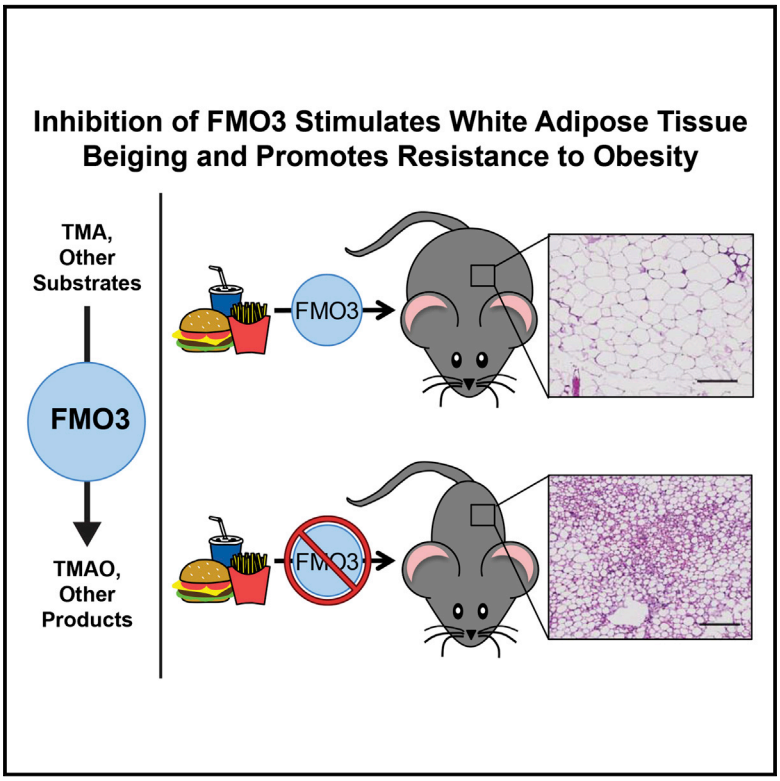
Cell Reports

The TMAO-Producing Enzyme Flavin-Containing Monooxygenase 3 Regulates Obesity and the Beiging of White Adipose Tissue

Graphical Abstract



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In Brief

Microbes resident in the human intestine represent a key transmissible environmental factor contributing to obesity and related disorders. Schugar et al. now show that expression of the TMAO-producing enzyme FMO3 is linked to obesity and energy expenditure in both mice and humans.

Highlights

- Plasma TMAO levels are elevated in type 2 diabetic patients
- Levels of the TMAO-producing enzyme FMO3 in adipose tissue correlate with obesity
- Pharmacologic and genetic inhibition of *Fmo3* stimulates white adipose tissue beiging
- Inhibition of *Fmo3* promotes resistance to obesity

Schugar et al., 2017, Cell Reports *19*, 2451–2461
June 20, 2017
http://dx.doi.org/10.1016/j.celrep.2017.05.077

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Report

The TMAO-Producing Enzyme Flavin-Containing Monooxygenase 3 Regulates Obesity and the Beiging of White Adipose Tissue

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http://dx.doi.org/10.1016/j.celrep.2017.05.077

SUMMARY

Emerging evidence suggests that microbes resident in the human intestine represent a key environmental factor contributing to obesity-associated disorders. Here, we demonstrate that the gut microbiota-initiated trimethylamine N-oxide (TMAO)-generating pathway is linked to obesity and energy metabolism. In multiple clinical cohorts, systemic levels of TMAO were observed to strongly associate with type 2 diabetes. In addition, circulating TMAO levels were associated with obesity traits in the different inbred strains represented in the Hybrid Mouse Diversity Panel. Further, antisense oligonucleotide-mediated knockdown or genetic deletion of the TMAO-producing enzyme flavin-containing monooxygenase 3 (FMO3) conferred protection against obesity in mice. Complimentary mouse and human studies indicate a negative regulatory role for FMO3 in the beiging of white adipose tissue. Collectively, our studies reveal a link between the TMAO-producing enzyme FMO3 and obesity and the beiging of white adipose tissue.

INTRODUCTION

There is strong evidence that microbes resident in the human intestine represent a key environmental factor contributing to obesity and associated insulin resistance (Bäckhed et al., 2004; Ley et al., 2005; Turnbaugh and Gordon, 2009; Cox et al., 2014). However, the molecular mechanisms by which gut microbiota promote obesity and insulin resistance in humans are incompletely understood. Recently, several independent groups have identified the gut microbiota-initiated trimethylamine (TMA)/flavin-containing monooxygenase 3 (FMO3)/trimethylamine N-oxide (TMAO) pathway as a potential modulator of cardiometabolic phenotypes in the host (Wang et al., 2011; Warrier et al., 2015; Miao et al., 2015; Shih et al., 2015), although our mechanistic understanding of this meta-organismal pathway is still incomplete. The TMA/FMO3/TMAO pathway is a microbe-to-host endocrine axis by which gut microbial metabolism of nutrients common in Western diets (phosphatidylcholine, choline, and *L*-carnitine) results in the production of the metabolite TMA,

which is exclusively generated by certain communities of gut microbiota (Wang et al., 2011; Koeth et al., 2013; Gregory et al., 2015; Romano et al., 2015). Then, the host hepatic enzyme flavin-containing monooxygenase 3 (FMO3) further metabolizes gut microbe-derived TMA to produce TMAO (Wang et al., 2011; Bennett et al., 2013). Importantly, the end product of this meta-organismal nutrient metabolism pathway, TMAO, is both a prognostic biomarker and mechanistically linked to cardiovascular disease (CVD) pathogenesis in humans (Wang et al., 2011, 2014, 2015; Koeth et al., 2013, 2014; Tang et al., 2013, 2014; Suzuki et al., 2016; Missailidis et al., 2016; Mafune et al., 2016; Trøseid et al., 2015; Zhu et al., 2016). Given the strong link between gut microbiota and both obesity and obesity-related disease in humans, and the links between the TMAO pathway and cardiometabolic diseases, we hypothesized that the TMAO pathway may be mechanistically linked to the pathogenesis of obesity. Here, we show that both antisense oligonucleotide-mediated knockdown and genetic deletion of the TMAO-producing enzyme FMO3 protect mice against high-fat diet-induced obesity, in part by stimulating the beiging of white adipose tissue, which may reduce the adverse effects of increased adiposity and improve overall metabolic health (Bartelt and Heeren, 2014). Collectively, our studies have uncovered a link between the gut microbe-driven TMA/FMO3/TMAO pathway and adipose tissue function.

