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Research Article

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Thyrocyte-specific G_q/G_{11} deficiency impairs thyroid function and prevents goiter development

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The function of the adult thyroid is regulated by thyroid-stimulating hormone (TSH), which acts through a G protein-coupled receptor. Overactivation of the TSH receptor results in hyperthyroidism and goiter. The G_s -mediated stimulation of adenylyl cyclase-dependent cAMP formation has been regarded as the principal intracellular signaling mechanism mediating the action of TSH. Here we show that the G_q/G_{11} -mediated signaling pathway plays an unexpected and essential role in the regulation of thyroid function. Mice lacking the α subunits of G_q and G_{11} specifically in thyroid epithelial cells showed severely reduced iodine organification and thyroid hormone secretion in response to TSH, and many developed hypothyroidism within months after birth. In addition, thyrocyte-specific $G\alpha_q/G\alpha_{11}$ -deficient mice lacked the normal proliferative thyroid response to TSH or goitrogenic diet, indicating an essential role of this pathway in the adaptive growth of the thyroid gland. Our data suggest that G_q/G_{11} and their downstream effectors are promising targets to interfere with increased thyroid function and growth.

Introduction

Thyroid hormone plays a central role in maintaining the basal level of metabolism in the body. It regulates O_2 consumption as well as lipid and carbohydrate metabolism and is required for normal growth and maturation (1, 2). The primary regulator of thyroid gland growth and function in the adult organism is the thyroid-stimulating hormone (TSH). Lack of TSH or TSH action results in a reduced weight of the adult thyroid gland and almost abolishes thyroid function, leading to hypothyroidism (3–5). Conversely, pathologically elevated serum TSH levels stimulate thyroid hormone production and thyroid growth, leading to hyperthyroidism and goiter (6).

TSH regulates thyroid function through a G protein-coupled receptor on thyrocytes (7–9). TSH receptor-dependent activation of the G_s /adenylyl cyclase-mediated pathway has been suggested to account for most of the biological effects of TSH on thyroid cells, such as the stimulation of iodine uptake, hormone secretion, and proliferation (7). Consistent with this, thyroid glands of mice lacking the TSH receptor have defects in producing iodinated thyroglobulin, but the ability to take up iodine and to organify it can be restored by the adenylyl cyclase activator forskolin (4). Nongoitrous hypothyroidism has also been observed in patients with one defective allele of the gene encoding $G\alpha_s$ (*GNAS*) and pseudohypoparathyroidism type 1a (10) as well as in at least one mouse model with $G\alpha_s$ deficiency (11). In addition, constitutive activation of the thyrocyte cAMP cascade in humans carrying activating somatic mutations of *GNAS* or in transgenic mice overexpressing the G_s -coupled adenosine A_2 receptor, a constitutively

active mutant of $G\alpha_s$, or cholera toxin in thyroids causes hyperfunctioning thyroid adenomas (12–17).

In various species, including humans, TSH can also induce the G_q/G_{11} -mediated stimulation of phospholipase C- β (PLC- β), leading to mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) by inositol 1,4,5-trisphosphate and formation of diacyl glycerol (18–20). However, the role of the G_q/G_{11} -mediated signaling pathway in thyroid function is unclear. There is evidence that the constitutive activation of the G_q/G_{11} /PLC- β pathway in thyrocytes of mice overexpressing an active mutant of the α_{1B} adrenergic receptor further promotes malignant transformation of the thyroid gland (21), but it is unclear whether G_q/G_{11} mediate a growth-promoting effect under more physiological conditions.

In order to understand the role of G_q/G_{11} -mediated signaling in thyroid follicular cells, we have generated mice lacking the α subunits of G_q/G_{11} selectively in thyrocytes. Because $G\alpha_q/G\alpha_{11}$ -deficient mice die in utero (22), we used a floxed allele of the gene encoding $G\alpha_q$ (*gnaq*), which can be used to inactivate $G\alpha_q$ function in a $G\alpha_{11}$ -deficient background (23), using the Cre/loxP system. Our data indicate that the G_q/G_{11} -mediated signaling pathway is dispensable for thyroid development but is required for TSH-induced thyroid hormone synthesis and release in the adult and that the lack of G_q/G_{11} leads to hypothyroidism. In addition, G_q/G_{11} deficiency prevented the development of goiter induced by blockade of thyroid function or TSH treatment.

Results

Generation of thyroid-specific $G\alpha_q/G\alpha_{11}$ deficiency. In order to inactivate the G_q/G_{11} -mediated signaling pathway in thyrocytes, we generated a transgenic mouse line expressing Cre recombinase under the control of the thyrocyte-specific thyroglobulin promoter using a P1-derived bacterial artificial chromosome (PAC) harboring the thyroglobulin gene (see Methods). After crossing with the *Gt(ROSA)26Sor* Cre reporter mouse line (24), 3 of the 4 tested transgenic founder

Nonstandard abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} ; PAC, P1-derived bacterial artificial chromosome; Tc- $G\alpha_q/G\alpha_{11}$ -KO, thyrocyte-specific Cre transgenic crossed with *Gnaq^{lox/lox};Gna11^{-/-}*; TSH, thyroid-stimulating hormone.

