Thyroid hormone synthesis continues despite biallelic thyroglobulin mutation with cell death

Xiaohan Zhang, …, Viviana A. Balbi, Peter Arvan

*JCI Insight*. 2021. [https://doi.org/10.1172/jci.insight.148496](https://doi.org/10.1172/jci.insight.148496).

**Graphical abstract**

Normal Thyroid
Iodinated Tg in the follicle lumen

Homozygous mutant Tg (e.g., rdw/rdw rat)
Tg entrapped in engorged ER; protein delivered to follicle lumen via cell death

Find the latest version:
[https://jci.me/148496/pdf](https://jci.me/148496/pdf)
Thyroid hormone synthesis continues despite biallelic thyroglobulin mutation with cell death

Authors: Xiaohan Zhang¹, Aaron P. Kellogg¹, Cintia E. Citterio¹,²,³, Hao Zhang¹, Dennis Larkin¹, Yoshiaki Morishita¹,⁴, Héctor M. Targovnik²,³, Viviana Balbi⁵, and Peter Arvan¹*

Affiliations:

¹Division of Metabolism, Endocrinology & Diabetes, University of Michigan, Ann Arbor, MI 48105


³CONICET, Universidad de Buenos Aires, Instituto de Inmunología, Genética y Metabolismo (INIGEM), Buenos Aires, Argentina

⁴Division of Diabetes, Department of Internal Medicine, Aichi Medical University, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan

⁵Department of Endocrinology and Growth, Hospital de Niños Sor María Ludovica, La Plata, Argentina

Conflict of interest: The authors declare that no conflict of interest exists.

Short Title: Thyroxine synthesis from dead cells
Complete absence of thyroid hormone is incompatible with life in vertebrates. Thyroxine is synthesized within thyroid follicles upon iodination of thyroglobulin conveyed from the endoplasmic reticulum (ER), via the Golgi complex, to the extracellular follicular lumen. In congenital hypothyroidism from bi-allelic thyroglobulin mutation, thyroglobulin is misfolded and cannot advance from the ER, eliminating its secretion and triggering ER stress. Nevertheless, untreated patients somehow continue to synthesize sufficient thyroxine to yield measurable serum levels that sustain life. We demonstrate that $TG^{W2346R/W2346R}$ humans, $TG^{cog/cog}$ mice, and $TG^{rdw/rdw}$ rats exhibit no detectable ER export of thyroglobulin, accompanied by severe thyroidal ER stress and thyroid cell death. Nevertheless, thyroxine is synthesized and brief treatment of $TG^{rdw/rdw}$ rats with anti-thyroid drug is lethal to the animals. When untreated, remarkably, thyroxine is synthesized on the mutant thyroglobulin protein, delivered via dead thyrocytes that decompose within the follicle lumen, where they are iodinated and cannabilized by surrounding live thyrocytes. As the animals continue to grow a goiter, circulating thyroxine increases. However, when $TG^{rdw/rdw}$ rats age, they cannot sustain goiter growth that provides the dying cells needed for ongoing thyroxine synthesis, resulting in profound hypothyroidism. These results establish a disease mechanism wherein dead thyrocytes support organismal survival.
**Introduction**

In the body, the circulating thyroid hormone, thyroxine (also known as T$_4$) originates exclusively from biosynthesis within the thyroid gland. Thyroxine biosynthesis occurs by a common mechanism in all vertebrates. Specifically, a monolayer of thyrocytes (also known as thyroid follicular epithelial cells) surrounds a central apical (extracellular) lumen, into which thyrocytes deliver a nearly-pure secretion of thyroglobulin (Tg, encoded by the TG gene) (1), which comprises ≥ 50% of the total protein of the thyroid gland (2). Thyrocytes exhibit a polarized distribution of plasma membrane enzymes/activities that coordinate thyroid peroxidase-catalyzed apical iodination of extracellular protein in the luminal cavity (3). Iodination of various tyrosine residues on secreted Tg (4) triggers the formation of T$_4$ intramolecularly within the Tg protein (5, 6) prior to endocytic re-entry of the hormone-containing protein into surrounding thyrocytes for lysosomal digestion, resulting in the proteolytic liberation and release of T$_4$ from the basolateral membrane of thyrocytes to the bloodstream (7).

The first three-dimensional atomic structure of human Tg has recently been reported (8). Already 227 different TG gene mutations have been found to be linked to congenital hypothyroidism (9); as far as is known, essentially all of the structurally-defective Tg mutants are entrapped in the endoplasmic reticulum (ER), causing thyrocyte ER swelling and ER stress (10). Susceptibility to the many different pathogenic mutations is in part explained by the large and complex structure of the Tg protein (8), including its multiple repeat domains bearing internal disulfide bonds, and concluding with the Cholinesterase-Like (ChEL) domain (10). The C-terminal ChEL domain of Tg has no direct impact on thyroidal iodination machinery, but it: a) shares a similar structure with other ChEL family members (11), b) provides information necessary and sufficient for the noncovalent homodimerization needed for intracellular transport (12), c) functions as an intramolecular chaperone required to stabilize the folded structure of upstream repeat domains of Tg (13), and d) provides its own hormonogenic iodination site (14). A number of human patients have been reported with homozygous mutation in the Tg-ChEL domain (e.g., Tg-W2346R or Tg-G2322S; in the UNIPROT P01266 numbering system this would need to include the 19-residue signal peptide) with congenital hypothyroidism (15, 16).
In years past, cases of congenital hypothyroidism could go undiagnosed in early life due to insufficient neonatal screening (17). Classic studies of Marine and Lenhart showed that thyroid hyperplasia is induced as a consequence of primary hypothyroidism (experimentally-induced following partial thyroidectomy or iodide deficiency) in animals (18) or humans (19); i.e., the endocrine feedback of primary hypothyroidism results in chronically upregulated pituitary secretion of Thyroid Stimulating Hormone (TSH) and such chronic stimulation contributes to exuberant growth of the thyroid gland (20-22). Therefore, patients with bi-allelic TG mutations would be expected to present, ultimately, with goiter. Interestingly, however, by linkage analysis, variants of the TG gene are linked to human hypothyroidism with or without thyroid goiter (23). Why hypothyroid patients with bi-allelic TG mutation (and no defect in TSH response) would not develop a goiter is unknown, although in the clinical setting, the understanding of goiter development is often confounded in patients who may or may not have received exogenous thyroxine treatment (24-26).

On the one hand, increased goiter growth might help to overcome genetic or acquired inefficiency of thyroid hormone synthesis (27); on the other, growth of a large goiter in iodine deficiency has been proposed to be a maladaptation (28). In either case, the fundamental knowledge gap has been an understanding of how untreated patients bearing pathogenic, bi-allelic TG mutations could possibly be capable of synthesizing endogenous T$_4$.

