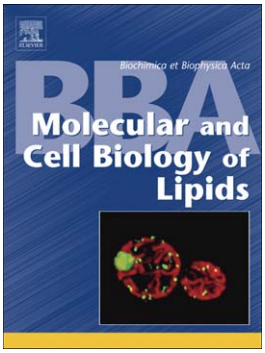

Browning deficiency and low mobilization of fatty acids in gonadal white adipose tissue leads to decreased cold-tolerance of transglutaminase 2 knock-out mice

András Mádi, Ixchelt Cuaranta-Monroy, Kinga Lénárt, Attila Pap, Zoltán András Mezei, Endre Kristóf, Anna Oláh, György Vámosi, Zsolt Bacsó, Péter Bai, László Fésüs

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TITLE PAGE

Title

Browning deficiency and low mobilization of fatty acids in gonadal white adipose tissue leads to decreased cold-tolerance of transglutaminase 2 knock-out mice

Authors

András Mádi[1,2], Ixchelt Cuaranta-Monroy[1], Kinga Lénárt[1], Attila Pap[1], Zoltán András Mezei[3], Endre Kristóf[1], Anna Oláh[3], György Vámosi[4], Zsolt Bacsó[4], Péter Bai[5], László Fésüs[1, 2]

Affiliations

[1]Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary

[2]MTA-DE Stem Cells, Apoptosis and Genomics Research Group of the Hungarian Academy of Sciences, Debrecen, Hungary

[3]Department of Laboratory Medicine, University of Debrecen, Debrecen, Hungary

[4]Department of Biophysics and Cell Biology, University of Debrecen, Debrecen, Hungary

[5]MTA-DE Lendület Laboratory of Cellular Metabolism Research Group, Research Center for Molecular Medicine, Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary

Corresponding author

Correspondence to: László Fésüs, Address: H-4032, Debrecen, Egyetem tér 1, Hungary, Tel: +3652-416-432, Fax: +3652-314-989, E-mail: fesus@med.unideb.hu

Abstract

During cold-exposure 'beige' adipocytes with increased mitochondrial content are activated in white adipose tissue (WAT). These cells, similarly to brown adipocytes (BAT), dissipate stored chemical energy in the form of heat with the help of uncoupling protein 1 (UCP1). We investigated the effect of TG2 ablation on the function of ATs in mice. Although $TG2^{+/+}$ and $TG2^{-/-}$ mice had the same amount of WAT and BAT, we found that $TG2^{+/+}$ animals could tolerate acute cold exposure for 4h, whereas $TG2^{-/-}$ mice only for 3h. Both $TG2^{-/-}$ and $TG2^{+/+}$ animals used up half of the triacylglycerol content of subcutaneous WAT (SCAT) after 3h treatment; however, $TG2^{-/-}$ mice still possessed markedly whiter and higher amount of gonadal WAT (GONAT) as reflected in the larger size of adipocytes and lower free fatty acid levels in serum. Furthermore, lower expression of 'beige' marker genes such as UCP1, TBX1 and TNFRFS9 was observed after cold exposure in GONAT of $TG2^{-/-}$ mice, paralleled with a lower level of UCP1 protein and a decreased mitochondrial content. The detected changes in gene expression of Resistin and Adiponectin did not provoke glucose intolerance in the investigated $TG2^{-/-}$ mice, and TG2 deletion did not influence adrenaline, noradrenilen, glucagon and insulin production. Our data suggest that TG2 has a tissue-specific role in GONAT function and browning, which becomes apparent under acute cold exposure.

Keywords:

browning, uncoupling protein-1, beige adipocytes, differentiation, cold exposure, TG2

Abbreviations

ADIPOQ: adiponectin

AT: adipose tissue

BAT: brown adipose tissue

WAT: white adipose tissue

SCAT: subcutaneous white adipose tissue

FFA: free fatty acid

GONAT: gonadal white adipose tissue

CIDEA: cell death-inducing DFFA-like effector a

DIO2: Type II iodothyronine deiodinase

FABP4: fatty acid binding protein 4

FC: fold change

GLUT4: glucose transporter 4

HSL: hormone sensitive lipase

LPL: lipoprotein lipase

PPARg: peroxisome proliferator-activated receptor gamma

PREF1: preadipocyte factor 1

PRDM16: PR domain containing 16

ROD: relative optical density

TG2: tissue transglutaminase

TMEM26: transmembrane protein 26

TBX1: T-box protein 1

TNFRSF9: Tumor Necrosis Factor Receptor Superfamily Member 9

TOM20: Mitochondrial import receptor subunit TOM20

UCP1: uncoupling protein 1

ZIC1: Zic family member 1

1. Introduction

Adipose tissue (AT) is present in mammals in two different forms: white and brown AT (WAT and BAT), with WAT being abundant. WAT serves as a site of calorie storage and the source of free fatty acids (FFA) released from triacylglycerol during fasting. BAT is specialized to dissipate stored chemical energy in the form of heat and plays a role in the regulation of body temperature [1]. Thermogenic function of BAT relies on the activity of mitochondrial uncoupling protein 1 (UCP1), which facilitates a proton leak across the inner mitochondrial membrane and dissociates substrate oxidation from ATP production [2-4]. More recently, it has been discovered that cold exposure or adrenergic stimuli can provoke the appearance of clusters of UCP1-positive heat producing cells in WAT through the process of browning [5]. These inducible cells have been named 'beige' adipocytes and have an overlapping but distinct gene expression pattern compared to classical brown adipocytes [6, 7]. Beige cells can be generated both from distinct precursors via de novo differentiation or from "masked" adipocytes that appear morphologically white, but have the capacity to reinitiate the thermogenic program in response to cold [8].

Obesity increases the risk of metabolic syndrome, a cluster of multiple conditions including hypertension, cardiovascular disease, type 2 diabetes and higher incidence of certain types of cancer [9, 10]. Obesity is defined as an excessive amount of body AT that stores energy taken in as food but not used for activity [11]. Moreover, AT produces regulatory adipokines modulating several target organs, and acts as an endocrine system at the centre of energy homeostasis. Thus, AT has crucial role in the development of various diseases when overproduced [12, 13]. Obesity epidemic has highlighted interest in exploring whether activation of browning may benefit weight control [14]. Therefore, identification of beige activators may open new directions in the development of obesity and type 2 diabetes therapeutics [15].

Tissue transglutaminase (TG2, EC 2.3.2.13) is already known to stand in differentiation processes of several cell types [16]. It covalently cross-links proteins producing an isopeptide bond between glutamine and lysine residues in a Ca^{2+}-dependent manner [17]. TG2 can incorporate primary amines into glutamine residues of proteins, and it is also able to cleave the produced cross-links by isopeptidase activity [18]. In addition, it has GTPase activity and can act as a G protein in transmembrane signaling [19]. Functions of TG2 have been implicated in various biological processes including regulation of the cytoskeleton, cell adhesion and cell death as a catalytically active or just an interacting protein partner [16, 18, 20, 21]. To unfold the complex biological function of TG2, a KO mouse model has been generated [22]. $TG2^{-/-}$ animals were found viable, and grew up to normal size and weight with no apparent abnormalities in organ functions. However, detailed investigations pointed out that TG2 participated in the crosstalk between dying and phagocytic cells to ensure tissue integrity [23-25] and was also required for proper differentiation and bacterial killing of neutrophils [26, 27]. Although, more careful examinations revealed several abnormalities, even more important alterations were expected under certain stresses and pathological conditions [28]. Indeed, ablation of TG2 in mice resulted in impaired wound healing [29], autoimmunity [30] and diabetes [31].

Studies on the gene expression profile of human AT revealed 6-fold higher TG2 expression in BAT compared to WAT raising the possibility that TG2 may be involved in thermogenic functions of AT [32]. To substantiate a potential role of TG2 in development and operation of AT, we studied its effects on AT formation and function in a mouse model. We did not find any robust difference between AT of $TG2^{+/+}$ and $TG2^{-/-}$ animals without treatments. However, $TG2^{-/-}$ animals had decreased tolerance to acute cold exposure compared to $TG2^{+/+}$ littermates. This phenomenon could be clearly explained with insufficient utilization of their gonadal WAT (GONAT). Lower

GONAT mobilization was very likely caused by hampered browning process of the tissue in TG2$^{-/-}$ mice possessing lower expression of browning markers including UCP1.

2. Materials and Methods

2.1. Materials

All chemicals were from Sigma-Aldrich (Munich, Germany) except indicated otherwise.

