

Non-canonical thyroid hormone signaling mediates cardiometabolic effects *in vivo*

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Thyroid hormone (TH) and TH receptors (TRs) α and β act by binding to TH response elements (TREs) in regulatory regions of target genes. This nuclear signaling is established as the canonical pathway for TH action. In addition, however, TRs can activate intra-cellular second messenger signaling pathways. Whether such non-canonical TR signaling is physiologically relevant *in vivo* is unknown. Here we generated knock-in mice with a mutation in the TR DNA-binding domain that abrogates binding to DNA and TREs and leads to a complete loss of canonical TH action. We show that several important physiological TH effects are preserved despite disrupted DNA-binding, most notably heart rate, body temperature, blood glucose and triglyceride concentration, all of which were regulated by non-canonical TR signaling. Additionally, we confirm that TRE-binding defective TR β leads to disruption of the hypothalamic-pituitary-thyroid axis with resistance to TH, while mutation of TR α causes a severe delay in skeletal development, showing TRE-mediated and tissue-specific canonical signaling. Our findings provide first *in vivo* evidence that non-canonical TR signaling exerts important cardiometabolic effects, which are clearly separated from canonical actions. Consequently, these data challenge the current paradigm that TH action is exclusively mediated through regulation of gene transcription at the nuclear level.

thyroid hormone action | non-canonical signaling | thyroid hormone receptor

Introduction

Thyroid hormone (TH) plays an essential role in organ development and homeostatic regulation. The effects of 3,3',5-triiodo-L-thyronine (T₃), the active TH, are mediated by TH receptors (TRs) α and β . Canonical TR signaling controls gene expression in the nucleus directly. This mechanism was recently classified as type 1 TR-dependent TH signaling (1) (Figure 1A): TRs bind to thyroid hormone response elements (TREs) in regulatory regions of target genes. In the absence of T₃, TRs recruit corepressors with histone deacetylase activity, preventing transcription. T₃ binding to TRs causes the release of corepressors, which are replaced by coactivators that engage histone acetylases and RNA polymerase II to activate gene transcription (2, 3). Thus, TRs act as hormone-dependent transcription factors. Consequently, the current paradigm of TH action attributes its physiological effects to the genes directly regulated via canonical TR signaling on DNA.

We know now that additional and very different mechanisms of TR signaling exist. T₃ and TR β can modulate intra-cellular second messenger signaling, e.g. the phosphatidylinositol-3-kinase

(PI3K) pathway (TR-dependent signaling of TH without DNA binding, type 3) (1, 4-7). Such TH/TR action is considered non-canonical, because it is independent from DNA- and TRE-binding, does not require gene transcription or protein synthesis as the initial event and may, therefore, also be more rapid (Figure 1B). However, non-canonical TRE-independent TR signaling remains ill-defined and its physiological importance is unknown.

To address this issue directly and to determine physiological effects of non-canonical TH/TR action *in vivo*, we generated TR α ^{GS} and TR β ^{GS} mice that lack canonical TR signaling by replacing glutamic acid and glycine (EG) in the first zinc finger of the DNA-binding domain of TR α and TR β with glycine and serine (GS). The GS mutation severely impairs TR binding to TREs and thus abrogates canonical TR signaling (8, 9). Since TRE-binding is abolished, only the non-canonical TH actions are preserved in TR GS mutant mice. By contrast, the TR can mediate canonical and non-canonical actions of TH in WT mice, whereas both actions are absent in TR knockout (KO) mice (Figure 1C).

Significance

This study changes our understanding of how thyroid hormones act. Thyroid hormone receptors are considered typical nuclear receptors that are attached to DNA and, after thyroid hormone binding, alter expression of their target genes and, subsequently, physiology. We show in mice with mutant thyroid hormone receptors that cannot bind to DNA that these receptors nevertheless mediate important physiological effects. These effects are mostly linked to energy metabolism, e.g. glucose and triglyceride concentration, body temperature, activity and heart rate. This study provides first *in vivo* evidence that thyroid hormone receptors mediate physiologically relevant effects independent from DNA-binding and direct modification of gene expression.

Reserved for Publication Footnotes

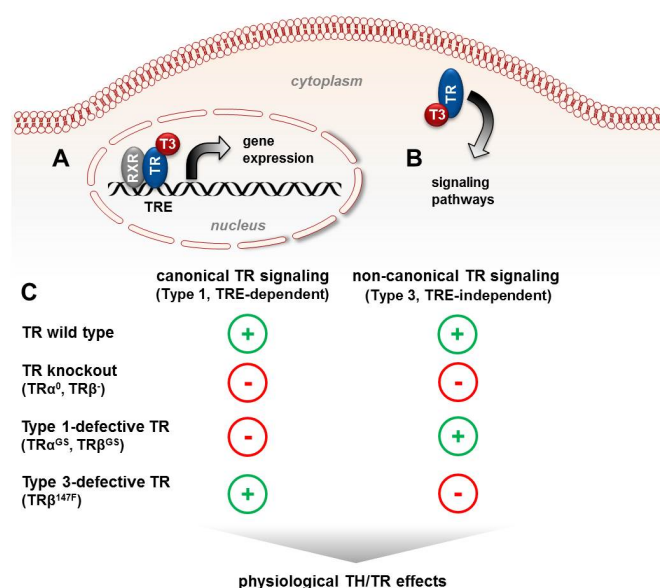


Fig. 1. Canonical and non-canonical TR signaling. (A) Canonical TR signaling requires binding of TR to regulatory DNA sequences, the thyroid hormone response elements (TREs), mostly as a heterodimer with retinoic-X-receptor (RXR). Binding of T3 leads to an exchange of cofactors which initiates or represses transcription of the target gene. (B) Non-canonical action of TRs involves rapid activation of signaling pathways without DNA-binding. (C) Present (+) and absent (-) TR signaling in mouse models. In TR WT mice, the TR can mediate both canonical and non-canonical signaling. In TR knockout mice, both effects are absent. In mice with TRE-binding deficient TRs, canonical signaling is abolished and only non-canonical signaling is preserved. Reciprocally, in mice with selective abrogation of PI3K signaling, this non-canonical TH effect is missing, while canonical signaling is preserved. Thus, a comparison of these mice allows to determine whether the signaling mechanism responsible for TH effects is canonical or non-canonical and PI3K mediated.

Therefore, we hypothesized that a comparison of WT, TR KO and TR GS mice will determine whether canonical or non-canonical TR signaling underlie a range of physiological TH responses. A phenotypic difference between WT and TR KO mice and similarity of TR KO and TR GS mice, will indicate that canonical TR signaling is required for a particular phenotype (Figure 1C). Conversely, if a phenotype is concordant between TR GS and WT mice but discordant from TR KO mice, then only non-canonical signaling is required.

As an additional control for TR β signaling, we used TR β^{147F} mice. In this mouse model, tyrosine 147 of TR β is changed to phenylalanine (Y147F). Tyrosine 147 is crucial for PI3K activation by TR β . The change to phenylalanine completely abrogates PI3K activation, but leaves the classical TR β action on TREs intact (7). Therefore, the TR β^{147F} mouse model serves as a perfect negative control for non-canonical, PI3K mediated TH/TR β effects, which are absent in this mouse (Figure 1C).

Here we provide *in vivo* evidence that non-canonical TR signaling is physiologically important and contributes significantly to TH action, specifically to regulation of body temperature, activity, heart rate and lipid and glucose concentrations, thus relevant parameters of cardiometabolic homeostasis and energy balance.

Results

The GS mutation abrogates canonical TR signaling *in vitro* and *in vivo*

Canonical TR signaling consists of TR binding to TREs in regulatory regions of target genes and subsequent activation of gene expression. ChIP-seq analyses in mouse liver reported a DR-

4 motif, a direct repeat of two 5'AGGTCA3' half-sites in the same orientation separated by 4 nucleotides, as the most prevalent TRE in T3 induced genes (10-12). This consensus DR-4 TRE was used in a fluorescent electrophoretic mobility shift assay (EMSA) to test DNA binding affinity of the TR α and TR β variants (TR β , TR β^{GS} , TR β^{147F} , TR α and TR α^{GS}). Only TR β , TR β^{147F} and TR α bound to the DR+4 probe, whereas the TR β^{GS} and TR α^{GS} receptors did not bind to the TRE (Fig. 2A). The GS mutation abrogated DNA binding of TR α and TR β . Next, we tested the transcriptional activity of TR α^{GS} , TR β^{GS} and TR β^{147F} mutants *in vitro* on a thyroid hormone-responsive luciferase reporter plasmid (DR-4-TKLuc) in comparison to WT TRs. Empty vector and TR mutants TR α G291R and TR β G354R served as controls. These mutant TRs cannot bind T3 and cannot activate gene transcription after T3 treatment (13). T3 increased luciferase activity eightfold with WT TR α and TR β , but not with empty vector or TR β G354R and TR α G291R (Fig. 2B). Luciferase activity was not increased by T3 the TR α 71GS and TR β 125GS mutants, demonstrating *in vitro* that the GS mutation in the DNA-binding domain abolishes the canonical TRE-mediated transcriptional activity of TR α and TR β . This was confirmed by experiments with a common variant of the 3' half-site (AGGACA) (Fig. SXY). Of note, already the results from vehicle treated cells demonstrate that TR α 71GS and TR β 125GS mutants do not bind to the TRE: unlike the WT and T3 binding deficient receptors, the unliganded GS mutants did not exert a dominant negative effect on basal luciferase expression.

To abolish canonical TR action *in vivo*, we introduced the GS mutation into the murine WT *Thra* and *Thrb* gene loci, generating TR α^{GS} (*Thra*^{GS/GS}) and TR β^{GS} (*Thrb*^{GS/GS}) mice. We determined expression of known TH responsive genes in these mice in comparison to WT and the respective TR KO mice (TR α^0 and TR β^0 mice, genotypes are *Thra*^{0/0} and *Thrb*^{-/-}, respectively). As TR α is predominantly expressed in heart and TR β in liver, we studied these two tissues. In heart, expression of *Myh6* and *Myh7* mRNAs in TR α^{GS} mice differed significantly from expression in WT mice and was comparable to that in TR α^0 mice (Fig. 2C), demonstrating that the GS mutation in TR α abrogates canonical TH/TR α action *in vivo*. For TR β mutant mice, the effects of different systemic TH concentrations in WT, TR β^{GS} and TR β^0 mice had to be mitigated, thus we determined TH-induced gene expression in hypothyroid animals without and with T3 treatment. In livers of WT mice, T3 induced expression of *Dio1*, *Spot14*, *Me1* and *Bcl3* mRNAs (Fig. 2D, SXY). This induction was equally reduced in TR β^0 mice and TR β^{GS} mice, which demonstrates that the GS mutation has the same effect on canonical TH/TR mediated gene expression as complete absence of TR in TR KO mice.

