ORIGINAL RESEARCH

CURRENT DEVELOPMENTS IN NUTRITION

Nutritional Biochemistry and Physiologic Mechanisms

TRB3 Deletion Has a Limited Effect on Milk Fat Synthesis and Milk Fat Depression in C57BL/6N Mice

Rachel E Walker,[1] Liying Ma,[2] Chengmin Li,[2] Yun Ying,[2] and Kevin J Harvatine[2]

[1]Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA, USA and [2]Department of Animal Sciences, The Pennsylvania State University, University Park, PA, USA

ABSTRACT

Background: Regulation of the endoplasmic reticulum (ER) stress pathway is critical to mammary epithelial cell function throughout pregnancy, lactation, and involution. Treatment with *trans*-10, *cis*-12 conjugated linoleic acid (t10c12CLA) suppresses mammary lipogenesis and stimulates the ER stress pathway. The ER stress pathway includes tribbles pseudokinase 3 (TRB3), a protein that regulates cellular energy and insulin signaling.

Objectives: Our objective was to describe the effect of TRB3 deficiency on milk fat synthesis and determine if TRB3 deficiency protects against suppression of mammary lipogenesis.

Methods: First, mammary *Trb3* expression was observed throughout pregnancy and lactation using ancillary microarray data ($n = 4$/time point). Second, intake, litter growth, and milk clot fatty acid (FA) profile of *Trb3* knockout (KO) C57BL/6N mice were compared with wild-type (WT) and heterozygous (HET) mice throughout first ($n \geq 8$/group) and second ($n \geq 6$/group) lactation. Lastly, the interaction between *Trb3* genotype and 2 treatments that suppress mammary lipogenesis, t10c12CLA and high safflower oil (HO) diet, was investigated in a 2 × 2 factorial design ($n \geq 6$/group).

Results: *Trb3* expression was higher during late pregnancy and lactation. *Trb3* KO and HET mice had lower feed intake, dam weight, and litter growth throughout first, but not second, lactation than WT mice. Treatment with t10c12CLA decreased litter growth (28%; $P < 0.0001$) and feed intake (8%; $P < 0.0001$) regardless of *Trb3* genotype. When fed the HO diet, *Trb3* KO mice had 17% higher mammary de novo synthesized FAs (<16 carbons; $P_{int} = 0.002$) than WT mice. Mammary ER stress and lipogenic genes were mostly unaltered by *Trb3* deficiency.

Conclusions: Overall, TRB3 plays a minor role in regulating mammary lipogenesis, because *Trb3* deficiency had only a limited protective effect against diet-induced suppression of lipogenesis. *Curr Dev Nutr* 2022;6:nzab142.

Keywords: tribbles pseudokinase 3, *trans*-10, *cis*-12 conjugated linoleic acid, milk fat synthesis, endoplasmic reticulum stress, milk fat depression

Manuscript received September 8, 2021. Initial review completed November 4, 2021. Revision accepted November 16, 2021. Published online January 27, 2022.
Supported by USDA National Institute of Food and Agriculture Federal Appropriations, project number PEN04664 (to KJH) and accession number 1017181. The funder had no role in study design, implementation, analysis, or interpretation.
Author disclosures: The authors report no conflicts of interest.
Supplemental Table 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/cdn/.
Address correspondence to KJH (e-mail: kjh182@psu.edu).

Abbreviations used: ATF, activating transcription factor; c/EBPβ, CCAAT enhancer binding protein β; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FA, fatty acid; FASN, fatty acid synthase; HET, heterozygous; HO, high safflower oil; HMBS, hydroxymethylbilane synthase; HSPA5, heat shock protein A5; IRE1, inositol requiring enzyme 1; KO, knockout; LFD, semipurified low-fat diet; MCFA, mid-chain fatty acid; MFD, milk fat depression; PERK, PKR-like eukaryotic initiation factor 2α kinase; SCD1, stearoyl CoA desaturase 1; Spot14, thyroid hormone responsive protein; SREBP1c, sterol regulatory-element binding protein 1; TRB3, tribbles pseudokinase 3; t10c12CLA, *trans*-10, *cis*-12 conjugated linoleic acid; UPR, unfolded protein response; WT, wild-type; XBP1s, X-box binding protein-1.

Introduction

During the course of pregnancy, lactation, and involution the mammary gland goes through extensive cell proliferation, differentiation, remodeling, and turnover. Throughout lactation, the mammary gland also performs high rates of protein, carbohydrate, and lipid synthesis and export, processes that largely take place within the endoplasmic reticulum (ER). ER stress, triggered by the unfolded protein response (UPR), stimulates apoptosis, or programmed cell death, and maintains protein integrity (1). Considering the role of the ER in nutrient synthesis and packaging, the UPR and ER stress may be critical in the regulation of milk synthesis.

PKR-like eukaryotic initiation factor 2α kinase (PERK) is stimulated by the ER stress response and induces synthesis of tribbles pseudokinase 3 (TRB3) through activating transcription factor (ATF)-4 to mediate apoptosis (2). TRB3 also directly inhibits the insulin signaling pathway (3), resulting in increased release of glucose from the liver (4), decreased glucose oxidation, and increased body weight (5, 6). Increased expression of TRB3 has been identified as part of the pancreatic adaptation during the transition to lactation (7). In an initial investigation we ob-

served that TRB3 was specifically increased in mammary tissue of mice at the start of lactation in publicly available microarray data, whereas other ER stress signals were increased during involution, suggesting a potential role in lactation physiology (see Results).

The ER stress signals also activate the sterol regulatory-element binding protein 1 (SREBP1) transcription factor, which activates expression of many lipogenic genes, including stearoyl CoA desaturase 1 (SCD1) and fatty acid synthase (FASN) (8). Because TRB3 is active in regulating insulin signaling and energy utilization, it may also contribute to regulation of mammary lipogenesis. PERK knockout (KO) mice have decreased milk fat content compared with wild-type (WT) mice, mediated by decreases in SREBP1, FASN, and SCD1 (9). It is reasonable to speculate that the effects of PERK on lipogenesis may be mediated by TRB3. In addition, TRB3 is directly involved in the down-regulation of acetyl coenzyme carboxylase 1 (ACC1) through ubiquitination (6), a critical rate-limiting enzyme in fatty acid (FA) synthesis. Therefore, TRB3 is positioned to be a key link connecting ER stress to mammary lipogenesis.

In dairy cattle, milk fat depression (MFD) is a marked decrease in milk fat caused by bioactive intermediates of unsaturated FA biohydrogenation produced by rumen microbes (10). Although multiple bioactive FAs exist, *trans*-10, *cis*-12 conjugated linoleic acid (t10c12CLA) is the most well studied in multiple species and appears to be linked to suppression of lipogenic genes in the mammary gland (10–13). In addition, t10c12CLA stimulates expression of ER stress pathway genes in mice (14, 15), ovarian cancer cells (16), and mammary epithelial cells (17, 18), and stimulation of the PERK/TRB3 branch of the ER stress pathway results in decreased lipogenesis (19). We have also observed elevated TRB3 expression in dairy cattle with diet-induced MFD (data not published). It is unknown if TRB3 activation is functionally involved in the mammary response to t10c12CLA during lactation. Therefore, we hypothesize that TRB3 may be an important regulator of milk synthesis and link ER stress to inhibition of lipogenesis during t10c12CLA-induced MFD.

Objectives

This study had 3 main objectives. The first objective was to describe the expression of *Trb3* during normal pregnancy and lactation in mice. The second objective was to characterize the lactational performance of mice deficient in *Trb3*. The final objective was to examine the interaction of *Trb3* with mammary lipogenesis using t10c12CLA and a high safflower oil (HO) diet, 2 treatments known to suppress de novo lipogenesis in the mammary gland. In this study we observed a minimal effect of *Trb3* on lactational performance or in mediating changes in milk fat by t10c12CLA and a high-fat diet.

Methods

Expression of *Trb3* and *Atf4* during the murine lactation cycle

To investigate the role of *Trb3* and *Atf4* in the mammary gland throughout the lactation cycle, we analyzed longitudinal gene expression during pregnancy, lactation, and involution using ancillary microarray data retrieved from studies by Rudolph et al. (20) (National Center for Biotechnology Information Gene Expression Omnibus GSE8191). Briefly, in the original study, the authors collected mammary tissue from FVB mice at 9 time points throughout pregnancy, lactation, and involution (n = 4/time point). Mammary epithelial cells were enriched by centrifugation and gene expression analyzed using Affymetrix Mu74Av2microarray chips. Data were normalized using the GC-RMA algorithm of GeneSpring (Agilent Technologies). For the current project, probesets for ER stress-related genes including C/EBP homologous protein (*Chop*), CCAAT enhancer binding protein β (*c/Ebp*β), and heat shock protein A5 (*Hspa5*) were averaged for analysis as we will describe.