2.2. Mice, treatments and obtained samples

TG2 deficient mice ($TG2^{-/-}$) [22] and wild-type littermates ($TG2^{+/+}$) with C57BL/6J genetic background were obtained from heterozygous breeding couples and were genotyped in the Animal Facility at the University of Debrecen. Mice were housed separately, had ad libitum access to water and chow and were kept in a 12h dark/light cycle at 22+/-1°C. 16 week old males were sacrificed and their body mass was measured. Interscapular brown (BAT), inguinal subcutaneous white (SCAT) and gonadal (intra-abdominal) white (GONAT) adipose tissues were removed and weighed. Portions were snap-frozen in liquid nitrogen for DNA, RNA and protein extraction, and paraffin sections were prepared for histology. Blood was collected from the heart, serum was obtained and stored at −80°C. During cold exposure experiments, animals were kept at 4°C for maximum 4h without chow but with cold (4°C) water, and their rectal body temperature was measured at 30 min intervals as described [33]. Glucose tolerance test was carried out as previously described [31]. All the animal experiments were performed according to local, national, and EU ethical guidelines (license numbers: 14/2010/DEMAB and 1/2014/DEMAB).

2.3. Gene expression studies

Total RNA was isolated from tissue portions using TRIzol Reagent according to the manufacturer's instructions (Invitrogen Life Technologies), and nucleic acid concentration was quantified by spectrometry. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to

generate cDNA from isolated RNA fractions. mRNA expression levels were determined by RT-qPCR in a LightCycler 480 (Roche Diagnostics). Primers for AT marker genes are listed in Supplementary Table 1 [6, 7, 34-37]. Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used applying a program of 10 min at 95°C, followed by 50 cycles of 10 sec at 95°C, 1 min at 60°C and 50 sec at 72°C. Single-product amplification was verified by an integrated post-run melting curve analysis. Gene expression was quantified by the comparative C_p method and normalized to cyclophilin expression; fold change (FC) was calculated as $2^{-\text{deltadeltaCT}}$ relative to WT values. Values are expressed as mean ± SD of the mean calculated from 3 parallel measurements for each tissue sample of 6-6 KO and WT animals. It was verified that cyclophilin expression did not change during the cold exposure.

2.4. Western blot

Frozen AT portions were heat-treated in 2x Laemmli Buffer (5 min, 100°C) and sonicated (Branson Sonifier 450, 2 min, max intensity and cycle control 40%) followed again by heat treatment (5 min, 100°C) and centrifugation (15 min, 14 000 g). Proteins were separated on 10% or 12% 12SDS-PAGE and blotted onto a PVDF membrane. The membranes were probed by polyclonal anti-TG2 H-237 (dilution: 200x, Santa Cruz Biotechnology Inc, TX), polyclonal anti-PPARg H-100 (dilution: 500x, Santa Cruz Biotechnology Inc, TX), polyclonal anti-FABP4 (dilution: 500x, Cayman Chemical Company, MI), polyclonal anti-Adiponectin (dilution: 500x, EMD Millipore Corporation, CA), polyclonal anti-UCP1, polyclonal anti-HSL (dilution and origin for both: 500x, Sigma-Aldrich Chemie GMBH, Germany), polyclonal anti-TOM20 (dilution: 500x, Sigma-Aldrich Chemie GMBH, Germany) and monoclonal anti-β-Actin (dilution: 1000x, Sigma-Aldrich Chemie GMBH, Germany) antibodies overnight at 4°C, followed by incubation with horseradish-peroxidase (HRP)-conjugated species-corresponding secondary antibodies (Covalab) for 1h at

room temperature. Immunoblots were developed with Immobilon Western chemiluminescent substrate (Millipore). Densitometry was carried out using the Image J software, and expression of proteins was normalized to actin expression (relative optical density: ROD).

2.5. Histological analysis and metabolic parameters

The size of adipocytes in AT sections was determined by staining with hematoxylin/eosin under standard conditions. Images of 5 sections/mouse were taken with EVOS Cell Imaging System (Thermo Fisher Scientific) and analyzed with Fiji software [38, 39]. Average size of cells is expressed as mean ± SD calculated from 3-3 KO and WT animals. After deparaffinization, 3 sections/mouse were stained with anti-Ucp1 primary antibodies for 6h at room temperature followed by 1h incubation with Alexa 488 goat anti-rabbit IgG (Invitrogen Life Technologies). Expression of UCP1 was studied with a FluoView 1000 confocal microscope (Olympus) as described previously [40]. Mean expression per single cell values ± SD from 3-3 cold-treated KO and WT mice were calculated using the Fiji software after counting propidium iodide-stained nuclei.

Triglycerides (TG), total cholesterol (C), HDL cholesterol (HDL-C) levels of serum were determined by colorimetric enzyme assay (Cobas6000, Roche Ltd, Germany) and free fatty acid (FFA) by a standard laboratory assay [41, 42].

2.6. Mitochondrial DNA measurement

DNA of tissue portions was isolated using the standard proteinase K digestion following phenol-chloroform extraction. Mitochondrial and genomic DNA was determined using qPCR. The measurements were performed with mtDNA-specific (fw: ccgcaagggaaagat; rev: tcgtttggtttcggggtttc) and nuclear DNA-specific (fw: gccagcctctcctgattttagtgt; rev:

gggaacacaaaagacctcttctgg) primers using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) in a LightCycler 480 (Roche Diagnostics) applying a program of 20 min at 95°C, followed by 50 cycles of 15 sec at 95°C, 20 sec at 58°C and 20 sec at 72°C. Single-product amplification was verified by an integrated post-run melting curve analysis. Results were calculated from the difference in threshold cycle (CT) values for mtDNA and nuclear specific amplification. Data are expressed as mitochondrial genomes per diploid nuclei. Measurements were carried out in triplicates on diluted DNA of each of 6-6 KO and WT samples [33].

2.7. Detection of hormone levels

Plasma of mice was collected after 3h cold exposure. Insulin content was measured using Mouse Insulin ELISA Kit (Mercodia, Sweden), glucagon level was measured using Glucagon EIA Kit (Sigma-Aldrich Chemie GMBH, Germany) according to the manufacturers' descriptions from 6-6 KO and WT samples. Adrenaline and noradrenaline were measured using p-Catecholamines by HPLC Kit (Bio-Rad Laboratories, Germany) according to the instructions of the manufacturer from 3-3 KO and WT samples.

2.8. Statistical analyses

GraphPad Prism version 5.02 and Microsoft Excel 14.0 were used for data interpretation and calculation of significance. Results are expressed as the mean ± SD for the assays indicated. For comparing two groups Student's t-test was used. Values of $p<0.05$ were considered statistically significant with *, ** and *** corresponding to $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

3. Results

3.1. TG2$^{-/-}$ mice have decreased tolerance to acute cold exposure

In accordance with the previous findings [22, 31] the investigated TG2$^{+/+}$ and TG2$^{-/-}$ mice had similar body weight (TG2$^{+/+}$: 31.4+/-2.4 g; TG2$^{-/-}$: 31.2+/-1.5 g). They possessed the same amount of BAT, SCAT and GONAT on normal diet at 22°C. However, we observed that the same amount of AT in mice could not support physiological functions similarly under stressful condition induced by low temperature. The rectal body temperature of TG2$^{-/-}$ mice dropped to the ethically still acceptable 28.8+/-0.57°C after 3h spent at 4°C, whereas TG2$^{+/+}$ animals could still maintain 31.8+/-0.61°C rectal body temperature at the same time (Figure 1A). To explore how AT contributes to cold tolerance of TG2$^{-/-}$ mice, we isolated BAT, SCAT, and GONAT from animals after 3h of acute cold exposure. While TG2$^{-/-}$ mice utilized their SCAT similarly to TG2$^{+/+}$ animals during the cold exposure, they still had a significantly higher remaining mass of GONAT (258+/-28 mg) as compared to TG2$^{+/+}$ animals (172+/-16 mg) (Figure 1B). Although we found that TG2 protein was strongly expressed in BAT as compared to SCAT and GONAT of TG2$^{+/+}$ mice, cold exposure had no effect on the expression profile (Supplementary Figure 1).

