Interdependence of thyroglobulin processing and thyroid hormone export in the mouse thyroid gland

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Abstract: Thyroid hormone (TH) target cells need to adopt mechanisms to maintain sufficient levels of TH to ensure regular functions. This includes thyroid epithelial cells, which generate TH in addition to being TH-responsive. However, the cellular and molecular pathways underlying thyroid auto-regulation are insufficiently understood. In order to investigate whether thyroglobulin processing and TH export are sensed by thyrocytes, we inactivated thyroglobulin-processing cathepsins and TH-exporting monocarboxylate transporters (Mct) in the mouse. The states of thyroglobulin storage and its protease-mediated processing and degradation were related to the levels of TH transporter molecules by immunoblotting and immunofluorescence microscopy. Thyroid epithelial cells of cathepsin-deficient mice showed increased Mct8 protein levels at the basolateral plasma membrane domains when compared to wild type controls. While the protein amounts of the thyroglobulin-degrading cathepsin D remained largely unaffected by Mct8 or Mct10 single-deficiencies, a significant increase in the amounts of the thyroglobulin-processing cathepsins B and L was detectable in particular in Mct8/Mct10 double deficiency. In addition, it was observed that larger endo-lysosomes containing cathepsins B, D, and L were typical for Mct8- and/or Mct10-deficient mouse thyroid epithelial cells. These data support the notion of a crosstalk between TH transporters and thyroglobulin-processing proteases in thyroid epithelial cells. We conclude that a defect in exporting thyroxine from thyroid follicles feeds back positively on its cathepsin-mediated proteolytic liberation from the precursor thyroglobulin, thereby adding to the development of auto-thyrotoxic states in Mct8 and/or Mct10 deficiencies. The data suggest TH sensing molecules within thyrocytes that contribute to thyroid auto-regulation.

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Research paper

Interdependence of thyroglobulin processing and thyroid hormone export in the mouse thyroid gland

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ABSTRACT

Thyroid hormone (TH) target cells need to adopt mechanisms to maintain sufficient levels of TH to ensure regular functions. This includes thyroid epithelial cells, which generate TH in addition to being TH-responsive. However, the cellular and molecular pathways underlying thyroid auto-regulation are insufficiently understood. In order to investigate whether thyroglobulin processing and TH export are sensed by thyrocytes, we inactivated thyroglobulin-processing cathepsins and TH-exporting monocarboxylate transporters (Mct) in the mouse. The states of thyroglobulin storage and its protease-mediated processing and degradation were related to the levels of TH transporter molecules by immunoblotting and immunofluorescence microscopy. Thyroid epithelial cells of cathepsin-deficient mice showed increased Mct8 protein levels at the basolateral plasma membrane domains when compared to wild type controls. While the protein amounts of the thyroglobulin-degrading cathepsin D remained largely unaffected by Mct8 or Mct10 single-deficiencies, a significant increase in the amounts of the thyroglobulin-processing cathepsins B and L was detectable in particular in Mct8/Mct10 double deficiency. In addition, it was observed that larger endo-lysosomes containing cathepsins B, D, and L were typical for Mct8- and/or Mct10-deficient mouse thyroid epithelial cells. These data support the notion of a crosstalk between TH transporters and thyroglobulin-processing proteases in thyroid epithelial cells. We conclude that a defect in exporting thyroxine from thyroid follicles feeds back positively on its cathepsin-mediated proteolytic liberation from the precursor thyroglobulin, thereby adding to the development of auto-thyrotoxic states in Mct8 and/or Mct10 deficiencies. The data suggest TH sensing molecules within thyrocytes that contribute to thyroid auto-regulation.

1. Introduction

Thyroid dysfunctions caused by altered thyroid hormone (TH)[2] generation or disturbed TH action are considered to be among the most frequent endocrine disorders (Brix et al., 2011; Führer et al., 2015). TH are of crucial importance for the proper functioning of nearly every organ, including the thyroid gland itself. Hence, all TH target cells need to adopt mechanisms to maintain sufficient levels of TH to ensure regular functions. Since the thyroid gland itself not only produces TH but also responds to TH levels in a self-sustaining manner, auto-protection of the thyroid gland is vital not only for thyrocytes but also for all other TH-dependent cells. Proper thyroid states of an organism are centrally regulated by the hypothalamus-pituitary-thyroid (HPT)–axis, whereby low serum TH levels trigger hypothalamic thyrotropin-releasing hormone (TRH) release which then promotes release of thyroid stimulating hormone (TSH) from the pituitary (Fliers et al., 2014), thereby triggering TH generation in the thyroid gland, and TH release into the blood.

To fulfill and maintain its tasks, the thyroid gland is composed of functional units, the so-called thyroid follicles, that are built by

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[2] Abbreviations: CMF-PBS, calcium- and magnesium-free phosphate buffered saline; Cy3, indocarbocyanine; HPT, hypothalamus-pituitary-thyroid; Mct, monocarboxylate transporters; PTC, papillary thyroid carcinoma; Tg, thyroglobulin; TH, thyroid hormones; TRH, thyrotropin-releasing hormone; TSH, thyroid stimulating hormone; WT, wild type.

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Table 1
Specific functions of different cathepsins expressed in the thyroid regarding their processing of Tg.

	extracellular Tg solubilisation from cross-linked storage forms	extracellular Tg processing	intracellular limited Tg processing (endosomal)	intracellular Tg degradation (lysosomal)	direct T_4 liberation from Tg	exhaustive Tg degradation for Tg utilization (endo-lysosomal TH liberation)	References
Cathepsin B	x	x	x	x		involved to major extent	Brix et al., 1996; Dunn et al., 1996; Tepel et al., 2000; Friedrichs et al., 2003; Jordans et al., 2009
Cathepsin D				x		involved to minor extent	Brix et al., 1996; Dunn et al., 1996; Friedrichs et al., 2003
Cathepsin K		x	x		x		Tepel et al., 2000; Friedrichs et al., 2003; Jordans et al., 2009
Cathepsin L	x	x	x	x		involved to major extent	Brix et al., 1996; Dunn et al., 1996; Tepel et al., 2000; Friedrichs et al., 2003; Jordans et al., 2009

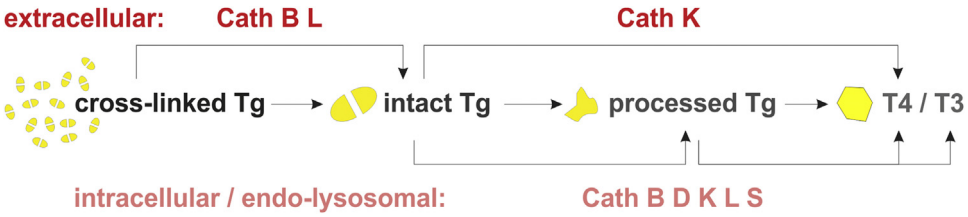

Fig. 1. Cathepsin-mediated solubilization, processing, and degradation of Tg.
Schematic drawing summarizing the sequential steps of Tg processing and degradation by endo-lysosomal cathepsins (Brix et al., 1996; Tepel et al., 2000; Brix et al., 2001; Linke et al., 2002a; Friedrichs et al., 2003; Jordans et al., 2009; Dauth et al., 2011). Cathepsins B and L involve in extracellular Tg solubilization from its covalently cross-linked storage forms, while cathepsin K is instrumental in Tg utilization for T4 liberation. Upon re-internalization of intact and partially degraded Tg, it is further processed and degraded by aspartic and cysteine cathepsins within endo-lysosomes of thyrocytes.

a monolayer of thyroid epithelial cells (Fujita, 1988; Nilsson and Fagman, 2013). The macromolecular TH precursor thyroglobulin (Tg) is stored in covalently cross-linked form in the extracellular thyroid follicle lumen (Herzog et al., 1992; Berndorfer et al., 1996; Klein et al., 2000; Saber-Lichtenberg et al., 2000). Covalently cross-linked Tg is processed for solubilization and degradation by proteases, which are secreted in a TSH-regulated fashion into the extra-cellular follicle lumen upon a demand of TH in the body periphery (for reviews, see Brix et al., 2001; Dauth et al., 2011). Thus, solubilization of Tg from its covalently cross-linked storage forms is enabled through limited, extracellular protein processing facilitated by cathepsins B and L, while TH liberation from Tg is mediated by cathepsins K and L, and also initiated in the thyroid follicle lumen (Brix et al., 1996; Linke et al., 2002a; Friedrichs et al., 2003; Tepel et al., 2000). Subsequently, partially degraded Tg is internalized and reaches endo-lysosomal compartments, where T_3 and T_4 liberation is completed for Tg utilization through exhaustive proteolysis mediated by a variety of hydrolytic enzymes including aspartic and cysteine cathepsins B, D, K, L, and S (Brix et al., 1996; Tepel et al., 2000; Linke et al., 2002a; Friedrichs et al., 2003; Jordans et al., 2009). Cathepsin K is special among the Tg-processing proteases as it is able to liberate T_4 directly from Tg (Tepel et al., 2000). In brief summary, thyroid functions are enabled by sequential proteolytic processing of Tg (Table 1, Fig. 1), resulting in the liberation of T_4 and, to a lower extent, T_3.

Considering that thyroid epithelial cells need to release TH through transmembrane translocation into the blood circulation, and keeping in mind that thyrocytes are TH target cells themselves, a regulatory mechanism must exist that determines how much of the T_4 liberated from Tg is either retained in the thyrocytes or transported back into the same. We propose that intra-thyroidal TH transporters like the monocarboxylate transporter molecules Mct8 and Mct10 may act as such sensors of (i) the extent of proteolytic TH liberation, (ii) the amounts of TH released from thyroid follicles, and (iii) the levels of re-uptake of circulating TH back into thyroid follicles.

Mice with a global Mct8-deficiency (Mct8 knock-out mice) exhibit central TH-resistance, *i.e.* TRH levels are dramatically elevated despite highly increased serum T_3 levels, whereas serum T_4 levels are decreased, and TSH is slightly elevated (Dumitrescu et al., 2006; Trajkovic et al., 2007; Wirth et al., 2011). This very unusual thyroid status (Table 2) is in part explained by the fact that Mct8 is localised at the basolateral plasma membrane domain of thyroid epithelial cells, and thus, its functional absence in mice leads to diminished T_4 export from thyroid follicles (Trajkovic-Arsic et al., 2010a,b; Di Cosmo et al., 2010). Moreover, intra-thyroidal levels of non-Tg-bound and Tg-bound TH are 2- to 3-fold elevated over WT in Mct8-deficient mice (Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b; Liao et al., 2011; Muller et al., 2014), which also feature enlarged thyroid follicles, enhanced thyroid epithelial extensions, and develop pathological abnormalities like papillary structures characterized by nuclear alterations, in particular, at older age (Wirth et al., 2011). Consequently, the Mct8-deficient mouse thyroid gland is considered to be characterized by autothyrotoxic as well as thyrotoxic states affecting peripheral TH target organs (for review, see Müller and Heuer, 2014; Heuer and Visser, 2013). The transporter Mct10 (Slc16a10/Tat1) is expressed to much lower amounts in the thyroid gland but in a pattern similar to Mct8; however, a lack of Mct10 alone did not alter the serum TH states in comparison to wild type mice (Mariotta et al., 2012; Muller et al.,

Table 2
Thyroid function parameters, peripheral and intra-thyroidal thyroid states, TH generation and TH release in Mct8/10-deficient mice.

