nature portfolio

Peer Review File

Title: Hepatic thyroid hormone signalling modulates glucose homeostasis through the regulation of GLP-1 production via bile acid-mediated FXR antagonism

REVIEWER COMMENTS</B>

Reviewer #1 (Remarks to the Author):

This manuscript proposes a four-organ interaction (a thyroid-liver-ileum-pancreas axis) whereby T3 signaling increases non-12a-hydroxylated BA production in the liver. This results in increased GLP-1 synthesis and secretion from the ileum due to local reduction in FXR signaling by those non-hydroxylated BAs. Ultimately, this increase in GLP-1 is responsible of the T3-induced improvement in glucose tolerance.

This is an interesting premise that links different cellular mechanisms playing key roles in the control of glucose homeostasis that are susceptible of drug targeting. This undeniably adds interest to the work. However, there also significant limitations, which are explained below:

Most experiments are performed in the context of T3 replacement in pharmacologically (MMI)-induced hypothyroidism and conclusions are drawn based on simple pair-wise comparisons between minimally powered groups. Lack of back-to-back comparison with euthyroid controls limits the potential relevance of the findings a pathophysiological state. There is also some lack of lack of systematic comparability across otherwise similar experiments (e.g. groups or parameters are selectively shown).

Although increased GLP-1 levels are at the center of their hypothesis, conclusions are inferred out of small changes in ambient levels of GLP-1 or Gcg expression. Considering the low number of replicates and the small size of the effect, this is a significant weakness that challenges reproducibility. Sometimes conclusions are derived from overinterpretation of indirect assessments. For example, in Fig1n if the T3-improvement in tolerance were due to solely to increased GLP-1, then the effect of the blockade of GLP1R with Ex9 between T3 (difference between green vs yellow) the untreated groups (difference between blue vs red) should be statistically significant. Given that additional experiments providing a more accurate determination of the GLP-1 secretion kinetics would be desirable.

The T3-induced improvement in glucose tolerance were mimicked by treatment with a liver-biased TRb agonist and were is significantly attenuated in mice lacking TRb expression in the liver (although, and again, critical Albcre+ control mice were not investigated; additional details about the generation including targeting methodology, genomic context and general phenotype of the flox mouse should provided).
Nonetheless, this is an important experiment that compellingly demonstrate the role of hepatic T3 signaling in modulating glucose metabolism. There is no question that this TRb mediates some of the benefits of T3 and MB07811 on glycemic control. However, significant unknowns arise from this experiment that question the main hypothesis. For instance, loss of hepatic TRb3 signaling in liver does not result in dramatic increases in Cyp8b1 levels compared to controls, as it would be expected in light of the effect of MMI treatment. Since changes in BA composition were not investigated either, it is hard to attribute the loss of effect of T3 in the KO to changes in BA composition. Considering this, MMI treatment of liver-specific TRb KO mice, plus minus T3, would shed light on the actual contribution of

hepatic TRb signaling on the benefits seen with the T3 replacement in the MMI treated mice. If worsening in glycemic control (note that the TRb KO exhibit normal glucose tolerance) is seen and that can be normalized with T3 treatment, then other options should be considered.
Administration of TbMCA acid to MMI-treated mice recapitulated a similar effect to that of T3 replacement on glucose tolerance and Gcg expression, GLP-1 and insulin levels. Conversely, treated with CA or the gut-bias FXR agonist Fexeramine reverted some of the effects of T3 replacement in MMI-treated mice that are consistent with enhanced GLP-1 secretion. However, due to the concern manifested above, this interaction between FXR signaling and T3 could be due to independent events acting in parallel with different contributions. T3 treatment of MMI-treated, villin-cre:FXR flox mice would be certainly more convincing. This is not unreasonable considering that other modalities of BA signaling play a meaningful role regulating L-cell differentiation (https://doi.org/10.2337/db19-0764)

Reviewer #2 (Remarks to the Author):

In the current study by Yan et al., hepatic activation of T3 signaling is sufficient to promote the insulin secretion and lower the glucose levels. Furthermore, the team identified that this is due to reduction in CYP8B1 and increase in bile acids that are FXR antagonists. Based on one study reported in 2015 that inhibition of FXR in L cells increase GLP1 production and secretion, the authors concluded that the T3-mediated induction in proglucagon (precursor of GLP-1) in L cells is due to intestinal inhibition of FXR. While the conclusion is exciting, there are some concerns:
1. How about the regulation of T3 on other genes involved in bile acid synthesis? Wat about levels of bile acids that are TGR5 activators?
2. What is the effects of T3 on carbohydrate ingestion and other incretin secretion in addition to GLP-1?
3. Validation of TGR5 activation. How about comparing to report showing that FXR can induce TGR5? How about the activation status in the intestine epithelial cells and also in L cells
4. In humans, inhibition of CYP8B1 will lead to the bile acid pool enriched in CDCA, which is the most FXR endogenous ligand---opposite to rodents.
5. How about using T4 than T3? which is more commonly used in the clinic for the treatment of hypothyroidism.
6. In fig 6, it will be interesting to have additional control groups treated with CA or FEX with or without MMI

Reviewer #3 (Remarks to the Author):

The authors demonstrate that euthyroid animals have improved glucose tolerance and increased circulating insulin and circulating and intestinal GLP-1 levels compared to hypothyroid animals. Using a

wide range of models the authors demonstrate that liver TRB is required for (the majority of) this effect. Intestinal FXR is shown to modulate the beneficial effect of T3 on GLP-1 levels, plasma insulin and plasma glucose in hypothyroid mice. The authors postulate that the link between (hepatic) thyroid hormone signaling and changes in bile acid composition, resulting in increased GLP-1 secretion, is the enzyme CYP8B1 in liver. The data is novel, interesting and clinically relevant given the development of liver specific TRB agonists for the treatment of metabolic disease.

Specific Comments:

1. Is hyperglycemia commonly found in hypothyroid mouse models. To this reviewers knowledge the answer is no. What is the reason for this here. Other examples should be brought forward.
2. The increased levels of GLP-1 described in Figure 1J are not clear.
3. Why was GLP-1 not given directly to hypothyroid animals?
4. Ideally, further experiments should be provided to demonstrate KO of the TR in the liver. ie TH mediated gene expression of classic TR target genes. The model is under-described in all aspects.
5. In Figure 2 and Figure 3 the induction of hypothyroidism leads to a rise in blood glucose in both WT and KO animals and T3 or the analog reduces it only in WT or floxed animals. Why is there an induction of BG in hypothyroidism in the absence of the TR?
6. In Figure 3 there is no data on the high fat diet mice ie food intake, body weight etc. It is impossible to determine the effects of the MB compound in this setting.
7. In Figure 4 where is the RNA-Seq data of the genes regulated by feeding. What does Cyp8b1 do with increased feeding.
8. Also, in Figure 4, T3 is known to regulate many other genes involved in bile acid transport that also regulate bile acid hydrophobicity including cyp27a1 and cyp3a11 and as well bile acid transporters. These genes are not included in the analysis shown. Interestingly, Cyp8b1 has been identified previously as a target of TH signaling: Andersson U, Yang YZ, Bjorkhem I, Einarsson C, Eggertsen G, Gafvels M. Thyroid hormone suppresses hepatic sterol 12α-hydroxylase (CYP8B1) activity and messenger ribonucleic acid in rat liver: failure to define known thyroid hormone response elements in the gene. Biochim Biophys Acta. 1999;1438(2):167–174. Additionally, thyroid hormone signaling has also been shown to alter bile acid composition previously and thus regulate intestinal cholesterol absorption (Astapova et al, JCI 2014).
9. The explanation of the data in Figure 7 is not clear. TSH is used to determine TH action. Do the TSH levels inversely correlate with the TH levels. Additionally do the T3 levels correlate with other actions of TH on the liver ie serum cholesterol.
10. Is there a CYP8B1-KO model available or specific inhibitor that would allow you to study the effect of T3 in the absence of CYP8B1?
11. Does the attenuated change in CYP8B1 in MMI+T3 treated L-TRBKO mice result in measurable changes in intestinal bile acid composition compared to controls?
Other comments:

12. Figure 2: . Please specify if these mice were rendered hypothyroid before being treated with T3 as this isn't mentioned in the figure legends or corresponding results section

13. The other bile acid receptor TGR5 has been described to be crucial for GLP-1 induction and several other metabolic effects. Did you find any evidence for a role of TGR5 in this pathway? Why did you choose to focus only on FXR?
14. In general the paper is well written but the discussion would benefit from a thorough read-through to correct several grammatical errors.
15. Please specify where the MB07811 TRB agonist was purchased and include data or references that demonstrate its liver and TRB specificity.
16. Have you done any of these experiments in female mice? If so please show (in supplemental)
17. Please state whether your human subjects provided informed consent in accordance with the declaration of Helsinki.

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This manuscript proposes a four-organ interaction (a thyroid-liver-ileum-pancreas axis) whereby T3 signaling increases non-12a-hydroxylated BA production in the liver. This results in increased GLP-1 synthesis and secretion from the ileum due to local reduction in FXR signaling by those non-hydroxylated BAs. Ultimately, this increase in GLP-1 is responsible of the T3-induced improvement in glucose tolerance.

This is an interesting premise that links different cellular mechanisms playing key roles in the control of glucose homeostasis that are susceptible of drug targeting. This undeniably adds interest to the work. However, there also significant limitations, which are explained below:

Most experiments are performed in the context of T3 replacement in pharmacologically (MMI)-induced hypothyroidism and conclusions are drawn based on simple pair-wise comparisons between minimally powered groups. Lack of back-to-back comparison with euthyroid controls limits the potential relevance of the findings a pathophysiological state. There is also some lack of lack of systematic comparability across otherwise similar experiments (e.g. groups or parameters are selectively shown).

Response to the Reviewer's Comments: We thank the reviewer for the comments. We agree with the reviewer that lack of comparison with euthyroid controls limits the potential pathophysiological relevance. Previously, we did not include such a comparison for following reasons. 1) TRs are ligand (T3)-dependent nuclear receptors. For positively regulated genes, in the absence of T3, unliganded TRs (apoTRs) sit on the promoter of its target gene and repress transcription by recruiting corepressors rather than sit there doing nothing. In the presence of T3, liganded TRs (holoTRs) release corepressors and recruit coactivators, thereby stimulating the transcription. Thus, in the TR field, to identify T3 target genes or T3-regulated pathways that have maximal T3 responsiveness, mice were normally rendered hypothyroid before T3 treatment, while cultured cells were normally pre-cultured in Td (TH-deficient) medium before T3 treatment. 2) The role of TH in metabolism is profound, as TH normally coordinates metabolic pathways by targeting multiple enzymes and affect systemic homeostasis via its regulatory action in different metabolic tissues. The severity or duration of thyrotoxicosis or hormone treatment may yield inconsistent results. Based on available evidence, we speculate that TH may exert beneficial effects on glucose homeostasis at least within the normal range of thyroid function, while extremely high levels of TH may have deleterious effects on metabolic homeostasis. Furthermore, because MMI may have unknown effects on metabolism independent of its actions on TH synthesis in the thyroid, to minimize the effects of potential confounding factors and focus on the regulatory action of T3, we employed MMI-treated mice with detectable but low T3 levels as a control group and MMI-treated mice receiving T3 injections as an experimental group.

As suggested, we provided the date showing the differences between hypothyroid MMI-treated mice (MMI mice) and untreated euthyroid control mice (CT mice). Consistent with our hypothesis, MMI mice exhibited elevated blood glucose levels, impaired intraperitoneal and oral glucose tolerance, decreased insulin levels, and reduced GLP-1 expression and production as compared to CT mice (Supplementary Fig. 1k-n). It is worth noting that, as the half-life of T3 in mouse serum is about 2

hours, MMI mice receiving daily injection of T3 are not an authentic mouse model for clinical hyperthyroidism. Thus, we should be cautious when they were compared directly to euthyroid mice. To avoid potential confusion and interpret the data correctly in a straightforward way, we decided to show the results of comparison between MMI mice and CT mice (euthyroid controls) rather than the results of comparison between T3-treated MMI mice (MMI+T3 mice) with CT mice in our revised manuscript (Supplementary Fig. 1k-n, 4h, i, 5b-e). Similar comparisons could also be found in Supporting data (Supporting Fig. 1e, f, 2i-k, 3a).

As the reviewer suggested, to substantiate our notion by increasing sample numbers in Fig. 1b,1d,1e, and 1h (previous version), we repeated these experiments and provided the new data in our revised manuscript (Fig. 1a, c, d, and the left panel of f). In addition, more control groups were included for better systematic comparability and more detailed information for general phenotype of the mice and experimental conditions were also provided in our revised manuscript (Supplementary Fig. 1i, k-n, 2g-i, 4h, i, 5b-e and Supporting Fig. 1d-f, 2a, i-k).

Although increased GLP-1 levels are at the center of their hypothesis, conclusions are inferred out of small changes in ambient levels of GLP-1 or Gcg expression. Considering the low number of replicates and the small size of the effect, this is a significant weakness that challenges reproducibility. Sometimes conclusions are derived from overinterpretation of indirect assessments. For example, in Fig1n if the T3-improvement in tolerance were due to solely to increased GLP-1, then the effect of the blockade of GLP1R with Ex9 between T3 (difference between green vs yellow) the untreated groups (difference between blue vs red) should be statistically significant. Given that additional experiments providing a more accurate determination of the GLP-1 secretion kinetics would be desirable.

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. Regarding the reproducibility, all animal experiments in our previously submitted manuscript had been repeated at least two to three times. Indeed, the regulation of GLP-1 production by either T3 or MB07811 treatment can be easily detected in mice under various conditions (Fig.1f, k, 2b, 3a, e, i, 4j, 6d, 7b, d, g and Supplementary Fig 1i, 2h, 3b, 7c, e). As the reviewer suggested, to substantiate our conclusion, we repeated the experiment by using a larger sample size and obtained similar results (Fig. 1n). Since Ex-9 treatment could attenuate the T3 effect on oral glucose tolerance, we speculate that GLP-1 is critically involved in the T3 action on glucose metabolism. As suggested, we also compared the changes in AUC after Ex-9 treatment between MMI+T3 mice and MMI mice. In line with the data that the GLP-1 levels were higher in MMI+T3 mice than those in MMI mice, a significantly larger effect of Ex-9 treatment was observed in MMI+T3 mice than that in MMI mice, further supporting our notion that GLP-1 is critically involved in the T3 action on glucose metabolism (Fig. 1n and Supporting data Fig. 1a-c).

To be noted, as the T3 levels were decreased but still detectable after MMI treatment, therefore, we speculate the retained T3 action might contribute to the observed effect of Ex-9 in MMI mice (Fig. 1n and Supporting data Fig. 1a). Nevertheless, since the sensitivity and specificity of different assays vary and systemic administration of T3 would affect other metabolic tissues, we could not totally rule out the possibility that other mechanisms might also be involved here. Indeed, in this study, we intended not to claim that the observed beneficial effect of systemic T3 treatment were solely due to the increased GLP-1 production. Based on our findings in this study, we prefer to hypothesize

that the beneficial effect of hepatic TRβ-mediated T3 effect might be primarily attributed to the regulation of GLP-1 production by T3 via BA-mediated FXR antagonism.

Furthermore, as the reviewer suggested, we determined the kinetics by measuring the GLP-1 and insulin levels during the oGTT assay. We found that the GLP-1 levels were higher before glucose oral ingestion and elevated more markedly after glucose oral ingestion in MMI+T3 mice than those in MMI mice (Supplementary Fig. 1e). Similar results were observed for the insulin levels during the oGTT assay (Supplementary Fig. 1f). These data indicate that the capacity of GLP-1 production was enhanced in MMI+T3 mice as compared to MMI mice.

The T3-induced improvement in glucose tolerance were mimicked by treatment with a liver-biased TRb agonist and were is significantly attenuated in mice lacking TRb expression in the liver (although, and again, critical Albcre+ control mice were not investigated; additional details about the generation including targeting methodology, genomic context and general phenotype of the flox mouse should provided).