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

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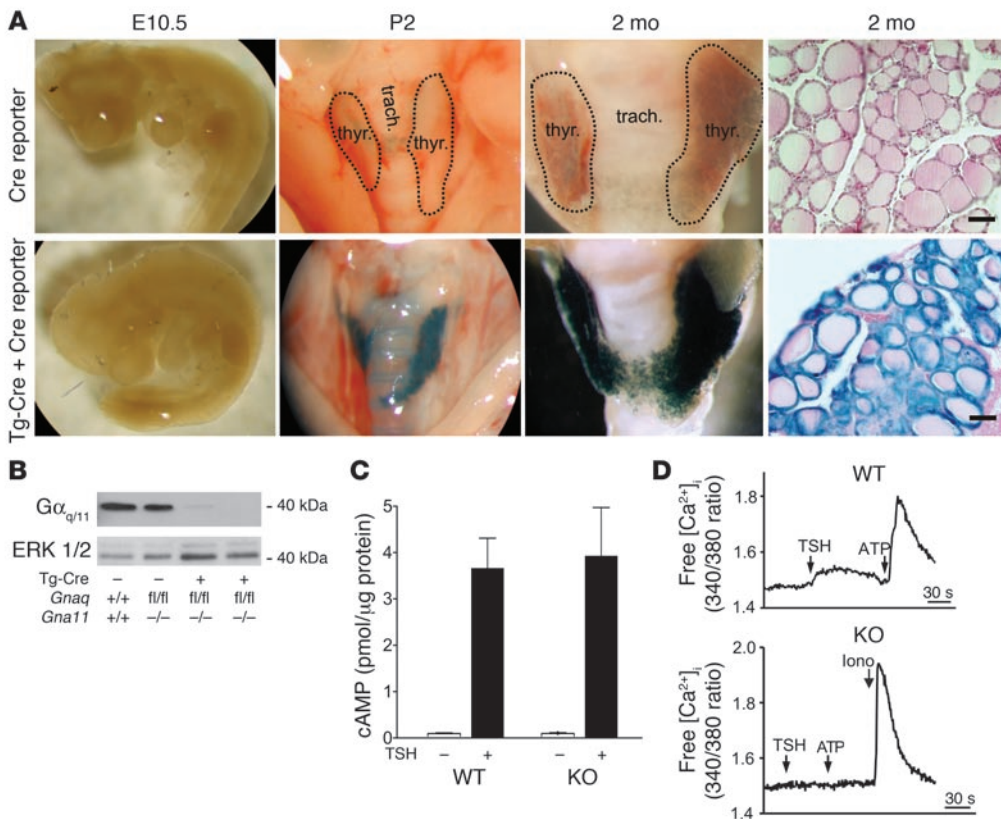


Figure 1

Validation of thyrocyte-specific deletion of the genes encoding $G\alpha_q$ and $G\alpha_{11}$. (A) *Gt(ROSA)26Sor* Cre reporter animals (Cre reporter) carrying no Cre gene (top row) or carrying the Cre gene under the control of the thyroglobulin promoter (Tg-Cre; bottom) were analyzed at the indicated ages, and β -galactosidase staining was performed on whole-mount preparations as well as on sections (far right panels). trach., trachea; thy., thyroid gland. Scale bars: 50 μ m. Original magnification, $\times 12$. (B) Western blot of thyroid gland lysates from 4-week-old wild-type (*Gnaq*^{+/+}*Gna11*^{+/+}), $G\alpha_{11}$ -deficient (*Gnaq*^{fl/fl}*Gna11*^{-/-}) and Tc- $G\alpha_q/G\alpha_{11}$ -KO mice (Tg-Cre;*Gnaq*^{fl/fl}*Gna11*^{-/-}) probed with antibodies recognizing $G\alpha_q/G\alpha_{11}$ ($G\alpha_{q/11}$) or ERK1/2. (C) cAMP levels in primary thyrocytes from wild-type and Tc- $G\alpha_q/G\alpha_{11}$ -KO animals treated (+) or not (-) with 50 mU/ml TSH. Values are mean \pm SEM of experiments performed in triplicate. (D) Effect of TSH (10 mU/ml), ATP (10 μ M), and ionomycin (iono; 1 μ M) on $[Ca^{2+}]_i$ in thyrocytes prepared from wild-type or Tc- $G\alpha_q/G\alpha_{11}$ -KO animals. y axis values are measured 340/380-nm fluorescence ratios as an indicator of free $[Ca^{2+}]_i$.

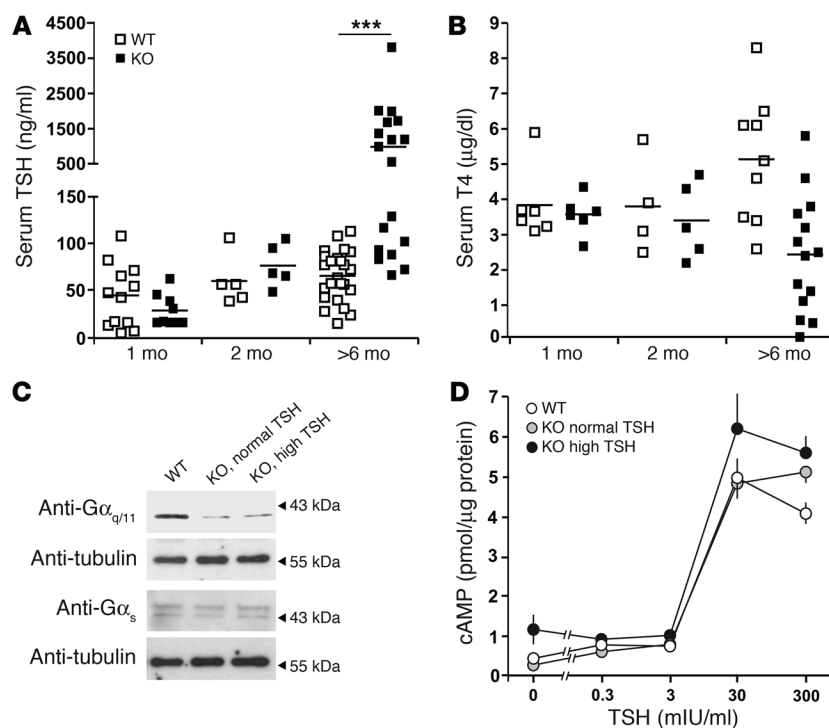
lines showed Cre-mediated recombination exclusively in the thyroid gland (see Supplemental Figure 1, available online with this article; doi:10.1172/JCI30380DS1). Cre activity was observed in virtually all thyrocytes, but not in other cells of the thyroid gland, like parathyroid cells or stromal cells (Figure 1A and data not shown). As expected from the time course of thyroglobulin promoter activity, no recombination was seen on E10.5 (Figure 1A). However, Cre-mediated recombination was observed by P2. There was no indication of altered thyroid histology or serum TSH and thyroid hormone levels in mice expressing Cre compared with wild-type littermates (Figure 1A and data not shown).

