Noncanonical thyroid hormone signaling mediates cardiometabolic effects in vivo

G. Sebastian Hönes[a], Helena Rakov[a], John Logan[b], Xiao-Hui Liao[c], Eugenie Werbenko[b], Andrea S. Pollard[b], Stine M. Præstholm[d], Majken S. Siersbæk[d], Eddy Rijntjes[e], Janina Gassen[a], Sören Latteyer[a], Kathrin Engels[a], Karl-Heinz Strucksberg[a], Petra Kleinbongard[f], Denise Zwanziger[a], Jan Rozman[g,h], Valerie Gailus-Durner[g], Helmut Fuchs[g], Martin Hrabe de Angelis[g,h,i], Ludger Klein-Hitpass[j], Josef Köhrle[e], David L. Armstrong[k], Lars Grøntved[d], J. H. Duncan Bassett[b], Graham R. Williams[b], Samuel Refetoff[c,l,m], Dagmar Führer[a], and Lars C. Moeller[a,1]

[a]Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University of Duisburg-Essen, 45147 Essen, Germany; [b]Molecular Endocrinology Laboratory, Department of Medicine, Imperial College London, London W12 0NN, United Kingdom; [c]Department of Medicine, The University of Chicago, Chicago, IL 60637; [d]Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense, Denmark; [e]Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institut für Experimentelle Endokrinologie, 10117 Berlin, Germany; [f]Institute for Pathophysiology, West-German Heart and Vascular Center Essen, University Hospital Essen, University of Duisburg-Essen, 45147 Essen, Germany; [g]German Mouse Clinic, Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany; [h]German Center for Diabetes Research, 85764 Neuherberg, Germany; [i]Chair of Experimental Genetics, School of Life Science Weihenstephan, Technische Universität München, 85354 Freising, Germany; [j]Institute of Cell Biology (Cancer Research), Faculty of Medicine, University of Duisburg-Essen, 45147 Essen, Germany; [k]Laboratory of Neurobiology, National Institute of Environmental Health and Sciences, National Institutes of Health, Research Triangle Park, NC 27709; [l]Department of Pediatrics, The University of Chicago, Chicago, IL 60637; and [m]Committee on Genetics, The University of Chicago, Chicago, IL 60637

Edited by David W. Russell, University of Texas Southwestern Medical Center, Dallas, TX, and approved November 15, 2017 (received for review April 25, 2017)

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 TRα and TRβ, 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 TRβ leads to disruption of the hypothalamic–pituitary–thyroid axis with resistance to TH, while mutation of TRα 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 (T_3), 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. 1*A*) (1): TRs bind to thyroid hormone response elements (TREs) in regulatory regions of target genes. In the absence of T_3, TRs recruit corepressors with histone deacetylase activity, preventing transcription. T_3 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. T_3 and TRβ can modulate intracellular 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 TRβ 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 TRβ (7). Noncanonical signaling by TRα 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. 1*B*).

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.

Author contributions: G.S.H. and L.C.M. designed research; G.S.H., H.R., J.L., X.-H.L., E.W., A.S.P., S.M.P., M.S.S., E.R., J.G., S.L., K.E., K.-H.S., D.Z., J.R., V.G.-D., H.F., M.H.d.A., L.K.-H., L.G., and L.C.M. performed research; S.M.P., M.S.S., P.K., J.R., V.G.-D., H.F., M.H.d.A., L.K.-H., J.K., D.L.A., L.G., J.H.D.B., G.R.W., and S.R. contributed new reagents/analytic tools; G.S.H., S.M.P., M.S.S., J.R., V.G.-D., H.F., M.H.d.A., L.K.-H., J.K., L.G., J.H.D.B., G.R.W., S.R., D.F., and L.C.M. analyzed data; and G.S.H., J.H.D.B., G.R.W., and L.C.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession nos. GSE93864 for microarray data and GSE104877 for H3K27 ChIP-seq Illumina sequencing).

[1]To whom correspondence should be addressed. Email: lars.moeller@uni-due.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1706801115/-/DCSupplemental.

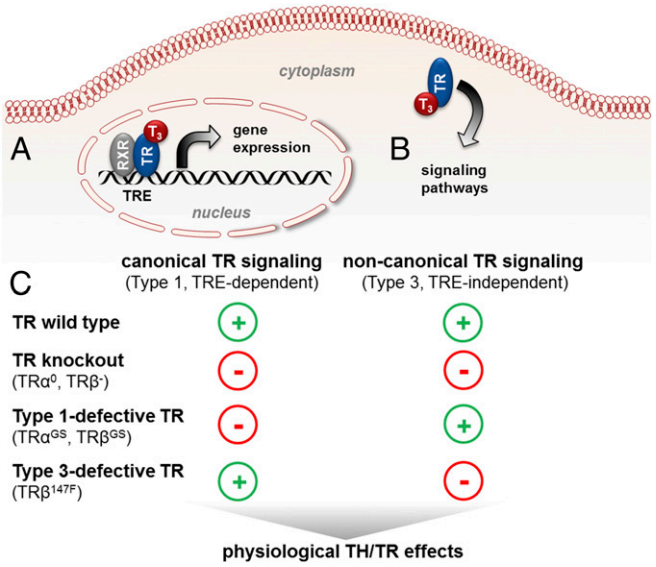

Fig. 1. Canonical and noncanonical TR signaling. (*A*) Canonical TR signaling requires binding of TR to regulatory DNA sequences, the TREs, mostly as a heterodimer with RXR. Binding of T_3 leads to an exchange of cofactors that initiates or represses the transcription of the target genes. (*B*) Noncanonical 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 $TR\alpha^{GS}$ and $TR\beta^{GS}$ 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 TRα and TRβ 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. 1*C*).