Animals and treatments

Experimental procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee (#41628). A colony of *Trb3* KO mice in the C57BL/6N genetic background (21) was established from founders obtained from Regeneron Pharmaceuticals. All animals were maintained with a 12-h light/dark cycle. Heterozygous (HET) breeding pairs were used to produce WT, HET, and *Trb3* KO mice for the following experiments. To conduct the lactation experiments, female WT mice were bred to KO males and female *Trb3* HET and KO mice were bred to WT males. Litters were cross-fostered on the first day of lactation to provide ≥6 pups/litter. Treatments were assigned by alternating between treatments based on whelping date. No experimental animals were removed after treatment assignment. Cage location was not randomly assigned. Pups and dams were killed by carbon dioxide asphyxiation followed by cervical dislocation.

Based on our analysis of the microarray *Trb3* data, we first aimed to compare the full lactation curve in *Trb3* KO dams with WT dams. Female WT (n = 8), *Trb3* HET (n = 11), and *Trb3* KO (n = 11) lactating dams fed an unpurified diet (diet #8640; Harlan Teklad) were first used to characterize lactation performance. Dams were followed through the full duration of their first lactation with litter weaning at 21 d. Feed, dams, and pups were weighed on 2 consecutive days multiple times throughout lactation (days 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, and 21) to obtain estimates for feed intake, pup growth, and dam body weight changes through a full lactation period. In order to obtain gene expression and milk fat profile at peak lactation, a portion of the dams were rebred after weaning for further investigation during their second lactation (WT, n = 6; *Trb3* HET, n = 7; *Trb3* KO, n = 6). Feed, dams, and pups were weighed throughout lactation (days 2, 3, 5, 6, 8, 9, 11, 12, and 13) to estimate intake and growth. Pups and dams were killed at approximately peak production (day 14) of the second lactation to obtain dam tissue samples and pup stomach milk clots.

Next, the interaction with treatments known to suppress mammary lipogenesis was investigated in a new cohort of mice. Our objective in these trials was to determine if *Trb3* deficiency would protect mice from suppression of lipogenesis. Lactating *Trb3* KO mice and WT mice maintained by a standard unpurified diet (diet #8640, Harlan Teklad) were used to test the interaction between *Trb3* genotype and t10c12CLA and an HO diet (**Table 1**). On day 2 of their first lactation, mice were switched to a semipurified low-fat diet (LFD; Research Diets) as described previously (22). To test an interaction with t10c12CLA and *Trb3*, on day 11 of lactation dams were randomly assigned to dietary treatment in a 2 × 2 factorial design (genotype × dietary treatment). In this trial, mice were treated with 22.5 mg t10c12CLA/d [WT (Control, n = 6; t10c12CLA, n = 7) and *Trb3* KO (Control, n = 7; t10c12CLA, n = 7)].

TABLE 1 Composition of experimental diets[1]

	Standard unpurified diet[2]		LFD		HO	
	Wt %	% kcal	Wt %	% kcal	Wt %	% kcal
Ingredients						
Casein (80 Mesh)	—	—	19.0	19.7	23.7	20.8
l-Cystine	—	—	0.28	0.30	0.36	0.31
Corn starch	—	—	29.9	31.1	21.5	18.9
Maltodextrin 10	—	—	3.3	3.5	—	—
Sucrose	—	—	33.2	34.5	21.5	18.9
Cellulose	—	—	4.7	—	5.9	—
Soybean oil	—	—	2.4	5.5	3.0	5.9
Lard	—	—	1.9	4.4	2.4	4.7
High-linoleic safflower oil	—	—	—	—	15.0	29.7
Mineral mix (S10026)	—	—	0.95	—	1.2	—
Dicalcium phosphate	—	—	1.2	—	1.5	—
Calcium carbonate	—	—	0.52	—	0.65	—
Potassium citrate, 1 H_2O	—	—	1.6	—	2.0	—
Vitamin mix (V10001)	—	—	0.95	0.99	1.2	0.83
Choline bitartrate	—	—	0.19	—	0.24	—
FD&C yellow dye #5	—	—	0.005	—	0.006	—
Nutrient composition						
Protein, %	22.0	29.0	19.2	20.0	24.0	21.3
Carbohydrate, %	40.6	54.0	67.3	70.0	42.9	38.1
Fat, %	5.5	17.0	4.2	10.0	20.3	40.6

[1]Composition provided as weight percentage of diet and as a percentage of calories (% kcal). HO, high safflower oil diet; LFD, semipurified low-fat diet.
[2]Standard unpurified diet (Harlan Teklad #8640) ingredients in descending order of inclusion: dehulled soybean meal, ground corn, wheat middlings, flaked corn, fish meal, cane molasses, soybean oil, ground wheat, dried whey, dicalcium phosphate, calcium carbonate, brewers dried yeast, iodized salt, choline chloride, kaolin, magnesium oxide, ferrous sulfate, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, copper sulfate, zinc oxide, niacin, thiamin mononitrate, vitamin A acetate, vitamin D_3 supplement, calcium pantothenate, pyridoxine hydrochloride, riboflavin, vitamin B-12 supplement, folic acid, calcium iodate, biotin, cobalt carbonate.

Treatment was administered orally by pipette twice per day (08:00 and 16:00) on days 11 and 12 of lactation as described by Harvatine et al. (11). In the second trial, to test the interaction with a high-fat diet, on day 9 of lactation dams were randomly assigned to either the LFD or an HO diet that contained 15% high linoleic acid (18:2n-6) safflower oil substituted for carbohydrate (cane sugar and corn starch) in the LFD [WT (LFD, $n = 6$; HO, $n = 7$) and *Trb3* KO (LFD, $n = 7$; HO, $n = 6$)]. The t10c12CLA and high-fat diet trials were conducted concurrently so dams fed only the LFD were used as controls in both trials. Feed, dams, and pups were weighed on 2 consecutive days multiple times throughout lactation (days 2, 3, 5, 6, 8, 9, 11, 12, and 13) to obtain estimates for intake and growth. Pups and dams were killed on day 14 for collection of pup stomach milk clots.

Sample collection

After killing dams, the #4 mammary gland (this gland is not intertwined with muscle), liver, gonadal adipose, and mesenteric adipose tissues were dissected and weighed. Tissues were snap frozen in liquid nitrogen and stored at −80°C for gene expression analysis. Stomach milk clots were collected from pups, composited within litter, and stored frozen at −20°C and freeze dried before analysis of FA profiles.

Real-time qPCR

To quantify gene expression, total RNA was extracted from ∼35 mg pulverized mammary tissue using the E.Z.N.A.® total RNA Kit with on-column DNase treatment (Omega Biotek). RNA concentration and integrity were then assessed using an Experion Automated Electrophoresis Station (Bio-Rad). RNA was reverse transcribed and quantified by SYBR green real-time qPCR as described previously (10) using gene-specific forward and reverse primers (**Supplemental Table 1**). Relative gene expression was determined using the geometric mean of 3 reference housekeeping genes (*β-actin*, *18S*, and hydroxymethylbilane synthase (*Hmbs*)) after standardizing each to a mean of 1.

FA profile analysis

Stomach milk clot FAs were extracted in hexane isopropanol, methylated with a dual methylation procedure using sodium methoxide followed by methanolic HCl, and quantified by GC with flame ionization detection as previously described (23). Tridecylic acid (13:0) methyl ester and margaric acid (17:0) triglyceride ester were used as internal standards to determine FA concentration.

Statistical analysis

Sample sizes were sufficient to provide >80% statistical power at a significance of 0.05 with an expected difference of 2.5 percentage units in milk fat concentration based on previous experiments conducted with similar procedures. Single time-point production variables and gene expression were assessed by ANOVA using genotype and treatment as fixed effects. Production variables at the baseline time points were considered as covariates, and included in the model if parameter estimates were significant ($P < 0.05$). Residuals were plotted by predicted values and were uniformly distributed. Differences between groups were assessed by protected least squares differences ($P < 0.05$). Time course analysis was performed by repeated-measures ANOVA using days as

the repeated variable. Compound symmetry, unstructured, and spatial power covariance structures were considered. The covariance structure that resulted in the lowest Bayesian information criterion was selected. The geometric mean of 3 housekeeping genes (*β-actin*, *18S*, and *Hmbs*) was included as a covariate in ANOVA models for all gene expression analysis to determine relative expression. All statistical analysis was performed using SAS 9.4 (SAS Institute, Inc.). The assumption of uniform distribution of residuals was determined by plotting residuals by predicted values.

Results

Trb3 and *Atf4* gene expression throughout pregnancy and lactation

In a retrospective analysis of microarray data of FVB mice available from Rudolph et al. (20), mammary expression of both *Trb3* and *Atf4* increased in late pregnancy, remained elevated and peaked at day 9 of lactation ($P < 0.05$) before returning to baseline during mammary involution (**Figure 1**A, B). In contrast, mammary expression of *Chop* and *c/Ebpβ* increased only during involution ($P < 0.05$) and expression of the protein chaperone *Hspa5* did not change across stages of lactation (Figure 1C). This suggested that TRB3 and ATF4 were more likely to be involved in regulation of milk synthesis than the other ER stress genes.