To evaluate recruitment of TR β mutants to chromatin we performed ChIP against TR β and its heterodimerization partner RXR α in liver tissue from hyperthyroid animals and evaluated occupancy of known TR binding sites previously identified in mouse liver tissue, e.g. in the *Dio1* gene (Fig. 2E and SXY). Both WT and TR β 147F mutant occupy these TR binding sites together with RXR α , whereas the TR β^{GS} mutant and TR β KO show only little or no enrichment, demonstrating that the GS mutation in the DNA-binding domain of TR β disrupts TR β interaction with chromatin. Moreover, evaluation of histone 3 lysine 27 acetylation (H3K27Ac) at these binding sites showed that impaired occupancy with TR β leads to reduced histone acetylation (Fig. 2E). To evaluate the effect on histone acetylation genome-wide, we performed ChIP-seq against H3K27Ac in the liver of hypo and hyperthyroid WT and TR mutant animals. Sequenced tags were quantified at all previously identified DNase accessible regions and we identified more than 500 regions where H3K27 acetylation was differently regulated by T3 in WT animals (Fig. 2F). Next, we quantified H3K27 acetylation at these differentially

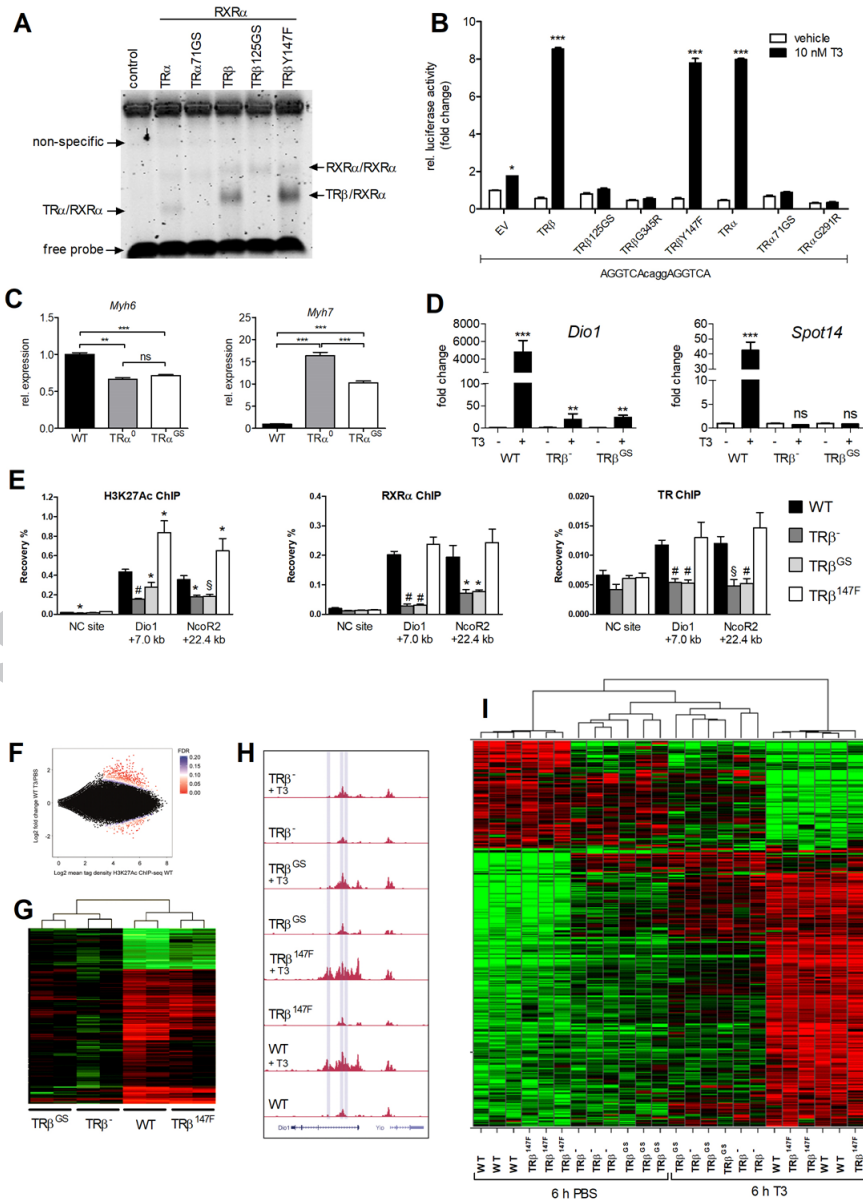


Fig. 2. The GS mutation abolishes TR binding to DNA and canonical TR signaling in vitro and in vivo. (A) Fluorescent EMSA was performed with 4 μ l of reticulocyte translated TR α^{WT} , TR α^{71GS} , TR β^{WT} , TR β^{125GS} , TR β^{147F} and 2 μ l of RXR α on a 5'-Cy5-labeled DR+4 TRE probe. DNA binding of heterodimers of TRs and RXR α was only observed for TRs with intact DBD (TR α^{WT} , TR β^{WT} and TR β^{147F}). (B) HEK293 cells transfected with plasmids encoding for TR β , TR β^{125GS} , TR β^{147F} , TR α , TR α^{71GS} and the indicated DR+4-luciferase reporter plasmids. TR mutants without T₃ binding served as negative controls (TR α^{G291R} and TR β^{G345R}). Cells were treated with 10 nM T₃ for 48 h (black bars) to induce luciferase expression via canonical TR/TRE-mediated action for TR α and TR β variants. (C) Relative expression of *Myh6* and *Myh7* in hearts of male WT (black bars), TR α^0 (grey bars) and TR α^{GS} mice (open bars) ($n=6$; mean $\pm$ SEM; ANOVA and Tukey's *post hoc* test; ns = not significant, $**P<0.01$, $***P<0.001$). (D) Response of TH target genes (*Dio1* and *Spot14*) to T₃ in livers of hypothyroid WT, TR $\beta^{-/-}$ and TR β^{GS} mice. Hypothyroid mice were either injected with vehicle (open bars) or with 5 μ g/100 g BW T₃ (black bars) for 4 consecutive days. ($n=6$; mean $\pm$ SEM). (E) qPCR data show repressed T₃-induced H3K27Ac levels and reduced recruitment of RXR α and TR to TR binding sites located +7.0 kb on *Dio1* and +22.4 kb on *NcoR2* gene loci for WT, TR $\beta^{-/-}$, TR β^{GS} and TR β^{147F} mice injected with T₃. ($n=4-6$, mean $\pm$ SEM; based on Student's *t*-test; $*P<0.05$, $\#P<0.005$, $\$P<0.001$). (F) H3K27Ac ChIP-seq experiments were performed on liver material from hypothyroid WT mice treated with PBS or T₃. H3K27Ac was quantified at 79,938 DNase accessible regions previously identified in mouse liver tissue. FDR of differentially regulated H3K27Ac in response to T₃ was calculated by DESeq2 and visualized by a heatmap. A total of 537 DNase accessible regions showed differential H3K27Ac at FDR<0.1 and log2 fold change of least 1 ($n=2$). (G) ChIP-seq experiments were performed on liver material from hypothyroid WT, TR $\beta^{-/-}$, TR β^{GS} and TR β^{147F} mice treated with PBS or T₃. The heatmap illustrates log2 fold change of H3K27Ac comparing T₃ treatment with PBS for all genotypes at 537 DNase accessible regions with differential H3K27Ac. (H) UCSC Genome browser tracks of H3K27Ac enrichment profiles at the *Dio1* gene locus for all four genotypes injected with PBS or T₃. Blue bars mark TR binding sites. (I) Hierarchical clustering of gene expression data from livers of hypothyroid WT, TR $\beta^{-/-}$, TR β^{GS} and TR β^{147F} mice treated either with 20 μ g/100 g BW T₃ or PBS for 6 h. ($n=3$).

regulated regions in liver samples from hypo- and hyperthyroid WT and TR $\beta^{-/-}$ animals and animals carrying the GS and 147F TR β mutants. Hierarchical clustering revealed that T₃-induced H3K27 acetylation in animals with the 147F mutation is very

similar to what can be observed in WT animals. In contrast, the TR β^{GS} mutation as well as TR β knockout resulted in attenuated H3K27 acetylation after T₃ treatment (Fig. 2G). As an example, H3K27Ac is increased at previously identified TR binding sites

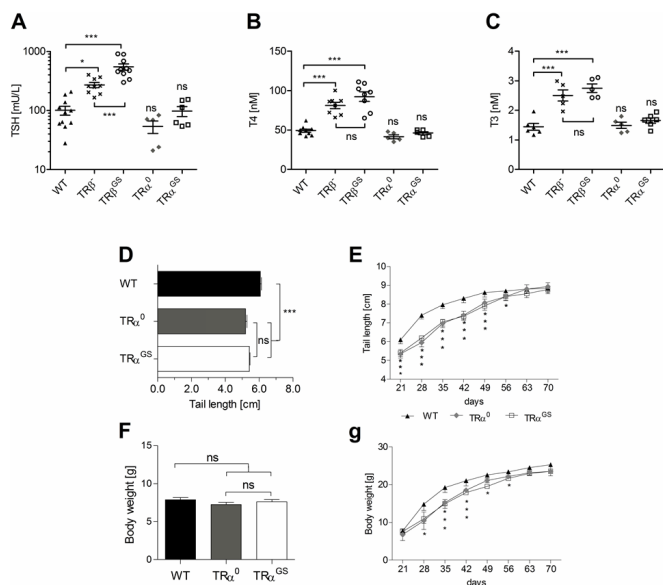


Fig. 3. Canonical TR β action is required for TSH transcriptional repression and normal growth. (A-C) TSH, T₄ and T₃ in serum of 15-week old WT (p; n=11) TR β ⁻ (x; n=9), TR β ^{GS} (TM; n=10), TR α ⁰ (z; n=5) and TR α ^{GS} (E; n=6) male mice (mean $\pm$ SEM; ANOVA and Tukey's post hoc test; *P<0.05, ***P<0.001, ns = not significant). (D) Tail length (TL) of 21-d old WT (black bar), TR α ⁰ (grey bar) and TR α ^{GS} (open bar) mice. (E) Linear growth was recorded until the age of 70 d. (F) Bodyweight (BW) on post-natal day 21 (P21) and (G) recorded until the age of 70 d (n=6; mean $\pm$ SD for TL and BW at P21 and follow-up; ANOVA and Tukey's post hoc test; ns = not significant, *P<0.05, ***P<0.001).

at the Dio1 gene locus in response to T₃ (Fig. 2H). This increase is not observed in animals carrying the GS mutant nor in TR β ⁻ animals. Interestingly, increased as well as decreased H3K27Ac in response to T₃ was affected in the GS mutant and KO, demonstrating that disruption of TR β binding to chromatin impairs T₃ regulated H3K27Ac in liver.