RESULTS

Elevated Systemic Levels of TMAO Are Associated with Type 2 Diabetes in Humans

To first establish clinical relevance, we investigated the relationship of fasting plasma levels of choline or TMAO with type 2 diabetes mellitus (T2DM) risk in two independent cohorts of subjects undergoing elective cardiac risk factor evaluation and recommendations in our preventative cardiology clinic (n = 187) or evaluation for suspected non-alcoholic fatty liver disease in our hepatology clinic (n = 248). Patient demographics, laboratory values, and clinical characteristics for both cohorts (n = 435 combined) are provided in Tables S1–S3. Plasma concentrations of TMAO were significantly higher in subjects with T2DM in each of the individual cohorts and when the cohorts were combined (Figure 1A). Fasting choline levels were significantly higher only in T2DM subjects from the hepatology cohort (Figure 1B). Similarly, we observed a dose-dependent association between higher TMAO concentrations and the presence of T2DM (Figure 1C), while the association between choline and T2DM was seen only in the hepatology cohort (Figure 1D). After adjustments for multiple comorbidities, prevalent CVD and CVD risk factors, medications, and renal function, TMAO remained a strong predictor of T2DM risk in both cohorts analyzed alone, as well as when the cohorts were combined (Figure S1). Collectively, these data suggest that circulating levels of the meta-organismal metabolite TMAO are closely correlated with T2DM risk in humans.

Plasma TMAO Levels in Mice and *FMO3* mRNA Expression in Men Demonstrate Positive Correlations with Obesity

First, using a systems genetics approach in mice, we examined various obesity-related traits and circulating TMAO levels in mice from the Hybrid Mouse Diversity Panel (HMDP) fed an obesogenic high-fat and high-sucrose diet (Parks et al., 2013). Across the different inbred strains represented in the Hybrid Mouse Diversity Panel, circulating levels of TMAO were positively associated with body weight, fat mass, mesenteric adiposity, and subcutaneous adiposity (Figures 2A–2D). Given the observed associations between TMAO and obesity across the diverse inbred strains of mice, we next set out to determine whether expression of *FMO3*, which encodes the TMAO-producing enzyme, was differentially expressed in the adipose tissue of overweight or obese humans. To do this, we first examined a random sampling (n = 770) of a large population-based study of Finnish men known as the METSIM study (Stancáková et al., 2009). This study performed dense phenotypic characterization of subjects for characteristics related to adiposity and insulin sensitivity, including adipose biopsies and microarray expression analysis (Stancáková et al., 2009; Civelek et al., 2017). When we examined the correlation between expression levels of all members of the FMO family in adipose tissue with metabolic traits in this population, we found that *FMO3* was positively correlated with body mass index (BMI) and waist-to-hip ratio and negatively correlated with the Matsuda Index (Matsuda and DeFronzo, 1999), which is a measure of insulin sensitivity (Figure 2E). Interestingly, *FMO3* mRNA expression levels in human adipose tissue were significantly negatively correlated with several genes that represent selective markers of beige or brown adipocytes that have recently been reported (Wu et al., 2012; Ussar et al., 2014) (Figure 2E). These data suggest that *FMO3* expression is negatively associated with beiging signatures in human subcutaneous white adipose tissue.

Given that the METSIM study only includes Finnish men, we set out to validate microarray expression data in several distinct cohorts spanning both men and women of European American and African American ethnicity. Importantly, these validation cohorts were also chosen for their gender, ethnic and racial diversity, and because each has been extensively characterized for obesity and cardiometabolic phenotypes (Das et al., 2015; Sharma et al., 2016). The first cohort included 99 white, non-Hispanic Americans (42 males and 57 females) (Das et al., 2015). The second cohort included 260 African Americans (139 males and 121 females) (Sharma et al., 2016). In agreement with findings from the METSIM study (Figure 2E), we found that *FMO3* was positively correlated with BMI and adiposity and negatively correlated with insulin sensitivity in both European American and African American men and women (Figure S2). Also, the primary transcript variant of *FMO3* was negatively correlated with the beige/brown marker genes uncoupling protein 1 (*UCP1*) and PR domain-containing 16 (*PRDM16*) (Figure S2).

In separate studies, we sought to examine whether similar associations were observed between FMO3 expression in human liver and metabolic traits using liver biopsies from obese patients undergoing bariatric surgery and normal weight controls. In contrast to our findings in human adipose tissue (Figures 2E and S2), we did not find significant correlations between liver FMO3 protein levels and metabolic traits in this cohort (Figure S3). Of note, the protein expression of FMO3 in human liver does not significantly differ between males and females in the cohort under study (n = 15 males, n = 35 females; p = 0.79) (Figure S3).

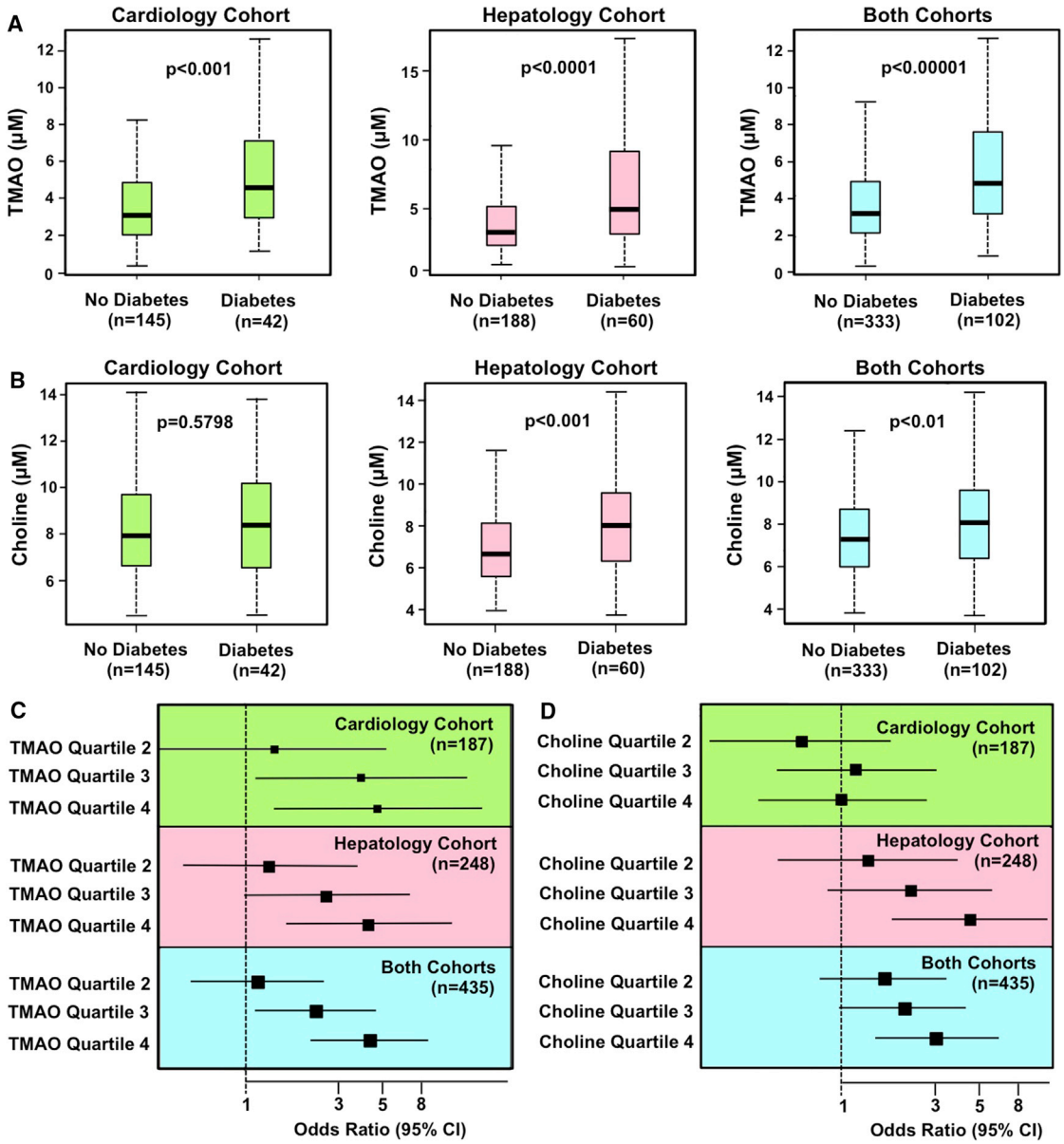


Figure 1. Elevated Circulating Levels of TMAO Are Associated with Type 2 Diabetes Mellitus in Humans

