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Thyroid Hormone Receptor Beta Sumoylation is Required for Thyrotropin Regulation and Thyroid Hormone Production

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Conflict of interest statement: The authors have declared that no conflict of interest exists.
Abstract

Thyroid hormone receptor beta (THRB) is post-translationally modified by small ubiquitin-like modifier (SUMO). We generated a mouse model with a mutation that disrupts sumoylation at lysine 146 (K146Q) and resulted in desumoylated THRB as the predominant form in tissues. The THRB K146Q mutant mice had normal serum thyroxine (T4), markedly elevated serum thyrotropin (TSH) (81-fold above control), and enlargement of both the pituitary and the thyroid gland. The marked elevation in TSH, despite a normal serum T4, indicated blunted feedback regulation of TSH. The THRB K146Q mutation altered transcription factors (TFs) recruitment to the TSHβ gene promoter, compared to wild-type, in hyperthyroidism and hypothyroidism. Thyroid hormone content (T4, T3 and rT3) in the thyroid gland of the THRB K146Q mice was 10-fold lower (per gram tissue) than control, despite normal TSH bioactivity. Expression of Thyroglobulin and Duox2 genes in the thyroid was reduced and associated with modifications of CREB DNA binding and cofactor interactions in the presence of the desumoylated THRβ. Thyroid hormone production, therefore, has both TSH-dependent and -independent components. We conclude that THRB sumoylation at K146 is required for normal TSH feedback regulation and TH synthesis in the thyroid gland, by a TSH-independent pathway.

Introduction

Thyroid hormone (TH) exert its biological and physiological actions primarily through interaction with the nuclear Thyroid Hormone Receptor (THR) (1, 2). There are two thyroid hormone receptor genes, THRA and THRB, and each gene produces functional THR protein. THRA is expressed predominantly in brain, heart, skeletal
muscle and adipose tissues. THRB is expressed in liver, heart and pituitary and mediates regulation of cholesterol metabolism, as well as the feedback regulation of thyrotropin stimulating hormone (TSH) in the pituitary. Mouse models with hemagglutinin (HA)-tags introduced into the THR genes to uniformly detect TR protein, demonstrate that THBR1 is the predominant TR isoform in liver and THR2 protein, which contains an extended amino terminus, is the predominant form in the pituitary (3).

Thyrotropin stimulating hormone (TSH) is a glycoprotein that consists of two subunits, α and β. The α subunit is a common to all of the pituitary glycoproteins. The β subunit (TSHβ) is directly regulated by thyroid hormone (TH)/THRB2-mediated feedback mechanism (4, 5). The regulation of TSHβ gene expression involves multiple transcription factors (TFs) in addition to THRB, including GATA binding protein 2 (GATA2) and Pit1/POU1F1 (6-12). The role of these TFs, however, has largely been based on transient reporter expression assays rather than direct determination of in vivo binding to the TSHβ gene regulatory regions (11). Pit 1 is essential for normal anterior pituitary development and individuals with a Pit 1 gene mutation in a region that disrupts function, have anterior pituitary insufficiency, including central hypothyroidism and reduced growth hormone (13). GATA2 is important for both stimulation and repression of TSHβ gene expression (9, 14). GATA-mediated transcription regulation has two modules: FOG (friend of GATA)-dependent and FOG-independent. FOG1 contains 8 zinc fingers, and is the best characterized GATA factor. FOG1 heterodimerizes with GATA, recruits Nucleosome Remodeling Factor (NURF) and deacetylase, and then activates or represses transcription depending on the
promoter context (15-18). FOG-dependent GATA action is involved in a range of diverse cellular processes (19, 20).

We previously showed that THRA and THRB were post-translationally modified by small ubiquitin-like modifier (SUMO), important for TH/THRs-mediated transcription (21). A number of TF’s are sumoylated, including nuclear receptors, as well as kinases and phosphatases (22-25). SUMO modification involves attachment of a SUMO, through covalently binding to a specific lysine motif in the target protein. The modification is reversible by SUMO peptidase that break the bond, called de-sumoylation. Sumoylation and de-sumoylation dynamically influences protein-protein interactions, protein structural conformation and protein binding to DNA. Sumoylation is involved in a range of biological processes including DNA replication, RNA transcription, as well as cell proliferation and differentiation, metabolism, immune response, and neuronal function (26-29). Sumoylation defects have been associated with several diseases.

We previously reported that THRA has two sumoylation sites and THRB has three (21). Mutations at each of these sites reduces sumoylation in vitro and disrupts THR interaction with coactivators and corepressors. Reduction in THRA sumoylation was shown to impair Wnt signaling, resulting in reduced preadipocyte proliferation and interference with PPARγ-mediated adipogenesis (21, 30). In vitro studies showed that sumoylation of THRs are important for the receptor function and TH signal-mediated cellular functions. To determine the physiologic role of THR sumoylation in vivo, we generated a mouse carrying a mutation at lysine (K) 146 in THRB that is required for SUMO conjugation. In this mouse model, we demonstrate that sumoylation of THRB at
K146 is essential for the regulation of $TSH\beta$ gene expression and for normal TH production in the thyroid. Desumoylated THRB directly disrupts $TSH\beta$ gene regulation as well as disrupting cAMP Response Element Binding Protein (CREB)-mediated regulation of genes important for thyroid hormone synthesis, including thyroglobulin and duox2.

RESULTS

Generation of THRB sumoylation mutant mice. THRB1 has three sumoylation sites located at lysines (K) 50, 146 and 438 (Figure 1A). The K50 residue is located in the amino terminus (A/B domain), residue K438 is in the ligand binding domain (LBD), and K146 is located in the 2$^{nd}$ zinc figure of the DNA binding domain (DBD) (Figure 1B). Based on crystallographic data, however, when THRB is DNA bound, K146 does not contact DNA (Figure 1C). Mutation of any of the 3 sites reduces THRB sumoylation $in vitro$, but the greatest functional disruption in our previous studies of adipocyte differentiation, was from mutations at K146, suggesting a dominant role for this site (21, 30). We chose to substitute glycine for lysine at 146 (K146Q) to generate a sumoylation-site mutation that would disrupt THR-SUMO conjugation without reducing ligand binding. We evaluated the influence of the K146Q mutation on the ability of THRB1 to mediate T3-induced transcription, utilizing a luciferase reporter with a consensus direct repeat thyroid hormone response element (DR4-TRE). The THRB K146Q mutant had the same ability as wild type THRB to stimulate T3-mediated luciferase gene expression (Figure 1D), indicating that the THRB K146Q mutation
binds ligand and DNA and the mutations does not alter T3-stimulated gene transcription, as mediated by a DR4-TRE.

We generated a mouse model with a global THRB K146Q mutation, using traditional gene targeting techniques (S Figure 1). The mutation was generated by a single nucleoside substitution, changing AAG (K) to CAG (Q). The *Thrb* isoforms-1, -2 and -3 are transcribed from the same gene by alternative start codons, such that all three THRB receptors isoforms were mutated simultaneously for the lysine (Gene bank accession # *Thrb1* NM_001113417, *Thrb2* NM_009380; *Thrb3* XM_011244745). The mutation position of nucleoside 1516 corresponds to the K146 residue in the THRB protein. The genotyping was based on the location in *Thrb1* and the mutation was confirmed by High Resolution Melting PCR and direct DNA sequencing (Figure 1E). The same gene mutation results in a substitution at K143 for THRB2 and at K115 for THRB3 and the mutations in the THRB2 and THRB3 receptor isoforms are located in the 2nd zinc finger, as it is for THRB1. To simplify the presentation of the data, the THRBI mutant mouse will be described as THRBI K146Q, to represent the mutation in the analogous residue for all three THRB isoforms.