	Mct8$^{-/y}$	*Mct10*$^{-/-}$	*Mct8*$^{-/y}$/*Mct10*$^{-/-}$	References
Thyroid function parameters, HPT axis				
• **serum T_4**	↓	=	=	Dumitrescu et al., 2006; Trajkovic et al., 2007; Di Cosmo et al., 2010; Liao et al., 2011; Wirth et al., 2011; Trajkovic-Arsic et al., 2010b; Muller et al., 2014
• serum T_3	↑↑↑	=	↑↑↑	Dumitrescu et al., 2006; Trajkovic et al., 2007; Di Cosmo et al., 2010; Liao et al., 2011; Wirth et al., 2011; Trajkovic-Arsic et al., 2010b; Muller et al., 2014
• serum TSH, or TSH mRNA (pituitary)	↑	=	↑	Dumitrescu et al., 2006; Trajkovic et al., 2007; Trajkovic-Arsic et al., 2010b; Liao et al., 2011; Wirth et al., 2011; Muller et al., 2014
• TRH mRNA (hypothalamus)	↑↑	=	=	Trajkovic et al., 2007; Trajkovic-Arsic et al., 2010b; Muller et al., 2014
peripheral Dio1 activity (liver, kidney)	↑	=	↑↑	Dumitrescu et al., 2006; Trajkovic et al., 2007; Liao et al., 2011; Trajkovic-Arsic et al., 2010a; Trajkovic-Arsic et al., 2010b; Muller et al., 2014
intra-thyroidal thyroid states				
• intra-thyroidal TH • (non-Tg-bound and Tg-bound)	↑↑	=	↑↑↑	Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b; Liao et al., 2011; Muller et al., 2014
• intra-thyroidal Dio1 activity	=			Di Cosmo et al., 2010; Wirth et al., 2011; Muller et al., 2014
• auto-thyrotoxic state, i.e. papillary structures with nuclear changes; or altered tumour marker like Galectin-3	↑↑	=	↑↑↑	Wirth et al., 2011; Muller et al., 2014
• thyroid follicle and/or luminal areas	↑	=	↑↑	Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b; Wirth et al., 2011; Muller et al., 2014
• thyroid epithelial extensions	↑			Di Cosmo et al., 2010; Wirth et al., 2011
TH generation				
• *Nis* (sodium iodide symporter), *Ttf1* (transthyretin)	↑ not signifi-cant	=	↑↑	Di Cosmo et al., 2010; Muller et al., 2014
• *Tshr* (TSH receptor), *Tpo* (thyroid peroxidase), *Tg* (thyroglobulin), *Dehal1* (dehalogenase 1)	=	=	↑↑	Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b; Muller et al., 2014
TH release from thyroid follicles				
• TSH-stimulated T_4 release	↓			Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b
• TSH-stimulated T_3 release	↑			Trajkovic-Arsic et al., 2010b
• *Mct10*, *Lat1*, *Lat2* (TH transporters)	=			Di Cosmo et al., 2010

2014). Interestingly, mice deficient in both Mct8 and Mct10 proteins (Muller et al., 2014) showed a partial rescue of the unusual serum TH status observed in Mct8-deficient animals, whereas the intra-thyroidal auto-thyrotoxic states were even more pronounced than in Mct8 deficiency alone. Therefore, both Mct8 and Mct10 appear to be responsible for TH export from thyrocytes (Muller et al., 2014).

The astonishing changes in tissue-specific TH states in mice with TH transporter-deficiencies indicate that TH levels are regulated in an organ-specific manner, and not entirely governed by systemic factors such as regulation through the HPT axis. With regard to the thyroid gland itself, such organ-specific mechanisms must comprise of intra-thyroidal self-regulatory pathways to manage its thyroid state. In this study, pathways of thyroid auto-regulation were studied in Mct8- and/or Mct10-deficient male mice in comparison to wild type (WT) controls. In addition, *Ctsk*$^{-/-}$ male mice were used, because they provide a suitable animal model for analyzing intra-thyroidal pathways of thyroid auto-regulation, in particular with regard to Tg proteolysis (Dauth et al., 2011). Serum TH levels are not altered in cathepsin K deficiency, and hence, TSH levels are likely not enhanced in this mouse model, despite developing an enlarged thyroid gland and featuring changes in Tg-processing abilities in that cathepsin L is up-regulated (Friedrichs et al., 2003). This means that *Ctsk*$^{-/-}$ mice show signs of thyroid dysfunction without an accompanying dysregulation of the HPT axis. Thus, the main objective of this study is to elucidate cellular and molecular pathways underlying auto-regulation of thyroid functions in animals with functional defects in Tg-processing and in TH release from thyroid follicles.

2. Materials and methods

2.1. Animals

All studies were performed with 10–14 weeks and 20–28 weeks old male *Ctsk*$^{-/-}$, *Mct8*$^{-/y}$, *Mct10*$^{-/-}$, *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ or WT C57Bl/6J mice. The mice were back-crossed to a congenic C57Bl/6J background in the animal facility of Jacobs University Bremen, Germany, licenced under SfAFGJS Az. 513-30-00/2-15-32 and 522-27-11/3-1, 05-A20 and A21. The generation and genotyping procedures of mouse strains lacking *Mct8* (Trajkovic et al., 2007; Wirth et al., 2009), *Mct10* (Mariotta et al., 2012), *Mct8* and *Mct10* (Muller et al., 2014), or *Ctsk* (Saftig et al., 1998) were described previously. *Ctsb*$^{-/-}$, *Ctsk*$^{-/-}$, *Ctsl*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsk*$^{-/-}$ male mice were bred and housed as previously described (Friedrichs et al., 2003). In general, mice were housed under standard conditions, with a 12 h/12 h light/dark cycle with lights out at 07:00 p.m. and *ad libitum* water and food.

2.2. Tissue sampling and sectioning

Mice were deeply anesthetized by CO_2 inhalation or by using the inhalation-anesthetic IsoFlou (Abbott GmbH & Co. KG, Wiesbaden-Delkenheim, Germany) before intra-peritoneal injection of 100 μl of 10% ketamine (CEVA Sante Animale, Düsseldorf, Germany). The abdominal and thoracic cavities were opened and the abdominal aorta was cut. Perfusion was carried out through the heart with 0.9% NaCl supplemented with 200 IU heparin (Braun Melsungen AG, Melsungen, Germany). The thyroid was immediately removed, while still attached to the trachea, either snap-frozen in liquid nitrogen, or incubated in 4% PFA in 200 mM HEPES, pH 7.4, and left overnight at 4 °C. Then, cryo-preservation was carried out by embedding in Tissue Freezing Medium (Jung, through Leica Microsystems, Wetzlar, Germany) overnight, before tissue was frozen in the gas phase of liquid nitrogen and stored at -80 °C until sectioning. Thyroid samples were then mounted on the sectioning tableau of the cryotome, and sections of 5 μm were prepared at −20 °C (Leica Microsystems).

2.3. Indirect immunofluorescence

Embedding material was washed out of the tissue sections with PBS prior to the staining procedure. Blocking was performed with 3% bovine serum albumin (Albumin Fraction V, Roth, Karlsruhe, Germany) for 60 min at 37 °C. Samples were incubated overnight with specific antibodies diluted in 0.1% BSA in calcium- and magnesium-free PBS (CMF-PBS) at 4 °C in a moisturized chamber. Specific antibodies were rabbit anti-Mct8 (HPA003353; 1 in 250 ATLAS Antibodies, Stockholm, Sweden), sheep anti-cathepsin L (06-0008; 1 in 20, RD antibodies through Merck-Millipore, Darmstadt, Germany), goat anti-cathepsin B (GT15047; 1 in 200, Neuromics, through Acris, Himmelstadt, Germany), sheep anti-cathepsin D (06-0003; 1 in 50, Bio-Ass, Diessen, Germany), and rabbit anti-bovine Tg (Brix et al., 1998). Secondary antibodies and counter-stains were incubated for 60 min at 37 °C. As secondary antibodies, indocarbocyanine- (Cy3) or Alexa488-conjugated goat anti-rabbit IgG, donkey anti-sheep and rabbit anti-goat IgG, and Alexa Fluor 546 coupled to donkey anti-sheep were used. Draq5™ (DR05500, 1 in 500, BioStatus, Leicestershire, UK) was used as counter-stain to visualize nuclear DNA.

2.4. Image acquisition of mouse thyroid tissue and densitometry

Confocal images were taken using a Zeiss LSM 510 META laser scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss Jena GmbH, Jena, Germany). Optical sections were taken with a pinhole opening of 1 Airy unit and at a resolution of 1024 × 1024 pixels or 2048 × 2048, using LSM 5 software (version 3.2; Carl Zeiss GmbH). For quantitation of cysteine cathepsin and Mct8 levels, thyroid tissue from at least three different animals was analyzed such that images from tissue sections were obtained at the same detector gain and pinhole settings. The number of follicles analyzed is given in the respective figure legends. By using Cell Profiler software, signals of immunostaining intensities were quantified and normalized to the number of cells by enumerating the corresponding Draq5™-positive nuclei (Kamentsky et al., 2011).

2.5. Protein extraction

Thyroid tissue samples were homogenized and lysed in ice-cold PBS containing 0.5% Triton X-100, 1 mM EDTA, and protease inhibitors (0.2 μg/ml aprotinin, Sigma-Aldrich, 1153; EDTA; 10 μM E-64, Enzo Life Science, Farmingdale, NY, USA, ALX 260007-M005; 1 μM pepstatin A, Sigma-Aldrich, 77170). After clearing at 10,000 × *g* and 4 °C for 10 min, the lysates were stored at −20 °C. The protein content of all samples was determined using the Neuhoff assay (Neuhoff et al., 1979) as described before (Arampatzidou et al., 2012).

2.6. Gel electrophoresis and immunoblotting

Tissue lysates were normalized to equal amounts of protein, heated at 95 °C in sample buffer and separated on self-cast 12.5% acrylamide gels or horizontal SDS Gradient 8–18 Excel-Gel gels (GE Healthcare, Uppsala, Sweden). In some experiments, proteins of tissue lysates were separated under non-reducing conditions. Gels were either stained for total protein by silver staining (Heukeshoven and Dernick, 1988), or separated proteins were further transferred onto a nitrocellulose membrane by a semi-dry blotting procedure (Kyhse-Andersen, 1984).

Immunodetection was performed with primary antibodies rabbit anti-ß-tubulin (ab6046, 1 in 500, Abcam), goat anti-cathepsin B (GT15047; 1 in 1000, Neuromics through Acris, Herford, Germany), rabbit anti-cathepsin D (IM16; 1 in 80, Calbiochem through Merck Millipore), goat anti-cathepsin L (GT15049; 1 in 1000, Neuromics through Acris), rabbit anti-Mct8 (HPA003353; 1 in 2500 ATLAS Antibodies), and rabbit anti-bovine Tg (W.S. 3, Brix et al., 1998) applied for 1.5 h (see, Friedrichs et al., 2003). The respective secondary antibodies, HRP-conjugated goat anti-rabbit IgG (H + L sp.) and rabbit anti-goat IgG (H + L sp.) were applied for 1 h at room temperature. Visualization was performed by chemiluminescence ECL Western Blotting substrate onto XPosure™ film (Pierce through Thermo Scientific, Schwerte, Germany), which were scanned using a transmitted light scanner device. Densitometry analyses were performed by using ImageJ as described elsewhere (Tan and Ng, 2008; Gassmann et al., 2009). Samples were normalized to equal amounts of protein and bands were normalized to WT controls or to ß-tubulin loading if not stated otherwise.

2.7. Statistical analyses

Levels of statistical significance of non-paired observations were calculated by two-tailed Student's *t*-test or one-way ANOVA with Dunnett's correction for multiple comparisons using Microsoft® Excel® 2016 (Microsoft Corp.) or Graph Pad Prism™ (GraphPad Software Inc., San Diego, CA, USA).

3. Results

Mice deficient in Mct8 and/or Mct10 are characterized by thyrotoxic states in several TH target organs, including the thyroid gland itself. The two- to three-fold elevated TH levels in thyroid

tissue of Mct8- and Mct10-double-deficient mice (Muller et al., 2014) can, in principle, be explained by the inability of thyroid follicles to properly release TH into the blood stream due to lacking Mct8- and/or Mct10-mediated transport across the basolateral plasma membrane domain of thyroid epithelial cells, leading to TH accumulation within these cells. In addition, reduced T_4 levels in the blood stream stimulate TSH production, in principle, possibly affecting Tg solubilization and utilization by means of its extra- and intracellular proteolysis mediated by the cysteine cathepsins. Thus, altered Tg turnover and degradation may further contribute to the enhanced levels of TH detectable in thyrocytes of Mct8- and/or Mct10-deficient mice. Therefore, the status of Tg cross-linking and degradation was correlated to the amounts of Tg-processing proteases in thyroid tissue of TH transporter-deficient mice, and *vice versa*, the expression and localization of the TH transporter Mct8 was determined in thyroid tissue of cathepsin-deficient mice.

3.1. Altered thyroglobulin status in Mct8- and/or Mct10-deficient mouse thyroid tissue

The Tg status of Mct8 and/or Mct10-deficient thyroid glands was analyzed morphologically by staining thyroid tissue sections with antibodies directed against Tg. Luminal Tg depositions are heterogeneous but show the typical multilayered appearance (Berndorfer et al., 1996; Friedrichs et al., 2003) (Fig. 2). Regions representing solubilized Tg can be identified by higher immuno-staining intensities, because these Tg forms are more easily accessible, whereas compact, covalently cross-linked forms of Tg are stained less intense by the antibodies (Friedrichs et al., 2003). While covalently cross-linked forms of Tg are prevalent in the centre of follicle lumina, the soluble and more easily accessible Tg forms are typically detected next to the apical plasma membrane (Fig. 2, panels A and B, WT; cf. panel C). The differences in appearance of luminal Tg-staining thus depend on the accessibility of Tg for the antibodies, and we used that effect to quantify the proportion of follicles containing tightly compacted Tg (Fig. 2C, a, black), multilayered Tg (Fig. 2C, b, grey) and highly solubilized, multilayered Tg apically with dense, compact Tg in the centre of that follicle (Fig. 2C, c, light grey; diamonds).

