Maintaining the thyroid gland in mutant thyroglobulin–induced hypothyroidism requires thyroid cell proliferation that must continue in adulthood

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Congenital hypothyroidism with biallelic thyroglobulin (Tg protein, encoded by the TG gene) mutation is an endoplasmic reticulum (ER) storage disease. Many patients (and animal models) grow an enlarged thyroid (goiter), yet some do not. In adulthood, hypothyroid TGcog/cog mice (bearing a Tg-L2263P mutation) exhibit a large goiter, whereas adult WIC rats bearing the TGrdw/rdw mutation (Tg-G2298R) exhibit a hypoplastic thyroid. Homozygous TG mutation has been linked to thyroid cell death, and cytotoxicity of the Tg-G2298R protein was previously thought to explain the lack of goiter in WIC-TGrdw/rdw rats. However, recent studies revealed that TGcog/cog mice also exhibit widespread ER stress–mediated thyrocyte death, yet under continuous feedback stimulation, thyroid cells proliferate in excess of their demise. Here, to examine the relative proteotoxicity of the Tg-G2298R protein, we have used CRISPR–CRISPR-associated protein 9 technology to generate homozygous TGrdw/rdw knock-in mice in a strain background identical to that of TGcog/cog mice. TGrdw/rdw mice exhibit similar phenotypes of defective Tg protein folding, thyroid histological abnormalities, hypothyroidism, and growth retardation. TGrdw/rdw mice do not show evidence of greater ER stress response or stress-mediated cell death than TGcog/cog mice, and both mouse models exhibit sustained thyrocyte proliferation, with comparable goiter growth. In contrast, in WIC-TGrdw/rdw rats, as a function of aging, the thyrocyte proliferation rate declines precipitously. We conclude that the mutant Tg-G2298R protein is not intrinsically more proteotoxic than Tg-L2263P; rather, aging-dependent difference in maintenance of cell proliferation is the limiting factor, which accounts for the absence of goiter in adult WIC-TGrdw/rdw rats.

The vertebrate thyroid gland supplies the entire supply of thyroxine (also known as T4) to the body (1). T4 biosynthesis normally occurs upon secretion of thyroglobulin (Tg protein, encoded by the TG gene) (2) from thyroid epithelial cells into the (extracellular) thyroid follicle lumen, in which the secreted Tg protein is stored (3). The iodination of proteins contained within the follicle lumen (4) includes tyrosyl residues on secreted Tg (5), triggering a coupling reaction that promotes formation of T4 within Tg (6–8). Hundreds of different TG gene mutations altering the primary structure of the Tg protein have been linked to defective thyroid hormone biosynthesis (9). Tg is synthesized within the endoplasmic reticulum (ER); most if not all mutant Tg is thought to misfold and become entrapped within the ER (3). As Tg is the single most abundant protein within the thyrocyte translome, the misfolding of mutant Tg induces significant ER stress (10–15). On the one hand, primary hypothyroidism with reduced circulatory thyroid hormone levels results in a compensatory upregulation of the pituitary secretion of thyroid stimulating hormone (TSH) to induce hyperplastic (i.e., proliferative) thyroid gland growth (16–20); on the other hand, the ER stress caused by misfolded mutant Tg has been found to be associated with thyroid cell death in both animal models and human patients (21). Indeed, we recently demonstrated that, albeit inefficient, T4 can be synthesized on mutant Tg released in the follicle lumen from dead thyrocytes, and thyroid goiter growth (proliferation in excess of cell death) helps to provide the cells that sustain this mechanism (21).