Effect of *Trb3* on lactational performance and milk fat

Data were collected over the first complete lactation in C57BL/6N WT and *Trb3* KO mice to characterize the effect of *Trb3* on dam intake and pup growth because these are indicators of lactation performance. During first lactation, KO and HET mice had lower feed intake (KO, $P = 0.03$; HET, $P = 0.008$), body weight (KO, $P = 0.008$; HET, $P = 0.007$), and litter weight (KO, $P = 0.0005$; HET, $P = 0.0001$) than WT. These differences were seen throughout the lactation period, but feed intake and body weight began to converge between genotypes around day 21 of lactation (**Figure 2**). Dams were rebred and a second lactation observed for tissue collection at peak lactation. During the second lactation, there were no significant differences in dam feed intake and litter growth during the first 14 d (**Table 2**). Expression of *Trb3* mRNA was not detectable in KO mice, but there was no difference in mammary expression of *Trb3* between WT and HET mice. There were no detectable differences between genotypes in the expression of other ER stress pathway genes or lipogenic genes in the mammary gland (**Figure 3**). Similarly, there were no detectable differences in pup stomach milk clot fat content or FA profile between genotypes (**Table 3**).

Interaction of TRB3 and t10c12CLA inhibition of milk fat synthesis

t10c12CLA is a bioactive FA that decreases milk fat primarily through inhibition of mammary de novo lipid synthesis. With oral administration of t10c12CLA, dam intake was decreased by 27% ($P < 0.0001$) and dam gonadal adipose tissue was reduced by 24% ($P = 0.04$). Final litter weight and litter weight gain were also decreased by t10c12CLA (8% and 59%, respectively; $P < 0.0001$), but there was no detectable effect of genotype or interaction between diet and genotype (**Table 4**). t10c12CLA decreased pup stomach milk fat concentration by 11% ($P < 0.0001$) and increased preformed FA (>16 carbons) content

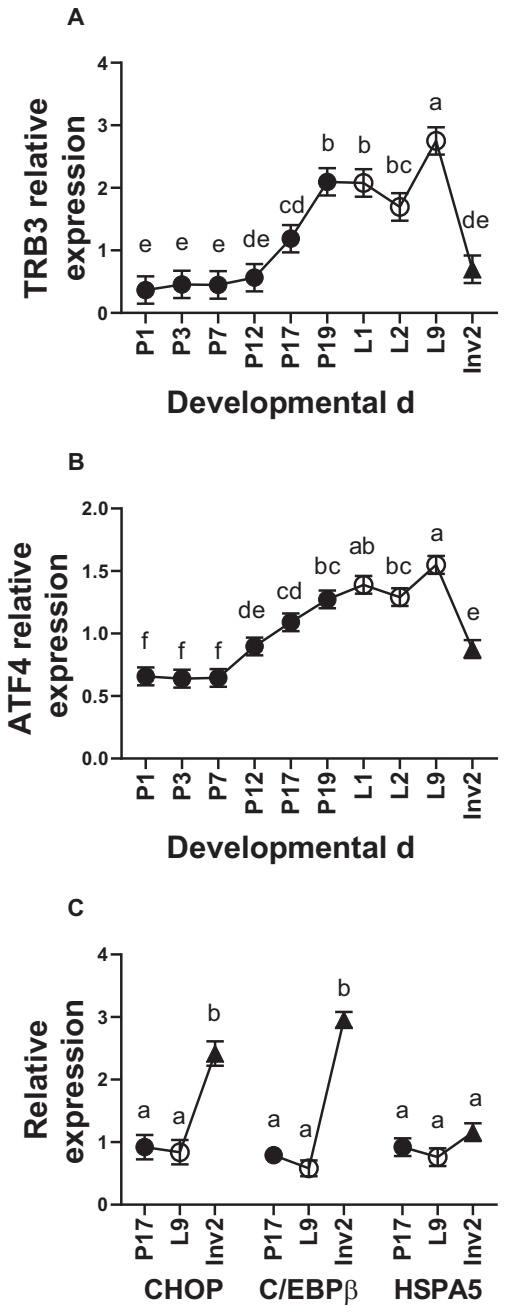

FIGURE 1 *Trb3* and *Atf4* expression data in FVB mice from early pregnancy through involution. These data represent ancillary analysis of microarray data from studies by Rudolph et al. (20). (A) Changes in the mRNA expression of *Trb3* in the mammary gland during pregnancy and lactation. (B) *Atf4* expression in the mammary gland during pregnancy and lactation. (C) *Chop*, *c/Ebpβ*, and *Hspa5* expression in the mammary gland during pregnancy and lactation. Levels not connected by the same letter are significantly different by ANOVA with protected least significant difference post hoc test ($P < 0.05$). Black circles, pregnancy; open circles, lactation; triangles, involution. *Atf4*, activating transcription factor-4; *c/Ebpβ*, CCAAT enhancer binding protein *β*; *Chop*, C/EBP homologous protein, *Hspa5*, heat shock protein A5; Inv, involution day; L, lactation day; P, pregnancy day; *Trb3*, tribbles pseudokinase 3.

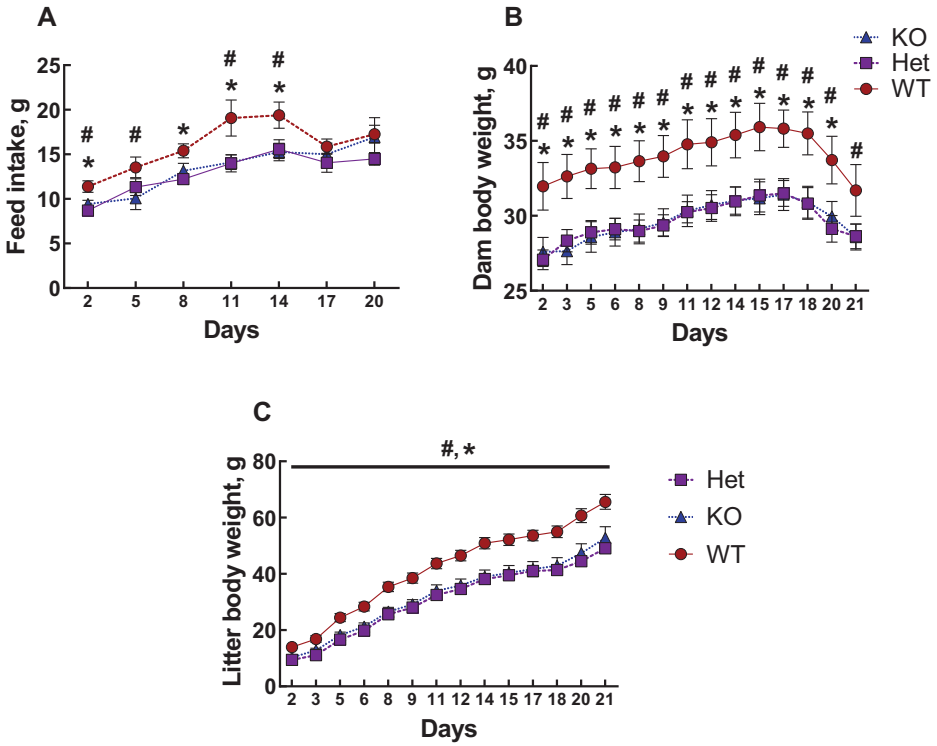

FIGURE 2 Effects of *Trb3* genotype on dam intake (A) and body weight (B) and pup growth (C) across the first lactation. *Trb3* KO, HET, and WT dams were maintained by a standard unpurified diet through their full first lactation. Litters were cross-fostered and balanced to 6–9 pups/litter. (A) Dam feed intake across full lactation. (B) Dam body weight across full lactation. (C) Litter body weight across full lactation. Data are presented as least square mean ± SD. Differences were assessed using repeated-measures ANOVA with protected least significant difference post hoc test comparing genotypes within each day ($P < 0.05$). *WT mice significantly different from HET. #WT mice significantly different from KO. HET, heterozygous; KO, knockout; *Trb3*, tribbles pseudokinase 3; WT, wild-type.

by 29% ($P < 0.0001$). There was a main effect of genotype and interaction of genotype and t10c12CLA for some individual FAs, although the overall genotype effect on FA profile was small. Specifically, KO mice on the placebo diet had lower concentrations of caprylic acid (8:0) and capric acid (10:0) (caprylic acid: −20%, $P_{int} = 0.03$; capric acid: −13%, $P_{genotype} = 0.04$) and higher palmitic acid (16:0) (7%, $P_{genotype} = 0.008$) than WT mice; also, t10c12CLA treatment resulted in a greater reduction in palmitoleic acid (16:1n-7) in KO mice than in WT ($P_{int} = 0.04$) (**Table 5**). We were unable to obtain gene expression data for the animals treated with t10c12CLA owing to sample loss from a freezer failure.