3.2. Classical BAT of TG2$^{-/-}$ mice does not contribute to decreased cold tolerance

As UCP1 has a prominent role in the regulation of thermotolerance, we investigated its expression at protein level in BAT and found that it was similarly present at 22°C and after 3h cold exposure in BAT of both strains (Figure 2A and 2B). Assuming that differences in acute cold tolerance indicated altered development of BAT in TG2$^{-/-}$ mice, we measured the expression of some adipocyte markers with RT-qPCR. Most of the investigated genes were expressed similarly in the strains at 22°C and after the cold exposure, such as UCP1, PRDM16, DIO2, ZIC1, Adiponectin,

and GLUT4. Expression of CIDEA (FC: 0.158+/-0.14) and Resistin (FC: 0.426+/-0.189) was lower in BAT of $TG2^{-/-}$ animals kept at 22°C (Figure 2C). CIDEA inhibits degradation of triglycerides and promotes lipid accumulation in brown adipocytes [43, 44], while Resistin is an important adipokine accelerating the development of insulin resistance [45]. However, the differences between wild-type and TG-/- mice in expression patterns disappeared after 3h cold exposure (Figure 2D, Supplementary Figure 2 and 3). In addition, we did not detect any differences either in mass values (Figure 1B) or in morphological properties of tissue sections prepared from BAT of the cold-treated and non-treated strains (data are not shown). Hence, we concluded that there were no obvious dysfunctions of BAT in $TG2^{-/-}$ animals which contribute to their decreased cold tolerance.

3.3. Expression of the thermogenic UCP1 protein is impaired in $TG2^{-/-}$ GONAT during cold exposure

After observing that the decreased cold tolerance could not be explained by deficient function of BAT in $TG2^{-/-}$ mice, we turned our attention to the browning of WAT. Surprisingly, while SCAT browned similarly during 3h cold exposure in the strains (Figure 3A and 3B), GONAT of $TG2^{-/-}$ mice remained visibly lighter than the $TG2^{+/+}$ tissue after the treatment (Figure 3C and 3D). Interestingly, we did not detect UCP1 protein in SCAT of animals kept at 22ºC (Figure 3A), but cold exposure resulted in its induction in both $TG2^{-/-}$ and $TG2^{+/+}$ mice equally (Figure 3B). However, protein expression of UCP1 was markedly different in GONAT of the two strains. It was detectable at 22ºC in both strains with lower expression in $TG2^{-/-}$ GONAT (ROD: 0.39+/-0.056) compared to $TG2^{+/+}$ (ROD: 0.58+/-0.044) (Figure 3C). The reduction in UCP1 protein expression in GONAT of the strains was so prominent after cold exposure that it was under the detection limit in $TG2^{-/-}$ GONAT on immunoblots (Figure 3D). Cold-treated $TG2^{-/-}$ GONAT expressed some

UCP1 as we detected it with immunohistochemistry (Figure 3E); however, the mean UCP1 intensity/cell of $TG2^{-/-}$ adipocytes (17+/-7 $x10^{-6}$) was approximately half of that detected in $TG2^{+/+}$ GONAT cells (38+/-11 $x10^{-6}$) (Figure 3F)**.**

In accordance with the expression of mitochondrial UCP1, mtDNA-specific qPCR revealed that cells in GONAT of $TG2^{-/-}$ animals contained a lower normalized mtDNA content (628+/-83) compared to the $TG2^{+/+}$ tissue (1063+/-284) after 3h cold exposure (Figure 3G). Meanwhile, both UCP1 protein and mtDNA increased similarly in SCAT of strains during the treatment, and there was no difference in tissues isolated from animals kept at 22ºC neither**.**

3.4. $TG2^{-/-}$ mice poorly mobilize their GONAT triacylglycerol content during cold exposure

The observed difference in mass values between $TG2^{-/-}$ and $TG2^{+/+}$ GONAT after 3h cold exposure (Figure 1B) was reflected in the analysed tissue sections. While the average size of adipocytes was similar in non-treated strains (Figure 4A), and it decreased equally in SCAT tissues after 3h cold exposure (Figure 4B), the size of adipocytes remained larger in the $TG2^{-/-}$ GONAT (479+/-51 μm^2) compared to the $TG2^{+/+}$ GONAT (296+/-14 μm^2) (Figure 4B and 4C). To learn more about the function of AT in $TG2^{-/-}$ mice in cold we investigated the lipid fractions of serum samples collected after 3h spent at 4˚C. We failed to detect any differences in total cholesterol, HDL-cholesterol and triglyceride content, but there was a momentous alteration in FFA contents. Both $TG2^{-/-}$ and $TG2^{+/+}$ animals increased FFA concentrations in their response to cold; however, very likely as a consequence of lower GONAT mobilization, the FFA concentration was significantly lower in the sera of $TG2^{-/-}$ mice (1.28+/-0.12 mM) as compared to $TG2^{+/+}$ ones (2.12+/-0.1 mM) at that time point (Figure 4D).

3.5. TG2$^{-/-}$ mice make up GONAT with different molecular characteristics compared to TG2$^{+/+}$ animals

Interestingly, expression of the investigated marker genes in SCAT did not show any differences at 22^{o}C (Figure 5A). In contrast, we found that expression of Resistin was higher in GONAT of TG2$^{-/-}$ animals than in the TG2$^{+/+}$ mice (FC: 1.688+/-0.318). Furthermore, expression of Adiponectin was lower in GONAT of TG2$^{-/-}$ mice (FC: 0.411+/-0.187) (Figure 5B, Supplementary Figure 3). Adiponectin acts opposite to Resistin and maintains insulin sensitivity of cells [45]. As reciprocal expression of Adiponectin and Resistin in GONAT of TG2$^{-/-}$ mice suggested their susceptibility for insulin resistance, we carried out glucose tolerance tests. While it was described that the investigated TG2$^{-/-}$ strain develops insulin resistance by the age of 6 months [31], we did not detect glucose intolerance in younger 16-week-old TG2-/- mice (Figure 7A), and there was no difference in the expression of GLUT4 insulin-dependent glucose transporter either (Figure 5A and 5B, Supplementary Figure 3). Of note, the possible role of TG2 in glucose tolerance is still contradictory, as this phenomenon was not observed in another TG2$^{-/-}$ strain [46]. Most importantly, we measured lower mRNA expression of the heat generator UCP1 in the GONAT of TG2$^{-/-}$ animals (FC: 0.196+/-0.178) (Figure 5B, Supplementary Figure 2) explaining the detected lower protein expression (Figure 3C).

3.6. TG2$^{-/-}$ GONAT has deficient general reaction to cold

The apparent colour difference of GONAT in strains after 3h cold exposure prompted us to investigate the expression of marker genes and the browning process in AT upon cold treatment. In parallel with previous results we obtained, qPCR measurements suggested that SCAT (such as BAT) could function normally during the cold exposure as we detected no change in the expression of most of the genes (Figure 5C). The only exception was the case of UCP1 in TG2$^{-/-}$ SCAT (Figure

5C, Supplementary Figure 2); however, it did not result in any change at the protein level in this tissue (Figure 3B). Strikingly, most of the marker genes were differently expressed in GONAT of $TG2^{-/-}$ mice compared to $TG2^{+/+}$ after 3h cold exposure, which was possibly the reason or consequence of low fat mobilization. Out of the 4 investigated beige marker genes we measured low level of UCP1 (FC: 0.236+/-0.11), TBX1 (FC: 0.357+/-0.086), and TNFRFS9 (FC: 0.416+/-0.174) in $TG2^{-/-}$ GONAT compared to the $TG2^{+/+}$ tissue (Figure 5D, Supplementary Figure 4); only TMEM26 encoding a transmembrane protein remained unchanged. TBX1 is a transcription factor, and TNFRSF9 encodes a selective cell surface marker on ’beige’ cells; both of them are accepted ’beige’ markers in mice similarly to UCP1 [7].

We found that cold treatment induced decrease in mRNA levels of other adipocyte marker genes in both strains; however, some of them did not decrease in $TG2^{-/-}$ GONAT to such an extent as in $TG2^{+/+}$ GONAT during cold exposure (Supplementary Figure 3). This phenomenon resulted in their higher FC values when we compared expression to $TG2^{+/+}$. Among them there were leptin (FC: 3.39+/-0.67), an adipokine which inhibits food intake and appetite in the central nervous system [47], LPL (FC: 7+/-0.882) facilitating lipid uptake and storage in AT [48], and FABP4 (FC: 4.61+/-0.41) activating fatty acid uptake and metabolism in adipocytes [49]. Also expression of PPARg (FC: 4.67+/-0.24), a transcription factor necessary for adipocyte differentiation and function [50], and the insulin-dependent glucose transporter GLUT4 (FC: 2.49+/-0.71) acting under well-fed conditions [51] in addition to PREF1 (FC: 2.56+/-0.87), a characteristic preadipocyte marker [52] was higher in GONAT of $TG2^{-/-}$ mice compared to $TG2^{+/+}$ after cold exposure (Figure 5D, Supplementary Figure 4). Interestingly, the gene expression of the hormone sensitive lipase (HSL) was similar in $TG2^{+/+}$ and $TG2^{-/-}$ mice even under cold exposure (Figure 5D).