It is not guaranteed that net goiter growth will occur in all individuals with hypothyroidism from biallelic TG mutations. Whereas TGcog/cog (congenital goiter) mice (bearing Tg-L2263P) are famous for their adult goiter (22), WIC-TGrdw/rdw rats (bearing Tg-G2298R) do not develop a goiter (23); this was the first model of the disease in which thyrocyte cell death was noted (24). This absence of goiter has been attributed to increased rdw-Tg proteotoxicity (25). However, original descriptions of the thyroid phenotype of TGcog/cog mice and WIC-TGrdw/rdw rats were limited to the very different AKR/J mouse and Wistar–Imamichi rat strain backgrounds, respectively, in which species- and strain-specific genetic interactions might distinctly alter the balance of thyroid cell growth and death. JAX laboratories currently distribute the TGcog allele in the C57BL6J background, and here, we have used CRISPR/CRISPR-associated protein 9 (Cas9) technology to generate homozygous TGrdw/rdw knock-in mice in the same strain background. The vertebrate thyroid gland supplies the entire supply of thyroxine (also known as T4) to the body (1). T4 biosynthesis normally occurs upon secretion of thyroglobulin (Tg protein, encoded by the TG gene) (2) from thyroid epithelial cells into the (extracellular) thyroid follicle lumen, in which the secreted Tg protein is stored (3). The iodination of proteins contained within the follicle lumen (4) includes tyrosyl residues on secreted Tg (5), triggering a coupling reaction that promotes formation of T4 within Tg (6–8). Hundreds of different TG gene mutations altering the primary structure of the Tg protein have been linked to defective thyroid hormone biosynthesis (9). Tg is synthesized within the endoplasmic reticulum (ER); most if not all mutant Tg is thought to misfold and become entrapped within the ER (3). As Tg is the single most abundant protein within the thyrocyte translome, the misfolding of mutant Tg induces significant ER stress (10–15). On the one hand, primary hypothyroidism with reduced circulatory thyroid hormone levels results in a compensatory upregulation of the pituitary secretion of thyroid stimulating hormone (TSH) to induce hyperplastic (i.e., proliferative) thyroid gland growth (16–20); on the other hand, the ER stress caused by misfolded mutant Tg has been found to be associated with thyroid cell death in both animal models and human patients (21). Indeed, we recently demonstrated that, albeit inefficient, T4 can be synthesized on mutant Tg released in the follicle lumen from dead thyrocytes, and thyroid goiter growth (proliferation in excess of cell death) helps to provide the cells that sustain this mechanism (21).

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background. Our analysis indicates that the gene product of the $TGrdw$ allele is not intrinsically more proteotoxic than that of the $TGcog$ allele, and our data suggest an alternative explanation for the incapacity of WIC-$TGrdw/rdw$ rats to maintain their hypothyroid goiter in adulthood.

**Results**

**Generation of $TGrdw/rdw$ mice**

Using CRISPR/Cas9 with a suitable guide RNA, we introduced within C57BL6J mouse $TG$ exon 40, the point mutation that encodes Tg-G2298R (Fig. 1A; in the UniProt P01266 system, this numbering would need to add 19 to account for the signal peptide), just 35 residues from the site of the cog mutation in the chaperone domain of Tg (Fig. S1). After crossing with C57BL6J, animals were bred to homozygosity so that $TGrdw/rdw$ mice could be compared with $TGcog/cog$ mice in the identical genetic background. Resection of the thyroid gland of adult animals was followed by tissue homogenization in buffer lacking all denaturants, followed by nondenaturing polyacrylamide gel electrophoresis (i.e., in the absence of SDS, mobility is dependent not on protein mass but rather on charge/mass ratio and hydrodynamic radius (26), and this has been used to separate unfolded and folded Tg monomers from native Tg dimers (27, 28)). After electrotransfer to nitrocellulose and Tg Western blotting of thyroid from B6, $TGrdw/rdw$, and $TGcog/cog$ mice (n = 3 animals per group). Cas9, CRISPR-associated protein 9; gRNA, guide RNA; mAb, monoclonal antibody; Tg, thyroglobulin gene; Tg, thyroglobulin protein.
(Fig. 1B) indicating a less compact structure consistent with an unfolded state (28). When the same samples were analyzed under denaturing conditions by nonreducing SDS-PAGE—although the molecular masses of cog-Tg-L2263P, rdw-Tg-G2298R, and WT-Tg are identical—the mobility of WT-Tg still appeared slightly faster (Fig. 1C), suggesting a more tightly disulfide-linked structure (29, 30). Moreover, in WT thyroid glands, nearly all Tg proteins had migrated out of the ER so that most glycans on those Tg molecules were processed to complex sugars resistant to digestion with endoglycosidase H (Fig. 1D, marked as “R”), whereas all Tg proteins in TG<sup>cog/cog</sup> and TG<sup>rdw/rdw</sup> mouse thyroids remained fully endoglycosidase H-sensitive (Fig. 1D, “S”), indicating failure of the mutant Tg protein to arrive at the Golgi complex.