Chronic, unremitting and unresolved ER stress is a factor that can promote cell death (29-34). Chronic ER stress is known to occur in the thyrocytes of $TG^{cog/cog}$ (congenital goiter) mice (encoding Tg-L2263P) which are famous for their hyperplastic goiter — and also in $TG^{rdw/rdw}$ rats (35) that do not develop a goiter (36). In all cases of bi-allelic TG mutation, it is thought that massive quantities of mutant Tg protein are blocked in forward advance from the ER, as in the $TG^{cog/cog}$ and $TG^{rdw/rdw}$ thyroid glands, triggering a dramatic ER stress response that is also seen in the thyroid glands of human patients with this disease (35, 37-39). Thyrocyte cell death has never been considered in goitrous $TG^{cog/cog}$ mice or humans with bi-allelic TG mutations, but in $TG^{rdw/rdw}$ rats we posited that thyroid follicular cell death might block the development of goiter (40).
Importantly, untreated $\text{TG}^{\text{cog/cog}}$ mice spontaneously increase their levels of serum $\text{T}_4$ that parallels growth of the thyroid gland, ultimately achieving nearly-normal levels (41). With this in mind, in this report we have analyzed both human and rodent thyroid glands of individuals expressing bi-allelic $\text{TG}$ missense mutations that render Tg incapable of forward trafficking from the ER. Remarkably, we find that thyrocyte cell death and disintegration within the thyroid follicle lumen provides the Tg substrate needed for synthesis of endogenous $\text{T}_4$. The life of untreated individuals depend on this unusual mechanism of endogenous $\text{T}_4$ synthesis, as even a brief exposure of such animals to antithyroid drugs is lethal. Most remarkably, we have uncovered compelling evidence that in this disease, goiter growth is needed to provide an ongoing supply of dead cells in order that thyroid hormonogenesis can be sustained.
Results

Mutant mice and humans bearing bi-allelic TG missense mutations endogenously synthesize T₄ using substrate derived from dead thyrocytes. With age, hypothyroid TG⁺⁺ mice (expressing homozygous Tg-L2263P) spontaneously increase their serum T₄ to eventually reach nearly-normal levels (41), which is perplexing because the mutant cogTg protein cannot exit the ER (37, 38). We performed routine histology of WT and TG⁺⁺ mouse thyroid glands. Whereas thyroid follicles from WT mice show the normal epithelial monolayer of thyrocytes surrounding an abundant proteinaceous extracellular lumen (Fig 1A panels at left), thyrocytes from TG⁺⁺ mice exhibited enormous intracellular distention [comprised of massive ER expansion (42)] with nuclei abnormally “pushed” into the apical cytoplasm (Fig. 1A panels at right). Immunofluorescence of Tg in the thyroid follicles of WT mice revealed densely packed Tg protein within the extracellular lumen (Fig. 1B upper panels) whereas in TG⁺⁺ mice, Tg was detectable in an abnormal, patchy distribution in the follicle lumen (Fig. 1B lower panels). On the one hand, one might expect to find Tg in the follicle lumen, because TG⁺⁺ mice do produce T₄, and Tg is the protein from which T₄ is synthesized (10). Indeed, detectable T₄-containing protein was generated in the lumen of 92.4 ± 10.9% (SD, n = 7 animals) of TG⁺⁺ thyroid follicles, surrounded by a ring of thyrocytes [nucleated cells positive for the thyrocyte-specific transcription factor, Pax8 (Fig. 1C lower panels)]. Thus in TG⁺⁺ mice, mutant Tg can reach the lumen of thyroid follicles, in which T₄ is synthesized. On the other hand, mutant Tg is not thought to be competent for anterograde transport from the ER (37). For each Tg molecule that successfully undergoes anterograde transport from the ER, ~2/3rd of the N-glycans on each molecule acquire Golgi sugar modifications (43, 44), enabling those glycans to acquire resistance to digestion with endoglycosidase H (Endo H) (45). We confirmed that in WT mice, nearly all thyroidal Tg molecules (which, in the steady-state, reside primarily in the extracellular follicle lumen) have acquired Golgi-based ‘complex’ N-glycans and thus have become Endo H-resistant (“R”, Fig. 1D). In contrast, in TG⁺⁺ thyroid glands, no Tg acquires Endo H-resistance (and instead remains completely Endo H-sensitive, marked as “S”, Fig. 1D). These data indicate that no cogTg undergoes intracellular
trafficking to the Golgi complex. Although exosomes cannot convey Endo H-sensitive protein directly from the ER to the extracellular space (46, 47), the foregoing data do suggest that in the $T^cog/cog$ thyroid gland, Tg must arrive in the lumen of thyroid follicles via a delivery mechanism other than the conventional secretory pathway. Interestingly, the patchy distribution of mutant Tg in the follicle lumen appeared associated with additional cellular material, including nuclear chromatin (Fig. 1A, B).

The synthesis of misfolded Tg is accompanied by ER stress (38, 48-51). Ongoing ER stress in the thyroids of $T^cog/cog$ mice was demonstrable (Fig. 1E) with dramatic elevation of ER stress markers including the ER hsp70 chaperone BiP (52) and its co-chaperone p58ipk [encoded by DNAJC3 (53)]. Additionally, ER stress-induced upregulation of CHOP (Fig. 1E) can promote cell death (54, 55). These data (and additional evidence, below) led us to consider that $T_4$ synthesis in $T^cog/cog$ mice might be based on mutant Tg being delivered to the thyroid follicle lumen via thyrocyte cell death. Such a possibility is not without precedent; indeed, upon cell death in other epithelia including renal tubular epithelial cells, mammary epithelial cells, and bronchial epithelial cells, dead cells are extruded selectively to the apical side of the epithelium (56-58), which, in the thyroid, would correspond to the follicular lumen.