The thyrocyte-specific Cre transgenic mouse line (Tg-Cre) was crossed with mice lacking the $G\alpha_{11}$ gene (*Gna11*^{-/-}) and carrying the floxed $G\alpha_q$ allele (*Gnaq*^{flax/flax}), resulting in the generation of Tg-Cre;*Gnaq*^{flax/flax}*Gna11*^{-/-} mice (Tc- $G\alpha_q/G\alpha_{11}$ -KO). As shown in Figure 1B, the amount of $G\alpha_q/G\alpha_{11}$ proteins was strongly reduced in thyroids from Tc- $G\alpha_q/G\alpha_{11}$ -KO mice compared with wild-type littermates. The small amount of remaining $G\alpha_q/G\alpha_{11}$ protein was most likely derived from cells other than thyrocytes. We also ana-

lyzed $G\alpha_q/G\alpha_{11}$ expression in nonthyroid tissues including brain, liver, spleen, and platelets of Tc- $G\alpha_q/G\alpha_{11}$ -KO mice, but did not observe any difference compared with levels in wild-type mice (data not shown). No significant difference in basal or TSH-induced cAMP levels was seen between thyrocytes prepared from Tc- $G\alpha_q/G\alpha_{11}$ -KO mice and wild-type littermates at 1–2 months of age, indicating no significant alteration in the G_s -mediated signaling pathway in the absence of $G\alpha_q/G\alpha_{11}$ (Figure 1C). In contrast, thyrocytes from Tc- $G\alpha_q/G\alpha_{11}$ -KO mice lacked a functional G_q/G_{11} -mediated signaling pathway, as demonstrated by determination of $[Ca^{2+}]_i$ concentrations using the fluorescent calcium indicator Fura-2. While in wild-type thyrocytes, TSH, ATP, and other stimuli acting via G_q/G_{11} -coupled receptors induced an increase in $[Ca^{2+}]_i$ (Figure 1D and data not shown), in thyrocytes from Tc- $G\alpha_q/G\alpha_{11}$ -KO mice, only the Ca^{2+} ionophore ionomycin induced a response (Figure 1D).

Hypothyroidism in thyrocyte-specific $G\alpha_q/G\alpha_{11}$ -deficient mice. The development of the thyroid gland in the absence of the G_q/G_{11} -mediated signaling pathway was normal, as

indicated by normal thyroid histology and normal thyroid hormone and TSH plasma levels during the first 2 months of age (Figures 2 and 3). Similarly, no change in the size or form of thyroid follicles was observed in mice up to 2 months of age (Figure 3 and data not shown). There was also no difference in body weight or reproductive performance in Tc- $G\alpha_q/G\alpha_{11}$ -KO mice compared with littermate controls (data not shown). However, after 2 months of age, the TSH plasma levels in the thyrocyte-specific $G\alpha_q/G\alpha_{11}$ -KO mice gradually increased, differing significantly at 6 months of age. Eventually, about half of the Tc- $G\alpha_q/G\alpha_{11}$ -KO animals developed overt hypothyroidism, with low T_4 levels and strongly increased TSH levels, at 6 months of age or older (Figure 2 and data not shown). The proportion of males to females was very similar in groups with normal and altered TSH and T_4 plasma levels (data not shown), which indicates that there were no sex-specific differences. There was also no difference in thyroid weights of Tc- $G\alpha_q/G\alpha_{11}$ -KO mice with normal (<150 ng/ml) and elevated TSH levels (>500 ng/ml) (0.12 ± 0.01 and 0.11 ± 0.01 mg/g body weight, respectively).

**Figure 2**

Physiological consequences of the thyrocyte-specific $G_{\alpha_q}/G_{\alpha_{11}}$ deficiency. Serum TSH (A) and T_4 levels (B) in wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO animals at the indicated ages. *** $P < 0.001$. (C) Western blot of thyroid gland lysates from wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice with normal (<150 ng/ml) and elevated (>500 ng/ml) TSH levels probed with antibodies recognizing $G_{\alpha_q}/G_{\alpha_{11}}$, G_{α_s} or tubulin. (D) Effect of increasing concentrations of TSH on cAMP formation in thyrocytes prepared from wild-type or Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice with normal or high TSH levels. Values are mean \pm SEM.

$G_{\alpha_q}/G_{\alpha_{11}}$ -mediated signaling pathway is not required for TSH-stimulated iodine uptake. To test whether the stimulation of iodine coupling to thyroglobulin by TSH is dependent on $G_{\alpha_q}/G_{\alpha_{11}}$, we measured the amount of iodine incorporated into thyroid proteins. While basal levels of iodine incorporation were the same in wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO animals, stimulation of incorporation by TSH was abrogated in the absence of $G_{\alpha_q}/G_{\alpha_{11}}$ (Figure 4B).

In order to evaluate the role of G_q/G_{11} -mediated signaling for thyroid hormone release, TSH was administered to 4-week-old mice, which are still euthyroid with no apparent alteration in thyroid histology. At this stage, there was also no difference in the T_4 content of thyroids from wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice (7.5 ± 0.6 and 8.05 ± 2.1 $\mu\text{g}/\text{dl}$, respectively). As expected, TSH led to an increase in total T_4 plasma levels in wild-type mice, with a maximal effect after 6 hours (Figure 4C). However, in Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice, the TSH-induced thyroid hormone release was almost completely abrogated (Figure 4C). There was no difference between wild-type and Tg-Cre mice (data not shown).

To test whether the impaired thyroid hormone release in Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice was due to an impaired pinocytotic uptake of colloid, we challenged control and euthyroid Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice with TSH and measured the formation of intracellular pinocytotic vesicles by counting the colloid droplets in the thyrocytes. In wild-type animals, TSH stimulation resulted in the formation of multiple pinocytotic vesicles in thyroid follicular epithelium cells within 5 hours (Figure 4, D and E). However, the number of pinocytotic vesicles after TSH treatment was severely reduced in thyrocytes from Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice and amounted to less than 20% that of wild-type animals.