Next, we applied transcriptome analysis to test whether TR β ^{GS} and TR β ^{147F} mutants possess residual transcriptional activity affecting known target genes or acquire new target genes. Hypothyroid WT, TR β ⁻, TR β ^{GS} and TR β ^{147F} mice were treated with a single dose of T₃ and hepatic gene expression was measured after 6 hours by microarray. Fig. 2I shows the results in a dendrogram with mice grouped not by genotype, but clustered by similarity of their gene expression patterns. WT and TR β ^{147F} mice were clearly different from TR β ⁻ and TR β ^{GS} mice and formed their own branch in the hypothyroid and T₃ treatment groups. Importantly, TR β ⁻ and TR β ^{GS} mice were grouped together because of the similarity of their hepatic gene expression patterns. In fact, T₃-treated TR β ⁻ and TR β ^{GS} mice could not be distinguished by gene expression pattern. The GS mutation renders the TR transcriptionally nonfunctional *in vivo*.

Regulation of the hypothalamic-pituitary-thyroid axis requires canonical TH/TR signaling

TH production and secretion is regulated by the hypothalamic-pituitary-thyroid (HPT) axis negative feedback loop. Inhibition of TSH is mediated by TR β (14-16) and depends on DNA-binding of TR β (9). In accordance with these reports, basal serum TSH was significantly higher in TR β ⁻ and in TR β ^{GS} mice compared to WT mice (Fig. 3A). As a consequence of elevated TSH, T₃ and thyroxine (T₄) concentrations were also significantly higher in TR β ⁻ and TR β ^{GS} mice compared to WT mice (Fig. 3B and C). The combination of elevated circulating TH concentrations and elevated TSH demonstrates central resistance to thyroid hormone due to lack of the receptor in TR β ⁻ mice and, more importantly, also lack of canonical TR β

action in the HPT axis of TR β ^{GS} mice. TSH, TT₄ and TT₃ concentrations in TR α ⁰ mice (devoid of TR α 1 and TR α 2) and TR α ^{GS} mice were not different from those in WT mice.

Skeletal development requires canonical TH/TR signaling

We measured growth curves of mice from all genotypes. The growth curves of TR β ⁻ and TR β ^{GS} mice were not different from that of WT mice (data not shown). However, TR α ⁰ and TR α ^{GS} mice showed a similar delay in linear growth compared to WT mice (Figures 3D-G). To investigate the underlying cause, skeletal analysis of TR α ⁰ and TR α ^{GS} mice was performed after weaning at postnatal day 21 (P21). X-ray microradiography revealed a similar decrease in bone length and vertebral height in both TR α ^{GS} and TR α ⁰ mice, together with a reduction in bone mineral content in vertebrae but no difference in long bones (Figures 4A and 4B). Histological analysis of the growth plate revealed a delay in endochondral ossification similarly affecting both TR α ^{GS} and TR α ⁰ mice (Figure 4C). This delay comprised a decrease in the size of the secondary ossification center together with an increase in the reserve zone width and a decrease in the hypertrophic zone, features that are characteristic of impaired T₃ action (17, 18). High resolution micro-CT imaging demonstrated epiphyseal dysgenesis, increased trabecular bone mass and reduced metaphyseal inwaisting consistent with a bone modelling defect and delayed endochondral ossification (Figures 4D-F). These are all classical features of impaired T₃ action in bone (18, 19). In summary, these data demonstrate an equivalent delay in skeletal development due to similar loss of TRE-mediated canonical TR α signaling in both TR α ⁰ and TR α ^{GS} mice. These findings establish that T₃ actions in the skeleton are mediated by canonical actions of TR α on TREs.

Non-canonical TH action decreases blood glucose concentration

T₃ treatment has been shown to rapidly reduce serum glucose concentration in lean and adipose mice (20). We hypothesized that such a rapid TH effect could be non-canonically mediated by TRs and thus be present in WT and either TR α ^{GS} or TR β ^{GS} mice. We tested this hypothesis first in WT, TR β ⁻, TR β ^{GS} and TR β ^{147F} mice. Basal serum glucose after 1h of fasting was not different between the TR β genotypes (suppl. Table SXY). T₃ injection rapidly decreased blood glucose within 60 minutes in WT mice (Figure 5A, suppl. table SXY). Strikingly, this glucose-lowering effect of T₃ was abolished in TR β ⁻ mice, but fully preserved in TR β ^{GS} mice. We conclude that the decrease in blood glucose after T₃ treatment is TR β mediated, because it is absent in TR β ⁻ mice. The similarity of the phenotype with an identical decrease in blood glucose in WT and TR β ^{GS} mice demonstrates that non-canonical TR β signaling mediates this effect. This is supported by the absence of a T₃ effect on glucose in TR β ^{147F} mice, demonstrating that PI3K activation by TR β is required for lowering of glucose concentration. Furthermore, this effect occurs within 60 minutes, which is likely too rapid to depend on RNA transcription and translation into new proteins. TR α , on the other hand, plays no role in glucose decrease as it cannot compensate lack of TR β in TR β ⁻ mice.

Non-canonical TR β signaling maintains normal serum triglyceride concentration

Serum triglyceride concentration is under TH control (21, 22). Previous studies have shown elevated serum triglyceride concentration in TR β knock-in mice with resistance to TH (TR β ^{PV}), indicating that TR β mediates TH regulation of triglycerides (23). We therefore measured serum triglyceride concentration in untreated WT, TR β ⁻, TR β ^{GS} and TR β ^{147F} mice. Triglyceride concentration was significantly higher in TR β ⁻ and TR β ^{147F} mice compared to WT mice (300 $\pm$ 61 and 264 $\pm$ 26 vs. 152 $\pm$ 23 mg/dl; P<0.05), but not in TR β ^{GS} mice (123 $\pm$ 34 mg/dl; n.s.) (Figure 5B).

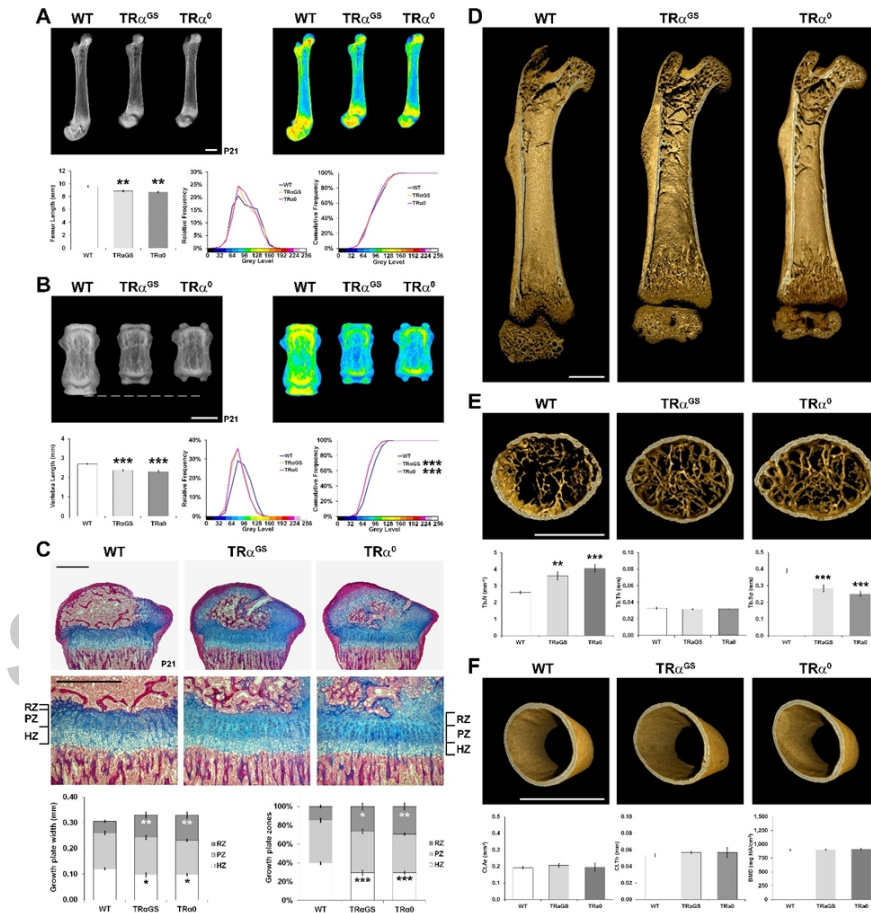


Fig. 4. Canonical TRα action is necessary for normal skeletal development of mice at post-natal day 21 (P21). (A) Grey scale images of femurs from P21 WT (n=5), TRα^{GS} (n=5) and TRα⁰ (n=3) mice (Bar = 1000 μm) were pseudocolored according to a 16-colour palette in which low mineral content is blue and high mineral content is red. Graph (left) demonstrates femur length of WT (open column) TRα^{GS} (light gray) and TRα⁰ (dark gray) mice (mean ± SEM; ANOVA and Tukey's post hoc test; **P<0.01). Relative (middle) and cumulative frequency (right) histograms display bone mineral content (BMC) of femurs from TRα^{GS} and TRα⁰ mice vs WT. (Kolmogorov-Smirnov test, not significant). (B) Images of caudal vertebrae from WT, TRα^{GS} and TRα⁰ mice (Bar = 1000 μm). Graph (left) demonstrates caudal vertebra length in WT, TRα^{GS} and TRα⁰ mice. (mean ± SEM; ANOVA and Tukey's post hoc test; ***P<0.001). Histograms (middle and right) show BMC of vertebrae (Kolmogorov-Smirnov test; ***P<0.001). (C) Proximal tibia growth plate sections stained with alcian blue (cartilage) and van Gieson (bone) (magnification ×50 and ×100) (Bars = 500 μm; RZ, reserve zone; PZ, proliferative zone; HZ, hypertrophic zone). Growth plate chondrocyte zone measurements (left) and relative proportions corrected for total growth plate height (right) are shown for WT (n=5), TRα^{GS} (n=5) and TRα⁰ (n=3) (mean ± SEM; ANOVA and Tukey's post hoc test; *P<0.05; **P<0.01; ***P<0.001). (D) Micro-CT images of longitudinal femur midline sections demonstrate bone morphology (Bar = 1000 μm). (E) Micro-CT images showing transverse sections of the distal metaphysis (Bar = 1000 μm). Graphs demonstrate trabecular number (Tb.N; left), trabecular thickness (Tb.Th; middle) and trabecular spacing (Tb.Sp; right) (mean ± SEM; ANOVA and Tukey's post hoc test; **P<0.01; ***P<0.001). (F) Micro-CT images showing transverse sections of mid-diaphyseal cortical bone (Bar = 1000 μm). Graphs display cortical bone area (Ct.Ar; left), cortical thickness (Ct.Th; middle) and bone mineral density (BMD; right) (mean ± SEM; ANOVA and Tukey's post hoc test, not significant).