**Metabolic phenotype of THRB K146Q Mice.** The body weight and body composition of the THRB K146Q mice (K146Q mice) was not different from the wild type (Wt) mice (Figure 2A). Markers of thyroid hormone action on metabolism, including fasting serum lipids (triglycerides, free fatty acid and cholesterol), serum insulin and glucose levels, showed no significant differences in the K146Q mutant mice, compared to Wt mice (Figures 2B and 2C). The levels of hepatic T3-responsive marker genes, important for
lipid and glucose metabolism, were not significantly different in the K146Q mutant mice, compared to Wt mice (Figure 2D), indicating no difference in the T3-mediated pathways that mediate lipolysis and lipogenesis.

**Thyroid status and changes in the pituitary and thyroid of THRβ K146Q mice.**
Serum thyroxine T4 and T3 levels in the K146Q mice were not significantly different from control. The serum TSH level in the mutant mice, however, was markedly elevated, the mean level increased 81-fold, compared to the Wt mice (Figure 3A). The thyroid in mutant mice was enlarged 5-fold by weight, compared to Wt mice (Figure 3B). Histological analysis of thyroid tissue showed hyperplastic thyroid follicles with scant colloid in the K146Q mice, compared to the normal follicles in the Wt mice (Figure 3C). Previously reported mouse models of Resistance to Thyroid Hormone, due to mutation in the THRβ gene (RTHβ), also show an elevated TSH and thyroid enlargement, but to a much smaller magnitude than seen in the K146Q mice (31-35). The pituitary gland in the K146Q mice was significantly enlarged, 20%, compared with control (Wt 0.911 mg ± SD 0.028 and K146Q 1.093 mg ± SD 0.055, n=13, p<0.005) (Figures 3D and E). Pituitary hyperplasia and expansion of thyrotrophs were clearly visible in pituitary histology sections (Figure 3F). The marked increase in the number of thyrotrophs in the pituitary of K146Q mice was demonstrated by TSH antibody-stained pituitary sections (Figure 3G). In K146Q mice, 56% (SD ± 5.4%) of the pituitary cells was stained positively for TSH, shown as green fluorescence, compared to 14% (SDV ± 3.2%) in Wt mice (p <0.002) (Figure 3H). The protein levels of common α subunit (α-glycoprotein) and TSHβ subunits were also increased, 1.65-fold and 5.6-fold
respectively, in pituitaries of mutant mice, compared to Wt (Figures 3I and J and S Figure 2). These data are consistent with the marked elevation of serum TSH in mutant mice. Mutation at the THRβ sumoylation site K146 reduced TH feedback regulation of TSH, resulting in a marked elevation of TSH and promotion of thyroid growth, but did not increase thyroid hormone production proportionate to the increase in serum TSH.

We investigated THRβ protein expression in the Wt and K146Q mice to determine if there were significant changes in THRβ protein content. The total THRβ (combined sumoylated and desumoylated) in the thyroid and pituitary, detected by Western blot and quantitated by pixel density relative to protein loaded, was similar in the Wt and K146Q mutant mice (Figures 3K and L and S Figure 3). In the thyroid, the mean total THRβ protein was 1.63% of the loaded protein in Wt and 1.51% in K146Q mutant mice. In the pituitary, the mean total THRβ was 2.06% of the loaded protein in Wt mice and 1.98% in the K146Q mutant. The total THRβ content in thyroid and pituitary, was similar (within less than 8%) in the Wt and K146Q mice. We then compared the proportion of sumoylated and desumoylated THRβ. The desumoylated THRβ molecular weight, as detected by Western blot, is 52-55 KDa. Addition of SUMO increases the molecular weight of protein with the predominant sumoylated THRβ protein to 75 kDa in size (Figures 3K-L). In Wt mice, the mean sumoylated THRβ was 44.7% of the total THRβ protein in the thyroid and 78.8% of the total THRβ in the pituitary. In the K146Q mice, sumoylated THRβ was not detectable by WB in the thyroid or pituitary, all THRβ was desumoylated. There are two additional sumoylation
sites in THRβ that were not mutated, but based on this analysis, the K146 site, under the conditions we studied, is the only active sumoylation site in thyroid and pituitary.

**Regulation of TSH and TSH Action.** TH feedback regulation occurs at both the hypothalamus, regulating TRH, and at the pituitary, regulating TSH, which then stimulates the thyroid gland to produce TH. The hypothalamic mRNA levels of Dio2, TRH and TRH Receptor (TRH-R) in the K146Q mice were not different from the levels in Wt mice (Figures 4A-B). We additionally performed dynamic testing by administering bovine TRH and measuring the serum TSH and T3 in response. Serum TSH was measured at 30 and 120 minutes after TRH injection and was stimulated in both mutant and Wt mice, but to a greater level in Wt mice, 19-fold, compared to 1.8-fold, in the mutant mice (Figure 4C). Serum T3 was measured at baseline and 2 h after TRH injection and there was no significant difference in the response between Wt and K146Q mice (Figure 4D). Similar levels of TRH and TRH-R mRNAs in the mutant mouse hypothalamus, and preserved responsiveness of TSH to exogenous TRH, indicate that disordered TRH regulation was not likely to be a significant contributor to the abnormal phenotype.

The 5-fold enlargement of the thyroid gland in the K146Q mice indicated that the elevated TSH was bioactive, with respect to thyroid growth, but the normal levels of serum T4 suggested reduced sensitivity to TSH action on hormone production. We directly evaluated TSH bioactivity by determining TSH-stimulated c-AMP production in CHO cells transfected with mouse TSH receptor (TSH-R). Serum was collected from Wt and THRβ K146Q mice. The TSH level of K146 mice was 81-fold greater than that
of Wt mice, so to compare bioactivity of the same TSH concentration, serum from K146Q mice was diluted (0-, 10-, 80-, and 100-fold), with TSH-depleted mouse serum. The undiluted serum of mutant mice stimulated c-AMP production 2.9-fold greater ($p < 0.001$), compared to stimulation from Wt mouse serum. There was not a significant difference in c-AMP production between Wt serum and K146Q serum with 10-fold and 80-fold dilution. At 100-fold dilution, c-AMP production was reduced 29% in K146Q mutant mice ($p < 0.003$), compared to Wt serum (Figure 4E). The TSH bioactivity at equal concentrations was not significantly reduced in mutant mice compared to Wt.