Lack of goiter development in the $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient thyroid. To study the role of the G_q/G_{11} -mediated signaling pathway in thyroid gland growth, weights of thyroid glands were determined. At the age of 1 month, when there is no significant difference in TSH levels between Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO and control mice, there was no significant difference in thyroid weight either. Interestingly, at the age of 1 year, despite the highly elevated serum TSH levels in the thyrocyte-specific $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient mice, there was no significant increase in thyroid weight compared with control animals (data not shown). This suggested that thyroids from Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice did not respond to elevated levels of TSH by growing. To test the acute effects of TSH on thyroid growth, we treated 6- to 8-week-old wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO animals for 1 week

To test whether the slowly progressing hypothyroidism in some animals was due to incomplete recombination of the floxed G_{α_q} allele or a defect in the G_{α_s} -mediated regulation of cAMP levels, we compared thyrocytes from 5- to 6-month-old Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice with normal and elevated TSH levels. As shown in Figure 2C, $G_{\alpha_q}/G_{\alpha_{11}}$ as well as G_{α_s} protein levels were indistinguishable between the groups. Also, the ability of TSH to induce an increase in cAMP levels in wild-type mice was similar to that in Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice with normal and elevated TSH levels (Figure 2D).

The histology of the Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO thyroid glands at 1 month of age showed no obvious difference in follicle size or form or staining of the colloid compared with control mice. However, at 6 months of age, concomitant with elevated TSH and reduced thyroid hormone levels, the thyroid histology of the thyroid-specific $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice was altered (Figure 3). In these animals the normal thyroid follicular structure was disturbed, with few normal follicles left. Follicle cells were often enlarged and columnar and had large nuclei. Despite the long-term elevation of TSH levels and the follicular cell changes, the thyroids of the Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice were not significantly larger than those of controls. Nor was the thyroid weight increased: thyroid weights were 1.65 ± 0.09 mg in control mice ($n = 14$) and 1.58 ± 0.15 mg in Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice ($n = 8$).

Defect of TSH-induced regulation of thyroid function in the absence of $G_{\alpha_q}/G_{\alpha_{11}}$. To analyze potential defects in thyrocytes resulting from $G_{\alpha_q}/G_{\alpha_{11}}$ deficiency, we determined several cellular functions required for thyroid hormone formation, storage, and release. The functional studies were performed at the age of 1–2 months, when thyroid morphology was still normal and animals were euthyroid with normal TSH levels. TSH has previously been shown to increase iodine uptake in thyrocytes (6). As shown in Figure 4A, there was no significant difference between basal and TSH-stimulated iodine uptake between 6-week-old control and thyrocyte-specific $G_{\alpha_q}/G_{\alpha_{11}}$ -deficient mice, indicating that the

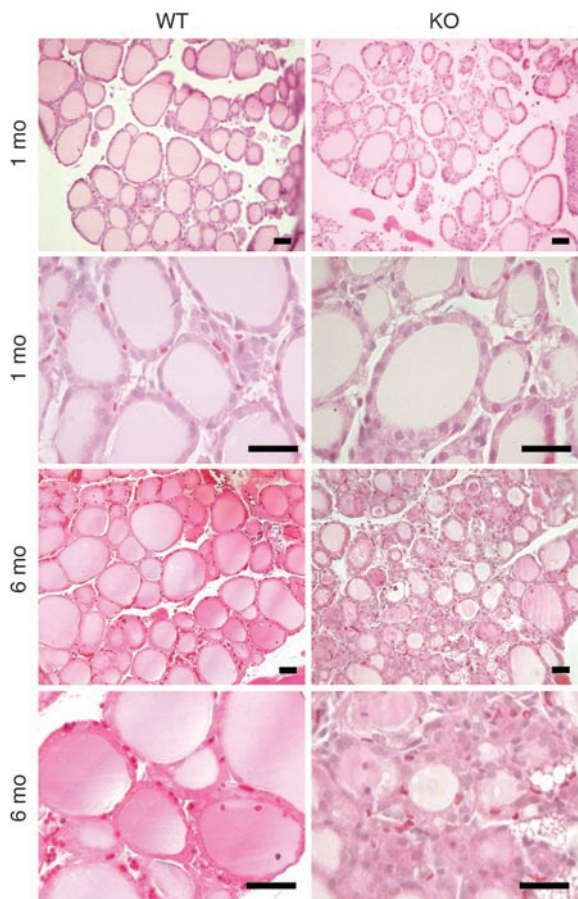


Figure 3

Histological analysis of thyroids from wild-type and Tc-G α_q /G α_{11} -KO animals at the indicated ages. Shown are representative sections from thyroids of at least 10 mice of each genotype and age group. Scale bars: 50 μ m.

with TSH. While the weight of wild-type thyroid increased by about 100%, no increase was observed in G α_q /G α_{11} -deficient thyroids in response to TSH (Figure 5, A and B). Instead, thyroids of Tc-G α_q /G α_{11} -KO mice showed decreased weight, caused by a slight reduction in follicle lumen size (see below). In wild-type animals, TSH treatment resulted in an increase in cell number as well as in cell size, while in Tc-G α_q /G α_{11} -KO mice, no increase in cell proliferation was observed (Figure 5C). While the average number of thyrocytes per follicle increased from 13.3 to 17.2 after treatment of wild-type animals with TSH, the number of thyroids per follicle found under basal conditions in Tc-G α_q /G α_{11} -KO animals did not increase after treatment with TSH (11.8 and 10.9, respectively). However, G α_q /G α_{11} -deficient thyrocytes still showed a hypertrophic response to TSH (Figure 5D). In addition, unlike wild-type thyroids, the colloid area in Tc-G α_q /G α_{11} -KO mice was reduced after TSH treatment (Figure 5E).

To test the response of G α_q /G α_{11} -deficient thyrocytes to inhibition of thyroid function, 1- to 2-month-old animals were fed a goitrogenic diet containing sodium perchlorate and methimazole. After 3 weeks of goitrogenic diet, the thyroid weight of the wild-type animals increased more than 2-fold compared with animals that received control diet (Figure 6A). Interestingly, in the Tc-G α_q /G α_{11} -KO mice, there was no significant difference in thyroid weight between the treated and untreated groups, indicating that G α_q /G α_{11} proteins are required for goiter development. TSH values increased 20-fold in wild-type and about 30-fold in Tc-G α_q /G α_{11} -KO mice 2–3 weeks after starting the diet (data not shown).