Manual counting revealed that there was a trend of a decline in follicle counts with tightly compacted Tg in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice (60.8 ± 5.7%) compared to the WT (87.4 ± 2.5%), while no significant differences were observed in *Mct10*$^{-/-}$ mice (72.0 ± 11.3%) and in *Mct8*$^{-/y}$ mice (69.4 ± 7.7%) (Fig. 2, bar charts beneath panel B). Additionally, follicles with peripheral regions of well-accessible Tg were not detected in WT (0.3%) or *Mct10*$^{-/-}$ mice (0.0%), but 4.3% of all follicles in Mct8-deficient mice contained those Tg appearances which were even more pronounced in Mct8/Mct10-deficient animals (9.0%) (Fig. 2, bar charts beneath panel B). Thus, as deduced from antibody accessibility, Tg states are altered with regard to the well-accessible Tg appearances in the peri-cellular region at the apex of thyrocytes, in particular in *Mct8*$^{-/y}$ and in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice, indicating more solubilized Tg than in WT animals (Fig. 2, panels A and B, cf. *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ with WT).

3.2. Thyroglobulin degradation is enhanced in Mct8- and/or Mct10-deficient mice

To further investigate the effects of TH transporter deficiency on the status of the TH precursor Tg, we analyzed whole thyroid lysates prepared from young (<2.5 month, see Fig. 3) and adult (5–8 month, see Fig. 4) mice.

Immunoblots revealed an increase of soluble Tg fragments (<330 kDa) in young *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice compared to all other genotypes (Fig. 3A and B), and less abundant multimeric forms of Tg in silver-stained SDS-gels of the same genotype (Fig. 3C), indicating enhanced solubilization of Tg in Mct8- and Mct10-double-deficient animals. The presence of more Tg fragments points towards an enhanced utilization of the TH precursor Tg by thyrocytes upon deficiencies in Mct8 and Mct10. Since a decrease of mono- and dimeric Tg forms in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice in comparison to Mct8-deficient mice was detected under reducing conditions (Fig. 3D), and at the same time, the soluble dimeric form of Tg was increased when thyroids lysates were separated under non-reducing conditions (Fig. 3C), it must be assumed that Tg cross-linking is altered in thyroid follicles of young Mct8- and Mct10-double-deficient mice.

To confirm this interpretation, adult animals were analyzed. In accordance with the Tg status observed in young animals (see Fig. 3), significantly enhanced amounts of Tg fragments were detected in both Mct8- and Mct8/Mct10-deficient adult mice when the protein lysates were separated under reducing conditions (Fig. 4A). However, it was found that the effect of increased dimeric Tg in Mct8/Mct10-deficiency of young animals was not present anymore when thyroid lysates of adult animals were analyzed under non-reducing conditions (Figs. 4B, cf. 3C). Moreover, Tg multimerization and the amounts of dimeric Tg were observed to occur to comparable extents in thyroid tissue lysates of all genotypes analyzed under non-reducing conditions (Fig. 4B), while less di- and monomeric Tg was observed in Mct8- and/or Mct10-deficient thyroid tissue lysates in comparison to WT when inspected under reducing conditions (Fig. 4C).

Thus, gross degradation of Tg is enhanced in Mct8- and/or Mct10-deficient mice at all ages, while cross-linking for Tg storage is affected primarily in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice at an age of up to 2.5 months, but by far less prominently altered in 5–8 month old mice. These results indicate that intra-thyroidal regulation of Tg turnover affects enzymes involved in cross-linking of Tg (for review, see Brix et al., 2001) to a lower degree than the proteases involved in extra- and intracellular processing of Tg for its solubilization and utilization, eventually resulting in TH liberation.

3.3. Tg-processing proteases are differentially affected by Mct8 and/or Mct10 deficiencies

To investigate whether the observed alterations in Tg status might be due to altered levels of proteolytic enzymes we analyzed the protein levels of the cathepsins B, D, and L in thyroid tissue of young and adult animals. In young mice (<2.5 month), protein levels of cathepsin B were significantly increased in Mct8/Mct10-double-deficient animals, only (Fig. 5). In adult mice (5–8 months), the protein amounts of cathepsins B and L in thyroid lysates were significantly increased in Mct8- and/or Mct10-deficiencies (Fig. 6, top and bottom panels), whereas cathepsin D protein levels were significantly increased in Mct8/Mct10-double-deficient animals, only (middle panel) when compared to the WT.

Since the localization of proteases, and therefore their possible access to the substrate Tg, defines their impact on maintaining thyroid function, we used immunohistochemistry to investigate whether the intra-follicular and subcellular localization patterns of the cathepsins B, D, and L changed in TH transporter deficiency.

Thyroid tissue cryo-sections from WT or Mct8/10-deficient animals were immunolabeled with antibodies against cathepsin B. In all genotypes inspected, a staining pattern of largely endocytic cathepsin B, and, in few follicles, staining in the follicle lumen as well as at the apical plasma membrane domain of thyrocytes was observed (Fig. 7A–D). Moreover, the appearance of thyrocytes with enlarged cathepsin B positive vesicles in *Mct8*$^{-/y}$ was detected (Fig. 7G, arrows), which was even more prominent in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice (Fig. 7H, arrows). However, the overall cathepsin B signal intensity was increased only slightly in *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice when compared to the WT (Fig. 7J).

In WT thyroid epithelial cells, staining of cathepsin D was restricted to intra-cellular vesicular structures (Fig. 8A and E). The

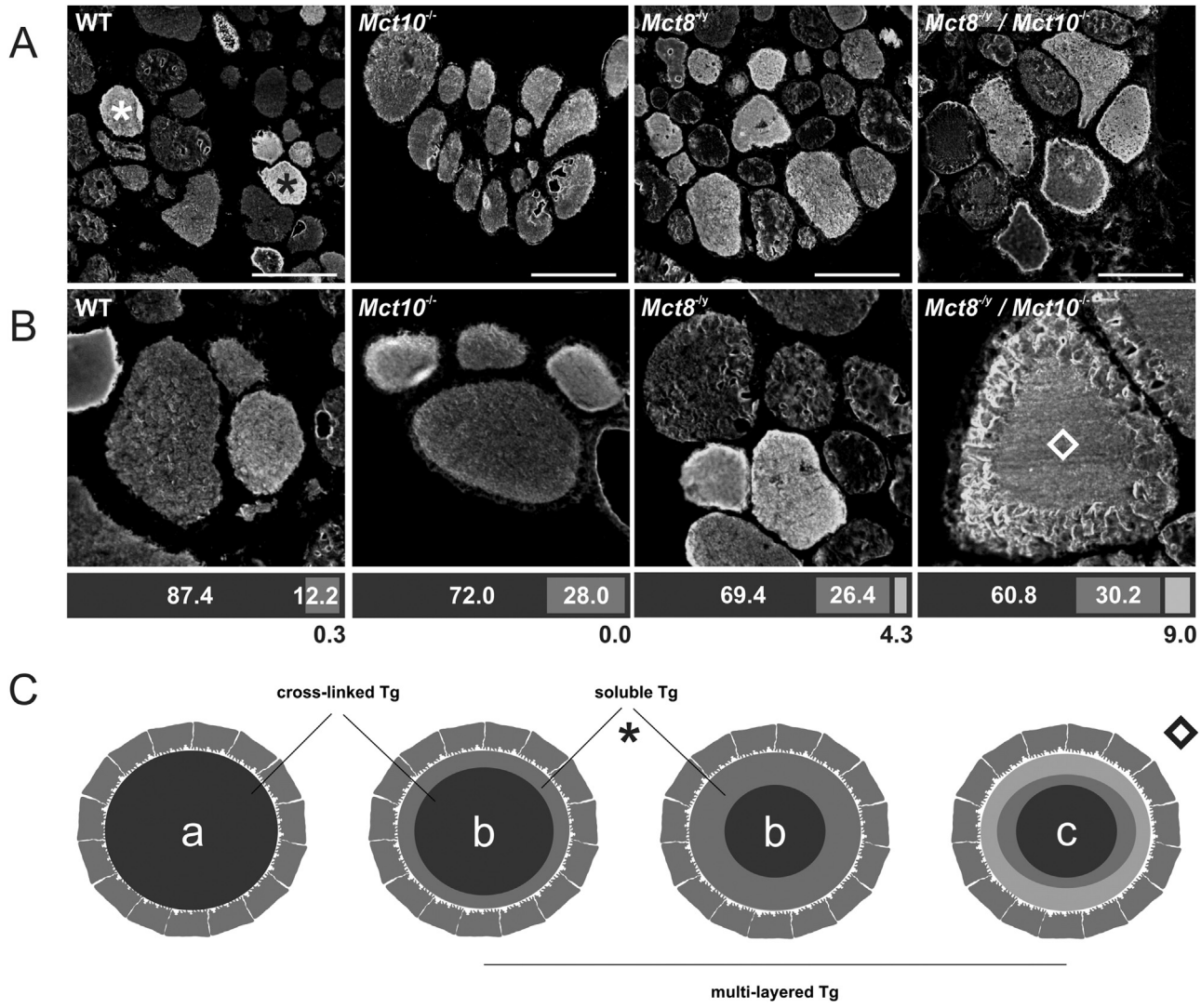

Fig. 2. Assessment of Tg states in thyroid follicles of Mct8/Mct10-deficient mice.
Confocal fluorescence images of cryo-sections of thyroid glands from adult WT or TH transporter-deficient mice (5–8 months old) of the indicated genotypes immunolabeled with antibodies against Tg. Tg was detected in multilayered form in the follicle lumen of WT mice with increased labelling in proximity to the apical plasma membrane domain of thyrocytes, thus indicating soluble forms of Tg (A, asterisks). Abundance of follicles without prominent labelling of soluble Tg (C, a) was decreased in Mct8- and Mct8/Mct10-double deficient animals (A and B). Likewise, the proportion of follicles with intensely immuno-labeled soluble Tg (C, b) is increased in Mct8-deficient animals and even more increased in Mct8/Mct10-double deficient animals. Interestingly, 4.3% and 9.0% of all analyzed follicles of Mct8 or Mct8/Mct10-deficient animals, respectively, featured highly solubilized Tg (C, c) in the luminal periphery but a dense mass of covalently cross-linked Tg in the central region of large follicles (B, diamond). A: representative, overview images used for quantification, n = 250, 72, 190, and 127 follicles, respectively. B: close-up images displaying follicles with different forms of Tg. Bar charts in B and C: black (a), percentage of follicles without labeled soluble Tg; grey (b), percentage of follicles with labeled soluble Tg; light grey (c), percentage of follicles with highly solubilized Tg given as means. Scale bars: 100 μm.

signal intensity of cathepsin D staining was elevated in thyroid tissue of *Mct10*$^{-/-}$, *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice (Fig. 8B and F, C and G, D and H, respectively) when compared to the WT. Interestingly, cathepsin D positive vesicles in Mct8- and/or Mct10-deficient mice appeared larger than those of the respective WT controls and in some cases, vesicles with prominently enhanced diameters were found (Fig. 8F–H, cf. E, dashed arrows). A striking difference between *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice was the occurrence of cathepsin D staining at the basal surface of thyrocytes, which could either be due to cathepsin D staining in endothelial cells (Fig. 8G, asterisks), secretion of cathepsin D at the basal pole, and its re-association with the basolateral plasma membrane domains of thyrocytes (arrows), or both.

In WT animals, cathepsin L is localised in vesicular structures, but in few follicles, association with the apical plasma membrane domain of thyrocytes was also observed (Fig. 9A). In Mct10-deficient mice, the localization of cathepsin L did not change when compared to the WT control, however a slight increase of signal intensity was measured indicating elevated protein expression levels (Fig. 9B and H). In *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice, cathepsin L was detected intracellularly within vesicular structures that were much larger than those in WT tissue, in some cases reaching diameters comparable of the nuclei of the respective cells, *i.e.* up to 6 μm (Fig. 9C and D, E and F, E' and F', arrows). Additionally, localization of cathepsin L at the apical plasma membrane domain of *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ thyrocytes was increased and luminal staining next to the apical plasma membrane domain was much more abundant than in the WT (Fig. 9F, cf. E, arrows).