The observed reduced TSH action in stimulation of thyroid hormone production could also be the result of reduced TSH-Receptor (TSH-R) signaling. We analyzed short-term TSH-R function by dynamic testing, administering exogenous bovine TSH. Mice were rendered hyperthyroid by administration of T3 for 7 days to suppress endogenous T4 production from the thyroid. A single dose of bovine TSH was then administrated ip. After exogenous TSH stimulation, serum T4 was compared to the baseline before stimulation (0 h). Serum T4 levels increased 2.9-fold ($p <0.045$) in Wt mice after bovine TSH injection and 2.6-fold ($p< 0.033$) in K146Q mice, compared to the baseline level. There was no significant difference between Wt and K146Q mice, indicating that TSH receptor signaling was not altered. After prolonged stimulation, up to 5 hours, T4 production did not change significantly in either genotype (Figure 4F). These data indicate that the response to short-term TSH-R stimulation was normal in the K146Q mice. Both in vivo and in vitro data indicated that the bioactivity of TSH and short-term TSH-R signaling was not different in mutant compared to wt mice.
**TSH Regulation by T3.** Reduced feedback response to T4 in the mutant mice could be the result of reduced Dio2 activity in the pituitary. In K146Q mice, however, Dio2 mRNA was significantly increased (log FC=2.86, \( p < 1.97E-10 \)), compared to wt mice. Pituitary feedback by thyroid hormone was further assessed by graduated exogenous T3 suppression, as has been performed in evaluation of animals and humans with RTH beta. Mice were rendered hypothyroid after 6 weeks on a low iodine/PTU diet to stimulate TSH (**Figure 5A, Table 1**), and then treated with T3 for 3 weeks with a progressive increase in dose each week. At the end of the first week of T3 treatment (0.25 ng/100 g body weight/day), serum TSH of K146Q mice was reduced 76% (421 to 99 ng/ml), compared to baseline. Serum TSH in Wt mice was reduced 87% (from 52.5 to 6.8 ng/ml), significantly lower than the levels in K146Q mice (\( p < 0.0015 \)). Serum TSH in the K146Q mice was further suppressed after 3 weeks of T3 treatment to 1.99 ng/ml, significantly higher than the level in Wt mice, 0.054 ng/ml, \( (p< .001) \) (**Figure 5B**). These data show that TSH in the K146Q mice was responsive to T3 suppression, but the response was blunted, even at the highest doses of exogenous T3. The pituitary TSH\( \beta \) mRNA content in K146Q mice was significantly increased (log2FC=4, \( p < 3E-08 \)) compared to Wt mice, consistent with the marked increase in serum TSH (**Figure 5C**).

**The mechanism of K146Q and wild type THRBP regulation of TSH\( \beta \) gene**

**transcription** Sumoylation modifies protein-protein interactions of TF’s required for gene transcription (36). We hypothesized that the desumoylated THRBP altered interaction with TF’s required for regulating TSH\( \beta \) gene transcription, including GATA2, Pit-1, THRBP and NCoR (6, 11, 12, 37). We utilized mouse pituitary tissue to directly determine the factors binding to the TSH\( \beta \) gene regulatory region, by ChIP assay, and
compared the pattern in K146Q mutant mice to that in Wt mice. Mouse THSβ gene 5’-flanking region (from 1847-2120 nt) contains multiple potential transcription response elements including those that bind GATA2, Pit-1 and a putative negative TRE (nTRE) that binds THRB. To determine transcription factor binding, the TSHβ gene promoter was divided into two regions (R1, R2) based on the distribution of the transcription factors and previously published data (Figure 6A). Since GATA2 is the key factor involved in TSHβ gene regulation, we first determined the GATA2 active binding site in pituitary tissue by ChIP assay. GATA2 binding was not detected in the further upstream segment of 1847-1947. Therefore, TF-binding in the ChIP assays was studied only in the R1 and R2 segments.

Mice (Wt and K146Q) were treated with T3 to suppress TSH and the binding of TFs to the TSHβ gene regulatory region was evaluated by ChIP assay in pituitary tissue. Wt mice treated with T3 had a low level of binding of TFs (THRB, NCoR1, GATA2, FOG1 and Pit1) in the R1 region, while these TFs were highly bound to the R2 region (Figures 6B and C). GATA2 recruited FOG1 to the promoter, indicating that FOG1-dependent GATA2 repression was involved in inhibition of TSHβ gene. In K146Q mice, GATA2 binding to the R1 region was enriched 8-fold, compared to the Wt mice in the same region (Figures 6D and E). In the R2 region, enrichment of THRB, and GATA2 were significantly increased 2-fold and 5-fold, respectively, compared to Wt, at the same region. However, FOG1 enrichment was not detected (Figure 6D). In summary, TH repression of TSHβ gene expression in the Wt mice was associated with FOG-dependent GATA2 binding to R2, as well as binding of THRB, Pit1 and NCoR1.
In the THR B K146Q mutant mice, there was enhanced GATA binding to R1 and R2, but diminished FOG1 bound to R2, compared to binding in the Wt mice.

Mice (Wt and THR B K146Q) were placed on a low iodine/PTU diet to examine ligand-independent activation of the TSHβ gene in hypothyroid mice. In Wt hypothyroid mice, the enrichment of the TFs, THR B2, GATA2, NCoR1 and Pit1, to the R1 region was significantly greater (from 5- to 18-fold), compared to the R2 region (Figures 6F-G). The enrichment data indicated that R1, but not the R2 region, was involved in activation of the TSHβ gene in Wt mice. In THR B K146Q hypothyroid mice, the R1 region enrichment pattern was similar to that in Wt mice, although with a significant increase in Pit1 binding (3-fold greater, P < 0.05). In the R2 region, the enrichment of GATA2 and FOG1, but not other factors, was significantly increased, 5-fold for both factors, compared to Wt mice (Figures 6H-I), indicating FOG1-dependent GATA2 binding. In summary, in Wt mice, activation of TSHβ gene transcription primarily involved factors binding to the R1 region. In contrast, in the K146Q mice, TSHβ gene regulation involved binding to both the R1 and R2 regions. The TSH level in K146Q mice was high at baseline and increased further (2.6-fold) from weeks 2-6, with the low iodine/PTU hypothyroid diet. In comparison, serum TSH in Wt mice on the hypothyroid diet increased 1.4 fold in the same period (S Figure 4). These data indicate that enhanced TSHβ gene expression in the hypothyroid K146Q mice was associated with increased TF binding to both the R1 and R2 regions of the TSHβ gene.

Highly elevated serum TSH levels promote thyroid gland growth but does not enhance TH production. The mutant mice require a higher level of TH to
suppress the TSH than Wt mice, associated with reduced TH-feedback inhibition of $TSH_\beta$ transcription in the K146Q mutant mice. The K146Q mutant mice displayed thyroid hyperplasia, but normal serum T4 levels, despite highly elevated serum TSH concentration. We investigated whether this was due to increased TH degradation in the thyroid, reduced TH synthesis, or both. We directly analyzed TH species (T4, T3, rT3, 3,3'T2, 3,5'T2 and 3'-T1) content in the thyroid and found that the level of all were reduced, more than 10-fold in the K146Q mutant thyroid, compared to the levels in the thyroid from wt mice (Figure 7A and Table 2). The expression of TH metabolism gene mRNA in the thyroid by RNA-seq data, showed no significant change in $Dio1$ and reduced $Dio2$ mRNA expression in mutant mice, compared to Wt. $Dio3$ expression was increased in the mutant mice, compared to Wt, although its overall expression level was very low and there was no significant increase in thyroidal rT3 content, which would be expected if the elevated $Dio3$ was a factor reducing T4.