TABLE 2 Effect of tribbles pseudokinase 3 (*Trb3*) deletion on dam intake, growth, and tissue weight and pup growth from day 12 to day 14 of their second lactation[1]

Mass,[2] g	WT	HET	KO	SE	*P* values
Dam					
Feed intake	17.1	16.3	16.8	1.8	0.95
Final BW	35.4	35.4	35.7	0.80	0.95
BW gain	0.24	0.11	0.29	0.26	0.87
Liver	2.85	2.37	2.65	0.16	0.13
Gonadal adipose	0.18	0.13	0.13	0.03	0.40
Mesenteric adipose	0.61	0.50	0.59	0.04	0.12
Litter[3]					
Final BW	43.0	43.6	43.6	2.09	0.98
BW gain	2.10	2.31	2.17	0.24	0.82

[1]Values are means unless indicated otherwise. BW, body weight; HET, heterozygous; KO, knockout; WT, wild-type.
[2]Weights are averaged from the last 2 d before killing in the second lactation.
[3]Litters were cross-fostered and balanced to 6–9 pups/litter. Pups and dams were killed at day 14 of lactation.

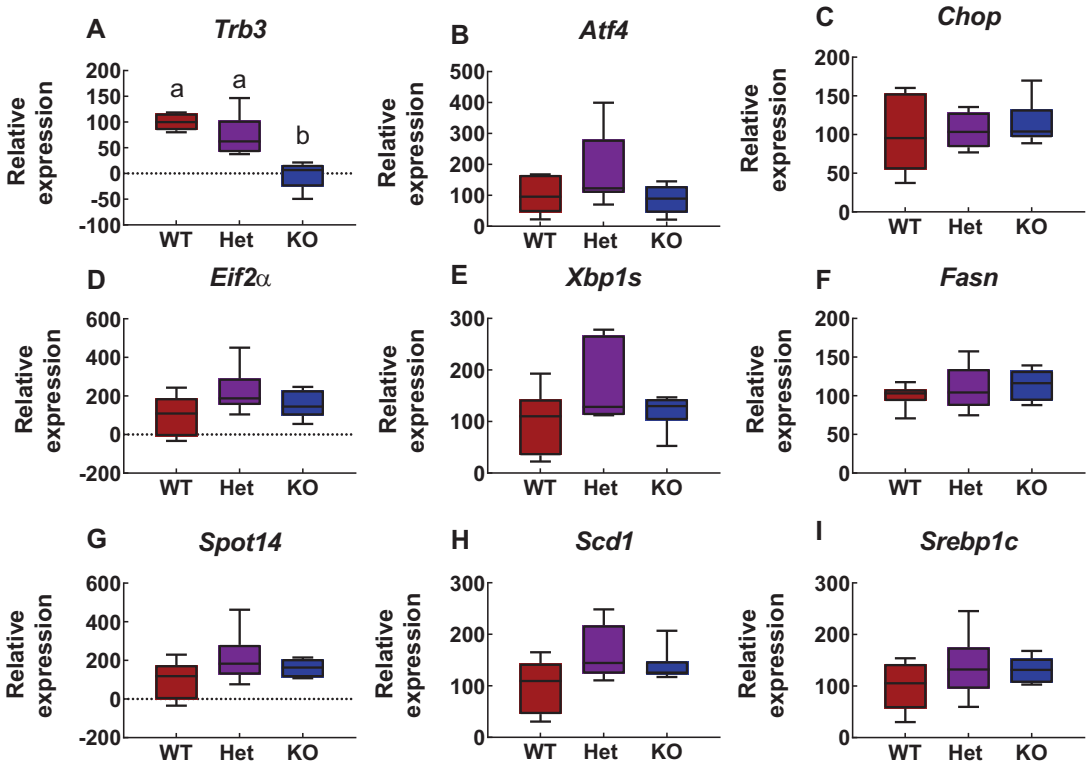

FIGURE 3 Effect of *Trb3* deletion on mammary gene expression of ER stress pathway and lipid metabolism genes during second lactation. *Trb3* KO, HET, and WT dams were maintained by a standard unpurified diet through to their second lactation. Litters were cross-fostered and balanced to 6–9 pups/litter. Mice were killed at day 14 of lactation and the #4 mammary gland was dissected and gene expression analyzed by RT-PCR. Expression of ER stress pathway genes: (A) *Trb3* (*Trb3* mRNA expression was not above background in KO mice), (B) *Atf4*, (C) *Chop*, (D) *Eif2α*, (E) *Xbp1s*. Expression of lipogenic genes: (F) *Fasn*, (G) *Spot14*, (H) *Scd1*, (I) *Srebp1c*. Data are presented as median, quartiles, and range normalized to a mean WT mRNA expression of 100. Differences were assessed using ANOVA with protected least significant difference post hoc test ($P < 0.05$). Levels without a common letter are significantly different. *Atf4*, activating transcription factor-4; *Chop*, C/EBP homologous protein; *Eif2α*, eukaryotic initiation factor 2α; *Fasn*, fatty acid synthase; HET, heterozygous; KO, knockout; *Scd1*, stearoyl CoA desaturase 1; *Spot14*, thyroid hormone responsive protein; *Srebp1c*, sterol regulatory-element binding protein 1; *Trb3*, tribbles pseudokinase 3; WT, wild-type; *Xbp1s*, X-box binding protein-1.

Interaction of TRB3 and an HO diet on milk fat synthesis

Increasing dietary fat generally increases milk fat concentration and pup growth, but decreases de novo lipogenesis in the mammary gland. In this study, the HO diet had no significant effect on litter growth or weight, but decreased dam intake ($P = 0.001$), body weight ($P = 0.005$), liver weight ($P = 0.01$), and mesenteric adipose weight ($P = 0.004$) (**Table 6**). There was no interaction of *Trb3* genotype and HO diet on dam feed intake or body and tissue weights. There were interactions observed in pup stomach milk clot fat concentration and FA profile. The HO diet increased milk fat by 8% in WT mice, but not in *Trb3* KO mice ($P_{int} = 0.04$). Preformed FA content (>16 carbons) was increased by 89% ($P < 0.0001$), and de novo FAs (<16 carbons) were decreased by 37% ($P < 0.0001$) with the HO diet. However, *Trb3* KO had a smaller decrease in de novo FAs with the HO diet than had WT (KO decreased 31% compared with 44% in WT; $P_{int} = 0.002$). *Trb3* KO also appeared to have mixed effects on the long-chain PUFAs. For example, only *Trb3* KO mice fed the HO diet had lower concentrations of milk DHA (22:6n–3; $P_{int} = 0.04$), not WT mice or those fed the LFD (**Table 7**).

Trb3 mRNA expression was not detectable above background in *Trb3* KO mice. Expression of other ER stress genes was less affected, with no change in *Chop* or X-box binding protein-1 (*Xbp1s*) mRNA expression. However, *Atf4* expression was decreased in *Trb3* KO compared with WT, particularly when fed the HO diet ($P = 0.01$). The HO diet decreased expression of the lipogenic genes, *Scd1* ($P < 0.0001$) and *Fasn* ($P = 0.04$), but did not change thyroid hormone responsive protein (*Spot14*) or *Srebp1c* expression. *Trb3* KO mice had increased expression of *Scd1* ($P = 0.02$) compared with WT mice when fed the LFD (**Figure 4**).