We recruited two separate cohorts of stable subjects in preventative cardiology (n = 187) or hepatology clinics (n = 248) to evaluate the association between fasting circulating choline or trimethylamine N-oxide (TMAO) levels and prevalent type 2 diabetes (T2DM). The total number of subjects recruited in both studies was 435. Patient demographics, laboratory values, and clinical characteristics are provided in Tables S1–S3 and Figure S1.

(A) Relationship of fasting plasma TMAO concentrations and prevalent T2DM. Boxes represent the 25th, 50th, and 75th percentiles of plasma TMAO concentration, and whiskers represent the 10th and 90th percentiles.

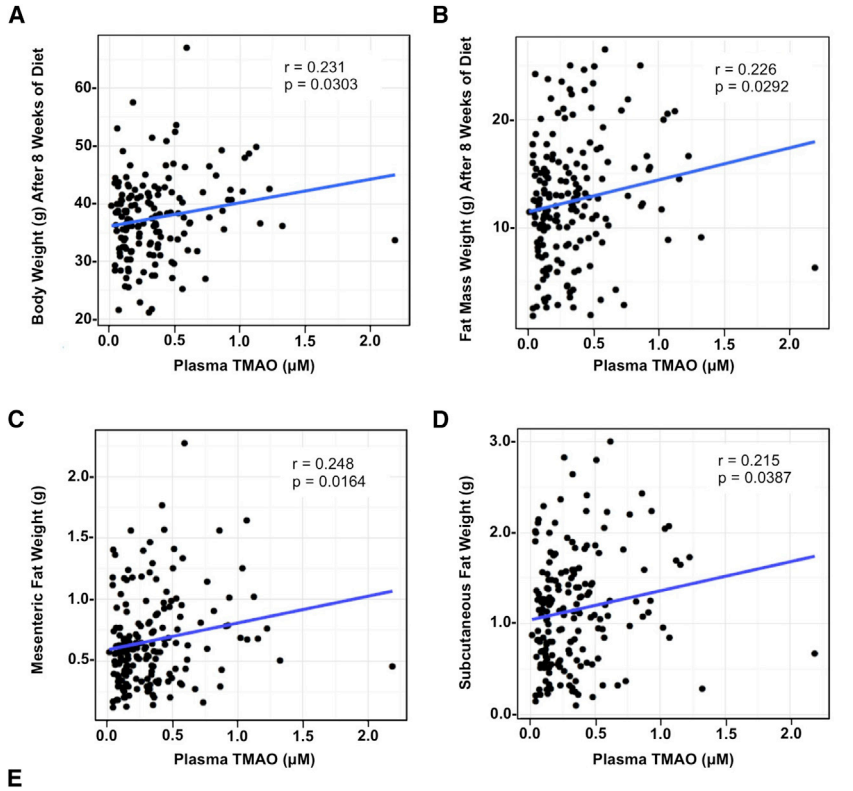
(B) Relationship between fasting plasma choline concentrations and prevalent T2DM. Boxes represent the 25th, 50th, and 75th percentiles of plasma choline concentration, and whiskers represent the 10th and 90th percentiles.

(C) Forest plots of the odds ratio of prevalent T2DM and quartiles of TMAO; bars represent 95% confidence intervals.

(D) Forest plots of the odds ratio of prevalent T2DM and quartile of choline; bars represent 95% confidence intervals.

Knockdown of FMO3 Protects Mice from High-Fat Diet-Induced Obesity by Stimulating the Beiging of White Adipose Tissue

To further examine the potential of the meta-organismal TMAO pathway to impact obesity, we utilized a second-generation antisense oligonucleotide (ASO) to inhibit the expression of *Fmo3* in mice challenged with a high-fat diet (HFD) (Figure 3). *Fmo3* ASO treatment resulted in >90% knockdown of *Fmo3* mRNA levels in mouse liver in both chow-fed and high-fat diet-fed mice (Figure 3A). In line with this, FMO3 knockdown caused accumulation of the FMO3 substrate TMA in mice on both diets, while reductions in its product, TMAO, were seen only in chow-fed mice



E

Association Between WAT *FMO3* Expression and Metabolic Traits or Beige Adipocyte Marker Genes in 770 men

	Metabolic Traits						Brown/Beige Adipocyte Marker Genes									
	Body Mass Index		Waist-to-Hip Ratio		Matsuda Index		*PRDM16* 11742187_a_at		*P2RX5* 11721388_a_at		*KCNK3* 117726446_a_at		*TMEM26* 11734401_a_at		*TBX1* 11732880_at	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
FMO3	0.233	6.1*E*-11	0.185	2.3*E*-07	-0.110	2.3*E*-03	-0.124	5.6*E*-04	-0.229	1.3*E*-10	-0.177	8.2*E*-07	-0.161	7.6*E*-06	-0.131	2.7*E*-04

Figure 2. Plasma TMAO Levels in Mice and *FMO3* mRNA Expression in Men Demonstrate Positive Correlations with Obesity

(A–D) Correlation of plasma trimethylamine N-oxide (TMAO) levels with obesity-related traits in 180 male mice from 92 inbred strains within the Hybrid Mouse Diversity Panel (HMDP) after 8-week feeding of a high-fat and high-sucrose diet. Correlation coefficient (r) and p value (p) are indicated for each obesity trait. (A) Correlation between plasma TMAO and body weight. (B) Correlation between plasma TMAO and fat mass. (C) Correlation between plasma TMAO and mesenteric fat weight. (D) Correlation between plasma TMAO and subcutaneous fat weight.

(E) Correlations between human white adipose tissue flavin monooxygenase 3 (*FMO3*) mRNA expression and metabolic traits or brown/beige adipocyte marker gene expression (n = 770). The gene name and probe set ID is provided for each of the brown/beige adipocyte marker genes.

