SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the role of tissue transglutaminase in the process of browning of white adipose tissue and the activation of beige adipocytes

by Kinga Fedor-Lénárt

Supervisor: András Mádi

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

DEBRECEN, 2022

Investigation of the role of tissue transglutaminase in the process of browning of white adipose tissue and the activation of beige adipocytes

By Kinga Fedor-Lénárt, Master of Science (MSc) in Biology

Supervisor: András Mádi (PhD)

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Defense Committee**:	Prof. Dr. Gábor Szabó, PhD, DSc
Reviewers:	Dr. Krisztián Kvell, PhD
	Dr. Dániel Törőcsik, PhD
Members of the Defense Committee:	Prof. Dr. Attila Bácsi, PhD, DSc
	Dr. Attila Ambrus, PhD
	Dr. Krisztián Kvell, PhD
	Dr. Dániel Törőcsik, PhD

The PhD defense will be organized online at 1:00 pm 23rd of March, 2022.
Participation requires registration. For registration and further information please email to kinga.lenart@med.unideb.hu until the 22nd of March 2022, 4 pm.

1. INTRODUCTION

The human body has an exceptional ability to adapt to internal and external changes, including nutritional status, environmental temperature, infections, circadian rhythm, and aging. These adaptations involve dynamic reprogramming of cellular metabolism in the peripheral tissues. For example, cold exposure is associated with metabolic adaptation in skeletal muscle and adipose tissue by inducing a switch from carbohydrate metabolism to fatty acid oxidation.

The main player during this adaptation process is the highly dynamic adipose tissue, which can transform rapidly under environmental change and its cells coordinate energy storage and oxidation properly. In the case of a persistent positive energy balance, the lipid storing white adipose tissue increases its volume by enlarging the volume of adipocytes (hypertrophy) and increasing the number of adipocytes (hyperplasia), which can lead to obesity eventually.

Obesity, now considered a common disease, can be attributed to an increase in the incidence of many comorbidities that decrease the quality of life, such as type 2 diabetes, liver steatosis, atherosclerosis, and hypertension. An excellent strategy for treating these diseases could be the reduction of adipose tissue masses by targeted drug activation of an existing natural mechanism. This process is the browning of white adipose tissue, during which an adipocyte type specializing in heat production appears and is activated as an adaptive response to the cold environment to ensure the body's thermotolerance. These so-called 'beige' (after their color: beige) cells are similar to brown fat cells in their properties, they also use their fat stores for heat production, but they are not the same. Because the development of beige adipocytes is highly inducible, special emphasis is being placed on this cell type today as an attractive cellular target for new anti-obesity therapeutic interventions.

To make it possible, the completeness of beige cell differentiation processes that could be manipulated to reduce white adipose tissue should be explored. Particular attention is therefore paid to the study of new proteins capable of regulating differentiation processes; based on previous literature data, this may also be the case for tissue transglutaminase. According to our studies presented in this dissertation, this enzyme plays an important role in the browning process, so its drug activation could be an effective target in the treatment of obesity and its comorbidities.

1.1.Types of fat cells: white, brown, beige, and pink adipocytes

There are two anatomically distinct forms of adipose tissue in mammals: the larger amount of white adipose tissue, which contains different cell types in its stromal vascular fraction, and its stored energy is mobilized for ATP production by starvation or stress; and the lower amount of

brown adipose tissue (BAT), which, with lower levels of storage, is uniquely able to turn its fat content into heat production during a process called nonshivering thermogenesis. The brown color of the cells is due to a large amount of cytochrome C in this tissue, which is very rich in mitochondria.

Their heat production is triggered by the fact that the cells of brown adipose tissue contain much more mitochondria in which the function of the electron transport chain is uncoupled from oxidative phosphorylation, resulting in proton efflux into the mitochondrial matrix. As a result, decomposing fats do not produce energy-storing ATP, but instead, release their energy content in the form of heat. Uncoupling protein 1 (UCP-1) in the inner membrane of the mitochondria is responsible for heat production. The third type of adipocyte has also been discovered in rodents, appearing in white adipose tissue as an adaptive response to a cold environment or adrenergic treatment, ensuring the body's thermotolerance. These cells, called "beige" or "brite" cells, represent a bifunctional phenotype in that they are similar to brown adipocytes in their properties and also use their fat stores for heat production.