The data from immunofluorescence staining of thyroid tissue (Figs. 7–9) confirmed the immunoblotting results (Fig. 6) such that cathepsin B protein levels were increased in thyroid lysates of *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ animals, while cathepsin L protein

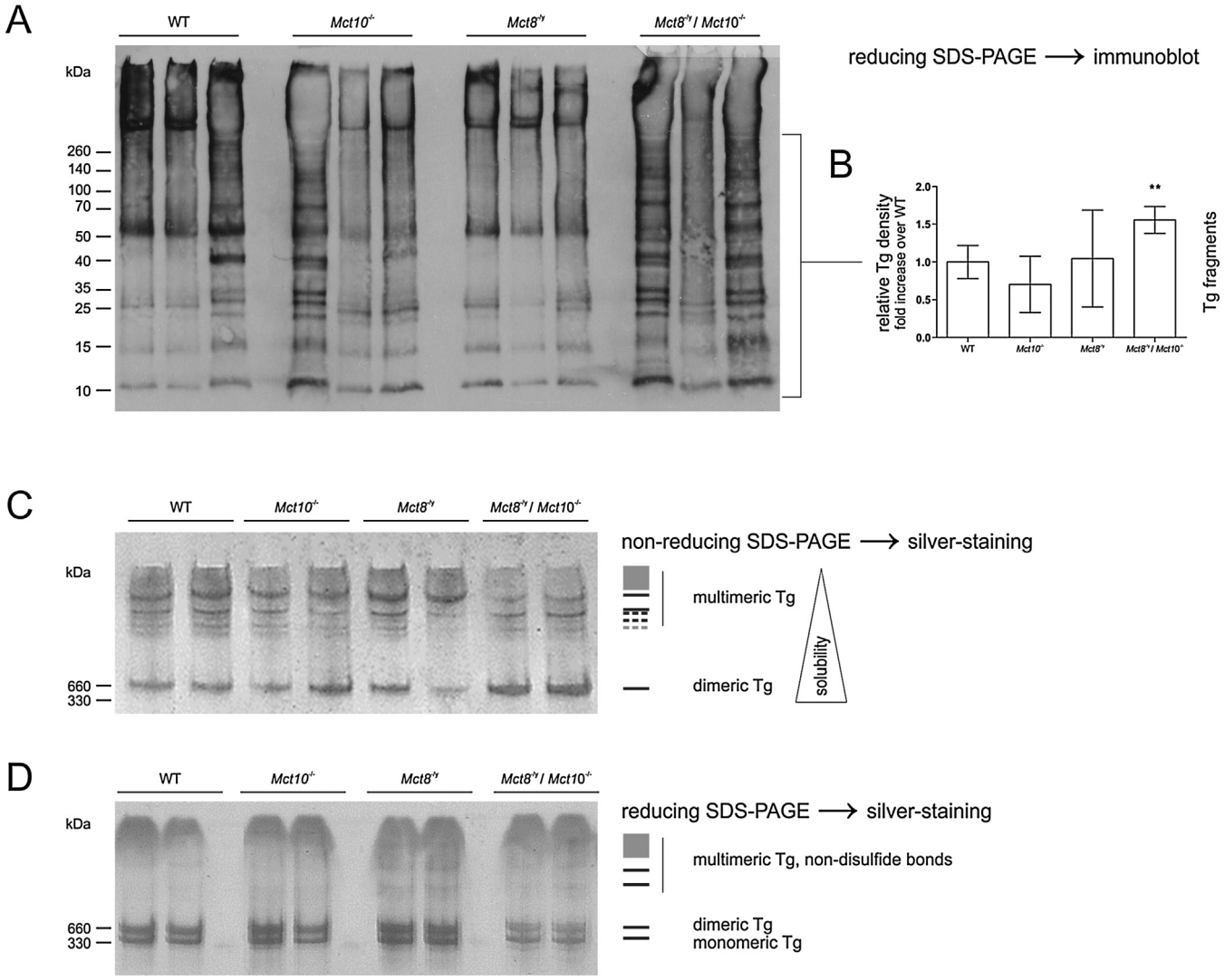

Fig. 3. Thyroglobulin degradation pattern and cross-linkage in <2.5 months old mice.
Whole thyroid lysates of WT mice or mice deficient in Mct10, Mct8 and/or Mct10 were separated on horizontal SDS-gels, silver-stained or transferred to nitrocellulose for subsequent incubation of the blots with antibodies against Tg as indicated, and compared to the WT. (A) Shown is a representative immunoblot with the indicated genotypes. (B) Low molecular mass fragments of Tg are representative of degradation products which were revealed by densitometry to be significantly increased in Mct8/Mct10-deficient animals. (C) Under non-reducing conditions, the amounts of the soluble form of Tg are increased in Mct8/Mct10-deficient animals, whereas the multimeric Tg forms were less abundant compared to the WT, Mct10- and Mct8-deficient animals. Mct8-deficient mice were observed to have more total Tg amounts including the multimeric form. Upon reducing samples with DTT before SDS-PAGE, the persistence of dimeric Tg was reduced in Mct8/Mct10-deficient animals (E), thus indicating altered solubilization of Tg, whereas Mct8-deficient animals featured increased dimeric and monomeric Tg when compared to the WT. Animals analyzed: n = 5, 3, 5, and 5, respectively. In B, means ± SD are given; *, $P < 0.05$; **, $P < 0.005$.

levels were enhanced in thyroid tissue of all genotypes, and most prominently in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mice, which were further characterized by enhanced amounts of cathepsin D. Thus, the data point to most significantly enhanced amounts of endo-lysosomal cathepsin L in particular in *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mouse thyrocytes, *i.e.* in the animal models featuring the most severe intra-thyroidal thyrotoxic states (Muller et al., 2014). Considering cathepsin L's main function in Tg processing, the enhanced amounts of this protease in *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ thyroid tissue explain the largely enhanced extent of Tg degradation observed in Mct8/Mct10-deficient mice (Figs. 3 and 4), and suggest that T_4 accumulates in thyrocytes of these animals. Thus, the enhanced amounts of cathepsin L, which is also vital for T_4 liberation from Tg, in thyroids of mice lacking the TH-transporting Mct8, which is mainly responsible for T_4 release from thyroid follicles, is counterintuitive.

3.4. A lack of the T_4-liberating cysteine cathepsin K is associated with enhanced amounts of the main T_4-exporting transporter Mct8

The cysteine cathepsins K and L together are critical for TH liberation (see Table 1; Friedrichs et al., 2003). Cathepsin K, in particular, mediates direct liberation of T_4 from Tg (Tepel et al., 2000). A loss of cathepsin K can be compensated by other cathepsins; this notion was derived from analyses of mice shortly after establishing the *Ctsk*$^{-/-}$ model, *i.e.* when the animals were still on a mixed 129Ola:C57BL/6J background (Friedrichs et al., 2003). However, in this study, we used congenic animals on the C57BL6/J background. Immunoblotting revealed that the loss of cathepsin K led to an increase of cathepsin L expression, whereas protein amounts of cathepsins B and D were not, or only marginally elevated in *Ctsk*$^{-/-}$ when compared to WT controls (Fig. 10). These data confirmed our

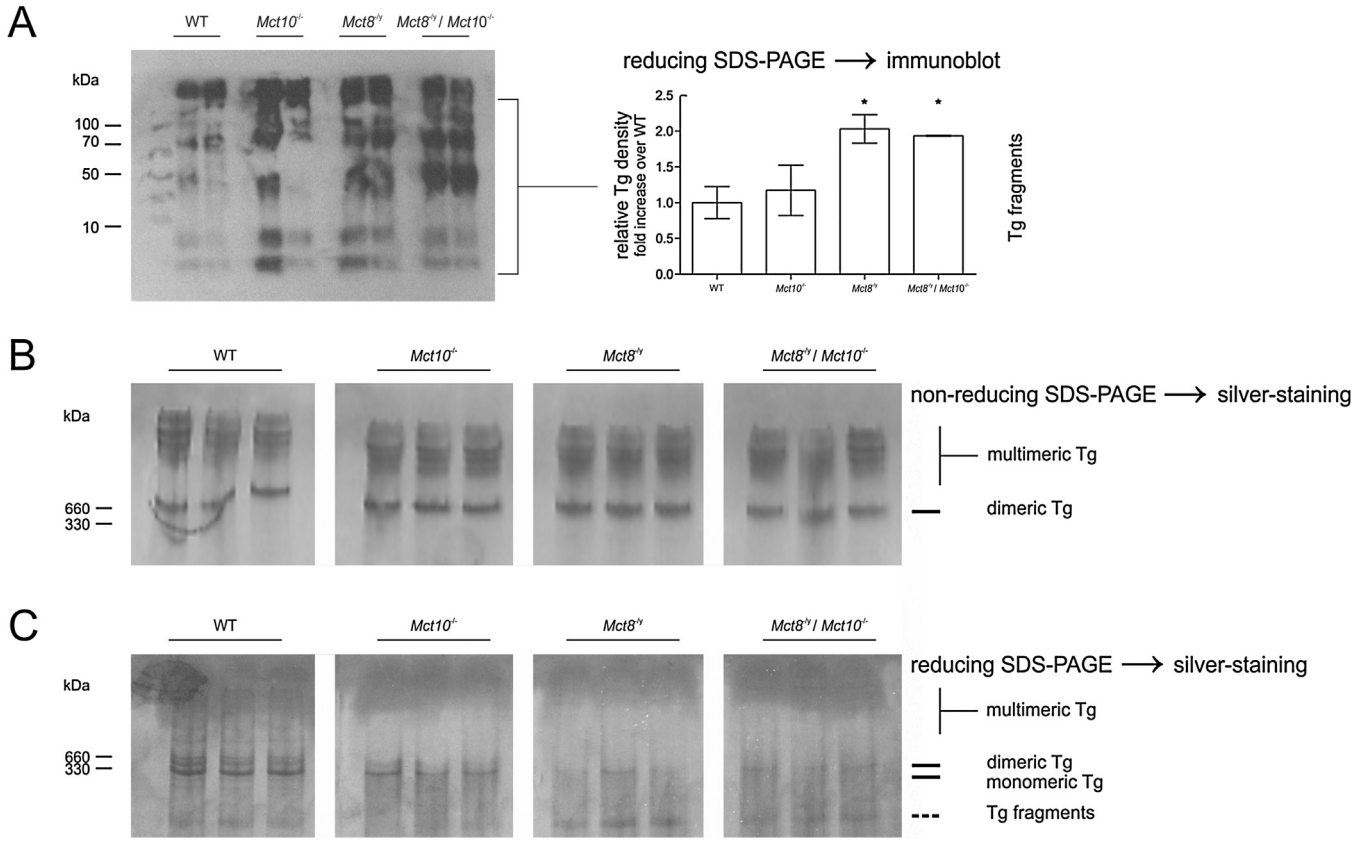

Fig. 4. Thyroglobulin degradation pattern and cross-linkage in 5–8 months old mice.
Whole thyroid lysates of mice deficient in Mct10, Mct8 and/or Mct10 were separated on vertical or horizontal SDS-gels, silver-stained or transferred to nitrocellulose for subsequent incubation of the blots with antibodies against Tg, as indicated, and compared to the WT. (A) Displayed is a representative immunoblot result and the corresponding densitometry data showing increased amounts of smaller Tg fragments, with molecular masses below 330 kDa, in Mct8- and/or Mct10-deficient mice as compared to the WT mice. (B) When separating whole thyroid lysates in non-reducing conditions, the band of the dimeric Tg was comparable in all genotypes investigated, however the multimeric forms of Tg were less abundant in Mct8- and Mct8/Mct10-deficient mice. (C) Reducing samples with DTT before SDS-PAGE yielded in decreased intensity of bands representing the mono- and dimeric Tg in Mct8- and/or Mct10-deficient animals, however most striking in Mct8- and Mct8/Mct10-deficient mice, which may indicate increased solubilization and proteolytic degradation of Tg by cysteine cathepsins. Animals analyzed: n = 2–3. In A, means ± SD are given; *, $P < 0.05$.

previous findings (Friedrichs et al., 2003), and further validated the importance of cathepsin L-mediated Tg processing.

Loss of both cathepsins K and L leads to decreased T_4 levels in the blood; however, loss of cathepsin K alone does not affect serum TH levels (Friedrichs et al., 2003). While endo-lysosomal Tg processing is compensated for in *Ctsk*$^{-/-}$ mice by cathepsin L, the function of cathepsin K in extracellular T_4 liberation directly from Tg is still lacking, leading us to wonder how normal serum T_4 levels are maintained in *Ctsk*$^{-/-}$ mice. We reasoned that one possible mechanism explaining normal serum TH levels may be an upregulation of TH transporters, and to this extent we investigated Mct8 expression in thyroids of *Ctsk*$^{-/-}$ mice.

The signal detected after incubation of cryo-sections with antibodies against Mct8 (Fig. 11A and B) was quantified, and a 3-fold increase in thyroid tissue of *Ctsk*$^{-/-}$ animals was found (Fig. 11F). Likewise, in immunoblots, Mct8 protein amounts were found to be increased in thyroid tissue lysates of cathepsin K-deficient over WT animals (Fig. 11G and H). However, the localization of Mct8 at the basolateral plasma membrane domain of the thyrocytes did not change in *Ctsk*$^{-/-}$ animals (Fig. 11D and E).

As described above, alterations in the levels of TH transporters feedback on the gross degradation of Tg mediated by endo-lysosomal proteases (see Figs. 3 and 4). Therefore, we further investigated whether the reverse situation, namely a loss of the Tg-degrading cathepsins B and L, is accompanied by different expression of the TH transporter Mct8. In mice with single-deficiencies in the cathepsins B or L, or in cathepsin B- and K-double-deficient animals, similar observations were made, as such, that Mct8 staining intensities were increased over WT, but its localization at the basolateral plasma membrane domain of thyrocytes did not change (Fig. 12). However, anti-Mct8-positive puncta were additionally observed in the cytoplasm of thyrocytes of cathepsin B-, K-, or L-single- and cathepsin B/K-double-deficient mice (Fig. 12, arrows).