**Thyroid histology of TG<sup>rdw/rdw</sup> mice**

Careful examination of the histology of TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mouse thyroid glands revealed important differences with that of the WT thyroid. While all three tissues exhibited prominent eosinophilic staining consistent with accumulation of Tg protein, in WT (TG<sup>+/+</sup>) thyroid, the Tg protein was primarily localized extracellularly in the follicle lumen, whereas in TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mouse thyroid glands, most Tg proteins were found in an expanded and a distorted cytoplasm within the thyrocytes (Fig. 2A), although there was some aberrant material in the follicle lumen (that we have recently established is comprised of the detritus of dead cells (21)). From multiple images of thyroid sections derived from 3-month-old animals of each genotype, the cross-sectional area of individual thyrocytes was abnormally expanded approximately eightfold in TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mouse thyroid glands (Fig. 2A) with a fraction of total cellular area occupied by cytoplasm increased to >90% (Fig. 2B) plus an increase of cross-sectional nuclear area (Fig. 2C). With the huge expansion of cytoplasm, the number of thyrocytes actually accommodated within each follicle profile shrank nearly in half (Fig. 2D).

To independently confirm whether Tg protein in mouse thyroid glands was primarily extracellular or intracellular, we coimmunostained these tissues for Tg and ezrin, which delimits the follicle lumen at the apical plasma membrane of thyrocytes. Whereas Tg in WT glands was contained within the ezrin ring (i.e., extracellular, in the follicle lumen), the

![Figure 2. Thyroid histology of TG<sup>rdw/rdw</sup> mice.](image-url)
mutant Tg protein in TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mice was largely excluded from within the ezrin ring, indicating that it had not been transported from thyrocytes to the follicle lumen (Figs. 2B and S3). Indeed, in WT (TG<sup>+/+</sup>) thyroid, the Tg protein distribution was largely nonoverlapping with the ER molecular chaperone, BiP (immunoglobulin heavy-chain binding protein); whereas in TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mouse thyroid glands, the Tg protein was essentially exclusively contained within the swollen ER, colocalized with BiP (Fig. 2C).

**Hypothyroidism of TG<sup>rdw/rdw</sup> mice**

As TG<sup>cog/cog</sup> mice have goiter from hypothyroidism (31), we wished to determine if TG<sup>rdw/rdw</sup> mice were even more hypothyroid by comparing them to TG<sup>cog/cog</sup> mice at 3 months of age and again at 11 months. At 3 months, both sets of mutant animals had >75% inhibition of circulating total T<sub>4</sub> and >65% inhibition of circulating total triiodothyronine (T<sub>3</sub>), accompanied by dramatic elevation of murine TSH that averaged ~25,000 mU/l (Fig. 3A). Accompanying the thyroid hormone defect was body growth retardation for both sets of mutant animals (Fig. 3B), which has been reported in other models of congenital hypothyroidism (32).

In both TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mice, the highly elevated TSH levels tended to decline by more than 50% by 11 months (Fig. 3), either as an aging-related phenomenon or possibly consistent with a small improvement in thyroid hormone output by the mutant thyroid glands over time (which, in the case of TG<sup>cog/cog</sup> mice, is known to be accompanied by goiter growth (22)). Notably, when compared with TG<sup>cog/cog</sup> mice, TG<sup>rdw/rdw</sup> mice never had more elevated TSH (or diminished T<sub>4</sub> or T<sub>3</sub>) levels or more severe growth retardation, indicating that TG<sup>rdw/rdw</sup> did not have more severe hypothyroidism than TG<sup>cog/cog</sup> mice at any age examined (Fig. 3).

**Chronic ER stress in the thyroid glands of TG<sup>rdw/rdw</sup> mice**

In the thyroid gland at 3 months of age, TG<sup>rdw/rdw</sup> mice showed elevated levels of BiP (normalized to tubulin as a loading control), as was also the case in TG<sup>cog/cog</sup> mice (Fig. 4A)—indicative of ongoing ER stress. In addition, the BiP co-chaperone p58<sup>ipk</sup> was also clearly elevated in the mutant thyroid glands compared with WT (Fig. 4A). The ratio of phosphorylated eukaryotic initiation factor 2α (phospho-eIF2α) to total eIF2α, suggestive of activation of the ER stress sensor, PERK—was elevated in the thyroid glands of both