Discussion

Because of the dramatic increase in mammary *Trb3* expression observed during lactation in the retrospective analysis of microarray data, we ex-

TABLE 3 Effect of tribbles pseudokinase 3 (*Trb3*) deletion on pup stomach milk clot FA profile[1]

FA (% of total)	WT	HET	KO	SE	*P* values
Total fat, %	57.9	59.1	56.8	0.01	0.43
FA profile, % of FA					
8:0	0.36	0.41	0.37	0.03	0.32
10:0	6.79	7.40	6.59	0.31	0.18
12:0	12.3	12.9	11.9	0.36	0.19
14:0	14.6	15.1	14.7	0.37	0.66
14:1	0.10	0.12	0.12	0.01	0.33
16:0	24.1	23.7	25.1	0.43	0.09
16:1	1.07	1.11	1.26	0.10	0.43
18:0	2.41	2.22	2.32	0.06	0.13
18:1, c9	13.5	12.6	13.1	0.50	0.43
18:1, c11	1.20	1.13	1.25	0.07	0.48
18:2n–6	16.1	15.9	15.4	0.27	0.27
18:3n–3	1.80	1.78	1.85	0.06	0.68
20:2	1.31	1.31	1.22	0.07	0.61
20:3n–6	0.59	0.60	0.56	0.04	0.80
20:4n–6	0.48	0.48	0.46	0.05	0.93
20:5n–3	0.13	0.14	0.15	0.02	0.84
24:0	0.03	0.02	0.03	0.01	0.74
24:1	0.04	0.04	0.04	0.01	0.90
22:4n–6	0.25	0.26	0.22	0.03	0.61
22:5n–3	0.21	0.23	0.21	0.02	0.84
22:6n–3	0.15	0.16	0.16	0.02	0.83
Unknown	2.50	2.53	2.42	0.09	0.66
FAs by source,[2] % of FA					
<16 carbons	34.2	35.9	33.7	0.97	0.26
16 carbons	25.1	24.8	26.3	0.47	0.08
>16 carbons	38.2	36.8	37.6	1.04	0.65
DI C14[3]	0.007	0.008	0.008	0.001	0.41
DI C16[4]	0.044	0.047	0.051	0.004	0.57

[1]DI, desaturase index; FA, fatty acid; HET, heterozygous; KO, knockout; WT, wild-type.
[2]FAs by source: FAs with <16 carbons originate from mammary de novo synthesis, FAs with >16 carbons originate from plasma, and 16-carbon FAs originate from both sources.
[3]DI C14 = 14:1/(14:0 + 14:1).
[4]DI C16 = 16:1/(16:0 + 16:1).

pected that *Trb3* KO mice would have impaired lactation and decreased litter growth. Growth and intake were moderately decreased in *Trb3* KO and HET mice compared with WT mice. This may be due to mammary growth limitation during the first lactation that may catch up by the second lactation. However, these changes, although significant, should be interpreted with caution because these results were not replicated in

TABLE 4 Interaction of tribbles pseudokinase 3 (*Trb3*) deletion and t10c12CLA on dam intake, growth, and tissue weight and litter growth[1]

Treatment[2]	LFD		t10c12CLA			*P* values		
Mass,[3] g	WT	KO	WT	KO	SE	Diet	GT	Diet*GT
Dam								
Intake	12.0[a]	11.8[a]	8.89[b]	8.49[b]	0.28	<0.0001	0.28	0.75
BW	28.7	29.1	28.3	28.5	0.27	0.11	0.34	0.69
BW gain	−0.04	0.13	−0.06	−0.11	0.18	0.56	0.73	0.55
Liver weight	2.12	2.30	2.35	2.21	0.10	0.52	0.87	0.14
Gonadal adipose	0.22	0.23	0.16	0.18	0.02	0.04	0.57	0.77
Mesenteric adipose	0.45[a,b]	0.48[a]	0.39[b]	0.42[a,b]	0.02	0.009	0.08	0.95
Litter								
BW	46.4[a]	45.8[a]	43.0[b]	42.1[b]	0.68	<0.0001	0.27	0.89
BW gain	2.92[a]	2.74[a]	1.27[b]	1.04[b]	0.19	<0.0001	0.31	0.89

[1]Levels not connected by the same letter ([a,b]) are significantly different by ANOVA with protected least significant difference post hoc test ($P < 0.05$). BW, body weight; GT, genotype; KO, knockout; LFD, semipurified low-fat diet; t10c12CLA, *trans*-10, *cis*-12 conjugated linoleic acid; WT, wild-type. .
[2]All mice were fed an LFD. Controls had no additional treatment; t10c12CLA treatment consisted of a daily dose of 22.5 mg t10c12CLA on days 11 and 12 of lactation.
[3]Weights are averaged from the last 2 d before killing in the first lactation (days 12–14).

TABLE 5 Interaction of tribbles pseudokinase 3 (*Trb3*) deletion and t10c12CLA on pup stomach milk clot FA profile[1]

Treatment[2]	LFD		t10c12CLA			Treatment	GT	Treatment*GT
FA, g/100 g FA	WT	KO	WT	KO	SE	*P* values	*P* values	*P* values
Total fat %	61[a]	62[a]	55[b]	54[b]	1.2	<0.0001	0.83	0.19
FA profile								
8:0	0.42[a]	0.33[b]	0.32[b]	0.34[b]	0.02	0.08	0.15	0.03
10:0	6.81[b]	5.91[c]	8.54[a]	8.25[a]	0.28	<0.0001	0.04	0.28
12:0	13.1	12.4	13.5	13.0	0.46	0.27	0.21	0.75
14:0	18.2[a]	18.0[a]	13.8[b]	14.1[b]	0.53	<0.0001	0.90	0.69
14:1	0.27[a]	0.29[a]	0.12[b]	0.12[b]	0.01	<0.0001	0.60	0.49
16:0	27.6[b]	29.6[a]	21.5[c]	22.6[c]	0.54	<0.0001	0.008	0.45
16:1	2.19[a,b]	2.41[a]	2.01[b,c]	1.85[c]	0.08	0.0002	0.77	0.04
18:0	2.15[b]	2.25[b]	2.79[a]	2.90[a]	0.07	<0.0001	0.13	0.96
18:1, c9	14.6[b]	14.6[b]	18.3[a]	18.0[a]	0.68	<0.0001	0.83	0.80
18:1, c11	1.75[b]	1.76[b]	2.24[a]	2.02[a]	0.09	0.0002	0.26	0.19
18:2	7.03[b]	6.90[b]	8.64[a]	8.88[a]	0.23	<0.0001	0.81	0.43
18:3n–6	0.07[b]	0.06[b]	0.10[a]	0.10[a]	0.01	<0.0001	0.25	0.71
20:1	0.69	0.67	0.80	0.82	0.05	0.02	0.98	0.71
18:3n–3	0.58[b]	0.59[b]	0.66[a,b]	0.68[a]	0.03	0.008	0.53	0.95
CLA, c9, t11	0.04[b]	0.04[b]	0.69[a]	0.73[a]	0.05	<0.0001	0.75	0.68
CLA, t10, c12	0.00[b]	0.00[b]	0.51[a]	0.54[a]	0.04	<0.0001	0.73	0.73
20:2	0.64[b]	0.60[b]	0.76[a]	0.78[a]	0.04	0.0006	0.87	0.43
20:3n–6	0.42	0.40	0.41	0.38	0.02	0.64	0.14	0.28
20:4n–6	0.35[b]	0.28[b]	0.59[a]	0.59[b]	0.03	<0.0001	0.25	0.30
20:5n–3	0.10	0.09	0.09	0.09	0.02	0.79	0.87	0.73
24:0	0.04[b]	0.05[b]	0.10[a]	0.10[a]	0.02	0.004	0.79	0.81
24:1	0.09	0.09	0.13	0.09	0.01	0.16	0.12	0.11
22:4n–6	0.17	0.15	0.16	0.19	0.01	0.29	0.88	0.06
22:5n–3	0.10	0.09	0.16	0.15	0.007	<0.0001	0.34	0.48
22:6n–3	0.12[a,b]	0.11[b]	0.14[a,b]	0.16[a]	0.02	0.02	0.63	0.25
Unknown	2.43[b]	2.34[b]	2.95[a]	2.49[a,b]	0.16	0.05	0.10	0.27
FAs by source[3]								
<16 carbons	38.8	36.9	36.3	35.8	1.2	0.14	0.34	0.56
16 carbons	29.8[b]	32.0[a]	23.5[c]	24.5[c]	0.56	<0.0001	0.01	0.30
>16 carbons	28.9[b]	28.7[b]	37.3[a]	37.2[a]	1.12	<0.0001	0.91	0.95
DI C14[4]	0.015[a]	0.016[a]	0.009[b]	0.008[b]	0.0005	<0.0001	0.52	0.30
DI C16[5]	0.074	0.075	0.086	0.076	0.003	0.06	0.20	0.10

[1]Levels not connected by the same letter ([a,b,c]) are significantly different by ANOVA with protected least significant difference post hoc test ($P < 0.05$). DI, desaturation index; FA, fatty acid; GT, genotype; KO, knockout; LFD, semipurified low-fat diet; t10c12CLA, *trans*-10, *cis*-12 conjugated linoleic acid; WT, wild-type.
[2]All mice were fed an LFD. Controls had no additional treatment; t10c12CLA treatment consisted of a daily dose of 22.5 mg t10c12CLA on days 11 and 12 of lactation.
[3]FAs by source: FAs with <16 carbons originate from mammary de novo synthesis, FAs with >16 carbons originate from plasma, and 16-carbon FAs originate from both sources.
[4]DI C14 = 14:1/(14:0 + 14:1).
[5]DI C16 = 16:1/(16:0 + 16:1).

other experiments. There were no significant differences in growth or intake by genotype in second lactation or the t10c12CLA and high-fat treatment experiments, even though these experiments were conducted during the first lactation. It is possible that differences in the first lactation results can be explained by dietary differences between the unpurified and semipurified diets. In second lactation animals, there were also no differences in lipogenic gene expression, milk clot fat concentration, or FA profile with *Trb3* KO. This lack of detectable change in multiple