Thus, the notion of enhanced amounts of the T_4-exporting Mct8 in conditions of Tg-processing cathepsins lacking from mouse thyroid epithelial cells (this study) is counterintuitive, as it most likely means that the thyroid gland itself becomes hypothyroid, especially in the absence of the T_4-liberating cathepsin K (Tepel et al., 2000; Friedrichs et al., 2003), in order to maintain proper serum levels of TH. The blood serum must therefore be viewed as a sink for free T_4 (Heuer and Visser, 2013).

4. Discussion

Keeping in mind that the thyroid itself is a target of the hormones it produces, regulation of the process of liberation and release of TH is crucial to overall thyroid function (Brix et al., 2011; Dauth et al., 2011). It is well known that thyroid gland tasks are directed by the HPT axis (Nussey and Whitehead, 2001). Thus, to prevent TH over-production, circulating TH carry out negative feedback by blocking TSH release from the anterior pituitary and decreasing the production of TRH by the hypothalamus. However, regulation through the HPT axis is largely impaired in

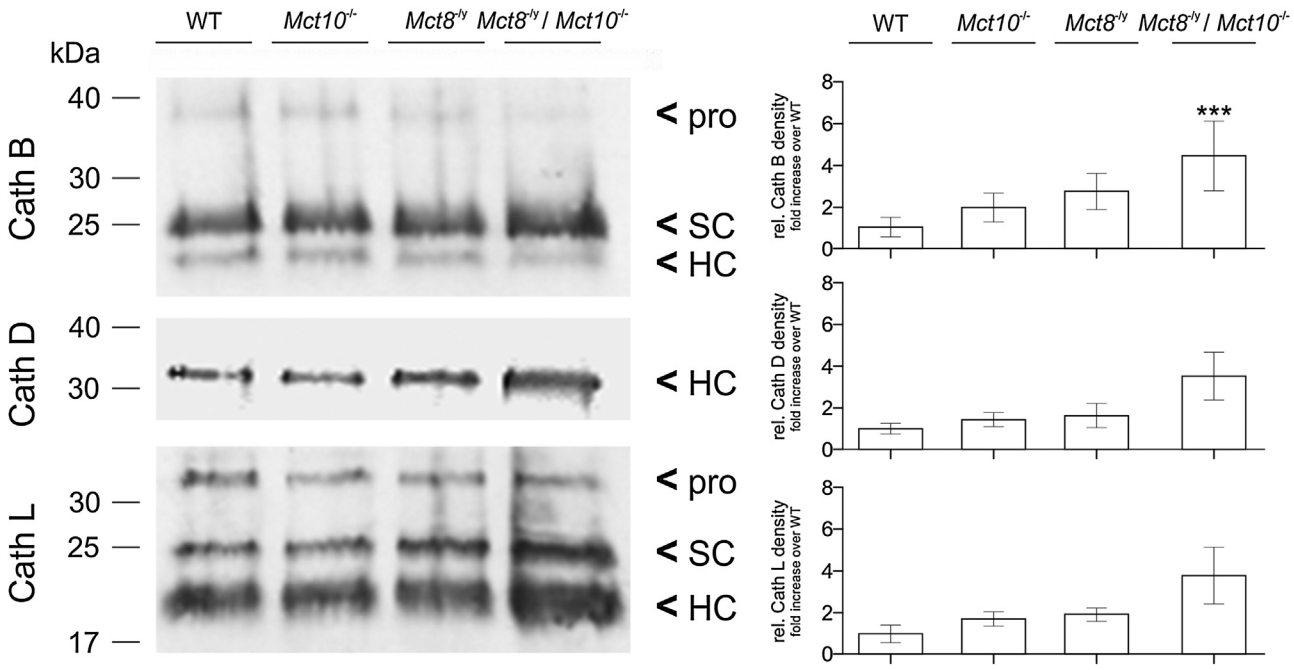

Fig. 5. Protein levels of cathepsin B, D, and L in thyroids of TH transporter-deficient young mice.
Whole thyroid lysates of young mice (less than 2.5 months old) were separated on horizontal SDS-gels, transferred to nitrocellulose and incubated with antibodies against the respective proteins (left panels). Densitometry of the immunolabeled bands revealed that cathepsin B, D, and L levels were elevated significantly in Mct8/Mct10-deficient animals, however thyroid lysates of Mct8- or Mct10-deficient animals did not exhibit elevated protein amounts when compared to the WT (right panels). Animals analyzed: n = 6, 3, 6 and 10, respectively. HC = heavy chain; SC = single chain; pro = pro form. Data are given as means ± SD; ***, P < 0.001.

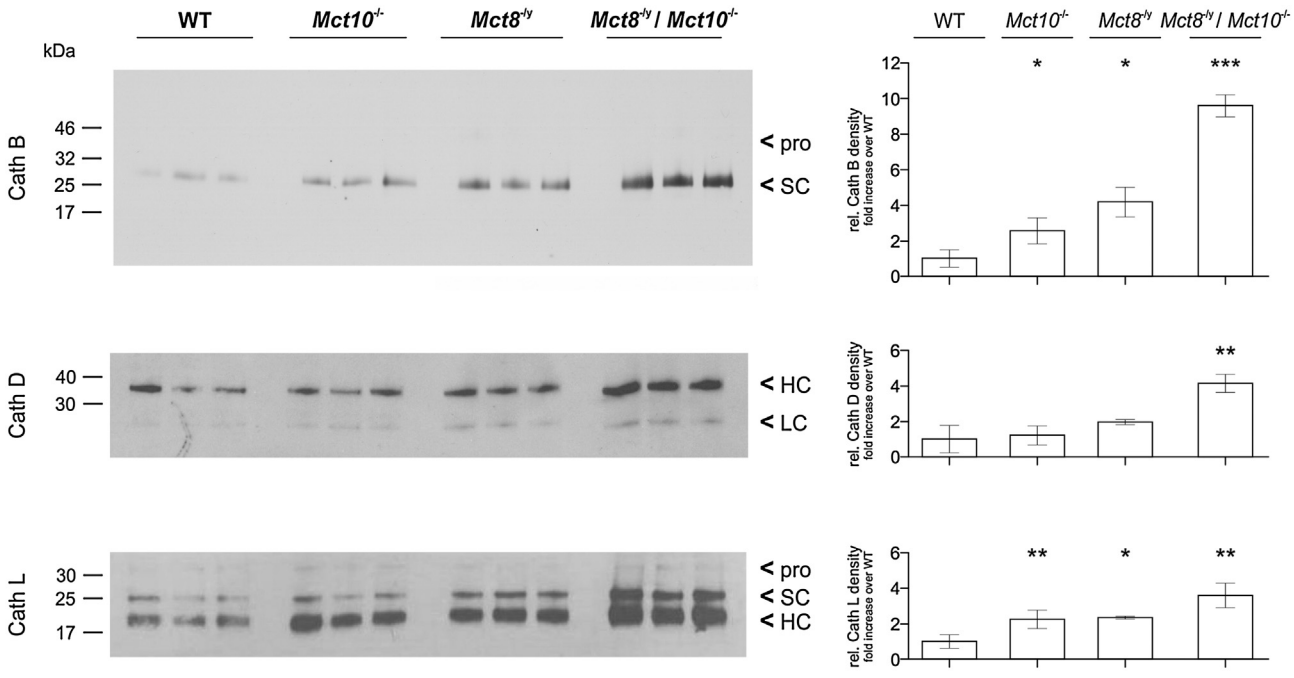

Fig. 6. Protein levels of cathepsin B, D, and L in thyroids of TH transporter-deficient adult mice.
Whole thyroid lysates of adult mice (5–8 months old) were separated on horizontal SDS-gels, transferred to nitrocellulose and incubated with antibodies against cathepsins B, D or L (left panels). Densitometry of the indicated bands revealed that cathepsin D protein levels were elevated significantly in Mct8/Mct10-deficient mice, whereas cathepsin B and L levels were increased in all investigated genotypes when compared to WT (right panels). Animals analyzed: n = 3 per genotype. HC = heavy chain; SC = single chain; pro = pro form. Data are given as means ± SD; *, P < 0.05; **, P < 0.005.

TH-resistant Mct8- and Mct8/Mct10-deficient mice (Trajkovic et al., 2007; Muller et al., 2014). In this study, we propose that TH transporters like Mct8 and Mct10 collectively meet the demands of facilitating the release of TH into the blood in order to properly support peripheral TH target organs, while also involving in intra-thyroidal TH sensing that affects the amounts and localization of Tg-processing proteases, and thereby, contributes to auto-regulation of the functional activities of thyrocytes. Indeed, the thyroid gland of mice lacking globally the TH transporters Mct8 and Mct10 is characterized by high amounts of the Tg-processing cys-

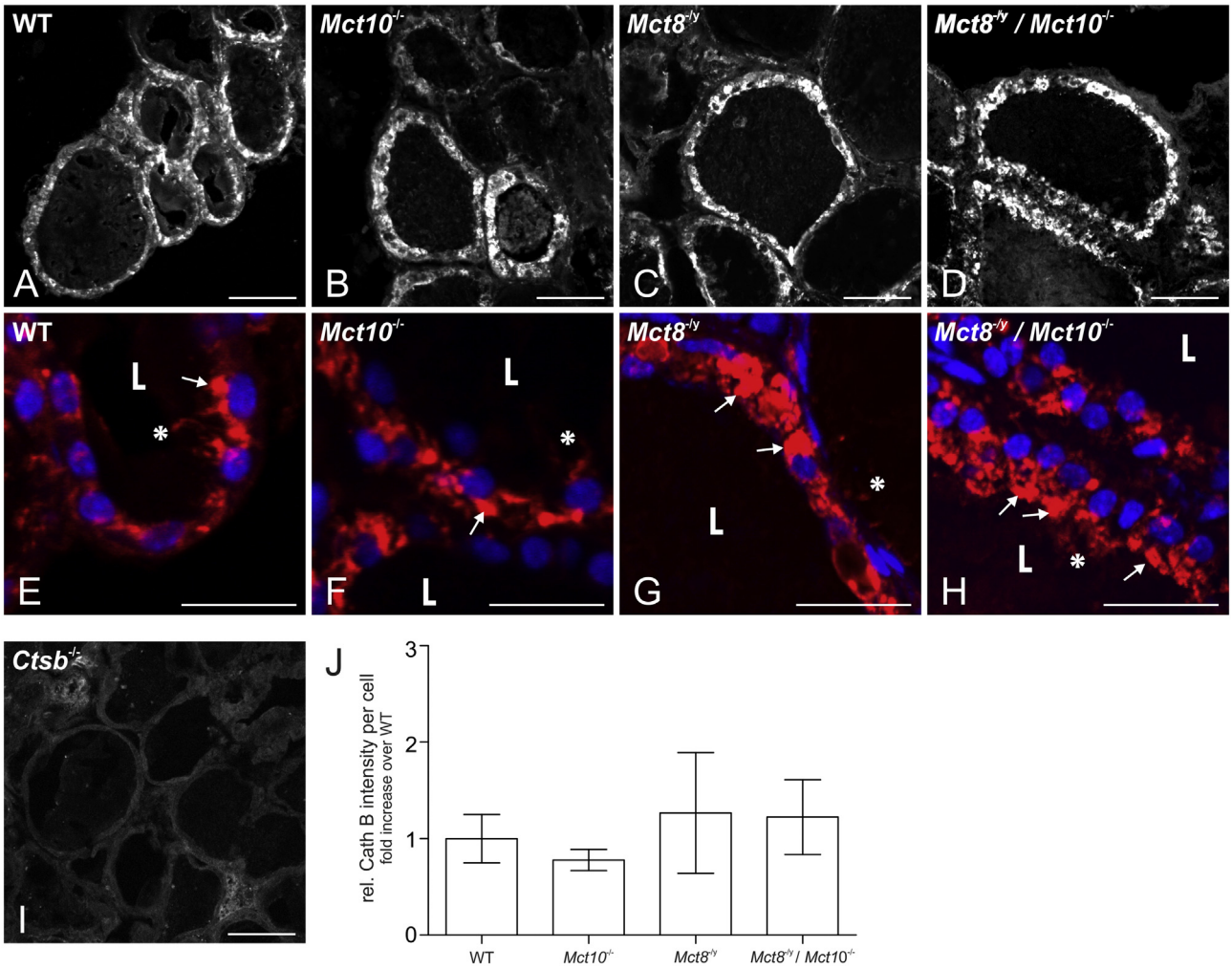

Fig. 7. Expression and localization of cathepsin B in thyroid glands from TH transporter-deficient mice.
Cathepsin B (white, red) was mainly localised in vesicles of thyrocytes of WT (A) and Mct10- (B) Mct8- (C), and Mct8/Mct10-deficient (D) animals but also associated with the apical plasma membrane domain and within the lumen of thyroid follicles (E to H, asterisks). Thyroid tissue taken from cathepsin B-deficient animals served as controls for antibody specificity, which was confirmed by lack of primary antibody reactivity (I). Vesicular staining (arrows) of cathepsin B is more pronounced and signal intensities (J) are slightly increased in both, Mct8- (G) or Mct8/Mct10-deficient (H) mouse thyroids when compared to WT (E). Representative images of 3 animals (5–8 months old) per genotype are displayed. J: normalized signal intensity per cell given as means ± SD; n = 12, 8, 8, and 12, respectively. Nuclei were counter-stained with Draq5™ (blue). L denotes follicle lumen. Scale bars: 50 μm (A–D, I) and 25 μm (E–H).

teine cathepsins, while the reverse situation, cathepsin deficiency, results in up-regulated T_4-exporting capabilities of thyrocytes, since Mct8 is present in enhanced amounts. Although counterintuitive, this notion contributes to explanations, why Mct8 deficiency leads to intra-thyroidal hyperthyroid states and to the unusual thyroid states in the blood circulation (see below; Müller and Heuer, 2014). In stark contrast, single deficiencies in the cathepsins B, K, and L feature eu- or only very mildly hypothyroid states (Friedrichs et al., 2003). Cathepsin K and L double-deficiency, only, results in hypothyroidism in mice (Friedrichs et al., 2003).

