Noncanonical 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 or type 1 pathway for TH action. Nevertheless, TRs also rapidly activate intracellular second-messenger signaling pathways independently of gene expression (noncanonical or type 3 TR signaling). To test the physiological relevance of noncanonical TR signaling, we generated knockin mice with a mutation in the TR DNA-binding domain that abrogates binding to DNA and leads to complete loss of canonical TH action. We show that several important physiological TH effects are preserved despite the disruption of DNA binding of TR α and TR β , most notably heart rate, body temperature, blood glucose, and triglyceride concentration, all of which were regulated by noncanonical 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, thus demonstrating tissue- and TR isoform-specific canonical signaling. These findings provide in vivo evidence that noncanonical TR signaling exerts physiologically important cardiometabolic effects that are distinct from canonical actions. These data challenge the current paradigm that in vivo physiological TH action is mediated exclusively via regulation of gene transcription at the nuclear level.

thyroid hormone receptor | noncanonical signaling | thyroid hormone action | skeleton | cardiometabolic effects

Thyroid hormone (TH) plays an essential role in organ development and metabolic homeostasis. 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 (Fig. 1A) (1): 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.

Beyond that, additional and distinct mechanisms of TR signaling have been demonstrated. T₃ and TR β can modulate in-

tracellular second messenger signaling, e.g., the PI3K pathway (TR-dependent signaling of TH without DNA binding, type 3) (1, 4–7). A specific molecular mechanism for PI3K activation by TR β has been identified and demonstrated to be essential for normal synapse development and plasticity in mice by mutating a specific tyrosine residue to phenylalanine in TR β (7). Noncanonical signaling by TR α remains ill-defined, and its physiological importance is unknown. Such non-type 1 TH/TR action is considered noncanonical, because it is independent of DNA binding and TRE binding, does not require gene transcription or protein synthesis as the initial event, and may, therefore, also be more rapid (Fig. 1B).

Significance

This study changes our understanding of how thyroid hormone acts. Thyroid hormone receptors are considered typical nuclear receptors that bind to DNA and, after binding, alter the expression of their target genes and regulate physiological responses. Nevertheless, we show that thyroid hormone still mediates important physiological effects in mice expressing mutant receptors that cannot bind DNA. These are predominantly linked to energy metabolism and include glucose and triglyceride concentrations, body temperature, locomotor activity, and heart rate. This study provides in vivo evidence that thyroid hormone receptors mediate physiologically relevant effects that are independent of DNA binding and direct activation of gene expression.

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Fig. 1. Canonical and noncanonical TR signaling. (A) Canonical TR signaling requires binding of TR to regulatory DNA sequences, the TREs, mostly as a heterodimer with RXR. Binding of T_3 leads to an exchange of cofactors that initiates or represses the transcription of the target genes. (B) Noncanonical 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 noncanonical signaling. In TR-KO mice, both effects are absent. In mice with TRE-binding-deficient TRs, canonical signaling is abolished, and only noncanonical signaling is preserved. Conversely, in mice with selective abrogation of PI3K signaling, this noncanonical TH effect is missing, while canonical signaling is preserved. Thus, a comparison of these mice can determine whether the signaling mechanism responsible for TH effects is canonical or noncanonical and PI3K mediated.

To determine the physiological consequences of noncanonical TH/TR action in vivo, we generated TR α^{GS} and TR β^{GS} mice that lack canonical TR signaling by replacing glutamic acid (E) and glycine (G) in the first zinc finger of the DNA-binding domain of TR α and TR β with glycine and serine (S). This GS mutation severely impairs TR binding to TREs and abrogates canonical TR signaling (8, 9). Since TRE binding is abolished, only the noncanonical TH actions are preserved in TR-GS-mutant mice. By contrast, the TR can mediate both canonical and noncanonical actions of TH in WT mice, whereas both actions are absent in TR-KO mice (Fig. 1C).

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

We also included TR β^{147F} mice, in which tyrosine 147 of TR β is changed to phenylalanine (Y147F), as an additional control for TR β signaling. Tyrosine 147 is crucial for TR β -mediated activation of PI3K, and replacement by phenylalanine abrogates PI3K activation. Nevertheless, the classical TRE-mediated actions of TR β are unaffected in TR β^{147F} mice (7). Thus, the TR β^{147F} mice are a specific negative control for noncanonical, PI3K-mediated TH/TR β effects, as TR β -mediated PI3K signaling is absent in these mice (Fig. 1C).