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

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 TRα and TRβ variants (TRβ, TRβ125GS, TRβY147F, TRα, and TRα71GS). Only TRβ, TRβY147F, and TRα bound to the DR+4 probe; the TRβ125GS and TRα71GS receptors did not (Fig. 2*A*). The GS mutation abrogated DNA binding of both TRα and TRβ. Next, we tested the transcriptional activity of TRα71GS, TRβ125GS, and TRβY147F mutants in vitro using a TH-responsive luciferase reporter plasmid (DR+4-TKLuc) in comparison with WT TRs. Empty vector and TR mutants TRαG291R and TRβG345R 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 TRα, TRβ, and TRβY147F but not with empty vector or TRβG345R and TRαG291R (Fig. 2*B*). Luciferase activity was not increased by T_3 with the TRα71GS and TRβ125GS mutants, demonstrating in vitro that the GS mutation in the DNA-binding domain abolishes the canonical TRE-mediated transcriptional activity of TRα and TRβ. 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 $TR\alpha^{GS}$ ($Thra^{GS/GS}$) and $TR\beta^{GS}$ ($Thrb^{GS/GS}$) mice. We determined the expression of known TH-responsive genes in these mice in comparison with WT and the respective TR-KO mice ($TR\alpha^0$ and $TR\beta^-$ mice; genotypes are $Thra^{0/0}$ and $Thrb^{-/-}$, respectively). As TRα is predominantly expressed in heart and TRβ in liver, we studied these two tissues. In heart, expression of *Myh6* and *Myh7* mRNAs in $TR\alpha^{GS}$ mice differed significantly from their expression in WT mice and was comparable to that in $TR\alpha^0$ mice (Fig. 3*A*), demonstrating that the GS mutation in TRα abrogates canonical TH/TRα action in vivo. For TRβ-mutant mice, the different systemic TH concentrations in WT, $TR\beta^{GS}$, and $TR\beta^-$ 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. 3*B* and Fig. S1 *C* and *D*). This induction was equally reduced in both $TR\beta^-$ mice and $TR\beta^{GS}$ mice, demonstrating that the GS mutation in TRβ has the same effect on canonical TH/TR-mediated gene expression as the complete absence of TRβ in $TR\beta^-$ mice.

To evaluate recruitment of TRβ mutants to DNA, we performed ChIP against TRβ and its heterodimerization partner retinoid X receptor alpha (RXRα) 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. 3*C* and Fig. S1*E*). Both WT and the $TR\beta^{147F}$ mutant occupy these TR-binding sites together with RXRα, whereas the $TR\beta^{GS}$ mutant and $TR\beta^-$ show little or no enrichment, confirming that the GS mutation in the DNA-binding domain of TRβ disrupts TRβ's interaction with chromatin. Moreover, evaluation of histone 3 lysine 27 acetylation (H3K27Ac) at these binding sites showed that impaired occupancy with TRβ in $TR\beta^-$ and $TR\beta^{GS}$ mice results in reduced histone acetylation (Fig. 3*C* and Fig. S1*E*). 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 TRβ mutant animals.

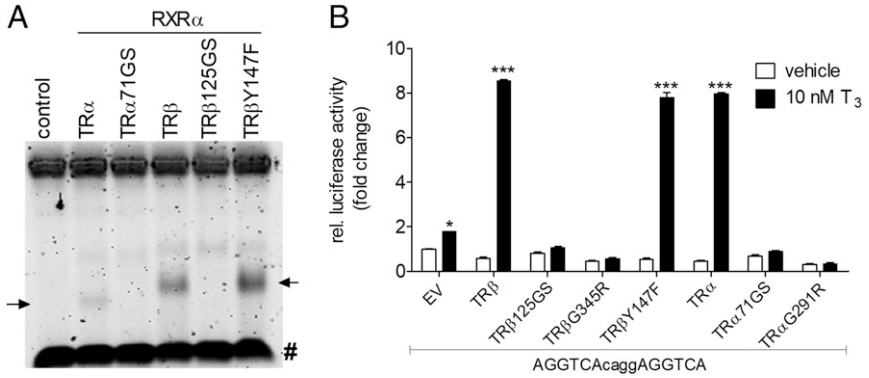

Fig. 2. The GS mutation abolishes TR binding to DNA and canonical TR signaling in vitro. (*A*) Fluorescent EMSA was performed with 4 μL of reticulocyte-translated TRαWT, TRα71GS, TRβWT, TRβ125GS, and TRβY147F and 2 μL of RXRα on a 5′-Cy5–labeled DR+4 TRE probe. Arrows indicate bands of TRα and TRβ binding to probe; #, free probe. (*B*) HEK293 cells transfected with plasmids encoding for TRβ, TRβ125GS, TRβY147F, TRα, and TRα71GS and a DR+4-luciferase reporter plasmid. Empty vector (EV) and TR mutants without T_3 binding (TRαG291R and TRβG345R) served as negative controls. Cells were treated with vehicle (open bars) or with 10 nM T_3 for 48 h to induce luciferase expression via canonical TR/TRE-mediated action for TRα and TRβ variants (black bars); $n = 3$. Data are shown as mean ± 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 T_3 in WT animals (Fig. S1*F*). We quantified H3K27Ac at these differentially regulated regions in liver samples from hypo- and T_3-treated hyperthyroid WT and TRβ$^-$ animals and animals carrying the GS and 147F TRβ mutants. Hierarchical clustering revealed that T_3-induced H3K27Ac in animals with the 147F mutation is very similar to that observed in WT animals. By contrast, the TRβGS mutation or TRβ deletion resulted in attenuated H3K27Ac after T_3 treatment (Fig. 3*D*). Consistent with this, H3K27Ac is increased in response to T_3 at previously identified TR-binding sites in the *Dio1* gene loci in WT and TRβ^{147F} mice but not in TRβ^{GS} or TRβ$^-$ animals (Fig. 3*E*).

We then used transcriptome analysis to determine whether TRβ125GS and TRβ147F mutants possess residual transcriptional activity affecting known target genes or had acquired new target-gene specificity. Hypothyroid WT, TRβ$^-$, TRβ^{GS}, and TRβ^{147F} mice were treated with a single dose of T_3, and hepatic gene expression was determined after 6 h by microarray analysis. Fig. 3*F* 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 TRβ^{147F} mice were clearly different from those in TRβ$^-$ and TRβ^{GS} mice and formed their own branch in the hypothyroid and T_3-treatment groups. Importantly, TRβ$^-$ and TRβ^{GS} mice were grouped together because of the similarity of their hepatic gene-expression patterns. In fact, T_3-treated TRβ$^-$ and TRβ^{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 TRβ 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 TRβ (14–16) and depends on DNA binding of TRβ (9). In accordance with these reports, basal serum TSH was significantly higher in TRβ$^-$ and TRβ^{GS} mice than in WT mice (Fig. 4*A*). As a consequence of elevated TSH, serum thyroxine (T_4) and T_3 concentrations were also significantly higher in TRβ$^-$ and TRβ^{GS} mice than in WT mice (Fig. 4*B* and Fig. S2*A*). The combination of elevated circulating TH concentrations and elevated TSH demonstrates central resistance to TH due to the lack of the receptor in TRβ$^-$ mice and, importantly, a corresponding absence of canonical TRβ action in the HPT axis of TRβ^{GS} mice. In TRβ^{147F} mice with intact DNA binding and canonical signaling, serum T_3 and T_4 concentrations and pituitary TSHβ expression were normal (7). TSH, T_4, and T_3 concentrations in TRα^0 mice (devoid of TRα1 and TRα2) and TRα^{GS} mice were not different from those in WT mice (Fig. 4 *A* and *B* and Fig. S2*A*).