We also checked for protein expression levels in case of some adipocyte markers whether acute cold exposure can result in notable changes. Parallel to mRNA levels expression of HSL, Adiponectin, PPARg and FABP4 was the same in SCAT and GONAT of $TG2^{+/+}$ and $TG2^{-/-}$ animals without cold treatment, and we did not find any differences in their SCAT after 3h cold exposure neither (Figure 6A-C). While PPARg and FABP4 mRNA levels were higher in $TG2^{-/-}$ GONAT after the treatment (Figure 5D, Supplementary Figure 3), we observed corresponding change at the protein level only in case of FABP4 ($TG2^{-/-}$ROD: 0.62+/-0.12; $TG2^{+/+}$ROD: 0.19+/-0.04) (Figure 6D). Results indicate that faster decrease in mRNA levels of some marker genes during 3h cold exposure is not followed necessarily by decrease in the level of proteins probably having longer half-life than mRNAs.

In addition to investigation on classical adipocyte markers, we detected significantly lower expression of mitochondrial TOM20 after 3h cold exposure in the GONAT of $TG2^{-/-}$ mice (ROD: 0.48+/-0.08) compared to $TG2^{+/+}$ (ROD: 1+/-0.18) (Figure 6D). TOM20 is a subunit of the mitochondrial import receptor complex responsible for the recognition and translocation of cytosolically synthesized mitochondrial preproteins [53]. Its expression is similar in SCAT and GONAT of both untreated strains and in the SCAT of treated animals (Figure 6A-C); however, its low expression in cold-exposed $TG2^{-/-}$ GONAT is in significant accordance to the low UCP1 expression and mitochondrial DNA content in this tissue (Figure 3D-G).

3.7. Deficient response of $TG2^{-/-}$ GONAT to cold is an autonomous reaction

The whole metabolism of WAT, and especially the browning mechanism is a strictly hormone regulated process. This is raising the question whether low mobilization of fat and browning deficiency of GONAT in $TG2^{-/-}$ animals during acute cold exposure are due to low sensitivity of the tissue to important hormones, or low hormone levels secreted to the plasma. According to our

measurements, the 1.1 nmol/l adrenaline and 4.1 nmol/l noradrenaline levels thought to be physiological [54] are momentously elevated after 3h cold exposure, but were very similar in $TG2^{+/+}$ and $TG2^{-/-}$ animals (Figure 7B); like circulating glucagon and insulin concentrations (Figure 7C). The same level of hormones in plasma of $TG2^{+/+}$ and $TG2^{-/-}$ mice during cold exposure was also reflected in the similar browning and mobilization of their SCAT (Figure 1, 3 and 4).

Discussion

Mouse models resistant to weight gain through increased brown and beige fat activity have clearly indicated that activation of thermogenesis can be a powerful strategy to improve metabolic health and prevent weight gain [55, 56]. Alteration in UCP1 expression have pathological consequences: ablation of UCP1 positive cells in transgenic mice causes an increased propensity to obesity and diabetes [57], and conversely, UCP1 KO mice develop obesity under thermoneutral conditions when fed a high-fat diet [58]. Characteristics of thermogenic human BAT still have to be determined in detail, but several studies have demonstrated that cells of supracavicular human BAT are more similar to the beige cells of rodents making the use of mouse browning models more rational [7, 36]. Hence, controlled augmentation of the thermogenic potential of human AT could be a future therapeutic target for obesity and associated diseases [14, 15, 59].

Upregulation of TG2 has been shown in human BAT compared to WAT [32] suggesting that it may have a role in thermogenic functions. To explore the possible task of TG2 in the thermogenesis of AT, we carried out cold tolerance assays and found that $TG2^{-/-}$ mice had lower tolerance to cold exposure than $TG2^{+/+}$ animals (Figure 1A). According to our results, normal BAT and SCAT functions could support $TG2^{-/-}$ animals to a certain degree at unusually low temperature; however, limited utilization of GONAT led to their increased sensitivity to cold (Figure 1B and 4). The

browning process was also curbed in GONAT of TG2$^{-/-}$ mice contributing to lower consumption of fat, which we did not observe in SCAT (Figure 3).

Beige cells can be activated not only by cold exposure or physical exercise, but also by the β-adrenergic signaling pathway [60, 61]. However, β-adrenergic receptor deficient mice can also increase the thermogenic function of their WAT in response to mild cold exposure [62] suggesting the existence of other contributing pathways. It is important to note that TG2 in some cells can act as a G (Ghα) protein [19]. This TG2 function can transmit the α1B-adrenoceptor (AR) signal to phospholipase C (PLC) through its GTPase activity [63, 64]. Action of PLC activates protein kinase C (PKC), and PKC may promote the browning process by phosphorylation of mitogen-activated protein kinase (MAPK) [65-69] possessing a central role in activation of UCP1 expression [70, 71]. In this manner, TG2 could be a key protein in the possible cross-talk between the α-adrenergic pathway activated by both adrenaline and noradrenaline [72] and the β-adrenergic pathway during the browning process. Although G protein function of TG2 has been revealed in several cell types (e.g. heart and smooth muscle cells, fibroblasts, endothelial cells, hepatocytes) [73], it is still unclear whether TG2 may have a similar function in adipocytes or preadipocytes, and if so, why it is restricted to GONAT.

Cold promotes not only de novo beige fat differentiation, but also induction of thermogenic characteristics in some "masked beige" adipocytes. These adipocytes appear morphologically and also functionally white but have beige identity and the capacity to reactivate the thermogenic program quickly in response to cold [74]. These cold-induced UCP1-positive cells acquire a white-like unilocular morphology after the animals are warmed [75]. Instead of differentiation from precursor cells, β-agonist treatment or cold exposure simply activate the thermogenic properties of these existing "masked beige" adipocytes [8]. As we could not detect significant differences in expression of beige marker genes in non-treated animals (Figure 5A and 5B), it is more likely that

TG2 deficiency leads to a lower activation rate of "masked beige" cells in GONAT decreasing the thermogenic capacity of this tissue during cold exposure.

Changes in the expression of beige marker genes in WAT, particularly the expression of UCP1 during the applied acute cold exposure in our experiments, are comparable to the changes detected in beige cell cultures treated with the adenylyl cyclase activator forskolin for 4h [7], in fully differentiated 3T3-F442A cells cooled to 27°C for 4h [59] or 5h after β-adrenergic agonist activation in fat pads generated from a beige cell line [7]. Increase in both mitochondrial UCP1 and TOM20 expressions and mtDNA (Figure 3 and 6) in $TG2^{+/+}$ ATs and $TG2^{-/-}$ SCAT indicates that the mass/function of mitochondria starts to increase after 3h cold exposure which does not occur in $TG2^{-/-}$ GONAT. Mitochondrial dynamics refers to the movement of mitochondria along the cytoskeleton and also to the regulation of mitochondrial morphology and distribution, which depend on fusion and fission events [76]. Little is known about mitochondrial functions in beige adipocytes yet [77]; however, it is clarified that mitochondrial thermogenesis/uncoupling in BAT is activated by a combination of adrenergic stimuli and FFA. Complete mitochondrial fragmentation is induced in brown adipocytes within 1h, and their swelling is clearly detectable after 4h spent at 6°C [78]. Interestingly, autophagic clearance of mitochondria (mitophagy) is well-known to allow the rapid adaptation of mitochondrial activities to physiological demands. The beige adipocyte maintenance is regulated by autophagy induced mitochondrial clearance during beige-to-masked adipocyte transition after withdrawal of external β-adrenergic stimuli [74]. As these cells can be reactivated at any time, it is possible that increased mitochondrial biogenesis and mitophagy cycle balances mitochondrial functions in resting forms; and when thermogenesis is needed, immediate inhibition of autophagy can activate mitochondrial functions quickly. Some data indicate that TG2 could participate in such regulatory mechanism as it can inhibit autophagy [79], although it is also needed for maturation of autophagosomes [80]. Moreover, TG2 may have

other activator effect, as it potentiates mitochondrial function by stabilization of the respiratory complex 1 [81]; hence, ablation of TG2 might also participate in low cold tolerance of $TG2^{-/-}$ mice via decreased β-oxidation of FFAs in mitochondria.