Here we provide in vivo evidence that noncanonical TR signaling is physiologically important and contributes significantly to TH action, specifically for the regulation of body temperature,

oxygen consumption (VO_2), locomotor activity, heart rate, and triglyceride and glucose concentrations, important 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 the regulatory regions of target genes and subsequent activation of gene expression. ChIP-sequencing (ChIP-seq) analyses in mouse liver reported a DR+4 motif, a direct repeat of two 5'-AGGTCA-3' half-sites in the same orientation separated by four nucleotides, as the most prevalent TRE in T_3 -induced genes (10–12). This consensus DR+4 TRE was used in a fluorescent EMSA to test DNA binding affinity of the TR α and TR β variants (TR β , TR β 125GS, TR β Y147F, TR α , and TR α 71GS). Only TR β , TR β Y147F, and TR α bound to the DR+4 probe; the TR β 125GS and TR α 71GS receptors did not (Fig. 2A). The GS mutation abrogated DNA binding of both TR α and TR β . Next, we tested the transcriptional activity of TR α 71GS, TR β 125GS, and TR β Y147F mutants in vitro using a TH-responsive luciferase reporter plasmid (DR+4-TKLuc) in comparison with WT TRs. Empty vector and TR mutants TR α G291R and TR β G345R served as controls. These mutant TRs cannot bind T_3 and cannot activate gene transcription after T_3 treatment (13). T_3 increased luciferase activity eightfold with WT TR α , TR β , and TR β Y147F but not with empty vector or TR β G345R and TR α G291R (Fig. 2B). Luciferase activity was not increased by T_3 with 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. S1 A and B).

To abolish the 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 the expression of known TH-responsive genes in these mice in comparison with WT and the respective TR-KO mice (TR α^0 and TR β^0 mice; genotypes are *Thra*^{0/0} and *Thrb*^{0/0}, 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 their expression in WT mice and was comparable to that in TR α^0 mice (Fig. 3A), demonstrating that the GS mutation in TR α abrogates canonical TH/TR α action in vivo. For TR β -mutant mice, the different systemic TH concentrations in WT, TR β^{GS} , and TR β^0 mice had to be equalized. Thus, we determined TH-induced gene expression in hypothyroid animals without and with T_3 treatment. In livers of WT mice, T_3 induced expression of *Dio1*, *Spot14*, *Me1*, and *Bcl3* mRNAs (Fig. 3B and Fig. S1 C and D). This induction was equally reduced in both TR β^0 mice and TR β^{GS} mice, demonstrating that the GS mutation in TR β has the same effect on canonical TH/TR-mediated gene expression as the complete absence of TR β in TR β^0 mice.

To evaluate recruitment of TR β mutants to DNA, we performed ChIP against TR β and its heterodimerization partner retinoid X receptor alpha (RXR α) in liver tissue from T_3 -treated animals and evaluated the occupancy of known TR-binding sites previously identified in mouse liver tissue, e.g., in the *Dio1*, *NcoR2*, *H13*, and *Strbp* genes (Fig. 3C and Fig. S1E). Both WT and the TR β^{147F} mutant occupy these TR-binding sites together with RXR α , whereas the TR β^{GS} mutant and TR β^0 show little or no enrichment, confirming that the GS mutation in the DNA-binding domain of TR β disrupts TR β 's interaction with chromatin. Moreover, evaluation of histone 3 lysine 27 acetylation (H3K27Ac) at these binding sites showed that impaired occupancy with TR β in TR β^0 and TR β^{GS} mice results in reduced histone acetylation (Fig. 3C and Fig. S1E). To evaluate the effect on histone acetylation genome-wide, we performed ChIP-seq against H3K27Ac in the liver of hypo- and T_3 -treated hyperthyroid WT and TR β mutant animals.

Fig. 2. The GS mutation abolishes TR binding to DNA and canonical TR signaling in vitro. (A) Fluorescent EMSA was performed with 4 μ L of reticulocyte-translated TR α WT, TR α 71GS, TR β WT, TR β 125GS, and TR β Y147F and 2 μ L of RXR α on a 5'-Cy5-labeled DR+4 TRE probe. Arrows indicate bands of TR α and TR β binding to probe; #, free probe. (B) HEK293 cells transfected with plasmids encoding for TR β , TR β 125GS, TR β Y147F, TR α , and TR α 71GS and a DR+4-luciferase reporter plasmid. Empty vector (EV) and TR mutants without T₃ binding (TR α G291R and TR β G345R) served as negative controls. Cells were treated with vehicle (open bars) or with 10 nM T₃ for 48 h to induce luciferase expression via canonical TR/TRE-mediated action for TR α and TR β variants (black bars); $n = 3$. Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; * $P < 0.05$; *** $P < 0.001$.