Skeletal Development Requires Canonical TRα Signaling. We measured growth curves from WT, TRα^0, TRα^{GS}, TRβ$^-$, and TRβ^{GS} mice. Growth curves of TRβ$^-$ and TRβ^{GS} mice were not different from those of WT mice (Fig. S2 *B* and *C*). However, TRα^0 and TRα^{GS} mice had a similar delay in linear growth and gain in body weight (BW) compared with WT mice (Fig. 4 *C*–*F*). To investigate the underlying cause, skeletal analysis of TRα^0 and TRα^{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 TRα^{GS} and TRα^0 mice, together with a reduction in bone mineral content in vertebrae but no difference in long bones (Fig. 5*A* and Fig. S3). Histological analysis of the growth plate revealed a delay in endochondral ossification similarly affecting both TRα^{GS} and TRα^0 mice (Fig. 5*B*). 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 T_3 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. 5 *C* and *D*). These are all classic features of impaired T_3 action in bone (18, 19). In summary, these data demonstrate an equivalent delay in skeletal development due to similar loss of TRE-mediated canonical TRα signaling in TRα^0 and TRα^{GS} mice. These findings establish that T_3 actions in the skeleton are mediated by canonical actions of TRα on TREs.

Noncanonical TRβ Action Decreases Blood Glucose Concentration. T_3 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 TRα^{GS} and TRβ^{GS} mice. We tested this hypothesis first in WT, TRβ$^-$, TRβ^{GS}, and TRβ^{147F} mice. Basal serum glucose after 1 h of fasting was not different among the TRβ genotypes (Table S1). T_3 injection rapidly decreased blood glucose within 60 min in WT mice (Fig. 6*A* and Table S1). Strikingly, this hypoglycemic effect of T_3 was abolished in TRβ$^-$ mice but was fully preserved in TRβ^{GS} mice. We conclude that the decrease in blood glucose after T_3 treatment is mediated by TRβ, as it is absent in TRβ$^-$ mice. The similar decrease in blood glucose