The key factors required for thyroid hormone synthesis are the sodium-iodide symporter (NIS), pendrin, thyroid peroxidase (TPO), dual oxidase (DUOX2) and thyroglobulin (Tg). We found that RNA expression of $Tg$ and $Duox2$ were significantly down regulated, logFC 2 and logFC 0.95 respectively, in the K146Q mutant mice, compared to Wt. $Tpo$ was increased (logFC 0.63, $p <0.05$), and mRNA of $NIS$, $sclc26a4$ (pendrin) and $slc5a5$ (MCT8) had no significant change (Figure 7B). DUOX2 is the primary dual oxidase to provide peroxide to TPO for iodination in thyroid. Thyroglobulin is the matrix substrate for thyroid hormone synthesis and thyroid hormone stores. Down regulation of these genes would be expected to impair TH synthesis. The transcriptional regulation of $Tg$, and $Duox2$ involves multiple factors,
however, they are primarily regulated by cAMP through TSH/TSH-R signaling (38-41). Previous studies have shown that THRβ interacts directly with CREB, as demonstrated by co-IP and ChIP assays, and influences CREB signaling (42-44). We hypothesized that the THRβ K146 mutation may alter the normal pattern of THRβ interaction with CREB, and interfere with CREB-mediated \textit{Tg} and \textit{Duox2} gene regulation. To investigate, we performed ChIP assays using thyroid tissue collected from Wt and mutant mice under euthyroid, hyperthyroid (T3-treated), and hypothyroid (Low Iodine/PTU diet) conditions. In mouse, the gene organization of \textit{Tg} and \textit{Duox2} are similar to human and rat (45-47), and CREB response element (CRE) were identified in the promoter region of both genes (\textbf{Figures 7C and D}).

In Wt mice, the enrichment of CREB was 14.5% and THRβ was not detected bound to the \textit{Tg} promoter, indicating that THRβ is not normally involved in regulation of \textit{Tg} transcription in this region (\textbf{Figure 7C}). However, in the K146Q mutant mice, THRβ was recruited to the Tg promoter ($p < 0.026$ compared to Wt mice) and CREB recruitment was lower than the IgG control. The reduced CREB enrichment in mutant mice, compared to Wt, is consistent with the K146Q mutation interfering with CREB-mediated \textit{Tg} transcription. Similarly, in hyperthyroid mice, CREB binding was also significantly reduced in K146Q mice. In hypothyroid mice, CREB and NCoR binding was highly enhanced in K146Q mutant mice, compared to Wt mice, but was associated with marked blunting of Tg mRNA expression (\textbf{S Figure 5}). NCoR has been shown to inactivate CREB (48) and in hypothyroid conditions, the desumoylated THRβ promotes NCoR and CREB binding. These findings are consistent with THRβ K146Q mutant altering CREB binding and action and reducing TSH-mediated Tg gene expression.
In the Duox2 promoter, THRβ, CREB and NCoR are enriched at a similar level in euthyroid mice (Figures 7D). In the K146Q mutant mice, the enrichment of TFs to the promoter was not detectable after subtracting baseline IgG levels. The TF DNA-binding pattern in Duox2 promoter was similar to that seen on the Tg promoter, reduced binding in hyperthyroidism and enhanced NCoR and CREB binding with hypothyroidism, also associated with reduced expression of Duox2 mRNA (S Figure 5). The ChIP data shows a predominant pattern of reduced CREB binding in euthyroid and hyperthyroid conditions in K146Q mice and enhanced in hypothyroidism, and blunted mRNA response to TSH stimulation. The THRβ K146Q mutant modifies CREB DNA-binding and reduced CREB-mediated transcription of Tg and Duox2.

Discussion

The K146Q mutant mouse model permitted us to study the physiologic function of THRβ-SUMO conjugation at K146 in vivo, without disrupting THRβ binding to DNA. The mutation at residue K146 in THRβ did not disrupt T3-induced gene expression of a DR+4 TRE in transient transfection, but in vivo, impaired TSH regulation at the level of the pituitary and reduced thyroid hormone synthesis. TH feedback suppression of TSH was diminished, resulting in a highly elevated serum TSH that was incompletely suppressed by exogenous T3, and thyrotroph hyperplasia in the pituitary. The highly elevated TSH promoted thyroid growth, but TSH action promoting TH production was diminished. Direct measurement of thyroidal TH (T4, T3 and rT3) levels were significantly reduced in K146Q mice, calculated as THs per mg thyroid tissue, as well as the total TH content per mouse thyroid. ChIP assays of select genes important for thyroid hormone synthesis showed that CREB-mediated expression of thyroglobulin
and Duox2 were disrupted by the THRβ K146Q mutant. The mutation in THRβ, tyrosine 147 to phenylalanine, also in the second zinc finger, was previously shown to disrupt THRβ augmentation of PI3K signaling, but did not impair TH signaling (49). At least two residues contained in the THRβ DNA binding domain, therefore, K146 and Y147, have regulatory roles distinct from mediating THRβ binding to DNA.

We utilized pituitary tissue from the Wt and mutant mice to determine, by ChIP assay, the pattern of THRβ and other TFs binding to the TSHβ gene promoter. Previous studies have mapped TSHβ regions important for TH-mediated feedback by deletion and mutations of regulator regions in transient transfections, but direct evaluation of binding of TFs pituitary extract, even in Wt mice, has been limited. The relative role of direct TR binding to DNA in TH-mediated downregulation of the TSHβ gene has varied by the model used, but in vivo models with mutation of the DBD, support that TR binding is required (5). We showed that two regions (R1 and R2) in the TSHβ gene promoter were differentially involved in repression and stimulation of gene expression. We also demonstrated that the TF’s, THRβ2, GATA2, Pit1, NCoR1 and FOG1, were involved in the transcription regulation of TSHβ. In hyperthyroid mice, suppression of TSHβ was mediated by the R2 region with FOG1-dependent GATA2 binding in Wt mice. In contrast, FOG1-independent GATA2 binding was observed in K146Q mice, resulting in impaired feedback suppression of the TSHβ gene. When mice were made hypothyroid, TF’s binding was enriched to the R1 region promoting TSHβ transcription in Wt mice. In \K146Q mice, however, TF’s bound to both R1 and R2, associated with excessive stimulation of the TSHβ gene and high levels of serum TSH.
The ChIP data showed that the THRβ K146Q mutation disrupted TH feedback regulation of TSH by altering the relative amount and configuration of TF DNA binding and interaction with regions R1 and R2. The level of THRβ2 recruitment was similar in the Wt and THRβ K146Q mice, so sumoylation did not directly influence THRβ binding to DNA. Although there is no consensus TRE in the R2 region, THRβ2 was largely enriched to this region in hyperthyroid mice, suggesting THRβ2 was attached to GATA2 and required for TSH suppression, perhaps in a Type 2 tethering role (50). NCoR1 was enriched to the R1 in hypothyroidism when TSHβ gene transcription was increased and enriched to R2 in hyperthyroidism for repression of TSHβ gene transcription. NCoR is a corepressor and recruited for inhibiting gene transcription. It was previously reported that mice with the NCoR ΔID mutation, which blocks interaction with THRβ, have reduced serum TH, normal TSH levels and increased T3-target gene expression in tissues (51), indicating an in vivo role of nuclear receptor corepressors in TH action. This phenotype suggests that NCoR1 is not involved in regulating THRβ TSHβ gene expression, but does modulate TH sensitivity in tissues (52). In our model, NCoR was bound to the TSHβ regulatory region with similar enrichment rate to that of wildtype THRβ. The role of the interaction of NCoR and THRβ on the TSHβ promoter needs to be further investigated.