Sequenced tags were quantified at all previously identified DNase-accessible regions (12), and we identified more than 500 regions where H3K27Ac was differently regulated by T₃ in WT animals (Fig. S1F). We quantified H3K27Ac at these differentially regulated regions in liver samples from hypo- and T₃-treated hyperthyroid WT and TR β [−] animals and animals carrying the GS and 147F TR β mutants. Hierarchical clustering revealed that T₃-induced H3K27Ac in animals with the 147F mutation is very similar to that observed in WT animals. By contrast, the TR β GS mutation or TR β deletion resulted in attenuated H3K27Ac after T₃ treatment (Fig. 3D). Consistent with this, H3K27Ac is increased in response to T₃ at previously identified TR-binding sites in the *Dio1* gene loci in WT and TR β ^{147F} mice but not in TR β ^{GS} or TR β [−] animals (Fig. 3E).

We then used transcriptome analysis to determine whether TR β 125GS and TR β 147F mutants possess residual transcriptional activity affecting known target genes or had acquired new target-gene specificity. 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 determined after 6 h by microarray analysis. Fig. 3F shows the results in a dendrogram with mice grouped by similarity of their gene-expression patterns, not by genotype. The gene-expression patterns in WT and TR β ^{147F} mice were clearly different from those in 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. Thus, the GS mutation renders the TR transcriptionally nonfunctional in vivo.

Regulation of the Hypothalamic–Pituitary–Thyroid Axis Requires Canonical TR β Signaling. TH production and secretion is regulated by the hypothalamic–pituitary–thyroid (HPT) axis negative-feedback loop. Inhibition of thyroid-stimulating hormone (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 TR β ^{GS} mice than in WT mice (Fig. 4A). As a consequence of elevated TSH, serum thyroxine (T₄) and T₃ concentrations were also significantly higher in TR β [−] and TR β ^{GS} mice than in WT mice (Fig. 4B and Fig. S2A). The combination of elevated circulating TH concentrations and elevated TSH demonstrates central resistance to TH due to the lack of the receptor in TR β [−] mice and, importantly, a corresponding absence of canonical TR β action in the HPT axis of TR β ^{GS} mice. In TR β ^{147F} mice with intact DNA binding and canonical signaling,

serum T₃ and T₄ concentrations and pituitary TSH β expression were normal (7). TSH, T₄, and T₃ 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 (Fig. 4A and B and Fig. S2A).

Skeletal Development Requires Canonical TR α Signaling. We measured growth curves from WT, TR α ⁰, TR α ^{GS}, TR β [−], and TR β ^{GS} mice. Growth curves of TR β [−] and TR β ^{GS} mice were not different from those of WT mice (Fig. S2B and C). However, TR α ⁰ and TR α ^{GS} mice had a similar delay in linear growth and gain in body weight (BW) compared with WT mice (Fig. 4C–F). 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 TR α ^{GS} and TR α ⁰ mice, together with a reduction in bone mineral content in vertebrae but no difference in long bones (Fig. 5A and Fig. S3). Histological analysis of the growth plate revealed a delay in endochondral ossification similarly affecting both TR α ^{GS} and TR α ⁰ mice (Fig. 5B). 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-computed tomography (micro-CT) imaging demonstrated epiphyseal dysgenesis, increased trabecular bone mass, and reduced metaphyseal inwaisting consistent with a bone-modeling defect and delayed endochondral ossification (Fig. 5C and D). These are all classic 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 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.

