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

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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 ≥ 8/group) and second (n ≥ 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 ≥ 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; Pint = 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

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Abbreviations used: ATF, activating transcription factor; c/EBPα, CCAAT enhancer binding protein α; 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 steraryl 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. 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/EBPβ), 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 among 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 on 2 consecutive days multiple times 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 semi-purified 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

| Ingredients                        | WT % | % kcal | LFD % | % kcal | HO % | % kcal |
|------------------------------------|------|--------|-------|--------|------|--------|
| 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 H2O           |      |        | 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|        |
| 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 | 20.3   |

1Composition provided as weight percentage of diet and as a percentage of calories (% kcal). HO, high safflower oil diet; LFD, semipurified low-fat diet.
2Standard 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, brewers dried yeast, dicalcium phosphate, calcium carbonate, menadione sodium bisulfite complex (source of vitamin K activity), manganese oxide, copper sulfate, zinc oxide, niacin, thiamin mononitrate, vitamin A acetate, vitamin D3 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 one-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 genespecific 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 1A, 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](https://academic.oup.com/cdn/article-6/1/nzab142/6515538)

**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%, Pgenotype = 0.03; capric acid: −13%, Pgenotype = 0.04) and higher palmitic acid (16:0) (7%, Pgenotype = 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 (Pint = 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.
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 pup growth, but decreases de novo lipogenesis in the mammary gland. Increasing dietary fat generally increases milk fat concentration and FA profile. The HO diet increased milk fat by 8% in WT mice, but not in Trb3 KO mice (P<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<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<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-

![Figure 3](https://academic.oup.com/cdn/article/6/1/nzab142/6515538/download)

**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.
TRB3 deletion does not modify milk fat

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

| 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         | 0.007 | 0.008 | 0.008 | 0.001 | 0.41    |
| Di C16         | 0.044 | 0.047 | 0.051 | 0.004 | 0.57    |

1DI, desaturase index; FA, fatty acid; HET, heterozygous; KO, knockout; WT, wild-type.
2FAs 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.
3DI C14 = 14:1/(14:0 + 14:1).
4DI C16 = 16:1/(16:0 + 16:1).

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

| Treatment2 | LFD | t10c12CLA | P values |
|------------|-----|-----------|---------|
|            | WT  | KO        | WT      | KO      | SE | Diet | GT | Diet × GT |
| Mass,3 g   |     |           |         |         |    |      |    |          |
| Dam        |     |           |         |         |    |      |    |          |
| Intake     | 12.0a | 11.8a | 8.89b | 8.49b | 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 |
| Mesentric adipose | 0.45ab | 0.48a | 0.39b | 0.42ab | 0.02 | 0.009 | 0.08 | 0.95 |
| Litter     |     |           |         |         |    |      |    |          |
| BW         | 46.4a | 45.8a | 43.0b | 42.1b | 0.68 | <0.0001 | 0.27 | 0.89 |
| BW gain    | 2.92a | 2.74a | 1.27b | 1.04b | 0.19 | <0.0001 | 0.31 | 0.89 |

1Levels 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.
2All 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.
3Weights 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