In the thyroids of $T^cog/cog$ mice, we could detect abnormal nuclear material in the lumen of 74.0 ± 22.8% (SD, n = 4 animals) of thyroid follicles including DAPI staining suggesting karyolysis and karyorrhexis — swollen nuclei retained in dead cell ghosts with less intense DAPI staining, consistent with a gradual disintegration of dead cells and their chromatin. Images of cell ghosts revealed positivity for CHOP (detected in 33 ± 9.7% of thyroid follicles, Supplemental Fig. S1A) and cleaved caspase-3 (an executioner caspase) was detected in 30 ± 11.4% (SD, n = 4 animals) of total follicles, Supplemental Fig. S1B] and were positive by TUNEL staining, indicating cleaved DNA (Fig. 1F, a dashed line drawn on the merged image highlights the apical luminal cavity). Crucially, >96% of TUNEL-positive cell ghosts were associated with the presence of $T_4$ synthesis (n = 4 animals; and see Fig. 1F). No DAPI-positive, CHOP-positive, cleaved caspase-3-positive, nor TUNEL-positive cells were observed within the thyroid follicle lumen of WT mice (Fig. 1F, Supplemental Fig. S1A, B).
Some of the T₄ synthesized in Tg can occur within small peptide regions that do not require the native globular structure of the entire molecule (8, 59), and in pilot studies using a recently-developed assay for thyroid hormone formation after in vitro iodination (14) of transfected cell lysates, we observed that ER-entrapped recombinant mutant cogTg and rdwTg (described below) have the potential to serve as substrate for T₄ synthesis. Immunoblotting of unpurified thyroid homogenates with anti-T₄ to identify T₄-containing proteins revealed the major Tg hormone-containing fragment [~250 kD (38)] and its degradation products (7) in WT mouse thyroid tissue, whereas TG⁵⁴⁰⁵⁴⁰ thyroid glands did not immediately reveal a clear predominant species (Supplemental Fig. S1C lanes 2-4).

We selectively concentrated T₄-containing protein from TG⁵⁴⁰⁵⁴⁰ thyroid tissue by immunoprecipitation with anti-T₄, followed by immunoblotting of the recovered samples with a mAb that specifically favors recognition of intact Tg (epitope located between Tg residues 1000-1100). As expected, when no tissue sample was included in the anti-T₄ immunoprecipitation, no T₄-containing Tg protein was recovered (Supplemental Fig. S1C lane 8). However, both WT (lane 7) and TG⁵⁴⁰⁵⁴⁰ thyroid glands clearly demonstrated Tg bearing T₄ (Supplemental Fig. S1C lanes 9-11). Moreover, whereas secreted WT Tg is entirely Endo H-resistant, the Tg that was specifically immunoprecipitated from TG⁵⁴⁰⁵⁴⁰ thyroid tissue by virtue of its T₄ content was still fully Endo H-sensitive (Fig. 1G). Thus, albeit inefficient, in the thyroid glands of untreated TG⁵⁴⁰⁵⁴⁰ mice, T₄ is synthesized in vivo on mutant Tg protein that has never traversed the Golgi complex.

Similar to TG⁵⁴⁰⁵⁴⁰ mice, a human patient bearing homozygous Tg-W2346R in the ChEL domain developed a large hypothyroid goiter, leading to thyroidectomy (15). Histological analysis of the patient’s thyroid tissue indicated the presence of abnormal nuclear and cytoplasmic profiles in follicle lumen (Supplemental Fig. S2A). Cross sections of the patient’s goiter were immunostained with anti-Tg. Unlike the normal human thyroid (Fig. 2A), >50% of thyroid follicle lumina contained the mutant Tg, in an abnormal, patchy distribution that was associated with cellular material including nuclei (Fig. 2A), similar to that seen in TG⁵⁴⁰⁵⁴⁰ mice. Additionally, 49% of the patient’s thyroid follicles exhibited detectable cleaved caspase-3 (Fig. 2B, an inner dashed line highlights the apical luminal cavity in the
WT control which is negative for cleaved caspase-3) and abnormal, weakly DAPI-positive material in the thyroid follicle lumen, consistent with various stages of nuclear disintegration (Fig. 2A-C). Indeed, residual positive signal for the Pax8 transcription factor was detectable (Fig. 2C) — albeit usually at a lower level than that seen in surrounding living follicular cells (but clear enough to identify the dead cells as thyrocytes). Remarkably, ongoing T₄ synthesis was detected in all of the follicles containing dead thyrocytes with evidence suggesting endocytic recapture of T₄-containing protein in an apically-concentrated ring near the boundary between living thyrocytes and the luminal cavity (Fig. 2C). Indeed, T₄-containing substrate was prominently seen on cytoplasmic protein adjacent to the nuclei of TUNEL-positive dead thyrocytes. Additionally, the dead cell ghosts were cannibilized (endocytosed) by surrounding live cell neighbors, highlighting a positive ring of T₄- immunostaining lining the apical region of live thyrocytes (Supplemental Fig. S2B-D). Together the data, both in rodents, and humans with mutant Tg, indicate a pathological salvage mechanism of T₄ synthesis that is built not upon Tg secretion but upon mutant Tg reaching the follicle lumen via exfoliation of ER-stressed, dead thyrocytes.

*In hypothyroidism with bi-allelic mutant TG, thyroid cell mass is the critical factor regulating thyroid hormone synthesis.* The adult homozygous TG<sup>rdw/rdw</sup> rat (encoding Tg-G2298R) is well-known for congenital hypothyroidism, although rather than goiter, the animal develops a hypoplastic thyroid gland (36, 60-62). As in other vertebrates, the normal rat thyroid exhibits a classic monolayer of epithelial thyrocytes surrounding a central cavity filled with secreted eosinophilic Tg (Fig. 3A left). TG<sup>rdw/rdw</sup> rat thyroid glands also form follicles surrounding a central cavity with eosinophilic content (Fig. 3A right). Although suitable antibodies were not available to confirm the apical distribution of (rat) thyroid peroxidase and DUOX2 (two enzymes that help to catalyze T₄ synthesis), we could confirm that aminopeptidase-N — also known to be an apical membrane marker in thyrocytes (63, 64) was still delivered to its correct destination in TG<sup>rdw/rdw</sup> thyroid follicles (Fig. 3B, a dashed yellow line highlights the basal membrane outlining the outer boundary of thyroid follicles). Nevertheless, the
TG<sup>rdw/rdw</sup> thyroid histology was far from normal — the cytoplasm was massively engorged with eosinophilic “vacuoles” displacing nuclei under the apical plasmalemma, and the staining of the follicle lumen was abnormally heterogeneous (Fig. 3A right). The eosinophilic “vacuoles” are in fact ER (60) filled with the ER molecular chaperone, BiP (Fig. 3C). Whereas >85% of Tg molecules in WT rats was endoglycosidase H-resistant, analysis of TG<sup>rdw/rdw</sup> thyroid glands (n = 4) showed that the fraction of Tg molecules bearing Endo H-resistance was zero, indicating an inability of mutant Tg to undergo intracellular transport to the Golgi complex (e.g., Fig. 3D), as previously reported (65). Nevertheless, 64.5 ± 18.5% (SD, n = 6 animals) of TG<sup>rdw/rdw</sup> thyroid follicles exhibited mutant Tg in the lumen — associated with whole cell profiles (Fig. 3E) that upon close histological inspection indicated the presence of thyrocytes at different stages of cell death (Supplemental Fig. S3A).