These results demonstrate that in the absence of non-canonical TRβ action in TRβ⁻ and TRβ^{147F} mice serum triglyceride concentration is elevated. A possible explanation for increased serum triglyceride concentration was increased triglyceride synthesis in the liver. Compared to WT mice, the expression of several key enzymes of triglyceride synthesis (Scd1, Me1, Fasn) was increased in livers of TRβ⁻ and TRβ^{147F} mice, but not or less in TRβ^{GS} mice (Fig. 5D, E). Accordingly, we also found higher triglyceride concentration in livers of TRβ⁻ and TRβ^{147F} mice, but not of TRβ^{GS} mice, replicating the pattern found in serum (Fig. 5 C, F). The observed phenotype similarity of WT and TRβ^{GS} mice on one hand and TRβ⁻ and TRβ^{147F} mice on the other hand demonstrates that absence of non-canonical TRβ signaling, preserved in TRβ^{GS} but not in TRβ⁻ and TRβ^{147F} mice, leads to higher triglyceride concentration. Cholesterol, albumin and total protein concentrations were not different between WT, TRβ⁻, TRβ^{GS} and TRβ^{147F} mice (suppl. table SXY).

Non-canonical TRβ signaling contributes to regulation of body temperature and locomotor activity

Body temperature homeostasis is crucially linked to TH, and deletion of either TRα or TRβ reduced body temperature, although not always significantly (24). In our experiments, mean body temperature of TRβ^{GS} mice was 0.9 °C higher than that of TRβ⁻ mice (Figure 5E). Our interpretation of this unexpected finding is that TRβ⁻ and TRβ^{GS} mice have equally elevated TH concentrations due to abrogated feedback to the pituitary, but only in TRβ^{GS} mice, TRβ can mediate non-canonical effects of these higher TH concentrations, resulting in higher body temperature. To further sustain our interpretation, we repeated body temperature measurements in TRβ WT, TRβ⁻ and TRβ^{GS} mice with a TRα⁰ genetic background. In TRα⁰β⁻ double KO mice, temperature was markedly reduced from 37.0 °C in WT mice to 34.9 °C (Figure 5E), in line with previous reports (24-26). However, also in the TRα⁰ genetic background, TRβ^{GS} mice

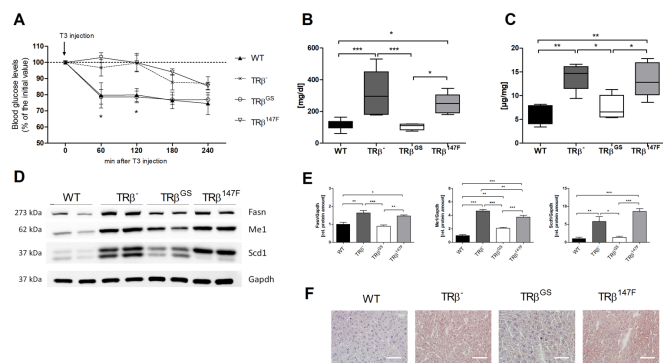


Fig. 5. Non-canonical TR β signaling influences blood glucose and hepatic triglyceride synthesis. (A) Under fasting conditions WT (black triangles), TR β^{GS} (open circles), TR β^{-} (x) and TR β^{147F} (open triangles) mice received a single injection of T3 (7 ng/g BW) and blood glucose concentration was measured at indicated time points ($n=4$; mean $\pm$ SEM; Student's t -test; $*P<0.05$). (B) Serum triglyceride concentration in untreated WT (black), TR β^{-} (grey), TR β^{GS} (open) and TR β^{147F} (light grey) mice. (C) Triglyceride concentration in liver tissue of desired genotypes was assessed after lipid extraction ($n=4-6$; box plot [whiskers min to max] and mean; ANOVA and Bonferroni's *post hoc* test for multiple comparison; $*P<0.05$; $**P<0.01$; $***P<0.001$). (D) Representative western blot against Fasn, Me1 and Scd1 from liver samples. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as loading control ($n=2$). (E) Immunoblots against Fasn, Me1 and Scd1 with a group size of $n=4$ was used for densitometric measurements ($n=4$; ANOVA with Tukey's *post hoc* test; $*P<0.05$, $**P<0.01$, $***P<0.001$). (F) Oil red O staining of liver samples from untreated WT, TR β^{-} , TR β^{GS} and TR β^{147F} mice confirmed triglyceride accumulation in livers of TR β^{-} and TR β^{147F} mice (white bar = 50 μ m).

had an approximately 1 °C higher body temperature than TR β^{-} mice (Figure 5E). These results suggested a TR β specific effect on energy metabolism that is independent from TR α and non-canonically mediated. In indirect calorimetry, minimum oxygen consumption as an approximate of resting metabolic rate of TR β^{GS} mice was not different from TR β^{-} mice and WT controls (Fig. XYA, B). Food consumption and metabolic fuel utilization also did not differ by genotype. But average and maximum oxygen consumption as well as distance travelled were increased in TR β^{GS} mice (Fig. XYZ). Thus, the noncanonical TR β specific effect of TH does not increase metabolic rate but increases locomotor activity.

Non-canonical TR α signaling contributes to central regulation of heart rate

TR α is the predominant TR isoform in the heart and regulation of heart rate (HR) is a well-known physiological effect of TH and TR α (26). We determined HR with a non-invasive ECG in untreated WT, TR α^0 and TR α^{GS} mice. HR was significantly reduced in TR α^0 mice compared to WT mice (Figure 6A). Importantly, HR was not reduced in TR α^{GS} mice, despite expression of TH responsive genes involved in regulation of HR being similarly reduced in hearts of TR α^{GS} and TR α^0 mice (e.g. *Hcn2*, *Hcn4* and *Kcne1*, Figure 6B). The phenotype concordance between WT and TR α^{GS} mice demonstrates that non-canonical TR α signaling contributes to normal HR in TR α^{GS} mice. To distinguish between an intrinsic vs. an extrinsic effect, we measured basal heart rate *ex vivo* in a Langendorff apparatus. *Ex vivo*, heart rate was the same in TR α^{GS} and TR α^0 mice, suggesting non-canonical TR α signaling contributes to regulation of heart rate centrally, e.g. in the autonomous nervous system, and not in the heart itself.

Discussion

The physiological role of TRs has been studied *in vivo* for more than fifteen years by comparing WT with TR KO mice. As canonical and non-canonical (type 1 and type 3 (1)) TR signaling

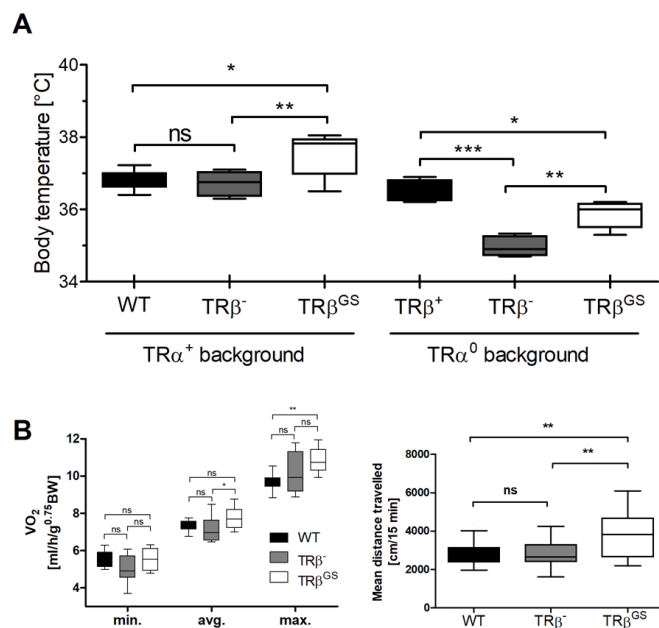


Fig. 6. Non-canonical action of TR β increases body temperature and locomotor activity. (A) Body temperature of male mice in a TR α WT as well as TR α^0 genetic background ($n=6$; box plot [whiskers min to max] and mean; ANOVA and Bonferroni's *post hoc* test for multiple comparison; ns = not significant; $*P<0.05$; $**P<0.01$; $***P<0.001$). (B) Indirect calorimetry (minimum [min.], maximum [max.] and average [avg.] oxygen consumption) revealed only subtle differences between TR β^{-} and TR β^{GS} mice as well as WT and TR β^{GS} mice regarding average and maximum oxygen consumption, respectively ($n=10$; ANOVA with Tukey's *post hoc* test; $*P<0.05$, $**P<0.01$, ns = not significant). (C) Locomotor activity was significantly increased in TR β^{GS} mice compared to WT, and TR β^{-} mice ($n=10$; ANOVA with Tukey's *post hoc* test; $**P<0.01$, ns = not significant).

are both present in WT and absent in TR KO mice, such a comparison could not distinguish between the two mechanisms and the role of putative non-canonical TR signaling remained largely unrecognized. To separate both modes of TH/TR signaling, we abolished DNA-binding of TRs in TR α^{GS} and TR β^{GS} mice. Strikingly, despite complete loss of canonical TR signaling, the TR α^{GS} and TR β^{GS} did not present a TR KO phenotype. Rather, for several TH effects their phenotype was indistinguishable from WT mice. Thus, these results provide the first *in vivo* evidence for the physiological relevance of non-canonical TR signaling. Furthermore, they demonstrate that non-canonical TR signaling can be mediated by both TR α and TR β in an isoform- and tissue-specific manner. These results have profound implications for the role of TRs in metabolism and physiology and, most likely, disease.

In general, a loss of function phenotype in TR KO mice and discordance between TR KO and TR GS mice, but concordance between TR GS and TR WT mice, indicated that non-canonical TR signaling is required for a TH effect. Conversely, phenotype concordance between TR KO and TR GS mice and discordance between TR GS and TR WT indicated that an effect is canonically mediated. TR β^{GS} mice showed a complete KO phenotype with lack of TR β mediated negative feedback in the pituitary, constituting resistance to TH, congruent with earlier reports (9). Delayed longitudinal growth and bone mineralization are typical features of TR α^0 mice and were identically replicated in TR α^{GS} mice. Negative regulation of the HPT axis in TR β^{GS} mice and bone development in TR α^{GS} mice therefore are solely dependent on canonical TR/TRE mediated transcriptional regulation.