| Treatment | LFD | t10c12CLA | Treatment | FA, g/100 g FA | P values | GT Values | Treatment*GT P values |
|-----------|-----|-----------|-----------|----------------|----------|-----------|----------------------|
| FA profile | WT | KO | WT | KO | SE | WT | KO | SE | WT | KO | SE |
| Total fat % | 61b | 62a | 55a | 54b | 1.2 | <0.0001 | 0.83 | 0.19 | |
| FA profile | 8:0 | 0.42b | 0.33b | 0.32b | 0.34b | 0.02 | 0.08 | 0.15 | 0.03 | |
| 10:0 | 6.81b | 5.91a | 8.54a | 8.25a | 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 | 18.2b | 18.0a | 13.8b | 14.1b | 0.53 | <0.0001 | 0.90 | 0.69 | |
| 14:1 | 0.27b | 0.29a | 0.12b | 0.12b | 0.01 | <0.0001 | 0.60 | 0.49 | |
| 16:0 | 27.6b | 29.6a | 21.5 | 22.6a | 0.54 | <0.0001 | 0.008 | 0.45 | |
| 16:1 | 2.19b | 2.41a | 2.01a | 1.85a | 0.08 | 0.0002 | 0.77 | 0.04 | |
| 18:0 | 2.15b | 2.25a | 2.79b | 2.90a | 0.07 | <0.0001 | 0.13 | 0.96 | |
| 18:1, c9 | 14.6b | 14.6b | 18.3b | 18.0a | 0.68 | <0.0001 | 0.83 | 0.80 | |
| 18:1, c11 | 1.75b | 1.76b | 2.24a | 2.02a | 0.09 | 0.0002 | 0.26 | 0.19 | |
| 18:2 | 7.03b | 6.90b | 8.64a | 8.88a | 0.23 | <0.0001 | 0.001 | 0.81 | |
| 18:3–6 | 0.07b | 0.06b | 0.10a | 0.10a | 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 | |
| 20:3–6 | 0.05b | 0.59a | 0.66b | 0.68a | 0.03 | 0.008 | 0.53 | 0.95 | |
| CLA, c9, t11 | 0.04b | 0.04a | 0.69b | 0.73a | 0.05 | <0.0001 | 0.75 | 0.68 | |
| CLA, t10, c12 | 0.00b | 0.00a | 0.51b | 0.54a | 0.04 | <0.0001 | 0.73 | 0.73 | |
| 20:2 | 0.64b | 0.60b | 0.76a | 0.78a | 0.04 | 0.0006 | 0.87 | 0.43 | |
| 20:3–6 | 0.42 | 0.40 | 0.41 | 0.38 | 0.02 | 0.64 | 0.14 | 0.28 | |
| 20:4–6 | 0.35b | 0.28b | 0.59b | 0.59b | 0.03 | <0.0001 | 0.25 | 0.30 | |
| 20:5–3 | 0.10 | 0.09 | 0.09 | 0.09 | 0.02 | 0.79 | 0.87 | 0.73 | |
| 24:0 | 0.04b | 0.05a | 0.10a | 0.10a | 0.02 | 0.004 | 0.79 | 0.79 | |
| 24:1 | 0.11 | 0.09 | 0.13 | 0.09 | 0.01 | 0.16 | 0.12 | 0.11 | |
| 24:2–6 | 0.17 | 0.15 | 0.16 | 0.19 | 0.01 | 0.29 | 0.88 | 0.86 | |
| 24:3–5 | 0.10 | 0.09 | 0.16 | 0.15 | 0.007 | <0.0001 | 0.34 | 0.48 | |
| 26:2–3 | 0.12b | 0.11b | 0.14b | 0.16a | 0.02 | 0.02 | 0.63 | 0.25 | |
| 26:3–5 | 0.24b | 0.24a | 0.29b | 0.29b | 0.16 | 0.05 | 0.10 | 0.27 | |

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). FA, fatty acid; GT, genotype; KO, knockout; LFD, semipurified low-fat diet; t10c12CLA, trans-10, cis-12 conjugated linoleic acid; WT, wild-type.

### Table 6: Interaction of tribbles pseudokinase 3 (Trb3) deletion and an HO diet on growth and milk production

| Treatment | LFD | HO | SE | p values | Diet | GT | Diet*GT |
|-----------|-----|----|----|---------|------|----|--------|
| Mass, g | WT | KO | WT | KO | SE | Diet | GT | Diet*GT |
| Dam Intake | 11.8b | 11.6a | 10.0b | 8.77b | 0.57 | 0.001 | 0.21 | 0.38 |
| BW | 28.7a | 29.1a | 27.6b | 26.7b | 0.52 | 0.005 | 0.65 | 0.20 |
| BW gain | 0.14 | 0.27 | 0.005 | 1.23 | 0.55 | 0.015 | 0.35 | 0.25 |
| Liver | 4.22b | 4.30a | 1.95b | 1.80b | 0.12 | 0.001 | 0.93 | 0.21 |
| Gonadal adipose | 0.22 | 0.23 | 0.10 | 0.16 | 0.03 | 0.009 | 0.78 | 0.60 |
| Mesenteric adipose | 0.45b | 0.48a | 0.41b | 0.41b | 0.02 | 0.004 | 0.28 | 0.32 |

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).
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 adipose.