As in humans and mice with congenital hypothyroidism with mutant Tg, T<sub>4</sub>-containing protein — while significantly diminished relative to that found in WT thyroid follicles — was apparent in the lumen of 89.7 ± 13.3% (SD, n = 5 animals) of the thyroid follicles of untreated TG<sup>rdw/rdw</sup> rats (Fig. 4A,B) — surrounded by a monolayer of Pax8-positive cells (indicative of thyrocytes, Fig. 4A). This T<sub>4</sub>-containing protein signal was specific, as it: a) could not be detected in the parotid salivary gland (that expresses sodium-iodide symporter but cannot iodinate proteins, Supplemental Fig. S3B); b) was fully blocked when adding soluble levothyroxine competitor during the immunofluorescence protocol (Fig. 4B, a dashed white line highlights the apical luminal cavity and a dashed yellow line highlights the basal membrane outlining the outer follicle boundary); and c) was diminished in the thyroids of TG<sup>rdw/rdw</sup> rats fed chow containing propylthiouracil (PTU, which inhibits thyroid iodination, Supplemental Fig. S3C). We could not continue the experiment to complete depletion of thyroidal T<sub>4</sub>-containing protein because within 4 weeks of treatment with the antithyroid drug, the animals became moribund and died spontaneously, indicating that ongoing endogenous T<sub>4</sub> synthesis is required to avoid postnatal lethality.

We examined ER stress responses in TG<sup>rdw/rdw</sup> thyroid glands. PERK phosphorylation of eIF2α stimulates increased translation of ATF4 that upregulates CHOP, which (as noted above) has been
strongly implicated in cell death (29). In addition to a dramatic increase of BiP and p58ipk, $TG^{rdw/rdw}$ thyroid glands were observed to have increased phosphorylated eIF2α (Fig. 5A), accompanied by a > 10-fold increase of CHOP mRNA (Fig. 5B). A second ER stress-related death pathway involves IRE1 hyperactivation that can trigger a ‘terminal UPR’ from exuberant RNAse activity (“RIDD”), which typically develops only in cells exhibiting demonstrably high levels of stress-induced IRE1 splicing of XBP1 mRNA (66). We observed that roughly half of thyroidal XBP1 mRNA was spliced to the active form in $TG^{rdw/rdw}$ animals (Fig. 5C), which is impressive considering that thyrocytes and “C-cells” together comprise only ~60% of resident cells in the mouse thyroid (67); i.e., these are conditions that can favor a ‘terminal UPR’. Moreover, TUNEL-positive cells were present in the follicular lumina of $TG^{rdw/rdw}$ thyroid glands; indeed, the thyroid follicles of untreated $TG^{rdw/rdw}$ rats exhibited ongoing $T_4$ synthesis (Fig. 5D) with 27.2 ± 5.3% (SD, n = 3 animals) of all follicles triply-positive for weak DAPI staining, TUNEL, and $T_4$-containing protein — a positive signal for cleaved caspase-3 was also detected in 24.8 ± 9.4% of thyroid follicles (SD, n = 5 animals), although none of these features were detected in WT thyroid (Fig. 5E). Caspase activity can cleave the DNA repair enzyme PARP [Poly(ADP-Ribose) Polymerase], and unlike in WT thyroid tissue, PARP was extensively cleaved in $TG^{rdw/rdw}$ thyroid glands (Fig. 5F).

We performed Western blotting of total thyroidal proteins with anti-$T_4$. Despite the presence of background bands, a ~330 kDa band co-migrating with WT Tg was the clearest $T_4$-containing protein specifically identified in the thyroid tissue of untreated $TG^{rdw/rdw}$ rats, and the intensity of this band was completely eliminated by the addition of free $T_4$ competitor to the antibody incubation during Western blotting (Fig. 5G left). The efficiency of $T_4$-formation in this protein indicative of mutant Tg was much less than in the Tg protein from WT rat thyroid glands, especially when considering that more Tg protein was loaded for the mutant sample (Fig. 5G right). Altogether, the data in Figs. 3-5 support that $TG^{rdw/rdw}$ rats also use dead thyrocytes for endogenous $T_4$ synthesis on mutant Tg.

To more clearly examine the disintegration of dead thyrocytes bearing mutant Tg, fixed/post-fixed WT and $TG^{rdw/rdw}$ thyroid tissue were plastic-embedded for semi-thin sectioning. As expected,
WT thyroid revealed dense, uniformly-stained “colloid” (Tg protein) in the follicle lumen (Fig. 6A). In contrast, in the thyroid of \( TG^{rdw/rdw} \) rats, in addition to large ‘vacuoles’ in the basal cytoplasm with apically-displaced nuclei, the lumen of different follicles varied, with contents ranging from whole cells to cellular debris (Fig. 6B). Moreover, electron microscopy revealed that living follicular thyrocytes had massively swollen ER with unusual nuclear morphology, and most remaining organelles were crowded into the apical cytoplasm — ultimately limited by the apical plasma membrane bearing microvilli that extend into the follicle lumen (Fig. 6C). Cell ghosts with disintegrating organelles were readily apparent in many of the follicle lumina examined (e.g., Fig. 6D), surrounded by epithelial cells bearing dense endo-lysosomes (Figs. 6E,F), suggesting that dead-cell material from the follicle lumen enters surrounding living thyrocytes via endocytic internalization, with progressive clearance of the detritus of dead-cell ghosts (Figs. 6G, H).

A great puzzle in the field has been to understand why some human patients (and some animal models) with bi-allelic \( TG \) mutations that grow a large goiter can yield a survivable serum \( T_4 \) level without treatment, yet other human patients and animals models with an intact hypothalamic-pituitary-thyroid axis are unable to do so (68). With this question specifically in mind, we examined thyrocyte proliferation in hypothyroid \( TG^{rdw/rdw} \) rats. Indeed, in early life we observed that \( TG^{rdw/rdw} \) rats did indeed exhibit active proliferation of thyrocytes, similar to that observed in \( TG^{cog/cog} \) mice (Supplemental Fig. S4A). Indeed, although never previously described, we observed that in early life \( TG^{rdw/rdw} \) rats do in fact develop thyroid enlargement (i.e., goiter) by 9 weeks of age (Fig. 6I) and this parallels a significant increase of endogenous \( T_4 \) synthesis that supports serum \( T_4 \) levels (Fig. 6J). However, as the \( TG^{rdw/rdw} \) animals aged, the enlarged thyroid gland size could not be sustained (Fig. 6I) and with this (61), the animals could not maintain their serum \( T_4 \) levels (Fig. 6J). Untreated profound hypothyroidism is ultimately incompatible with life in rodents [noted above, and (69, 70)] as well as in humans. It thus appears that only patients and animal models that can support a sufficient goiter are able to provide the continuous supply of dead thyrocytes needed for ongoing \( T_4 \) synthesis.
— a mechanism that can allow some individuals the chance to sustain endogenous thyroid hormone levels in adulthood (24).
Discussion