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. Liver-selective TRβ knockout (LTRβKO) mice were generated by cross-breeding of TRβ flox/flox mice with loxP sites flanking the fifth TRβ exon (TRβ Floxed mice), developed by Shanghai Model Organism Center, Inc., with mice harboring Cre-recombinase under the control of albumin promoter (Alb-Cre mice) (Supplementary Fig. 2d, e). We provided detailed information, including targeting methodology, genetic background, and general phenotype of the TRβ Floxed ($TR\beta^{f/f}$) mice in our revised manuscript (Supplementary Fig. 2d and Page 16, Line 528-532). Briefly, TRβ Floxed ($TR\beta^{f/f}$) mice were fertile and appeared indistinguishable from $TR\beta^{+/+}$ control littermates. There was no significant difference in body weight, food intake, glucose and insulin levels between $TR\beta^{f/f}$ mice and $TR\beta^{+/+}$ controls (Supporting data Fig. 1d). As the reviewer suggested, we also compared the LTRβKO mice ($TR\beta^{f/f}$, Alb-Cre^{+}) with Alb-Cre^{+} control mice ($TR\beta^{+/+}$, Alb-Cre^{+}). As expected, the MB07811 effects on GLP-1, insulin, and glucose levels were abolished in LTRβKO mice lacking hepatic TRβ as compared to Alb-cre^{+} control mice (Supplementary Fig. 3b).

Nonetheless, this is an important experiment that compellingly demonstrate the role of hepatic T3 signaling in modulating glucose metabolism. There is no question that this TRb mediates some of the benefits of T3 and MB07811 on glycemic control. However, significant unknowns arise from this experiment that question the main hypothesis. For instance, loss of hepatic TRb3 signaling in liver does not result in dramatic increases in Cyp8b1 levels compared to controls, as it would be expected in light of the effect of MMI treatment. Since changes in BA composition were not investigated either, it is hard to attribute the loss of effect of T3 in the KO to changes in BA composition. Considering this, MMI treatment of liver-specific TRb KO mice, plus minus T3, would shed light on the actual contribution of hepatic TRb signaling on the benefits seen with the T3 replacement in the MMI treated mice. If worsening in glycemic control (note that the TRb KO exhibit normal glucose tolerance) is seen and that can be normalized with T3 treatment, then other options should be considered.

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. In our study, loss of TRβ in liver only slightly increased the mRNA levels but not the protein levels of CYP8B1 (Fig. 4i, f), suggesting compensatory mechanisms may exist. In line with these results, no differences in ileal BA composition (percentages of 12α-OH BAs and non-12α-OH BAs) (Fig.

4n, 5d) and GLP-1 production (Fig. 2b, 3e and Supplementary Fig. 2h) were observed between LTRβKO and Floxed mice. In contrast, MMI treatment led to an elevation of CYP8B1 levels, accompanied with altered BA composition (Supplementary Fig. 4h, i) and GLP-1 production (Supplementary Fig. 1n). This is not surprising because it has been proposed that, unlike steroid hormone receptors, TRs can act in the absence of the ligand as aporeceptors (apoTRs), which have an intrinsic activity rather than being silent. These apoTRs repress basal transcription of positively regulated genes and stimulate that of negatively regulated genes. In agreement with this notion, unliganded TRs (apoTRs) in MMI mice might act to increase the mRNA expression of CYP8B1, thereby altering the BA composition and impairing glucose metabolism (Supplementary Fig. 1k, l, 4h, i). It is worth noting that, as a matter of fact, more severe defects were normally observed in hypothyroid subjects than those subjects harboring TR mutations.

As the reviewer suggested, we rendered LTRβKO mice hypothyroid followed by T3 treatment. In line with our previous findings, T3 treatment markedly repressed the mRNA expression of CYP8B1 and lowered the glucose levels in MMI-treated Floxed mice, while loss of hepatic TRβ could block these effects in MMI-treated mice, further supporting our hypothesis that hepatic TRβ signaling contributes to the benefits seen with the T3 treatment (Supporting data Fig. 1e). It has been proposed that TH deprivation induces a strong apoTR activity, while the effect of the absence of TH can be attenuated by the removal of TR [1]. Interestingly, the mRNA levels of CYP8B1 were not reduced in MMI-treated LTRβKO mice compared to MMI-treated Floxed mice. Moreover, the glycemic control was not improved after the removal of hepatic TRβ in MMI-treated mice (Supporting data Fig. 1e). These observations indicate that apoTRα but not apoTRβ may play a more important role at least in the control of CYP8B1 transcription, which agrees with the previous notion that TRβ may have no aporeceptor activity in liver [1]. Notably, our findings are similar to those observed for TSH, another negatively regulated target gene of T3. It has been proposed that TRα but not TRβ seems to be responsible for aporeceptor-mediated activation of TSH. Because whether apoTRs are silent or whether they have an intrinsic activity should be determined by measuring the gene activity in the absence of TH and in the absence of individual TR isoforms, to fully understand the role of hepatic apoTRs in metabolic regulation, a more careful examination is needed in the future. Anyway, to the best of our knowledge and according to our data, we believe that physiological homeostasis depends on a precise balance between apoTRs and holoTRs, the apoTRs participate in the fine-tuning of T3-target genes, and the combination of holoTRs with active apoTRs permits a larger amplitude of transcriptional responses to moderate variations in T3 concentrations. Because we mainly focus on the beneficial T3 effect and the role of liganded TRβ (holoTRβ) in current study, we provided the related data from MMI-treated LTRβKO mice as supporting data (Supporting data Fig. 1e, f) to avoid confusion due to the potential contribution from apoTR.

Additionally, we noticed that lacking hepatic TRβ abolished the T3 effect on CYP8B1 expression and glucose tolerance in MMI-treated mice, which further supports our proposed model involving the action of hepatic TRβ and incretin GLP-1 (Supporting data Fig. 1e, f). We also noticed a small reduction of glucose levels in MMI-treated LTRβKO mice after T3 treatment, although the difference did not reach statistical significance ($p=0.06$) (Supporting data Fig. 1e, left panel). These results indicate that systemic administration of T3 might also affect glucose metabolism via other mechanisms, which seemed to be not as dominant as those mediated by hepatic TRβ under these experimental conditions. Based on our findings in this study, we believe we have discovered a novel

role of T3 and hepatic TRβ in modulating glucose homeostasis, which involves the regulation of GLP-1 production via BA -mediated FXR antagonism. Nevertheless, we did not intend to exclude the possibility that other mechanism exists that also contribute the profound role of T3 in glucose homeostasis.

Administration of TbMCA acid to MMI-treated mice recapitulated a similar effect to that of T3 replacement on glucose tolerance and Gcg expression, GLP-1 and insulin levels. Conversely, treated with CA or the gut-bias FXR agonist Fexeramine reverted some of the effects of T3 replacement in MMI-treated mice that are consistent with enhanced GLP-1 secretion. However, due to the concern manifested above, this interaction between FXR signaling and T3 could be due to independent events acting in parallel with different contributions. T3 treatment of MMI-treated, villin-cre:FXR flox mice would be certainly more convincing. This is not unreasonable considering that other modalities of BA signaling play a meaningful role regulating L-cell differentiation (https://doi.org/10.2337/db19-0764)

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. We agree with the reviewer that other mechanisms might also be involved in the regulation of glucose metabolism by systemic administration of T3 in mice. Our findings in LTRβKO mice and MB07811-treated animals support our hypothesis that hepatic TRβ signalling plays a critical role in modulating glucose homeostasis through the regulation of GLP-1 production via BA-mediated FXR antagonism. As the reviewer suggested, to test whether intestinal FXR signalling was essential for the T3 action observed in this study, intestine-specific FXR-null mice (IFXRKO) were employed [24]. We found that the T3 effects on the mRNA expression of ileal proglucagon, GLP-1, insulin, and glucose levels, and oral glucose tolerance were all abolished in IFXRKO mice in hypothyroid state (Fig. 7a-c). Similar results were observed when MB07811 was used for treatment in IFXRKO mice in hypothyroid state (Fig. 7d-f). We also found that the T3 effects on the GLP-1, insulin, and glucose levels, and oral glucose tolerance were all attenuated in euthyroid IFXRKO mice (Supplementary Fig. 7c, d). Similar data were obtained when MB07811 was used for treatment in IFXRKO mice in euthyroid state (Supplementary Fig. 7e, f). These results indicate that intestinal FXR is indispensable for the beneficial effect of either T3 or MB07811 on GLP-1 production and glucose homeostasis. Additionally, consistent with our current knowledge of intestinal FXR signalling and our working hypothesis, the GLP-1 production was increased, while the glucose metabolism was improved in MMI-treated IFXRKO mice as compared to MMI-treated Floxed mice (Fig. 7a-f). Notably, in line with the previous findings that intestinal FXR is not required for maintaining the normal glucose homeostasis [2], the glucose metabolism was normal in IFXRKO mice in the euthyroid state, suggesting that compensatory mechanisms may exist (Supplementary Fig. 7c-f).

We also agree with the reviewer that other modalities of BA signaling might also play a role here. We then tested whether increased TβMCA could have impact on L-cell differentiation. In cultured enteroendocrine cells and intestinal organoids, TβMCA treatment not only increased the GLP-1 expression and secretion (Fig. 6e, g and Supplementary Fig. 6c-e) but also elevated the mRNA expression of Ngn3, NeuroD1, and Arx, which are key genes associated with L-cell differentiation and endocrine specification, indicating that the increased TβMCA might also be able to promote the L-cell differentiation (Supplementary Fig. 6g). Accordingly, elevated mRNA levels of Ngn3, NeuroD1, and Arx were observed after T3 or MB07811 treatment in the ileum of MMI mice

(Supporting data Fig. 1g). Given that paracrine GLP-1 signalling has been implicated in L-cell differentiation [3], based on our data, we speculate that activation of hepatic TRβ signalling might increase the TβMCA levels in ileum, subsequently promoting the GLP-1 secretion in L-cells, which would act in both endocrine and paracrine manners to modulate insulin secretion and glucose metabolism and enhance the endocrine function of L-cell by recruiting more L-cells, respectively.

Reviewer #2 (Remarks to the Author):

In the current study by Yan et al., hepatic activation of T3 signaling is sufficient to promote the insulin secretion and lower the glucose levels. Furthermore, the team identified that this is due to reduction in CYP8B1 and increase in bile acids that are FXR antagonists. Based on one study reported in 2015 that inhibition of FXR in L cells increase GLP1 production and secretion, the authors concluded that the T3-mediated induction in proglucagon (precursor of GLP-1) in L cells is due to intestinal inhibition of FXR.

While the conclusion is exciting, there are some concerns:

1. How about the regulation of T3 on other genes involved in bile acid synthesis? What about levels of bile acids that are TGR5 activators?

Response to the Reviewer's Comments: We thank the reviewer for raising these questions. In line with previous findings, the mRNA expression of other enzymes involved in BA synthesis, such as CYP7A1, CYP27A1, and CYP7B1, was positively regulated by T3 treatment (Supporting data Fig. 2a). Based on our knowledge and our findings, we hypothesize that T3 treatment not only promotes BA synthesis by regulating the expression of these enzymes but also modulates the BA composition by targeting CYP8B1. Our new data demonstrate that knockdown of CYP8B1 could attenuate the effect of T3 treatment on the GLP-1 production and glucose metabolism in MMI mice, strongly supporting the notion that CYP8B1 might be the primary BA synthetic enzyme responsible for the observed glucose-lowering effect of T3 (Fig. 4j-l and Supplementary Fig. 4g).

Additionally, as the reviewer suggested, we analyzed the levels of BAs with TGR5 agonist activities. We found that T3 treatment decreased the total percentage of BAs with TGR5 agonist activities in the ileum of MMI mice, while the percentage of DCA, which is abundant in ileum and exhibits potent TGR5 agonist activity, was not altered (Supplementary Fig. 5f). As we did not observe an elevation in the levels of these TGR5-agonistic BAs, we speculate that FXR-mediated pathway rather than TGR5-mediated pathway is critically involved after T3 treatment in mice. Consistently, in human feces, positive correlation was also not observed between the T3 level and the percentage of potent TGR5-agonistic BAs (LCA and DCA), while a negative correlation was observed between T3 level and the percentage of total TGR5-agonistic BAs (Supplementary Fig. 8c, d), further indicating that TGR5 signalling might not play a considerable role here.

2. What is the effects of T3 on carbohydrate ingestion and other incretin secretion in addition to GLP-1?

Response to the Reviewer's Comments: We thank the reviewer for raising this question. In order to investigate whether T3 could impact GLP-1 production directly in intestinal L-cells, we employed enteroendocrine STC-1 and NCI-H716 cells and mouse intestinal organoids. We found that T3 had no effect on GLP-1 production and expression in enteroendocrine cells and intestinal organoids

(Supplementary Fig. 2a-c), suggesting that T3 might regulates GLP-1 production in a cell-nonautonomous manner. As the reviewer suggested, we examined the expression of Sglt1 and Glut2, two key transporters involved in carbohydrate ingestion in these enteroendocrine cells and intestinal organoids after T3 treatment. We found that T3 treatment had no effect on Sglt1 and Glut 2 mRNA levels (Supporting data Fig. 2b, c). Similar results were observed in the ileum of T3 or MB-treated MMI mice (Supporting data Fig. 2d). These data suggest that glucose absorption might not play a significant role here. We also would like to point out that the plasma levels of GLP-1 were higher in MMI+T3-5d mice than those in MMI mice before glucose challenge in oGTT assay (0 min, Fig. 1e and Supplementary Fig. 1e). Moreover, the GLP-1 expression and plasma levels of GLP-1 were also higher in MMI+T3-5d mice than those in MMI mice without any treatment (Fig. 1f, g, k, 4j, 6d, 7a, b, g and Supplementary Fig. 1g). These data suggest that the basal level of GLP-1 production or the capacity of GLP-1 production has been already increased or enhanced by T3 treatment, which is independent of glucose ingestion.

Additionally, as suggested, we investigated the T3 effect on the expression of PYY, another gut hormone produced by L-cells. We found that the mRNA expression of PYY was not altered in the enteroendocrine STC-1 and NCI-H716 cells, mouse intestinal organoids, as well as the ileum of mice after T3 or MB07811 treatment as indicated (Supporting data Fig. 2d, e). We also examined the production of another incretin, glucose-dependent insulinotropic polypeptide (GIP). We found that, in contrast to GLP-1, the plasma GIP levels and the ileal GIP mRNA levels were not altered after T3 or MB07811 treatment in mice (Supplementary Fig. 1o). We also could not observe any changes in GIP mRNA expression in the intestinal organoids after T3 administration (Supplementary Fig. 1o). These results suggest that GIP might not be involved in the regulation of glucose metabolism by TH observed in this study. Based on these results and others including the new data obtained from *in vitro* experiments using BAs (Fig. 6e-h and Supplementary Fig. 6c-h), we propose that T3 regulates GLP-1 production in a cell-nonautonomous manner via BA-mediated FXR antagonism.

3. Validation of TGR5 activation. How about comparing to report showing that FXR can induce TGR5? How about the activation status in the intestine epithelial cells and also in L cells.

Response to the Reviewer's Comments: We thank the reviewer for the suggestion. As the reviewer suggested, to test the TGR5 activation, we first measured the cAMP levels in the ileum of mice and found that either T3 or MB07811 treatment could not affect the cAMP levels (Supplementary Fig. 5h). We also employed enteroendocrine STC-1 and NCI-H716 cells and mouse intestinal organoids to investigate the effect of T3 and BAs on TGR5 activation. Interestingly, we found that T3 treatment had no effect on cAMP levels in these enteroendocrine cells and intestinal organoids (Supplementary Fig. 5i), suggesting that T3 could not affect the GLP-1 production in L-cells by directly activating TGR5 signalling. We then treated enteroendocrine cells and intestinal organoids with TβMCA. We found that TβMCA could increase GLP-1 expression and production, and decrease the mRNA expression of SHP, a downstream target of FXR, in STC-1 cells, NCI-H716 cells, and intestinal organoids (Fig. 6e, g and Supplementary Fig. 6c-e). Meanwhile, we found that, in contrast to potent TGR5-agonistic BAs (DCA etc.), TβMCA treatment did not elevate the cAMP levels in STC-1 cells, NCI-H716 cells, and intestinal organoids (Supplementary Fig. 6h), suggesting that the increased TβMCA after T3 treatment would not activate TGR5 signalling in mice. These results together with other findings in this study, suggest that the increased ileum TβMCA after T3

treatment might promote the GLP-1 production in L-cells through FXR inhibition but not through TGR5 activation.