GATA2-mediated transcription regulation has dual actions, FOG-dependent and FOG-independent. In FOG-dependent action, it recruits the nucleosome remodeling and deacetylase (NuRD) complex to remodeled chromosomal landscape, which may include DNA looping (16, 53). Although our current model did not address looping, FOG1 recruitment of NuRD is likely. Chromatin looping has been implicated to promote
interaction between factors separate by more than 10kb. Currently there are no suitable tools available to precisely demonstrate looping. Pit1 has been shown to be involved in TRH regulation of $TSH\beta$ expression (6, 54). In the pituitary nuclear extract ChIP assay, Pit1 was also involved in transcription inhibition of $TSH\beta$ in Wt mice, but not in K146Q mice.

K146Q mice had thyroid hyperplasia, but the thyroid hormone levels, including T4, T3 and rT3, in the thyroid were low. The main factors important for the TH synthetic pathway (NIS, TPO, Pendrin, DUOX2 and TG), are all stimulated by TSH and most are regulated by TSH-activated c-AMP signaling though CREB. The CREB binding sites have been studied and characterized in these genes using in vitro models (40, 47, 55-58). Our data showed that K146Q selectively interfered with CREB-mediated Tg and Duox2 transcription, but not all CREB signaling, such as regulation of $Tpo$ and $Slc5A5$ expression. The extent to which desumoylated THRB interferes with CREB signaling may depend on the DNA sequence context for the CRE and the position in the promoter. In most of cases, THRB does not directly contact the CRE, but is tethered with CREB and disrupts CREB function (43, 44, 59, 60). Besides THRB interaction with CREB, Kruppel-like zinc figure transcription factor GLI-similar (GLIS3) have recently been identified as a critical factor for activation of $Slc26a4$ (pendrin) and $Slc5A5$ gene transcription. Deficiency in GLIS3 protein is linked to insufficient follicular cell proliferation, low thyroid hormone production and hypothyroidism in human (61). In K146Q mice GLIS3 was reduced (LogFC = -0.6, p <0.003). However, the mRNA level of $Slc5A5$ (LogFC = 0.26) p < 0.11) and $Slc26a4$ (logFC = -0.25, p < 0.72) were not changed in our THRB K146Q mice.
Local regulation of thyroid hormone production, independent of TSH, has been shown to involve THR (62) and in another model, direct feedback by follicular Tg on genes important for thyroid hormone synthesis (63). Throcyte-selective knockout of THR resulted in reduced thyroid gland size and reduced thyroid hormone synthesis. Thyroid hormone content, in contrast to the desumoylated model, was increased, likely due to a marked reduction in the thyroid hormone transporters, Mct8 and Mct10, important for transporting TH out of the thyroid gland. Local feedback of Tg on thyroid hormone synthesis, has been linked to direct effects of Tg on reducing gene expression of genes important in TH synthesis (62).

In K146Q mice, highly elevated TSH with normal bioactivity did not stimulate TH production, but TH levels were reduced 10-fold. TH production is directly associated with TSH-stimulated thyroid transcription factors (TTFs), paired box 8 (Pax8) and SLC5a5 (coding for NIS), which in turn stimulates Tg, Tpo and Duox2 gene. THR selectively decreased CREB-mediated Tg and Duox2 transcription in euthyroid and hypothyroid conditions and reduced cellular Tg protein. It is known that the shape and size of thyroid follicles are important for intracellular colloid content and thyroid function. Reduced TH production in K146Q mice may be due in part to the inappropriate morphological shape of the follicles from reduced intracellular colloid (64-66).

The robust phenotype resulting from disruption of a single sumoylation site in THR, indicates the likely importance of posttranslational THR modifications. Thyroid hormone signaling plays a central role in the balance of lipogenesis and lipolysis, but also impacts glucose homeostasis. Our study showed that THR sumoylation at K146
plays a key role in TH regulation of TSH expression in the pituitary and TSH/TSHR-regulated TH production in thyroid. The presence of a regulatory system that could modulate central sensitivity to TH feedback and thyroid hormone synthesis, perhaps from feeding or nutrient signals, may be relevant for normal physiology, as well as response to stress, systemic illnesses and in metabolic diseases. It will be important to understand and characterize those factors that regulate THR sumoylation, as well as the role of other THR posttranslational modifications.

**Materials and Methods**

**Reporter Assay.** Human choriocarcinoma JEG-3 cells (ATCC) express low levels of THRB. JEG-3 cells were maintained in EMEM cell culture media supplemented with 10% Fetal Bovine Serum (FBS). Cells were plated to 24 well plates, 24 hours prior to transient transfection, and grown in serum-free media. Cells were transfected with plasmids using Attractene (Qiagen) in Opti-MEM (Thermofisher Inc). There were 6 replicates in each transfection. T3 was added to the medium at various concentrations, as indicated (Figure 1). Luciferase activity was determined 12 hours after transfection using a multifunction plate reader. The cells were co-transfected with the reporter and plasmids expressing THRB and/or THRB K146Q. The reporter vector carried 3 copies of a consensus direct repeat 4 (DR4) thyroid hormone response element (TRE), upstream of the luciferase gene. For each transfection, an equal amount of DNA was used (0.2 µg reporter and 0.2 µg THRB or THRBK146Q). The reporter-only transfection included 0.2 µg of empty vector plasmid. In co-transfections, 0.1 µg of
each THRBK146Q and THRB were used. The results were expressed as % maximum luciferase activity of cells transfected with THRB (control).

**Generation of THRB Sumoylation mutant mice.** All animal experiments were approved by the institutional animal care and use committee. The mutant mice were generated as whole-body “Knock-In” with a single nucleotide change at position 1516 (A to C), resulting in mutation of one THRB sumoylation site at lysine 146 (Supplemental figure 1). The mutation was in a region common to all THRB isoforms (TRB-1, -2 and -3). The mutant mice were generated in a C57BL/NTC background and were then backcrossed to the same genetic background at least 3 times. All mutant mice used in the study were homozygous for the mutation. The wild type (Wt) mice were the same sub-strain as the mutant mice. Male mice were used in all studies, unless otherwise stated.

**Body composition analysis using Echo-MRI.** Mice were maintained on regular chow diet and a 12 hour light and dark cycle. Body composition of Wt and THRB K146Q mice (n=10/genotype) were determined by Echo-MRI at 9-10 weeks of age.

**Thyroid and pituitary histology and immunofluorescence (IF) staining.** Mice (n=4/genotype) at 10 weeks of age were perfused by a transcardiac route with 4% paraformaldehyde. The thyroid and pituitary were then removed and underwent automated histology tissue processing including hematoxylin-eosin (H&E) stain and imaging in the UCLA Pathology Core Facility. The pituitary frozen sections were stained with anti-TSHβ antibody conjugated to Alexa Fluor 488 (green) and the Prolong
Gold/ DAPI mountant applied. Each mouse had 3 sections evaluated with representative images shown.