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**Figure 3. Hypothyroidism of TG<sup>rdw/rdw</sup> mice.** A, serum total T<sub>4</sub>, T<sub>3</sub>, and TSH levels of B6, TG<sup>rdw/rdw</sup> and TG<sup>cog/cog</sup> mice at 3 months (black) and 11 months (red) of age (males shown as squares and females as circles). Data are mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 compared with B6 mice of 3 months old, &p < 0.001 compared with B6 mice of 11 months old. B, body weight of B6 (black), TG<sup>rdw/rdw</sup> (red) and TG<sup>cog/cog</sup> mice (blue). The shaded area indicates the body weights of dwarf mice. Data are mean ± SD; **p < 0.01, ***p < 0.001 compared with B6 mice. T<sub>3</sub>, triiodothyronine; T<sub>4</sub>, thyroxine; TG, thyroglobulin gene; TSH, thyroid stimulating hormone.
mouse mutants. Importantly, none of these ER stress markers were more elevated in the thyroid glands of TG<sup>rdw/rdw</sup> mice than in TG<sup>cog/cog</sup> mice (Fig. 4A). Compared with WT mice, elevation of CCAAT/enhancer-binding protein homologous protein (CHOP) mRNA level in the thyroid gland was increased in both TG<sup>rdw/rdw</sup> mice and TG<sup>cog/cog</sup> mice (Fig. 4B) but again was not more elevated in TG<sup>rdw/rdw</sup> mice (4.1 ± 2.2 fold) than in TG<sup>cog/cog</sup> mice (13.2 ± 8.7 fold). Moreover, because chronic continuous ER stress has been associated with subtle signs of dedifferentiation in thyrocytes (11, 33) as well as Ire1-mediated mRNA decay (in various cell types (34–36)), we examined thyroidal TG mRNA levels and observed an average decrease of 56 ± 8% of WT levels in TG<sup>rdw/rdw</sup> mice and 62 ± 14% in TG<sup>cog/cog</sup> mice—both significantly different from WT but not different from each other (Fig. 4C). Finally, in conjunction with the chronically increased ER stress, cell death signaling as measured by poly(ADP-ribose) polymerase (PARP) cleavage was apparent in the thyroid glands of both mutant animals—but with no evidence of greater PARP cleavage in TG<sup>rdw/rdw</sup> mice than in TG<sup>cog/cog</sup> mice (Fig. 4A).

Because both TG<sup>cog/cog</sup> mice and WIC-TG<sup>rdw/rdw</sup> rats are known to exhibit intrathyroidal cell death (21), we performed TUNEL staining on the newly engineered TG<sup>rdw/rdw</sup> mice. As expected, the thyroid glands of TG<sup>rdw/rdw</sup> mice revealed 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen)–positive nuclear material within the lumen of thyroid follicles, associated with positive TUNEL staining (Fig. 5A). While thyrocyte cell death in the mutant mice was significantly greater than background TUNEL staining seen in WT mouse thyroid glands, the TG<sup>rdw/rdw</sup> mice did not exhibit greater TUNEL staining than that found in TG<sup>cog/cog</sup> thyroid (Fig. 5B). Altogether from the results of Figs. 4 and 5, although homozygous expression of Tg-G2298R does indeed produce hypothyroidism, we found no evidence to suggest that this mutant Tg yields more ER stress or more proteotoxicity than the Tg-L2263P expressed in TG<sup>cog/cog</sup> mice.

**Thyroid cell proliferation during chronic ER stress in the thyroid glands of animals bearing TG mutations**

We recently reported that in WIC rats, TG<sup>rdw/rdw</sup> homozygotes do begin to develop thyroid enlargement within the first two postnatal months, but their absence of goiter in adulthood involves an inability to maintain the goiter as a function of aging (21). As TG<sup>cog/cog</sup> mice exhibit thyroid cell death (Fig. 5) but nevertheless manage to grow a large goiter in adulthood, we turned attention to thyroid cell proliferation. By Ki-67 immunostaining of mouse thyroid glands, it was apparent that both TG<sup>rdw/rdw</sup> mice and TG<sup>cog/cog</sup> mutants exhibited sustained enhancement of thyroid cell proliferation well into adulthood (11 months, Fig. 6A). When quantified, TG<sup>rdw/rdw</sup> mice, on average, definitely did not exhibit less thyroid cell proliferation than that observed in TG<sup>cog/cog</sup> mice—and both exhibited significantly greater proliferation than that found in the thyroids of WT animals (Fig. 6B). As ER stress–mediated cell death was not increased (Figs. 4 and 5) and thyroid cell proliferation was not diminished (Fig. 6B), there was no reason why TG<sup>rdw/rdw</sup> mice would not grow a goiter as large as that found in TG<sup>cog/cog</sup> mice. Indeed, when allowed to survive over the first postnatal year of life, multinodular goiters in TG<sup>rdw/rdw</sup> mice at least as large as those found in TG<sup>cog/cog</sup> mice appeared (Fig. S4); vastly larger than the thyroid glands seen in WT animals (Fig. 6C).