Additionally, as suggested, we checked the expression of TGR5 after FXR inhibition or activation. In agreement with previous reports showing the regulation of TGR5 by FXR [4, 5], we found that ileal TGR5 mRNA expression was decreased in MMI-treated IFXRKO mice (mice lacking intestinal FXR) regardless of T3 administration (Supporting data Fig. 2f). Consistently, either CA or FEX treatment led to an elevation of TGR5 mRNA levels in the ileum of mice (Supporting data Fig. 2g). Also consistent with above-mentioned reports and our current findings, the TGR5 mRNA expression was decreased by TβMCA treatment in STC-1 cells, NCI-H716 cells, and intestinal organoids (Supporting data Fig. 2h).

4. In humans, inhibition of CYP8B1 will lead to the bile acid pool enriched in CDCA, which is the most FXR endogenous ligand---opposite to rodents.

Response to the Reviewer's Comments: We thank the reviewer for the comments. We agree with the reviewer that inhibition of CYP8B1 will lead to the BA pool enriched in CDCA in human. To test whether BAs with FXR antagonist activities have any effects on the FXR activities in the presence of CDCA, we determined the effect of BAs with FXR antagonist activities on the FXR signalling and GLP-1 production in the presence of CDCA *in vitro* using human enteroendocrine NCI-H716 cells. Interestingly, we found that treatment of FXR-antagonistic BAs could reduce the SHP mRNA levels and increase the GLP-1 production (Fig. 6h and Supplementary Fig. 6f). These data suggest that FXR-antagonistic BAs can antagonize the effect of CDCA on FXR activities. Thus, based on these data, we speculate that changes in FXR-antagonistic BA levels would influence FXR activities, thereby modulating the production of GLP-1 in L-cells.

5. How about using T4 than T3? which is more commonly used in the clinic for the treatment of hypothyroidism.

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. As the reviewer suggested, we tested the effect of T4. To avoid the adverse effect of hyperthyroidism induced by T4 treatment, a low dose of T4 (at a dosage of 60ug/100g BW) was used. We found that treatment of T4 for 5 days decreased glucose levels, increased oral glucose tolerance, and enhanced insulin and GLP-1 production in MMI mice, which were very similar to the effects observed for T3 treatment (Supplementary Fig. 1j).

6. In fig 6, it will be interesting to have additional control groups treated with CA or FEX with or without MMI.

Response to the Reviewer's Comments: We thank the reviewer for the suggestion. We provided the data showing the effects of CA and FEX in mice with or without MMI treatment (Supporting data Fig. 2i-k). In agreement with our working hypothesis, MMI treatment resulted in a decrease in GLP-1 production and insulin levels but an increase in glucose levels. Either CA or FEX treatment did not affect the levels of GLP-1, insulin, and glucose in MMI mice. These data suggest that activating FXR by administration of endogenous or synthetic agonist would not further suppress the GLP-1 production and worsen the glucose metabolism in MMI mice probably because FXR was already activated due to the decreases in FXR-antagonistic BAs in enteroendocrine L-cells. In the mice without MMI treatment (CT mice), FEX had no significant effect on these parameters, while

CA slightly decreased glucose levels, increased GLP-1 expression and production, and insulin level, although some of the differences did not reach statistical significance. We interpret these two results as follows.

We did not observe any FEX effect on the glucose and GLP-1 levels in normal mice, which is not surprising (Supporting data Fig. 2i, k). It has been reported that 5 weeks of FEX treatment, which resulted in intestinally restricted FXR activation, could affect glucose homeostasis in obese mice but not in normal mice, which might involve the browning of adipose tissues and weight loss [6]. It has also been reported that 7 days of FEX treatment could increase GLP-1 production and oral glucose tolerance in mice in an either FXR- or TGR5-dependent manner, which might involve the action of gut microbiota [5]. Notably, in the same study, 9 days of FEX treatment could increase the GLP-1 production and improve oral glucose tolerance in obese mice, which was accompanied by a decrease in body weight [5]. In a recent paper, 28 days of FEX treatment did not affect GLP-1 production and oral glucose tolerance in mice (6 weeks old) [7]. In line with these data, we also did not observe the effects of FEX treatment on the levels of glucose, insulin, and GLP-1 and body weight in normal mice (CT mice) (Supporting data Fig. 2i, k), suggesting that some of FEX effects observed in obese mice might be attributed to the weight loss after long-term FEX treatment, which might involve gut microbiota-mediated TGR5 signaling. Interestingly, in our study, 5 days of FEX treatment could attenuate the T3 effects on the levels of glucose, insulin, and GLP-1 in MMI-treated mice without changing the body weight (Fig. 7g). Based on these findings and our other data, we speculate that inactivation of FXR signalling might be critically involved in hepatic T3 signalling-mediated regulation of GLP-1 production and glucose metabolism, which did not require either the weight loss or the involvement of TGR5 signaling. Nevertheless, we did not intend to exclude the possibility that other mechanism exists that also contribute the profound role of T3 in glucose homeostasis.

BAs function as endogenous ligands for FXR, which has a complex role in metabolic homeostasis. FXR total knockout mice developed hyperglycemia on a normal chow diet, while they exhibited improved glucose homeostasis on a high-fat diet [8, 9]. Although contradictory observations have been reported using systemic FXR agonists, beneficial effects were normally observed in chow-fed mice, whereas exacerbated glucose intolerance is seen under pathophysiological conditions such as obesity [4, 10-12]. Consistent with a previous report showing that CA feeding could decrease fasting glucose by approximately 50% in mice [13], in this study, we also detected a beneficial effect of CA treatment in normal mice, as evident from reduced glucose levels and increased GLP1-1 expression and production, although some of the differences did not reach statistical significance (Supporting data Fig. 2j). However, in MMI mice, CA lost its beneficial effect, probably due to the already enhanced intestinal FXR signalling (Supporting data Fig. 2j). Importantly, consistent with current knowledge for intestinal FXR, administration of CA significantly attenuated T3-induced elevation of GLP-1 and insulin levels and the glucose-lowering effect of T3 in MMI mice (Fig. 6d). These data further support the notion that FXR is essential for normal glucose homeostasis and inhibition of FXR might serve as an approach in glycemic control under disease states.

Reviewer #3 (Remarks to the Author):

The authors demonstrate that euthyroid animals have improved glucose tolerance and increased circulating insulin and circulating and intestinal GLP-1 levels compared to hypothyroid animals.

Using a wide range of models the authors demonstrate that liver TRB is required for (the majority of) this effect. Intestinal FXR is shown to modulate the beneficial effect of T3 on GLP-1 levels, plasma insulin and plasma glucose in hypothyroid mice. The authors postulate that the link between (hepatic) thyroid hormone signaling and changes in bile acid composition, resulting in increased GLP-1 secretion, is the enzyme CYP8B1 in liver. The data is novel, interesting and clinically relevant given the development of liver specific TRB agonists for the treatment of metabolic disease. Specific Comments:

1. Is hyperglycemia commonly found in hypothyroid mouse models. To this reviewers knowledge the answer is no. What is the reason for this here. Other examples should be brought forward.

Response to the Reviewer's Comments: We thank the reviewer for the comments and suggestion. As we mentioned in the introduction of our manuscript, the role of TH in glucose metabolism is profound. Abnormal glucose homeostasis has been noticed in hypothyroid patients and animal models of hypothyroidism, although the proposed underlying mechanisms sometimes differ among different studies [14-20]. As the reviewer suggested, we searched literatures published recently and listed just a few here: 1) a PNAS paper contributed by Dr. Carrasco, reviewed by Dr. Hollenberg and Dr. Moore, showed impaired glucose tolerance in LID mice in Fig. 3A; 2) similar result could be found in a Diabetes paper by Dr. Kieffer; 3) increased fasting glucose levels in hypothyroid rats were shown in a paper by Dr. Ayuob; 4) in addition to animal studies, low fasting glucose levels were observed in patients with SCH in two papers reported by Dr. Gao.

As the suggested, we employed propylthiouracil (PTU) to induce hypothyroidism in mice. We also observed increased glucose levels, impaired oral glucose tolerance, and decreased insulin and GLP-1 levels in these hypothyroid mice induced by PTU administration (Supporting data Fig. 3a).

2. The increased levels of GLP-1 described in Figure 1J are not clear.

Response to the Reviewer's Comments: We thank the reviewer for the comments. As the reviewer suggested, we provided another image with stronger staining intensity showing the increased GLP-1 levels (Fig. 1g). To substantiate our conclusion, we performed immunohistochemistry for GLP-1 and obtained similar results (Supplementary Fig. 1g). We have provided these data in our revised manuscript.

3. Why was GLP-1 not given directly to hypothyroid animals?

Response to the Reviewer's Comments: We thank the reviewer for the comments. As suggested, we tested whether GLP-1 administration could improve the glucose homeostasis in hypothyroid mice (MMI mice). In agreement with our working model, GLP-1 treatment increased insulin levels, decrease glucose levels, and enhance oral glucose tolerance in MMI mice (Fig. 1h-j), suggesting that T3-induced elevation of GLP-1 levels had the capacity to improve glucose metabolism in hypothyroid mice.

4. Ideally, further experiments should be provided to demonstrate KO of the TR in the liver. ie TH mediated gene expression of classic TR target genes. The model is under-described in all aspects.

Response to the Reviewer's Comments: We thank the reviewer for the comments. As the reviewer suggested, we examined the classic T3 targe genes (Dio1, Me, Scd1, and Sp14) in the liver of LTRβKO mice with or without T3 treatment (Supporting data Fig. 3b). We found that the mRNA

expression of positively regulated genes (Dio1, Me, and Sp14) was decreased in the liver of LTRβKO mice. T3 could increase the mRNA expression of these positively regulated genes in the liver of Floxed mice but not LTRβKO mice. These data agree with the notion that TRβ is the major TR isoform in liver. For Scd1, a T3 negatively regulated gene, the expression pattern is very similar to that of CYP8B1 (Fig. 4i). As suggested, we provided more information for Floxed mice and LTRβKO mice in our revised manuscript, including details about targeting methodology, genomic context, and general phenotype (Supplementary Fig. 2d-i, 3b and Supporting data Fig. 1d, Page 6, Line 185-188 and Page 16, Line 528-532).

5. In Figure 2 and Figure 3 the induction of hypothyroidism leads to a rise in blood glucose in both WT and KO animals and T3 or the analog reduces it only in WT or floxed animals. Why is there an induction of BG in hypothyroidism in the absence of the TR?

Response to the Reviewer's Comments: We thank the reviewer for the comments. The data shown in Fig. 2 and Fig. 3 (previously version) were not from hypothyroid animals. In this study, we did not observe abnormal glucose levels in LTRβKO mice (Fig. 2d, 3g and Supplementary Fig. 2i), which was consistent with the observation that loss of TRβ in liver only slightly increased the mRNA levels but not the protein levels of CYP8B1 (Fig. 4f, i) and had no effect on ileal BA composition (Fig. 4n, 5d) and GLP-1 production (Fig. 2b, 3e and Supplementary Fig. 2h), indicating that compensatory mechanisms may exist. To investigate the blood glucose levels and T3 effect in LTRβKO mice in hypothyroid state, we treated LTRβKO mice with MMI (Supporting data Fig. 1e). Our observations are as follows: 1) In line with data obtained in wild type mice (Supplementary Fig. 1k, l, 4h), MMI treatment could increase CYP8B1 mRNA expression and glucose levels and impair oral glucose tolerance in Floxed mice (Supporting data Fig. 1e, f). The finding that MMI treatment had a more deleterious effect than loss of TR is not surprising, because unlike steroid hormone receptors, TRs can act in the absence of the ligand as aporeceptors (apoTRs), which have an intrinsic activity rather than being silent. These apoTRs repress basal transcription of positively regulated genes and stimulate that of negatively regulated genes. We speculate that unliganded TRs (apoTRs) in MMI mice might act to increase CYP8B1 mRNA expression, thereby altering the BA composition and impairing glucose metabolism. 2) Consistent with our previous findings in euthyroid state (Fig. 2a-e, 4i), T3 treatment markedly repressed the mRNA expression of CYP8B1 and lowered the glucose levels in MMI-treated Floxed mice, while loss of hepatic TRβ could block these effects in MMI-treated mice (Supporting data Fig. 1e, f), further supporting our hypothesis that hepatic TRβ signaling contributes to the benefits seen with the T3 treatment. 3) It has been proposed that TH deprivation induces a strong apoTR activity, while the effect of the absence of TH can be attenuated by the removal of TR. Here, we found that the mRNA levels of CYP8B1 were not reduced in MMI-treated LTRβKO mice compared to MMI-treated Floxed mice (Supporting data Fig. 1e). Moreover, the glycemic control was not improved after the removal of hepatic TRβ in MMI-treated mice (Supporting data Fig. 1e, f). These observations indicate that apoTRα but not apoTRβ may play a more important role at least in the control of CYP8B1 transcription, which agrees with the previous notion that TRβ may have no aporeceptor activity in liver. Since we mainly focus on the beneficial T3 effect and the role of liganded TRβ (holoTRβ) in current study, we provided these data from hypothyroid LTRβKO mice as supporting data to avoid confusion due to the potential contribution from apoTR.

Additionally, we noticed that loss of hepatic TRβ abolished the T3 effect on CYP8B1 expression

and oral glucose tolerance in MMI-treated mice, which further supports our proposed model involving the action of hepatic TRβ and incretin GLP-1 (Supporting data Fig. 1e, f). We also noticed a small reduction of glucose levels in MMI-treated LTRβKO mice after T3 treatment, although the difference did not reach statistical significance (p=0.06) (Supporting data Fig. 1e, left panel). These data indicate that systemic administration of T3 might also affect glucose levels via other pathways or mechanisms, which seemed to be not as dominant as those mediated by hepatic TRβ under these experimental conditions. Collectively, we believe we have discovered a novel role of T3 and hepatic TRβ in glucose homeostasis, which involves the regulation of GLP-1 production via BA -mediated FXR antagonism. Nevertheless, we did not intend to exclude the possibility that other mechanism exists that also contribute the profound role of T3 in glucose homeostasis.

6. In Figure 3 there is no data on the high fat diet mice ie food intake, body weight etc. It is impossible to determine the effects of the MB compound in this setting.

Response to the Reviewer's Comments: We thank the reviewer for the comments. In line with previous data reported by Dr. Erion et. al. [21], we found that MB07811 treatment had no effects on food intake but could reduce the body weight and white fat mass in HFD-fed mice (Supplementary Fig. 3a, c-g). We have added these data to our revised manuscript.

7. In Figure 4 where is the RNA-Seq data of the genes regulated by feeding. What does Cyp8b1 do with increased feeding.

Response to the Reviewer's Comments: We thank the reviewer for the comments. As the reviewer suggested, we provided the accession code of our microarray data for the genes regulated by feeding (GSE184055) in our revised manuscript. CYP8B1 is highly regulated under various physiological and pathological conditions. CYP8B1 is known to be increased by fasting and downregulated by feeding through multiple mechanisms, including FXR-SHP/MAFG-mediated or FGFR4-mediated negative feedback on BA *de novo* synthesis, thereby modulating the BA profile and the hydrophobicity of BA pool to adapt to the feeding status or nutritional environment. Based on current evidence, we speculate that fasting-induced CYP8B1 expression induces an increase in CA production, which will facilitate the dietary lipid absorption for the next meal. On the other hand, refeeding-induced downregulation of CYP8B1 can lead to an increase in GLP-1 production to enhance the insulin action after the meal [22, 23]. Evidence also indicates that FoxO1 can regulate the CYP8B1 expression in an FXR-independent manner, thereby modulating lipid homeostasis [24]. Regarding the expression of CYP8B1 upon increased feeding or HFD feeding, current evidences are not conclusive. We speculate that it is probably due to the differences in the nutritional status (as it is very sensitive to feeding), the protocol of HFD treatment, and the facility conditions. As the altered expression of CYP8B1 could affect systemic insulin action by modulating GLP-1 and insulin production, which might have either beneficial or deleterious effect on glucose and lipid metabolism dependent on the feeding pattern or condition or disease status or stage. Nevertheless, based on available knowledge, it has been proposed that targeting BA profile by inhibiting CYP8B1 might be a promising therapeutic strategy for metabolic diseases, including T2D.