**Western Blot (WB).** Three pituitaries from each genotype were pooled and lysed using RIPA buffer. The total lysate (30 µg/lane) was separated on an SDS-PAGE gel and then a Western blot performed using a standard protocol. The antibodies used included, anti-TSHβ monoclonal antibody at 1:500 dilution for WB (Life Span Biosciences, LS-C334949), and anti-Chorionic Gonadotropin α at 1:1,000 dilution for WB (Thermofisher Scientific Inc, PA5-88517). THRB protein was assessed in the pituitary. Pituitaries were lysed in RIPA buffer with protease inhibitors and 20mM N-Ethylmaleimide. Two 10% SDS gels were prepared, one for WB and another for Coomassie blue staining (L). Protein (20 µg) was loaded two each lane and gels were run in the same tank. Membrane was first blotted with anti-THRB antibody at 1:250 dilution (Abcam, ab104417). THRB protein was assessed in the thyroid. Crude lysate (30µg) of thyroid gland from each mice was loaded on a 10% SDS-PAGE gel. Detection of THRB and sumoylated THRB in the thyroid gland was carried out using ab-THRB1 (Abcam Inc, ab180612) at 1:500 dilution.

**Hormones, lipids and insulin.** Serum TSH, triiodothyronine (T3), and thyroxine (T4) levels were determined using the Rat Thyroid Hormone Elisa Kit (Millipore Catalog # RTHYMAG-30K) by Luminex 200 Bioanalyzer and Miliplex analyst v5.1 software. Serum fasting free fatty acid (FFA), total cholesterol and triglycerides were analyzed by Elisa Kit (Cayman Chemical). Serum insulin was measured by Elisa Kit (Invitrogen).
**TSH suppression by exogenous T3.** Mice at 9 weeks of age (wild-type and THRBB K146Q sumoylation mutant) were divided into two groups: low iodine+0.15%PTU diet for 6 weeks and control. Serum was collected for measurement of T4, T3, and TSH. Mice were then given T3 (Sigma-Aldrich) by intraperitoneal (ip) injection for 3 weeks with an increasing dose each week, 25 µg, 50 µg and 100 µg /100 g body weight, per day. Serum was collected for the measurement of T4, T3, and TSH levels at the end of each week.

**Assay of TSH stimulation of T4 production.** Mice were given a T3 injection (4 µg/100 g body weight/day, ip) for 7 days to suppress endogenous TSH. Serum TSH and T4 were analyzed before and 20 h after the final injection. Mice were then given bovine-TSH (Millipore Inc.) 200 mIU/100 g body weight (ip) to directly stimulate the thyroid gland. The TSH and T4 levels were analyzed at 3 h and 5 h after the injection of bovine-TSH.

**Thyrotropin Releasing Hormone (TRH) stimulation of TSH.** Wild-type and THRBB K146Q mice, 9-weeks of age, were given a single ip dose of bovine TRH (5.0µg/kg body weight). After injection, serum samples were collected at 30 min for TSH and at 2 h for measurement of T4 and T3. TRH was purchased from Sigma-Aldrich.

**TSH bioactivity test with cAMP ELISA assay.** Chinese hamster ovary (CHO) cells were maintained with Hanks F-12K medium supplemented with 10% FBS and an antibiotic mixture of penicillin and streptomycin (ATCC). CHO cells (2 million cells/reaction) were transfected with a vector expressing mouse TSH receptor and/or adrenergic receptor beta 3 (ADR-3) using a Nucleofector 4D device and cell type-
specific reagent (Lonza Inc). After the electroporation, cells were plated to a 24-well plate (~2 x 10^5 cells/well). The assay was based on a previously described method (67) with modifications. In brief, on the day of testing, mouse serum was collected from whole blood centrifuged at 8,000 g for 3.3 minutes at 4°C, and kept on ice. The assay buffer was freshly made and was composed of Krebs-Ringer HEPES buffer (pH 7.4), 8mM glucose 0.5 g/L BSA (KRPH-DG-BSA) and filtered through a 0.2 µm membrane. Cells were washed twice with 300 µl KRPH-DG-BSA, then incubated in the same buffer for 30 minutes in culture incubator. Medium was then removed and fresh KRPH-DG-BSA buffer containing 30 µl of mouse serum and 25 µM Rolipram, a cAMP phosphodiesterase inhibitor, added and incubated for 1h. In the positive control, mouse serum was replaced by 10µM Forskolin. At the end of the incubation, cells were washed twice with 200 µl cold PBS and then lysed in 100 µl lysis buffer. A cAMP Elisa assay was performed following kit instructions (Cell Signaling). Because of the very high TSH level in mutant mice, the serum of mutant mice used in the assay was diluted with TSH-depleted serum at ratios of 1:10 and 1:100.

**Preparation of TSH-depleted mouse serum.** TSH-depleted serum was prepared by treatment of mice with Levothyroxine 5 µg/100g body weight for 10 days, which suppresses endogenous TSH production. Blood was drawn and serum TSH level measured.

**RNA-seq (DGE).** Mice (Wt and THRBK146Q sumoylation mutant mice) at 3 months of age (n=3/group) were euthanized and the pituitary and hypothalamus were collected. Total RNA was isolated from both tissues. RNA-seq was performed in the HiSeq 3000
system and data analyzed by the UCLA Genetics Core. The RNA-seq data for hypothalamus, pituitary and thyroid has been deposited to NCBI Gene Expression Omnibus (GEO) repository (GEO # GSE167105).

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of thyroid hormone in the thyroid gland.** The method used was as previously described (68). In brief, mouse thyroid glands were dissected from Wt and THRB K146Q mice (n=18/genotype) and snap frozen in liquid N2. Tissues were quickly digested using Pronase. Thyroid hormones were extracted from the thyroid gland with a solid-phase extraction method and analyzed by LC-MS/MS.

**Chromatin immunoprecipitation (ChIP) analysis of TSHβ gene in the pituitary.** Age- and gender-matched Wt and mutant mice were divided into three groups: control (n=13/group), hypothyroid (low iodine and 0.15% PTU diet for 3 weeks), and hyperthyroid (T3 10 µg/100 g body weight/day for 3 days), groups. Serum TSH, T4, and T3 levels were measured after treatment. Pituitary and thyroid tissues were collected and analyzed using the ChIP Kit (Millipore), following the manufacturer’s instructions. In brief, tissues were first incubated with tissue chromatin stabilization buffer and the DNA-protein complex was cross-linked using 1.3% formaldehyde (final concentration) for 10 min and stopped by addition of glycine to a final concentration of 125 mM. Tissue were lysed in ChIP lysis buffer with complete protease inhibitors (Roche Inc.) and 2 mM PMSF. We optimized the sonication procedure to obtain 150- to 200-bp DNA fragments. The sonicated lysate was centrifuged for 15 min at 12,000 g at 4°C and the supernatant collected for immunoprecipitation. A 10% fraction of the supernatant was used as the input control. Antibodies used are listed in Table 3. Each
antibody was pre-tested by immunoprecipitation (IP) using GH3 cell lysate. The same antibody lot # was utilized across multiple experiments to ensure uniformity of results. The negative control in the ChIP assay was rabbit IgG. Purified DNA from the ChIP assay was used in q-PCR, 2µl DNA in 10µl PCR reaction. For PCR primer sequences, see Table 4. The assay (PCR) data represents DNA-bound TFs with 10% input and is labeled as relative enrichment.