Because WIC-TG<sup>rdw/rdw</sup> rats have a dramatic loss of thyroid tissue mass during adulthood (21), the goitrogenesis in TG<sup>rdw/rdw</sup> mice seemed perplexing. We therefore returned to WIC-TG<sup>rdw/rdw</sup> rats to examine thyroid cell proliferation as a function of postnatal age. As noted previously, it is established
that WIC-\textit{TGrdw/rdw} rats do exhibit exuberant thyroid gland growth during the first two postnatal months of life (21); indeed, at 8 weeks of age, there was a dramatic increase of thyroidal Ki-67 immunostaining (Fig. 7, A and B). To our surprise, however, unlike in \textit{TGrdw/rdw} mice (in the C57BL6J genetic background, Fig. 6, A and B), by early adulthood, the hypothyroid WIC-\textit{TGrdw/rdw} rats no longer exhibited thyroid cell proliferation beyond control levels, and this feature persisted as the animals aged further (Fig. 7, A and B). It has been repeatedly reported that WIC-\textit{TGrdw/rdw} rats during this period of life (18–40 weeks) do maintain markedly elevated circulating TSH levels (23, 37–39), and indeed, we found that older WIC-\textit{TGrdw/rdw} rats (>30 weeks) exhibited a circulating TSH (32.7 ± 10.7 ng/ml; n = 10) that was 16.6-fold elevated over that of their WT counterparts (1.97 ± 0.11 ng/ml; n = 3).

To confirm that the elevated TSH in adult WIC rats should be sufficiently bioactive to stimulate thyroid cell proliferation, we challenged 14-week-old WT WIC rats to induce hypothyroidism, by introducing propylthiouracil (PTU) continuously for 3 weeks before euthanasia and thyroid tissue analysis at 17 weeks. This treatment yielded a similar elevation of circulating TSH (35.8 ± 1.01 ng/ml; n = 3), and these older hypothyroid WT WIC rats exhibited exuberant thyroid cell proliferation well beyond control levels (Fig. 8, A and B). The data suggest that the WIC strain background is specifically deficient neither for a central nervous system response to hypothyroidism nor for the bioactivity of TSH in older animals. Thus, we conclude that the inability of WIC-\textit{TGrdw/rdw} rats to expand thyroidal mass in adulthood is caused neither by a genetic deficiency of the central nervous system axis to provide adequate feedback stimulation nor solely by ER stress–mediated thyroid cell death, but rather by an inability of the adult WIC-\textit{TGrdw/rdw} rat strain to maintain the TSH-driven thyroid cell proliferative response to hypothyroidism that is needed for net thyroid gland growth.

**Discussion**

Chronic, unremitting, and unresolved ER stress is a factor that can promote cell death (40); thus, the field of medicine is just beginning to attack ER stress–mediated cell death as a potential therapeutic approach to address various clinical disorders (41, 42). However, from studies of hypothyroid patients and animal models bearing biallelic \textit{TG} mutations (10) as well as cell culture models of the disease (43), we have been mostly impressed by continued thyrocyte proliferation (11) despite the ongoing stress-mediated thyrocyte cell death (21). In such a case, the entire thyroid gland overgrows, that is, beyond normal size (22). Nevertheless, in individuals with hypothyroidism from biallelic \textit{TG} mutations, net thyroid growth is not always observed. This is also the case in WIC-\textit{TGrdw/rdw} rats, which initiate thyroid growth during the first one or two postnatal months (21) but in adulthood lose the ability to further expand or even maintain the goiter, leading ultimately to thyroid atrophy (23). Despite the remarkably close physical proximity of the encoded mutation from that seen in \textit{TGrdw/rdw} mice (Fig. S1), we considered the possibility that the \textit{TG}–encoded Tg-G2298R protein might be intrinsically more proteotoxic than the \textit{TG}–encoded Tg-L2263P protein. Such a hypothesis can only be tested by comparing the cell biological and physiological impact of the two mutant proteins in the identical genetic background. This was made possible using CRISPR/Cas9 technology to engineer the \textit{TG} allele into C57BL6J mice (Fig. 1A).