Noncanonical TR β Action Decreases Blood Glucose Concentration. T₃ treatment has been shown to reduce serum glucose concentration rapidly in lean and obese mice (20). We hypothesized that such a rapid TH effect could be mediated noncanonically by TRs and thus be present in WT and in TR α ^{GS} and 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 1 h of fasting was not different among the TR β genotypes (Table S1). T₃ injection rapidly decreased blood glucose within 60 min in WT mice (Fig. 6A and Table S1). Strikingly, this hypoglycemic effect of T₃ was abolished in TR β [−] mice but was fully preserved in TR β ^{GS} mice. We conclude that the decrease in blood glucose after T₃ treatment is mediated by TR β , as it is absent in TR β [−] mice. The similar decrease in blood glucose

Fig. 4. Canonical TR action is required for TSH repression and normal growth. (A and B) TSH (A) and T₄ (B) in serum of 15-wk-old WT (black triangles; $n = 11$) TR β ⁻ (x; $n = 9$), TR β ^{GS} (open circles; $n = 10$), TR α ⁰ (gray diamonds; $n = 5$), and TR α ^{GS} (open squares; $n = 6$) male mice. Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; * $P < 0.05$; *** $P < 0.001$; ns, not significant. (C) Tail length of 21-d-old WT (black bar), TR α ⁰ (gray bar), and TR α ^{GS} (open bar) mice. (D) Linear growth was recorded until the age of 70 d. (E and F) Bodyweight on P21 (E) and recorded until the age of 70 d (F); $n = 6$; mean $\pm$ SD for tail length and BW at P21 and follow-up; ANOVA and Tukey's post hoc test; ns, not significant; * $P < 0.05$; *** $P < 0.001$.

seen in both WT and TR β ^{GS} mice demonstrates that this effect is mediated by noncanonical TR β signaling. Furthermore, this effect occurs within 60 min, which is likely too rapid to be dependent on RNA transcription and new protein translation. This conclusion is further supported by the failure of T₃ to induce hypoglycemia in TR β ^{147F} mice, which also demonstrates that PI3K activation by TR β is required to lower the glucose concentration. By contrast, TR α is not involved in hypoglycemic response to T₃, as it is unable to compensate for the lack of TR β in TR β ⁻ mice.

Noncanonical TR β Signaling Maintains Normal Serum and Liver Triglyceride Concentration. Serum triglyceride concentration is under TH control (21, 22). Previous studies have shown elevated serum triglyceride concentration in TR β knockin mice with resistance to TH (TR β ^{PV}), indicating that TR β mediates TH regulation of triglycerides (23). We therefore measured serum triglyceride concentrations in untreated WT, TR β ⁻, TR β ^{GS}, and TR β ^{147F} mice. Triglyceride concentrations were significantly higher in TR β ⁻ and TR β ^{147F} mice than in WT mice (300 ± 61 and 264 ± 26 vs. 152 ± 23 mg/dL; $P < 0.05$) but not in TR β ^{GS} mice (123 ± 34 mg/dL; n.s.) (Fig. 6B). Cholesterol, albumin, and total protein concentrations were not different among WT, TR β ⁻, TR β ^{GS}, and TR β ^{147F} mice (Table S2). These results demonstrate that in the absence of noncanonical TR β action the serum triglyceride concentration is ele-

vated in TR β ⁻ and TR β ^{147F} mice. A possible explanation for increased serum triglyceride concentration was increased triglyceride synthesis in the liver. Compared with WT mice, the expression of several key enzymes involved in triglyceride synthesis (Scd1, Me1, Fasn) was increased in livers of TR β ⁻ and TR β ^{147F} mice but was not increased or was increased to a lesser extent in TR β ^{GS} mice (Fig. 6C and E and Fig. S4). Accordingly, we found higher triglyceride concentrations in the livers of TR β ⁻ and TR β ^{147F} mice but not in TR β ^{GS} mice, replicating the pattern found in serum (Fig. 6D and F). The increased serum and hepatic triglyceride concentrations and hepatic triglyceride synthesis in TR β ⁻ and TR β ^{147F} mice and the similarity of the phenotype in TR β ^{GS} mice to that of WT mice demonstrates that the hypertriglyceridemia is due to the absence of noncanonical TR β signaling in TR β ⁻ and TR β ^{147F} mice.