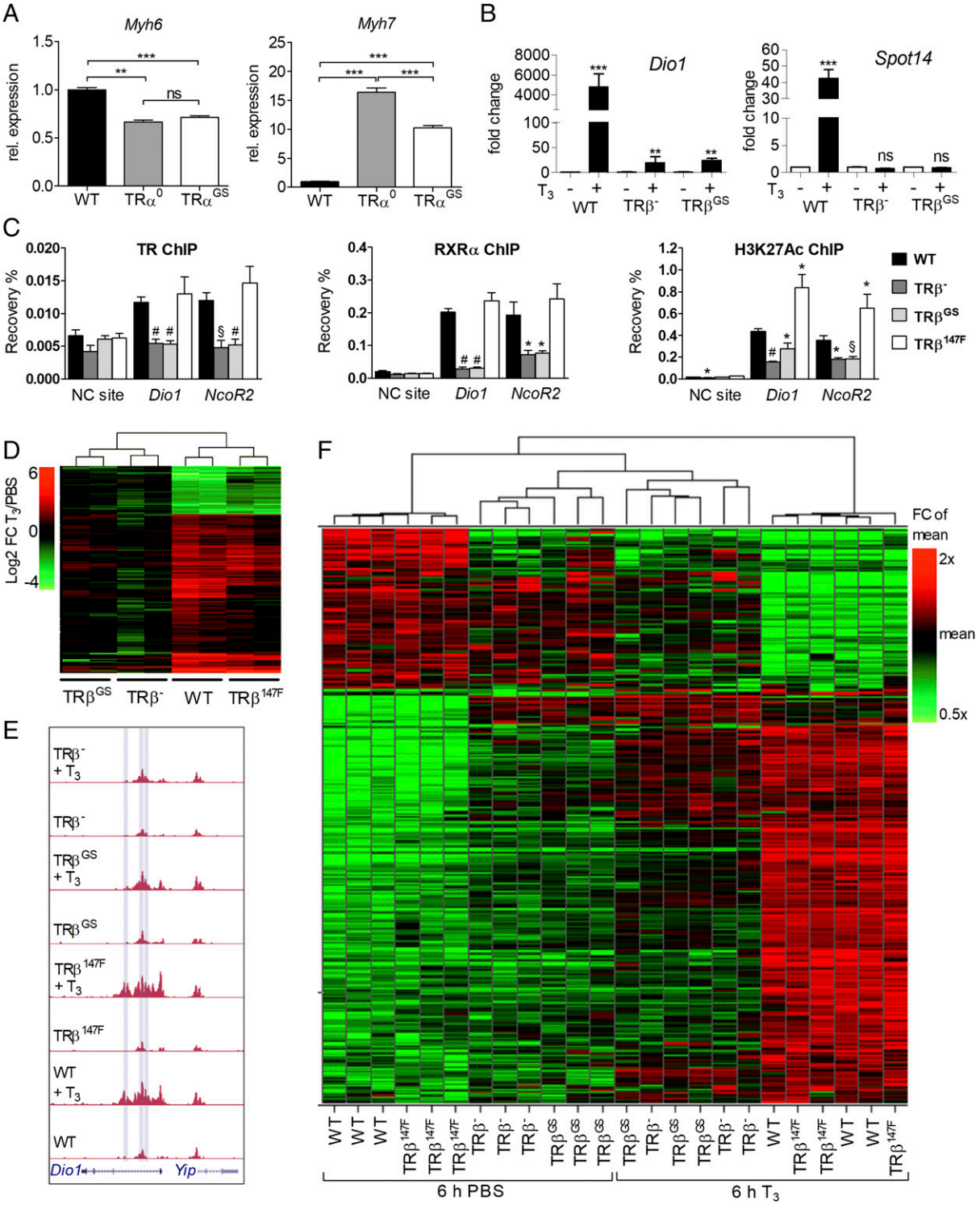

Fig. 3. The GS mutation abolishes TR binding to DNA and canonical TR signaling in vivo. (*A*) Relative expression of *Myh6* and *Myh7* in hearts of male WT (black bars), $TR\alpha^0$ (gray bars), and $TR\alpha^{GS}$ (open bars) mice; $n = 6$. Data are shown as mean ± SEM; ANOVA and Tukey's post hoc test; ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$. (*B*) Response of TH target genes (*Dio1* and *Spot14*) to T_3 in livers of hypothyroid WT, $TR\beta^-$, and $TR\beta^{GS}$ mice. Hypothyroid mice were injected either with vehicle (open bars) or with 50 ng/g BW T_3 (black bars) for four consecutive days; $n = 6$ mice per genotype. Data are shown as mean ± SEM; ANOVA and Tukey's post hoc test; ns, not significant, $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) ChIP of TRβ, RXRα, and H3K27Ac was followed by qPCR to determine H3K27 acetylation and recruitment of RXRα and TR to TR-binding sites located +7.0 kb and +22.4 kb from the transcriptional start site of *Dio1* and *NcoR2*, respectively, in T_3-treated $TR\beta^{147F}$, $TR\beta^-$, and $TR\beta^{GS}$ mice compared with WT mice; $n = 4$–6. Data are shown as mean ± SEM; Student's *t* test; $^*P < 0.05$; $^{\#}P < 0.005$; $^{\S}P < 0.001$; NC, negative control. (*D*) H3K27Ac ChIP-seq experiments were performed using livers from hypothyroid WT mice treated with PBS or T_3 for 6 h. A heatmap illustrates log2 fold change of H3K27Ac comparing T_3 treatment with PBS for all genotypes at 537 DNase-accessible regions with differential H3K27Ac calculated by DEseq2 (Bioconductor). (*E*) University of California, Santa Cruz Genome browser tracks of H3K27Ac enrichment profiles at the *Dio1* gene locus for all four genotypes injected with PBS or T_3. Vertical blue bars mark TR-binding sites. (*F*) Hierarchical clustering of gene-expression data (microarray) from livers of hypothyroid WT, $TR\beta^-$, $TR\beta^{GS}$, and $TR\beta^{147F}$ mice treated with either 200 ng/g BW T_3 or PBS for 6 h ($n = 3$).

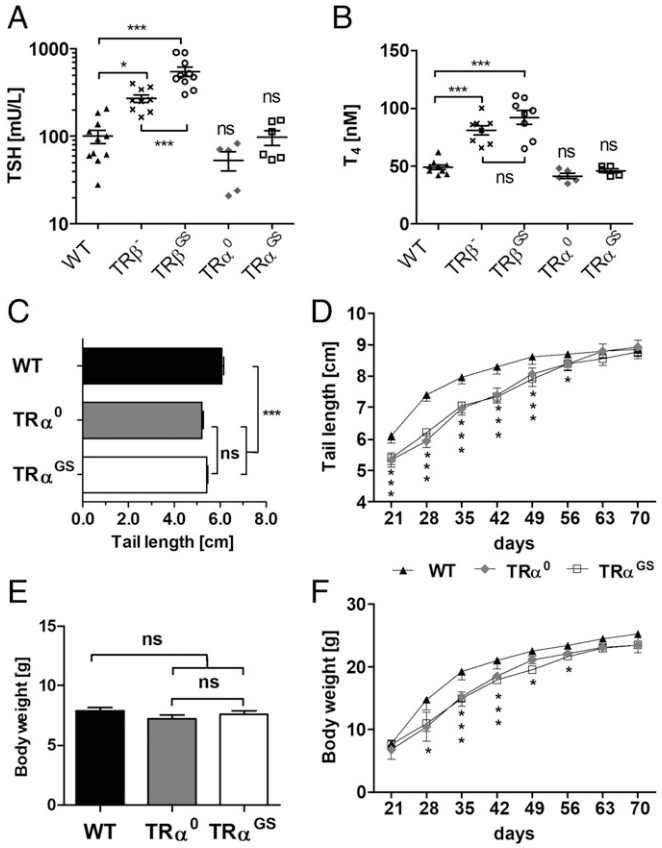

Fig. 4. Canonical TR action is required for TSH repression and normal growth. (*A* and *B*) TSH (*A*) and T_4 (*B*) in serum of 15-wk-old WT (black triangles; $n = 11$) TRβ$^-$ (x; $n = 9$), TRβ^{GS} (open circles; $n = 10$), TRα^0 (gray diamonds; $n = 5$), and TRα^{GS} (open squares; $n = 6$) male mice. Data are shown as mean ± 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), TRα^0 (gray bar), and TRα^{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 ± 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 TRβ^{GS} mice demonstrates that this effect is mediated by noncanonical TRβ 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 T_3 to induce hypoglycemia in TRβ^{147F} mice, which also demonstrates that PI3K activation by TRβ is required to lower the glucose concentration. By contrast, TRα is not involved in hypoglycemic response to T_3, as it is unable to compensate for the lack of TRβ in TRβ$^-$ mice.

Noncanonical TRβ 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 TRβ knockin mice with resistance to TH (TRβ^{PV}), indicating that TRβ mediates TH regulation of triglycerides (23). We therefore measured serum triglyceride concentrations in untreated WT, TRβ$^-$, TRβ^{GS}, and TR^{β147F} mice. Triglyceride concentrations were significantly higher in TRβ$^-$ and TRβ^{147F} mice than in WT mice (300 ± 61 and 264 ± 26 vs. 152 ± 23 mg/dL; $P < 0.05$) but not in TRβ^{GS} mice (123 ± 34 mg/dL; n.s.) (Fig. 6*B*). Cholesterol, albumin, and total protein concentrations were not different among WT, TRβ$^-$, TRβ^{GS}, and TRβ^{147F} mice (Table S2). These results demonstrate that in the absence of noncanonical TRβ action the serum triglyceride concentration is elevated in TRβ$^-$ and TRβ^{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 TRβ$^-$ and TRβ^{147F} mice but was not increased or was increased to a lesser extent in TRβ^{GS} mice (Fig. 6 *C* and *E* and Fig. S4). Accordingly, we found higher triglyceride concentrations in the livers of TRβ$^-$ and TRβ^{147F} mice but not in TRβ^{GS} mice, replicating the pattern found in serum (Fig. 6 *D* and *F*). The increased serum and hepatic triglyceride concentrations and hepatic triglyceride synthesis in TRβ$^-$ and TRβ^{147F} mice and the similarity of the phenotype in TRβ^{GS} mice to that of WT mice demonstrates that the hypertriglyceridemia is due to the absence of noncanonical TRβ signaling in TRβ$^-$ and TRβ^{147F} mice.