From our analysis, it was immediately apparent that \textit{TGrdw/rdw} mice did not exhibit greater misfolding of the
mutant Tg protein (Fig. 1, B and C) or greater failure of Tg export from the ER to that seen in TGcog/cog mice (Fig. 1D). In addition, the ER entrapment of mutant Tg led to enormous ER swelling that was comparable in the two mouse models (Fig. 2). Furthermore, TGrdw/rdw mice exhibited profound primary hypothyroidism with body growth retardation and initial elevation of circulating TSH level to \( \geq 25,000 \) mU/l, which was not greater than that in TGcog/cog mice (Fig. 3).

Unlike what has been reported in the original AKR/J mouse strain (22), neither TGrdw/rdw nor TGcog/cog mice in the C57BL6/J background (from JAX laboratories; fed Formulab Diet 5008 bearing 0.8–0.97 ppm iodine) could spontaneously restore circulating T4 to euthyroid levels by 11 months of age; instead showing only marginal improvement of their dramatically elevated levels of circulating TSH (Fig. 3A). A dashed line shows the linear regression of the thyroid size in WT, TGrdw/rdw, and TGcog/cog mice. No significance difference was observed between thyroid glands of TGrdw/rdw and TGcog/cog mice. Tg, thyroglobulin gene.

It is the failure to sustain thyroid cell proliferation in adult WIC-TGrdw/rdw rats (Fig. 7) that leads to the loss of thyroid tissue mass in these animals (21). What could be the cause of this failure to maintain cell proliferation in WIC-TGrdw/rdw rats? We considered the possibility that either the extent of rat
TSH elevation (44)—or the bioactivity of the rat TSH protein itself (45)—might be insufficient in adult rats of the WIC strain. However, when we challenged older WIC rats with chemically induced hypothyroidism, we observed exuberant thyroid cell proliferation (Fig. 8), which seems to exclude that adults of the WIC strain background are deficient in adequate TSH production or action. Second is the possibility that under conditions of chronic continuous ER stress in adult WIC-TG<sup>rdw/rdw</sup> rats, those thyrocytes that do not die may adapt by developing a state of quiescence. Precedence for such a behavior has been noted in secretory cell tumor metastases that exist in a state of “antiproliferative dormancy” (46) with a highly activated ER stress response including markedly upregulated BiP levels (47) similar to what we have observed (this report, Fig. 4A). Indeed, dormant/latent metastatic pancreatic ductal adenocarcinoma cells exhibit a transcriptomic signature in which the single most upregulated pathway is “ER stress response” (including CHOP, linked to stress-mediated cell death; similar to what we have seen herein [Fig. 4B]), and the single most downregulated pathway is “cell division” (48). Indeed, deficiency of the MYRF transcription factor has been linked precisely to the same phenotype (49). Even in non-tumorous pancreatic beta cells, sustained ER stress (from genetically encoded misfolded proinsulin—a secretory protein expressed abundantly as Tg protein is in thyrocytes) or impaired ER stress response (from defective PERK; a kinase that phosphorylates eIF2α) has been linked to impaired cell proliferation (50–52). Thus, loss of thyrocyte proliferation in adult WIC-TG<sup>rdw/rdw</sup> rats may in many ways be an expected result. What is remarkable is that neither hypothyroid TGrdw/rdw mice (this report) nor TG<sup>cop/cop</sup> mice (22)—nor many human patients with biallelic TG mutations (14, 53)—share this growth-arrest phenotype.

Why then, would the proliferative thyroid cell response to TSH be self-limited during extended concurrent ER stress in WIC-TG<sup>rdw/rdw</sup> rats?

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**Figure 7. Thyroid cell proliferation in WIC-TG<sup>rdw/rdw</sup> rats.**

A, Ki67 immunohistochemistry of the thyroid gland of WT and WIC-TG<sup>rdw/rdw</sup> rats at 8 weeks, 14 weeks, and over 30 weeks of age. The scale bars represent 20 μm. B, quantification of immunostained sections like those shown in A, presented as Ki67-positive nuclei in the proportion of total nuclei in the thyroids of WT WIC and WIC-TG<sup>rdw/rdw</sup> rats (n = 3–4 animals per group; each color represents a single animal; each point is an independent section; square = male, circle = female). Data are mean ± SD; ***p < 0.001. TG, thyroglobulin gene.