Noncanonical TR β Signaling Contributes to Regulation of Body Temperature, Oxygen Consumption, and Locomotor Activity. Body temperature homeostasis is crucially linked to TH, and deletion of either TR α or TR β results in reduced body temperature, although not always significantly (24). By contrast, the mean body temperature of TR β ^{GS} mice was 0.9 °C higher than that of TR β ⁻ mice and 0.5 °C higher than in WT mice (Fig. 7A), suggesting a noncanonical TR β effect on body temperature. To support this conclusion further, we repeated body temperature measurements in TR β WT, TR β ⁻, and TR β ^{GS} mice on a TR α ⁰ genetic background. Body temperature in TR α ⁰ double-KO mice was markedly reduced compared with WT mice (to 34.9 °C vs. 37.0 °C) (Fig. 7A), in line with previous reports (24–26). However, the body temperature of TR β ^{GS} mice on the TR α ⁰ background was ~1 °C higher than in TR α ⁰ double-KO mice (Fig. 7A). These results demonstrate a TR β -specific effect on energy metabolism that is independent of TR α and is mediated via noncanonical actions. We then determined VO₂ by indirect calorimetry and compared the groups using linear regression models including body mass as a covariate. Average VO₂ was increased in TR β ^{GS} mice and reduced in TR β ⁻ mice compared with WT controls (Fig. 7C and D and Table S3). Minimum VO₂ as a proxy of resting metabolic rate was also increased in TR β ^{GS} mice. Food consumption did not differ between genotypes in this short period (Table S3). Furthermore, distance traveled was increased in TR β ^{GS} mice (Fig. 7B). Thus, the noncanonical TR β -specific effects of TH increase the metabolic rate and locomotor activity.

Noncanonical TR α Signaling Contributes to Extrinsic Regulation of Heart Rate. TR α is the predominant TR isoform in the heart, and regulation of heart rate is a well-known physiological effect of TH and TR α (26). We determined heart rate using a noninvasive ECG in untreated WT, TR α ⁰, and TR α ^{GS} mice. Heart rate was significantly reduced in TR α ⁰ mice compared with WT mice but not in TR α ^{GS} mice (Fig. 8A). The heart rate in TR α ^{GS} mice was normal despite similar reductions in TH target genes known to be involved in heart rate regulation in both TR α ⁰ and TR α ^{GS} mice (Fig. 8B). The similar phenotype in WT and TR α ^{GS} mice demonstrates that noncanonical TR α signaling contributes to normal heart rate in TR α ^{GS} mice. To distinguish between intrinsic and extrinsic cardiac effects, we determined basal heart rate in isolated perfused hearts using a Langendorff apparatus. Ex vivo, the cardiac rhythm was similar in TR α ^{GS} and TR α ⁰ mice (Fig. 8C), indicating that noncanonical TR α signaling controls heart rate by an extrinsic mechanism, likely via central regulation of the autonomous nervous system.

Discussion

The physiological role of TRs has been studied in vivo for more than 15 y by comparing WT with TR-KO mice. As both canonical and noncanonical, or type 1 and type 3 (1), TR signaling are present in WT mice, but both are absent in TR-KO mice, such a comparison could not distinguish between the two mechanisms.

Fig. 5. Canonical TR α action is necessary for normal skeletal development of mice at P21. (A, Upper Left) Gray-scale images of caudal vertebrae from P21 WT ($n = 8$), TR α^{GS} ($n = 5$), and TR α^0 ($n = 3$) mice were pseudocolored according to a 16-color palette in which low mineral content is blue and high mineral content is red. (Scale bar, 1,000 μm .) (Upper Right) The graph demonstrates caudal vertebra length in WT, TR α^{GS} , and TR α^0 mice. Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; $***P < 0.001$. (Lower) Relative (Left) and cumulative (Right) frequency histograms display bone mineral content of vertebrae from TR α^{GS} and TR α^0 mice vs. WT mice; Kolmogorov-Smirnov test, $***P < 0.001$. (B, Upper) Proximal tibia growth plate sections stained with Alcian blue (cartilage) and van Gieson (bone) (magnification: 50 $\times$ and 100 $\times$, respectively). HZ, hypertrophic zone; PZ, proliferative zone; RZ, reserve zone. (Scale bars, 500 μm .) (Lower) Growth plate chondrocyte zone measurements (Left) and relative proportions corrected for total growth plate height (Right) are shown for WT ($n = 8$), TR α^{GS} ($n = 6$), and TR α^0 samples; $n = 6$. Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (C) Micro-CT images of longitudinal femur midline sections demonstrate bone morphology. (Scale bar: 1,000 μm .) (D, Upper) Micro-CT images showing transverse sections of the distal metaphysis. (Scale bar: 1,000 μm .) (Lower) Graphs demonstrate trabecular number (Tb.N) (Upper) and trabecular spacing (Tb.Sp.) (Lower). Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; $**P < 0.01$; $***P < 0.001$.