Statistics. Comparisons between 2 independent groups were assessed with a 2-tailed Student’s t test. One-way ANOVA was used for comparing one factor in multiple groups. Two-way ANOVA was used in comparison of multiple groups. A p value 0.05 or less was shown in the graphs. The data mean ± SD was shown in the figures. Prism version 9.02 was used for statistical analysis.

Study Approval. All experimental procedures and animal use were approved by the institutional Animal Care Committee in compliance with federal guidelines.

Author contribution
SK performed the majority of experiments. RK and KK analyzed TH species in thyroid tissue. JJ, FL, KA and AM assisted in critical experiments. YL performed ChIP assays. YYL and GAB designed research, analyzed data, made all figures and co-wrote the manuscript.

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Acknowledgement

This work is supported by NIH RO1 DK98576 to GAB.

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Figure 1. Location of sumoylation site K146 in the THR1B protein and functional assay of the THR1B K146Q mutant receptor (A) Diagram of three sumoylation sites, K50, K146, and K438, in the THR1B protein indicating the amino terminus (A/B domain), DNA Binding Domain (DBD), and the Ligand Binding Domain (LBD). (B) Residue K146 is located within the 2nd zinc finger (Zn 2) of the THR1 DBD (mutated K shown in red and sumoylation motif highlighted). (C) Ribbon diagram based on crystallographic data showing the THR1 and Retinoid X Receptor (RXR) heterodimer bound to DNA Direct Repeat, 4 base pair gap, Thyroid Hormone Response Element (DR4 TRE) and the location of the mutated K146, outside of the region of direct DNA contact. (D) The THR1 K146Q mutant was analyzed for its T3-mediated gene
transcription in a reporter assay. The reporter contained 3 copies of the consensus DR4 TRE upstream of a luciferase gene. JEG3 cells were co-transfected with reporter and plasmids expressing THRBP1 or THRBP K146Q or a combination of THRBP and K146Q. The amount of DNA in each transfection was kept constant. The luciferase activity was determined 12 hours after transfection using a multifunction plate reader. Results are presented as luciferase expression relative to the maximal THRBP control transfection induction (shown as 100%). (E) The THRBP point mutation in the mutant mice, C to A, resulting in substitution of glutamine for lysine (K146Q), was confirmed by direct DNA sequencing. The THRBP K146Q gene targeting strategy is shown in S Figure 1. Statistical analysis was performed for the reporter assay (Panel D), using multiple paired t test in Prism statistical software.
Figure 2. Metabolic phenotype of THRBP1 K146Q mice. Mice (10 weeks old) were maintained at a normal light cycle on a regular chow diet and fasted for 6 hours before blood collection. Results shown for Wild-type (Wt) (blue) and THRBP1 K146Q mice (orange), dots show individual values and bars indicate average ±SD and. (A) Body weight (BW) is shown, as well as body composition, fat and lean body mass as a percentage of BW, as determined by Echo-MRI. (B) Serum concentrations of
cholesterol, triglycerides and free fatty acids. (C) Fasting glucose and serum insulin levels. (D) Transcriptome sequencing data shown the mRNA expression level of known T3 target genes in the liver of THRB K146Q and Wt mice. One-way ANOVA was used for statistical analysis, except transcriptome sequencing data ($p < 0.05$ is shown). Gene symbols: Thrsp/Spot14, thyroid hormone responsive protein; Cyp7a1, cytochrome P450 member 7a1; Slc2a2/Slut2, solute carrier family 2 member a2; G6pc, Glucose-6-Phosphatase Catalytic Subunit; Hmgcr, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; Ldlr, low density lipoprotein receptor; Acaca/ACC1, Acetyl-CoA carboxylase 1; Cpt1a, Carnitine Palmitoyltransferase 1A; GSK3b, Glycogen Synthase Kinase 3 Beta; Pdk2, Pyruvate Dehydrogenase Kinase 2. Paired t test with normal distribution was used in statistical analysis. The statistical analysis for data shown in all panels was performed using Student $t$ test for paired analysis.
Figure 3. Thyroid status, thyroid gland and pituitary findings in THRB K146Q mice. (A) Serum T4, T3 and TSH concentrations in Wt and THRB K146Q mice (n=13/}
(genotype) is shown as mean (±SD) and paired T test for statistical analysis. TSH is shown in log_{10} scale because of the wide differences in the levels in Wt and K146Q mice. (B) Thyroid was rinsed with saline, patted dry and weighed. The weight is shown as wet weight per mouse (n=18/genotype). (C) Representative histology of thyroid gland stained with H&E. Transverse section of thyroid gland (top panel) and thyroid follicles (lower panel). (D) Dissected pituitaries were rinsed with saline, patted dry and weighed, and values are shown as wet weight of pituitary from each mouse (n=13/genotype). (E) Pituitaries are shown from Wt and THRβ K146Q mice. (F) Image of representative pituitary tissue histology with H&E stain from Wt and THRβ K146Q mutant mice. (G) Immunofluorescent staining for TSHβ (green) and for nuclei (DAPI blue). Frozen sections of the pituitaries were incubated with anti-TSHβ antibody at 1:50 dilution and conjugated with Alexa Fluor 488. (H) The TSHβ expressing cells and total cell numbers were counted using green and blue filters. (I) Western blot detection of TSHβ and common glycoprotein α-subunit (CGα) proteins. Pituitaries (n = 3) were lysed in RIPA buffer and 30 µg of protein loaded on an 8% SDS gel. Membranes were Ponceau S-stained (S Figure 2) prior to blot with anti-TSHβ and anti-CGα. (J) Quantification of TSHβ and CGα protein band in Western blot using Li-Cro Image Studio Lite. (K - L) WB detection of THRβ protein in the thyroid and pituitary. Protein (30 µg) was loaded to 10% SDS gel, transferred to PVDF membrane, and blotted with anti-THRβ antibody. The protein loading is shown (S Figure 3). Statistical analysis was performed using paired Student t test (panels A, B, D and H).
Figure 4. Exogenous TRH and TSH stimulation, hypothalamic gene expression and TSH bioactivity in wild type and THRβ K146Q mice (A-B) The mean (±SD) mRNA expression of hypothalamic TRH-associated genes by RNA-seq are shown in bar figures and a heat map using three biologic replicates (1, 2, 3) of Wt and THRβ K146Q mice. The scale of intensity bar shows normalized counts per million (CPM). The gene symbols: TRH, thyroid releasing hormone; TRHr-1, TRH receptor-1; TRHr-2,
TRH receptor-2; Dio2, Type 2 5’-deiodinase. The statistical analysis embedded in RNA-seq data analysis software showed no significant differences. (C-D) Serum TSH and T3 in Wt and THRBK146Q mice after TRH stimulation. A single dose of Bovine TRH (5.0µg/kg body weight) was injected (ip). Blood samples were collected at 0 and 30 minutes for TSH measurement and at 0 and 2 hours for T3 measurement. Individual values and the mean ±SD are shown. Statistical analysis was performed with Two-way ANOVA to compare time points. *, P < 0.005 compared Wt-0 to K146Q-0; **, p< 0.05 compared Wt-30 to K146Q-30. (E) TSH bioactivity of serum from Wt and THRB K146Q mice. CHO cells were transfected with TSHβ expression vector +/- β3-ADR expression vector. Sera of THRB K146Q mice was serially diluted with TSH-depleted sera. Mouse sera was added to the cells and incubated for 1 hour and production of cAMP in CHO cells was determined. Statistical analysis was performed using One-way ANOVA, * shows p <0.01 as compared to Wt. (F) Mice were given a T3 injection (4ug/100g body weight/day) for 7 days to suppress TSH, and an ip injection of Bovine TSH (200 mIU/100g body weight) 20 hrs after the last T3 injection. Serum T4 was analyzed at baseline and then 3 and 5 hours after Bovine TSH injection. Statistical analysis was performed using paired student t test.
Figure 5. Response to exogenous T3 Suppression in Wild Type and THRB K146Q Mice and Pituitary mRNA Profile. Mice (n=10/genotype) were made hypothyroidism with a low iodine PTU-containing diet. Mice were then given T3 (ip) daily for a total of 3 weeks with increasing dose each week. Blood was collected at end of each week and TSH was assayed using Elisa kit (see details in materials and methods). (A) TSH level before and after mice rendered hypothyroid (Hypo). TSH is shown on a log_{10} scale because of the wide differences in the TSH levels in Wt and THRB K146Q mice. (B) TSH level shown as % baseline at week 0 of T3 treatment. Insert shows the actual TSH level at end of 3 weeks of T3 suppression. Statistical analysis was performed using paired student t test (panels A and B) (C) The anterior pituitary gene expression
analyzed by RNA-seq is shown as log$_2$ fold change (Log FC). The p value of the log$_2$ FC is shown in parallel graph for upregulated and downregulated genes. Gene symbols: TSHb, TSH$\beta$; CG$\alpha$, common glycoprotein alpha subunit; Gh, growth hormone; Prl, Prolactin; Trhr, TRH receptor; Dio2, 5’ deiodinase type 2; Ghsr, growth hormone secretagogue receptor
Figure 6. Transcription factor binding patterns to the TSHβ gene promoter in hyperthyroid and hypothyroid Wt and THRβ K146Q mice. Mice (n=15/genotype) were made hyperthyroid with T3 injection or hypothyroid with PTU-Low Iodine diet. The
pituitaries were removed and pooled for the ChIP assay to detect TFs (THRB2, GATA2, FOG1 and NCoR) binding to DNA regions (R1 and R2). (A) Diagram of the TSHβ gene regulatory region mediated by T3. The numerical labeling of the regulatory region is corresponding to mouse thyrotropin gene (Gene Bank accession # AH002109.2). The segment is divided into two regions (R1, R2) that bind TF’s, with the R1 region containing the transcription start site (TSS) and negative Thyroid Hormone Response Element (nTRE). GATA, Pit 1 and THRB binding sites, identified by sequence, are shown. (B and C) ChIP analysis of transcription factor binding in R2 and R1 regions of TSHβ gene promoter in hyperthyroid Wt mice. Inset shows modified Y axis scale with the same data. Graphic summary of T3-induced TF DNA binding in suppression of TSHβ gene transcription. The transcription start site is shown with a green arrow and the magnitude of transcription factor binding by the size of the TF symbol. (D and E) ChIP analysis of transcription factor binding to the TSHβ gene promoter in the pituitary of hyperthyroid K146Q mice. (F-G) ChIP analysis of transcription factor binding to the TSHβ gene promoter in the pituitary of hypothyroid Wt mice. (H-I) ChIP analysis of transcription factor binding to the TSHβ gene promoter in the pituitary of hypothyroid K146Q mice. The Chromatin assay (PCR) data represents DNA-bound TFs and a 10% fraction of the chromatin lysate supernatant was used as the input control and is labeled as “Relative enrichment”. The statistical analysis described in the text utilized paired student t test.
Figure 7. Characterization of thyroid hormone content, gene expression and transcription factor binding in Thyrogbolin (Tg) and Dual Oxidase 2 (Duox2) gene regulator regions, in thyroid glands from THBR K146Q mutant and wild-type (Wt) mice. (A) Thyroid hormone (T4, t3, rT3) levels in the thyroid gland were analyzed using liquid chromatography-tandem mass spectrometry. The data is
normalized using average thyroid gland weight and total thyroid hormone content in Wt and K146Q mouse and expressed per mouse (ng/mouse). The statistical analysis was performed with paired student $t$ test and $p$ values are shown, wt compared to K146Q mice  