**Figure 8. Thyroid cell proliferation in PTU-induced hypothyroid adult WT rats.**

A, Ki67 immunohistochemistry of the thyroid gland of control WT and PTU-treated WT rats at 17 weeks of age. Treated rats received PTU chow at 14 weeks of age for 21 days. The scale bars represent 20 μm. B, quantification of A presented as Ki67-positive nuclei in the proportion of total nuclei in the thyroids of control WT and PTU-treated WT rats (n = 3–4 animals per group; each color represents a single animal; each point is an independent section, males shown as squares and females as circles). Data are mean ± SD; ***p < 0.001. PTU, propylthiouracil.
Multiple factors contribute to the development and maintenance of a large goiter. Congenital hypothyroidism with biallelic mutations, among other environmental factors, underscores the importance of maintaining a balance of thyroid hormone levels to prevent the formation of goiters.

In the context of stress that underlies a variety of degenerative diseases, it is crucial to understand the mechanisms that regulate tissue regeneration and growth. The chronicity of endoplasmic reticulum (ER) stress plays a significant role in tissue regeneration and growth processes. While the exact mechanisms are still under investigation, recent studies have shed light on the role of various cell cycle genes and their impact on thyroid cell proliferation.

### Experimental procedures

**Primary antibodies**

- Anti-Ki67 (SP6) (catalog no.: ab16667; Abcam); monoclonal antibody anti-Tg (catalog no.: 365997, Santa Cruz; catalog no.: ab156008, Abcam); rabbit anti-Tg and rabbit anti-BiP were previously described (13, 27); rabbit anti-Ezrin (catalog no.: PA5-17518; Invitrogen); rabbit anti-p58ipk (catalog no.: 2940; Cell Signaling Technology); rabbit anti-phospho-eIF2α (Ser51) (catalog nos.: 3597 and 9721; Cell Signaling) and total eIF2α (catalog no.: 9722; Cell Signaling); mouse antitubulin (catalog no.: T5168; Sigma); and rabbit anti-PARP (catalog no.: 9542; Cell Signaling).

**Animals**

Six founder TGrdw/+ rats were generated using CRISPR/Cas9: C57BL/6J mice received injection of guide RNA (GGTGTACGCTGACATTGA) and donor vector with Tg-G2298R mutation (ACCATGGAGATGGAAGGCAGTGTTGGTCAGCTGACCATTGACAGCATCCTGGCTGCA) targeting Tg exon 40 (GenePharmatech). A 534-base PCR amplicon from genomic DNA (forward primer: 5'-GTATCAAGGCAGGCAAGA-3'; reverse primer: 5'-CAGGACTCAAGAGAGATCCCTGGGACTGCGAAG-3') was sequenced to confirm a male F0 generation mouse that harbored the heterozygous Tg+/rdw mutation, the ultimate extent of tissue regeneration and replicative growth within tissues experiencing the chronic ER stress that underlies a variety of degenerative diseases. In the meantime, what emerges from our current studies is that in congenital hypothyroidism with biallelic Tg mutation, the ultimate development and maintenance of a large goiter requires that thyroid cell proliferation must continue unabated in adulthood.

**Western blotting**

Mouse thyroid glands were mechanically homogenized either in nondenaturing lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% NP-40, and 2 mM EDTA) or in radioimmunoprecipitation assay buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS; Thermo Fisher Scientific). Protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific) were added to cell lysis buffer. Protein concentration was measured by bicinchoninic acid assay (Thermo Fisher Scientific). Gel sample buffer either omitted (Fig. 1B) or included SDS and boiling (Fig. 1C) and then were resolved by 3 to 8% PAGE in the absence (Fig. 1B) or the presence of SDS (Fig. 1C). For Figures 1D and 4A, lysates were boiled in SDS-gel sample buffer plus 50 mM DTT and then resolved by SDS—straight 4.5% or gradient 4 to 12% PAGE. Proteins were electrotransferred to nitrocellulose membranes, blocked with 5% milk, immunoblotted with the indicated antibodies and appropriate horseradish peroxidase–conjugated secondary antibody, and visualized by enhanced chemiluminescence. Endoglycosidase H digestion was performed as previously described (21).