Noncanonical TRβ Signaling Contributes to Regulation of Body Temperature, Oxygen Consumption, and Locomotor Activity. Body temperature homeostasis is crucially linked to TH, and deletion of either TRα or TRβ results in reduced body temperature, although not always significantly (24). By contrast, the mean body temperature of TRβ^{GS} mice was 0.9 °C higher than that of TRβ$^-$ mice and 0.5 °C higher than in WT mice (Fig. 7*A*), suggesting a noncanonical TRβ effect on body temperature. To support this conclusion further, we repeated body temperature measurements in TRβ WT, TRβ$^-$, and TRβ^{GS} mice on a TRα^0 genetic background. Body temperature in TRα^{0}β$^-$ double-KO mice was markedly reduced compared with WT mice (to 34.9 °C vs. 37.0 °C) (Fig. 7*A*), in line with previous reports (24–26). However, the body temperature of TRβ^{GS} mice on the TRα^0 background was ∼1 °C higher than in TRα^{0}β$^-$ double-KO mice (Fig. 7*A*). These results demonstrate a TRβ-specific effect on energy metabolism that is independent of TRα and is mediated via noncanonical actions. We then determined VO_2 by indirect calorimetry and compared the groups using linear regression models including body mass as a covariate. Average VO_2 was increased in TRβ^{GS} mice and reduced in TRβ$^-$ mice compared with WT controls (Fig. 7 *C* and *D* and Table S3). Minimum VO_2 as a proxy of resting metabolic rate was also increased in TRβ^{GS} mice. Food consumption did not differ between genotypes in this short period (Table S3). Furthermore, distance traveled was increased in TRβ^{GS} mice (Fig. 7*B*). Thus, the noncanonical TRβ-specific effects of TH increase the metabolic rate and locomotor activity.

Noncanonical TRα Signaling Contributes to Extrinsic Regulation of Heart Rate. TRα is the predominant TR isoform in the heart, and regulation of heart rate is a well-known physiological effect of TH and TRα (26). We determined heart rate using a noninvasive ECG in untreated WT, TRα^0, and TRα^{GS} mice. Heart rate was significantly reduced in TRα^0 mice compared with WT mice but not in TRα^{GS} mice (Fig. 8*A*). The heart rate in TRα^{GS} mice was normal despite similar reductions in TH target genes known to be involved in heart rate regulation in both TRα^0 and TRα^{GS} mice (Fig. 8*B*). The similar phenotype in WT and TRα^{GS} mice demonstrates that noncanonical TRα signaling contributes to normal heart rate in TRα^{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 TRα^{GS} and TRα^0 mice (Fig. 8*C*), indicating that noncanonical TRα 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.

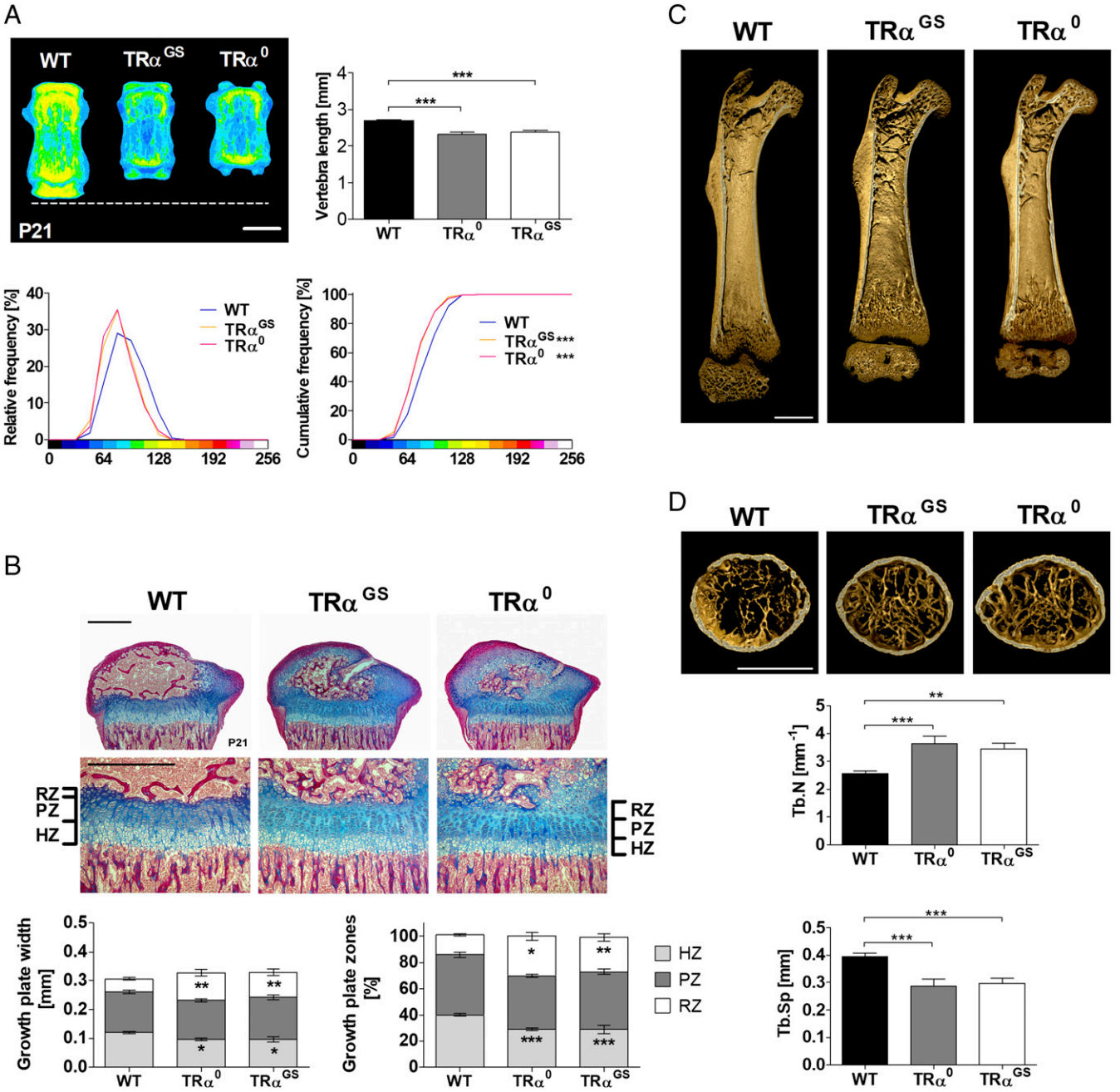

Fig. 5. Canonical TRα action is necessary for normal skeletal development of mice at P21. (*A*, *Upper Left*) Gray-scale images of caudal vertebrae from P21 WT ($n = 8$), TRα^{GS} ($n = 5$), and TRα^{0} ($n = 3$) mice were pseudocolored according to a 16-color palette in which low mineral content is blue and high mineral content is red. (Scale bar, 1,000 μm.) (*Upper Right*) The graph demonstrates caudal vertebra length in WT, TRα^{GS}, and TRα^{0} mice. Data are shown as mean ± 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 TRα^{GS} and TRα^{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× and 100×, 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 ($n = 8$), TRα^{GS} ($n = 6$), and TRα^{0} samples; $n = 6$. Data are shown as mean ± 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 ± 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 TRα^{GS} and TRβ^{GS} mice. Strikingly, despite complete loss of canonical TR signaling, the TRα^{GS} and TRβ^{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 TRα and TRβ 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.