Unlike "classic brown" adipocytes, mature, unstimulated beige adipocytes express lower levels of the genes needed for heat production. When the thermogenic signal disappears, white fat-like cells called "masked beige" remain *in vivo*. The dependence of UCP1 induction on external stimuli is an important distinguishing feature of beige fat. Beige adipocytes show extreme plasticity and can turn their heat generation programs on and off according to the needs of the body. Both brown and beige cells express a similar basic program of mitochondrial and thermogenic genes. The interscapular brown adipose tissue of infants is very similar to the classic brown fat of rodents. Based on these, beige cells are uniquely programmed for two functions: they can store energy in the absence of thermogenic stimuli, but they are fully capable of initiating heat production when they receive appropriate signals. Interestingly, in mouse models, the loss of classic brown fat induces compensatory induction of beige fat, thus providing physiological body temperature and resistance to diet-induced obesity, suggesting a significant overlap between brown and beige adipocytes in their function.

Intensive research in recent years has identified another, the fourth type of adipocyte; called pink fat cells. Pink adipocytes are milk-secreting alveolar cells that may result from transdifferentiation of white fat cells during pregnancy and lactation. These cells are characterized by cytoplasmic lipid droplets, microvilli on their apical surface, a round and large nucleus centrally located, and a robust, rough endoplasmic reticulum (RER), Golgi complex, and milk protein granules. In the post-lactation phase, pink adipocytes are converted to white and brown adipocytes, and evidence supports the hypothesis of white-pink transdifferentiation,

pink-brown transdifferentiation, and reversible brown-myoepithelial cell conversion, which again demonstrates high plasticity of adipose tissue.

1.2. Induction of heat production of beige adipocytes

Physiologically, cold exposure induces the browning process in white adipose tissue (WAT) through β3-adrenergic stimulation. The sympathetic nervous system secretes norepinephrine (NE), which binds to the cell surface β3 receptor, followed by intracellular signaling cascades leading to the breakdown of triglycerides into free fatty acids (FFAs). FFAs activate UCP1, resulting in uncoupled respiration from ATP synthesis. In addition to catecholamines, some circulating hormones are known to be involved in the activation of beige adipocytes, such as triiodothyronine (T3), hepatic bile acids, 'Fibroblast growth factor 21' (FGF21), 'Atrial natriuretic peptide' (ANP), 'B-type natriuretic peptide' (BNP), and cardiotropin-1. In addition, a hormone called irisin released by the skeletal muscle during exercise can also cause the browning of WAT. These ligands trigger the browning process through their receptors via different and often overlapping mechanisms. Bile acids, for example, activate the receptor protein 'G-protein-coupled bile acid receptor 1' (GPBAR1), which in turn induces the enzyme deiodinase that promotes the formation of intracellular T3. Thus, the activation of beige adipocytes and induction of heat production by pharmacological activation could be an effective target in the treatment of obesity and comorbidities.

1.3. Study of metabolism in a whole body by indirect calorimetry

The evaluation of human energy use originally focused on quantifying heat production (i.e., direct calorimetry) because the first law of thermodynamics predicted that all energy consumed in physiological processes would be distributed as heat under conditions of thermal stability and negligible energy storage. However, such a technically challenging process was soon triggered by methods that calculate heat production based on oxygen consumption and carbon dioxide production according to the classic Weir equation:

heat production (kJ / kg) = 4.186 x [3.9 x O_2 (L) consumed + 1.11 x CO_2 (L) produced].

By measuring oxygen consumption and carbon dioxide production, indirect calorimetry provides real-time data on energy use to detect sleep and resting metabolism, the effect of food on heat production, and the energy cost of physical activity. It is important that such measurements also provide information on the utilization of energy substrates. The use of indirect calorimetry can be a valuable tool in the treatment of obesity with personalized

interventions, but the way to regulate metabolism should not vary greatly from individual to individual.

1.4. Biological functions of tissue transglutaminase

As our knowledge of adipose tissue accumulates, so do the chances of developing new and effective therapies for obesity and related diseases. There is a need to explore cell differentiation processes that could be manipulated to activate beige adipocytes and thereby reduce white adipose tissue masses. This requires further investigation of proteins capable of regulating gene expression. One such protein is tissue transglutaminase (TG2, EC 2.3.2.13), a multifunctional protein that can be detected both extracellularly and intracellularly. It may also be localized in the nucleus, mitochondria, or plasma membrane in certain cell types. It also has protein cross-linking and guanosine-5'-triphosphate (GTP) hydrolyzing activity. In the presence of Ca^{2+}, TG2 cannot bind GTP and takes on an open conformation that covalently cross-links proteins producing isopeptide bonds between glutamine and lysine residues through transamidase activity. However, in GTP-bound conformation, TG2 takes on a closed conformation and may function as a GTPase. Besides, TG2 can also act as a protein disulfide isomerase and a protein kinase. Functions of TG2 have been implicated in various biological processes including regulation of the cytoskeleton, cell adhesion, and cell death as catalytically active or just as an interacting protein partner. Several reports have linked TG2 functions to different types of diseases, like cancer, type 2 diabetes, neurodegenerative disorders, and coeliac disease.