4.1. Thyroid function is maintained by TH release from thyroid follicles which is enabled by protease-mediated TH liberation from Tg

Mct8 knock-out mice exhibit an unusual pattern of altered TH serum levels with low T_4 and highly increased T_3 levels (Dumitrescu et al., 2006; Trajkovic et al., 2007; Wirth et al., 2011). Animals lacking both Mct8 and Mct10 exhibit partially normalized serum T_4 levels, despite TSH levels elevated over WT to an extent comparable to Mct8-deficient mice, but the elevated T_3 serum levels typical for Mct8-deficient mice are maintained (Muller et al., 2014). The reason for these highly unusual serum TH states are considered to reside in decreased T_4 release from thyroid follicles due to dysfunctional Mct8-facilitated export across the basolateral plasma membrane domain of thyrocytes (Muller et al., 2014). In the same animals, increased conversion of T_4 to T_3 mediated by deiodinase 1 (Dio1) in liver and kidney explains the abnormally high T_3 serum levels. In addition, we believe that the thyroid gland of Mct8 knock-out mice releases more T3 due to intact Mct10-facilitated transport, which contributes further to the unusually high T_3/T_4 ratios in the blood of Mct8-deficient mice (Muller et al., 2014).

Previously, an enlargement of thyroid follicles was found in mice with deficiencies in Mct8 alone, or in Mct8 and Mct10 (Di Cosmo et al., 2010; Trajkovic-Arsic et al., 2010b; Wirth et al., 2011; Muller et al., 2014). Moreover, transcript levels of the thyroid-specific genes coding for the sodium-iodide symporter NIS, the thyroid peroxidase TPO, and for the TH precursor protein Tg are increased in Mct8-deficient mice, and this increase was even more pronounced in Mct8/Mct10-double deficiency (Muller et al., 2014). Although TSH levels are similar in both mouse models, it is not yet known in how far bioactivity of TSH is altered in

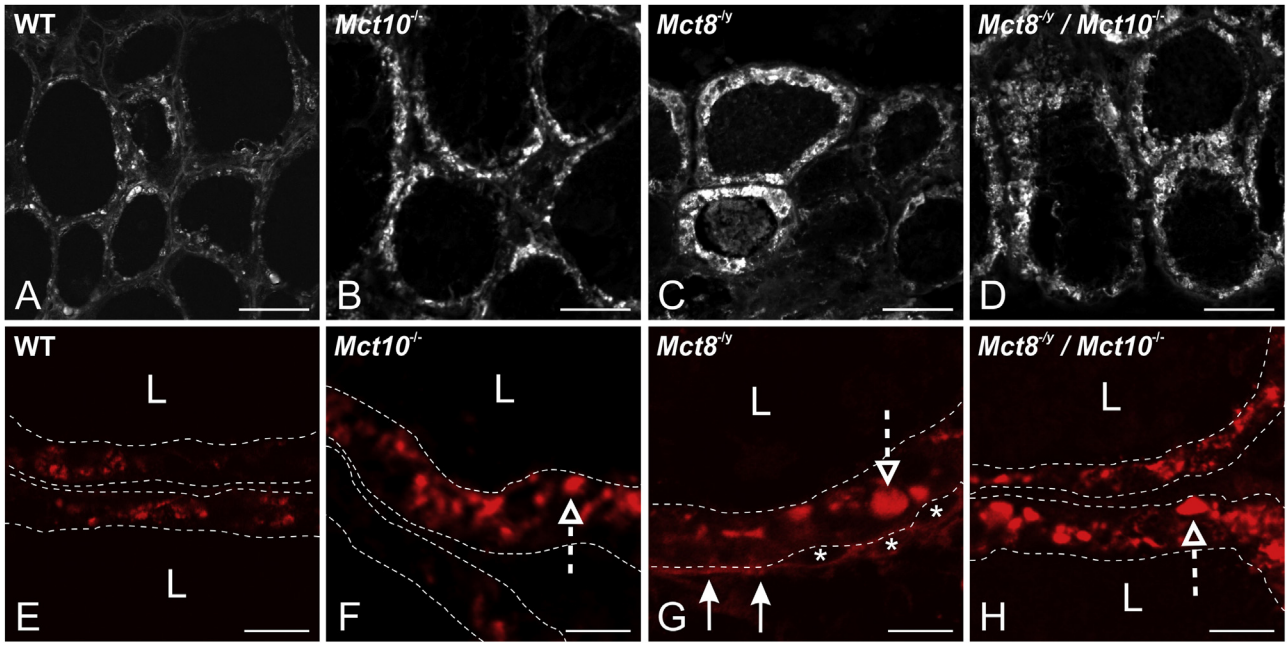

Fig. 8. Expression and localization of cathepsin D in thyroid glands from TH transporter-deficient mice.
Cathepsin D was localised in vesicles distributed evenly throughout the cytoplasm in thyrocytes of WT (A), Mct10- (B), Mct8- (C), and Mct8/Mct10-deficient (D) mice. Enlarged anti-cathepsin D-positive vesicles where found in Mct10- (F), Mct8- (G), and Mct8/Mct10-deficient (H) mice (dashed, empty arrows) when compared to the WT (E). In Mct8-deficient mice, cathepsin D staining was observed at the basal surface of thyrocytes (G, arrows), or at the plasma membrane domain of endothelial cells (G, asterisks). Dotted lines (E-H) represent outlines of follicle epithelia. Representative images of 3 animals (5–8 months old) per genotype are displayed. L denotes follicle lumen. Scale bars: 50 μm (A–D) and 10 μm (E–H).

the TH transporter-deficient mice. Indeed, it is likely that Mct8-deficient thyrocytes are stimulated more strongly than thyroid follicle cells of Mct8/Mct10-double deficient mice, because TRH levels are higher in Mct8-deficient animals (Muller et al., 2014). Thus, intra-thyroidal TH production and TH liberation from Tg is most likely de-regulated differently in *Mct8*$^{-/y}$ and *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ animals. Therefore, we investigated the Tg status and the molecular pathways of Tg utilization for TH liberation by analyzing the key players involved in proteolytic processing and degradation of Tg, the cathepsins (see Table 1 and Fig. 1).

The data collected in this study by inspecting the Tg status of TH transporter-deficient mice (see Figs. 2–4), suggests that both Tg solubilization from covalently cross-linked storage forms by extracellular Tg processing, and its utilization for TH liberation by extra- and intracellular means of proteolytic Tg degradation are enhanced in Mct8- and/or Mct10-deficiencies. Therefore, we postulated that Tg-processing enzymes may be differently expressed and localised in thyroid tissue of Mct8- and/or Mct10-deficient mice.

Secretion of cathepsin B at the apical pole of thyrocytes is increased upon acute TSH stimulation, *i.e.* 1–2 h after TSH stimulation, whereas enhanced biosynthesis of cathepsin B will occur only during long-term chronic TSH stimulation (Linke et al., 2002a,b). Accordingly, because the elevated TSH levels in Mct8- or Mct8/Mct10 animals are chronic (Di Cosmo et al., 2010; Muller et al., 2014), we expected increased protein levels of cathepsin B, which were indeed observed by immunoblotting (see Fig. 6). Moreover, the intra-follicular localization of cathepsin B differed from WT, in particular in Mct8- and Mct8/Mct10-deficient mice, such that the protease was almost exclusively present in enlarged endo-lysosomes of the thyroid epithelial cells (see Fig. 7).

Another important protein that is involved in endo-lysosomal Tg degradation, but less so in TH liberation from Tg, is the aspartic protease cathepsin D (Dunn et al., 1991; Jordans et al., 2009). In mouse thyrocytes, cathepsin D is usually localised in vesicles distributed throughout the cytoplasm and accumulating in the perinuclear region, *i.e.* in lysosomes of thyrocytes (Friedrichs et al., 2003). In this study, cathepsin D was found in immensely enlarged endo-lysosomal vesicles of Mct8- and Mct8-/Mct10-deficient mouse thyrocytes (see Fig. 8), indicating enhanced abilities of these follicle cells to completely degrade Tg by intracellular means of lysosomal proteolysis. Moreover, cathepsin D was found to be secreted from thyrocytes of Mct8-deficient mice at the basolateral pole. This finding is interesting when considering the role that cathepsin D plays in cancer (Brix et al., 2008; Brix et al., 2015). Mct8-deficient mice develop papillary thyroid carcinoma (PTC) at older age, possibly due to a loss of auto-regulative control of thyrocyte proliferation (Wirth et al., 2011). However, basolaterally secreted cathepsin D may further contribute to the progression of PTC development as it can, in principle, enhance extracellular matrix degradation, and thereby facilitate cancer progression.

Cathepsin L plays a vital role in thyrocyte function, and it was shown to be important for survival of thyrocytes, since the respective knock-out models showed luminal inclusions of dead cells and dead cell remnants (Friedrichs et al., 2003). Here we show that the availability of cathepsin L in the extracellular follicle lumen is more pronounced in Mct8/Mct10-deficient mice than in *Mct8*$^{-/y}$ mice (see Fig. 9), suggesting enhanced Tg solubilisation for subsequent endocytic uptake. In addition, this enzyme with a critical function in maintaining proper serum levels of T_4 (Friedrichs et al., 2003) was enhanced in protein amounts, in particular, in Mct8 and Mct10 deficient mouse thyrocytes.

Hence, the protein levels of the Tg-processing proteases correlate with the lack of TH transporter proteins like Mct8 and/or Mct10. These observations most likely contribute to explaining why among the TH transporter-lacking genotypes inspected in this study, especially the *Mct8*$^{-/y}$/*Mct10*$^{-/-}$ mouse model features the most severe auto-thyrotoxic states (Muller et al., 2014). In reverse, the main T_4-exporting transporter molecule Mct8 is up-regulated in *Ctsk*$^{-/-}$ mice, a Tg processing enzyme that is able to directly liberate T_4 from its protein precursor molecule Tg by extra- and intracellular means of proteolysis (Tepel et al., 2000). In addition, cathepsin L is up-regulated in the thyroid of *Ctsk*$^{-/-}$ mice (Friedrichs et al.,