. -> mention HPT axis in TR β^{147F} mice (7)

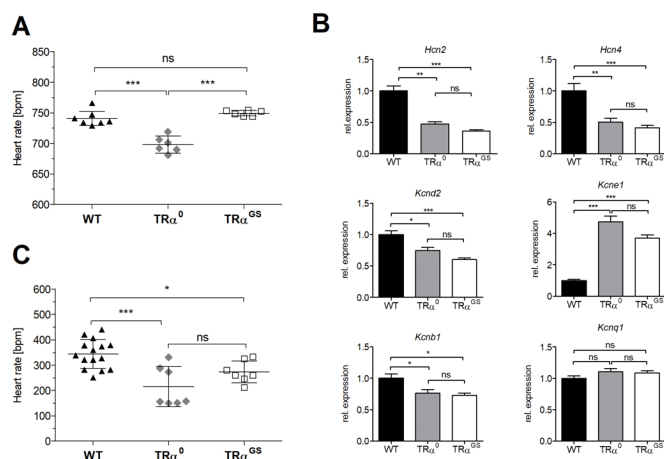


Fig. 7. Non-canonical TR β signaling maintains normal basal heart rate without altering cardiac pacemaker channel gene expression. (A) HR of non-sedated male WT (black triangles; $n=7$), TR α^0 (grey diamonds; $n=6$) and TR α^{GS} (open squares; $n=6$) mice (mean $\pm$ SD; ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons; *** $P<0.0001$; ns = not significant). (B) Relative expression of pacemaker channels *Hcn2* and *Hcn4* and of potassium channel subunits with importance for repolarization, *Kcnb1*, *Kcnd2*, *Kcne1* and *Kcnq1*, in hearts of TR α^0 (grey bars), TR α^{GS} (open bars) mice and WT mice (black bars) ($n=6$; mean $\pm$ SEM; ANOVA and Tukey's *post hoc* test; * $P<0.05$, *** $P<0.001$; ns = not significant). (C) *Ex vivo* HR from hearts isolated from untreated WT (black triangles; $n=15$), TR α^0 (grey diamonds; $n=8$) and TR α^{GS} (open squares; $n=7$) mice measured via Langendorff apparatus (mean $\pm$ SD; ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons; * $P<0.05$, *** $P<0.0001$; ns = not significant).

Kalyanaraman et al. reported that *in vitro* a short TR α variant, TR α p30, lacking an N-terminal part including the transcription activation domain and the DNA-binding domain, is sufficient and necessary for activation of the ERK and PI3K pathways (27). The proposed mechanism involves association of TR α p30 to the plasma membrane, activation of NO synthase, NO generation, cGMP production and ultimately Src kinase activation, which then activates PI3K and MEK-ERK. *In vivo* data are based on hypothyroid wild type mice that were treated with cinaciguat, which increases cGMP and restored mineral apposition and bone formation compared to untreated hypothyroid mice. From these data the authors concluded that this effect may be mediated by TR α p30-mediated GMP-increasing action.

-> age of Kalyanaramans mice? 16 weeks

-> age of our mice? Growth recorded through week 10, bone samples week 3.

-> while clearly classical in adolescent bones, ... discuss effects in adult bone

-> bone formation/ resorption / balance regulated by non-classical mechanisms of TR α

Importantly, several TH effects did not require DNA-binding of TRs.

HR was significantly reduced in TR α^0 mice compared to WT mice but not in TR α^{GS} mice, demonstrating that TR α influences HR via non-canonical signaling. Yet, cardiac expression of two so-called pacemaker channel genes, *Hcn2* and *Hcn4*, associated with bradycardia in hypothyroidism and tachycardia in hyperthyroidism (28), was identically reduced in TR α^{GS} and TR α^0 mice and cannot explain why HR in TR α^{GS} mice was normal. To exclude any external influence like stress on heart rate, we measured basal heart rate *ex vivo* in a perfused heart in a Langendorff apparatus. In this experiment, heart rate in TR α^{GS} hearts was the same as in TR α^0 hearts and significantly different from that of WT hearts. So, heart rate in TR α^{GS} mice *in vivo* was the same as that in WT mice, but *ex vivo* the same as that in TR α^0 mice.

mice (Fig. 7C). This aligns well with results from expression of cardiac pacemaker genes. Together, these results suggest that the mechanism maintaining normal heart rate *in vivo* is not heart intrinsic. Our interpretation is that TH/TR α influence heart rate via the autonomous nervous system and not in the heart itself. Interestingly, this seems to be similar to regulation of locomotor activity, another probably centrally mediated effect.

Thus, TH mediated regulation of HR cannot solely be attributed to canonical TR action. Rather non-canonical signaling of TR α seems to be a key component in the regulation of HR. As ECG was obtained without anesthesia, it is possible that the response to stress or the activity of the sympathetic nervous system is reduced in TR α^0 mice, but preserved in TR α^{GS} mice.

For triglyceride concentration and response of blood glucose to TH, the phenotype of TR β^{GS} mice was the same as that of WT mice.

-> better connection? These results not only demonstrate that presence of non-canonical signaling is sufficient to produce the WT phenotype, but strongly suggest that non-canonical action of TR β is closely related to energy-linked metabolic processes.

Locomotor activity was significantly increased in TR β^{GS} mice. Interestingly, running activity has recently been shown to be controlled in the ventromedial hypothalamus (VMH) by TR β (29). Thus, our results indicate that increased locomotor activity is a consequence of non-canonical TR β action in the VMH.

This is further supported by the finding that non-canonical action of TR β increased body temperature. The challenge is now to determine whether this TR β action contributes to the thermogenic program directly in brown, beige or white adipose tissue or centrally, e.g. in the hypothalamus.

-> connect the metabolic effects, point out that these functions appear to be centrally regulated (locomotor activity, heart rate)

Together, our findings suggest that non-canonical TR β signaling mainly influences energy metabolism and promotes the conversion of energy into heat with increased body temperature and locomotor activity, apparently increased glucose utilization and reduced storage of energy in form of triglycerides in TR β^{GS} mice. Support for this interpretation may be derived from evolution: TH stimulates thermogenesis in homeothermy, a feature of mammals. Interestingly, the tyrosine motifs in TR β , that are required for non-canonical PI3K activation by TR β , are found in all published mammalian TR β ortholog sequences, but not in that of poikilothermic animals, such as alligator, clawed toad or zebrafish (7). Hence, non-canonical TR signaling appears to be a newly acquired function in evolution that provides mammals with additional means for TH to regulate energy metabolism and body temperature. Development of non-canonical TR signaling relatively late in evolution, long after TRs assumed their canonical role as ligand-dependent transcription factors, may explain why canonical and non-canonical effects on physiology are so clearly separable from each other.

Still a matter of controversy is the negative regulation of gene expression by TRs, especially whether binding of TRs to DNA is required or not. The fact that TSH is elevated in TR β^+ and TR β^{GS} mice clearly demonstrates that TSH suppression requires DNA-binding, which is in agreement with previous reports (9). Results from our TR β^{GS} mice indicate that additional, non-canonical mechanisms for negative regulation by TH may exist. *Scd1* expression in mouse liver and in HepG2 cells is negatively regulated by TH (30) (suppl. Fig. 2). Compared to WT mice, hepatic *Scd1* expression was elevated in TR β^+ mice, but not in TR β^{GS} mice. Therefore, negative regulation of *Scd1* expression *in vivo* does not require DNA-binding of TR β . In our microarray experiment, however, we did not detect downregulation of *Scd1* within 6 hours. Two explanations seem plausible: Either *Scd1* is indeed

directly negatively regulated by TH/TR β without DNA binding by a mechanism that takes longer than 6 hours. Interestingly, recent ChIPseq analyses of TH regulated genes (10-12) show significant TR β enrichment near positively regulated genes, but less or no enrichment of TR β at negatively regulated genes, suggesting that DNA-binding of TR β may not be required for gene repression. The possibility of several mechanisms for negative gene regulation by TRs, canonical and dependent on DNA-binding (e.g. for *Tshb*) and non-canonical (e.g. for *Scd1*), should be considered. But it is also possible that *Scd1* downregulation in liver is a secondary effect in response to non-canonical TH metabolism. -> discuss induction of the other enzymes...

Our experiments cannot determine the precise molecular mechanism of non-canonical TR action in TR α ^{GS} and TR β ^{GS} mice for every TH effect. The best studied non-canonical action of TR β is rapid activation of PI3K (4-7): cytosolic TR β simultaneously binds p85 α and another kinase, Lyn, which allows Lyn to activate p85 α and consequently PI3K (7). TR α also promotes signaling pathway activation (31, 32), possibly as a short TR α variant, activating ERK and PI3K after T₃ binding (27). The underlying mechanism may not be the same for all physiological effects of non-canonical TR action.

As insulin also signals via PI3K to decrease blood glucose by increasing translocation of glucose transporter 4 (GLUT4) to the plasma membrane, it is conceivable that insulin and TH signaling converge at the PI3K pathway, both increasing glucose clearance from the blood, resulting in decreased glucose concentration. This interpretation is in agreement with previous observations of increased glucose uptake into the thymus in rats, which was independent from protein synthesis (33), increased glucose uptake into L6 muscle cells in a PI3K-dependent manner in vitro (34) and increased translocation of GLUT4 after T₃ treatment in skeletal muscle of rats (35).

In conclusion, non-canonical TR signaling significantly contributes to the physiological effects of TH, with a suggested preferential impact on energy homeostasis. These findings demonstrate for the first time that TR α and TR β mediate canonical and non-canonical signaling *in vivo*, establishing a paradigm shift for TH action.

Materials and Methods

TR expression vectors

Point mutations for TR α 71GS, TR α G291R, TR β 125GS, TR β G345R and TR β Y147F were introduced into pCDNA3 vectors expressing TR α 1 and TR β 1 by site-directed mutagenesis (Quikchange Site Directed Mutagenesis Kit, Agilent Technologies, USA; primer sequences will be provided upon request).

Cell culture and transfection

HEK293 cells, obtained from ATCC, were used for *in vitro* experiments. Cells were maintained in DMEM containing 4.5 g/L glucose, pyruvate (DMEM+GlutaMAX, Gibco, USA), 1% ZellShield (Minerva Biolabs GmbH, Germany) and 10% FCS (Gibco, USA). For transient transfection, 4x10⁴ HEK293 cells were grown to 80% confluence in 24-well plates. FuGENE6 (Promega, USA) transfection reagent was used in a 1:3 DNA:FuGENE ratio.

Electrophoretic mobility shift assay

TR α , TR α 71GS, TR β , TR β 125GS, TR β Y147F and RXR α proteins were generated using a reticulocyte transcription and translation kit (TNT[®] T7, Promega, USA) and 1 μ g of plasmid, respectively. 4 μ l of translated TR and 2 μ l of RXR α were preincubated in reaction buffer (10 mM Tris, 50 mM KCl, 1 mM DTT and 5% glycerol) with 1 μ g of poly-dI:dC for 10 min at 20 °C before 1 μ l of 5'-Cy5-labeled DR4 probe (Cy5-5'-GCTATGAGGTCACAGGAGGTCATGCAAC-3') was added to achieve a final concentration of 1 pmol/ μ l. After 30 min incubation at 20 °C protein-DNA complexes were analyzed on a 6% polyacrylamide gel (0.5x Tris-borate-EDTA, 89 mM Tris-base, 89 mM borate, 2 mM EDTA). The fluorescent probe was visualized using a VersaDoc imaging system (Bio-Rad, USA).