It has been reported that TG2 regulates adipocyte differentiation, so it may have effect somehow on the formation of 'beige'/masked 'beige' cells as well. TG2 controls the differentiation from MEFs via multiple factors of anti-adipogenic pathways [82]. Interestingly, this regulatory role of TG2 could be related to GONAT (but not investigated in SCAT). Namely, the GONAT contains more but smaller adipocytes in 24-week-old $TG2^{-/-}$ animals compared with $TG2^{+/+}$. The increase in adipocyte number in $TG2^{-/-}$ GONAT suggests increased proliferation of precursor cells and unduly fast differentiation into mature but smaller adipocytes [82]. It should be noted that formation of beige cells is known to be induced from specific precursor cells [83]; and, although we did not observe any differences in the size of adipocytes in younger 16-week-old animals at 22°C, we assumed that the regulation of TG2 was linked to balance between proliferation and differentiation of white versus 'beige'/masked 'beige' precursors in GONAT fundamentally effecting their thermogenic potential. The ratio of beige and white adipocytes in WAT is determined, at least partially, during early differentiation of mesenchymal progenitors into adipocyte subtypes [8, 60]. Such a well-known mechanism, for example, is the role of IRX3. Its inhibition in AT of mice reduced body weight and increased energy dissipation without changes in physical activity or appetite. This process seems to be evolutionary conserved, as in humans carrying the risk allele of Fat Mass and Obesity Related (FTO) locus proper repression of IRX3 and IRX5 is failed in their mesenchymal progenitors leading to obesity [84].

Cold-induced thermogenesis comprises non-shivering thermogenesis by BAT and 'beige' cells, and shivering thermogenesis that generates heat from muscle contractions [85]. Importantly, the latter requires mobilization of FFAs from WAT as well, The main pathway leading to lipolysis is

the cAMP-dependent protein kinase (PKA) pathway, through which the stimulation of Gs-coupled β-adrenergic receptors activate adenylate cyclase and the subsequent increase in intracellular level of cAMP leads to the activation of PKA, then phosphorylation and translocation of the hormone sensitive lipase (HSL) to fat droplets [86]. However, this process is clearly reduced in $TG2^{-/-}$ GONAT, again raising the probability for TG2 functioning as a G protein in GONAT. The TG2/Ghα-related signaling pathway initiated by adrenaline and noradrenaline could also participate in lipolysis by activated PKC inducing adenylate cyclase and resulting in subsequent elevation of cAMP levels [87] and further activation of the HSL by PKA. Defect in such an existing process might explain why we observed lower mobilization of $TG2^{-/-}$ GONAT compared to $TG2^{+/+}$. In this work we have identified TG2 as a regulator, which is involved in the browning process of GONAT in a tissue-specific manner in mice; however, further studies have to reveal the underlying exact molecular mechanisms. Nevertheless, the presented data have added transglutaminase related regulatory processes to the list of possible targets for pharmacological interventions in obesity and metabolic disorders.

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Conflict of Interest

The authors declare no competing financial interests.

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Figure Legends

Figure 1 $TG2^{-/-}$ mice have low tolerance to acute cold exposure and utilize a low amount of GONAT during the 3h treatment.

(A) Cold tolerance test on 16-week-old male mice. **(B)** Mass values of isolated adipose tissue specimens from animals kept at 22°C and exposed to 4°C for 3h, respectively. BAT: interscapular brown adipose tissue, SCAT: inguinal subcutaneous white adipose tissue, GONAT: gonadal (intra-abdominal) white adipose tissue. (Experiments were carried out as described in Materials and Methods.) Results are expressed as the mean ± SD. **p<0.01, n=6.

Figure 2 Alterations in BAT cannot explain the decreased cold tolerance of $TG2^{-/-}$ mice.

(A) Expression of TG2, UCP1 and β-actin proteins in BAT detected on Western blots from $TG2^{+/+}$ and $TG2^{-/-}$ untreated mice kept at 22°C and **(B)** after 3h cold exposure (n=4). **(C)** Relative gene expression of brown and general adipogenic markers in BAT of mice kept at 22°C and **(D)** after 3h cold exposure. Brown markers: UCP1, PRDM16, CIDEA, DIO2, ZIC1; General markers: Adiponectin, Resistin, GLUT4. All the target genes were normalized to cyclophilin after qPCR, and fold changes of $TG2^{-/-}$ samples were calculated in comparison to $TG2^{+/+}$ values (1+/-SD). (n=6, *p<0.05,**p<0.01). Normalized mRNA expressions of CIDEA and UCP1 are shown on Supplementary Figure 2; Adiponectin, Resistin and GLUT4 expressions on Supplementary Figure 3.

Figure 3 Browning is inhibited in the GONAT of $TG2^{-/-}$ mice.

Representative images on the portions of WAT specimens and their TG2, UCP1 and β-actin contents on Western blot (n=4) are shown **(A-D)**. **(A)** SCAT of untreated mice; **(B)** SCAT of mice

isolated after 3h cold exposure; **(C)** GONAT of untreated mice; **(D)** GONAT of mice isolated after 3h cold exposure. **(E)** Representative images of UCP1 detected with confocal microscopy in situ in WAT from cold-treated $TG2^{+/+}$ and $TG2^{-/-}$ mice using immunohistochemistry. UCP1 is green, and the nuclei are red, stained with propidium iodide. Scale bars represent 40 μm. **(F)** 5 mages were obtained from each cold-treated $TG2^{+/+}$ and $TG2^{-/-}$ mice, and were analysed with Fiji software. Mean+SD UCP1 fluorescent intensity values/cell are shown (n=3, *p<0.05). **(G)** Mitochondrial DNA content of cells from cold-treated and untreated SCAT and GONAT of strains was determined using qPCR as described in Materials and Methods (n=6, *p<0.05).

Figure 4 $TG2^{-/-}$ animals utilize their triacylglycerol content of GONAT at lower level during cold exposure.

Representative images of hematoxylin/eosin-stained SCAT and GONAT sections from mice without treatment and after 3h cold exposure are shown. Scale bars represent 200 μm **(A-B)**. **(A)** SCAT and GONAT from untreated animals, **(B)** SCAT and GONAT from cold-treated animals. **(C)** Average area of adipocytes without treatment and after 3h cold exposure in SCAT and GONAT of $TG2^{+/+}$ and $TG2^{-/-}$ mice. Bars represent the results obtained from 5 analyzed images for each investigated animal (n=3, *p<0.05, **p<0.01). **(D)** Serum lipid content of $TG2^{+/+}$ and $TG2^{-/-}$ mice without treatment and after 3h cold exposure. C: total cholesterol, LDL-C: LDL cholesterol, TG: triglyceride, FFA: free fatty acid (n=6, **p<0.01, ***p<0.001).

Figure 5 Expression of browning and adipogenic marker genes in WAT isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice without treatment and after 3h cold exposure. **(A)** SCAT of untreated mice, **(B)** SCAT of cold-treated mice, **(C)** GONAT of untreated animals, **(D)** GONAT of cold-treated animals.