8. Also, in Figure 4, T3 is known to regulate many other genes involved in bile acid transport that also regulate bile acid hydrophobicity including cyp27a1 and cyp3a11 and as well bile acid transporters. These genes are not included in the analysis shown. Interestingly, Cyp8b1 has been identified previously as a target of TH signaling: Andersson U, Yang YZ, Bjorkhem I, Einarsson C,

Eggertsen G, Gafvels M. Thyroid hormone suppresses hepatic sterol 12α-hydroxylase (CYP8B1) activity and messenger ribonucleic acid in rat liver: failure to define known thyroid hormone response elements in the gene. Biochim Biophys Acta. 1999;1438(2):167–174. Additionally, thyroid hormone signaling has also been shown to alter bile acid composition previously and thus regulate intestinal cholesterol absorption (Astapova et al, JCI 2014).

Response to the Reviewer's Comments: We thank the reviewer for the comments. We agree with the reviewer that systemic T3 treatment can regulate many hepatic genes involved in metabolism including BA metabolism. As the reviewer suggested, we analyzed our RNA-seq data (GSE184261) and provided these data in our revised manuscript (Supplementary Fig. 4a) and cited related papers [25, 26]. Indeed, as described in our manuscript, to understand the mechanism underlying the glucose-lowering effect of hepatic TH signalling, we performed RNA-seq followed by KEGG pathway analysis to identify hepatic genes or pathways regulated by T3 (Fig. 4a). KEGG pathway analysis of DEGs revealed that 72 pathways were regulated by T3 treatment, including primary BA biosynthesis (Fig. 4b). Given that intestinal GLP-1 is secreted postprandially and GLP-1-mediated incretin effect contributes to the glucose-lowering effect of T3, we compared the T3-reuglated pathways with those pathways altered in response to oral intake of nutrients and identified 10 pathways that were regulated by both T3 administration and nutrition ingestion (Fig. 4b, c). We then analyzed the overlapped gene sets in these 10 pathways and identified 22 genes (Supplementary Fig. 4b), including CYP8B1. As CYP8B1 has been implicated in the regulation of GLP-1 secretion and glucose homeostasis [22]. We then speculated that CYP8B1 might be involved in the regulation of glucose metabolism by T3. Furthermore, our new data demonstrate that AAV-mediated knockdown of CYP8B1 could block the T3 effect on GLP-1 production, insulin and glucose levels, oral glucose tolerance (Fig. 4j-l), further suggesting that CYP8B1 might be the primary regulator that mediates the metabolic effect of T3 observed in this study.

We also agree with the reviewer that CYP8B1 is known to be regulated by T3. Since an early study failed to define known TRE in the promoter region of rat CYP8B1 (about 2-kb upstream of the start site), whether CYP8B1 is transcriptionally by TRβ has been a longstanding mystery. To better understand the regulation of CYP8B1 by T3, we analyzed recent ChIP-seq data (GSE159648) reported by Lazar's lab [27] and identified a super-enhancer encompassing the mouse CYP8B1 gene (Supplementary Fig. 4f). Moreover, we found that two putative TR binding sites (DR1 and DR4) identified early are in this super-enhancer, which are in the intergenic region but not in the promoter region of mouse CYP8B1 (Supplementary Fig. 4f). Our ChIP analysis revealed that TRβ could be recruited to the super-enhancer region containing these two binding sites (Fig. 4h). In line with our above super-enhancer analysis, we observed H3K27 acetylation in the same region (Fig. 4h). These results suggest that TRβ might transcriptionally controls the CYP8B1 expression through a super-enhancer-mediated mechanism. Additionally, our data obtained by using LTRβKO mice suggest that the negative regulation of CYP8B1 by T3 requires TRβ (Fig. 4f, i).

Regarding the previous studies by Astapova et al from Hollenberg's lab, they employed a unique mouse model developed mice that express a mutant NCoR protein (L-NCoRΔID) that cannot interact with the TR in the liver to explore the role of NCoR and TR. In their PNAS paper [25], they found that positive T3 targets were up-regulated in L-NCoRΔID mice in the hypo- and euthyroid state. Interestingly, 326 genes were activated in hypothyroidism (representing negatively regulated TR/T3-target genes) in control mice, and only 3 of these genes were repressed (<1%) in hypothyroid

L-NCoRΔID mice. The authors proposed that NCoR is a specific regulator of T3 action and mediates repression by unliganded TR (apoTR) in hypothyroidism. Therefore, NCoRΔID mice might not be a suitable mouse model to study the T3 effect on negatively regulated T3 target genes or the role of liganded TRs (holoTRs) in the negative regulation by T3. Later, in their JCI paper[26], they found an alteration in the composition and hydrophobicity of BA pool in L-NCoRΔID mice upon 2% cholesterol feeding, accompanied with changes in the expression of genes involved in BA synthesis (CYP27A1 and CYP3A11) and transport (ABCB11), which might eventually lead to a decrease in cholesterol absorption. Consistent with their PNAS paper, they did not observe any changes in CYP8B1 expression. They also showed that the mRNA expression of CYP27A1 and CYP3A11 were downregulated in the hypothyroid state, however, unlike CYP27A1, the expression of CYP3A11 was further suppressed by T3 treatment in hypothyroid mice. Thus, the T3 action in BA metabolism, especially the T3 action mediated by liganded TRs (holoTRs) is still not fully understood. Notably, although CYP27A1 is the first enzyme in the alternative BA synthetic pathway, it also participates in classic BA synthesis. Although CYP3A11 has been proposed to participate in alternative bile acid synthesis, loss of CYP3A11 would not affect BA composition [28]. As the regulation by BA composition by NCoRΔID had not been investigated in the absence or after knockdown of downstream effectors, thus, whether these two enzymes are the primary players in hypothyroidism or the major players mediating the effect of apoTR in the euthyroid state requires further investigation.

It is also worth noting that these authors only investigated the BA profiles in L-NCoRΔID mice, which provides indirect evidence for the role of TH signalling in BA metabolism. Moreover, previous studies mainly focused on the role of BAs in nutrition absorption, whether TH signalling could use BAs as essential endocrine molecules to control metabolic homeostasis is unknown. Furthermore, given that NCoR only interacts with TR in the absence of T3, NCoR may also bind to other transcription factors, and therefore the profound T3 effects, especially those effects mediated by liganded TRs (holoTRs) or negatively regulated target genes of T3, on BA metabolism and corresponding physiological consequence remain unclear and require extensive investigation.

Indeed, we observed similar results for those genes reported by Astapova et al from our RNA seq analysis, as mentioned above, we provided these data with other genes involved in BA synthesis and transport in our revised manuscript (Supplementary Fig. 4a). Whether these genes involved in BA transport and the regulation of BA hydrophobicity also play a role in the regulation of glucose homeostasis by T3 requires future investigation. Nevertheless, since CYP8B1 is responsible for CA synthesis, it is destined to be a master regulator of BA pool composition. Moreover, as we mentioned above, CYP8B1 is regulated by nutritional status, loss of CYP8B1 has been shown to be able to promote GLP-1 production thereby modulating glucose homeostasis, and knockdown of CYP8B1 could attenuate the T3 effect on GLP-1 production and glucose metabolism (Fig. 4j-l), we propose that CYP8B1 might be the primary regulator that mediates the metabolic effect of T3 observed in this study.

9. The explanation of the data in Figure 7 is not clear. TSH is used to determine TH action. Do the TSH levels inversely correlate with the TH levels. Additionally do the T3 levels correlate with other actions of TH on the liver ie serum cholesterol.

Response to the Reviewer's Comments: We thank the reviewer for the comments. We agree with

the reviewer that TSH is normally used as an indicator for the changes of function in HPT axis, moreover, it is also the only diagnostic indictor for subclinical thyroid disease. However, the association between TSH and THs is weak or not significant or even "unexpected" within normal arrange, which is not surprising probably due to many compensatory mechanisms and feedback loops that maintains the levels of each hormone. In two cohorts used in our study, we did not detect any significant association between TSH and T3/T4 (Supporting data Fig. 3c, d, g, h). It is also worth noting that, in normal subjects, when the metabolic regulation is normal, the association between two hormones that are regulated reciprocally is hard to detect. For instance, the association between glucose and insulin is normally not seen in normal subject. Although we did not observe an association between TSH levels and the levels of GLP-1 or insulin, the levels of non-12α-OH BAs, or the ratios of 12α-OH BAs to non-12α-OH BAs in this study (Supporting data Fig. 3e, f, i, j), we could not rule out the possibility that we would detect such an association in a larger cohort. As suggested, we also determined the association between T3 and total cholesterol levels in two cohorts we used in this study. As expected, we observed an inverse correlation between T3 and cholesterol levels in the plasma in the larger cohort (Supplementary Fig. 8a). A similar trend could be observed in the small cohort, although the association was not statistically significant probably due to the limited sample size (Supplementary Fig. 8b).

10. Is there a CYP8B1-KO model available or specific inhibitor that would allow you to study the effect of T3 in the absence of CYP8B1?

Response to the Reviewer's Comments: We thank the reviewer for the comments. As CYP8B1-KO model is not available in the lab, knockdown of hepatic CYP8B1 was achieved by using adeno-associated virus (AAV) expressing shRNA specific for CYP8B1. As expected, AAV-mediated knockdown of hepatic CYP8B1 could block the T3 effect on GLP-1 production, insulin and glucose levels, oral glucose tolerance (Fig. 4j-l and Supplementary Fig. 4g), further supporting our notion that CYP8B1 might be the primary regulator that mediates the metabolic effect of T3 observed in this study.

11. Does the attenuated change in CYP8B1 in MMI+T3 treated L-TRBKO mice result in measurable changes in intestinal bile acid composition compared to controls?

Response to the Reviewer's Comments: Yes. To test whether the attenuated change in CYP8B1 expression levels in T3-treated LTRβKO mice, as shown in Fig. 4f, would result in measurable changes in intestinal BA composition, we determined the ileal BA composition in these four groups of mice. Consistent with the CYP8B1 expression levels as shown in Fig. 4f, we found that the ileal BA composition (percentages of non-12α-OH BAs and 12α-OH BAs) was not altered in LTRβKO mice as compared to Floxed mice, was changed after T3 treatment in Floxed mice, and loss of hepatic TRβ could abolished the T3 effect on BA composition (Fig. 4n, 5d).

Other comments:

12. Figure 2: . Please specify if these mice were rendered hypothyroid before being treated with T3 as this isn't mentioned in the figure legends or corresponding results section

Response to the Reviewer's Comments: We thank the reviewer for the comments. Regarding Figure 2, these LTRβKO mice and Floxed mice were not rendered hypothyroid before T3 treatment. We have performed new experiments and provided the data from MMI-treated LTRβKO mice with

or without T3 treatment in Supporting data Fig. 1e, f.

13. The other bile acid receptor TGR5 has been described to be crucial for GLP-1 induction and several other metabolic effects. Did you find any evidence for a role of TGR5 in this pathway? Why did you choose to focus only on FXR?

Response to the Reviewer's Comments: We thank the reviewer for the comments. As suggested, we explored the role of TGR5 in this study. Similar to FXR, we did not detect any changes in mRNA expression of TGR5 in the ileum of MMI mice after T3 or MB07811 treatment (Supplementary Fig. 5j). We then analyzed the levels of BAs with TGR5 agonist activities. We found that T3 treatment decreased the total percentage of BAs with TGR5 agonist activities in the ileum of MMI mice, while the percentage of DCA, which is abundant in ileum and exhibits potent TGR5 agonist activity, was not altered (Supplementary Fig. 5f). As we did not observe an elevation in the levels of these TGR5-agonistic BAs, we speculate that FXR-mediated pathway rather than TGR5-mediated pathway was critically involved after T3 treatment in mice. Consistently, positive correlation was not observed between the T3 level and the percentage of BAs with potent TGR5 agonist activities in human feces, further indicating that TGR5 signalling might not play a considerable role here (Supplementary Fig. 8c, d).

To further test whether TGR5 signalling were activated, we first measured the cAMP levels in the ileum of mice and found that either T3 or MB07811 treatment could not affect the cAMP levels (Supplementary Fig. 5h). We also employed enteroendocrine STC-1 and NCI-H716 cells and mouse intestinal organoids to investigate the effect of T3 and BAs on TGR5 activation. Interestingly, we found that T3 treatment had no effect on cAMP levels in STC-1 cells, NCI-H716 cells, and mouse intestinal organoids (Supplementary Fig. 5i), suggesting that T3 could not affect GLP-1 production in L-cells by directly activating TGR5 signalling. Meanwhile, we found that, in contrast to potent TGR5-agonistic BAs, TβMCA treatment did not elevate cAMP levels in these enteroendocrine cells and intestinal organoids (Supplementary Fig. 6h), suggesting that the elevated TβMCA levels after T3 treatment would not activate TGR5 signalling in mice. We also treated STC-1 and NCI-H716 cells and intestinal organoids with TβMCA. We found that TβMCA could increase GLP-1 expression and production (Fig. 6e, g and Supplementary Fig. 6c-e), and decrease the mRNA expression of SHP, a downstream target of FXR, in these enteroendocrine cells and intestinal organoids (Supplementary Fig. 6c-e). These results together with other findings in this study, further suggest that the increased ileal TβMCA after T3 treatment might promote the GLP-1 production in L-cells through FXR inhibition but not through TGR5 activation.

14. In general the paper is well written but the discussion would benefit from a thorough read-through to correct several grammatical errors.

Response to the Reviewer's Comments: We thank the reviewer for the comments. We have corrected a few mistakes and improved the discussion part in our revised manuscript.

15. Please specify where the MB07811 TRB agonist was purchased and include data or references that demonstrate its liver and TRB specificity.

Response to the Reviewer's Comments: We thank the reviewer for the comments. MB07811 can be either purchased from TargetMol or customized from Adooq. We recently synthesized MB07811 and its analogues with the help of Dr. Wenjun Tang from State Key Laboratory of Bio-Organic and

Natural Products Chemistry, Center for Excellence in Molecular Synthesis, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. We have validated its selectivity (for TRβ) by performing luciferase assay, its specificity (liver-targeting) by using THAI mice and its function (cholesterol-lowering) by using MMI mice (Supporting data Fig. 3k-m).

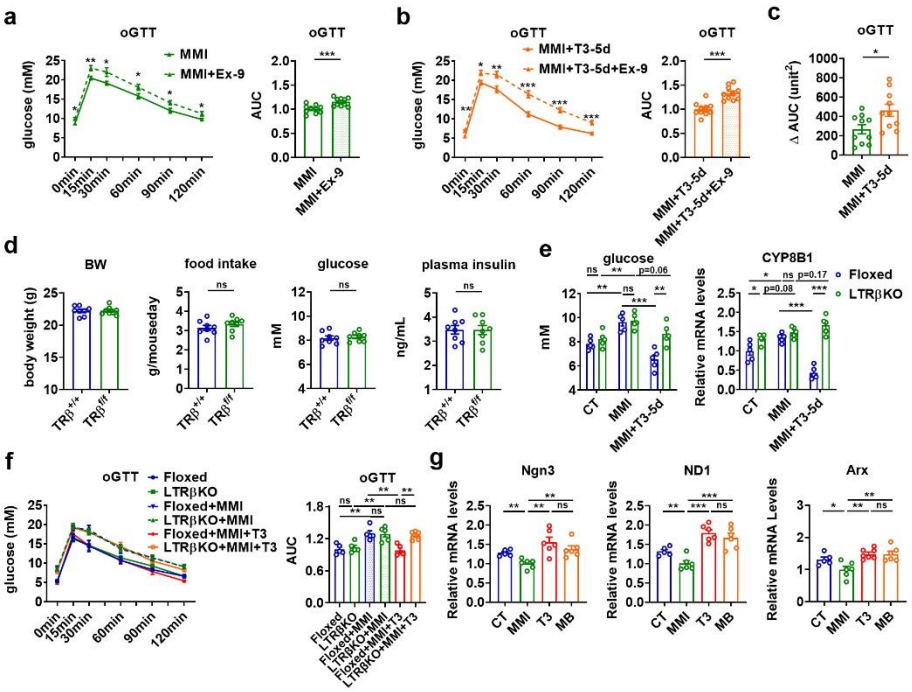
16. Have you done any of these experiments in female mice? If so please show (in supplemental)

Response to the Reviewer's Comments: Thanks for the comments. Indeed, we had performed a few key experiments in female mice and obtained similar results, suggesting that the regulation of GLP-1 production and glucose homeostasis by T3 treatment can also be observed in female mice (Supplementary Fig. 1i, 2h, i). Moreover, our recent data showed that deletion of hepatic TRβ in female mice could also diminish the T3 effects on the GLP-1 production and glucose homeostasis (Supplementary Fig. 2h, i). Thus, we speculate that the underlying mechanism for the regulation of glucose metabolism by T3 identified in this study is gender-independent.