TABLE 6 Interaction of tribbles pseudokinase 3 (*Trb3*) deletion and an HO diet on growth and milk production[1]

Treatment[2]	LFD		HO			*P* values		
Mass,[3] g	WT	KO	WT	KO	SE	Diet	GT	Diet*GT
Dam								
Intake	11.8[a]	11.6[a]	10.0[a,b]	8.77[b]	0.57	0.001	0.21	0.38
BW	28.7[a]	29.1[a]	27.6[a,b]	26.7[b]	0.52	0.005	0.65	0.20
BW gain	0.14	0.27	−0.05	−1.23	0.55	0.15	0.35	0.25
Liver	2.12[a,b]	2.30[a]	1.95[a,b]	1.80[b]	0.12	0.01	0.93	0.21
Gonadal adipose	0.22	0.23	0.19	0.16	0.03	0.09	0.78	0.60
Mesenteric adipose	0.45[a,b]	0.48[a]	0.41[b]	0.41[b]	0.02	0.004	0.28	0.32
Litter								
BW	45.2	44.6	45.4	44.0	0.94	0.82	0.29	0.69
BW gain	2.83	2.72	2.80	2.57	0.21	0.68	0.44	0.79

[1]Levels not connected by the same letter ([a,b]) are significantly different by ANOVA with protected least significant difference post hoc test ($P < 0.05$). BW, body weight; GT, genotype; HO, high–safflower oil diet; KO, knockout; LFD, semipurified low-fat diet; WT, wild-type.
[2]Diets were an LFD and an HO with 15% added high–linoleic acid safflower oil. Treatment diets were fed from day 9 to day 13 of lactation.
[3]Weights are averaged from the last 2 d before killing in the second lactation (days 12–14).

TABLE 7 Interaction of tribbles pseudokinase 3 (*Trb3*) deletion and an HO diet on pup stomach milk clot FAs[1]

Treatment[2]	LFD		HO			Diet	GT	Diet*GT
FA (% of total)	WT	KO	WT	KO	SE	*P* values	*P* values	*P* values
Total fat %	61[b]	62[b]	66[a]	62[b]	0.01	0.08	0.27	0.04
FA profile								
8:0	0.42[a]	0.33[b]	0.19[d]	0.28[c]	0.02	<0.0001	0.94	<0.0001
10:0	6.81[a]	5.91[b]	4.34[d]	5.12[c]	0.16	<0.0001	0.69	<0.0001
12:0	13.1[a]	12.4[a]	7.7[c]	8.9[b]	0.27	<0.0001	0.42	0.002
14:0	18.2[a]	18.3[a]	9.7[c]	11.2[b]	0.45	<0.0001	0.08	0.11
14:1	0.27[a]	0.29[a]	0.06[b]	0.08[b]	0.009	<0.0001	0.16	0.92
16:0	27.6[a]	29.6[a]	18.4[b]	19.7[b]	0.81	<0.0001	0.06	0.68
16:1	2.19[b]	2.41[a]	0.76[c]	0.82[c]	0.06	<0.0001	0.03	0.21
18:0	2.15[b]	2.25[b]	2.55[a]	2.47[a]	0.06	<0.0001	0.87	0.11
18:1, c9	14.6	14.6	15.2	14.1	0.52	0.93	0.34	0.33
18:1, c11	1.75	1.76	0.74	0.69	0.05	<0.0001	0.70	0.47
18:2n–6	7.03[c]	6.90[c]	33.0[a]	30.3[b]	0.83	<0.0001	0.10	0.13
18:3n–6	0.07[c]	0.05[c]	0.26[a]	0.17[b]	0.02	<0.0001	0.01	0.06
20:1	0.69[a]	0.67[a]	0.46[b]	0.42[b]	0.05	<0.0001	0.56	0.85
18:3n–3	0.58[a]	0.59[a]	0.40[b]	0.36[b]	0.02	<0.0001	0.57	0.14
CLA, c9, t11	0.04[a]	0.04[a]	0.00[b]	0.00[b]	0.003	<0.0001	0.51	0.51
20:2	0.64[b]	0.60[b]	1.92[a]	1.76[a]	0.10	<0.0001	0.32	0.54
20:3n–6	0.42[c]	0.38[c]	0.97[a]	0.70[b]	0.05	<0.0001	0.003	0.02
20:4n–6	0.35[b]	0.28[b]	0.90[a]	0.49[b]	0.09	0.0002	0.01	0.06
20:5n–3	0.10[a]	0.09[a]	0.03[b]	0.01[b]	0.005	<0.0001	0.03	0.58
24:0	0.04[b]	0.04[b]	0.08[a]	0.08[a]	0.004	<0.0001	0.20	0.66
24:1	0.09	0.09	0.10	0.11	0.008	0.13	0.49	0.50
22:4n–6	0.17[b]	0.15[b]	0.28[a]	0.18[b]	0.02	0.001	0.006	0.08
22:5n–3	0.10[a]	0.09[a]	0.04[b]	0.02[c]	0.007	<0.0001	0.008	0.27
22:6n–3	0.12[a]	0.11[a]	0.11[a]	0.05[b]	0.01	0.003	0.005	0.04
Unknown	2.43[a]	2.34[a]	1.76[b]	1.91[b]	0.12	0.0001	0.81	0.32
FAs by source[3]								
<16 carbons	38.8[a]	36.9[a]	21.9[c]	25.6[b]	0.80	<0.0001	0.28	0.002
16 carbons	29.8[a]	32.0[a]	19.2[b]	20.6[a]	0.83	<0.0001	0.05	0.62
>16 carbons	28.9[c]	28.7[c]	57.1[a]	52.0[b]	1.37	<0.0001	0.06	0.09
DI C14[4]	0.015[a]	0.016[a]	0.007[b]	0.006[b]	0.0005	<0.0001	0.14	0.87
DI C16[5]	0.074[a]	0.075[a]	0.040[b]	0.040[b]	0.003	<0.0001	0.82	0.82

[1]Levels not connected by the same letter ([a,b,c,d]) are significantly different by ANOVA with protected least significant difference post hoc test ($P < 0.05$). DI, desaturase index; FA, fatty acid; GT, genotype; HO, high–safflower oil diet; KO, knockout; LFD, semipurified low-fat diet; WT, wild-type.
[2]Diets were an LFD and an HO with 15% added high–linoleic acid safflower oil. Treatment diets were fed from day 9 to day 13 of lactation.
[3]FAs by source: FAs with <16 carbons originate from mammary de novo synthesis, FAs with >16 carbons originate from plasma, and 16-carbon FAs originate from both sources.
[4]DI C14 = 14:1/(14:0 + 14:1).
[5]DI C16 = 16:1/(16:0 + 16:1).

analyses suggests that the lipogenic effect of ER stress and the PERK pathway occurs upstream of *Trb3*.

We expected that *Trb3* deletion would be protective against suppressed lipogenesis caused by t10c12CLA and an HO diet. As expected, there were dramatic changes in litter growth and milk fat associated with t10c12CLA treatment. At the selected dose, the effects of t10c12CLA on milk fat production are expected to be predominantly due to inhibition of lipid synthesis pathways in mammary epithelial cells. t10c12CLA results in both ER stress (17) and alterations in mammary lipogenic gene expression (10–13, 22, 24). If *Trb3* was the critical link, then we expect that *Trb3* KO mice would be protected from t10c12CLA-induced ER stress and inhibition of lipogenesis. However, there were no differences in litter growth or milk fat observed between genotypes. Further, we found no evidence that mammary de novo lipogenesis was altered with *Trb3* deficiency, because the response in the concentration of de novo lipogenesis–derived FAs (<16 carbons) was unchanged with genotype and there was no interaction of treatment and genotype. It is noteworthy that *Trb3* KO mice had higher concentrations of 16-carbon FAs, considered a mix of preformed and de novo FAs, than controls in this trial. The mechanism to explain this change is not obvious, but may include modification of mammary thioesterase activity resulting in increased synthesis of palmitic acid during de novo synthesis or possibly increased synthesis in other lipogenic tissues like adipose.