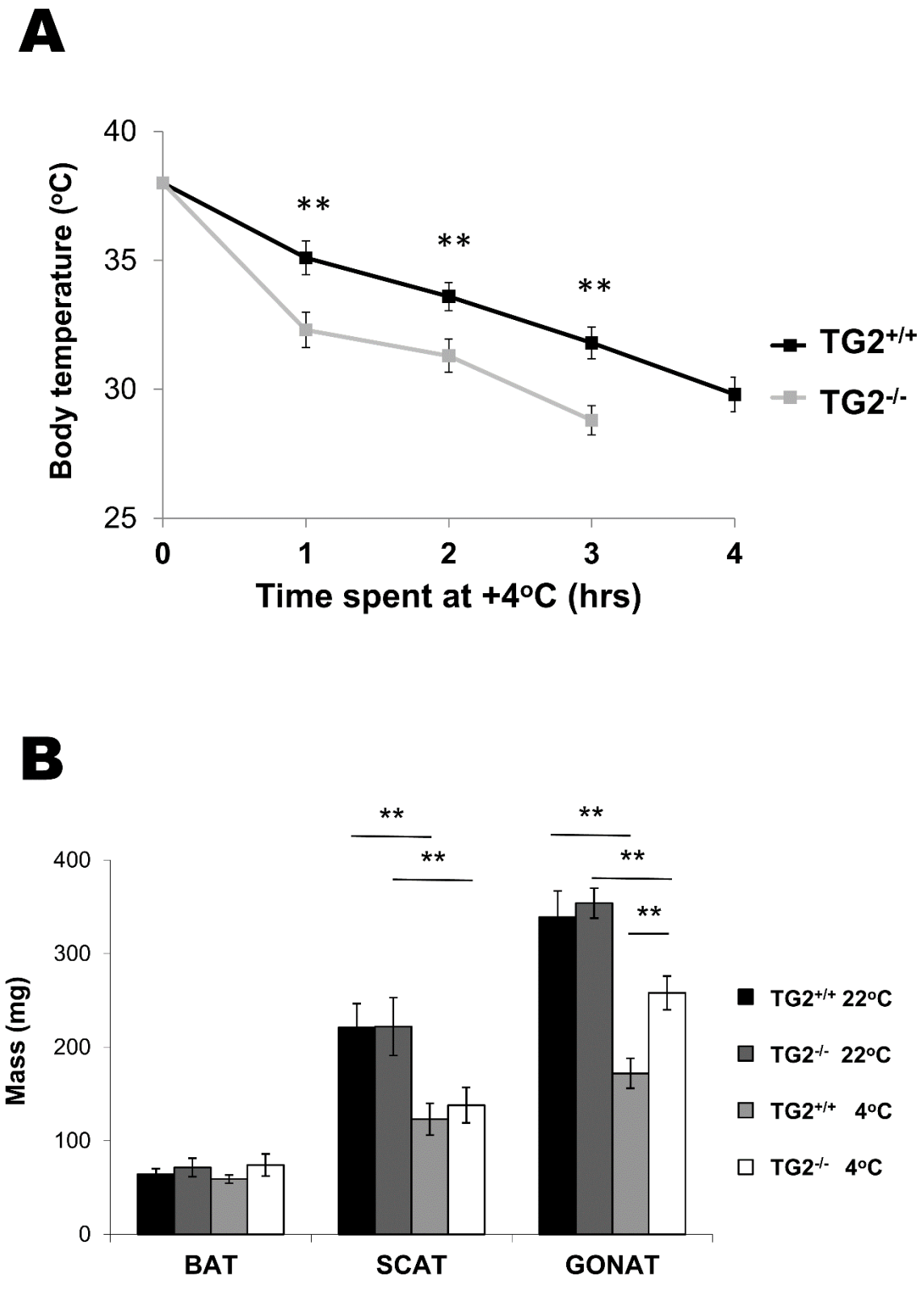
'Beige' markers: UCP1, TBX1, TNFRSF9, TMEM26; White markers: Leptin, LPL, FABP4, HSL, PPARg; General markers: Adiponectin, Resistin, GLUT4; Preadipocyte marker: PREF1.

All the target genes were normalized to cyclophilin, and fold changes of $TG2^{-/-}$ samples were calculated in comparison to $TG2^{+/+}$ values (1+/-sd). (n=6, *p<0.05, **p<0.01, ***p<0.001). Normalized mRNA expressions of UCP1 are shown on Supplementary Figure 2; expressions of Adiponectin, Resistin, GLUT4, PPARg and FABP4 on Supplementary Figure 3, expressions of TBX1, TNFRSF9, TMEM26 and PREF1 are on Supplementary Figure 4.

Figure 6 Detection of adipogenic marker proteins (HSL, ADIPOQ, PPARg, FABP4) and mitochondrial TOM20 protein in WAT isolated from $TG2^{+/+}$ and $TG2^{-/-}$ mice without treatment and after 3h cold exposure using Western blot and densitometric analyses (n=4, *p<0.05, **p<0.01). **(A)** SCAT of untreated mice, **(B)** SCAT of cold-treated mice, **(C)** GONAT of untreated animals, **(D)** GONAT of cold-treated animals.

Figure 7 $TG2^{+/+}$ and $TG2^{-/-}$ WAT are exposed to the same levels of hormones regulating metabolism.

(A) Glucose tolerance test on untreated 16-week-old male $TG2^{+/+}$ and $TG2^{-/-}$ mice. Experiments were carried out as described in Materials and Methods (n=6). **(B)** Detection of adrenaline and noradrenaline levels (n=3) and **(C)** detection of insulin and glucagon levels (n=6) in plasma of mice after 3h cold-exposure.


A
Body temperature (°C)
40
35
30
25
0
1
2
3
4
Time spent at +4°C (hrs)
**
**
**
TG2+/+
TG2-/-
B
Mass (mg)
400
300
200
100
0
BAT
SCAT
GONAT
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TG2+/+ 22°C
TG2-/- 22°C
TG2+/+ 4°C
TG2-/- 4°C

Figure 1

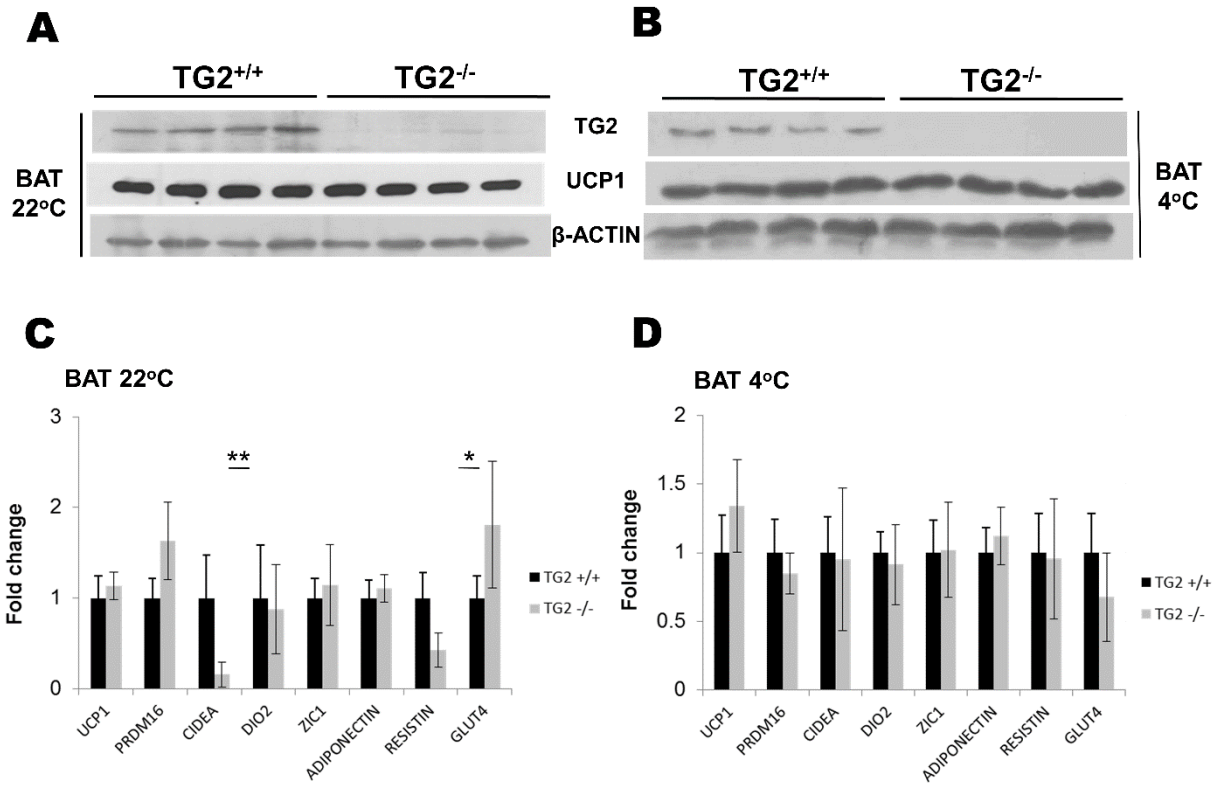
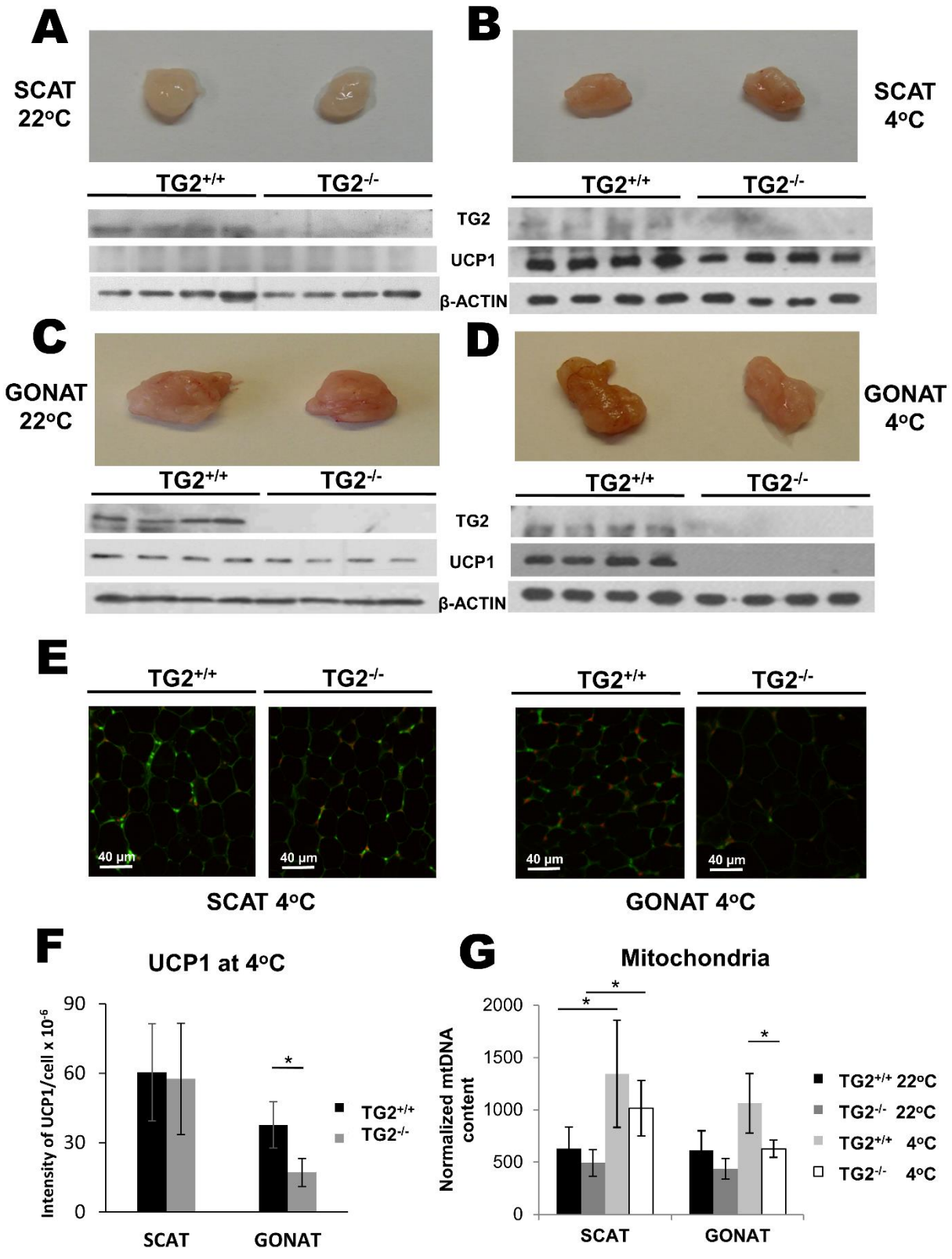