(Figures 3B and 3C). Indeed, the overall lower TMAO levels in the high-fat diet groups may be due to less overall choline substrate in the high-fat diet (Table S4). Despite documenting comparable food intake on high-fat diet (Figure S4D), FMO3 knockdown resulted in significantly decreased body weight gain (Figure 3D). The attenuation in body weight gain observed appeared to be largely attributed to decreased white adipose tissue weight (Figure 3E), with magnetic resonance imaging (Figure 3F) demonstrating that both peritoneal (Figure 3G) and subcutaneous (Figure 3H) adipose tissue mass were markedly reduced with *Fmo3* ASO treatment. Collectively, *Fmo3* ASO treatment altered body composition, reducing the overall percentage of fat mass (Figure 3I), while increasing the percent of lean mass (Figure 3J) in the high-fat diet-fed cohort. In additional studies, gonadal white adipose tissue *Fmo3* mRNA levels were observed to be ~1,000-fold lower than hepatic *Fmo3* mRNA levels (Figure S4A). Importantly, *Fmo3* ASO treatment protected against high-fat diet-induced obesity in both female (Figure 3) and male (Figure S4B) mice, despite sexual dimorphism in total FMO activity in liver and gonadal white adipose tissue (Figure S4C).

Given the fact that *FMO3* expression was negatively correlated with brown and beige adipocyte gene markers in human adipose tissue (Figure 2E), we next examined the effect of FMO3 knockdown on thermogenic reprogramming in adipose tissue (Figure 4). An initial clue that FMO3 knockdown may alter thermogenic programs was that gonadal adipose depots had morphologic (lipid droplet multi-locularity) and gene expression profiles that are consistent with the appearance of beige adipocytes (Wu et al., 2012) in a classically white adipose tissue depot (Figures 4A and 4B). In the gonadal white adipose tissue depots, FMO3 knockdown elicited a 5-fold upregulation of the β1 adrenergic receptor (*Adrb1*), a 150-fold increase of uncoupling protein 1 (*Ucp1*), and a 3-fold increase in transmembrane protein 26 (*Tmem26*) (Figure 4B). In parallel, plasma membrane-associated β1-AR abundance was increased 4.7-fold in *Fmo3* ASO-treated mice (Figure 4C), which was associated with a 3-fold increase in cyclic AMP levels in gonadal adipose tissue (Figure 4D). To examine the role of FMO3 in cold-induced transcriptional reprogramming, we housed mice in either room temperature (22°C) or cold (4°C) conditions. Interestingly, the normal cold-induced upregulation of the thermogenic transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*) (Puigserver et al., 1998) was much higher in mice treated with *Fmo3* ASO in multiple adipose depots (Figure 4E).

We next investigated the physiological role of FMO3 in cold-induced thermogenic reprogramming by performing indirect calorimetry in high-fat diet-fed *Fmo3* ASO-treated mice housed at thermoneutrality (30°C), room temperature (22°C), or under cold stress (4°C). FMO3 knockdown increased oxygen consumption (VO_2) and heat production under all temperature conditions (Figures 4F and 4G). Interestingly, the normal cold-induced increase in VO_2 and heat seen in control mice was significantly enhanced in the *Fmo3* ASO-treated mice housed at 4°C (Figures 4F and 4G). It is well documented that the

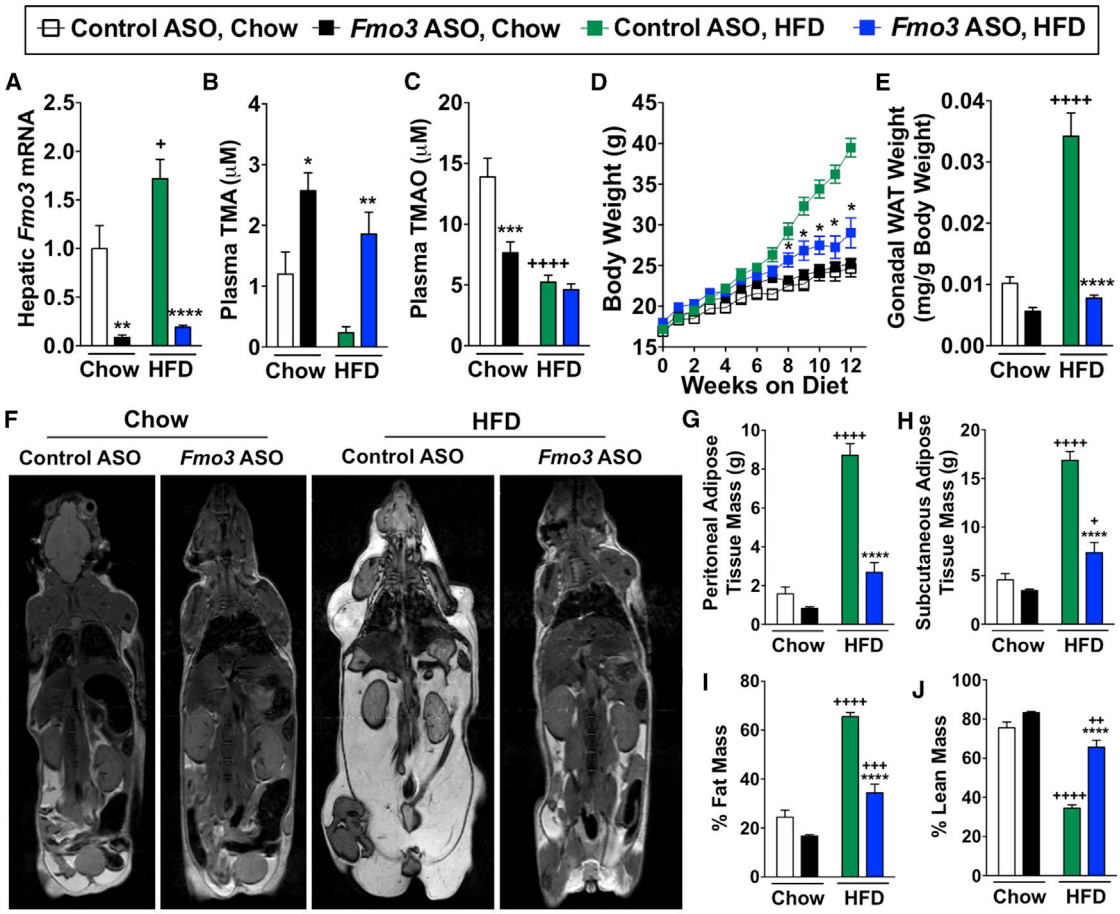


Figure 3. FMO3 Knockdown Protects Mice from High-Fat Diet-Induced Obesity by Stimulating the Beiging of White Adipose Tissue

At 6–8 weeks of age, female C57BL/6 mice were treated with either a non-targeting control ASO or *Fmo3* ASO in conjunction with either standard rodent chow or high-fat diet (HFD) feeding for the indicated times.

(A) Hepatic *Fmo3* mRNA expression was quantified by qPCR after 10 weeks.

(B) Plasma levels of TMA after 6 weeks.

(C) Plasma levels of TMAO after 6 weeks.

(D) Body weight changes over 12 weeks.

(E) Gonadal white adipose tissue (WAT) weight at necropsy.

(F–J) MRI images and subsequent quantification of adiposity in control and *Fmo3* ASO-treated mice maintained on diets for 14 weeks. (F) MRI images of control and *Fmo3* ASO-treated mice. (G) Peritoneal adipose tissue mass. (H) Subcutaneous adipose tissue mass. (I) Fat mass (%). (J) Lean mass (%).