**Histology and immunostaining of thyroid sections**

Thyroids from mice and rats were dissected, immersion fixed with 10% formalin, paraffin embedded, sectioned, and stained with hematoxylin–eosin (Vector). For immunofluorescence, thyroid sections (6 μm) were deparaffinized in CitriSolv, followed by antigen retrieval in citrate buffer (12.3 mM pH 6), and blocked in 1.5% normal goat serum for 30 min at room temperature before incubation with primary antibodies overnight at 4 °C. After washing, the sections were then incubated with Alexa Fluor–conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature. Sections were counterstained with Prolong-Gold and DAPI (Invitrogen) and imaged with a Nikon A1 confocal microscope. For immunohistochemistry, anti-Ki67 staining was performed as previously described (21). Images were obtained in a Leica DMI-3000B microscope (40× objective). Analysis and quantification (Ki67-positive nuclei as a proportion of total nuclei) were performed by observers blinded to the genotypes and groups.

**Serum hormone measurement**

Serum total T₄, total T₃, and TSH concentrations were measured using radioimmunoassays as previously described (61, 62).

**PCR**

Total RNA isolation from the mouse thyroid glands was performed using RNAeasy Plus kit (Qiagen), followed by reverse transcriptions into cDNA. This cDNA was then used as a template for PCR amplification.
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transcription using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). For real-time PCR, PowerUp SYBR Green PCR Master Mix (Applied Biosystems) was used on a StepOnePlus PCR system (Thermo Fisher Scientific). CHOP and Tg mRNA levels were normalized to 18S RNA; primers are as follows: CHOP (forward: 5’-CCTGAGAGAGGTGTCCAG-3’, reverse: 5’-GACA CGGTCTCAGGTTGAA-3’); Tg (forward: 5’-TGATCTGATCACAATCACAAACAG-3’, reverse: 5’-ATTCCAGTCCTGTCAGCC-3’); and 18S (forward: 5’-GGCGTGCCCCAACTTCTTA-3’, reverse: 5’-GGGCATCAGACCTGTATATC-3’).

**TUNEL labeling**

The In Situ Cell Death Detection Kit, Fluorescein (Roche) was used for TUNEL staining. Sections were counterstained and mounted with Prolong-Gold and DAPI. Fluorescence images were captured in a Nikon A1 confocal microscope. Quantification of TUNEL-positive nuclei in the proportion of total nuclei was performed using Imaris software (version 7.7.2; Imaris).

**Thyroid gland size measurement**

The areas of the thyroid glands were measured from photographic imaging at the time of dissection with an embedded millimeter rule included in each image, as previously described (21). Thyroid images for 11-month-old mice and older are shown in Fig. S4. Thyroid sizes were quantified using Imaris software (version 7.7.2; Imaris).

**Statistics**

Two-way ANOVA with Tukey’s comparison sample test was used for comparison of two factors (e.g., genotype plus age) and applied for statistical analysis of serum hormones, body weight, and rat thyroid Ki67 immunostaining. Unpaired two-tailed Student’s t test was used for direct comparison of two groups (e.g., CHOP or Tg mRNA level as well as TUNEL staining). One-way ANOVA followed by Tukey’s multiple comparison test was used for multigroup comparison of a single factor (e.g., effect of mouse genotype on thyroid Ki67 immunostaining). All statistical analyses were calculated using GraphPad Prism (GraphPad Software, Inc). All data were expressed as mean ± SD. Thyroid size measurements as a function of age were plotted by simple linear regression. Differences of p < 0.05 were considered significant.

**Data availability**

All data are contained within the article, with primary data available upon request (Dr Peter Arvan, University of Michigan, email: parvan@umich.edu).

**Supporting information**—This article contains supporting information.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: BiP, immunoglobulin heavy-chain binding protein; Cas9, CRISPR-associated protein 9; CHOP, CCAAT/enhancer-binding protein homologous protein; DAPI, 4’,6-diamidino-2-phenylindole; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; PARP, poly(ADP-ribose) polymerase; PTU, propylthiouracil; Tg, thyroglobulin protein; TG, thyroglobulin gene; TSH, thyroid stimulating hormone.

**References**


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