1.5. Tissue transglutaminase deficient mouse models

Strategies to unfold the complex biological functions of TG2 involve the application of small molecules that can inhibit TG2 activities and intensive studies on the identification and characterization of substrates and interacting partners. Additionally, two TG2 knock-out (KO) mouse models have been generated using different approaches. In the first model, by deleting parts of exons 5 and 6 containing the active site, and the intron 5 through homologous recombination; and in the second model, by the creation of a TG2-loxP knock-in mouse, which allowed inactivation of both alleles after cross-breeding with animals expressing Cre recombinase. Considering the multifunctionality of TG2, it is quite surprising to learn that the homozygous deletion of TG2 does not result in a lethal phenotype. The $TG2^{-/-}$ animals are viable and fertile and grow up to normal size and weight with no apparent abnormalities in development and organ functions. The most probable explanation for the lack of severe

differences is that other transglutaminases in mammalian tissues can compensate for the loss of TG2. However, detailed investigations of KO models have revealed that TG2 participates in the crosstalk between dying and phagocytic cells to ensure tissue integrity, and it is required for proper differentiation and bacterial killing of neutrophils. Ablation of TG2 results in impaired wound healing, autoimmunity, and hyperglycemia. Given the multifunctionality of TG2, it is surprising that homozygous deletion of TG2 does not cause a lethal phenotype. $TG2^{-/-}$ mice are viable and fertile growing to normal weight and size without obvious abnormalities in their development and organ functions. The most likely explanation for the lack of severe differences is that other transglutaminases can compensate for the loss of TG2 in mammalian tissues. However, a detailed study of KO models revealed that TG2 is involved in the interaction between apoptotic and phagocyte cells to ensure tissue integrity, and is required for neutrophil differentiation of NB4 promyelocyte cells and killing of bacteria by these cells. Elimination of TG2 can lead to imperfect wound healing and the development of autoimmunity, as well as glucose intolerance and hyperglycemia.

1.6. G protein function of tissue transglutaminase

In a GTP-bound closed conformation, TG2 can act as a G protein (Ghα) that can transmit signals from the α1B-adrenoceptor (α1B-AR), the oxytocin receptor, the thromboxane A2α receptor, and the follicle-stimulating hormone receptor. In general, G protein-coupled receptors (GPCRs) interact with heterotrimeric G proteins; however, TG2 / Ghα forms a heterodimer with calreticulin (Ghβ), which, however, acts similarly to heterotrimeric G proteins. Upon receptor activation, GDP on the TG2 protein is exchanged for GTP and G protein function is activated. GTP-bound TG2 / Ghα dissociates from Ghβ and then directly activates phospholipase Cδ 1 (PLC δ1) leading to inositol triphosphate release and increased intracellular Ca^{2+} concentration. In addition, TG2 / Ghα may regulate other signaling pathways, including adenylate cyclase (AC) activity and direct activation of highly conductive Ca^{2+} -activated K^{+} channels in vascular smooth muscle cells. The TG2 / Ghα signal is abolished when GTP is hydrolyzed to GDP by GTPase activity and TG2 / Ghα re-associates with Ghβ.

1.7. Role of tissue transglutaminase in mitochondrial function

Based on the *in vivo* results described in the literature, TG2 can also function as a protein disulfide isomerase (PDI) under physiological conditions and has been shown to contribute to the proper assembly of mitochondrial respiratory chain complexes through this activity. It is known that TG2-deficient mice show impairment in mitochondrial energy production as

evidenced by decreased ATP levels in the myocardium after exercise. This defect is phenotypically reflected in the dramatic decrease in ATP-dependent motor muscle work in animals. The molecular mechanisms leading to this phenotype involve the improper formation of disulfide bonds in NADH-ubiquinone oxidoreductase (complex I), succinate ubiquinone oxidoreductase (complex II), cytochrome oxidase (complex IV), and ATP synthase (complex V). Recent data suggest that TG2 activity may modify not only the assembly of respiratory chain complexes but also the transcription of critical genes, including PGC-1α and cytochrome C, that are important for mitochondrial function and biogenesis. In other cell types, TG2 has been shown to play a role in maintaining the homeostasis of the electron transport chain and energy production. Deletion of TG2 caused significant deregulation in the respiratory chain I and II and decreased ATP production in mouse embryonic fibroblasts (MEF).