Reports describe untreated adult patients with a large goiter who are biochemically and clinically nearly-euthyroid despite bi-allelic TG deficiency (71-73). Two longstanding but competing schools of thought are that a) a large hyperplastic goiter is a compensatory physiological adaptation in response to thyroidal genetic or environmental factors that disfavor thyroid hormone production (74), or b) growth of a large thyroid goiter is actually a maladaptation (28). On the one hand, because the Tg protein is the evolutionarily-preferred thyroid hormone precursor (1), and because the mutant TG alleles encode a Tg protein that cannot be exported via the secretory pathway from the ER to the site of iodination (2, 10), simply enlarging the thyroid gland does seem pointless. On the other hand, in this disease, there are reasons favoring a positive correlation between thyroid follicular cell mass and the overall synthesis of thyroxine needed to sustain serum T₄ levels (41).

The main finding of this report is that in humans and animals with bi-allelic mutant TG, T₄ biosynthesis continues as long as the thyroid follicle lumen is provided with a supply of dead or dying thyrocytes (see schematic cartoon, Supplemental Fig. S4B). Throughout the course of the disease, a fraction of the ER-stressed thyrocytes die and are extruded to the luminal cavity, temporarily including activation of caspase-3, cleavage of PARP, and DNA cleavage leading to nuclear fragmentation and karyolysis, leading ultimately to complete disintegration of cellular organelles within the iodination environment of the follicle lumen. Death of thyrocytes is asynchronous, chronic, and heterogenous during the course of the disease, such that at any moment in time, cells at various stages of demise are observed across the gland, surrounded by living cells that maintain a follicular architecture enclosing iodoproteins within the apical cavity. Our analysis did not include specific detection of inflammatory cell infiltration of thyroid follicles but rather ER stress throughout the entire population of living mutant thyrocytes, with remnant detection of ER stress (e.g., CHOP) in the dead cell ghosts. Crucially in this disease, it is the dead, disintegrating thyrocytes upon which T₄-containing protein can be detected, with the thyroid follicles cannibalizing (internalizing) the iodinated detritus of dead thyrocytes into the surrounding living follicular cells.
The spectrum of proteins upon which T₄ might be made inefficiently in humans with bi-allelic TG mutation has not yet been fully explored, although it has often been speculated that albumin, which can become highly iodinated (75) as a serum protein capable of transcytosis (76) or paracellular leakage (75) — could perhaps be a source of endogenous T₄. Given the mechanistic understanding presented in the current study, we recognize that the entire proteome leaked from dead thyrocytes becomes eventually exposed to the iodination environment. Here we show that even though the mutant Tg protein cannot be secreted via conventional intracellular trafficking (77), it is nevertheless conveyed to the lumen of thyroid follicles via dead thyrocytes, wherein T₄ is produced (albeit inefficiently) within the mutant Tg protein in TG<sup>cog/cog</sup> mice, TG<sup>rdw/rdw</sup> rats and, most likely, humans with the same disease.

Indeed, this pathological salvage mechanism of T₄ synthesis is observed in the goitrous thyroid gland of a patient with homozygous expression of Tg-W2346R. Moreover, in this study, we provide strong supporting evidence that total thyroid cell mass (i.e., the goiter) is important for the endogenous rescue from hypothyroidism. Specifically, increasing amounts of T₄ are produced as the thyroid begins to grow postnatally (41); however in TG<sup>rdw/rdw</sup> rats, profound hypothyroidism ensues in parallel with an atrophic thyroid gland (36, 61). Thus, our results demonstrate that TG<sup>rdw/rdw</sup> rats form a goiter but cannot sustain their goiter with aging. Evidently, without the growing goiter, the thyroid gland cannot provide sufficient dead cells needed to fuel ongoing thyroid hormone production. These considerations make clear that the balance of cell proliferation-versus-death is indeed a critical factor, as ultimately an unfavorable balance in TG<sup>rdw/rdw</sup> rats deprives the thyroid of sufficient substrate to maintain T₄ synthesis. In contrast, continued growth of the goiter in TG<sup>cog/cog</sup> mice allows this pathological salvage mechanism to endogenously self-correct the hypothyroidism (41), and human studies suggest a similar conclusion in goitrous patients with bi-allelic TG mutations.

More work is still needed to determine if rdwTg might somehow be more proteotoxic than cogTg (40) or if other factors that vary between species drive the enhanced capability of TG<sup>cog/cog</sup> mice for a net proliferation of thyrocytes (i.e., in excess of cell death) into adulthood. What is apparent in all
cases, however, is that the continuous contribution of dead and dying thyrocytes to provide substrate for T₄ production represents the critical compensatory response to congenital hypothyroidism with bi-allelic TG mutations. Moreover, as a brief exposure to antithyroid drug is lethal to TG^{rdw/rdw} rats, our findings appear consistent with the hypothesis that in the presence of bi-allelic TG mutations, survival of the organism (8, 68) does require this most unusual means of thyroid hormonogenesis.
Methods

Primary Antibodies. Anti-Ki67 [SP6] (ab16667, Abcam); anti-Cleaved Caspase-3 (Asp175) Antibody (9661, Cell Signaling); anti-CHOP (sc-7351, Santa Cruz); anti-Thyroxine (1H1) (sc-52247, Santa Cruz); mAb anti-Tg (365997, Santa Cruz; ab156008, Abcam); rabbit anti-Tg and rabbit anti-BiP were previously described (38, 45); rabbit anti-Pax8 (10336-1-AP, ProteinTech Group); mAb anti-Aminopeptidase N (1D7) was the kind gift of Dr. D. Fox, University of Michigan, Ann Arbor (78); rabbit anti-p58ipk (2940, Cell Signaling Technology); rabbit anti-phospho-eIF2α (Ser51) (3597, 9721, Cell Signaling) and total eIF2α (9722, Cell Signaling); mouse anti-tubulin (T5168, Sigma); rabbit anti-PARP (9542, Cell Signaling).

Human thyroid sections. A homozygous patient bearing the TG-W2346R mutation was previously described [including parental written informed consent, and appropriate IRB approval (15)]. Paraffin blocks of de-identified surgically-resected thyroid tissue, including large regions of normal human thyroid from three now-deceased patients, were obtained from the Molecular Pathology Research Laboratory, University of Michigan, and were sectioned and stained for H+E, or processed for immunofluorescence as described below.