All data represent the mean ± SEM for n = 5–10 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 versus control ASO-treated mice fed the same diet; +p ≤ 0.05, ++p ≤ 0.01, +++p ≤ 0.001, ++++p ≤ 0.0001 versus chow-fed mice treated with the same ASO.

preferred macronutrient fuel source (carbohydrates versus fats) differs under fasted and fed states as well as during cold stress, and dysregulation of this metabolic flexibility is thought to be a key component of the metabolic syndrome (Muoio, 2014). High-fat diet-fed *Fmo3* ASO-treated mice have a marked improvement in metabolic flexibility in response to feeding (during the dark cycle) and cold (Figure 4H). In fact, *Fmo3* ASO-treated mice have a slightly larger increase in glucose oxidation (indicated by increased respiratory exchange ratio) during the dark cycle at thermoneutrality, and this enhanced metabolic flexibility becomes much more striking at 22°C and 4°C (Figure 4H). These data suggest that FMO3 inhibition facilitates feeding-induced fuel switching (from fats to carbohydrates), especially under conditions of cold stress. Collectively, these data suggest that knockdown of FMO3 increases energy expenditure and enhances metabolic flexibility under conditions of cold stress.

Given that TMAO levels were linked to adiposity across multiple strains of mice (Figure 2), we hypothesized that TMAO itself may be directly involved in regulating adiposity. Therefore, we set out to determine the involvement of TMAO in the adipose tissue phenotype seen with FMO3 knockdown. To achieve this, we provided TMAO as a dietary supplement as previously described (Warrier et al., 2015) to control and *Fmo3* ASO-treated mice maintained on high-fat diet. Dietary provision of TMAO effectively raised levels of TMAO in the circulation, liver, and white adipose tissue in control and *Fmo3* ASO-treated mice (Figures S4E

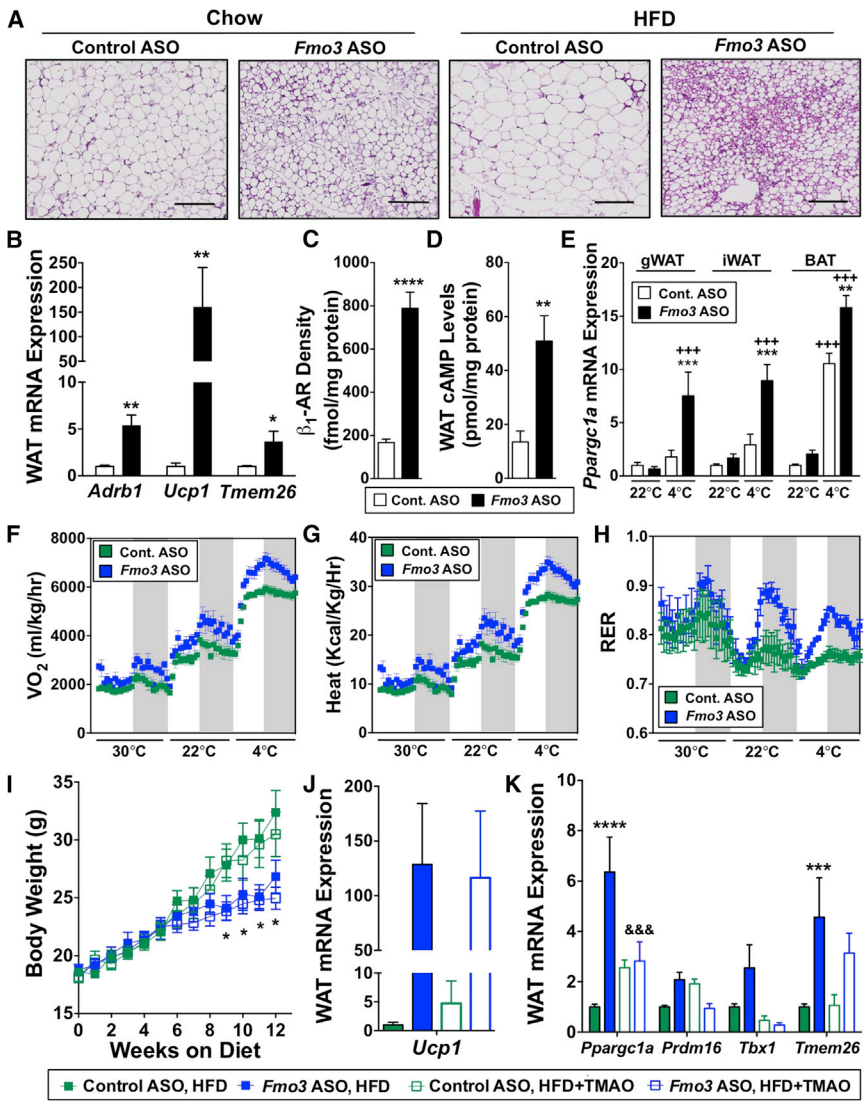


Figure 4. FMO3 Knockdown Stimulates the Beiging of White Adipose Tissue

(A–E) At 6–8 weeks of age, female C57BL/6 mice were treated with either a non-targeting control ASO or *Fmo3* ASO in conjunction with high-fat diet (HFD) feeding for 8–12 weeks. (A) Microscopic examination of H&E-stained gonadal white adipose tissue; scale bar represents 200 μm. (B) Gonadal white adipose tissue (WAT) mRNA expression of β1-adrenergic receptor (*Adrb1*), uncoupling protein 1 (*Ucp1*), and transmembrane protein 26 (*Tmem26*) quantified by qPCR. (C) Surface density of β1-adrenergic receptor (β1-AR) in gonadal white adipose tissue quantified by radio-ligand binding assay. (D) Cyclic AMP (cAMP) levels in gonadal white adipose tissue. (E) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(Ppargc1a)* mRNA expression was quantified by qPCR in gonadal (gWAT), inguinal (iWAT), and brown adipose tissue (BAT).

(F–H) Mice were housed in metabolic cages for indirect calorimetry measurements. Gray background denotes dark cycle. (F) Oxygen consumption (VO_2). (G) Heat production. (H) Respiratory exchange ratio (RER).

(I–K) At 6–8 weeks of age, female C57BL/6 mice were treated with either a non-targeting control ASO or *Fmo3* ASO in conjunction with feeding of HFD or HFD supplemented with 0.02% w/w TMAO for 12 weeks. See also Figure S4.

(I) Body weight changes over 12 weeks.

(J) Gonadal white adipose tissue (WAT) expression of uncoupling protein 1 (*Ucp1*).

(K) Gonadal white adipose tissue (WAT) expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*), PR domain-containing 16 (*Prdm16*), T-box transcription factor (*Tbx1*), and transmembrane protein 26 (*Tmem26*).