The failure of Tc-G α_q /G α_{11} -KO mice to respond to goitrogenic diet with an enlargement of the thyroid was accompanied by a lack of corresponding changes in the morphology of the thyroid (Figure 6B). While wild-type mice showed hyperplastic goiters characterized by colloid-depleted follicles, large vessels, and varying epithelial cell size, thyroids of Tc-G α_q /G α_{11} -KO animals did not show cell proliferation and showed much less vascularization than did thyroids from wild-type animals fed the goitrogenic diet (Figure 6B). The lack of cell proliferation in Tc-G α_q /G α_{11} -KO mice fed the goitrogenic diet was verified by the determination of BrdU incorporation (Figure 6C). While wild-type animals showed 10 ± 2.1 BrdU-positive cells per visual field in thyroid sections, less than 1 BrdU-positive cell per field was observed in Tc-G α_q /G α_{11} -KO mice and in mice fed normal diet. Because goiter-associated angiogenesis requires the synthesis of various vascular growth factors by thyrocytes (25), we determined the amount of VEGF in thyroids from wild-type and Tc-G α_q /G α_{11} -KO mice before and 3 weeks after starting the goitrogenic diet. As seen in Figure 6D, wild-type thyroids showed an increase in VEGF levels after treatment with goitrogenic diet. In contrast, in the absence of G α_q /G α_{11} there was already a lower basal level of VEGF in thyroid lysates, and treatment with goitrogenic diet did not induce any change in VEGF levels.

Discussion

In this study we have addressed the role of G $_q$ /G $_{11}$ -mediated signaling in the regulation of thyroid function by generating a conditional genetic mouse model lacking both G α_q and G α_{11} proteins in thyrocytes. While the deletion of genes encoding G α_q and G α_{11} proteins occurred perinatally, the lack of G $_q$ /G $_{11}$ did not lead to any obvious defect in postnatal development of the thyroid gland, as indicated by normal thyroid histology and normal serum TSH and thyroid hormone levels for up to 2 months. However, by that age, the thyroid-specific G α_q /G α_{11} -deficient mice showed impaired thyroid hormone formation and secretion when acutely challenged with TSH. Starting at 2 months of age, most of the Tc-G α_q /G α_{11} -KO animals slowly developed hypothyroidism, with elevated serum TSH levels and alteration in thyroid histology appearing later in life. Despite the highly elevated TSH levels at 6 months of age, the thyroid weight of the G α_q /G α_{11} -deficient mice was not increased. In addition, the lack of G α_q /G α_{11} proteins in thyrocytes prevented thyroid growth when challenged with TSH or goitrogenic diet.

While basal and TSH-stimulated iodine uptake was normal, the incorporation of iodine into thyroid proteins in response to TSH was impaired in Tc-G α_q /G α_{11} -KO mice. This indicates that the rapid TSH-dependent stimulation of iodine uptake via the Na $^+$ /I $^-$ symporter is not mediated by the G $_q$ /G $_{11}$ -dependent pathway, but rather involves G $_s$ -mediated cAMP formation (26). In contrast to the TSH-dependent regulation of iodine uptake, iodination in response to TSH requires signaling through the G $_q$ /G $_{11}$ -mediated pathway. This is consistent with earlier reports suggesting a regulation of peroxidase primarily through Ca $^{2+}$ and PKC (27, 28).

Mice lacking the G $_q$ /G $_{11}$ -mediated signaling pathway show impaired thyroid hormone secretion in response to TSH. TSH-