1.8. Dynamics of mitochondria

Mitochondria play a central role in the metabolism of eukaryotic cells like the Krebs cycle, fatty acid degradation, cellular respiration, antiviral signaling, aging, apoptosis, and they contribute to Ca^{2+} homeostasis. They are highly dynamic organelles that can undergo fusion and fission events, as well as transport processes and degradation, which are collectively referred to as "mitochondrial dynamics". Each of these dynamic processes is critical to maintaining a healthy mitochondrial population, so continuous monitoring of mitochondrial quality is essential to maintain cellular homeostasis. Given the central metabolic functions of the mitochondria, it is not surprising that mitochondrial dynamics and bioenergetics interact. Dysregulation of mitochondrial dynamics leads to the accumulation of dysfunctional mitochondria, which can be detected in several pathological processes, including neurodegenerative diseases, muscle wasting, sarcopenia, aging, and metabolic diseases such as type 2 diabetes and obesity. Fragmentation of mitochondria is often associated with deterioration in mitochondrial function, with severe stress effects such as loss of mitochondrial membrane potential, decreased ATP levels, and predominantly observed during apoptosis. However, there are also events where complete fragmentation of the mitochondrial network is required: such processes take place during cell division to ensure the mitochondrial inheritance of daughter cells and during the differentiation of post-mitotic cells such as neurons and cardiomyocytes. The fusion of mitochondria contributes to the distribution of components of the mitochondrial matrix within the network, increasing the homogeneity of the mitochondrial network and also providing complementarity. Fusion is a key mechanism by which mitochondria can save the damaged unit

of the network. Elongation of mitochondria may increase mitochondrial activity as well as protect the cells against autophagy.

The four major protein mediators of mitochondrial dynamics are members of the dynamin family of guanosine triphosphatases (GTPases): mitofusin 1 and 2 (MFN1 and 2), optical atrophy 1 (OPA1) protein, and dynamin-linked protein 1 (DRP1). DRP1 localizes almost entirely in the cytosol until it binds to any of the DRP1 receptor proteins in the outer membrane of the mitochondrion to induce fission, oligomerizes, and bisects the mitochondria to form a loop. The sites of division are designated by a transmembrane protein, mitochondrial fission protein 1 (FIS1). Mitochondrial cleavage factor (MFF) interacts with the FIS1 protein and serves as an adapter that recruits DRP1 to facilitate the polymerization process. Recent studies have shown that MFF is required for fission, but FIS1 is dispensable. This surprising fact may suggest that other proteins may override FIS1 and act as alternative receptors for MFF and DRP1. Unlike DRP1, MFN1, MFN2, and OPA1 are localized in mitochondria: MFN1 and MFN2 are located in the outer mitochondrial membrane with their N- and C-terminal facing the cytosol, and OPA1 located in the inner mitochondrial membrane.

2. AIMS

Our research group investigates the multifunctional biological role of TG2. Previously, six-fold higher TG2 gene expression was detected in human BAT compared to WAT, suggesting that the enzyme may play an important role in the heat-producing function of adipose tissue.

Based on these, the questions to be answered and the main goals of our research were the following:

- To investigate the cold tolerance of TG2 mice
- To explore the molecular mechanisms by which TG2 may be involved in adipose tissue browning
- To investigate how $TG2^{-/-}$ mice respond to different adrenergic stimuli compared to $TG2^{+/+}$ animals
- To show correlations between the function and metabolic role of Ghα in TG2
- To carry out comparative studies on $TG2^{+/+}$ and $TG2^{-/-}$preadipocytes and beige adipocytes (gene expression, laser scanning cytometry, functional studies with Seahorse measurement, immunocytochemical and protein level studies)
- To identify the role of TG2 in mitochondrial function and thermogenesis

3. MATERIALS AND METHODS

3.1. Animals and their treatments

$TG2^{-/-}$ mice and $TG2^{+/+}$ littermates with C57BL/6J genetic background were obtained from heterozygous breeding couples and were genotyped in the Animal Core Facility at the University of Debrecen. Mice were housed separately, had ad libitum access to water and chow, and were kept in a 12 h dark/light cycle at 22 ± 1 °C. All the animal experiments were carried out according to the national and EU ethical guidelines (license numbers: 14/2010/DEMAB and 1/2014/DEMAB). During the acute cold treatment, 6 $TG2^{+/+}$ and 6 $TG2^{-/-}$ 16-week-old male mice were kept at 4°C (cold room) for a maximum of 4 h without chow but with cold (4 °C) water and their rectal body temperature was measured at 30 min intervals and kept cold until their body temperature dropped to an ethically acceptable value of around 30 °C. The body mass of mice was measured and they were injected intraperitoneally with 60 nM/g body weight phenylephrine (specific alpha1-AR agonist) or CL-316,243 (specific beta-AR agonist). Mice were anesthetized and blood was collected from the heart, and the obtained serum samples were stored at -80 ° C.