Figure 2



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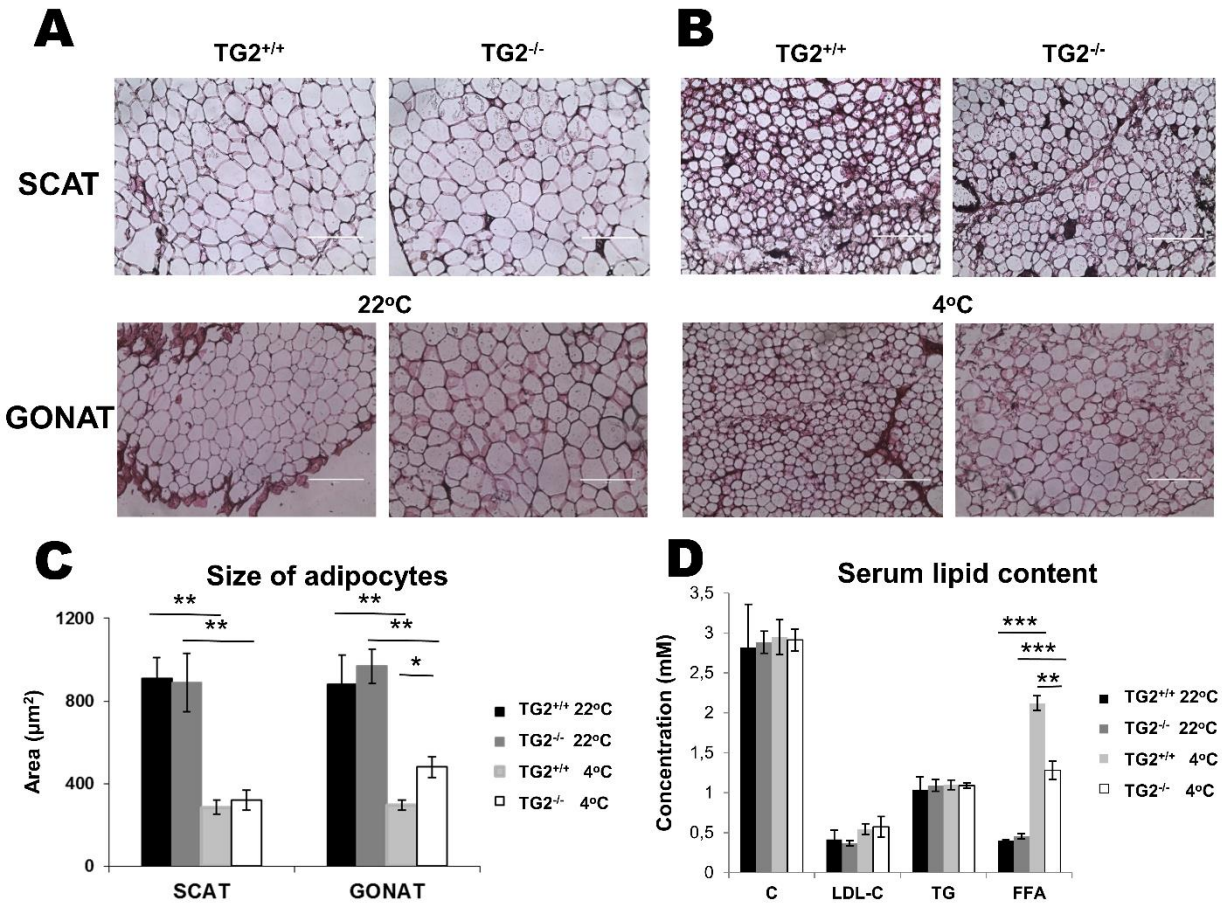