All data represent the mean ± SEM for n = 5–10 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 versus control ASO-treated mice fed the same diet; +++p ≤ 0.001 versus chow-fed mice treated with the same ASO; &&&p ≤ 0.001 versus *Fmo3* ASO-treated mice fed an HFD.

and S4F). Interestingly, despite commensurate food consumption (Figure S4D), *Fmo3* ASO-treated mice had significantly lower plasma TMAO levels compared to control ASO-treated mice following dietary supplementation (Figure S4E). Importantly, dietary provision of TMAO did not reverse the ability of *Fmo3* ASO treatment to attenuate high-fat diet-induced body weight gain (Figure 4I). Likewise, dietary TMAO provision did not alter the ability of *Fmo3* ASO treatment to elevate *Ucp1* expression in gonadal white adipose tissue (Figure 4J) However, *Fmo3* ASO-driven increases in gonadal white adipose tissue genes involved in energy metabolism and the development of brown adipose tissue, including *Ppargc1a*, *Prdm16*, and T-box transcription factor (*Tbx1*) were reversed by dietary TMAO provision (Figure 4K). Collectively, these data demonstrate that provision of dietary TMAO can reverse a portion, but not all, of the transcriptional reorganization observed in white adipose tissue driven by FMO3 knockdown and suggest that future studies should investigate the role of additional FMO3 products or substrates in energy metabolism.

Genetic Deletion of the TMAO-Producing Enzyme FMO3 Protects Mice from Obesity

To further examine the role of FMO3 in obesity, we examined global FMO3 knockout (*Fmo3*$^{-/-}$) mice generated using CRISPR-Cas9-mediated gene editing (Figure 5; Table S5). Hepatic FMO3 protein was undetectable by western blot in *Fmo3*$^{-/-}$ mice (Figure 5A). In initial studies, we maintained *Fmo3*$^{-/-}$ mice on a C57BL/6 background and fed a choline-supplemented chow-based diet. Under these non-obesogenic conditions, *Fmo3*$^{-/-}$ mice accumulate plasma TMA and have diminished TMAO as predicted, and while there were no differences in food intake or body weight, they do exhibit significantly reduced adiposity compared to *Fmo3*$^{+/+}$ mice (Figure 5B). To examine effects of FMO3 knockout on adiposity under

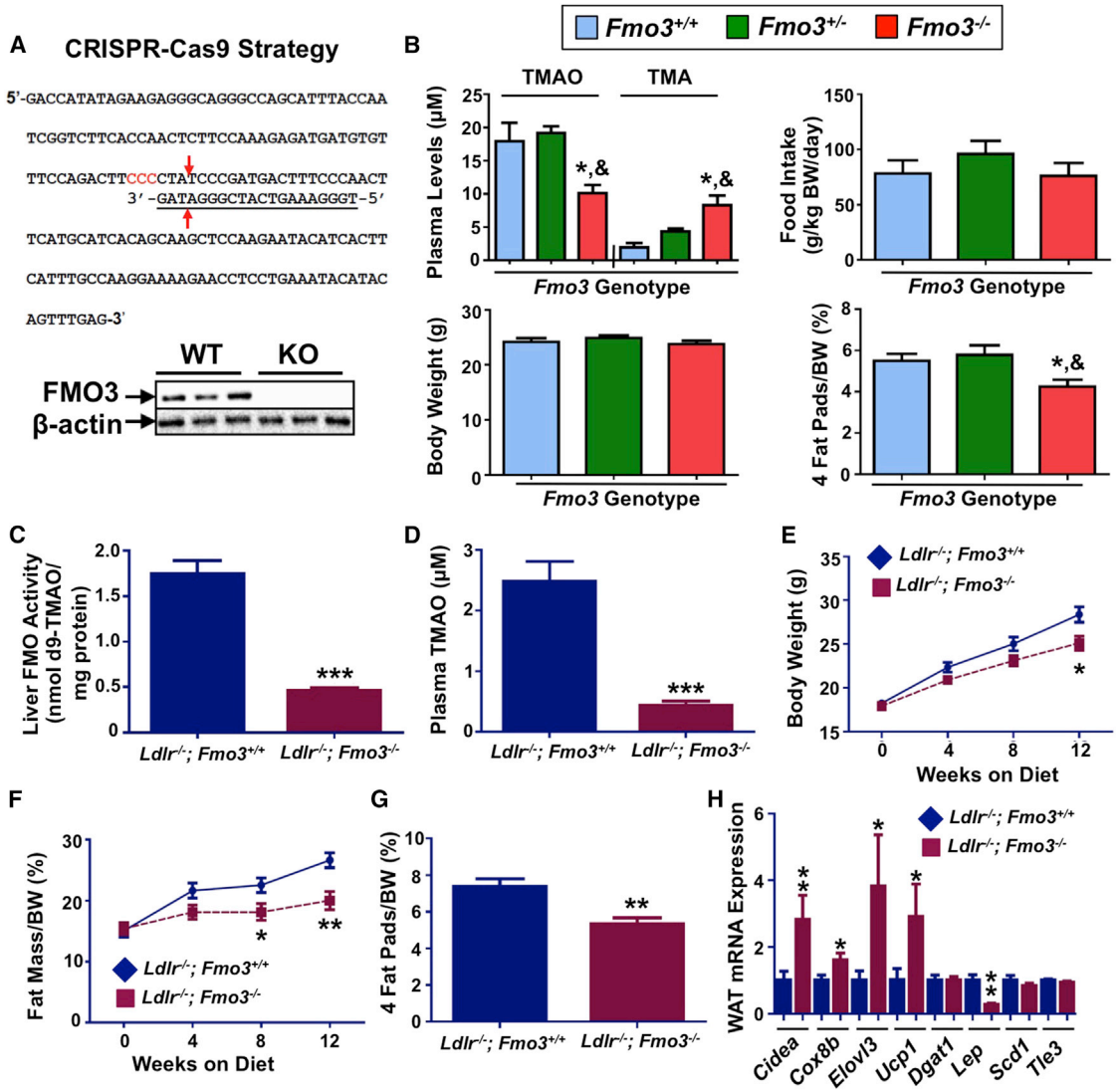


Figure 5. Genetic Deletion of FMO3 Protects Mice from Diet-Induced Obesity

(A) Top: CRISPR-Cas9 strategy for generating *Fmo3*$^{-/-}$ mice. The sequence of exon 2 of the murine *Fmo3* coding sequence is shown. The target sequence (underlined) used for construction of the guide RNA is shown with arrows indicating predicted cleavage sites by Cas9. Bottom: immunoblotting analysis of FMO3 protein levels in the livers of wild-type (WT) and *Fmo3*$^{-/-}$ (KO) mice.

(B) Decreased adiposity in *Fmo3*$^{-/-}$ mice. *Fmo3*$^{+/+}$ (n = 9), *Fmo3*$^{+/-}$ (n = 6), and *Fmo3*$^{-/-}$ (n = 11) mice were fed a 1.3% choline chloride (w/w) diet for 12 weeks before tissue collection. Plasma TMAO and TMA levels (top left), food intake (top right), body weight (bottom left), and four fat pads/body weight (%; bottom right) are shown. The four fat pads included in the four fat pads/body weight measurement were gonadal, mesentery, perirenal, and subcutaneous. *p $\leq$ 0.05 between *Fmo3*$^{+/+}$ and *Fmo3*$^{-/-}$ groups; &p $\leq$ 0.05 between *Fmo3*$^{+/-}$ and *Fmo3*$^{-/-}$ genotype groups.