There has been interest in t10c12CLA supplementation for weight management, consistently showing reductions in adipose tissue in rodent models (25, 26). However, t10c12CLA also induces pronounced MFD in dairy cattle and rodent models (10, 11, 22, 24), with mixed results in human lactation studies (27, 28). This suggests that the same mechanisms that reduce body weight may have detrimental effects on lactation. Mechanisms in adipose tissue are dose dependent with decreased lipid synthesis at low doses and insulin resistance, inflamma-

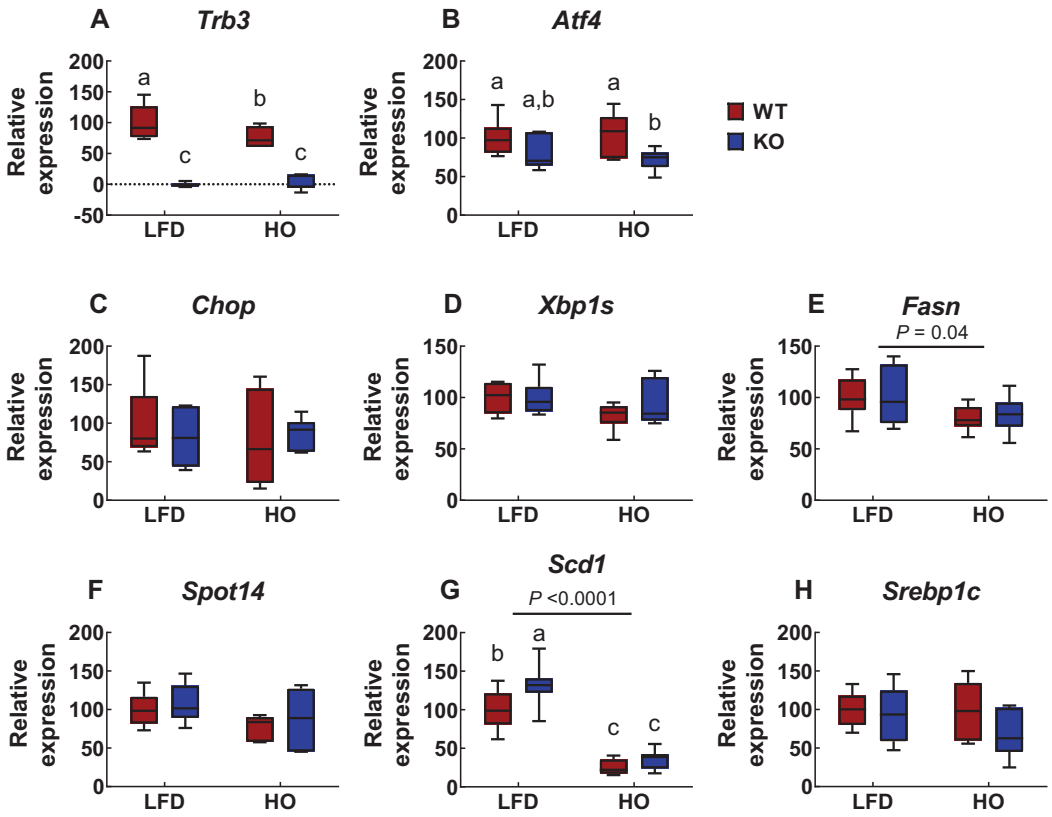

FIGURE 4 Interaction of *Trb3* deletion and an HO diet on mammary expression of ER stress and lipid metabolism genes. Dams were bred with males of differing genotype and assigned to diet in a 2 × 2 factorial design from day 9 to day 13 of lactation. Litters were cross-fostered and balanced to 6–9 pups/litter. Mammary gland #4 was dissected and mRNA gene expression was analyzed by RT-PCR. Expression of ER stress pathway genes: (A) *Trb3* ($P_{int} = 0.11$. *Trb3* mRNA expression was not above background noise in KO mice), (B) *Atf4* ($P_{int} = 0.42$), (C) *Chop* ($P_{int} = 0.52$), (D) *Xbp1s* ($P_{int} = 0.32$). Expression of lipogenic genes: (E) *Fasn* ($P_{int} = 0.85$), (F) *Spot14* ($P_{int} = 0.80$), (G) *Scd1* ($P_{int} = 0.25$), (H) *Srebp1c* ($P_{int} = 0.38$). Data are presented as median, quartiles, and range normalized to a mean WT LFD mRNA expression of 100. Differences were assessed using ANOVA with protected least significant difference post hoc test ($P < 0.05$). Levels without a common letter are significantly different. *Atf4*, activating transcription factor-4; *Chop*, C/EBP homologous protein; *Fasn*, fatty acid synthase; HO, high–safflower oil diet; KO, knockout; LFD, semipurified low-fat diet; *Scd1*, stearoyl CoA desaturase 1; *Spot14*, thyroid hormone responsive protein; *Srebp1c*, sterol regulatory-element binding protein 1; *Trb3*, tribbles pseudokinase 3; WT, wild-type; *Xbp1s*, X-box binding protein-1.

tion, and hepatic lipidosis at high doses that is similar to a lipodystrophy model (14, 15, 29–31). In lactating mice, low doses reduce mammary lipogenesis, likely through SREBP-1c regulation, whereas higher doses cause general inhibition of lactation (25). The current study replicates the t10c12CLA-induced MFD seen in other studies, but we found no evidence of a functional role of TRB3, because responses in *Trb3* KO mice were not different from those in WT mice.

Spot14 is a thyroid hormone responsive protein that regulates lipogenesis through increasing FASN activity (32). Production of mid-chain fatty acids (MCFAs) by FASN is especially critical during lactation. *Spot14* KO mice produce milk with reduced fat content, resulting in pup growth restriction, whereas overexpression of *Spot14* in mammary epithelial cells results in increased MCFA concentration, but no change in total milk fat (32). *Spot14* expression is markedly reduced in dairy cattle (10) and mice (11, 22) treated with t10c12CLA, and is associated with a reduction of both adipose tissue mass and milk fat content. This makes Spot14 a potential mechanistic link for MFD in dairy cows with ruminally produced t10c12CLA (10, 11). Results from this study show limited evidence to support *Trb3*-mediated effects on *Spot14* in mice.

Interestingly, the HO diet with greatly increased linoleic acid content increased fat concentration of pup stomach milk clots in WT mice, but this change was not observed in KO mice. The HO diet decreased milk de novo FAs (primarily ≤12 carbons) in WT mice by 44% compared with a 31% decrease in KO mice, indicating a mild protective effect. The HO diet resulted in decreased expression of *Trb3* and the lipogenic genes *Scd1* and *Fasn*. *Trb3* KO increased *Scd1* expression moderately when mice were fed the LFD, but this effect size was not as dramatic as expected with a KO model.

The ER stress response can be stimulated by 3 distinct signaling pathway arms that include activation of either the X-box binding protein-1 (XBP1s), ATF6, or ATF4/TRB3 endpoints by inositol requiring enzyme 1 (IRE1) and PERK (9). PERK activates phosphorylation of eukaryotic initiation factor 2α (eIF2α) which causes ATF4 and CHOP

to dimerize (8) and activate TRB3 to mediate apoptosis (2). IRE1 converts mRNA of XBP1s to a spliced version that improves protein folding and resolves ER stress (8). XBP1s regulates energy release and utilization by adipose and liver (33) and is a key regulator of mammary epithelial cell proliferation and ER formation (19). Expression of these ER stress–related genes was measured in this study, but there were no main effects of HO diet and minimal effects of *Trb3* deficiency. *Atf4* expression was decreased in *Trb3* KO mice. It is possible that TRB3 acts as a positive feedback promoter of *Atf4*, but the PERK ER stress pathway regulates mammary lipogenesis by a TRB3-independent mechanism. Previous work shows that high concentrations of unsaturated FAs induce mild increases in ER stress gene expression in cultured mammary epithelial cells (34), but the results of the current study do not provide any further insight into this connection.

Strengths and limitations

The KO model combined with analysis of gene expression in the mammary gland provided a robust test of the functional role of TRB3 in mammary lipogenesis. This experiment was built on strong evidence from microarray mRNA data that both *Atf4* and *Trb3* are elevated during lactation, and previous research indicating a role for ER stress in suppression of lipogenesis during MFD (17, 19). In addition, we used 2 different well-investigated treatments known to suppress expression of mammary lipogenesis: an HO diet and oral t10c12CLA. Both these treatments reduce mammary lipogenesis, but only the t10c12CLA was expected to reduce milk fat. If TRB3 mediated these responses the KO mice would have been protected from the effects.

One weakness was that the HO and t10c12CLA treatments shared the same LFD control animals to reduce animal numbers and expense. The control animals were maintained concurrently on the same semipurified base diet, but there was not a true handling control for the oral t10c12CLA treatment administered by pipette. We have previously investigated the effect of conjugated linoleic acid and our treatment responses were similar to those previously observed. In addition, the key comparison was t10c12CLA treatment in WT compared with KO, which was not confounded by handling. Finally, HO diets differ in FA profile; we selected to use a high–linoleic acid oil to minimize interaction of FAs. Each FA has its own bioactive properties and it is difficult to disentangle the effect of oil from the FA profile. The response to the high-fat diet was similar to that reported for other high-fat diets.

Conclusions

Based on these results, TRB3 appears to play a minimal role in the regulation of lipogenesis in the mammary gland. The *Trb3* KO is a powerful model that is expected to clearly reveal functional roles of TRB3 in mammary lipid synthesis if they exist. The absence of a clear response indicates that TRB3 is not a major driver of t10c12CLA-induced MFD. This supports the conclusion that regulation of lipogenesis by the UPR and the PERK pathway are likely occurring upstream of TRB3 through a different ATF4 target.