Figure 4

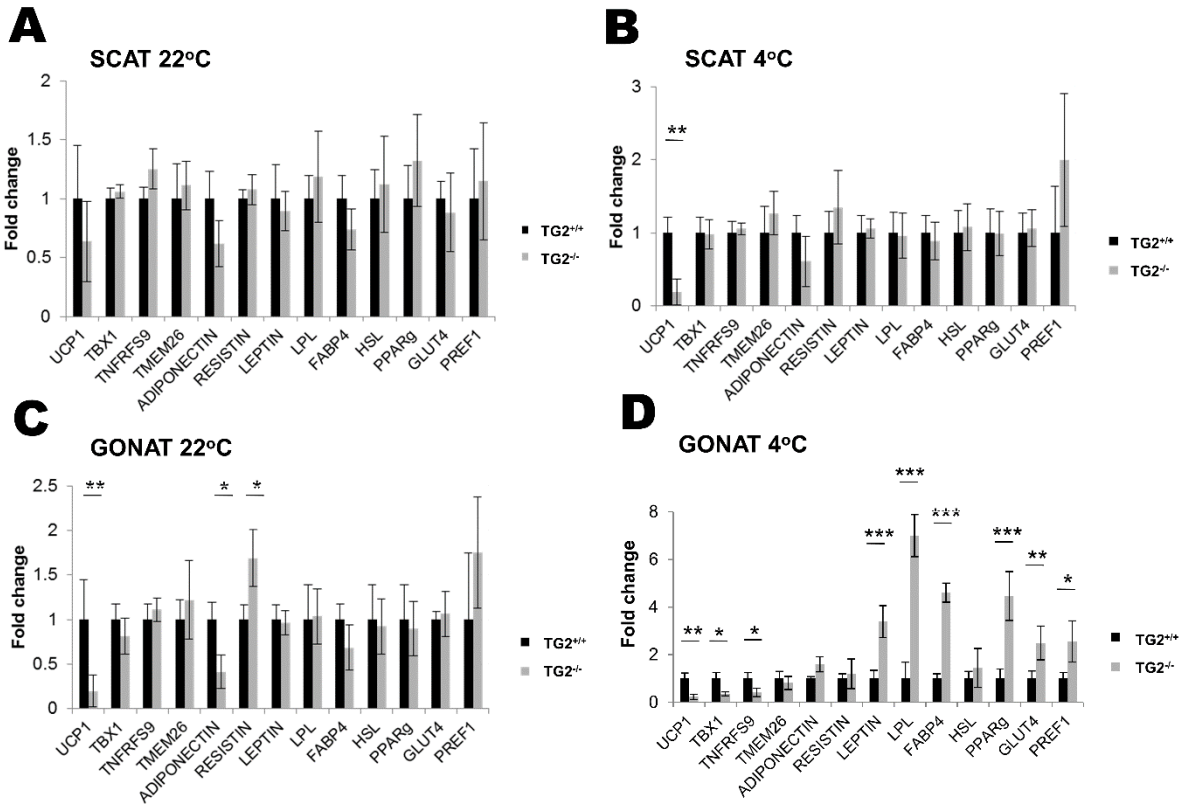

Figure 5

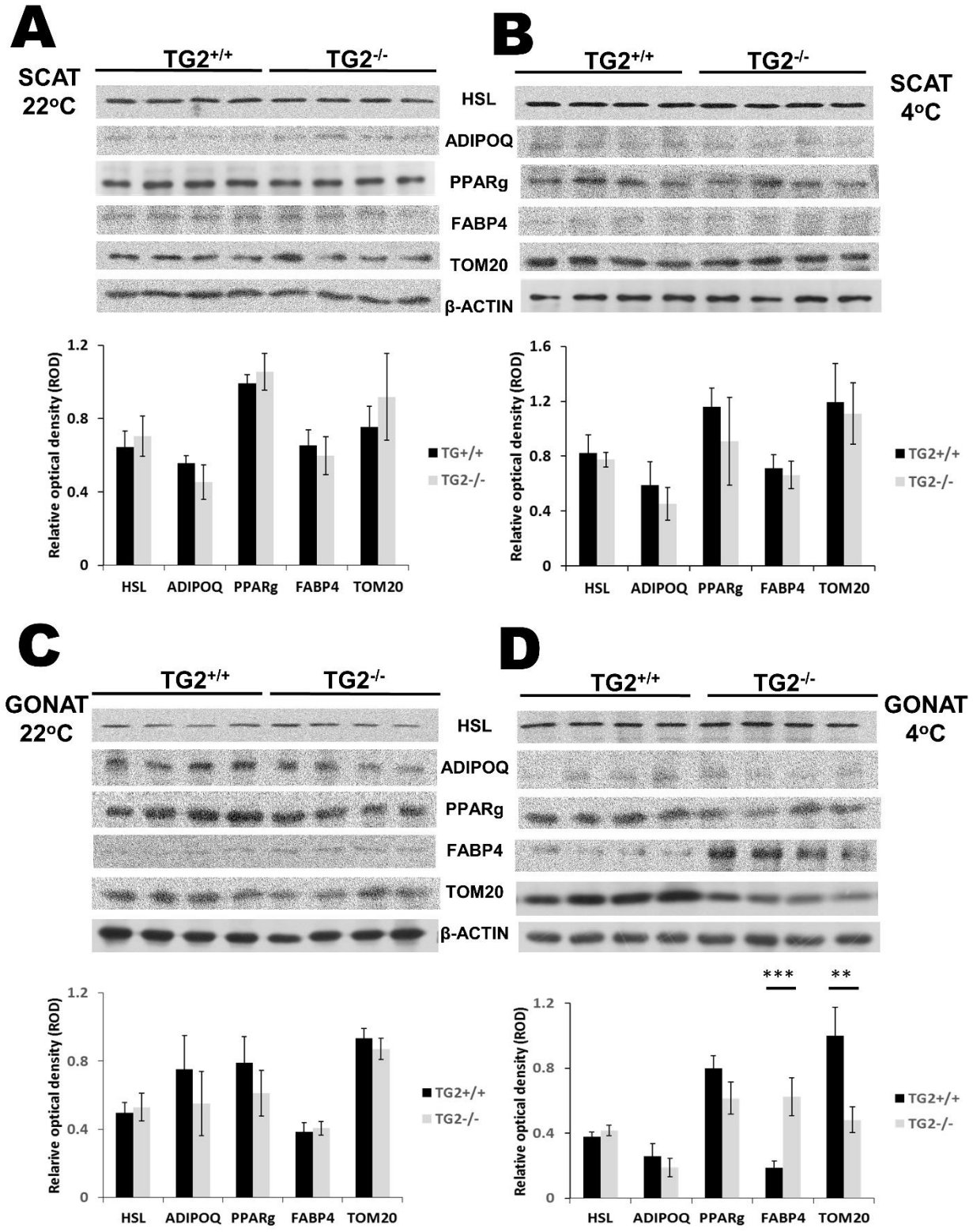

Figure 6

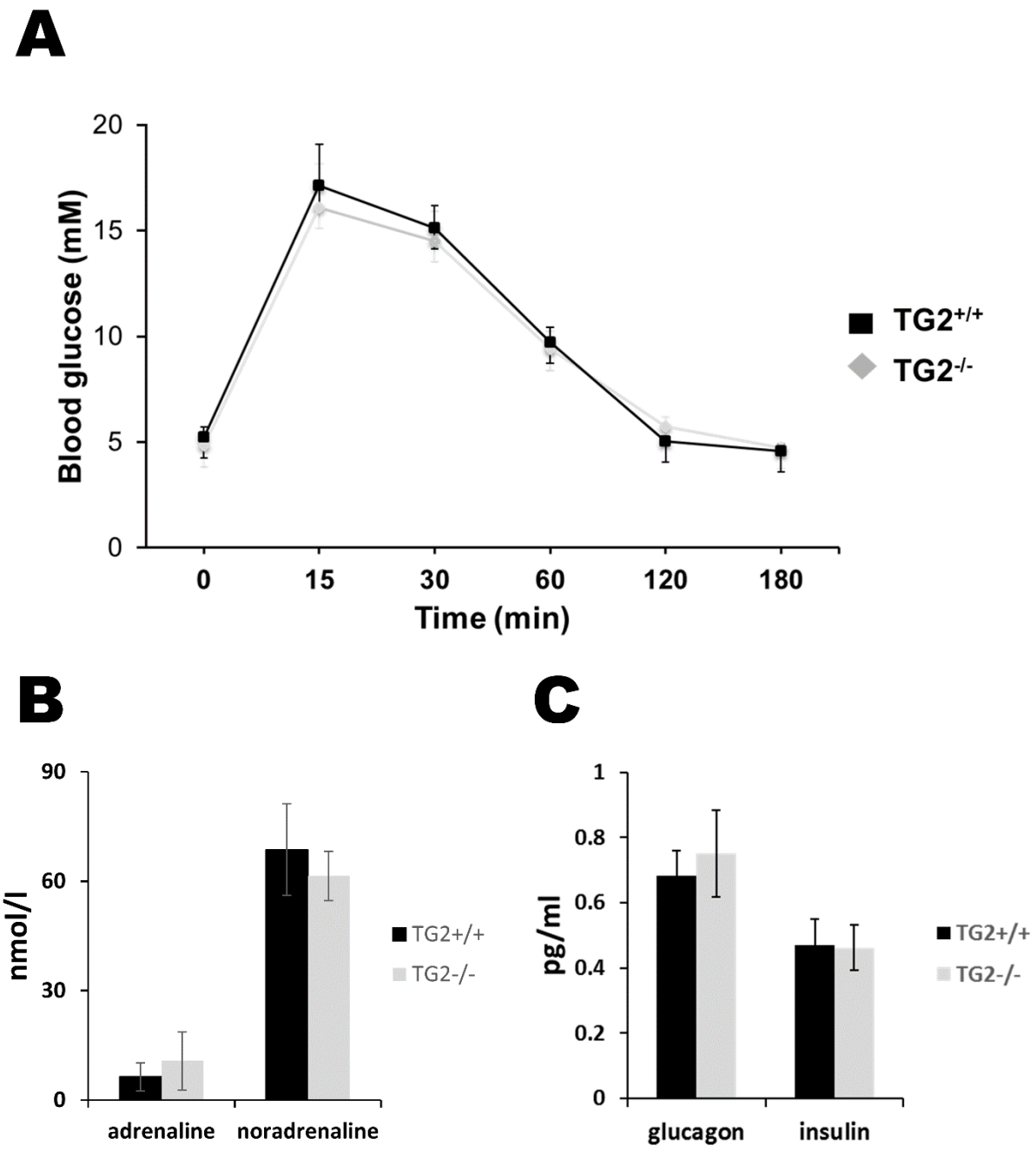

Figure 7

Highlights

Tissue transglutaminase knock-out mice have low tolerance to acute cold exposure.

Ablation of the enzyme has tissue-specific effect in the epididymal fat.

The enzyme is required for activation of the thermogenic potential in epididymal fat.