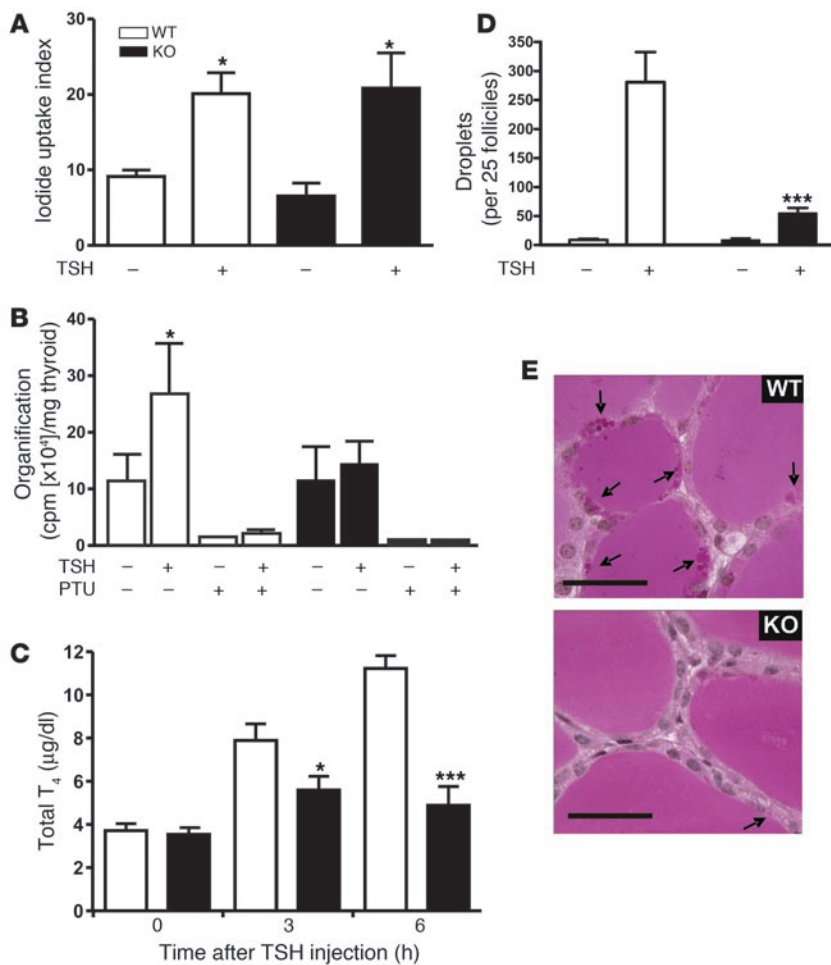


Figure 4

Cellular effects of TSH in wild-type and Tc-Gα_q/Gα₁₁-KO mice. (A–C) Iodine uptake (A), organification (B), and thyroid hormone release (C) were determined as described in Methods. TSH was administered i.p. at a dose of 100 mIU/100 g body weight. As a control, organification was suppressed by treatment of animals with propylthiouracil (PTU; 0.4 mg/10 g body weight i.p. –24, –12, and 0 h relative to TSH administration). **P* < 0.05, ****P* < 0.001 versus non-TSH-treated (A and B) or wild-type (C). Results in A–C are representative of experiments performed at least 3 times with 3–4 animals per group. (D) Periodic acid-Schiff–stained colloid droplets in follicle epithelial cells of thyroid from wild-type and Tc-Gα_q/Gα₁₁-KO animals treated or not with TSH (100 mIU/10 g body weight) for 5 hours (*n* = 6). ****P* < 0.001 versus wild-type. (E) Representative sections of wild-type and Tc-Gα_q/Gα₁₁-KO animals treated for 5 hours with TSH after staining with periodic acid-Schiff stain. Arrows indicate clusters of droplets. Scale bars: 50 μm.

induced thyroid hormone secretion is initiated by internalization of thyroglobulin via macropinocytosis (29–31). There is evidence that TSH-induced pinocytotic uptake of thyroglobulin and thyroid hormone release are mediated by the cAMP-dependent pathway (7, 32). However, other mediators have also been suggested to play a role in processes leading to thyroid hormone secretion. In sheep thyroid cells, for example, [Ca²⁺]_i has been shown to regulate hormone secretion in vitro (33). Our results indicate that the G_q/G₁₁-mediated signaling pathway in mice is required for the macropinocytotic uptake of thyroglobulin in response to TSH.

In addition to macropinocytosis, endocytotic processes can contribute to the uptake of thyroglobulin by thyrocytes (34). The recently described endocytosis of thyroglobulin via megalin is followed by the apical to basolateral transcytosis of thyroglobulin with low hormone content (35, 36). The transcytotic removal of hormone-poor thyroglobulin is believed to increase lysosomal degradation of hormone-rich thyroglobulin and hence hormone secretion. There is, however, no evidence that megalin or other endocytic receptors are regulated via G protein-mediated signaling pathways (37). During and after pinocytotic uptake of thyroglobulin, thyroid hormone is released via enzymatic digestion by cathepsins (38, 39). The effect of the Gα_q/Gα₁₁-mediated signaling pathway on proteolysis was not studied here, but our data indicate that the lack of Gα_q/Gα₁₁ proteins in thyrocytes impairs the process of secretion already at the level of colloid uptake via pinocytosis.

The fact that serum TSH levels in 2-month-old animals were normal while their response to TSH treatment was impaired suggests that under normal in vivo conditions the thyroid can fully compensate the partial defect in TSH responsiveness for a variable time period. However, challenge with high, supraphysiological TSH concentrations revealed the defect even when thyroid function in vivo was completely normal and the defect was compensated.

Abnormal thyrocyte cell proliferation underlies a variety of diseases, including various forms of goiter and thyroid neoplasia. In many cases thyroid proliferation has been shown to be under the control of TSH, and the cAMP-mediated signaling pathway is believed to play a predominant role in the mitogenic effects exerted by TSH (6, 40, 41). The induction of thyroid growth by a goitrogenic diet consisting of thyrostatic drugs is thought to be initiated by increased pituitary secretion of TSH, which results in thyroid cell hyperplasia accompanied by hypervascularization caused by angiogenesis within 1–2 weeks (42, 43). The lack of thyrocyte proliferation in Tc-Gα_q/Gα₁₁-KO mice in response to TSH or goitrogenic diet indicates that downstream mediators of the G_q/G₁₁-mediated signaling pathway are required for TSH-induced thyroid cell proliferation as well as for thyroid growth in response to goitrogenic diet. This is consistent with previous data indicating that G_q/G₁₁ can mediate mitogenic effects in different cell types (44, 45).

Other than the follicular cell hyperplasia, thyroid enlargement caused by nontoxic goiter is also characterized by an early vascu-