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} mice did not exhibit a TR-KO phenotype. Rather, for several established TH effects their phenotype was indistinguishable from WT mice. Thus, these results provide *in vivo* evidence for the

physiological importance of noncanonical TR signaling. Furthermore, they demonstrate that noncanonical TR signaling can be mediated by both TR α and TR β in an isoform- and tissue-specific manner. These findings have profound implications for the role of TRs in metabolism and physiology and explain the pathophysiology in diseases caused by the various TR mutations.

Fig. 6. Noncanonical TR β signaling influences blood glucose and hepatic triglyceride synthesis. (A) Under fasting conditions, WT (black triangles), TR β^{GS} (open circles), TR $\beta^{-/-}$ (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; $n = 4$. Data are shown as mean $\pm$ SEM; Student's t test; * $P < 0.05$. (B) Serum triglyceride concentration in untreated WT (black bar), TR $\beta^{-/-}$ (dark gray bar), TR β^{GS} (open bar), and TR β^{147F} (light gray bar) mice. (C) Representative immunoblots against *Fasn*, *Me1*, and *Scd1* from liver samples. *Gapdh* was used as loading control; $n = 2$. (D) Triglyceride concentration in liver tissue was assessed after lipid extraction; $n = 4$ –6 mice per genotype. Horizontal bars in the box plots indicate mean values, and whiskers indicate minimum and maximum values; ANOVA and Bonferroni's post hoc test for multiple comparison; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (E) Immunoblots against *Scd1* (Left), *Me1* (Center), and *Fasn* (Right), with a group size of $n = 4$ were used for densitometric measurements. Data are shown as mean $\pm$ SEM; ANOVA with Tukey's post hoc test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (F) Oil red O staining of liver sections from untreated WT, TR $\beta^{-/-}$, TR β^{GS} , and TR β^{147F} mice was done to visualize triglycerides in liver tissue. (Scale bar: 50 μ m.)

Canonical Actions of TRs. An important physiological role of TH is negative feedback in the HPT axis. TR β^{GS} mice had a phenotype similar to that of TR $\beta^{-/-}$ mice with lack of TR β -mediated negative feedback in the pituitary and TH resistance (9), whereas the HPT axis in TR β^{147F} mice was unaffected (7). A higher TSH in TR β^{GS} mice could indicate that they are slightly more resistant than the TR $\beta^{-/-}$ mice. A potential but highly speculative explanation is cofactor sequestration. TR β^{GS} does not bind to DNA but is still capable of cofactor binding. This could, theoretically, reduce cofactor availability for TR α and reduce a minimal repressive TR α effect on TSH expression, resulting in even higher TSH concentrations than in TR $\beta^{-/-}$ mice. Delayed longitudinal growth and bone mineralization are typical features of TR α^0 mice and were replicated in TR α^{GS} mice. Therefore negative regulation of the HPT axis in TR β^{GS} mice and bone development in TR α^{GS} mice are solely dependent on canonical TR/TRE-mediated transcriptional regulation. Nevertheless, a recently identified short variant of TR α , p30 TR α 1, associates with the plasma membrane, increases nitric oxide and cGMP production, and activates the ERK and PI3K pathways in vitro (27). Furthermore, it has been suggested that p30 TR α may have a role in the regulation of adult bone formation. Here, we studied bone development in 3-wk-old

mice and determined linear growth until cessation of the experiment at 10 wk of age. While skeletal development and linear growth were unequivocally canonical, we cannot exclude a contribution of noncanonical actions of TR α in adult bone remodeling.

Noncanonical Actions of TR β . Serum glucose decreased rapidly only in WT and TR β^{GS} mice but not in TR $\beta^{-/-}$ and TR β^{147F} mice despite intact canonical TR β signaling in the TR β^{147F} mice. The phenotype in TR β^{GS} mice demonstrates that noncanonical TR β action alone suffices to generate physiological responses. Similarly, triglyceride synthesis and serum and liver triglyceride content were elevated in TR $\beta^{-/-}$ and TR β^{147F} mice that lack noncanonical TR β action, suggesting reduced consumption of triglyceride substrates. Body temperature was higher in TR β^{GS} than in WT mice, probably as a consequence of elevated serum TH concentration due to the disruption of the HPT axis in these mice and the retained noncanonical action of TR β^{GS} . Additionally, VO₂ and distance traveled were higher in TR β^{GS} mice. Increased energy metabolism, body temperature, and locomotor activity and lower triglyceride concentrations are physiological consequences of noncanonical TR β action.