Animals. TGcog/cog mice (C57BL/6) were from JAX. TGrdw/+ rat heterozygotes were obtained from the National BioResource Project in Japan [NBRP Rat No: 0104] and bred to homozygosity; WT animals were littermates of the same strain background. All experiments performed with mice and rats were in compliance and approved by the University of Michigan Institutional Animal Care and Use Committee. Adult 2-4-month-old mice were used in all studies except in Fig. 1G, S1C and Fig. S4 (in which 9-16-month-old mice with larger goiter were used for better yield of T4-containing proteins and Ki67-positive thyrocytes). 4-18-week-old rats were used in all studies (i.e., post-weaning) except in Fig. 6I and J, in which detailed ages are shown. In all Figures, data from males and females were combined, except in Fig 6I and J, in which results from each individual animal are shown (males, squares; females, circles).
Cell culture. PCCL3 cells (79) [source: Dr. B. DiJeso, U. Salento, Lecce Italy] maintained in DMEM/F-12 with 5% fetal bovine serum, 1 mIU/mL thyrotropin, 1 µg/mL insulin, 5 µg/mL apo-transferrin, 1 nM hydrocortisone (4 hormones obtained from Sigma), and penicillin/streptomycin. PCCL3 conditioned media containing secreted TG were collected as a positive control for endo H-resistant TG.

Thyroid gland size measurement. Thyroids of euthanized animals were dissected with both lobes of the gland fully exposed. Images of the neck were captured with a calibrated size marker included in situ. The areas of the thyroid glands (correlated with volume) were measured using ImageJ and quantified as a fraction of body weight of each animal.

Serum total T₄ measurement. Whole blood was collected, clotted and centrifuged at 750 × g for 20 min to obtain serum. Total T₄ was assayed by ELISA (Diagnostic Automation / Cortez Diagnostics).

Preparation and immunostaining of thyroid sections. Thyroid glands from mice and rats were immersion-fixed with 10% formalin and processed for paraffin embedding, sectioning, and H&E staining. For immunofluorescence, 6-µm sections were deparaffinized in Citrisolv and an ethanol series, then heated in citrate buffer (12.3 mM, pH 6) for antigen retrieval, and blocked in 1.5% normal goat serum for 30 min at room temperature. Primary antibody incubation was performed overnight at 4°C, followed by incubation of AlexaFluor-conjugated secondary antibodies (Thermo Fisher). After washing, sections were counterstained and mounted with Prolong-Gold and DAPI (Invitrogen). Images were captured in a Nikon A1 confocal microscope. For anti-Ki67 immunohistochemistry, the VECTASTAIN ABC Kit (Vector) was used. After antigen retrieval, sections were treated with 3% H₂O₂, blocked in 1.5% normal goat serum for 20 min at room temperature, incubated with anti-Ki67 antibody for 1 h at room temperature and biotinylated secondary antibody for 30 min, followed by incubation with avidin-HRP. Staining was visualized by DAB reaction. Sections were counterstained with hematoxylin, dehydrated in a graded series of ethanol, and mounted with Permount. Images were obtained with a Leica DMI-3000B microscope.
T<sub>4</sub> immunofluorescence / TUNEL double labeling. The ApopTag In Situ Apoptosis Detection Kit (Millipore) was used for TUNEL staining of thyroid sections. Minor modifications were applied for double immunofluorescence labeling with anti-T<sub>4</sub>. Briefly, de-paraffinized thyroid tissue sections were pre-treated with proteinase K (20 µg/mL) and blocked in 1.5% normal goat serum for 30 min at room temperature. Incubation with anti-T<sub>4</sub> antibody was performed at room temperature for 1 h, followed by incubation with AlexaFluor-488-conjugated secondary antibody for 30 min at room temperature. After washing, TUNEL staining was performed. Sections were counterstained and mounted with Prolong-Gold and DAPI (Invitrogen). Fluorescence images were captured in a Nikon A1 confocal microscope.

Endoglycosidase H digest. Thyroid homogenates from WT or mutant mice and rats were boiled in denaturing buffer containing 0.5% SDS and 40 mM DTT at 95°C for 5 minutes, cooled, and then either mock-digested or digested with endoglycosidase H (1000 units, NEB) for 1 h at 37 °C.

Western blotting. Rodent thyroid glands were homogenized in RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Thermo Scientific) including either protease inhibitor cocktail (Roche) or protease-plus-phosphatase inhibitor cocktail (Thermo Scientific). Total protein concentration was determined by BCA assay (Thermo Scientific). Lysates were boiled in SDS-gel sample buffer with 50 mM dithiothreitol (DTT). Samples were then resolved by SDS- straight 4.5% or 4-12%-PAGE, electrotransferred to nitrocellulose, and blocked with 5% milk before immunoblotting with the indicated antibodies and appropriate HRP-conjugated secondary antibody, and visualized by enhanced chemiluminescence. Band quantitation was performed using ImageJ.

Immunoprecipitation analysis of T<sub>4</sub>-containing protein. Mouse thyroid glands were homogenized in RIPA buffer plus protease inhibitor cocktail (Roche). Thyroid homogenates were incubated with mAb anti-T<sub>4</sub> antibody and protein G-agarose (Exalpha Biologicals) overnight at 4 °C. Precipitates were washed three times in RIPA buffer (and for samples to be digested with Endo H, two additional washes in PBS) and then boiled in SDS gel-sample buffer containing 50 mM
dithiothreitol, resolved by SDS-straight 4.5% or 4-12%-PAGE, electrotransferred to nitrocellulose, and immunoblotted with anti-T4 or anti-Tg antibody.

**PCR.** Total RNA was purified from the thyroid gland tissue or PCCl3 cells using a Rneasy Plus kit (Qiagen). Synthesis of cDNA was performed using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) or High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). For XBP1 splicing analysis, the primers below were designed to encompass the IRE1 cleavage site of XBP1: forward primer 5'-TGGCCGGGTCTGCTGAGTCCG-3'; and reverse primer 5'-ATCCATGGGAAGATGTTCG-3'. The amplicons including the spliced (71 bp) and unspliced (97 bp) XBP1 were generated using the GoTaq Green Master Mix Kit (Promega). PCR products were resolved by a 3% agarose gel. Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used as a control, and amplified using the following primers and loaded as a loading control: forward primer 5'-CTCATGGACTGATTATGGACAGGA-3'; and reverse primer 5'-GCAGGTACAGGACTGATTATGGACAGGA-3'. Band quantitation was performed using ImageJ. For real-time PCR, TaqMan Universal Master Mix was used on a StepOnePlus PCR system (Thermo Fisher Scientific). CHOP gene expression was normalized to that of Ywhaz; both probes were from TaqMan Gene Expression Assays (Thermo Fisher Scientific).