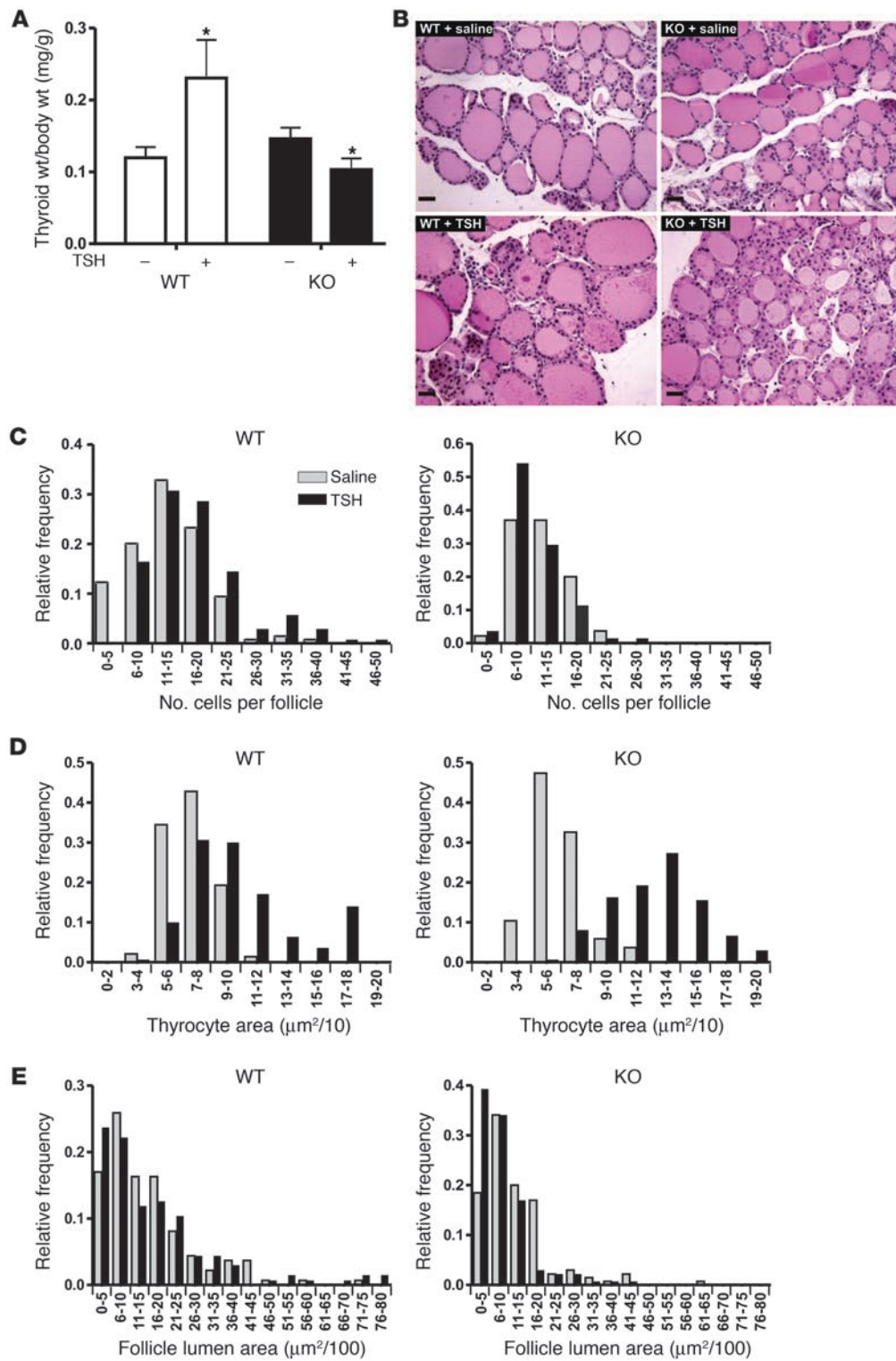
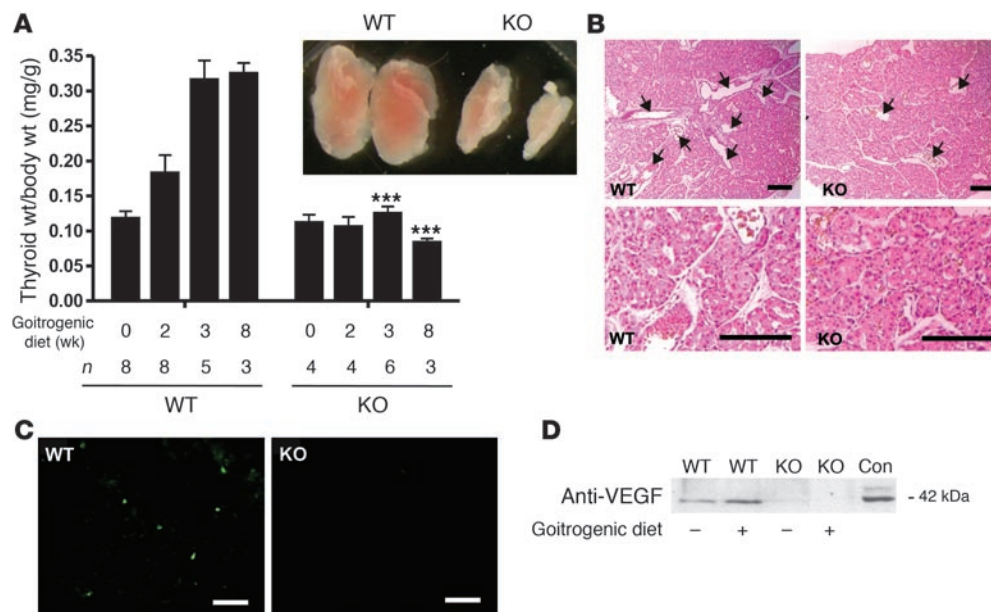


Figure 5
 Role of $G\alpha_q/G\alpha_{11}$ in TSH-induced thyroid growth. Wild-type and $Tc-G\alpha_q/G\alpha_{11}$ -KO mice were treated or not with TSH (50 mIU/10 g body weight) twice daily for 7 days. **(A)** Animals were sacrificed and thyroid weights were determined. Values are mean \pm SEM ($n = 4$ per group). **(B)** Thyroids were fixed, sectioned, and stained with hematoxylin and eosin. Scale bars: 50 μ m. **(C–E)** In stained sections, the number of cells per follicle **(C)**, the individual thyrocyte area **(D)**, and the follicle lumen area **(E)** were morphometrically analyzed. See Methods for details. * $P < 0.05$ versus non-TSH-treated.

lar response resulting in hypervascularization and abnormally enlarged blood vessels. There is good evidence that the goiter-associated angiogenesis is initiated by the production of endothelial growth factors including VEGF by thyrocytes exposed to increased TSH levels (25, 46, 47). The fact that no increase in VEGF levels in response to goitrogenic diet was observed in $Tc-G\alpha_q/G\alpha_{11}$ -KO

mice indicates that induction of VEGF expression requires an intact G_q/G_{11} -mediated signaling pathway.

The analysis of the intracellular signaling mechanisms regulating thyroid follicular cell function and growth has led to the concept that the G_s -dependent cAMP-mediated signaling pathway is the principal mechanism through which TSH and other factors acting via G pro-

**Figure 6**

Role of $G_{\alpha_q}/G_{\alpha_{11}}$ in goiter development. (A) Wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice were treated with goitrogenic diet 0–8 weeks, and thyroid weights were determined thereafter. Inset: thyroids of wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice after 8 weeks of treatment. Values are mean \pm SEM. *** $P < 0.001$ versus wild-type. (B) Sections of thyroids from wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice after 2 weeks of goitrogenic treatment. Arrows indicate blood vessels. (C) After 20 days of goitrogenic treatment, wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice were injected with BrdU, and thyroid sections were prepared and stained with anti-BrdU antibodies to determine cell proliferation. Scale bars: 125 μ m. (D) Thyroid homogenates from wild-type and Tc- $G_{\alpha_q}/G_{\alpha_{11}}$ -KO mice treated with normal or goitrogenic diet for 8 weeks were separated on SDS-PAGE and immunoblotted. Shown is an immunoblot with an anti-VEGF antibody. Con, control (Panc-1 cell lysates).

tein-coupled receptors increase thyroid function and growth. Our data, based on a thyrocyte-specific knockout of the G_{α_q}/G_{11} -mediated signaling pathway, reveal an essential role of these G proteins in mediating the regulation of thyroid function. We show that G_{α_q}/G_{11} -mediated signaling processes are required for thyroid hormone formation and release as well as for the adaptive growth of the thyroid. Thus, inhibition of G_{α_q}/G_{11} -mediated signaling processes or their downstream effectors may be a promising strategy to interfere with diseases characterized by increased thyroid function and/or growth.