(B) RNA-seq determination of the expression level of the genes relevant for thyroid hormone synthesis.  

(C-D) Chromatin Immunoprecipitation (ChIP) detection of transcription factor enrichment to the Tg (C) and Duox2 (D) 5' regulatory regions. Mice (n=6/group) were in euthyroid, hypothyroid or hyperthyroid conditions. The thyroid was dissected and pooled for ChIP assays using antibodies against THRβ, CREB, NCoR. The mapping of 5' regulatory regions of the Thyrogblobin and Duox2 genes are shown above the quantitation of transcription factor binding. The horizontal arrows indicate the position of primers utilized for the ChIP assays. The ChIP data for various thyroid conditions are shown in lower panels as the relative enrichment. *, Statistical analysis was performed using paired student $t$ test, comparison between Wt and K146Q value, * $p < 0.05$ (panels B, C and D).
Table 1. Serum hormone levels before and after 6 weeks of low iodine/PTU diet

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<tr>
<td></td>
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<td>After</td>
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<tr>
<td>TSH (pg/ml)</td>
<td>580 ± 25</td>
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<td>T4 (ug/dl)</td>
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<td>T3 (ng/ml)</td>
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Table 2. Thyroid hormone levels in thyroid tissue

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Table 3. Antibodies used in ChIP assays

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<td>Santa Cruz Bio Inc</td>
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<td>ab31387</td>
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<td>sc-393478</td>
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Table 4. ChIP primer sequences

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<tr>
<th>Primer name</th>
<th>Primer sequences (5’ to 3’ direction)</th>
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<tr>
<td>TSHb 1847F</td>
<td>CTGTTATTAACATTACAGAT ATTA</td>
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<td>TSHb 1947R</td>
<td>CTATATCCTCTCCCTGGAACCTCT</td>
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<tr>
<td>TSHb 1947F</td>
<td>GTGAACCTGTGTTGTCTAAACAA</td>
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<tr>
<td>TSHb 2017R</td>
<td>CATTCGAATTGCTGCTTCT</td>
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<td>TSHb 2037F</td>
<td>CAATTATATA AACAAAGGAATCAGA</td>
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<tr>
<td>TSHb 2120R</td>
<td>GAGTTACTGCTGTGATGACCCACT</td>
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<tr>
<td>Thyroglobulin F</td>
<td>CACCCTACTGACCACCTTAG</td>
</tr>
<tr>
<td>Thyroglobulin R</td>
<td>GCTCTCCTGTGTAAGGACAC</td>
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<tr>
<td>Duox2 F</td>
<td>GTCCTGTCTG GTTGAGGTCAT</td>
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<tr>
<td>Duox2 R</td>
<td>GGTGTAATGTGTGAATCATA</td>
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