Luciferase assay

HEK293 cells were transiently transfected using 280 ng/well of DR-4-TKLuc-reporter plasmid, 33 ng/well RL-TK control plasmid and 17 ng/well of plasmid encoding for TR α 1 or TR β 1 variants (TR α , TR α 71GS, TR α G291R, TR β , TR β 125GS, TR β G345R, TR β Y147F or empty vector pCDNA3) in the absence of FCS. After 24 hours, transfection medium was replaced by fresh medium containing 5% TH-depleted FCS, which was generated by treatment with anion exchange resin as previously described (36). Cells were stimulated with 10 nM T₃ (dissolved in 40 mM NaOH, 0.002% BSA) or treated with

vehicle for another 48 hours. Cells were harvested and the activities of firefly and renilla luciferases were determined (Dual-Glo Luciferase Assay System, Promega, USA) in a Sirius luminometer (Berthold Detection Systems GmbH, Germany). Firefly luciferase luminescence was normalized to renilla luciferase luminescence from the same transfection sample to control for transfection efficiency.

Generation of TR α ^{GS} and TR β ^{GS} knock-in mice

The GS point mutations were introduced into the *Thra* (NM.178060.3) and *Thrb* (NM.001113417.1) gene loci of C57BL/6J mice using custom made zinc finger nucleases (ZFNs) (CompoZr[™] Targeted Integration-Kit-AAVS, Sigma-Aldrich, USA) and a donor plasmid containing the desired sequence (37, 38). For *Thra* we chose a ZFN cutting site within exon 3, 42 bp upstream from the target codons, and for *Thrb* the ZFN cutting site was chosen downstream of exon 3 (ZNF recognition sequence for *Thra*, 5'-TCC CTA GTT ACC TGA CAAAGAC ACG AGC AGT GTG TCG TGT GTG-3'; for *Thrb*, 5'-CCA GAC AAC CAC TGGACAT CAGG CTT ACA GAA AGA GAT-3'; underlined = ZFN binding sites; *italic* = FokI cutting site). The donor vectors pTR α GS-donor and pTR β GS-donor (both in pUC57-Kan, GENEWIZ, UK) contained the mutations changing the amino acid sequence in the TR P-box from glutamic acid and glycine (EG) to glycine and serine (GS). The codons 71, GAG, and 72, GGC, of exon 3 of *Thra* were mutated to GGG and AGC, respectively. Corresponding codons in *Thrb* (125, GAA and 126, GGC) were changed to GGA and AGC, respectively. Additionally, silent mutations created artificial restriction sites for *EcoRV* (TR α ^{GS}) and *Mph1103I* (TR β ^{GS}) to facilitate genotyping by RFLP-PCR. C57BL/6J oocytes were injected with microinjection solution (MIS) containing 20 ng/ μ l ZFN mRNA and 1.3 ng/ μ l donor plasmid. Injection of 1 pl MIS per pronucleus was performed with Leica micromanipulators. Manipulated oocytes were transplanted into oviducts of 0.5 days p.c. pseudopregnant mice. Offspring were screened by PCR and sequencing, which confirmed the successful TR α ^{GS/+} and TR β ^{GS/+} knock-in mutations. One pup out of 29 for TR α ^{GS/+} and one out of 39 for TR β ^{GS/+} was positive for the targeted integration, which equals a mutation rate of 3.5 % and 2.6 %, respectively. Homozygous TR α ^{GS} and TR β ^{GS} mice were born in Mendelian proportions. These mice are viable and no effect on fertility was observed in breeding animals up to the age of eight months. Genotyping of TR β ^{GS} mice was performed by RFLP using forward primer mTR β -fwd (5'-TCT GAA GCA CCA ACA CTT TCC T-3'), located on the genomic *Thrb* sequence upstream of the 3'-homology region of the donor vector, and a reverse primer mTR β -rev (5'-AGA AAG GCT GTG GAG CAA AC-3'), binding downstream exon 3, generating a 1399 bp amplicon. For TR α ^{GS} we used mTR α -fwd (5'-GTT TTG GCC CCA TCT AAG CC-3') and mTR α -rev (5'-AGC CGT GCC AGG TGA ATT AG-3'). Digestion of the PCR product with *EcoRV* (TR α ^{GS/+}) or *Mph1103I* (TR β ^{GS/+}) indicated the GS allele (both enzymes were purchased from ThermoFisher Scientific, Germany). TR knockout (TR α ⁰ and TR β ⁰) mice were obtained from the European Mouse Mutant Archive (<https://www.infrafrontier.eu>). TR β ^{147F} mice were provided by Dr. D. L. Armstrong (7). TR α and TR β WT and knockout and TR β ^{147F} mice were genotyped as previously described (7, 24, 39). All mouse strains were kept on a C57BL/6Jcrl backcross for at least five generations. All experiments were performed with homozygotes.

Animal treatment and phenotyping

All mice were housed at 21 $\pm$ 1 °C and an alternating 12-hour light and 12-hour dark cycle. Standard chow (Sniff, Soest, Germany) and tap water were provided *ad libitum*, unless indicated otherwise. All animal studies were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). After 15 weeks WT, TR α ⁰, TR α ^{GS}, TR β ⁰ and TR β ^{GS} animals were sacrificed to obtain serum and tissue samples. Tail length and BW were recorded for all genotypes from postnatal day 21 through week 10. Bone samples were taken from WT, TR α ⁰ and TR α ^{GS} mice at postnatal day 21. Body temperature of 12 to 13-week-old male WT, TR β ⁰ and TR β ^{GS} mice was measured every other day between 9 and 10 a.m. with a rectal probe (TempJKT, Eutech Instruments Europe, Netherlands). Heart rate was determined in WT, TR α ⁰ and TR α ^{GS} mice without anesthesia using a non-invasive electrocardiography (ECG) and at least three recordings per mouse. ECG data were analyzed with *PicoScope6* (Pico Technology, UK). For *in vivo* studies of T₃ effects on gene expression, WT, TR β ⁰, TR β ^{GS} and TR β ^{147F} mice were rendered hypothyroid at the age of 10 to 11 weeks with an iodine-deficient diet (LID) (TD.95007, Harlan-Teklad, USA), 0.05% 2-mercapto-1-methylimidazole (MMI) and 1% potassium perchlorate (Sigma-Aldrich) administered in the drinking water. After three weeks, mice were separated into T₃ treatment and control groups for each genotype. T₃ treated animals received T₃ (Sigma-Aldrich) 20 μ g/100 g BW i.p. once (for microarray analysis) as a single dose or daily for 5 days (for real time PCR). Animals of the control group were injected with vehicle i.p. The mice were euthanized with CO₂ six hours after the single dose of T₃ or two hours after the last injection on day 5 and blood and tissue samples were collected. To measure the blood glucose response to T₃, WT, TR β ⁰, TR β ^{GS} and TR β ^{147F} mice were injected i.p. with T₃ (7 ng/g BW) or vehicle. Before and 60 and 120 minutes after T₃ injection, blood was drawn by tail vein puncture.

Indirect calorimetry and activity

Food intake, respiratory exchange ratio and locomotor activity were assessed using a combined indirect calorimetry system (TSE Systems, Germany). After 24 hours of adaptation, oxygen consumption and CO₂ production were

measured every 10 min for a total of 21 hours. Food intake was determined continuously for the same time as the indirect calorimetry assessments by integration of scales into the sealed cage environment. Locomotor activity was determined using a multidimensional infrared light beam system and expressed as distance travelled [cm/15 min].

Thyroid function tests, analysis of serum parameters and triglyceride concentration

Blood was collected by cardiac puncture. Serum thyroid-stimulating hormone (TSH) was measured with a sensitive, heterologous disequilibrium double-antibody precipitation RIA (40). Results are expressed in mU/L. Serum concentrations of total T₄ (TT₄) and total T₃ (TT₃) were measured by RIA (DRG Instruments, Germany), following the manufacturer's instructions. 30 µl of serum were used to analyze triglycerides (TG), cholesterol, albumin and total protein on an ADVIA 2400 Chemistry System (Siemens Healthcare, Germany). Blood glucose concentration was measured with a Contour[®]XT glucometer (Bayer, Germany). 50 mg of shock frozen liver tissue was homogenized in 300 µl PBS with 1% Triton-X100. Lysates were boiled for 10 min at 95 °C and the supernatant was collected after 10 min of centrifugation with 17.000xg. Whole lysates were used for analysis of TG with ADVIA2400.

Oil red O staining

Livers were embedded in TissueTek[®] O.C.T.™ (Sakura Finetek, Germany) and shock frozen in the gas phase of liquid nitrogen. 5 µm section were fixed in 10% formalin for 30 min at RT. Oil red O working solution consisted of three parts of an 0.3% Oil red O stock solution (in 99% isopropanol) and two parts of deionized water. Sections were preincubated with 60% isopropanol for 5 min at RT, followed by an incubation in Oil red O working solution for 15 min. After rinsing with tap water sections were counter stained with hematoxylin and covered immediately. Stained sections were imaged at the day of staining.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (41) with the following modifications. 25 mg frozen liver tissue from was used per IP. The procedure was performed on whole cell material or isolated nuclei involving 1.5 h incubation in lysis buffer 1 (1 % SDS, 20 mM EDTA, 50 mM Tris-HCl pH 8.0, 1 µg/µl BSA and proteinase inhibitors). Samples were resuspended in lysis buffer 2 (0.1 % SDS, 1 % Triton, 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 1 µg/µl BSA and proteinase inhibitors) and sonicated using Bioruptor[™] Twin Diagenode or Covaris[®] ME220. IPs using 2 or 1 µg H3K27Ac (Abcam, ab4729), RXRα D-20 (Santa Cruz, sc-553) or TR (12) were performed with overnight incubation followed by 2-hour incubation with 30 µl Protein A/G PLUS-Agarose beads (Santa Cruz, sc-2003). Beads were extensively washed and DNA-protein complexes isolated with elution buffer (1 % SDS and 0.1 M NaHCO₃). DNA-protein complexes were decrosslinked at 65°C for at least 6 hours and DNA was purified by phenol/chloroform extraction. Enrichment of TR, RXR and H3K27Ac at specific TR binding sites was evaluated by qPCR using the following primer pairs:

Dio1 +7.0kb F: 5'-CAGGCAAAGGGAGTCAATGT-3', R: 5'-GGTTCGGACAGTGGTCTTA-3'

NCoR2 +22.4kb F: 5'-AGGCGGTGTCTGTACTCTG-3', R: 5'-CACTAGGCA-CCAGAGGGAAG-3'

H13 +3.1kb F: 5'-GGGAGTGTCCGAAACCTGTA-3', R: 5'-CTGAAGCGAAA-GCCCAATAG-3'

Strbp +59.1kb F: 5'-TTGCATCATGCTCAGTCAAA-3', R: 5'-TGAATGCTCAG-GGTCAGATG-3'

Illumina sequencing and data analysis

H3K27Ac ChIP-seq libraries were constructed using the NEBNext DNA library prep kit according to manufacturer's (NEB) instructions. Sequencing was carried out on a HiSeq 1500 platform (Illumina). Sequenced tags were aligned to mm9 using STAR (42). Aligned tags were quantified at previously identified DNase accessible regions in liver tissue (12) using HOMER (43). Differentially regulated H3K27Ac in the liver from WT animals in response to T3 was identified by DESeq2 at FDR<0.1 and log2 fold change >1 or <-1 (44). Heat map was generated using the *pheatmap* R package using hierarchical clustering (45).