Methods

Experimental animals and treatments. Procedures of animal care and use in this study were approved by the Regierungspräsidium Karls-ruhe (Karls-ruhe, Germany). For the analysis of serum hormone levels, 100 μ l of blood was drawn from the retroorbital plexus under anesthesia with xylazine hydrochloride (3 mg/kg body weight) and ketamine hydrochloride (100 mg/kg body weight). For collecting tissue samples, mice were sacrificed with CO_2 . To induce goiter, a group of mice was treated with 5 g/l sodium perchlorate and 0.05 g/l methimazole (Sigma-Aldrich) in the drinking water.

Generation of mice expressing Cre recombinase in thyrocytes. In order to generate a mouse line expressing Cre recombinase specifically in thyroid epithelial cells, a 100-kb PAC containing the mouse thyroglobulin locus was isolated from the mouse genomic RPCI21 library using a mouse thyroglobulin EST probe (48). The coding sequence of the iCre recombinase (49), followed by the bovine growth hormone polyadenylation signal and an ampicillin resistance cassette (bla) flanked by *frt* sites (50), was amplified twice using nested PCR to fuse sequences from the proximal promoter and first intron

of the gene encoding thyroglobulin to the iCre-*frt*-bla-*frt* construct. By homologous recombination in *E. coli* (51, 52), this fragment was introduced into the Tg PAC in such a way that the ATG of the iCre matched the Tg ATG. After removal of the selection cassette by Flp-mediated recombination, the recombined PAC containing the Tg-Cre gene and the Cre cDNA was injected into pronuclei of fertilized mouse oocytes. Mice were analyzed for PAC insertion by Southern blot and genomic PCR amplification and backcrossed into the C57BL/6 mouse genomic background.

Histology, tissue staining, and BrdU labeling. Cre recombinase activity was evaluated by crossing Tg-Cre mice with the *Gt(ROSA)26Sor* Cre reporter mouse line (The Jackson Laboratory) (24). Animals were histologically analyzed for lacZ expression in different tissues using whole mount β -galactosidase staining as described previously (24). For the evaluation of thyroid histology, thyroids were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. For the counting of droplets, fixed and sectioned thyroids were treated with

periodic acid-Schiff stain as previously described by Gerber et al. (53). Histological morphometry was performed using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>) on 5 randomly selected slides from 3 thyroids of each group. From each slide, 30 thyroid follicles were analyzed, and the following parameters were determined and quantified: colloid-containing area, whole follicle area, thyrocyte area (whole follicle area minus colloid area), number of visible nuclei, and average thyrocyte size (thyrocyte cell area divided by number of visible nuclei). For BrdU labeling, mice were injected i.p. with 10 μ g BrdU per kg body weight and sacrificed 1 hour after injection. Paraffin sections of the thyroid glands were stained with anti-BrdU antibody (BD Biosciences).

Determination of TSH, thyroid hormones, and cAMP levels. Serum TSH concentrations were measured using a specific mouse TSH radioimmunoassay provided by A.F. Parlow (Pituitary and Antisera Center, Harbor-UCLA Medical Center, Los Angeles, California, USA). The serum thyroid hormones T_4 and T_3 were measured with a commercial ELISA (Trinity Biotech) according to the manufacturer's instructions.

Culture of mouse primary thyrocytes and determination of $[Ca^{2+}]_i$ and cAMP levels. Mouse primary thyrocytes were prepared as described previously (54). For cAMP determinations, the cells were washed with PBS, and the medium was replaced with modified Coon's F12 medium containing 0.1% bovine serum albumin and 0.5 mM 3-isobutyl-1-methylxanthine in the absence or presence of the indicated concentrations of bovine TSH. For cAMP measurements, media were collected after 1 or 3 hours, boiled for 5 minutes, and stored at $-80^\circ C$ until measurement. The cAMP concentration were determined using an ELISA system from Cayman Europe. Cells grown on 10-mm-diameter coverslips were washed twice with HBSS (pH 7.4) and incubated in the same buffer with 3 mM fura-2/AM containing 0.04% (w/v)



Pluronic F-127. After a 30-minute incubation at 37°C, the cells were washed twice and incubated for another 30 minutes. To monitor the changes in $[Ca^{2+}]_i$, the coverslips were mounted in a holder and the fluorescence at 510 nm was measured. The excitation wavelength alternated between 340 and 380 nm in intervals of 500 ms. Changes in $[Ca^{2+}]_i$ are given as the ratio of 340-nm to 380-nm intensities.

Western blotting. For Western blot analyses, thyroid gland lysates were analyzed by Western blot using anti- α_q/α_{11} and anti-VEGF antibodies (Santa Cruz Biotechnology Inc.) and antibodies against ERK1/2 (Cell Signaling Technology).

Iodine uptake and iodination. For iodine uptake experiments, Na ^{125}I was administered i.p. (0.1 mCi/10 g body weight) with or without TSH (100 mIU/100 g body weight) 3 hours before the mice were sacrificed. To analyze incorporation of iodine, animals were first treated with TSH (100 mIU/10 g body weight) twice, 24 and 12 hours before administration of ^{125}I , together with a third dose of TSH; 5 hours later, animals were sacrificed. The whole thyroid gland and a piece of liver were dissected, weighed, and counted in a γ counter. An uptake index was calculated as counts of thyroid per weight of thyroid tissue divided by counts of liver per weight of liver. Organification was determined after trichloroacetic acid (10% w/v) precipitation of thyroid homogenates as previously described (55).

Statistics. The Statview program (Windows version 4.57, Abacus Concepts Inc.) was used for ANOVA and for Fisher's protected least-significant-difference post-hoc tests. A *P* value less than 0.05 was considered significant. Results are presented as mean \pm SEM.

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