**Electron microscopy of rat thyroid glands.** WT and rdw/rdw rats were briefly perfusion-fixed with HEPES-buffered 2% glutaraldehyde before thyroid dissection and continuing as immersion fixation. The tissues were then washed in 100 mM Na cacodylate containing 2 mM CaCl2, before postfixation with 0.25% OsO4, further washing, and staining with 0.5% uranyl acetate. After additional washes, the tissue was dehydrated in a graded series of ethanol (50, 75, 95, and 100%) followed by a 30 min incubation in propylene oxide. The tissue was finally infiltrated with Araldite in propylene oxide, and then pure Araldite, which was polymerized under heating. 10 μm plastic sections were stained with 1% toluidine blue and examined by light microscopy (100x objective), followed by 0.5 μm sections picked up on Formvar-coated copper grids, post-stained with 1% lead citrate, and rinsed prior to examination in a JEOL-JEM-1400 transmission electron microscope.
Statistics. Statistical analyses were calculated using GraphPad Prism. Data were represented as mean ± S.D. Unpaired two-tailed Student’s t test was used for comparisons between 2 groups. One-way ANOVA with Bonferroni post hoc test was used for comparison of 3 groups. Thyroid size and serum T\textsubscript{4} measurements were analyzed by two-way ANOVA with Bonferroni post hoc test. Statistical significance was determined when p < 0.05.

Study approval. All experiments performed with mice and rats were in compliance and approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC, PRO00009936). The human patient thyroid block was obtained with parental written informed consent, and appropriate IRB approval.

Data and materials availability. All data and methods for this manuscript are included directly in the paper and/or Supplementary Figures. Materials are freely available upon request.

Author Contributions
X.Z., C.E.C., Y.M., and P.A. designed experiments; X.Z. and C.E.C. performed experiments; assistance was provided by H.Z. and D.L.; H.T. and V.B. provided key reagents; X.Z. wrote methods; P.A. supervised the work and wrote manuscript; all co-authors reviewed, edited, and approved the manuscript.

Acknowledgements
This work was supported by NIH R01DK40344 and the University of Michigan Protein Folding Diseases Initiative (to P.A.) and by ATA-2018-012.R1 (to C.E.C). We are deeply grateful to Drs. John E. Heuser and Tatyana Tenkova (Section on Integrative Biophysics, National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, MD) for their invaluable assistance with plastic embedding and imaging of semi-thin plastic sections (light microscopy) and transmission electron microscopy. We acknowledge the Michigan Histology Core Facility for assistance with
sample preparation. We also thank Drs. Wen Jing and other members of the Arvan lab for comments and help.

Address correspondence to: Dr. Peter Arvan, Division of Metabolism, Endocrinology & Diabetes, University of Michigan Brehm Tower rm 5112, 1000 Wall Street, Ann Arbor, MI 48105 USA; Phone: (734) 936-5505; Email: parvan@umich.edu
References


25


60. Sakai Y, Yamashina S, and Furudate SI. Missing secretory granules, dilated endoplasmic reticulum, and nuclear dislocation in the thyroid gland of rdw rats with hereditary dwarfism. *Anat Rec.* 2000;259(60-6).


Fig. 1. ER stress, cell death, and T₄ synthesis in TG<sup>cog/cog</sup> mice. **A.** Representative H&E thyroid images from WT and TG<sup>cog/cog</sup> (n=6 animals/group), showing thyrocyte distention with apically-displaced nuclei in TG<sup>cog/cog</sup> mice compared to a thin monolayer of thyrocytes in WT (+/+ ) mice. Scale bars = 20μm. **B.** Representative anti-Tg immunofluorescence in thyroid glands of WT (+/+ ) and TG<sup>cog/cog</sup> mice (n=8 animals/group), with DAPI counter-stain. Scale bars = 20μm **C.** Representative immunofluorescence of T₄-containing protein in thyroid follicles of WT (+/+ ) or TG<sup>cog/cog</sup> mice (n=6 animals/group). Thyrocytes are highlighted by PAX8-positive nuclear transcription factor, with DAPI counter-stain. Scale bars = 10μm. **D.** Endoglycosidase H digest of thyroid homogenates before SDS-PAGE and Tg Western blotting from WT (+/+ ) and TG<sup>cog/cog</sup> mice (n=3 animals/group; two of each kind shown). **E.** Upper panel: Western blotting BiP, p58ipk, and CHOP in the thyroids of TG<sup>cog/cog</sup> mice (n=3-4; each lane =1 animal). **Lower panel:** Quantitation of bands (normalized to tubulin), shown as mean ± S.D.; *** p < 0.001 (Unpaired two-tailed Student’s t test). **F.** Representative TUNEL staining and immunofluorescence of T₄-containing protein with DAPI counter-stain in thyroid sections of WT (+/+ ) and TG<sup>cog/cog</sup> mice (n=7 animals/group). For clarity in the merged image from TG<sup>cog/cog</sup> mice, a dashed white line delimits the thyroid follicle lumen. Scale bars (lower right) = 20μm. **G.** Thyroid homogenate from TG<sup>cog/cog</sup> mice (n=3) were immunoprecipitated with mAb anti-T₄ in the presence or absence of T₄ competitor, followed by either mock digest or Endo H digest and SDS-PAGE + immunoblotting with mAb antibody that recognizes intact Tg. As a positive control, WT Tg secreted from PCCL3 (rat) thyrocytes was digested for Endo H-resistance. The T₄-containing Tg protein of TG<sup>cog/cog</sup> mice was entirely Endo H-sensitive. The position of the 250 kDa molecular weight marker is shown.
Fig. 2. Tg and T4 synthesis in a homozygous patient bearing $\text{TG}^{W2346R/W2346R}$. A. Anti-Tg immunofluorescence (red) with DAPI counter-staining (blue) of human thyroid sections from a patient bearing $\text{TG}^{W2346R/W2346R}$, and a representative unaffected (Control) individual ($n = 3$). The diseased thyroid gland shows abnormal accumulation of intracellular Tg but also shows Tg in a patchy distribution in the thyroid follicle lumen. Scale bars (lower right) = 10 µm. B. Anti-cleaved caspase-3 immunofluorescence (red) with DAPI counter-staining (blue) in the thyroid gland of the individuals from panel A. For clarity, a dashed white line delimits the thyroid follicle lumen in the Control (in which cleaved caspase-3 is not seen); scale bars = 10 µm. C. Immunostaining of T4-containing protein (green) in thyroid follicles from the individuals in panel A. Thyrocyte identity is confirmed by PAX8-positive nuclei (red) with DAPI counter-stain (blue); scale bars = 10 µm.
Fig. 3. Tg is entrapped in the ER, yet reaches the thyroid follicle lumen in TG<sup>rdw/rdw</sup> rats.