17. Please state whether your human subjects provided informed consent in accordance with the declaration of Helsinki.

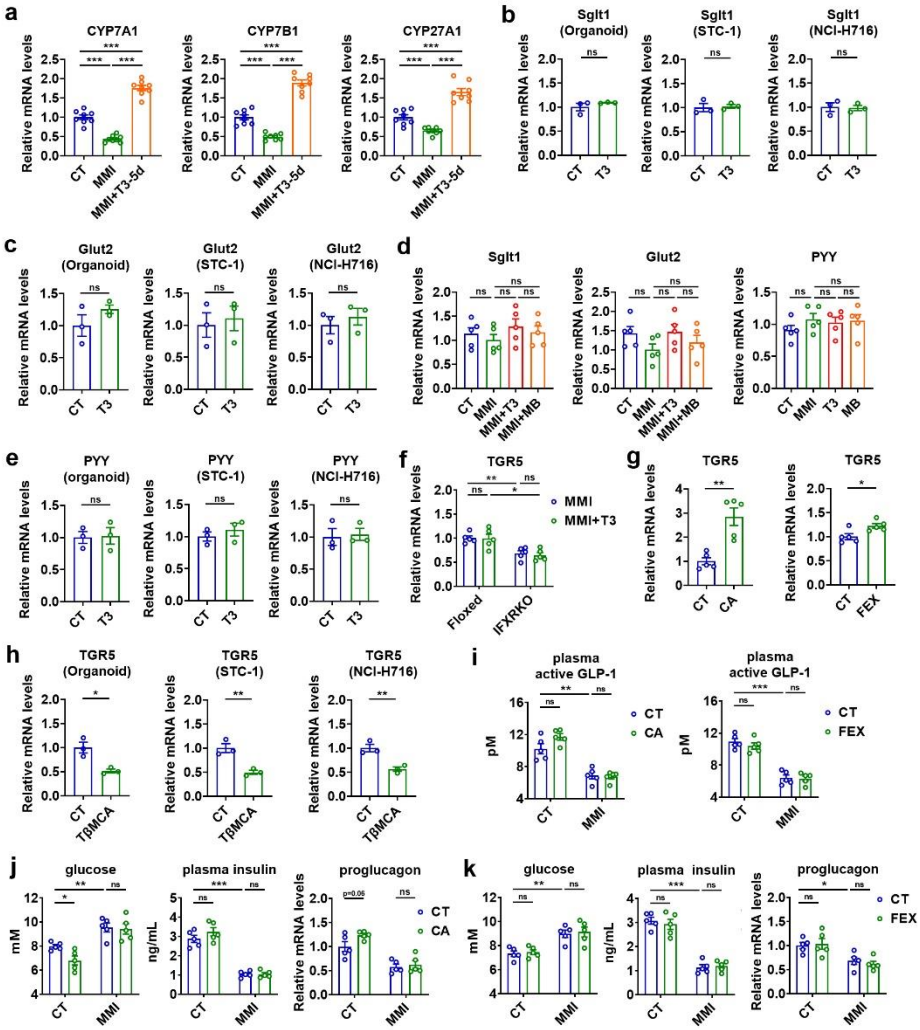
Response to the Reviewer's Comments: We thank the reviewer for the comments. Human subjects in this study provided informed consent in accordance with the declaration of Helsinki. We have included this information in our revised manuscript.

Supporting data



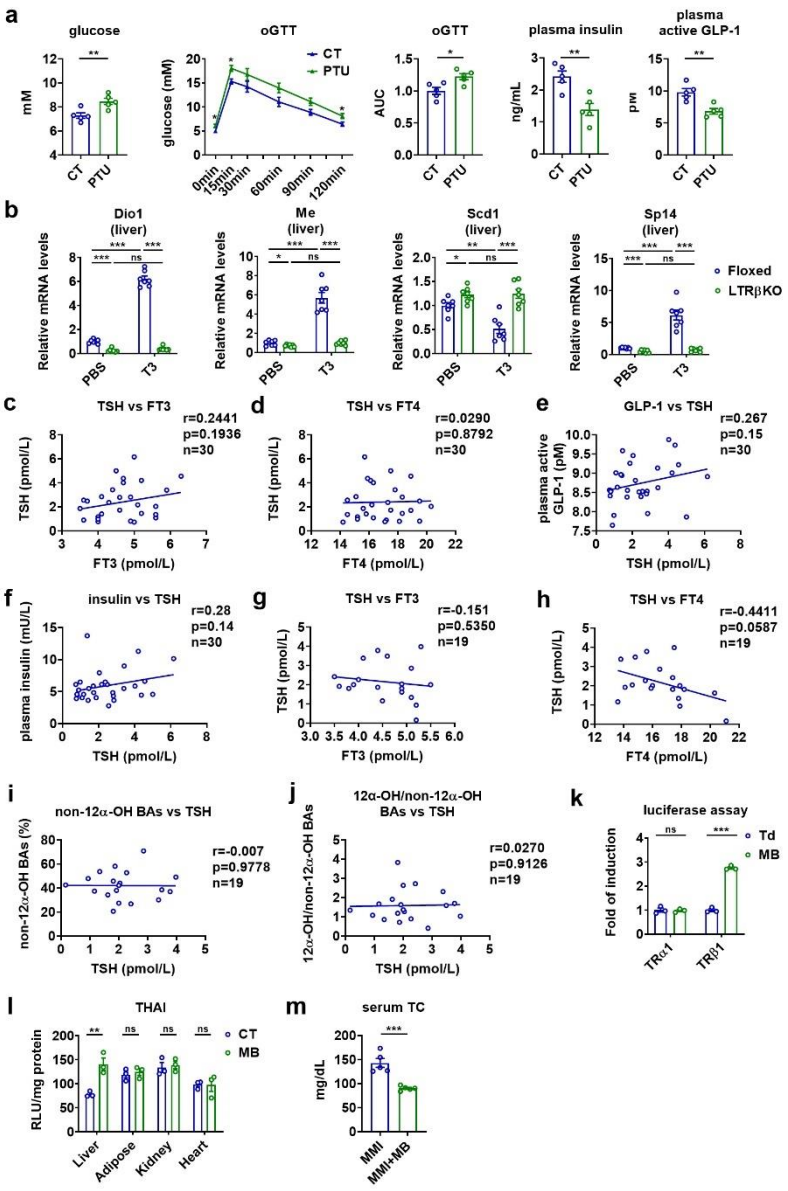
Supporting data Figure 1.

(a and b) The data in Figure 1 n are shown separately. oGTT for MMI mice (a) and MMI+T3-5d mice (b) treated with saline or Ex-9 for 5 days and the AUC (n=10). (c) ΔAUC are calculated as follows: ($AUC_{MMI+EX-9}$-AUC_{MMI}) and ($AUC_{MMI+T3-5d+EX-9}$-$AUC_{MMI+T3-5d}$), according to panel a and b. (d) The BW, food intake, glucose and plasma insulin levels in TRβ Floxed ($TR\beta^{f/f}$) mice and unfloxed mice ($TR\beta^{+/+}$) mice (n=8). (e) The blood glucose levels (left) and relative mRNA levels of CYP8B1 in the liver (right) of Floxed and LTRβKO mice treated with vehicle (CT), MMI or MMI and 5 days of T3 (n=5). (f) oGTT for Floxed and LTRβKO mice treated with vehicle (CT), MMI or MMI and 5 days of T3 (n=5) and the corresponding AUC for oGTT (n=5). (g) The relative mRNA levels of Ngn3, ND1, and Arx in the ileum of CT mice, MMI mice, and MMI mice after 5 days of T3 or MB treatment (n=5-6). Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.



Supporting data Figure 2.

(a) The relative mRNA levels of CYP7A1, CYP7B1 and CYP27A1 in the liver of CT, MMI and MMI+T3-5d mice (n=8). (b and c) Relative mRNA levels of Sglt1 (b) and Glut2 (c) in mouse intestinal organoids, STC-1 cells and NCI-H716 cells after T3 treatment (n=3). (d) The relative mRNA levels of Sglt1, Glut2, and PYY in the ileum of CT mice, MMI mice, MMI mice after 5 days of T3 or MB treatment (n=5-6). (e) Relative mRNA levels of PYY in mouse intestinal organoids, STC-1 cells and NCI-H716 cells after T3 treatment (n=3). (f) The relative mRNA levels of TGR5 in the ileum of Floxed and IFXRKO mice treated with MMI or MMI and 5 days of T3 (n=6). (g) The relative mRNA levels of TGR5 in the ileum of mice treated with or without CA or FEX for 5 days as indicated (n=5). (h) Relative mRNA levels of TGR5 in mouse intestinal organoids, STC-1 cells and NCI-H716 cells after TβMCA treatment (n=3). (i-k) Plasma active GLP-1 (i), blood glucose and plasma insulin levels, relative proglucagon mRNA levels (j and k) in the ileum of CT and MMI mice treated with or without CA or FEX for 5 days as indicated (n=5). Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.



Supporting data Figure 3.

(a) Blood glucose levels, oGTT, plasma insulin and plasma active GLP-1 levels in mice treated with PTU (n=5). (b) Relative mRNA levels of Dio1, ME, Scd1 and Sp14 in the liver of Floxed and LTRβKO mice treated with PBS or T3 for 5 days (n=6). (c-f) Correlation between free T3 and TSH levels (c), free T4 and TSH levels (d), TSH levels and plasma active GLP-1 levels (e) or plasma insulin levels (f) in a cohort of euthyroid subjects (n=30). (g-j) Correlation between free T3 and TSH levels (g), between free T4 and TSH levels (h), between TSH levels and the percentage of fecal non-12α-OH BAs (i), or between TSH levels and the fecal 12α-OH/non-12α-OH ratios (j) in another cohort of euthyroid subjects (n=19). (k) Luciferase assay showing that MB is an agonist with selectivity for TRβ as compared to TRα. Pal-luc reporter plasmid was co-transfected with TRα or TRβ plasmid in HepG2 cells followed by MB treatment. Reporter activity was analyzed and fold of induction by MB treatment was calculated. (l) Luciferase activity in tissue samples, including liver, adipose tissue (inguinal fat), kidney, and heart, from TH action indicator (THAI) mice treated with MB for 5 days (n=3). (m) Serum total cholesterol (TC) levels in MMI mice treated with MB for 5 days (n=5). Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.

Materials and Methods for Supporting data

Mice studies. Mice were rendered hypothyroid by adding 0.15% propylthiouracil (PTU) (T1309, TargetMol) in their drinking water for four weeks. TH action indicator (THAI) mice were developed previously [29], which harbor a TH-responsive luciferase reporting system. THAI mice received MB07811 treatment at a dose of 5mg/kg/day for five days. Tissue samples were lysed in luciferase lysis buffer, supernatant was collected after centrifugation at 14,000 g at 4°C 10 min. Luciferase activity was determined with luciferase assay system reagent (E1910, Promega) on a Luminoskan Ascent. Serum total cholesterol levels were determined according to the manufacturer's instructions (294-65801, Wako).

Cell luciferase assay. To analyze MB07811 is an agonist with selectivity for TRβ as compared to TRα, pal-luc reporter plasmid was co-transfected with TRα or TRβ expressing plasmid as indicated in HepG2 cells followed by MB07811 treatment (100 μM, 24h), the pRL-TK vector was used to normalize the luciferase activity. Cells were lysed 48 h after transfection and measured luciferase activity by using Dual-Luciferase® Reporter Assay System.

Reference

1. Chassande, O., *Do unliganded thyroid hormone receptors have physiological functions?* J Mol Endocrinol, 2003. **31**(1): p. 9-20.
2. Xie, C., et al., *An Intestinal Farnesoid X Receptor-Ceramide Signaling Axis Modulates Hepatic Gluconeogenesis in Mice.* Diabetes, 2017. **66**(3): p. 613-626.
3. Lund, M.L., et al., *L-Cell Differentiation Is Induced by Bile Acids Through GPBAR1 and Paracrine GLP-1 and Serotonin Signaling.* Diabetes, 2020. **69**(4): p. 614-623.
4. Pathak, P., et al., *Farnesoid X receptor induces Takeda G-protein receptor 5 cross-talk to regulate bile acid synthesis and hepatic metabolism.* J Biol Chem, 2017. **292**(26): p. 11055-11069.
5. Pathak, P., et al., *Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism.* Hepatology, 2018. **68**(4): p. 1574-1588.
6. Fang, S., et al., *Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance.* Nature Medicine, 2015. **21**(2): p. 159-165.
7. Zheng, X.J., et al., *Hyocholic acid species improve glucose homeostasis through a distinct TGR5 and FXR signaling mechanism.* Cell Metabolism, 2021. **33**(4): p. 791-+.
8. Sinal, C.J., et al., *Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis.* Cell, 2000. **102**(6): p. 731-44.
9. Prawitt, J., et al., *Farnesoid X receptor deficiency improves glucose homeostasis in mouse models of obesity.* Diabetes, 2011. **60**(7): p. 1861-71.
10. Zhang, Y., et al., *Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 1006-11.
11. Watanabe, M., et al., *Lowering bile acid pool size with a synthetic farnesoid X receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure.* J Biol Chem, 2011. **286**(30): p. 26913-20.
12. Trabelsi, M.S., et al., *Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells.* Nat Commun, 2015. **6**: p. 7629.
13. Ma, K., et al., *Farnesoid X receptor is essential for normal glucose homeostasis.* J Clin Invest,

2006. **116**(4): p. 1102-9.
14. Ferrandino, G., et al., *Pathogenesis of hypothyroidism-induced NAFLD is driven by intra- and extrahepatic mechanisms.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(43): p. E9172-E9180.
15. Bruin, J.E., et al., *Hypothyroidism Impairs Human Stem Cell-Derived Pancreatic Progenitor Cell Maturation in Mice.* Diabetes, 2016. **65**(5): p. 1297-1309.
16. Faddladdeen, K., et al., *Thymoquinone Preserves Pancreatic Islets Structure Through Upregulation of Pancreatic beta-Catenin in Hypothyroid Rats.* Diabetes Metab Syndr Obes, 2021. **14**: p. 2913-2924.
17. Song, Y., et al., *Thyroid-Stimulating Hormone Levels Are Inversely Associated with Serum Total Bile Acid Levels: A Cross-Sectional Study.* Endocr Pract, 2016. **22**(4): p. 420-6.
18. Xu, C., et al., *Abnormal Glucose Metabolism and Insulin Resistance Are Induced via the IRE1alpha/XBP-1 Pathway in Subclinical Hypothyroidism.* Front Endocrinol (Lausanne), 2019. **10**: p. 303.
19. Salazar, P., et al., *Induction of hypothyroidism during early postnatal stages triggers a decrease in cognitive performance by decreasing hippocampal synaptic plasticity.* Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2017. **1863**(4): p. 870-883.
20. Biondi, B., G.J. Kahaly, and R.P. Robertson, *Thyroid Dysfunction and Diabetes Mellitus: Two Closely Associated Disorders.* Endocrine Reviews, 2019. **40**(3): p. 789-824.
21. Erion, M.D., et al., *Targeting thyroid hormone receptor-beta agonists to the liver reduces cholesterol and triglycerides and improves the therapeutic index.* Proc Natl Acad Sci U S A, 2007. **104**(39): p. 15490-5.
22. Kaur, A., et al., *Loss of Cyp8b1 improves glucose homeostasis by increasing GLP-1.* Diabetes, 2015. **64**(4): p. 1168-79.
23. Li, T., et al., *Glucose and insulin induction of bile acid synthesis: mechanisms and implication in diabetes and obesity.* J Biol Chem, 2012. **287**(3): p. 1861-73.
24. Haeusler, R.A., et al., *Impaired generation of 12-hydroxylated bile acids links hepatic insulin signaling with dyslipidemia.* Cell Metab, 2012. **15**(1): p. 65-74.
25. Astapova, I., et al., *The nuclear corepressor, NCoR, regulates thyroid hormone action in vivo.* Proc Natl Acad Sci U S A, 2008. **105**(49): p. 19544-9.
26. Astapova, I., et al., *Hepatic nuclear corepressor 1 regulates cholesterol absorption through a TRbeta1-governed pathway.* J Clin Invest, 2014. **124**(5): p. 1976-86.
27. Shabtai, Y., et al., *A coregulator shift, rather than the canonical switch, underlies thyroid hormone action in the liver.* Genes Dev, 2021. **35**(5-6): p. 367-378.
28. Wahlström, A., et al., *Cyp3a11 is not essential for the formation of murine bile acids.* Biochem Biophys Rep, 2017. **10**: p. 70-75.
29. Mohacsik, P., et al., *A Transgenic Mouse Model for Detection of Tissue-Specific Thyroid Hormone Action.* Endocrinology, 2018. **159**(2): p. 1159-1171.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript has been significantly improved with this revised version. The authors have included new data from critical experiments requested by the reviewers. Issues related to comparability of effects across relevant groups has been partially mitigated by the inclusion of wild type controls treated with T3 in some of those newly added loss-of-function experiments. Those experiments are key to compellingly test the authors hypothesis and provide critical mechanistic insights. Additional plasma measurements also increase the overall confidence in the results. I have no further objections.