(C–H) *Ldlr*$^{-/-}$*; Fmo3*$^{-/-}$ mice are more resistant to obesity than *Ldlr*$^{-/-}$ littermates when fed a Western diet for 12 weeks. (C) Liver FMO activity. (D) Plasma TMAO levels. (E) Body weight changes over 12 weeks. (F) Fat mass/body weight (%). (G) Four fat pads weight/body weight (%); the four fat pads measured were gonadal, mesentery, perirenal, and subcutaneous. (H) Gene expression analysis of subcutaneous fat pads of *Ldlr*$^{-/-}$ (n = 17) and *Ldlr*$^{-/-}$*; Fmo3*$^{-/-}$ (n = 9) mice. Cell death-inducing DFFA-like effector A (*Cidea*), cytochrome *c* oxidase subunit 8b (*Cox8b*), elongation of very long chain fatty acids protein 3 (*Elovl3*), uncoupling protein 1 (*Ucp1*), diglyceride acyltransferase 1 (*Dgat1*), leptin (*Lep*), stearoyl coenzyme A (CoA) desaturase-1 (*Scd1*), transducin-like enhancer of split 3 (*Tle3*). *p $\leq$ 0.05, **p $\leq$ 0.01, and ***p $\leq$ 0.0001 between the two genotype groups.

obesogenic conditions we crossed *Fmo3*$^{-/-}$ mice to the low-density lipoprotein knockout (*Ldlr*$^{-/-}$) background and maintained mice on a Western diet (Figures 5C–5H). Western diet-fed *Ldlr*$^{-/-}$*;Fmo3*$^{-/-}$ mice had markedly reduced hepatic FMO activity (Figure 5C) and circulating TMAO levels (Figure 5D) when compared to *Ldlr*$^{-/-}$*;Fmo3*$^{+/+}$ control mice. In agreement with our studies in high-fat diet-fed *Fmo3* ASO-treated mice (Figures 3 and 4), Western-diet-fed *Ldlr*$^{-/-}$*;Fmo3*$^{-/-}$ mice were protected against diet-induced obesity (Figures 5E–5G) and had increased expression of brown/beige adipocyte marker genes in the subcutaneous fat depots compared to *Ldlr*$^{-/-}$*;Fmo3*$^{+/+}$ control mice (Figure 5H). Collectively, these data provide genetic

evidence that FMO3 is a negative regulator of beiging programs in white adipose tissue.

DISCUSSION

Obesity, insulin resistance, and atherosclerotic CVD are closely linked diseases that can be heavily impacted by the quantity and quality of dietary inputs. In a time where genetic and genomic approaches dominate clinical investigation, we are still constantly reminded that environmental factors such as diet can play a major role in disease pathogenesis. The meta-organismal TMAO pathway was initially discovered using untargeted metabolomics approaches to identify small molecules in plasma associated with CVD risk (Wang et al., 2011). In multiple follow-up studies, it has been shown that feeding atherosclerosis-prone mice diets enriched in either distinct nutrient precursors to TMA synthesis or TMAO itself enhances atherosclerotic CVD and thrombosis potential by altering host cholesterol metabolism and platelet hyper-reactivity (Koeth et al., 2013, 2014; Zhu et al., 2016). In parallel, ASO-mediated inhibition of the TMAO-producing enzyme FMO3 protects mice from atherosclerosis (Shih et al., 2015; Miao et al., 2015), possibly in part by stimulating an intestinal pathway of reverse cholesterol transport called transintestinal cholesterol excretion (TICE) and altering tissue sterol metabolism (Warrier et al., 2015; Shih et al., 2015). More recently, inhibition of the microbial enzymes responsible for generating TMA from choline, thereby reducing TMAO levels, was shown to similarly inhibit atherosclerosis in mice (Wang et al., 2015). Therefore, while significant mechanistic and clinical data indicate that the meta-organismal TMA/FMO3/TMAO pathway is closely linked to the pathogenesis of atherosclerosis in mice and humans (Brown and Hazen, 2015), here we provide evidence that the pathway can also impact the beiging of white adipose tissue. The key findings of the current study are that (1) circulating levels of the gut microbe-derived metabolite TMAO are associated with enhanced risk of T2DM in humans, (2) TMAO levels are associated with adiposity traits across mouse strains within the Hybrid Mouse Diversity Panel, (3) adipose tissue expression of *FMO3* is positively associated with obesity in humans, (4) *FMO3* mRNA expression is negatively associated with brown/beige adipocyte gene expression in white adipose tissue in humans, (5) FMO3 knockdown or genetic deletion protects mice against high-fat diet-induced obesity, and (6) FMO3 knockdown or genetic deletion is associated with the beiging of white adipose tissue in mice.

Although the vast majority of studies have focused on the TMA/FMO3/TMAO pathway in the context of CVD, several recent studies have linked this meta-organismal pathway to diabetes. In agreement with our findings here, several independent groups have found an association between circulating TMAO levels and both T2DM and the heightened adverse CVD outcomes in diabetics (Lever et al., 2014; Dambrova et al., 2016; Tang et al., 2017). Using a metabolomics platform, Miao and colleagues recently showed that mice with selective hepatic insulin resistance (liver insulin receptor knockout mice [LIRKO]) have elevated levels of circulating TMAO and a profound upregulation of the TMAO-producing enzyme FMO3 in the liver (Miao et al., 2015). This study also demonstrated that the hepatic expression of FMO3 is suppressed by the postprandial hormone insulin yet is reciprocally stimulated by the fasting hormone glucagon (Miao et al., 2015). In addition to these findings in LIRKO mice, dietary supplementation with the FMO3 product TMAO is reported to exacerbate glucose intolerance in high-fat diet-fed mice (Gao et al., 2014). Collectively, a growing body of evidence suggests that the gut microbial TMAO pathway may be an attractive drug target for subjects with T2DM. Of note, while TMAO has been causally linked to atherosclerosis and thrombosis (Wang et al., 2011; Koeth et al., 2013; Zhu et al., 2016) and associated with obesity here, there is also literature that shows diets high in the TMAO source nutrient choline are associated with beneficial effects on fetal development and cognitive function in adults (Jiang et al., 2012; Shaw et al., 2009; Poly et al., 2011).