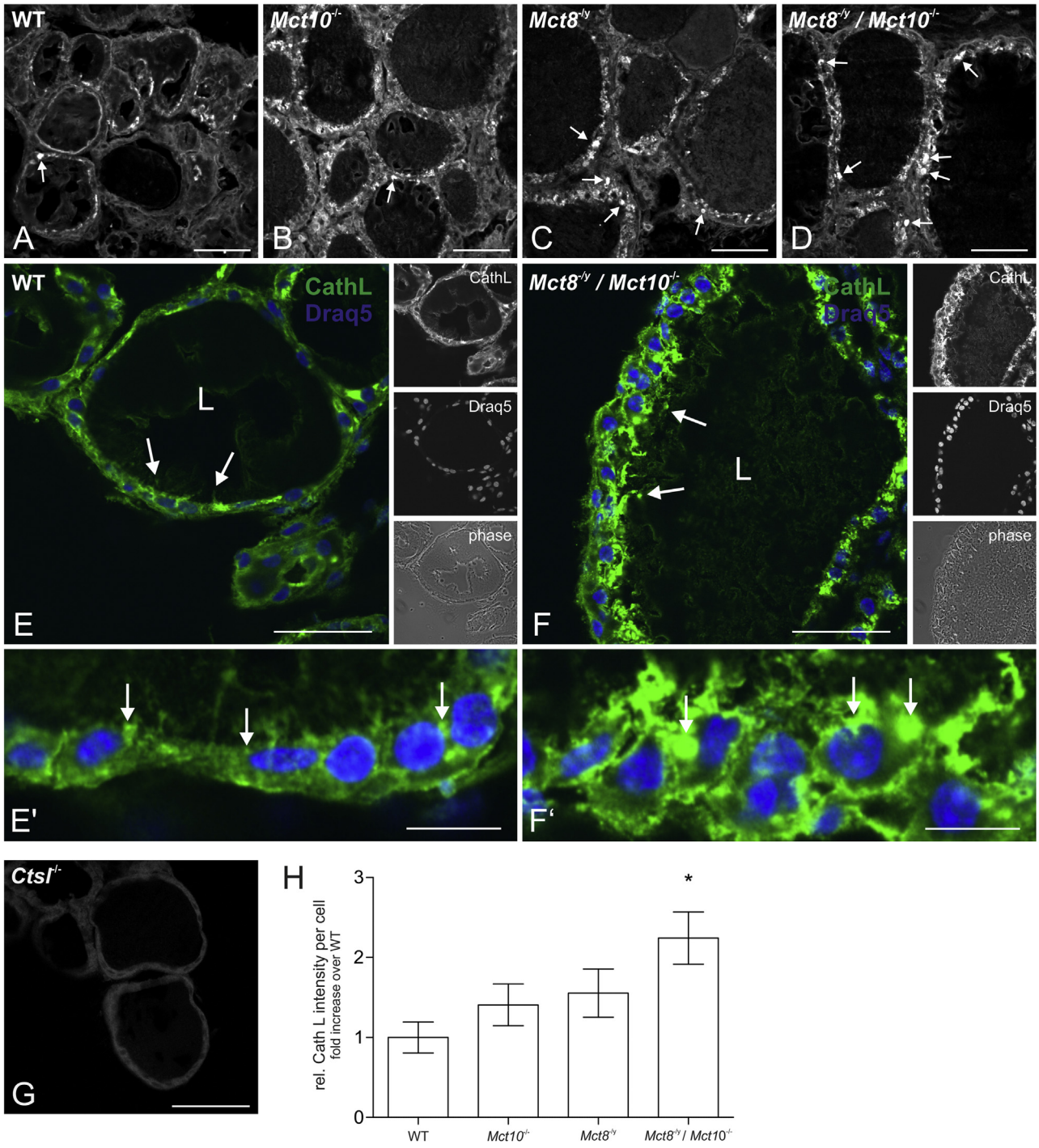

Fig. 9. Expression and localization of cathepsin L in thyroid glands from thyroid hormone transporter deficient animals.
Cathepsin L was detected in vesicular structures in all genotypes (A, B, C, and D), except in tissue from cathepsin L-deficient mice (G), which served as control for antibody specificity. Few follicles of WT (A) and Mct10-deficient (B) animals showed apical and luminal cathepsin L staining, however intensities of cathepsin L labelling (H) were increased in Mct8 and/or Mct10-deficient mice (B and C) when compared to WT animals (A). Note the increased luminal, apical and vesicular staining patterns and intensities of cathepsin L (green) in thyrocytes of Mct8/Mct10-deficient animals (F) compared to the WT (E), and in the respective higher magnifications (E' and F'). Representative images of 3 animals (5–8 months old) per genotype are displayed. Nuclei were counter-stained with Draq5™ (blue). H: normalized signal intensity per cell; mean +/− SEM; n = 1193, 1422, 1309, and 1638, respectively. L denotes follicle lumen. Scale bars: 50 μm (A–D, G) and 10 μm (E, F). *, $P < 0.05$.

2003), which also feature enhanced Mct8 at the basolateral plasma membrane domains of thyrocytes (this study).

Such correlations between TH-liberating proteases and TH transporters are somewhat counterintuitive, and indicate a loss of TH sensing capabilities of thyrocytes upon functional loss of either key-player enabling thyroid gland functions (see Fig. 13).

4.2. Thyroid auto-regulation tolerates minor changes in thyroid states by redundancy, but a self-destructive vicious cycle is switched on when the key-players cathepsin L and Mct8 are affected by disease

Physiologically, we suggest that thyrocytes can counter-act minimal functional disbalances of thyroid states by up-regulation

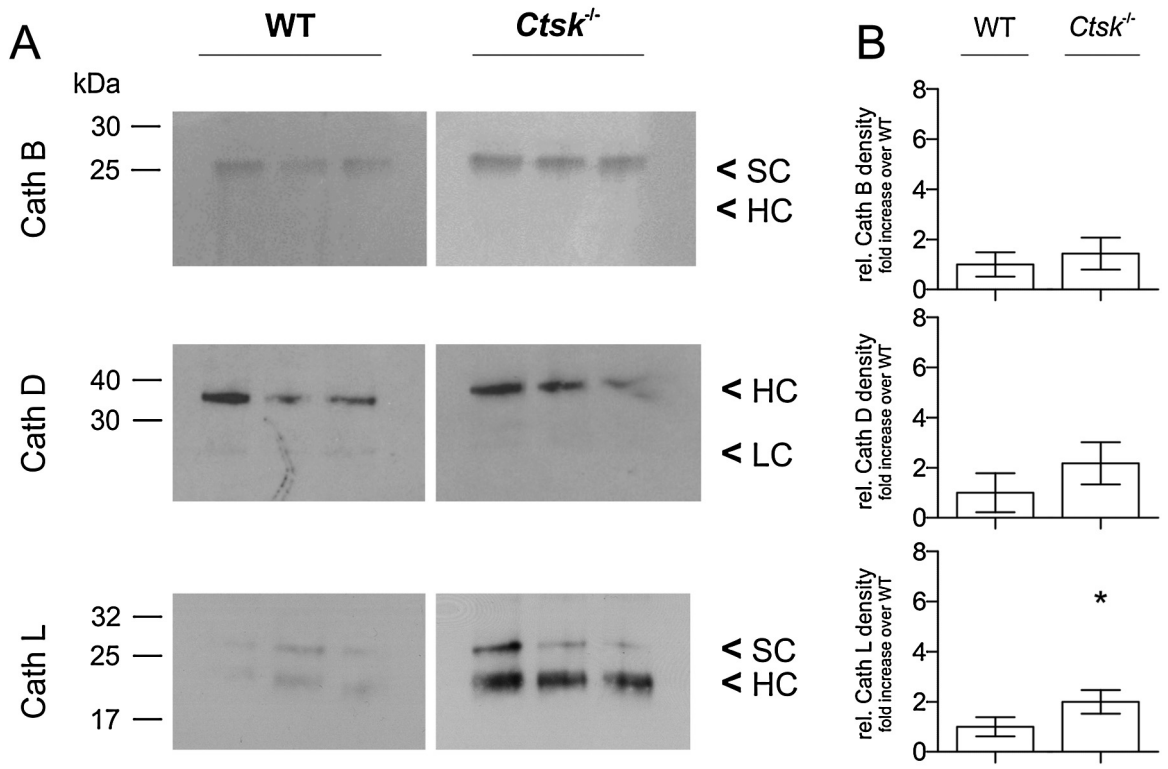

Fig. 10. Protein levels of cathepsins B, D, and L in thyroids of cathepsin K-deficient mice.
Whole thyroid lysates were separated on horizontal SDS-gels, transferred to nitrocellulose and incubated with antibodies against the respective proteins. (A) Immunoblots of cathepsins B, D, and L were quantified by densitometry (B). Cathepsin L levels were elevated significantly in *Ctsk*-deficient animals. Animals analyzed: n = 3 per genotype. HC = heavy chain; SC = single chain. Data are given as means ± SD; *, $P < 0.05$.

of TH transporters (as in $Ctsb^{-/-}$, $Ctsk^{-/-}$, $Ctsl^{-/-}$, or $Ctsb^{-/-}/Ctsk^{-/-}$ mice), or by enhancing cathepsin-mediated Tg-processing (as in $Ctsk^{-/-}$ mice), or both, without the necessity of TSH-triggered classical regulation (see Fig. 13). Such auto-regulatory pathways, driven exclusively by intra-thyroidal means, might be based upon thyrocyte-specific measures of TH-sensing. Thus, within thyroid follicles, the thyroid epithelial cells detect the amounts of TH liberated, and integrate these with those TH amounts exported from the follicles for global supply (Fig. 13, WT). Such a pathway of TH-sensing within thyroid epithelial cells of individual follicles most likely requires the involvement of TH transporters acting at different cellular membranes (Fig. 13). Interestingly, although different TH transporter proteins are expressed in mouse thyrocytes (Di Cosmo et al., 2010), compensatory up-regulation of TH transporters in Mct8-deficient animals has not been found (Di Cosmo et al., 2010; Wirth et al., 2011). In contrast, enhanced Tg degradation and utilization takes place when Mct8 and Mct10 are lacking (this study). Eventually, this leads to increased intra-thyroidal TH levels, which should feedback to the thyrocyte to stop the elevation of TH production at one point. Ironically, and against all logics, such a regulatory feedback does not happen in Mct8/Mct10-deficient animals.

In summary, the results of this study provide a molecular mechanistic explanation for the altered thyroid functional states of mice with Mct8- and/or Mct10-deficiencies in that enhanced Tg degradation by cathepsins B and L (acting extra- and intracellularly on Tg) and by intra-lysosomally acting cathepsin D occurs, although this is counterintuitive and likely worsens the intra-follicular thyrotoxic states.

At first glance, up-regulation of Mct8 in thyroid glands of animals lacking the Tg-processing cathepsin proteases (Figs. 11 and 12) makes more sense as the export of T_4 from the thyroid follicles would be facilitated in such conditions of enhanced Mct8 at the basolateral plasma membrane of thyrocytes. However, again counterintuitively, the thyroid gland itself might become hypothyroid, at least transiently, at the expense of maintaining proper TH supply to the peripheral organs, in particular, in $Ctsk^{-/-}$ mice (Friedrichs et al., 2003). Within the thyroid follicles of $Ctsk^{-/-}$ mice, such states of too little TH liberation due to lacking cathepsin K-mediated Tg-processing by extra- and intracellular means of proteolysis are obviously sensed and counter-acted such that they eventually lead to a compensatory up-regulation of the amounts of cathepsin L (Friedrichs et al., 2003).

4.3. Perspectives

In conclusion, we propose that the intra-thyroidal TH sensing mechanisms present in $Ctsb^{-/-}$, $Ctsk^{-/-}$, $Ctsl^{-/-}$, $Mct8^{-/y}$, and $Mct8^{-/y}/Mct10^{-/-}$ mice are enabled such that different TH transporters are present in specific subcellular locations of thyroid epithelial cells, thereby selectively monitoring individual steps of thyroglobulin processing and thus, the cellular TH status (see Fig. 13). Hence, future studies must elucidate the molecular nature of these proposed intra-thyroidal T_4-sensing mechanisms beyond Mct8, in order to unravel, how T_4-sensing by Mct8 and/or Mct10 at the basolateral plasma membrane domain is mechanistically connected to regulation of translation and trafficking of Tg-processing proteases within individual follicles, and how precisely the association with endo-lysosomal functions is maintained. We propose an involvement of the TH transporter Lat2, which is present at the endo-lysosomal membrane of thyrocytes in WT mice (McInnes et al., 2013; Weber et al., 2015).