Reviewer #2 (Remarks to the Author):

The authors have done a very comprehensive job in addressing my questions and comments. The work is novel and very intriguing, which will provide novel insights into understanding the effects and underlying molecular mechanism of hypothyroidism on glucose homeostasis.

Reviewer #4 (Remarks to the Author):

Yan et al. have described an interesting manuscript about a novel T3-mediated pathway in which T3 transcriptionally down-regulates CYP8B1 in the liver to generate bile acids that are FXR antagonists and cause increased GLP1 synthesis in the small bowel to cause increased insulin synthesis. This is an interesting pathway which highlights potential inter-organ communication and multi-hormone regulation to control insulin synthesis. It raises the possibility that blocking CYP8B1 could be a novel drug target to increase insulin secretion.

However, my major concern is an important one and is similar to Reviewer 1's concern. In Yan's model system, the mice are rendered hypothyroid with MMI. They are then injected with T3 daily for 5 days. However, since T3 has a half-life of 2 hours in mice, the mice are transiently hyperthyroid and then become hypothyroid again over the following 24 hours before their next T3 injection. Given this fluctuation, it is hard to know the precise thyroid state of the T3-injected mice. Thus, it would be useful to see whether this pathway plays a significant role in the euthyroid and hyperthyroid states where serum fT3 levels would be relatively constant. Performing such studies also would provide more clinical relevance to their studies. In Suppl. Fig. 1, the authors compare MMI-treated and euthyroid mice, and found decreased insulin and reduced GLP1 in MMI-treated mice compared to euthyroid mice. What happens to the bile acid composition in the euthyroid mice? Does AAV sh8B or FXR KO affect GLP1 and insulin secretion in euthyroid mice? The same types of questions could be asked for hyperthyroid mice. Note that the hyperthyroid mice would need to be treated with both T4 and T3 since T4 has a much longer half-life than T3.. As such, Yan's system describes chronic daily T3 injections over 5 days that repetitively stimulate or decrease target genes daily (reminiscent of a study by Ohba et al. on T3-treated

euthyroid mice. Endocrinology 157:1660–1672(2016)). It is interesting that one T3 injection did not generate effects on GLP1 and insulin secretion. Does MMI+T3-4h have any effects in CyP8B1 expression in comparison to 5d shown in Fig. 4d? The study by Ohba showed that acute vs. chronic stimulation by T3 has different effects on target genes.

Fig. 4. I would like to see transcriptome analysis of euthyroid vs MMI+T3-5d mice. Comparing hyperthyroid mouse transcriptome with MMI+T3-5d also would be useful. It would be useful to look at GLP1, insulin, GTT, and bile acid composition for euthyroid and hyperthyroid mice with MMI and MMI+T3-5d mice.

It would be interesting to examine insulin response in the liver in their studies. T3 typically stimulates gluconeogenesis in the liver, so does activation of this pathway by insulin change the direction towards glycolysis or are the livers of the hypothyroid mice still insulin resistant? The authors should look at IR phosphorylation, pAkt, pmTOR signaling for evidence of insulin signaling. The also could look at expression of T3 target genes, PEPCK, G6Pase, PDK4 mRNA, in the liver. It would be important to know whether this elaborate regulatory pathway is increasing insulin effects in the liver or is stimulating insulin secretion to serve other tissues.

Fig. 6. Does T3 have separate effects on intestinal cells? Could it regulate FXR levels, or GLP1 gene expression independently of FXR? Such studies could be done in cell lines or organoids described in Fig. 6e.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript has been significantly improved with this revised version. The authors have included new data from critical experiments requested by the reviewers. Issues related to comparability of effects across relevant groups has been partially mitigated by the inclusion of wild type controls treated with T3 in some of those newly added loss-of-function experiments. Those experiments are key to compellingly test the authors hypothesis and provide critical mechanistic insights. Additional plasma measurements also increase the overall confidence in the results. I have no further objections.

Response to the Reviewer's Comments: We thank the reviewer for the valuable and insightful comments and suggestions, which have improved our manuscript substantially.

Reviewer #2 (Remarks to the Author):

The authors have done a very comprehensive job in addressing my questions and comments. The work is novel and very intriguing, which will provide novel insights into understanding the effects and underlying molecular mechanism of hypothyroidism on glucose homeostasis.

Response to the Reviewer's Comments: We thank the reviewer for the detailed review and the constructive suggestions that have been helpful to improve our manuscript.

Reviewer #4 (Remarks to the Author):

Yan et al. have described an interesting manuscript about a novel T3-mediated pathway in which T3 transcriptionally down-regulates CYP8B1 in the liver to generate bile acids that are FXR antagonists and cause increased GLP1 synthesis in the small bowel to cause increased insulin synthesis. This is an interesting pathway which highlights potential inter-organ communication and multi-hormone regulation to control insulin synthesis. It raises the possibility that blocking CYP8B1 could be a novel drug target to increase insulin secretion.

However, my major concern is an important one and is similar to Reviewer 1's concern. In Yan's model system, the mice are rendered hypothyroid with MMI. They are then injected with T3 daily for 5 days. However, since T3 has a half-life of 2 hours in mice, the mice are transiently hyperthyroid and then become hypothyroid again over the following 24 hours before their next T3 injection. Given this fluctuation, it is hard to know the precise thyroid state of the T3-injected mice. Thus, it would be useful to see whether this pathway plays a significant role in the euthyroid and hyperthyroid states where serum fT3 levels would be relatively constant. Performing such studies also would provide more clinical relevance to their studies.

Response to the Reviewer's Comments: We thank the reviewer for the comments. We agree with the reviewer that, normally, the MMI or PTU-treated mice receiving T3 daily injections for several days are not considered as hyperthyroid mice [1]. Although the T3 levels are repetitively elevated by daily T3 injections, mice are thought to be transiently hyperthyroid, which are not suitable for modeling clinical hyperthyroidism. However, given that, after 24 hours of the last T3 injection, the T3 normally would return to baseline levels similar to those in euthyroid mice (Supporting Fig. 1a

and TABLE 1 in a published paper by Zavacki et al. [1]), we speculate that the mice may not become hypothyroid again over the following 24 hours before the next T3 injection.

Importantly, we'd like to point out that this canonical model is normally employed to identify genes with maximal T3 responsiveness. In this study, instead of exploring T3-regulated genes, we used this mouse model, aiming to discover novel metabolic pathways not only responding to the T3 treatment but also mediating the beneficial effects of T3. It is well accepted that the overall effects of thyroid hormone (TH) on glucose metabolism are complex, some of its effects are mediated via its action on multiple metabolic tissues, including liver, skeletal muscle, and adipose tissues, and some of its actions on different tissues may counteract [2]. Given that glucose intolerance is sometimes observed in thyrotoxicosis and hyperthyroidism normally worsens glycemic control, we think a hyperthyroid mouse model may be not suitable for this study that aims to explore the beneficial effect of T3 on glucose metabolism. Thus, we neither use the word "hyperthyroid" to describe the state of T3-treated MMI mice nor attempt to use this canonical "replenishment" model (MMI plus T3 treatment) to understand the dysregulated glucose metabolism in patients with clinical hyperthyroidism.

Indeed, based on our newly obtained data from a hyperthyroid mouse model (euthyroid mice treated with both T4 and T3, also referred to as TH-5d mice), we noticed that increased GLP-1 and insulin levels and reduced glucose levels could not be observed (Supporting Fig. 1c), although the elevated T3 and T4 levels in hyperthyroid mice (TH-5d mice) (Supporting Fig. 1b) could result in decreases in the hepatic CYP8B1 expression and the ratios of 12α-OH to non-12α-OH BAs and increases in the ileum FXR-antagonistic BA levels and proglucagon mRNA expression (Supporting Fig. 1d-h). Oral glucose tolerance was not significantly impaired, although the abnormal fasting glucose levels and higher glucose levels 15 minutes after oral glucose ingestion were noticed in hyperthyroid mice (TH-5d mice) (Supporting Fig. 1i). These results suggest that the sustained elevated or the pathological levels of T3 in these hyperthyroid mice may have deleterious effects. As hyperthyroidism can cause protein catabolism and increase renal blood flow and glomerular filtration rate [3], while kidney is thought to be a major site of GLP-1 extraction [4], we speculate that increased glomerular filtration in the hyperthyroid state may promote the renal catabolism of GLP-1, counteracting the effect of T3 on GLP-1 production. However, we cannot rule out the possibility that there may be more complex mechanisms involved. Due to the technical issues, the renal clearance of GLP-1 is difficult to study for the time being, although it deserves more attention. Anyway, we agree with the reviewer that the regulation of GLP-1 by TH in the hyperthyroid state requires further study, which will provide insights into the adverse events associated with clinical hyperthyroidism. We provided some discussion and cited related papers regarding these issues mentioned above in our revised manuscript.

In Suppl. Fig. 1, the authors compare MMI-treated and euthyroid mice, and found decreased insulin and reduced GLP1 in MMI-treated mice compared to euthyroid mice. What happens to the bile acid composition in the euthyroid mice? Does AAV sh8B or FXR KO affect GLP1 and insulin secretion in euthyroid mice? The same types of questions could be asked for hyperthyroid mice.

Response to the Reviewer's Comments: We thank the reviewer for the comments. Probably due to the limitation on the number of figures and the length of manuscript, we provided some data as supplementary figures. For example, we provided the data of BA levels and composition for

euthyroid mice in our manuscript submitted previously (Supplementary Fig. 4l (4i in previous version) and Supplementary Fig. 5b-e). We found that the decreased insulin and reduced GLP-1 levels (Supplementary Fig. 1m,n) were accompanied with increased ratios of 12α-OH to non-12α-OH BAs and reduced FXR-antagonistic BA levels in MMI mice compared to euthyroid control mice (Supplementary Fig. 4l (4i in previous version) and Supplementary Fig. 5b-e).

As the reviewer suggested, we determined the effect of AAV-sh8B on GLP-1 and insulin in euthyroid and hyperthyroid mice. We found that AAV-sh8B infection could increase GLP-1 and insulin levels and decrease glucose levels in euthyroid mice (Supplementary Fig. 4i), which are similar to those observed in MMI-treated mice (Fig. 4j,k), suggesting that hepatic CYP8B1 knockdown has beneficial effects in either euthyroid or hypothyroid mice. In contrast, in hyperthyroid mice (TH-5d mice), AAV-sh8B infection was unable to alter the levels of GLP-1, insulin, and glucose, although it could elevate the ileum GLP-1 expression (Supporting Fig. 2a). Notably, T4 and T3 treatment for 5 days decreased the GLP-1 and insulin levels and increased glucose levels in mice with hepatic CYP8B1 knockdown, further supporting our notion that the sustained elevated or pathological levels of T3 have deleterious effects (Supporting Fig. 2a), which is likely independent of the regulation of hepatic CYP8B1 by T3.

Regarding the effect of intestine-specific FXR knockdown on GLP-1 and insulin in a euthyroid state, we included these data in our manuscript submitted previously (Supplementary Fig. 7c). We found that the T3 effects on the GLP-1, insulin, and glucose levels were all attenuated in euthyroid IFXRKO mice (Supplementary Fig. 7c), which are similar to those observed in MMI-treated mice (Fig. 7a-c). These results indicate that intestinal FXR is indispensable for the beneficial effect of T3 on GLP-1 and insulin production and glucose homeostasis. As we described in detail in our previous rebuttal letter (Page5, Line 197-200), consistent with our current knowledge of intestinal FXR signalling and our working hypothesis, the GLP-1 production was increased, while the glucose metabolism was improved in MMI-treated IFXRKO mice as compared to MMI-treated Floxed mice (Fig. 7a-c). On the other hand, in line with the previous findings that intestinal FXR is not required for maintaining the normal glucose homeostasis [5], the glucose metabolism was normal in IFXRKO mice in the euthyroid state, suggesting that compensatory mechanisms may exist (Supplementary Fig. 7c,d).

As the reviewer suggested, we tested the effect of intestinal FXR deficiency in the hyperthyroid state. Again, we neither observed the T3 on the serum levels of GLP-1, insulin, and glucose in euthyroid Floxed control mice (Supporting Fig. 2b), consistent with the data from wild-type C57BL/6J with (Supporting Fig. 2a) or without (Supporting Fig. 1c) AAV-CT infection, nor detected the effect of deletion of intestinal FXR in the euthyroid group as we observed previously (Supplementary Fig. 7c). As expected, we did not observe the effect of intestinal FXR deficiency in mice treated with both T3 and T4 (TH-5d mice) (Supporting Fig. 2b).

Note that the hyperthyroid mice would need to be treated with both T4 and T3 since T4 has a much longer half-life than T3. As such, Yan's system describes chronic daily T3 injections over 5 days that repetitively stimulate or decrease target genes daily (reminiscent of a study by Ohba et al. on T3-treated euthyroid mice. Endocrinology 157:1660–1672(2016)). It is interesting that one T3 injection did not generate effects on GLP1 and insulin secretion. Does MMI+T3-4h have any effects

in CyP8B1 expression in comparison to 5d shown in Fig. 4d? The study by Ohba showed that acute vs. chronic stimulation by T3 has different effects on target genes.

Response to the Reviewer's Comments: We agree with the reviewer that mice need to be treated with both T4 and T3 to induce hyperthyroidism. As mentioned above, as requested, we employed this hyperthyroid mouse model (euthyroid mice treated with both T4 and T3, referred to as TH-5d mice) to test whether the T3 effects observed in MMI mice or euthyroid mice could be retained in the hyperthyroid state. We found that the elevated T3 and T4 levels could downregulate hepatic CYP8B1, decrease the ratios of 12α-OH BAs to non-12α-OH BAs, and increase the ileum FXR-antagonistic BA levels and proglucagon mRNA expression, but failed to elevate the GLP-1 and insulin levels and lower glucose levels in TH-5d mice (Supporting Fig. 1b-g), suggesting that the sustained elevated or pathological levels of T3 might have deleterious effects. According to our proposed model, the beneficial effect of T3 requires the downregulation of hepatic CYP8B1 and the alteration of BA composition to affect GLP-1 and insulin. We then speculate that it might take times to change the BA composition, therefore, the beneficial effect of T3 could not be observed 4 hours after one T3 injection (Supplementary Fig. 1b-d). In agreement with our hypothesis, we found that the BA composition (12α-OH and non-12α-OH BAs and FXR-antagonistic BAs) were not altered 4 hours after one T3 injection (Supplementary Fig. 4m and 5f,g).