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

| Treatment<sup>2</sup> | FA (% of total) | FA profile | LFD | HO | Diet<sup>1</sup> | GT<sup>1</sup> | Diet*GT<sup>1</sup> |
|----------------------|----------------|------------|-----|----|----------------|-------------|-----------------|
|                      | FA profile     |            | WT  | KO | LFD | HO          | SE | P values | P values | P values |
| Total fat %          | 61<sup>b</sup> | 62<sup>b</sup> | 66<sup>a</sup> | 62<sup>b</sup> | 0.01 | 0.08 | 0.27 | 0.04 |
| FA profile           | 8:0            | 0.42<sup>a</sup> | 0.33<sup>b</sup> | 0.19<sup>d</sup> | 0.28<sup>c</sup> | 0.02 | <0.0001 | 0.94 | <0.0001 |
|                      | 10:0           | 6.81<sup>a</sup> | 5.91<sup>b</sup> | 4.34<sup>d</sup> | 5.12<sup>c</sup> | 0.16 | <0.0001 | 0.69 | <0.0001 |
|                      | 12:0           | 13.1<sup>a</sup> | 12.4<sup>a</sup> | 7.7<sup>c</sup> | 8.9<sup>b</sup> | 0.27 | <0.0001 | 0.42 | 0.002 |
|                      | 14:0           | 18.2<sup>a</sup> | 18.3<sup>a</sup> | 9.7<sup>c</sup> | 11.2<sup>b</sup> | 0.45 | <0.0001 | 0.08 | 0.11 |
|                      | 14:1           | 0.27<sup>a</sup> | 0.29<sup>a</sup> | 0.06<sup>b</sup> | 0.08<sup>b</sup> | 0.009 | <0.0001 | 0.16 | 0.92 |
|                      | 16:0           | 27.6<sup>a</sup> | 29.6<sup>a</sup> | 18.4<sup>b</sup> | 19.7<sup>b</sup> | 0.81 | <0.0001 | 0.06 | 0.68 |
|                      | 16:1           | 2.19<sup>b</sup> | 2.41<sup>a</sup> | 0.76<sup>c</sup> | 0.82<sup>c</sup> | 0.06 | <0.0001 | 0.03 | 0.21 |
|                      | 18:0           | 2.15<sup>b</sup> | 2.25<sup>b</sup> | 2.55<sup>a</sup> | 2.47<sup>a</sup> | 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<sup>c</sup> | 6.90<sup>c</sup> | 33.0<sup>a</sup> | 30.3<sup>b</sup> | 0.83 | <0.0001 | 0.10 | 0.13 |
|                      | 18:3n–6        | 0.07<sup>c</sup> | 0.05<sup>c</sup> | 0.26<sup>a</sup> | 0.17<sup>b</sup> | 0.02 | <0.0001 | 0.01 | 0.06 |
|                      | 20:1           | 0.69<sup>a</sup> | 0.67<sup>a</sup> | 0.46<sup>b</sup> | 0.42<sup>b</sup> | 0.05 | <0.0001 | 0.56 | 0.85 |
|                      | 18:3n–3        | 0.58<sup>a</sup> | 0.59<sup>a</sup> | 0.40<sup>b</sup> | 0.37<sup>b</sup> | 0.02 | <0.0001 | 0.57 | 0.14 |
|                      | CLA, c9, t11   | 0.04<sup>a</sup> | 0.04<sup>a</sup> | 0.00<sup>b</sup> | 0.00<sup>b</sup> | 0.003 | <0.0001 | 0.51 | 0.51 |
|                      | 20:2           | 0.64<sup>b</sup> | 0.60<sup>b</sup> | 1.92<sup>a</sup> | 1.76<sup>a</sup> | 0.10 | <0.0001 | 0.32 | 0.54 |
|                      | 20:3n–6        | 0.42<sup>c</sup> | 0.38<sup>c</sup> | 0.97<sup>a</sup> | 0.70<sup>b</sup> | 0.05 | <0.0001 | 0.003 | 0.02 |
|                      | 20:4n–6        | 0.35<sup>b</sup> | 0.28<sup>b</sup> | 0.90<sup>a</sup> | 0.49<sup>b</sup> | 0.09 | 0.0002 | 0.01 | 0.06 |
|                      | 20:5n–3        | 0.10<sup>a</sup> | 0.09<sup>a</sup> | 0.03<sup>b</sup> | 0.01<sup>b</sup> | 0.005 | <0.0001 | 0.03 | 0.58 |
|                      | 24:0           | 0.04<sup>b</sup> | 0.04<sup>b</sup> | 0.08<sup>a</sup> | 0.08<sup>a</sup> | 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<sup>b</sup> | 0.15<sup>b</sup> | 0.28<sup>a</sup> | 0.18<sup>b</sup> | 0.02 | 0.001 | 0.006 | 0.08 |
|                      | 22:5n–3        | 0.10<sup>a</sup> | 0.09<sup>a</sup> | 0.04<sup>b</sup> | 0.02<sup>c</sup> | 0.007 | <0.0001 | 0.008 | 0.27 |
|                      | 22:6n–3        | 0.12<sup>a</sup> | 0.11<sup>a</sup> | 0.11<sup>a</sup> | 0.05<sup>b</sup> | 0.01 | 0.003 | 0.005 | 0.04 |
|                      | Unknown        | 2.43<sup>a</sup> | 2.34<sup>a</sup> | 1.76<sup>b</sup> | 1.91<sup>b</sup> | 0.12 | 0.0001 | 0.81 | 0.32 |

<sup>1</sup>Levels not connected by the same letter<sup>a,b,c,d</sup> are significantly different by ANOVA with protected least significant difference post hoc test<sup>P </sup>&lt;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.

<sup>2</sup>Diet was 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.

<sup>3</sup>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.

<sup>4</sup>Di C14 = 14:1/14:0 + 14:1.

<sup>5</sup>Di C16 = 16:1/16:0 + 16:1.
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 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 pathways 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. Afp4 expression was decreased in Trb3 KO mice. It is possible that TRB3 acts as a positive feedback promoter of Afp4, 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 Afp4 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.

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**Data Availability**

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

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