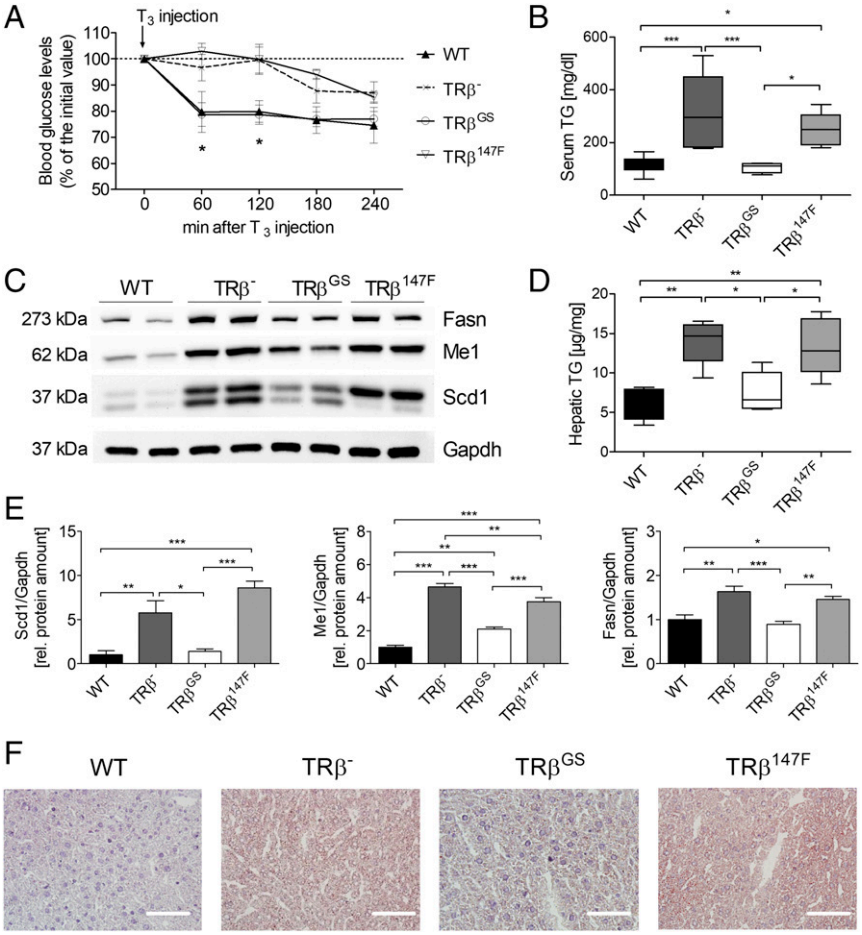

Fig. 6. Noncanonical TRβ signaling influences blood glucose and hepatic triglyceride synthesis. (*A*) Under fasting conditions, WT (black triangles), $TR\beta^{GS}$ (open circles), $TR\beta^{-}$ (**x**), and $TR\beta^{147F}$ (open triangles) mice received a single injection of T_3 (7 ng/g BW), and blood glucose concentration was measured at indicated time points; $n = 4$. Data are shown as mean ± SEM; Student's *t* test; $^{*}P < 0.05$). (*B*) Serum triglyceride concentration in untreated WT (black bar), $TR\beta^{-}$ (dark gray bar), $TR\beta^{GS}$ (open bar), and $TR\beta^{147F}$ (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 ± 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, $TR\beta^{-}$, $TR\beta^{GS}$, and $TR\beta^{147F}$ mice was done to visualize triglycerides in liver tissue. (Scale bar: 50 µm.)

Canonical Actions of TRs. An important physiological role of TH is negative feedback in the HPT axis. $TR\beta^{GS}$ mice had a phenotype similar to that of $TR\beta^{-}$ mice with lack of TRβ-mediated negative feedback in the pituitary and TH resistance (9), whereas the HPT axis in $TR\beta^{147F}$ mice was unaffected (7). A higher TSH in $TR\beta^{GS}$ mice could indicate that they are slightly more resistant than the $TR\beta^{-}$ mice. A potential but highly speculative explanation is cofactor squelching. TRβGS does not bind to DNA but is still capable of cofactor binding. This could, theoretically, reduce cofactor availability for TRα and reduce a minimal repressive TRα effect on TSH expression, resulting in even higher TSH concentrations than in TRβ-KO mice. Delayed longitudinal growth and bone mineralization are typical features of $TR\alpha^{0}$ mice and were replicated in $TR\alpha^{GS}$ mice. Therefore negative regulation of the HPT axis in $TR\beta^{GS}$ mice and bone development in $TR\alpha^{GS}$ mice are solely dependent on canonical TR/TRE-mediated transcriptional regulation. Nevertheless, a recently identified short variant of TRα, p30 TRα1, 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 TRα 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 TRα in adult bone remodeling.

Noncanonical Actions of TRβ. Serum glucose decreased rapidly only in WT and $TR\beta^{GS}$ mice but not in $TR\beta^{-}$ and $TR\beta^{147F}$ mice despite intact canonical TRβ signaling in the $TR\beta^{147F}$ mice. The phenotype in $TR\beta^{GS}$ mice demonstrates that noncanonical TRβ action alone suffices to generate physiological responses. Similarly, triglyceride synthesis and serum and liver triglyceride content were elevated in $TR\beta^{-}$ and $TR\beta^{147F}$ mice that lack noncanonical TRβ action, suggesting reduced consumption of triglyceride substrates. Body temperature was higher in $TR\beta^{GS}$ 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 $TR\beta^{GS}$. Additionally, VO_2 and distance traveled were higher in $TR\beta^{GS}$ mice. Increased energy metabolism, body temperature, and locomotor activity and lower triglyceride concentrations are physiological consequences of noncanonical TRβ action.