Fig. 7. Noncanonical action of TR β increases body temperature, VO $_2$, and locomotor activity. (A) Body temperature of male mice in a TR α^+ (WT) or a TR α^0 genetic background; $n = 6$. Box plots indicate mean values, and whiskers indicate minimum and maximum values; ANOVA and Bonferroni's post hoc test for multiple comparison; n , not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (B) Locomotor activity of WT, TR β^- , and TR β^{G5} mice expressed as centimeters traveled in 15 min; $n = 10$ –20 mice per genotype. Horizontal bars in the box plots indicate mean values, and whiskers indicate minimum and maximum values; ANOVA with Tukey's post hoc test; $**P < 0.01$, n , not significant. (C and D) Average VO $_2$ of TR β^{G5} (C) and TR β^- mice (D) and WT littermate controls was measured by indirect calorimetry and is plotted against body weight ($n = 10$).

Noncanonical Actions of TR α . Noncanonical TH effects on the cardiovascular system have been reported, especially a rapid decrease in blood pressure and PI3K signaling pathway activation in the heart (28–30). In TR α^{GS} mice the heart rate in vivo was similar to that in WT mice, but, consistent with the expression of cardiac pacemaker genes, the ex vivo cardiac rate in TR α^{GS} hearts was similar to that of TR α^0 hearts. These results suggest that the noncanonical TH/TR α mechanism contributing to normal heart rate in vivo is not intrinsic to the heart. As ECG data were obtained from mice without anesthesia, it is possible that the response to stress or the activity of the sympathetic nervous system was reduced in TR α^0 mice but preserved in TR α^{GS} mice. Therefore we propose that noncanonical TH/TR α action influences heart rate via the autonomous nervous system and not via direct actions in the heart. Interestingly, this appears to be similar to the regulation of locomotor activity, which also is likely centrally mediated. Where and how TR α acts has yet to be determined.

Evolution of Noncanonical TH Signaling. Our findings suggest that increased noncanonical TR β signaling increases energy metabolism and body temperature. 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 , which are required for noncanonical PI3K activation by TR β , are found in all mammalian TR β ortholog sequences but not in sequences from poikilothermic animals, such as alligator, clawed toad, or zebrafish (7). Hence, noncanonical TR signaling may be a recently acquired adaptation in evolution that provides mammals with a novel mechanism by which TH can regulate energy metabolism.

body temperature, and activity. Development of noncanonical TR signaling relatively late in evolution, long after TRs assumed their canonical role as ligand-dependent transcription factors, may also explain why the physiological effects of canonical and noncanonical TR signaling are so clearly distinct from one another.

Negative Regulation of Gene Expression. A persisting point of controversy is whether DNA binding of TRs is required for negative regulation of gene expression by TRs. TSH elevation in TR β^{-} and TR β^{GS} mice clearly demonstrates that, in agreement with previous reports (9), inhibition of TSH expression in vivo requires DNA binding. Results from TR β^{GS} mice indicate that noncanonical TH action may contribute to negative regulation. *Scd1* expression in HepG2 cells and in mouse liver is negatively regulated by TH (Fig. S5) (31). Compared with 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 microarray studies, however, we did not detect down-regulation of *Scd1* within 6 h. Two explanations seem plausible: Either *Scd1* is directly negatively regulated by TH/TR β without DNA binding by an unknown mechanism that takes longer than 6 h or down-regulation of *Scd1* in liver is only a secondary effect in response to noncanonical TR β effects on metabolism, which also take more than 6 h. The fact that, in addition to *Scd1*, several other genes involved in triglyceride synthesis also were negatively regulated by T $_3$ suggests the latter. Repression of these genes appears to be a coordinated response to reduced substrate availability for triglyceride synthesis, possibly due to increased metabolism. Repression of *Myh7* appeared to be partially preserved in hearts of

Fig. 8. Noncanonical TR α signaling maintains normal basal heart rate without altering cardiac pacemaker channel gene expression. (A) Heart rate of nonsedated male WT (black triangles; $n = 7$), TR α^0 (gray diamonds; $n = 6$), and TR α^{GS} (open squares; $n = 6$) mice. Data are shown as 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 (*Kcnd2*, *Kcne1*, *Kcnb1*, and *Kcnq1*) in hearts of WT (black bars), TR α^0 (gray bars), and TR α^{GS} (open bars) mice; $n = 6$. Data are shown as mean $\pm$ SEM; ANOVA and Tukey's post hoc test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. (C) Ex vivo heart rate measured in hearts isolated from untreated WT (black triangles; $n = 15$), TR α^0 (gray diamonds; $n = 8$), or TR α^{GS} (open squares; $n = 7$) mice. Data are shown as mean $\pm$ SD; ANOVA followed by Tukey's post hoc test; * $P < 0.05$; *** $P < 0.0001$; ns, not significant.