The involvement of the HPT-axis on such intra-thyroidal, regulatory pathways is most likely mild, but must still be considered, because TSH affects both, short-term trafficking and long-term translational regulation of Tg-processing abilities (Linke et al., 2002a,b), besides regulating the differentiated state of thyrocytes

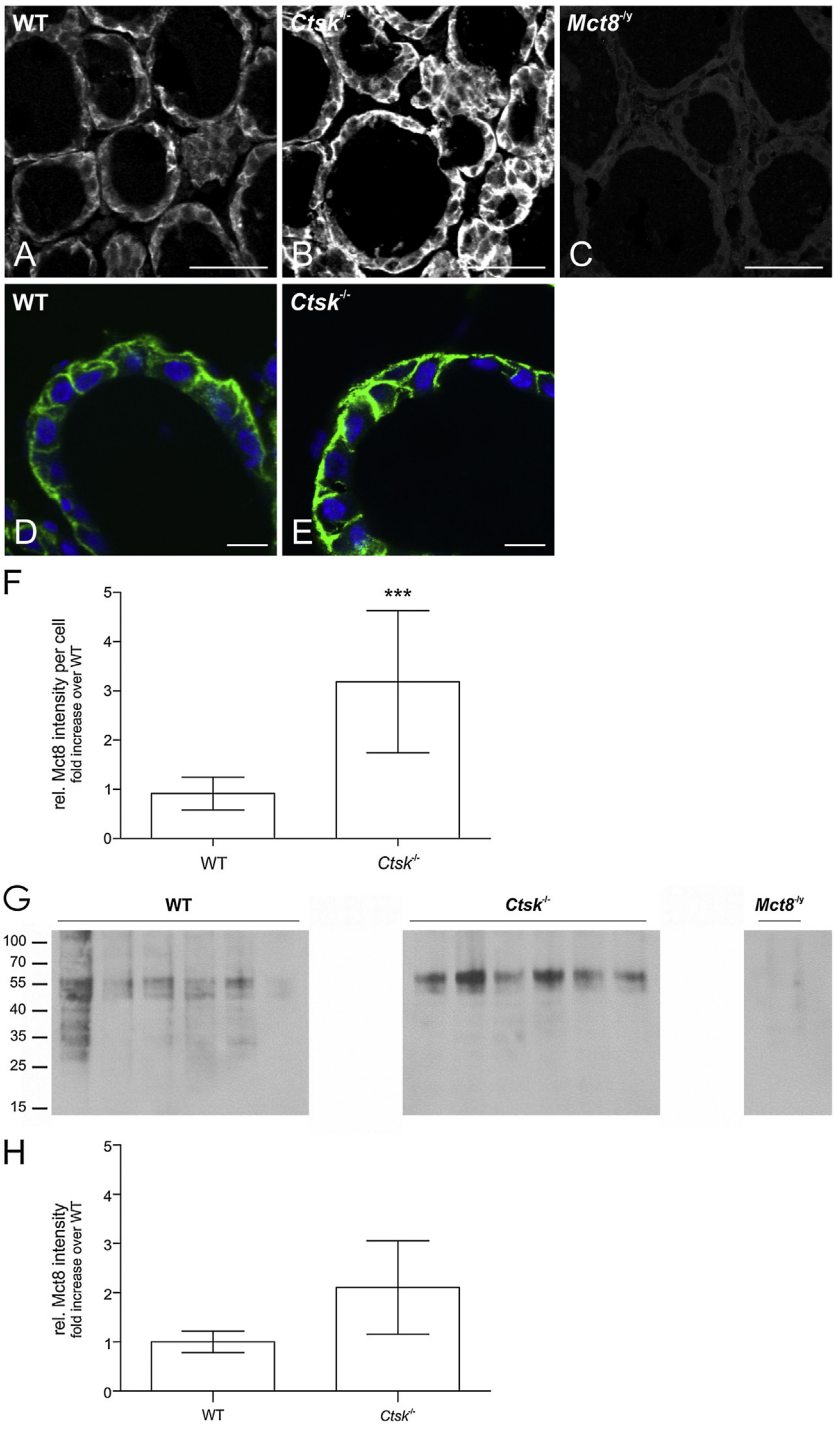

Fig. 11. Mct8 expression levels and localization in thyroid tissue of cathepsin K-deficient mice.
Signal intensities were quantified in micrographs of cryosections labeled with antibodies against Mct8 (A, B). Tissue of Mct8-deficient animals served to control for staining specificity of the primary antibody (C). Note the significant increase of Mct8 in cathepsin K-deficient mice when compared to the WT (F). However, the localisation of Mct8 (green) at the basolateral plasma membrane domain (D and E) did not change in cathepsin K-deficiency. Whole thyroid lysates were separated on horizontal SDS-gels, transferred to nitrocellulose for subsequent incubation of the blots with antibodies against Mct8 (G) and the quantification by densitometry revealed elevated Mct8 levels in *Ctsk*-deficient mice (H). F: animals analyzed were 8 and 9, respectively; data are given as means ± SD; n = 41 and 49 follicles, respectively. H: animals analyzed n = 6, and 6, respectively. Scale bars: 40 μm (A–C) and 10 mm (D and E). Nuclei were counter-stained with Draq5™ (blue). ***, P < 0.001.

and Tg biosynthesis (Brix et al., 2001). Thus, it will be important in future studies to discriminate between TSH-dependent regulation and thyroid auto-regulation that is independent of the systemic HPT-axis. In this regard, we are currently analyzing knock-out models featuring thyroid-specific Mct8-deficiency.

Lastly, it would be interesting to include studies on the Tg cross-linking mechanisms and the enzymes involved, with the aim to compare those in global *versus* thyroid-specific Mct8-deficiencies. In this context, it should be noted that, although Tg cross-linking in mice is under-explored, Tg folding must be considered a prerequisite for Tg trafficking along the secretory route and its exocytotic

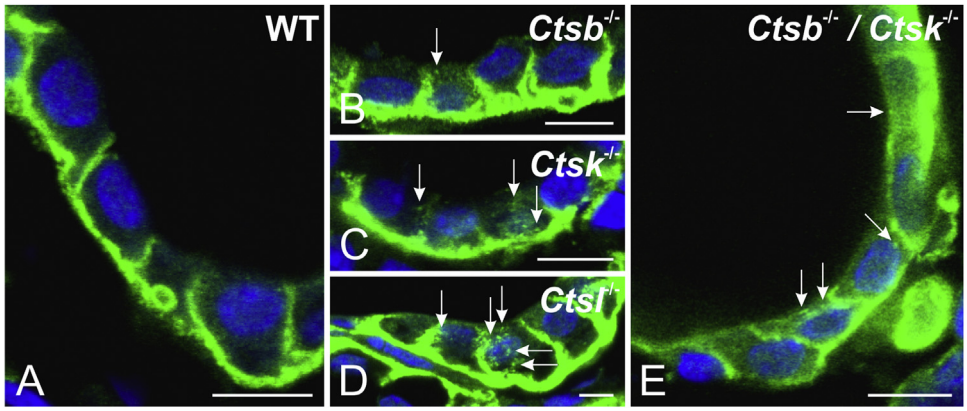

Fig. 12. Mct8 expression levels and localization in thyroid tissue of WT and cathepsin B, K, L, and B/K-deficient mice.
Confocal fluorescence micrographs of cryosections labeled with antibodies against Mct8 (green) revealed enhanced staining intensity in cathepsin-deficient mice (B–E) when compared to the WT (A). However, the localization of Mct8 at the basolateral plasma membrane domain did not change in cathepsin-deficiencies. Thyroid of cathepsin-deficient mice were characterized by anti-Mct8-positive puncta (arrows) which were most abundant in *Ctsl*$^{-/-}$ (D) and *Ctsb*$^{-/-}$/*Ctsk*$^{-/-}$ (E) mice. Scale bars: 10 μm. Nuclei were counter-stained with Draq5™ (blue).

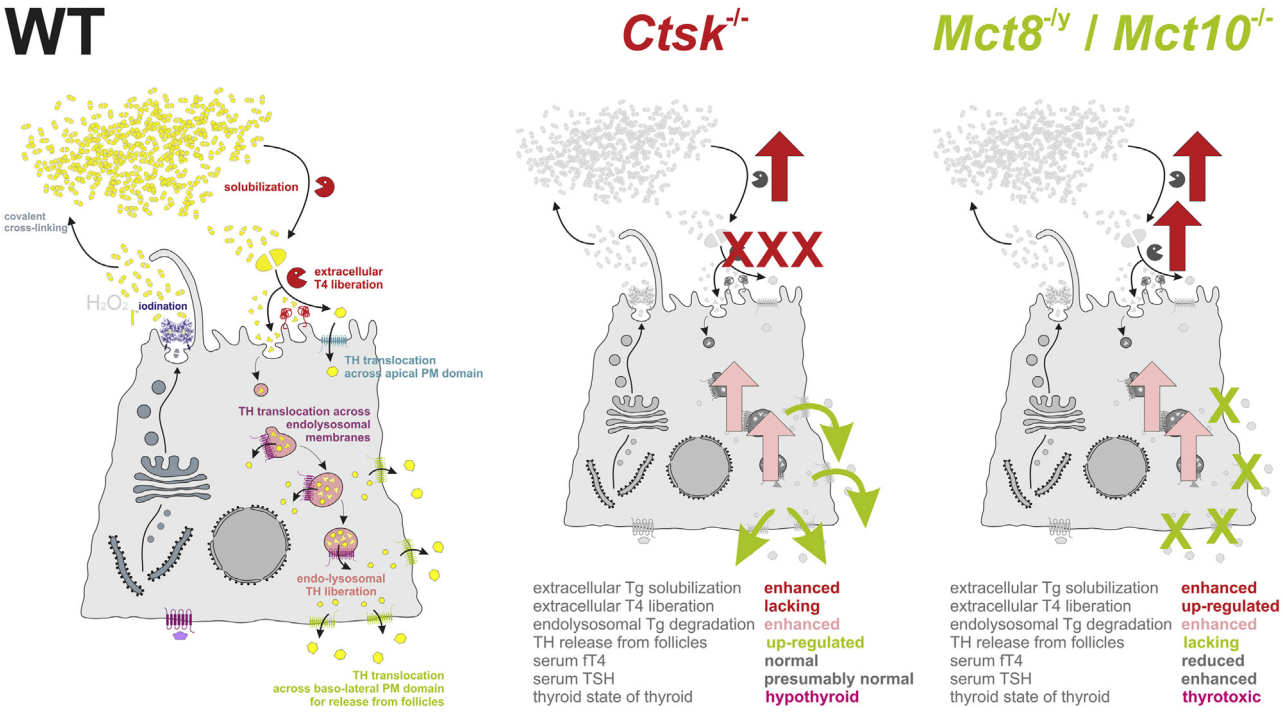

Fig. 13. The influence of cathepsin K and TH transporter deficiency on liberation of TH and their release from thyroid follicles.
Tg is synthesised and secreted at the apical plasma membrane of thyrocytes (WT, left) to be stored in covalently cross-linked form in the lumen of thyroid follicles. Upon TSH stimulation, cysteine cathepsins are secreted upon retrograde trafficking, and cathepsins B and L are involved in solubilization of Tg, whereas cathepsin K is involved in direct T_4 liberation in the follicle lumen (WT, upper portion, and lower panel), which may be transported across the apical plasma membrane domain by an as yet unknown T4 transporter (cyan). Then, Tg and partially degraded Tg molecules are endocytosed for delivery to early and late endosomes as well as lysosomes, where T_4 and T_3 can be liberated by the intracellular proteolytic action of cysteine cathepsins before being transported across the endo-lysosomal membranes into the cytosol (dark pink). T_4 may then be deiodinated and thus activated to T_3, or T_4 is released from the thyrocytes by transport across the basolateral plasma membrane domain (chartreuse).
Mice deficient in cathepsin K exhibit normal T_4 serum levels, while Mct8 deficiency results in reduced T_4 and slightly elevated serum TSH concentrations. TSH influences the increase of TH production and likewise, expression of cathepsins is elevated upon long-term TSH stimulation (Linke et al., 2002b). Mice with deficiencies in Mct8 and Mct10 exhibit similar TSH levels as Mct8-deficient mice but different serum T_4 levels. Upon loss of cathepsin K (*Ctsk*$^{-/-}$), we observed up-regulated cathepsin L expression and activity (Friedrichs et al., 2003; red arrows) and enhanced expression of the TH transporter Mct8 at the basolateral plasma membrane domain of the thyrocytes (this study; green arrows), suggesting T_4 release from thyroid follicles to be favored over self-sustaining regulation, thereby rendering the thyroid gland hypothyroid. Upon loss of both Mct8 and Mct10 (*Mct8*$^{-/y}$/*Mct10*$^{-/-}$), we observed altered cross-linking and multimerization of Tg which may have led to enhanced utilization of Tg for TH liberation (red arrows) and thereby increasing the intra-thyroidal TH levels (Muller et al., 2014). We speculate that the loss of Mct8 (green crosses) and Mct10 from the basolateral plasma membrane domain of thyrocytes led to a loss of sensing capability for the amount of liberated TH by cathepsins, which yielded in increased processing and degradation of Tg despite toxic intra-thyroidal TH concentrations, thereby however also contributing to the normal serum T_4 levels in Mct8/Mct10-deficient mice.

release from thyrocytes, before Tg is eventually stored in cross-linked form in the thyroid follicle lumen. Both, Tg folding and Tg cross-linking are chaperoned and mediated by protein disulfide isomerase (PDI; Berndorfer et al., 1996; Muresan and Arvan, 1998; Di Jeso and Arvan, 2016), at least in humans. However, Tg may also undergo self-assisted disulfide bond formation due to the thioredoxin-boxes present in the N-terminal half of the molecule (Klein et al., 2000; Schmitz et al., 2002). Interestingly,

the Tg molecule further features the presence of different internal homology domains (for review, see Brix et al., 2001), some of which contain the aforementioned CXXC-motifs of thioredoxins, while others are characterized by CWCV-motifs that are indicative of the so-called thyropins, i.e. small proteins or peptides acting as cysteine peptidase inhibitors (Lenarcic et al., 1997). Clearly, future studies are needed in mice to explore the significance of Tg cross-linking and the possible interconnections with its degradation, since both are potentially subject to intra-thyroidal auto-regulation, i.e. by Tg itself (Rousset and Mornex, 1991; Brix et al., 2001).

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Disclosure statement

The authors declare that no conflict of interest exists.

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