Gene expression analysis

Total RNA was isolated from liver and heart (RNeasy Kit, QIAGEN, Germany) and stored at -80 °C. 2 µg of total RNA were reverse transcribed into cDNA with SuperScriptIII (Invitrogen, USA) and random hexamer primers. qRT-PCR was performed using Roche SYBR Green I Master Mix on a Light-Cycler LC480 (Roche, Switzerland). Primer sequences will be provided upon request. In compliance with the MIQE guidelines for qRT-PCR (46), we used a set of three reference genes per tissue for accurate normalization and calculation (liver: *18S*, *Ppia*, *Rpl13a*; heart: *18S*, *Gapdh*, *Hprt1*). Ct values <35 were used for analysis and calculation of the fold change in gene expression by the efficiency corrected method (47).

Microarray

For microarray analyses, we used the Affymetrix GeneChip platform employing the Express Kit protocol for sample preparation and microarray hybridization. Total RNA (200 ng) was converted into biotinylated cRNA, purified, fragmented and hybridized to MG-430.2.0 microarrays (Affymetrix, Santa Clara, CA, USA). The arrays were washed and stained according to the manufacturer's recommendation and finally scanned in a GeneChip scanner 3000 (Affymetrix). Array images were processed using the PartekG5 software (RMA algorithm). Differentially expressed probesets were identified using

the implemented ANOVA method and the step-up procedure to correct for multiple testing. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (48) and are accessible through the following GEO Series accession number: GSE93864 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93864>).

Bone histology

Tibias were fixed in 10% neutral buffered formalin and decalcified in 10% EDTA, embedded in paraffin wax. Sections (5 µm) were stained with alcian blue and van Gieson (18). Measurements from at least 4 separate positions across the growth plate were obtained to calculate the mean height using a Leica DMLB2 microscope and DFC320 digital camera (Leica Microsystems). Results from 2 levels of sectioning were compared.

Faxitron digital x-ray microradiography

Femurs were imaged at 10 µm resolution using a Faxitron MX20 (Qados). Bone mineral content was determined relative to steel, aluminum, and polyester standards. Images were calibrated with a digital micrometer, and bone length was determined (18, 19).

Micro-computed tomography (CT)

Femurs were imaged by micro-CT (Scanco µCT50: SCANCO Medical AG) at 70kV, 200µA with a 0.5mm aluminium filter and a voxel resolution of 3 µm. Images were reconstructed and analyzed using SCANCO software. A 1-mm³ region of interest was selected 100 µm from the growth plate, and trabecular bone volume as proportion of tissue volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were determined (18). A 1-mm region of interest centred in the mid-shaft 56% along the length of the femur distal to the femoral head was used to determine cortical area (Ct.Ar), cortical thickness (Ct.Th) and cortical bone mineral density (BMD). Raw data (333 cross-sections per specimen in 450x450 pixel 16 bit DICOM files) from representative femurs from each genotype were imported using 32-bit Driшти v2.0.221 (Australian National University Supercomputer Facility, <http://anuf.anu.edu.au/Vizlab/driшти/>) and rendered using 64-bit Driшти v2.0.000 to generate high-resolution images.

Western blotting

Whole protein lysates were generated from snap frozen liver tissue homogenized in RIPA buffer (150 mM NaCl, 50 mM HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF) with protease inhibitor cOmplete (Roche). After 30 min incubation on ice with gentle agitation, lysates were sonicated with an ultrasound probe (SONOPLUS, Bandelin, Germany). After 10 min of centrifugation with 17.000xg at 4 °C, supernatant was collected and the cell debris was discarded. 20 µg of the samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roti-Fluoro PVDF, Roth). After blocking with 5% milk in TBS-T (Tris buffered saline with 0.5% Tween-20) for 1 h. Membranes were incubated for 16 h at 4 °C under gentle agitation with desired primary antibody against Scd1 (SCD H-300, sc-30081, Santa Cruz), Me1 (PAS-21550, ThermoFisher Scientific), Fasn (MA5-14887, ThermoFisher Scientific) and Gapdh (ACR001P, Acris antibodies). Horseradish peroxidase labeled secondary antibody against rabbit IgG and Alexa488 labeled antibody against mouse IgG were used in an appropriate dilution for detection with VersaDocMP4000 (BioRad).

Determining ex vivo heart rate via Langendorff apparatus

Isolation and cannulation of hearts were performed as describes elsewhere (Hildebrandt,2016; habe ich in EndNote hinzugefügt). Briefly, at a constant pressure of 80 mm Hg, hearts were perfused with modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.64 mM MgSO₄ × 7 H₂O, 0.2 g/L EDTA, 2 mM Na-pyruvate, 1.18 mM KH₂PO₄, 5.55 mM glucose, 24.88 mM NaHCO₃ and 2 mM CaCl₂). A water-filled balloon made out of saran wrap (Toppits, Cofresco Frischhalteprodukte GmbH & Co. KG, Minden, Germany) was inserted through the mitral valve into the left ventricular cavity and connected to a transducer (DPT-6000, Pvb Codan, Forstinning, Germany) to allow continuous monitoring of HR. After 10 min of acclimation basal ex vivo HR was recorded for 1 min and mean HR was calculated.

Statistics

We used one-way ANOVA with Tukey's *post hoc* test for statistical analysis for normally distributed data sets unless otherwise noted. Differences were considered significant when *P*<0.05. For gene expression data, statistical significance was calculated on log-transformed data (to obtain normal distribution) as recommended by the MIQE guidelines (46). Analysis and plotting were done with *GraphPad Prism6* (GraphPad Software, USA).

Study approval

All animal experiments were approved by the *Landesamt für Natur, Umwelt und Verbraucherschutz* (LANUV) of North Rhine-Westphalia, Germany.

Author contributions

GSH and LCM designed the studies; GSH, HR, JL, XHL, EW, ASP, SMP, MSS, ER, JG, SL, KE, KHS, DZ and LKH conducted experiments; GSH, LKH, JK, DLG, LG, JHDB, GRW, SR, DF and LCM analyzed data; GSH and LCM wrote and all authors contributed to the manuscript.

Helmholtz authors

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Supplemental information (SI)

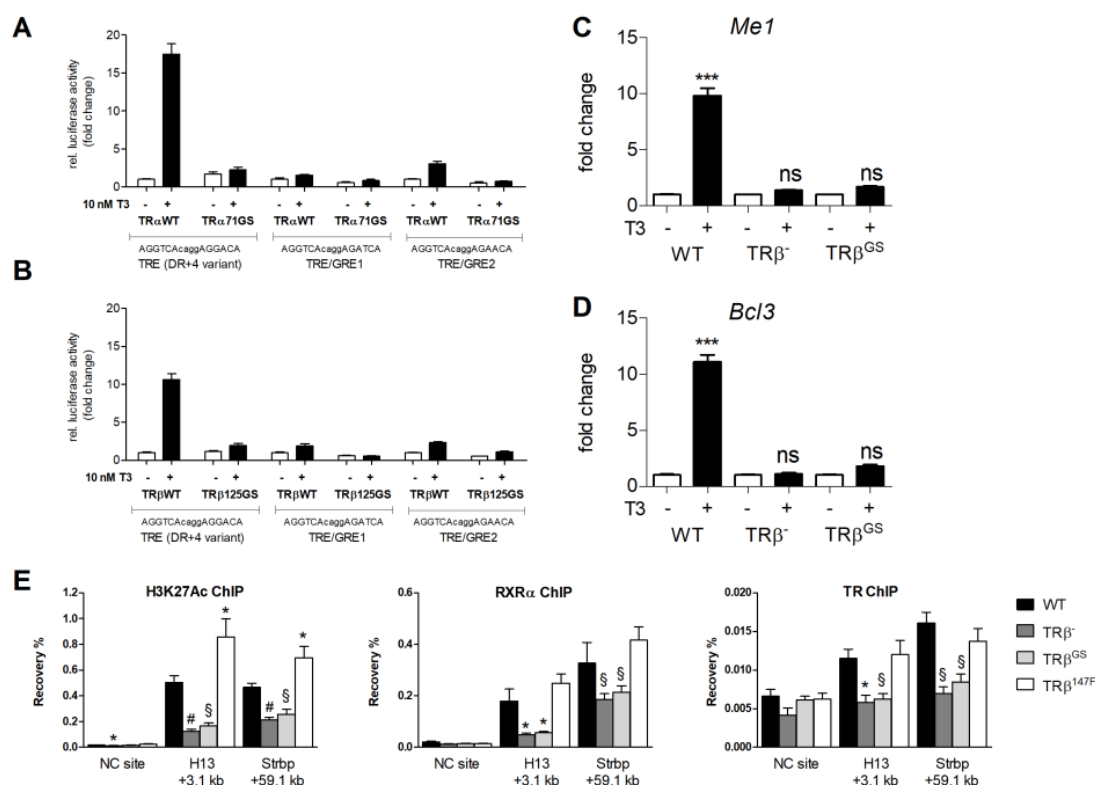


Fig. SI1. GS mutation mediates abrogation of DNA binding and canonical action of TRs.

HEK293 cells transfected with plasmids encoding for (A) TR β and TR β 125GS as well as (B) TR α and TR α 71GS and a luciferase reporter plasmid under control of an alternative DR+4 sequence or an hybrid TRE/GRE sequence. Cells were treated with 10 nM T₃ for 48 h (black bars) to induce luciferase expression via canonical TR/TRE-mediated action for TR α and TR β variants. (C-D) Response of TH target genes (*Me1* and *Bcl3*) to T₃ in livers of hypothyroid WT, TR $\beta^{-/-}$ and TR β^{GS} mice. Hypothyroid mice were either injected with vehicle (open bars) or with 5 μ g/100 g BW T₃ (black bars) for 4 consecutive days. (*n*=6; mean $\pm$ SEM). (E) Chromatin immunoprecipitation (ChIP) for H3K27Ac, RXR α and TR β was followed by qPCR for determining H3K27 acetylation and recruitment of RXR α and TR to TR binding sites located +7.0 kb on *Dio1* and +22.4 kb on *NcoR2* gene loci in T₃-treated TR β^{147F} , TR $\beta^{-/-}$ and TR β^{GS} mice compared to WT mice. (*n*=4-6, mean $\pm$ SEM; Student's t-test; **P*<0.05, #*P*<0.005, §*P*<0.001).