Acknowledgments

The authors' responsibilities were as follows—REW, LM, CL, and KJH: analyzed the data; REW and KJH: wrote the paper; LM and YY: conducted the research; KJH: designed the research and has primary responsibility for the final content; and all authors: read and approved the final manuscript.

Data Availability

Data described in the article, code book, and analytic code will be made available upon request.

References

1. Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 2000;101(5):451–4.
2. Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H. *TRB3*, a novel ER stress-inducible gene, is induced via ATF4–CHOP pathway and is involved in cell death. EMBO J 2005;24(6):1243–55.
3. Ding J, Kato S, Du K. PI3K activates negative and positive signals to regulate TRB3 expression in hepatic cells. Exp Cell Res 2008;314(7):1566–74.
4. Du K, Herzig S, Kulkarni RN, Montminy M. TRB3: a *tribbles* homolog that inhibits Akt/PKB activation by insulin in liver. Science 2003;300(5625):1574–7.
5. Zhang W, Wu M, Kim T, Jariwala RH, Garvey WJ, Luo N, Kang M, Ma E, Tian L, Steverson D, et al. Skeletal muscle TRIB3 mediates glucose toxicity in diabetes and high-fat diet-induced insulin resistance. Diabetes 2016;65(8):2380–91.
6. Qi L, Heredia JE, Altarejos JY, Screaton R, Goebel N, Niessen S, MacLeod IX, Liew CW, Kulkarni RN, Bain J, et al. TRB3 links the E3 ubiquitin ligase COF1 to lipid metabolism. Science 2006;312(5781):1763–6.
7. Bromati CR, Lellis-Santos C, Yamanaka TS, Nogueira TCA, Leonelli M, Caperuto LC, Gorjão R, Leite AR, Anhê GF, Bordin S. UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression. Am J Physiol Regul Integr Comp Physiol 2011;300(1):R92–R100.
8. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010;140(6):900–17.
9. Bobrovnikova-Marjon E, Hatzivassiliou G, Grigoriadou C, Romero M, Cavener DR, Thompson CB, Diehl JA. PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation. Proc Natl Acad Sci U S A 2008;105(42):16314–19.
10. Harvatine KJ, Bauman DE. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. J Nutr 2006;136(10):2468–74.
11. Harvatine KJ, Robblee MM, Thorn SR, Boisclair YR, Bauman DE. *Trans*-10, *cis*-12 CLA dose-dependently inhibits milk fat synthesis without disruption of lactation in C57BL/6J mice. J Nutr 2014;144(12):1928–34.
12. Kadegowda AKG, Connor EE, Teter BB, Sampugna J, Delmonte P, Piperova LS, Erdman RA. Dietary *trans* fatty acid isomers differ in their effects on mammary lipid metabolism as well as lipogenic gene expression in lactating mice. J Nutr 2010;140(5):919–24.
13. Baldin M, Gama MAS, Dresch R, Harvatine KJ, Oliveira DE. A rumen unprotected conjugated linoleic acid supplement inhibits milk fat synthesis and improves energy balance in lactating goats. J Anim Sci 2013;91(7):3305–14.
14. Kadegowda AKG, Khan MJ, Piperova LS, Teter BB, Rodriguez-Zas SL, Erdman RA, Loor JJ. *Trans*-10, *cis* 12-conjugated linoleic acid-induced milk fat depression is associated with inhibition of PPARγ signaling and inflammation in murine mammary tissue. J Lipids 2013:890343.
15. Adkins Y, Schie IW, Fedor D, Reddy A, Nguyen S, Zhou P, Kelley DS, Wu J. A novel mouse model of nonalcoholic steatohepatitis with significant insulin resistance. Lab Invest 2013;93(12):1313–22.
16. Shahzad MMK, Felder M, Ludwig K, Van Galder HR, Anderson ML, Kim J, Cook ME, Kapur AK, Patankar MS. Trans10,cis12 conjugated

linoleic acid inhibits proliferation and migration of ovarian cancer cells by inducing ER stress, autophagy, and modulation of Src. PLoS One 2018;13(1): e0189524.
17. Ou L, Wu Y, Ip C, Meng X, Hsu Y-C, Ip MM. Apoptosis induced by t10,c12-conjugated linoleic acid is mediated by an atypical endoplasmic reticulum stress response. J Lipid Res 2008;49(5):985–94.
18. Hsu Y-C, Ip MM. Conjugated linoleic acid-induced apoptosis in mouse mammary tumor cells is mediated by both G protein coupled receptor-dependent activation of the AMP-activated protein kinase pathway and by oxidative stress. Cell Signal 2011;23(12): 2013–20.
19. Davis KR, Giesy SL, Long Q, Krumm CS, Harvatine KJ, Boisclair YR. XBP1 regulates the biosynthetic capacity of the mammary gland during lactation by controlling epithelial expansion and endoplasmic reticulum formation. Endocrinology 2016;157(1):417–28.
20. Rudolph MC, McManaman JL, Hunter L, Phang T, Neville MC. Functional development of the mammary gland: use of expression profiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation, and involution. J Mammary Gland Biol Neoplasia 2003;8(3):287–307.
21. Okamoto H, Latres E, Liu R, Thabet K, Murphy A, Valenzeula D, Yancopoulos GD, Stitt TN, Glass DJ, Sleeman MW. Genetic deletion of Trb3, the mammalian *Drosophila tribbles* homolog, displays normal hepatic insulin signaling and glucose homeostasis. Diabetes 2007;56(5): 1350–6.
22. Robblee MM, Boisclair YR, Bauman DE, Harvatine KJ. Dietary fat does not overcome *trans*-10, *cis*-12 conjugated linoleic acid inhibition of milk fat synthesis in lactating mice. Lipids 2020;55(3):201–12.
23. Kramer JKG, Fellner V, Dugan MER, Sauer FD, Mossoba MM, Yurawecz MP. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids. Lipids 1997;32(11):1219–28.
24. Urrutia N, Harvatine KJ. Effect of conjugated linoleic acid and acetate on milk fat synthesis and adipose lipogenesis in lactating dairy cows. J Dairy Sci 2017;100(7):5792–804.
25. Wang YW, Jones PJH. Conjugated linoleic acid and obesity control: efficacy and mechanisms. Int J Obes 2004;28(8):941–55.
26. Foote MR, Giesy SL, Bernal-Santos G, Bauman DE, Boisclair YR. *t*10,*c*12-CLA decreases adiposity in peripubertal mice without dose-related detrimental effects on mammary development, inflammation status, and metabolism. Am J Physiol Regul Integr Comp Physiol 2010;299(6): R1521–8.
27. Masters N, McGuire MA, Beerman KA, Dasgupta N, McGuire MK. Maternal supplementation with CLA decreases milk fat in humans. Lipids 2002;37(2):133–8.
28. Mosley SA, Shahin AM, Williams J, McGuire MA, McGuire MK. Supplemental conjugated linoleic acid consumption does not influence milk macronutrient contents in all healthy lactating women. Lipids 2007;42(8):723–9.
29. Pang K, Zhu Z, Zhu S, Han L. A high dose of conjugated linoleic acid increases fatty liver and insulin resistance in lactating mice. PLoS One 2019;14(8):e0214903.
30. Cordoba-Chacon J, Sugasini D, Yalagala PCR, Tummala A, White ZC, Nagao T, Kineman RD, Subbaiah PV. Tissue-dependent effects of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers on glucose and lipid metabolism in adult male mice. J Nutr Biochem 2019;67:90–100.
31. Adkins Y, Belda BJ, Pedersen TL, Fedor DM, Mackey BE, Newman JW, Kelley DS. Dietary docosahexaenoic acid and *trans*-10, *cis*-12-conjugated linoleic acid differentially alter oxylipin profiles in mouse periuterine adipose tissue. Lipids 2017;52(5):399–413.
32. Rudolph MC, Wellberg EA, Lewis AS, Terrell KL, Merz AL, Maluf NK, Serkova NJ, Anderson SM. Thyroid hormone responsive protein Spot14 enhances catalysis of fatty acid synthase in lactating mammary epithelium. J Lipid Res 2014;55(6):1052–65.
33. Deng Y, Wang ZV, Tao C, Gao N, Holland WL, Ferdous A, Repa JJ, Liang G, Ye J, Lehrman MA, et al. The Xbp1s/GalE axis links ER stress to postprandial hepatic metabolism. J Clin Invest 2013;123(1):455–68.
34. Sharmin MM, Mizusawa M, Hayashi S, Arai W, Sakata S, Yonekura S. Effects of fatty acids on inducing endoplasmic reticulum stress in bovine mammary epithelial cells. J Dairy Sci 2020;103(9):8643–54.