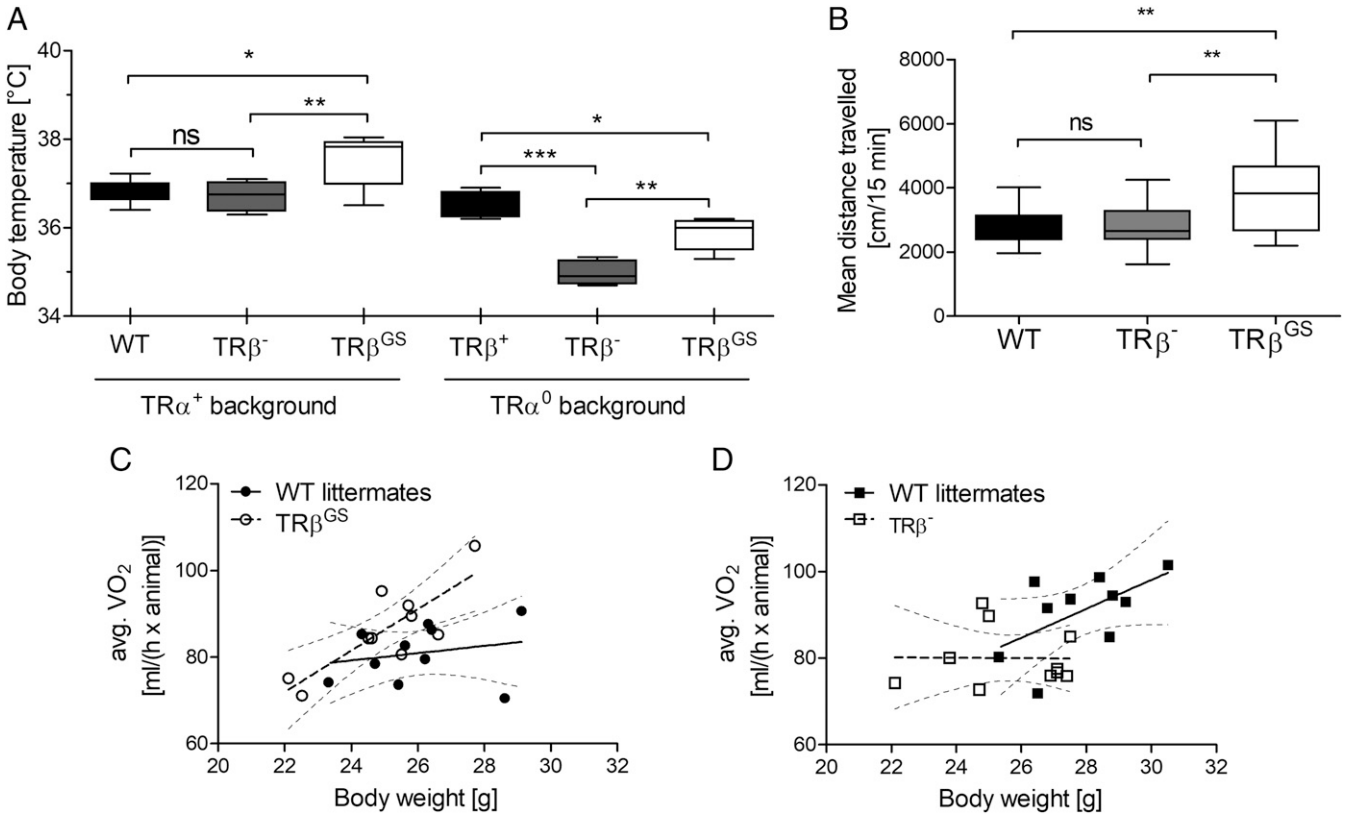

Fig. 7. Noncanonical action of TRβ increases body temperature, VO_2, and locomotor activity. (*A*) Body temperature of male mice in a TRα$^+$ (WT) or a TRα^0 genetic background; $n = 6$. Box plots indicate mean values, and whiskers indicate minimum and maximum values; ANOVA and Bonferroni's post hoc test for multiple comparison; ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*B*) Locomotor activity of WT, TRβ$^-$, and TRβ^{GS} 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; $**P < 0.01$, ns, not significant. (*C* and *D*) Average VO_2 of TRβ^{GS} (*C*) and TRβ$^-$ mice (*D*) and WT littermate controls was measured by indirect calorimetry and is plotted against body weight ($n = 10$).

Noncanonical Actions of TRα. 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 TRα^{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 TRα^{GS} hearts was similar to that of TRα^0 hearts. These results suggest that the noncanonical TH/TRα 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 TRα^0 mice but preserved in TRα^{GS} mice. Therefore we propose that noncanonical TH/TRα 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 TRα acts has yet to be determined.

Evolution of Noncanonical TH Signaling. Our findings suggest that increased noncanonical TRβ 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 TRβ, which are required for noncanonical PI3K activation by TRβ, are found in all mammalian TRβ 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 TRβ$^-$ and TRβ^{GS} mice clearly demonstrates that, in agreement with previous reports (9), inhibition of TSH expression in vivo requires DNA binding. Results from TRβ^{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 TRβ$^-$ mice but not in TRβ^{GS} mice. Therefore, negative regulation of *Scd1* expression in vivo does not require DNA binding of TRβ. 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/TRβ 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 TRβ 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