TR α^{GS} mice. Expression of *Myh7* is negatively regulated by miRNA miR-208a, which is encoded by an intronic sequence of *Myh6*. Repression of *Myh7* by increased TH concentration is an indirect effect of *Myh6* induction and, consequently, miR-208a expression. Our interpretation of the difference in *Myh7* expression in TR α^0 and TR α^{GS} mice is that the relationship between *Myh6* and *Myh7* may not be linear. However, a partial noncanonical repressive effect of TR α^{GS} cannot be ruled out.

Role of Noncanonical PI3K Activation. The best-studied noncanonical action of TR β is rapid activation of PI3K (5–7). We therefore studied the TR β^{147F} mouse as a control, because in this mouse model the activation of PI3K by TR β is selectively abrogated. In the blood glucose response to T₃ and serum and liver triglyceride concentrations, the phenotype of TR β^{147F} mice was similar to that of TR β^- mice, demonstrating that these effects are indeed mediated by TR β activation of PI3K. As insulin also signals via PI3K to decrease blood glucose by increasing the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, we hypothesize that insulin and noncanonical TR β signaling converge at the PI3K pathway. This mechanism of TR β action would explain previous observations in which T₃ treatment increased glucose uptake into the thymus in rats independent of protein synthesis (32), increased glucose uptake into L6 muscle cells in a PI3K-dependent manner in vitro (33), and increased the translocation of GLUT4 in skeletal

muscle of rats (34). TR α also activates signaling pathways (29, 35), possibly as a short p30 TR α variant that indirectly activates ERK and PI3K after T₃ binding and nitric oxide and cGMP production (27) or by direct protein–protein interaction between TR α and PI3K (29, 35, 36). The noncanonical mechanism by which TR α influences heart rate remains unknown.

In conclusion, noncanonical, DNA-independent TR signaling contributes significantly to the physiological actions of TH, and these noncanonical actions appear to predominantly regulate energy homeostasis. These findings demonstrate that TR α and TR β mediate both canonical and noncanonical signaling in vivo, establishing a paradigm shift for TH action.

Materials and Methods

Extended and detailed material and methods are provided in [Supporting Information](#). All animal studies were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). All tests performed at the German Mouse Center (GMC) were approved by the responsible authority of the Regierung von Oberbayern. TR α and TR β WT and KO and TR β^{147F} mice were genotyped as previously described (7, 24, 37). TR β^{147F} mice were provided by D.L.A. (7). TR-GS mice were generated via the zinc finger nuclease approach (CompoZr Targeted Integration-Kit-AAVS; Sigma-Aldrich) (38, 39). Mice were rendered hypothyroid by maintenance on a low-iodine diet and administration of methimazole and perchlorate via the drinking water. Animals were treated with experiment-specific concentrations of T₃ by i.p. injections. Serum TSH was measured as previously described (40), and TH serum concentrations were determined by ELISA (DRG Diagnostics). Bone morphology

and histology were investigated with standardized methods described in detail in [Supporting Information](#) and refs. 18 and 19. Body temperature was determined with a rectal probe, and blood glucose was measured with a Contour XT glucometer (Bayer). Indirect calorimetry was performed at the GMC (Munich) according to standardized in-house protocols. Heart rate was determined either with an ECG in fully conscious mice or ex vivo via Langendorff apparatus. Experimental procedures for transfection, luciferase reporter assay, fluorescent EMSA, qRT-PCR (41), Oil red O staining, and immunoblotting have been published previously and are described together with fluorescent DNA probes, primers, and antibodies in [Supporting Information](#). Microarray was done with a MG-430_2.0 gene chip (Affymetrix). ChIP and ChIP-seq were performed as previously described (12, 42) with minor modifications explained in [Supporting Information](#). Microarray and ChIP-seq data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (43) and are accessible through the following GEO Series accession numbers: GSE93864 (microarray) and GSE104877 (ChIP-seq). Statistical analysis was performed with GraphPad Prism6, and differences were considered significant when $P < 0.05$.

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