As suggested, we determined the expression of CYP8B1 four hours after one T3 injection. In contrast to MMI+T3-5d group, the mRNA levels of CYP8B1 were not reduced four hours after one T3 injection (Supplementary Fig. 4d). Importantly, the protein levels of CYP8B1 were not altered four hours after one T3 injection (Supplementary Fig. 4d). These results indicate that it might also take times to change the expression of CYP8B1 after T3 treatment. We then determined the CYP8B1 expression at different time points after single T3 injection (acute T3) (4 hours or 1 day after T3 injection) or daily T3 injections for 2 or 5 days (chronic T3) in euthyroid mice and found a trend of gradual decrease in CYP8B1 after T3 treatment (Supporting Fig. 3a). As the expression changes of CYP8B1 after acute and chronic T3 treatment did not follow the "desensitization" pattern observed by Ohba et al.[6], we speculate that the T3 responsiveness of CYP8B1 may have its own regulatory mechanism.

Our knowledge of the transcriptional regulation mediated by T3 and its receptor TR has been greatly expanded recently by taking advantage of the genome wide ChIP-seq analysis [7-11]. Growing evidence suggests that the dynamic TR binding and chromatin remodeling may be critically involved in the transcriptional regulation by T3. To better understand the regulation of CYP8B1 by T3, we performed ChIP analysis to investigate the TR occupancy and chromatin state after single T3 injection (acute T3) or daily T3 injections for 2 or 5 days (chronic T3) in euthyroid mice in the super-enhancer region we identified based on the latest data from Lazar's lab [10], using a mouse model newly developed in our lab with an epitope tag located at the C-terminal of TRβ protein (HA-TRβ mouse), which allows us to explore the recruitment of endogenous TRβ at the low physiological levels (Supporting Fig. 3b).

In agreement with the recent hypothesis based on the genome wide ChIP-seq data, we also observed that T3 treatment could affect the TR recruitment to chromatin, as evident by decreased TRβ occupancy around two putative TR binding sites (TRBS) (DR1 and DR4) (Supporting Fig. 3c,d). Moreover, consistent with the notion that H3K27ac, a hallmark for active enhancers, would decrease

in a T3-dependent manner at TRBSs near down-regulated genes, we found the occupancy of H3K27ac at the DR1 and DR4 sites was also decreased after T3 administration, suggesting a decrease in enhancer activity for CYP8B1 after T3 treatment (Supporting Fig. 3c,d).

Furthermore, for the DR1 site, H3K27ac was decreased 4 hours after T3 injection and maintained at a low level for at least 1 day after acute T3 treatment and during chronic T3 treatment. Decreased TRβ occupancy was only observed after chronic T3 treatment (Supporting Fig. 3c). For the DR4 site, H3K27ac was decreased until 1 day after T3 injection and maintained at a low level during chronic T3 treatment. TRβ occupancy was decreased as early as 4 hours after acute T3 treatment and maintained at a low level for at least 1 day after acute T3 treatment and during chronic T3 treatment (Supporting Fig. 3d). As the dynamics of TR recruitment and H3K27ac occupancy at DR1 and DR4 seem to be differentially regulated by T3, we speculate that the TR binding and chromatin remodeling at DR1 and DR4 sites may be independently regulated after T3 treatment. Although a decrease in TR binding was always accompanied with a reduction of H3K27ac during chronic T3 treatment, we speculate that decreased TR binding may not be required for the reduction of H3K27ac and vice versa. As the decrease in H3K27ac occurred prior to the decline in TR occupancy for the DR1 site, while the reduction of H3K27ac occurred after the decline in TR binding for the DR4 site, we speculate that binding site-dependent mechanisms may also exist. As the chromatin remodeling is highly dynamic and other chromatin modifications are also involved, the epigenetic landscape of the enhancer and promoter of CYP8B1 requires further investigation in different thyroid states and at more time points after T3 treatment in the future.

We provided some background information and cited important papers mentioned above (including the paper by Ohba et al.) in our revised manuscript, for a better understanding the current knowledge of the transcriptional regulation mediated by T3 and its receptor TR, especially for people not in TR field.

Fig. 4. I would like to see transcriptome analysis of euthyroid vs MMI+T3-5d mice. Comparing hyperthyroid mouse transcriptome with MMI+T3-5d also would be useful. It would be useful to look at GLP1, insulin, GTT, and bile acid composition for euthyroid and hyperthyroid mice with MMI and MMI+T3-5d mice.

Response to the Reviewer's Comments: As suggested, we first compared the 4428 differential expressed genes (DEGs) identified in MMI+T3-5d mice as compared to MMI mice and 2763 DEGs identified in euthyroid (CT) mice as compared to MMI mice and found 1412 overlapped DEGs (Supporting Fig. 3e) (RNA-seq data in this study, GSE184261), suggesting that the DEGs in euthyroid (CT) and MMI-T3-5d mice as compared to MMI mice exhibit a significant degree of overlap. The heatmap of the overlapped DEGs showed that these DEGs might be classic T3-regulated genes, although the responsiveness or sensitivity of individual DEGs to MMI or T3 treatment was different (Supporting Fig. 3f).

Also as suggested, we compared the DEGs identified in MMI+T3-5d mice as compared to MMI mice (RNA-seq data, GSE184261) and DEGs identified in hyperthyroid (Chronic T3) mice as compared to euthyroid (NT, no treatment) mice (microarray data from Ohba et al.). We found that the DEGs identified from these two datasets exhibit a significant degree of overlap (Supporting Fig. 3g), suggesting that quite a few of T3-regualted genes can be identified from both experimental models, including CYP8B1. Interestingly, a higher degree of overlap was observed for positively-

regulated genes as compared to negatively-regulated genes (Supporting Fig. 3g). Furthermore, KEGG pathway analysis of DEGs identified from these two datasets also revealed a large overlap, further suggesting that the T3 effects on quite a lot of pathways can be observed in both models (Supporting Fig. 3h).

Although there is considerable overlapping, alteration of some genes was exclusively detectable in one model but not in the other model, suggesting that these two experimental models are not exactly the same. Nerveless, the negatively regulation of CYP8B1 after chronic T3 treatment could be observed in either MMI-induced hypothyroid mice or euthyroid mice with no treatment.

As suggested, we determined the GLP1, insulin, and BA composition in hyperthyroid mice (TH-5d mice) as compared to euthyroid mice (CT mice). We have described these results above in response to the Reviewer's Comments (Page 2, Line 54-73). Briefly, increased GLP-1 levels and reduced glucose levels could not be observed, although the elevated T3 and T4 levels could also decrease the hepatic CYP8B1 expression and the ratios of 12α-OH to non-12α-OH BAs, and increase the ileum FXR-antagonistic BA levels and proglucagon mRNA expression (Supporting Fig. 1b-h). Oral glucose tolerance was not significantly impaired, although the abnormal fasting glucose levels and higher glucose levels 15 minutes after oral glucose ingestion were noticed in hyperthyroid mice (TH-5d) (Supporting Fig. 1i). Given that hyperthyroidism may increase renal blood flow and glomerular filtration [3] and the kidney is a site for GLP-1 extraction [4], we speculate that increased glomerular filtration in the hyperthyroid state may counteract the effect of T3 on GLP-1 production by promoting the renal clearance of GLP-1. Due to the technical issues, the clearance of GLP-1 is difficult to study for the time being, although it deserves more attention and requires further study in the future. We thus provided some discussion and cites related papers in our revised manuscript.

It would be interesting to examine insulin response in the liver in their studies. T3 typically stimulates gluconeogenesis in the liver, so does activation of this pathway by insulin change the direction towards glycolysis or are the livers of the hypothyroid mice still insulin resistant? The authors should look at IR phosphorylation, pAkt, pmTOR signaling for evidence of insulin signaling. The also could look at expression of T3 target genes, PEPCK, G6Pase, PDK4 mRNA, in the liver. It would be important to know whether this elaborate regulatory pathway is increasing insulin effects in the liver or is stimulating insulin secretion to serve other tissues.

Response to the Reviewer's Comments: We thank the reviewer for the suggestion. As requested, we examined the insulin signalling and the expression of insulin or T3-related genes in our mouse models. We found that the hepatic insulin signalling was decreased in hypothyroid mice (MMI mice) as compared to euthyroid mice (CT mice) (Supporting Fig. 4a). As acute T3 treatment (4 hours after T3 injection) did not affect the insulin signalling in the liver of MMI mice, suggesting that the hepatic insulin signalling remained impaired (Supporting Fig. 4a). In contrast, daily T3 injections for 5 days increased the insulin signalling in the liver of MMI mice, suggesting that chronic T3 treatment could restore the hepatic insulin signalling (Supporting Fig. 4a). Notably, although abnormal insulin levels were not observed in hyperthyroid mice (TH-5d mice) as compared to euthyroid mice (CT mice) (Supporting Fig. 1c), the hepatic insulin signalling was impaired in these hyperthyroid mice (TH-5d mice) (Supporting Fig. 4a). Collectively, we speculate that both hypothyroid and hyperthyroid mice had impaired hepatic insulin signalling and only chronic but not

acute T3 treatment could restore the hepatic insulin action in hypothyroid mice (MMI mice) with deficiency in TH production.

Gluconeogenic PERCK and G6Pase are responsible for glucose anabolism, while PDK4 is a negatively regulator for glucose catabolism, the overall intracellular effect of three genes is to promote the glucose production and reduce the glucose usage. PEPCK, G6Pase, PDK4 are considered as T3 target genes, which are also negatively regulated by insulin signalling. As suggested, we examined the mRNA expression of PEPCK, G6Pase, PDK4. In agreement with the notion that they are T3 target genes, we found that they were all downregulated in the liver of hypothyroid mice (MMI mice) as compared to euthyroid mice (CT mice) (Supporting Fig. 4b), while they were all upregulated 4 hours after T3 injection in the liver of MMI mice (Supporting Fig. 4c). In contrast, the mRNA expression of these T3 target genes tended to be downregulated rather than upregulated after daily T3 injections for 5 days in the liver of MMI mice (Supporting Fig. 4d). These results suggest that acute T3 treatment can upregulate PEPCK, G6Pase, PDK4 genes, thereby promoting gluconeogenesis and reduce glucose usage, while the expression of PEPCK, G6Pase, PDK4 would return to baseline expression after chronic T3 treatment.

Based on our finding that chronic but not acute T3 treatment could elevate insulin levels by promoting GLP-1 production in this study (Fig. 1c,f) and the increases in hepatic insulin signalling could be only observed after chronic but not acute T3 treatment (Supporting Fig. 4a), we speculate that insulin action might not be involved in the regulation of PEPCK, G6Pase, and PDK4 after acute T3 treatment, because the hepatic insulin signalling and insulin levels in MMI mice were not altered, while the chronic T3 treatment-induced and GLP-1-mediated increase in insulin production (as evident by the elevated insulin levels and the increased phosphorylation of key components of insulin signalling pathway) could counteract the effect of T3 on the expression of PEPCK, G6Pase, and PDK4. Therefore, rather than being elevated as observed after acute T3 treatment, the mRNA levels of PEPCK, G6Pase, and PDK4 returned to baseline expression levels in MMI mice after chronic T3 treatment. Collectively, our study might also provide new insight into the possible involvement of insulin action in the regulation of hepatic glucose metabolism by chronic T3 treatment.

Additionally, further analysis of our RNA-seq data (GSE184261) revealed that, in MMI mice, acute T3 treatment might not affect glycolysis, in contrast, chronic T3 treatment might promote glycolysis, indicating that glycolysis might be tightly controlled by inulin but not by T3 and the regulation of glycolysis by chronic T3 treatment might be attributed to the elevation of insulin levels. As the insulin can lower blood glucose levels through multiple metabolic tissues via multiple mechanisms, based on all available data, we speculate that insulin action in both liver and other tissues would contribute to the glucose-lowering effect of insulin after chronic T3 treatment, which requires further investigation in the future.

Fig. 6. Does T3 have separate effects on intestinal cells? Could it regulate FXR levels, or GLP1 gene expression independently of FXR? Such studies could be done in cell lines or organoids described in Fig. 6e.

Response to the Reviewer's Comments: We thank the reviewer for the comments. We had indeed explored the effect of T3 on GLP-1 production *in vitro* (Supplementary Fig. 2a). As we neither observed the effect of T3 on GLP-1 expression and secretion or FXR expression in STC-1 cells,

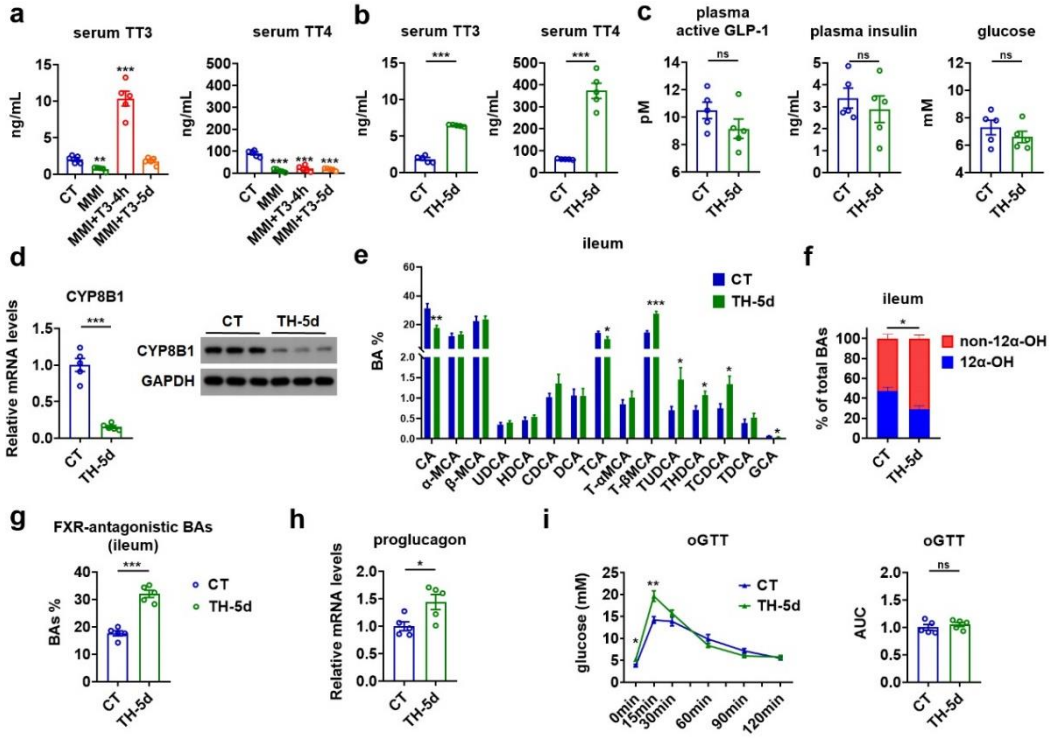
NCI-H716 cells, and mouse intestinal organoids (Supplementary Fig. 2a), nor detected the T3 effect on TGR5 mRNA levels in STC-1 cells, NCI-H716 cells, and mouse intestinal organoids (not included in our previous version, Supporting Fig. 5), we did not further explore the potential direct effect of T3 on these cells or organoids. As the reviewer suggested, we examined the FXR levels in these cell lines and organoids and found that the mRNA expression of FXR was not altered by T3 treatment, which agrees with our proposed model that T3 regulates GLP-1 production via a non-cell autonomous mechanism involving hepatic TR and the alteration of BA composition.

Attached please find the references, supporting figures and legends, and materials and methods associated with the Supporting data mentioned in this response letter.