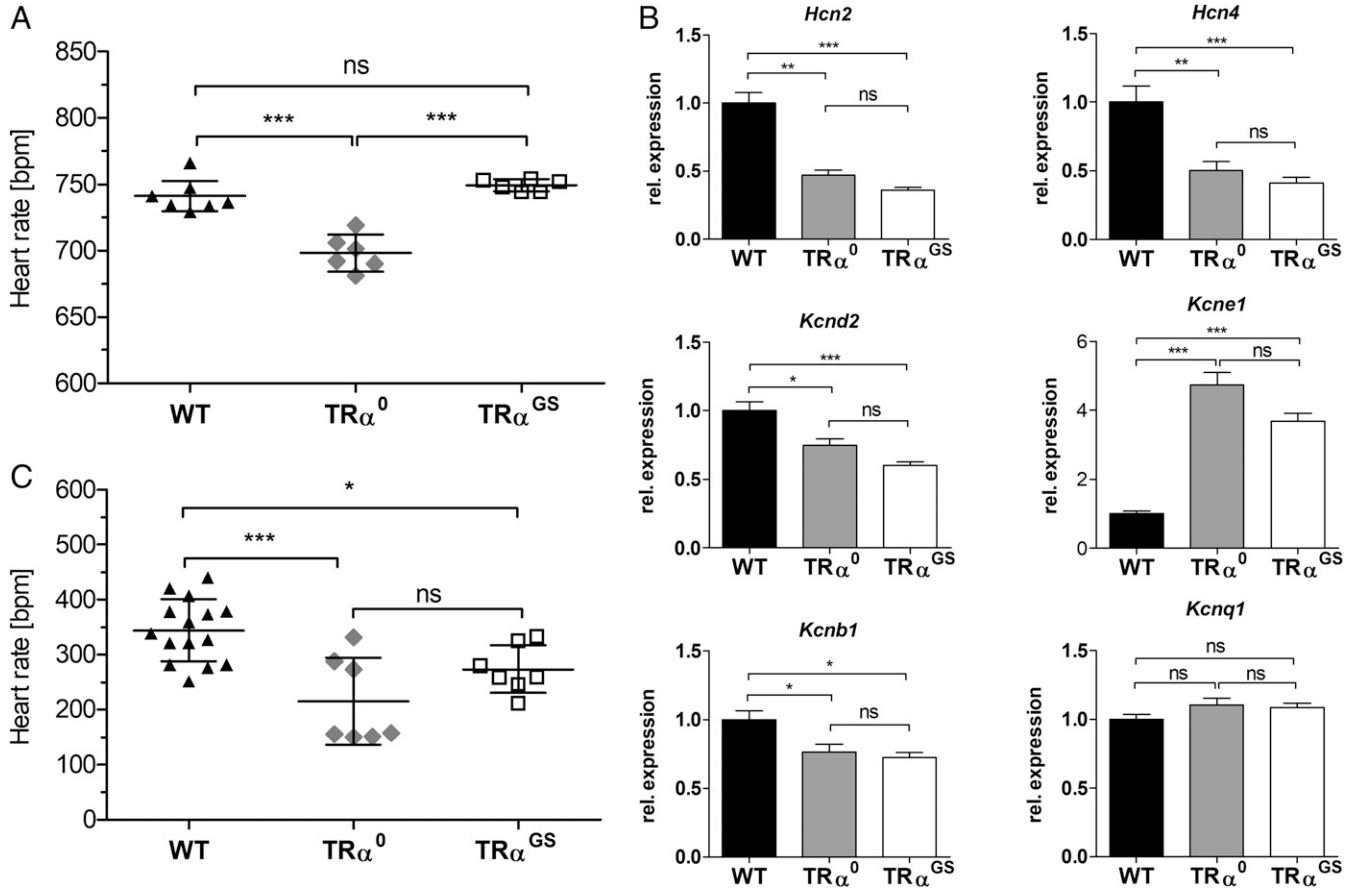

Fig. 8. Noncanonical TRα signaling maintains normal basal heart rate without altering cardiac pacemaker channel gene expression. (*A*) Heart rate of nonsedated male WT (black triangles; $n = 7$), $TR\alpha^0$ (gray diamonds; $n = 6$), and $TR\alpha^{GS}$ (open squares; $n = 6$) mice. Data are shown as mean ± 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),$TR\alpha^0$ (gray bars), and $TR\alpha^{GS}$ (open bars) mice; $n = 6$. Data are shown as mean ± 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; $n = 15$), $TR\alpha^0$ (gray diamonds; $n = 8$), or $TR\alpha^{GS}$ (open squares; $n = 7$) mice. Data are shown as mean ± SD; ANOVA followed by Tukey's post hoc test; *$P < 0.05$; ***$P < 0.0001$; ns, not significant.

$TR\alpha^{GS}$ 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 $TR\alpha^0$ and $TR\alpha^{GS}$ mice is that the relationship between *Myh6* and *Myh7* may not be linear. However, a partial noncanonical repressive effect of TRαGS cannot be ruled out.

Role of Noncanonical PI3K Activation. The best-studied noncanonical action of TRβ is rapid activation of PI3K (5–7). We therefore studied the $TR\beta^{147F}$ mouse as a control, because in this mouse model the activation of PI3K by TRβ is selectively abrogated. In the blood glucose response to T_3 and serum and liver triglyceride concentrations, the phenotype of $TR\beta^{147F}$ mice was similar to that of $TR\beta^-$ mice, demonstrating that these effects are indeed mediated by TRβ 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 TRβ signaling converge at the PI3K pathway. This mechanism of TRβ action would explain previous observations in which T_3 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). TRα also activates signaling pathways (29, 35), possibly as a short p30 TRα variant that indirectly activates ERK and PI3K after T_3 binding and nitric oxide and cGMP production (27) or by direct protein–protein interaction between TRα and PI3K (29, 35, 36). The noncanonical mechanism by which TRα 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 TRα and TRβ 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. TRα and TRβ WT and KO and $TR\beta^{147F}$ mice were genotyped as previously described (7, 24, 37). $TR\beta^{147F}$ 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 perchloride via the drinking water. Animals were treated with experiment-specific concentrations of T_3 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 $P < 0.05$.

ACKNOWLEDGMENTS. We thank B. Gloss at the National Institute of Environmental Health Sciences for providing breeding pairs of TRβ^{147F} mice. Illumina sequencing was performed by Ronni Nielsen with support from the VILLUM Center for Bioanalytical Sciences at the University of Southern Denmark. The TR C1 antibody was provided by Sheue-yann Cheng of the NIH. This work was supported by Deutsche Forschungsgemeinschaft Grants MO1018/2-1/2 (to L.C.M.), FU356/7-1 (to D.F.), and KO 922/17-1/2 (to J.K.) within the priority program Thyroid Trans Act SPP 1629; by Charité funds (J.K.); and in part by NIH Grant R37DK15070 (to S.R.), the Seymour J. Abrams Fund for Thyroid Research, Wellcome Trust Strategic Award 101123, and Wellcome Trust Project Grant 094134 (to J.H.D.B. and G.R.W.). Studies at the GMC were supported by German Federal Ministry of Education and Research Infrafrontier Grant 01KX1012 (to M.H.d.A.). L.G. was supported by grants from the Lundbeck Foundation and Novo Nordisk Foundation. D.L.A. was supported by Award Z01-ES102285 from the Intramural Program at the National Institute of Environmental Health Sciences of the NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the NIH.

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