Tab. S1. Absolute blood glucose concentrations before and 60 min after T3 injection.

	Blood glucose (mg/dl)				$\Delta\bar{x}$	$\Delta\%$
	before T3		60 min after T3			
WT	179	± 40	127 ^a	± 6	-52	-29%
TR β^-	150	± 17	151	± 21	+1	+0%
TR β^{GS}	137	± 26	102 ^a	± 10	-35	-26%
TR $\beta^{147\text{F}}$	158	± 12	158	± 12	+0	+0%

n=4-5; mean $\pm$ SD; paired Student's T-test; ^a $P \leq 0.02$.

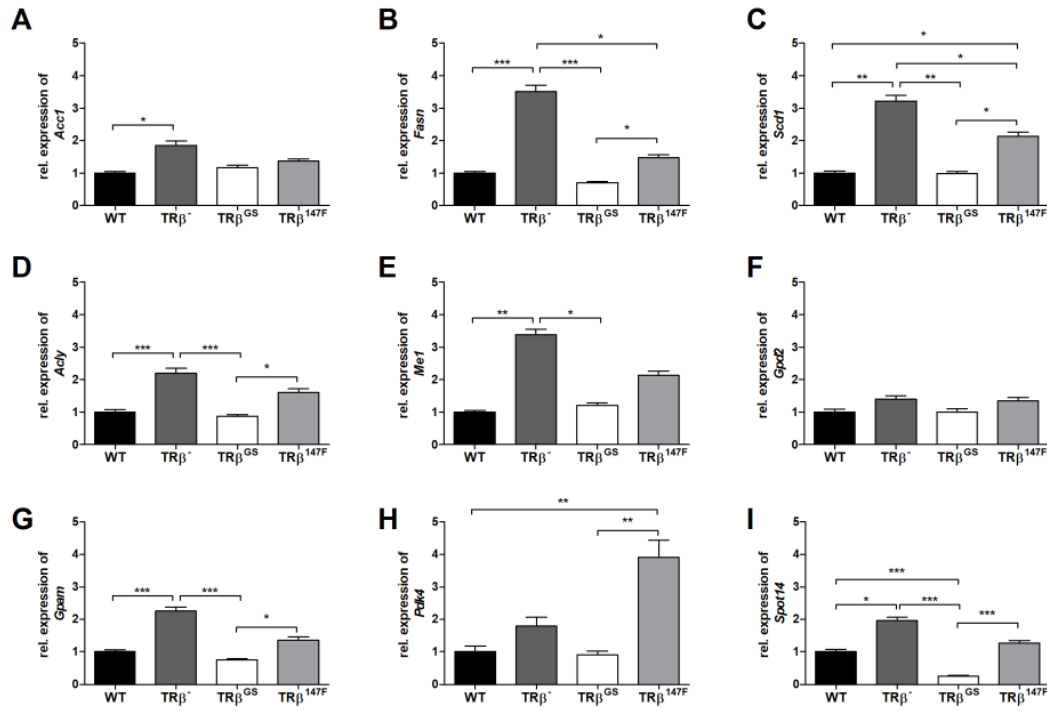


Fig. S12. Hepatic mRNA expression profile of key enzymes and regulatory proteins involved in triglyceride synthesis. (A-G) mRNA expression of enzymes (acetyl-CoA carboxylase (*Acc1*), fatty acid synthase (*Fasn*), Δ^9 -stearyl-CoA desaturase (*Scd1*), ATP-citrate lyase (*Acly*), malic enzyme (*Me1*), glycerol-3-phosphate dehydrogenase (*Gpd2*), mitochondrial glycerol 3-phosphate acyltransferase (*Gpam*) involved in TG synthesis in livers of WT (black bar), TRβ⁻ (dark gray bar), TRβ^{GS} (open bar) and TRβ^{147F} (light gray bar) mice. (H-I) mRNA expression of proteins regulating fuel demand (pyruvate dehydrogenase kinase 4; *Pdk4*) and lipogenesis (thyroid hormone responsive; *Spot14*). (n=4-6; ANOVA with Tukey's *post hoc* test on log-transformed qRT-PCR data; significant results are indicated as follows: **P*<0.05, ***P*<0.01, ****P*<0.001; non-significant differences are not stated)

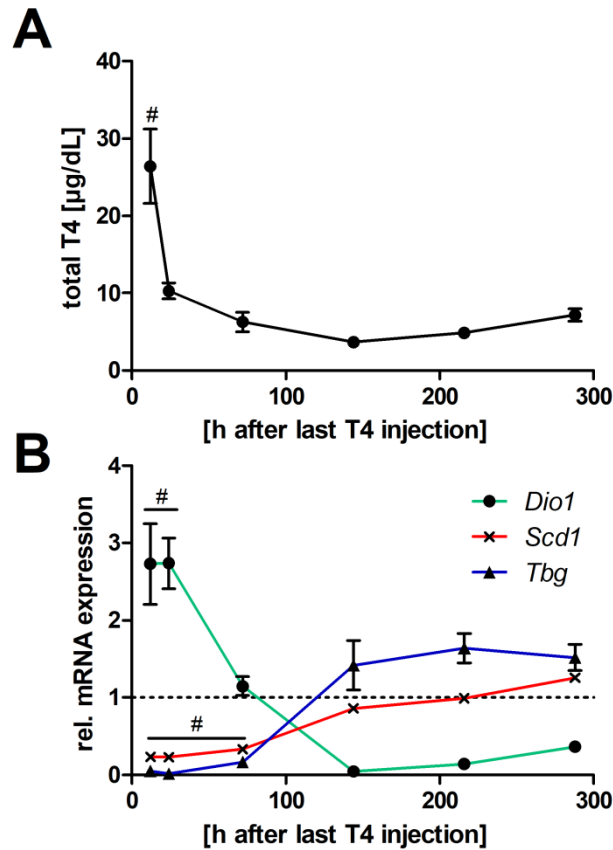
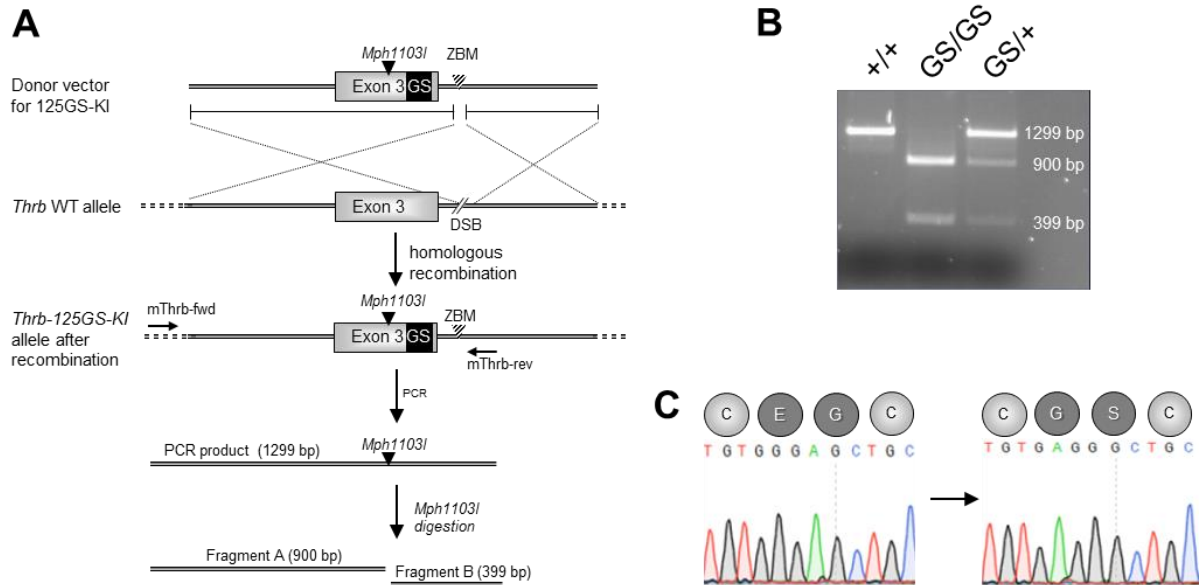


Fig. S13. Time course of T₄ serum concentration and hepatic TH target gene expression. (A) T₄ serum concentrations of hyperthyroid mice (i.p. injection of T₄ 1 $\mu\text{g/g}$ BW every second day for 6 weeks) was measured at indicated time points after the last injection. (B) Hepatic TH target gene expression: *Dio1* followed the T₄ kinetic, whereas negatively regulated TH target genes thyroxine binding globulin (*Tbg*) and Δ^9 -stearyl-CoA desaturase (*Scd1*) showed a negative correlation with T₄ serum concentrations. (n=5-6; mean $\pm$ SEM; ANOVA followed by Tukey's *post hoc* test; # $P < 0.001$ compared to untreated mice)

Table S2: Serum parameter.

	WT	TR β ⁻	TR β ^{GS}	TR β ^{147F}
total protein [g/dL]	6.0 $\pm$ 0.0	6,3 $\pm$ 0.3	5.8 $\pm$ 0,2	5,9 $\pm$ 0,3
albumin [g/dL]	3.4 $\pm$ 0.2	3.7 $\pm$ 0.2	3.6 $\pm$ 0,2	3,6 $\pm$ 0,2
cholesterol [mg/dL]	134.0 $\pm$ 10.8	163.3 $\pm$ 15.2	146.0 $\pm$ 3.3	155.2 $\pm$ 13.4

n=6; mean $\pm$ SEM; One-way ANOWA, Tukey's *post hoc* test.



Generation of TRP^{GS} knock-in mice. (A) A donor vector (DV) bearing the desired point mutations and zinc finger nuclease (ZFN) mRNA were microinjected into the pronucleus of an oocyte. A zinc finger binding mutation (ZMB) was introduced into the DV sequence to protect it from ZFN. The genomic WT allele was cut by ZFN and the double strand break (DSB) was repaired via homologous recombination using the DV sequence as a template. In addition to the point mutations generating the amino acid exchange from EG to GS, a silent mutation was introduced to generate an *Mph1103I* restriction site for genotyping by restriction fragment length polymorphism PCR (RFLP-PCR). For identification of positive founder animals, RFLP-PCR using a forward primer (ThrbGS-fwd), which binds to a sequence of the *Thrb* gene outside the left homologous sequence of the DV, and a reverse primer (ThrbGS-rev) located downstream of exon 3 was performed. (B) Restriction digestion of the PCR product determined homo- and heterozygosity for the mutated allele. (C) Integration of GS mutation was confirmed by sequencing. TRP^{GS} mice were generated in parallel using the same techniques.