A. Representative H&E images of thyroid glands from WT (+/+) and TG<sup>rdw/rdw</sup> rats (n=6 per group; showing abnormally heterogeneous eosinophilic content in the follicle lumen, surrounded by abnormally swollen thyrocytes in TG<sup>rdw/rdw</sup> rats; scale bars = 20 µm. B. Representative distribution of aminopeptidase N by immunofluorescence (green) with DAPI counter-stain (blue) in thyroid follicles of WT (+/+) and TG<sup>rdw/rdw</sup> rats (n=4 per group). For clarity, a yellow dotted line highlights the outer boundary of the thyroid follicular cells; scale bars = 10 µm. C. Representative distribution of BiP by immunofluorescence (red) with DAPI counter-stain (blue) in thyroid follicles of WT (+/+) and TG<sup>rdw/rdw</sup> rat (n=4 per group); scale bars = 10 µm. D. Representative thyroid homogenates from WT (+/+) and TG<sup>rdw/rdw</sup> rats were either mock-digested or digested with endoglycosidase H (Endo H), followed by SDS-PAGE and immunoblotting with anti-Tg (n=4 animals per group). No Tg from TG<sup>rdw/rdw</sup> rats is endo H-resistant. E. Representative distribution of Tg in the thyroid follicle lumen by immunofluorescence (green) with DAPI counter-stain (blue) from WT (+/+) and TG<sup>rdw/rdw</sup> rats (n=7 per group); scale bars = 20 µm.
Fig. 4. T₄ synthesis in TG²dw/²dw rats. A. Representative immunofluorescence of T₄-containing protein (green) in the thyroid follicle lumen of WT (+/+ and TG²dw/²dw rats (n=5 animals per group). Thyrocyte identity is confirmed by PAX8-positive nuclei (red) with DAPI counter-stain (blue); scale bars = 20 µm. B. Representative immunofluorescence detection of T₄-containing protein (green; with DAPI counter-stain in blue) in the thyroid of WT and TG²dw/²dw rats (n=9 animals per group) is specifically blocked by addition of T₄ competitor (1 µg/mL). For clarity, a dashed white line delimits the thyroid follicle lumen; a yellow dotted line highlights the outer boundary of the thyroid follicle; scale bars = 10 µm.
Fig. 5. ER stress, cell death, and T₄ synthesis in \(TG^{rdw/rdw}\) rats. A. Left: BiP, p58ipk, and phospho-eIF2α Western blotting in thyroids of WT (+/+) and \(TG^{rdw/rdw}\) rats (each lane =1 animal). Right: Quantification (BiP and p58ipk normalized to tubulin; phospho-eIF2α normalized to total eIF2α; mean ± S.D.) ** p <0.01, *** p <0.001 (Unpaired two-tailed Student’s t-test). B. CHOP mRNA levels (normalized to YWHAZ) in the thyroid glands of WT (+/+)–and \(TG^{rdw/rdw}\) rats (n=7-8 animals/group; each point =1 animal; mean ± S.D.) *** p < 0.001 (Unpaired two-tailed Student’s t-test). C. Upper: Representative samples showing spliced and unspliced XBP1 mRNA in the thyroids of WT, \(TG^{rdw/+}\) and \(TG^{rdw/rdw}\) rats (n=3-6 animals/group; each lane =1 animal). Hprt1 is a loading control. Lower: Quantitation of the fraction of spliced XBP1; mean ± S.D.) ** p < 0.01, *** p < 0.001 (One-way ANOVA, Bonferroni post-hoc test). D. Representative TUNEL staining and immunofluorescence of T₄-containing protein with DAPI counter-stain in the thyroids of WT (+/+)–and \(TG^{rdw/rdw}\) rat (n=4 animals/group); scale bars = 20µm. E. Representative immunofluorescence of cleaved caspase-3 with DAPI counter-stain in thyroids of WT (+/+)–and \(TG^{rdw/rdw}\) rats (n=5 animals/group). For clarity, a dashed white line delimits the thyroid follicle lumen in the WT (in which cleaved caspase-3 is not detectable); scale bars = 20µm. F. Western blotting of PARP in thyroid glands from WT (+/+) and \(TG^{rdw/rdw}\) rats (n=3-4; each lane =1 animal). G. Left panel: representative Western blotting of T₄-containing protein in thyroid homogenates of WT and \(TG^{rdw/rdw}\) rats (n=5 animals/group) ± soluble competitor T₄ to block specific bands (left of dotted red line). Right panel: the same samples immunoblotted with mAb anti-Tg showing intentional overloading of the \(TG^{rdw/rdw}\) rat sample.
Fig. 6. In congenital goiter with mutant TG, thyrocyte cell mass provides the dead cell-derived substrate for T4 synthesis. A-H. Microscopy of WT and TG<sup>rdw/rdw</sup> rat thyroid follicles. A. WT rat thyroid. Cross-section of several thyroid follicles are seen; each follicle lumen (F.L.) is acellular but filled with WT Tg protein (thin yellow arrows). Scale (upper left) = 10 µm in 1.0 µm increments. B. TG<sup>rdw/rdw</sup> rat thyroid. Yellow arrows point to the follicle lumina (F.L.); note enlarged cytoplasm and abnormal, cellular contents of the follicle lumina. Scale (upper left) = 10 µm in 1.0 µm increments. C-H. Transmission EM survey of TG<sup>rdw/rdw</sup> rat thyroid follicles (scale bars = 2 µm). Panel C highlights engorged ER vacuoles in the basal cytoplasm with apically-displaced nuclei. Panel D-H highlight dead-cell ghosts in various thyroid follicles, each at a different stage of cellular disintegration within the follicle lumen. Panel G also highlights living thyrocytes with abundant apical microvilli, which have internalized material from the follicle lumen into endo-lysosomes. Panel H highlights that until new dead cells enter the follicle lumen, there is progressive clearance of cellular debris from the luminal cavity. I. Thyroid gland size (normalized to body weight) in a cohort of young versus older animals (open symbols = 8.9 ± 1.7 wk; closed symbols = 33.4 ± 2.6 wk; males shown as squares and females as circles). Data are shown as mean ± S.D.; *** p < 0.001 (two-way ANOVA, Bonferroni post hoc test). J. Total T4 level in serum of WT (+/+)) and TG<sup>rdw/rdw</sup> rats as a function of age (males shown as squares and females as circles). Data are mean ± S.D.; **p < 0.01, ***p < 0.001 (two-way ANOVA, Bonferroni post hoc test).