacylglycerol; TG: triglyceride.

lar system, TGs, in the form of lipoproteins, are continuously being circulated in the bloodstream to be metabolized to provide a source of energy for oxidative tissues or saved in WAT, according to the body's needs [1, 3]. High TG levels in the blood (hypertriglyceridemia) are associated with a collection of disorders known as metabolic syndrome, increasing the risk of developing diabetes, stroke, or heart disease [29-31]. Therefore, TG metabolism, i.e., its synthesis (lipogenesis) and breaking down (lipolysis) is accurately and carefully orchestrated during the fed-fast cycle.

In the current study, we examined how the two pathways, the canonical and alternatively TG synthesis pathways, are regulated, at the mRNA levels, throughout the fed-fast cycle. We found *Tmem68* mRNA levels are relatively stable, without statistically significant changes by fed and fasting treatments. In contrast, Tmx1, the Tmem68 inhibitor, is induced by fasting in both the liver and WAT. Thus, it is likely that the alternative pathway is regulated mainly by altering the level of Tmx1.

Additionally, in the liver, during fasting, the canonical pathway is upregulated, while the alternative pathway is suppressed. These results support the notion that during fasting, the liver relies more on the canonical pathway to generate TG (Fig. 6). In contrast, in WAT, fasting dramatically reduces the expression of *Dgat2*, while feeding increases it, which is consistent with its role in TG synthesis in WAT. Additionally, fasting modestly increases the expression of *Tmx1*. These results are consistent with a notion that both pathways are needed for TG synthesis in WAT (Fig. 7).

Moreover, we found that HFD treatment does not significantly change levels of *Tmem68* and *Tmx1* in the liver. In contrast, in WAT, HFD dramatically suppressed the levels of *Dgat1* and *Dgat2*. This seems to suggest that the body downregulates the two TG synthetases as a compensatory mechanism in response to obesity. Interestingly, aging also downregulates the expression of *Dgat1* and *Dgat2* in WAT, but the implications of these aging-associated changes are not clear.

The current study has the following limitations. One limitation is that we examined the mRNA levels of these genes, but not the protein levels, which may have varied half-lives.

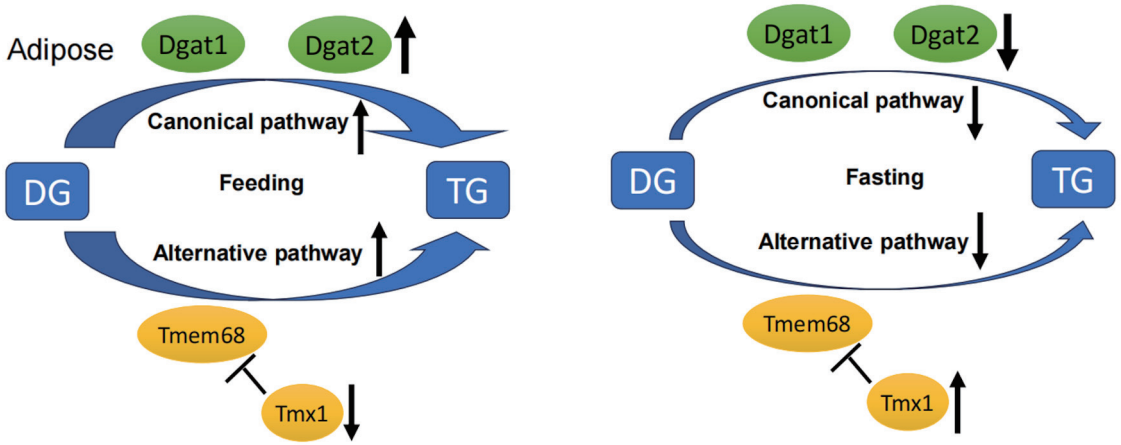

Figure 7. A model of how canonical and alternative pathways work during the fed-fasting cycle in white adipose tissue. DG: diacylglycerol; TG: triglyceride.

Therefore, in some cases, the mRNA levels do not reflect the relative or absolute protein abundance. Another limitation is that for enzymes, their activity is more important than their mRNA expression levels and protein abundance. Thus, an ideal readout would be enzyme activity, such as for Tmem68. However, comparing the mRNA levels of genes involved in the two pathways can be a starting point to address these questions. To the best of our knowledge, this study is the first attempt to compare and contrast these two pathways. Future studies ideally would need to examine the abundance of the proteins in the two pathways during the fed-fast cycle, enzymatic activities of the enzymes, and metabolite flux analysis in the relevant KO mice.

The *Tmem68* KO mice showed phenotypes of changes in fasting glucose and AST levels, but not in TG levels and adiposity. Because the regulation of TG synthesis is sensitive to nutrition states, it is possible that the phenotypes such as TG levels, fatty acid levels, and body composition, can only be revealed when the KO mice are challenged with fasting, refeeding, and HFD. The opposite to TG synthesis is fatty acid oxidation, which primarily happens during fasting. Fasting activates a series of mechanisms to increase fatty acid oxidation and ATP production. For example, in mitochondria, these mechanisms include increased expression of the mitochondrial NAD transporter [32], activation of sirtuin 3 [33], and the activation of mitochondrial NAD kinase [34-36]. Thus, another question to address is how the expression of TG synthetase genes and that of fatty acid oxidation genes are coordinated under various physiological and pathological conditions.

In summary, the study explores TG metabolism, with a focus on synthesis pathways in the liver and WAT, comparing the canonical and the newly discovered alternative pathways. The study investigates gene expression changes during the fed-fast cycle, fasting, refeeding, HFD treatments, and aging. Our results are consistent with a notion that fasting upregulates the canonical pathway in the liver, while in WAT, feeding upregulates both pathways, and that HFD and aging downregulate the canonical pathway in WAT. *Tmem68* KO mice exhibit liver and glucose homeostasis phenotypes. We showed that the regulation at the mRNA level of the newly identified alternative TG synthesis pathway is likely through Tmx1, but not Tmem68, during the fed-fast cycle. Our results are consistent with the notion that during fasting, the liver relies more on the canonical pathway than the alternative pathway. In WAT, there is a possibility that both pathways are upregulated and are needed following feeding.

Acknowledgments

None to declare.

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Conflict of Interest

The authors declare no conflict of interest.

Informed Content

Not applicable.

Author Contributions

RZ conceived the study, analyzed the data, and wrote the manuscript. ZF and QC performed the experiments. KZ analyzed the data and revised the manuscript.

Data Availability

All data supporting the findings of this study are available within the article.

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