As drug discovery forges ahead, identifying the mechanisms driving the links among TMA, FMO3, TMAO, and human health and disease will be needed to understand where to therapeutically intervene. It is important to note that FMO3 is a promiscuous xenobiotic metabolizing enzyme with many substrates and products (Cashman and Zhang, 2006). In fact, FMO3 knockdown-associated effects on metabolic disease may be driven by factors other than TMA or TMAO. Although provision of supplemental TMAO reversed a subset of the *Fmo3* ASO-induced effects on adipose gene expression, it also makes clear that not all of the phenotypes observed by FMO3 knockdown are mediated by either TMA or TMAO. Additional work is needed to understand the most therapeutically tractable targets in the entire TMA/FMO3/TMAO meta-organismal pathway. Based on the present studies, it appears that the phenotypic effects of FMO3 knockdown or deletion could be driven by a combination of factors, including (1) chronic increases in the levels of TMA, (2) chronic decreases in the levels of TMAO, and/or (3) effects driven by other FMO3 substrates or products. It is interesting to note that dietary provision of TMAO reversed *Fmo3* ASO-driven increases in *Ppargc1a* but did not rescue *Fmo3* ASO-driven reductions in body weight or expression of *Ucp1* in white adipose tissue (Figures 5I–5K). Such findings support a model where TMAO may be involved in specific transcriptional reprogramming in adipocytes, but how TMAO is being sensed is still an unanswered question. TMA is known to activate the G-protein-coupled receptor trace amine-associated receptor 5 (TAAR5); however, TAAR5 does not recognize TMAO (Li et al., 2013). It will be important in future studies to determine whether TMA-driven activation of TAAR5 is coupled to metabolic reprogramming in the host and to identify potential host TMAO receptor(s). In conclusion, this work highlights a role for the TMA/FMO3/TMAO meta-organismal pathway in the progression of obesity-related disorders. Given the numerous strong associations of the gut microbe-driven TMAO pathway with human disease, this work has broad implications for drug discovery efforts targeting gut microbes themselves instead of the human host in which they reside.

EXPERIMENTAL PROCEDURES

Human Studies

To examine whether circulating choline and TMAO levels were associated with T2DM risk, we recruited two unique cohorts including both men and women

with diverse cardiometabolic risk profiles in cardiology and hepatology clinics at the Cleveland Clinic. These studies were approved by the Cleveland Clinic Institutional Review Board, and every subject provided written informed consent. Extended patient demographics, laboratory values, and clinical characteristics are available in Supplemental Experimental Procedures. To examine the relationship between *FMO3* expression and metabolic traits, we took advantage of adipose biopsy microarray data (n = 770) within the Metabolic Syndrome in Men (METSIM) study, which has been previously described in detail (Stancáková et al., 2009). The study was approved by the ethics committee of the University of Eastern Finland and Kuopio University Hospital and was conducted in accordance with the Helsinki Declaration. All study participants gave written informed consent. To validate human adipose microarray findings from the METSIM cohort, we analyzed additional microarray data from two distinct cohorts spanning both men and women of European American and African American ethnicity, which have previously been described (Das et al., 2015; Sharma et al., 2016). These studies were approved by the University of Arkansas for Medical Sciences and the Institutional Review Board of Wake Forest School of Medicine. All study participants gave written informed consent. Finally, we examined the protein expression of FMO3 in human liver from normal BMI or bariatric surgery patients. This study was approved by the Institutional Review Board of Wake Forest School of Medicine, and all study participants gave written informed consent. Detailed information for all human studies is provided in Supplemental Experimental Procedures.

Animal Studies

To study the role of the TMAO-producing enzyme FMO3 in diet-induced obesity, we initially employed an in vivo ASO-mediated knockdown approach as previously described (Warrier et al., 2015; Shih et al., 2015). Because of the known sexual dimorphism of hepatic FMO3 expression in mice, all studies were conducted in adult female mice unless otherwise noted. Mice were maintained on either standard rodent chow (2918 Teklad Global 18% Protein Rodent Diet) or a custom high-fat diet composed of 45% kcal derived from fat (Brown et al., 2010). ASO-treated mice were then subjected to cold tolerance and indirect calorimetry studies using methods previously described (Thomas et al., 2013). To establish FMO3 knockout mice, we used CRISPR-Cas9 gene editing as described in Supplemental Experimental Procedures. Plasma TMA and TMAO quantification and FMO activity measurements were measured using stable isotope dilution mass spectrometry-based assays as previously described (Wang et al., 2014; Warrier et al., 2015) on a Shimadzu 8050 triple quadrupole mass spectrometer. All mouse studies were approved by Institutional Animal Care and Use Committees of the Cleveland Clinic, Case Western Reserve University, or University of California, Los Angeles. Detailed information for all mouse studies and biochemical workup of mouse tissue is provided in Supplemental Experimental Procedures.

Statistical Analysis

To examine the association between circulating choline and TMAO with T2DM, Wilcoxon rank-sum tests were used for continuous variables, and χ^2 tests were used for categorical variables. Multilogistic regression models were used to estimate odds ratio and 95% confidence interval for diabetes. All analyses were performed using R 3.1.0, and $p \leq 0.05$ was considered statistically significant. All mouse data were analyzed using either one-way or two-way ANOVA, where appropriate, followed by post hoc analysis. Differences were considered significant at $p \leq 0.05$. All mouse data analyses were performed using JMP Pro 10 (SAS Institute) or GraphPad Prism 6 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.077.

AUTHOR CONTRIBUTIONS

R.C.S., D.M.S., M.W., and J.M.B. planned the project, designed experiments, analyzed data, and wrote the manuscript; J.M.B., S.L.H., S.V.N.P., W.H.W.T., A.J.L., M.C., and M.L. designed experiments and provided useful discussion directing the project; R.C.S., D.M.S., M.W., R.N.H., A.B., D.F., A.L.B., A.D.G., M.H., A.C., L.L., X.S.L., Z.W., B.W., Y.M., H.K., N.C., C.P., R.E.M., C.D.L., S.K.D., L.L.R., N.Z., A.J.M., S.D., M.L., M.C., and J.H. either recruited human subjects or conducted mouse experiments, performed biochemical workup of mouse tissues, analyzed data, and aided in manuscript preparation; B.O.E and C.A.F. performed imagining studies in mice; R.G.L., R.M.C., and M.J.G. provided antisense oligonucleotides; and all authors were involved in the editing of the final manuscript.

ACKNOWLEDGMENTS

This work was supported by NIH and Office of Dietary Supplements grants R00 HL096166 (J.M.B.), R01 HL122283 (J.M.B.), P50 AA024333 (J.M.B.), R01 HL103866 (S.L.H.), R01 HL126827 (S.L.H. and W.H.W.T), R01 DK106000 (S.L.H. and W.H.W.T), R01 HL130819 (Z.W.), R01 DK090111 (S.K.D.), R00 HL12172 (M.C.), P01 HL028481 (A.J.L.), P01 HL030568-31A1 (A.J.L. and D.M.S.), and P01 HL49373 (L.L.R.), as well as by the Deutsche Forschungsgemeinschaft in the framework of SFB841 (J.H.), the Academy of Finland and the Finnish Cardiovascular Research Foundation (M.L.), and the American Heart Association (postdoctoral fellowship 15POST2535000 to R.C.S., postdoctoral fellowship 14POST18700001 to M.W., and postdoctoral fellowship 17POST3285000 to R.N.H.).

S.L.H. and Z.W. are named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. S.L.H. reports he has been paid as a consultant by Esperion and Procter & Gamble. S.L.H. has received research funds from Astra Zeneca, Procter & Gamble, Roche, and Takeda. S.L.H. has the rights to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics from Cleveland Heart Lab Inc., Frantz Biomarkers, and Siemens Healthcare. R.G.L., R.M.C., and M.J.G. are employees of Ionis Pharmaceuticals, Inc. (Carlsbad, CA).

Received: April 13, 2016
Revised: May 2, 2017
Accepted: May 23, 2017
Published: June 20, 2017

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