Reference

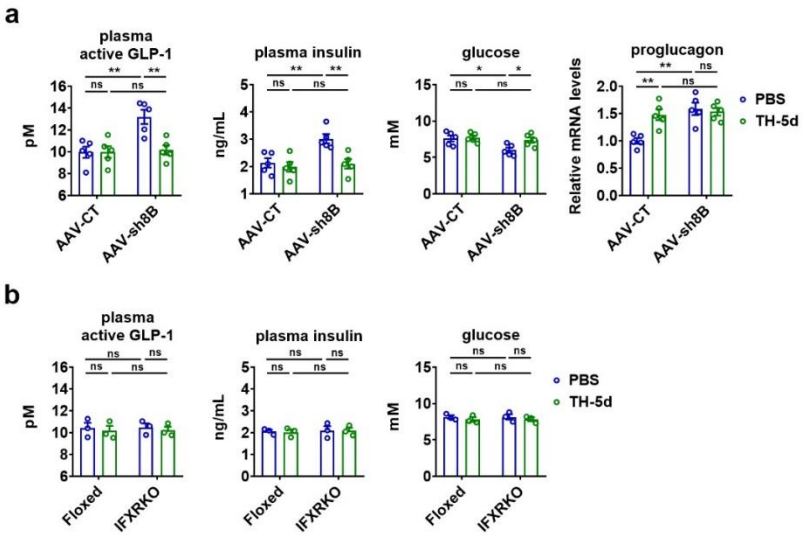
1. Zavacki, A.M., et al., *Type 1 iodothyronine deiodinase is a sensitive marker of peripheral thyroid status in the mouse.* Endocrinology, 2005. **146**(3): p. 1568-75.
2. Sinha, R.A., B.K. Singh, and P.M. Yen, *Thyroid hormone regulation of hepatic lipid and carbohydrate metabolism.* Trends Endocrinol Metab, 2014. **25**(10): p. 538-45.
3. Iglesias, P., et al., *Thyroid dysfunction and kidney disease: An update.* Rev Endocr Metab Disord, 2017. **18**(1): p. 131-144.
4. Deacon, C.F., *Circulation and degradation of GIP and GLP-1.* Horm Metab Res, 2004. **36**(11-12): p. 761-5.
5. Xie, C., et al., *An Intestinal Farnesoid X Receptor-Ceramide Signaling Axis Modulates Hepatic Gluconeogenesis in Mice.* Diabetes, 2017. **66**(3): p. 613-626.
6. Ohba, K., et al., *Desensitization and Incomplete Recovery of Hepatic Target Genes After Chronic Thyroid Hormone Treatment and Withdrawal in Male Adult Mice.* Endocrinology, 2016. **157**(4): p. 1660-1672.
7. Chatonnet, F., et al., *Genome-wide analysis of thyroid hormone receptors shared and specific functions in neural cells.* Proc Natl Acad Sci U S A, 2013. **110**(8): p. E766-75.
8. Grontved, L., et al., *Transcriptional activation by the thyroid hormone receptor through ligand-dependent receptor recruitment and chromatin remodelling.* Nat Commun, 2015. **6**: p. 7048.
9. Praestholm, S.M., et al., *Multiple mechanisms regulate H3 acetylation of enhancers in response to thyroid hormone.* PLoS Genet, 2020. **16**(5): p. e1008770.
10. Shabtai, Y., et al., *A coregulator shift, rather than the canonical switch, underlies thyroid hormone action in the liver.* Genes Dev, 2021. **35**(5-6): p. 367-378.
11. Ramadoss, P., et al., *Novel mechanism of positive versus negative regulation by thyroid hormone receptor beta1 (TRbeta1) identified by genome-wide profiling of binding sites in mouse liver.* J Biol Chem, 2014. **289**(3): p. 1313-28.

Supporting Figures and legends



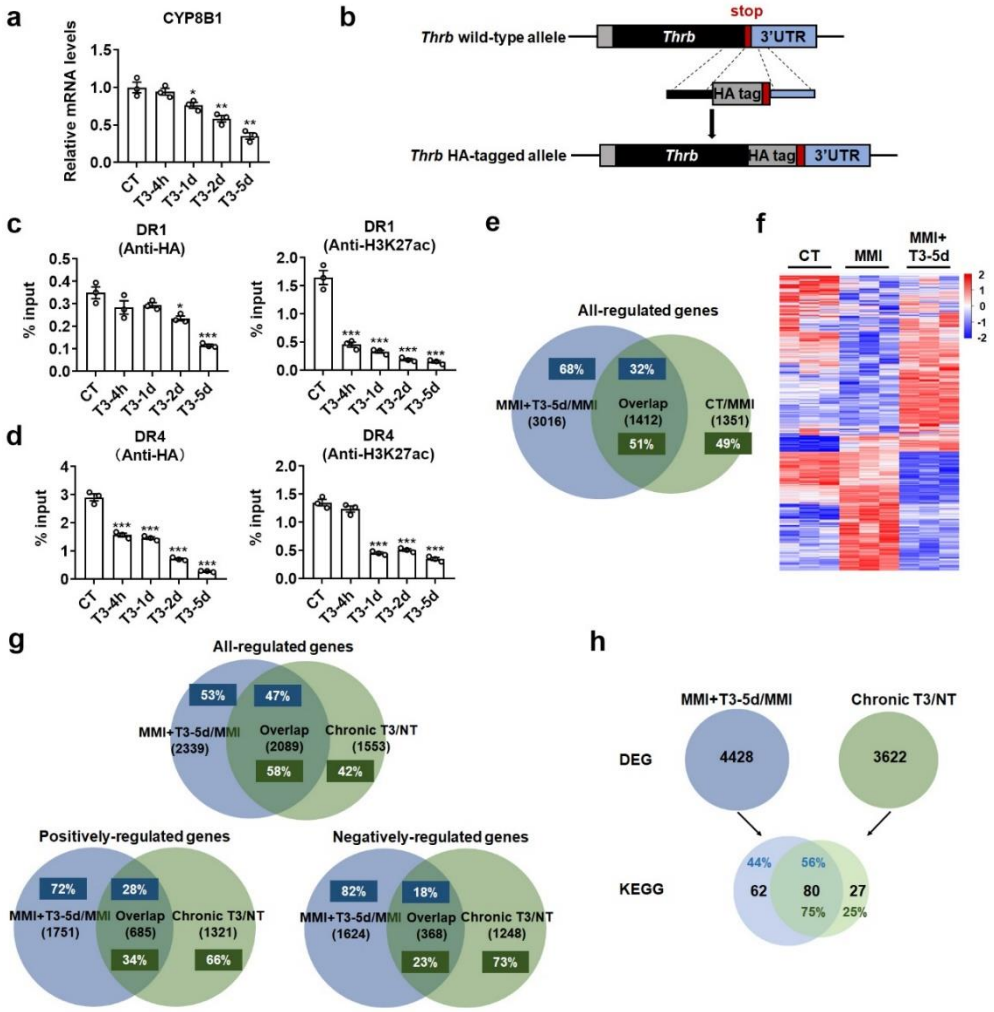
Supporting Figure 1. CYP8B1, BA, GLP-1, insulin, and glucose levels in hyperthyroid mice.

(a) The serum total T3 (left) and T4 (right) levels in CT, MMI, MMI+T3-4h and MMI+T3-5d mice (n=5) (right). (b) The serum total T3 (left) and T4 (right) levels in CT and TH-5d mice (n=5). (c) Plasma active GLP-1 levels, plasma insulin levels and blood glucose levels in CT and TH-5d mice (n=5). (d) Relative mRNA (left) and protein (right) levels of hepatic CYP8B1 in CT and TH-5d mice. (e) The percentage of individual BA in the ileum of CT and TH-5d mice (n=5). (f) Relative levels of 12α-OH (blue) and non-12α-OH (red) BAs in the ileum of CT and TH-5d mice (n=5). (g) The percentage of non-12α-OH FXR-antagonistic BAs, including T(α/β)MCA, (T/G)UDCA and (T)HDCA in the ileum of CT and TH-5d mice (n=5). (h) The relative mRNA levels of proglucagon in CT and TH-5d mice (n=5). (i) oGTT for CT and TH-5d mice (left) and the AUC (right) (n=5). CT mice (euthyroid) are untreated control mice, while TH-5d mice (hyperthyroid) are euthyroid mice treated with both T3 and T4 as described in Materials and Methods for Supporting data. Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.



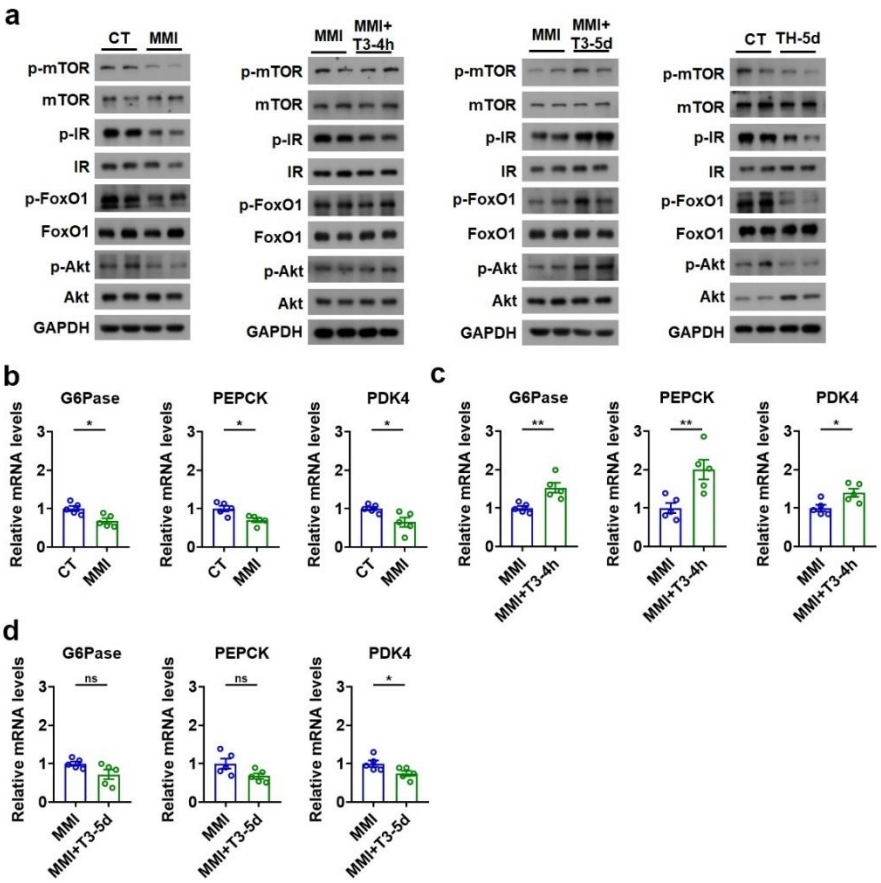
Supporting Figure 2. The effects of hepatic CYP8B1 knockdown or intestine-specific FXR knockout in euthyroid and hyperthyroid mice.

(a) Plasma active GLP-1 levels, plasma insulin levels, blood glucose levels and relative mRNA levels of proglucagon in CT and TH-5d mice administered with AAV-CT or AAV-shCYP8B1 (AAV-sh8B) (n=5). (b) Plasma active GLP-1, plasma insulin and blood glucose levels in Floxed and IFXRKO mice treated with PBS or TH for 5 days (n=3). Mice were treated with both T3 and T4 (TH) or PBS as described in Materials and Methods for Supporting data. Means ± SEM are shown. *p<0.05 and **p<0.01.



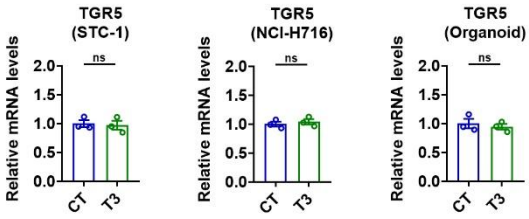
Supporting Figure 3. The occupancy TRβ and H3K27ac in super-enhancer region of CYP8B1 and the comparison between changes in transcriptome in different experimental models.

(a) Relative mRNA levels of hepatic CYP8B1 in mice treated with T3 (0.25 μg/g; 0h (CT), 4h, 1d, 2d, 5d) (n=3). (b) Schematic diagram of generating HA-TRβ mouse line that harbors a HA-tag in the C-terminal of *Thrb* gene. (c and d) ChIP-PCR showing enrichment (percent input normalized to CT) of HA-TRβ (left) and H3K27 acetylation (right) at the putative TRβ binding sites (c, DR1) (d, DR4) of the super-enhancer region of CYP8B1 in the liver of HA-TRβ mice treated with T3 (0.25 μg/g; 0h (CT), 4h, 1d, 2d, 5d) (n=3). (e) Venn diagrams showing overlapped DGEs between MMI+T3-5d vs MMI groups and MMI vs CT groups according to the RNA-seq data reported in this study. (f) Heatmaps of DEGs identified in the liver between CT, MMI and MMI+T3-5d groups (n=3) based on the RNA-seq data reported in this study. (g) Venn diagrams showing overlapped DGEs between MMI+T3-5d vs MMI groups (RNA-seq data reported in this study) and Chronic T3 vs NT groups (microarray data reported by Ohba et al.). (h) Venn diagrams showing the overlapped KEGG pathways identified from the DEGs between MMI+T3-5d vs MMI groups (RNA-seq data reported in this study) and Chronic T3 vs NT groups (microarray data reported by Ohba et al.). The detailed information for the Chronic T3 treatment group and no treatment (NT) group in the study by Ohba et al. could also be found in the Materials and Methods for Supporting data. Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.



Supporting Figure 4. Hepatic insulin signalling and the mRNA expression of G6pase, PEPCK, and PDK4 in different experimental models.

(a) Western blot analysis of key molecules of insulin signalling pathway in the liver of CT and MMI mice, MMI and MMI+T3-4h mice, MMI and MMI+T3-5d mice, CT and TH-5d mice. (b-d) Relative mRNA levels of G6Pase/PEPCK/PDK4 in the liver of CT and MMI mice, MMI and MMI+T3-4h mice, MMI and MMI+T3-5d mice (n=5). Means ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001.



Supporting Figure 5. The effect of T3 treatment on TGR5 mRNA expression in cultured cells.

Relative mRNA levels of TGR5 in STC-1 cells, NCI-H716 cells and mouse intestinal organoids after T3 treatment (n=3). Means ± SEM are shown.

Materials and Methods for Supporting data

Mice study

HA-TRβ mice are mice harboring a HA-tag in the C-terminal of *Thrb* gene in C57BL/6J mice, developed by the Genome Tagging Project (GTP) Center of Shanghai Institute of Biochemistry and Cell Biology. Male C57BL/6J mice between ages 8 and 10 weeks were made hyperthyroid by intraperitoneal injection of 40 μg/100 g T4 with 4 μg/100 g T3 for 5 days (TH-5d mice). Control mice were injected with the same volume of PBS alone.

Chromatin immunoprecipitation assay

Male HA-TRβ mice between ages 8 and 10 weeks received daily intraperitoneal injection of T3 (0.25 μg per gram BW) as indicated time in the figure. ~25mg of frozen liver material was used per IP sample from 3–4 mice in each group. Livers were homogenized in PBS containing 1% formaldehyde, incubated 10 min at room temperature and quenched with 0.125 M glycine. ChIP assays were performed using an EZ Magna ChIP G kit (Millipore) according to the manufacturer's protocol. 2μg/IP of antibody was used in HA (3724S, Cell Signaling Technology) and H3K27ac (ab4729, Abcam) ChIP experiments.

Analysis of mRNA and protein expression

For qRT-PCR analysis, primers for G6Pase, PEPCK and PDK4 are show below: mG6Pase-F, CTCTGGCCATGCCATG; mG6Pase-R, GCTGGCATTGTAGATGCC; m PEPCK -F, GAGAAAG CATTCAACGCCA; m PEPCK -R, AGTTGTTGACCAAAGGCTTTTTA; mPDK4-F, AGGGAG GTCGAGCTGTTCTC; mPDK4-R, GGAGTGTTCACTAAGCGGTCA. For western blot analysis, primary antibodies against p-Akt (9271S, Cell Signaling Technology), p-mTOR (2971S, Cell Signaling Technology), p-IR (3024S, Cell Signaling Technology), p-FoxO1(9461T, Cell Signaling Technology), mTOR (2983S, Cell Signaling Technology), FoxO1 (2880S, Cell Signaling Technology), IR (23413T, Cell Signaling Technology), Akt (9272S, Cell Signaling Technology) were used.

Hormone measurement

For the measurement of serum levels of total T3, a T3 (total) (Mouse/Rat) ELISA Kit (KA0925, Abnova) was used. For the measurement of serum levels of total T4, a Thyroxine (T4) ELISA Kit (MBS9711535, MYBiosource) was used.

Data availability

The liver RNA-seq data from CT, MMI and MMI+T3-5d groups generated in this study have been deposited in the Gene Expression Omnibus database under accession code GSE184261. Previously published liver microarray data from the Chronic T3 treatment group and no treatment (NT) group is from Gene Expression Omnibus: GSE68867.

Notes:

The other materials and methods were described in the paper.

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

I am satisfied with responses to my comments. I appreciate the extra effort and experiments to address my concerns.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

I am satisfied with responses to my comments. I appreciate the extra effort and experiments to address my concerns.

Response to the Reviewer's Comments: We thank the reviewer for the detailed review for helping us to improve our manuscript.