3.2. Indirect Calorimetry

Measurements were performed using the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA), which allows non-invasive, 24-hour, automated detection of various physiological parameters. Six $TG2^{+/+}$ control and six $TG2^{-/-}$, 18-week old male mice on a chow diet were placed individually in chambers for 4 consecutive days at ambient temperature (22 °C). Mice were provided free access to food and water, in a 12 h light (6 a.m. to 6 p.m.) and 12 h dark (6 p.m. to 6 a.m.) cycle. Measurements were made in 8 min intervals after the initial 18–20 h acclimation period. The acclimatization period was followed by a control measurement for 24 hours without treatment. Then, mice were injected intraperitoneally with 60 nM/g body weight phenylephrine (specific alpha1-AR agonist) or CL-316,243 (specific beta-AR agonist) and investigated for an additional 24 h. In the end, all the mice survived the experiment and recovered completely.

3.3. Measurement of arterial blood pressure and examination of metabolic parameters in blood

Based on an indirect calorimetric experiment, the blood pressure of the animals was measured using a CODA Standard Tail-cuff (Kent Scientific Corporation, Torrington, CT) non-invasive

method on an acclimatization day followed by a control day without any treatment and finally after phenylephrine treatment. Measurements were carried out on awake mice. The tails of the mice were fitted with a cuff equipped with a pneumatic pulse sensor to determine the blood pressure and heart rate of the animals.

Blood glucose concentration was measured as previously described. Serum samples were prepared from the plasma of mice collected 13 h after phenylephrine treatment through heart punctuation. Total cholesterol (C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), free fatty acid (FFA), and lactate levels were determined by colorimetric enzyme assays (Cobas6000, Roche Ltd., Mannheim, Germany) and free fatty acid (FFA) by a standard laboratory assay. Insulin content was detected using Mouse Insulin ELISA Kit (Mercodia, Sweden), glucagon level was measured using Glucagon EIA Kit (Sigma-Aldrich Chemie GMBH, Darmstadt, Germany) according to the manufacturers' instruction from 5–5 $TG2^{+/+}$ and $TG2^{-/-}$ 18-week old male mice serum samples.

3.4. Detection of LDH and CK-MB

LDH activity was determined with a UV kinetic method, and CK-MB activity was measured with the immunoinhibition UV kinetic method on the Cobas-501 analyzer (Roche). LDH isoenzymes were separated on Hydragel ISO-LDH with electrophoresis and the amounts of isoenzymes were determined by densitometry (Sebia Hydrasys, Sebia, Lisses, France). The activity of each LDH isoform was obtained by dividing the value of the total activity by the proportions of the amounts obtained in the densitometric determination.

3.5. Histochemical detection of UCP1

After deparaffinization, sections were stained with antiUCP1 primary antibody (Sigma-Aldrich, St. Louis, MO) for 6 hours at room temperature (1: 500 dilution) followed by 1 hour incubation with Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA). Expression of UCP1 was examined using a FluoView 1000 confocal microscope (Olympus, Tokyo, Japan), and quantified using Fiji image analysis software (https://imagej.net/Fiji/Downloads).

3.6. Preadipocyte isolation and beige differentiation

Gonadal adipose tissue from mice was prepared under sterile conditions. Stromal vascular fractions were placed in 1 ml of PBS in a centrifuge tube and 80 µl of collagenase (Sigma-Aldrich, St. Louis, MO) stock solution (120 U / ml) was added. This was followed by incubation for 1 hour at 37°C with shaking occasionally, and the centrifuge tube was filled with medium and centrifuged (10 min, 1300 rpm). Finally, another wash was performed and the cells were plated in 6-well Falcon cell culture plates (Corning Incorporated, Durham, NY). Cells were grown to 100% confluence in DMEM-F12 medium containing 10% FBS, 1% antibiotic /antimycotic (penicillin/streptomycin), 33 µM biotin, and 17 µM pantothenic acid (all Sigma-Aldrich, St. Louis, MO). Cell differentiation was induced for 2 days with 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 2 µg / ml dexamethasone, 850 nM insulin, 1 nM T3 (both Sigma-Aldrich, St. Louis, MO), and 0.5 µM with the addition of rosiglitazone (Cayman Chemicals, Ann Arbor, MI). From day 3 to day 9, cells were maintained in DMEM-F12 differentiation medium containing 10% FBS, 850 nM insulin, 1 nM T3, and 0.5 µM rosiglitazone. Finally, cells were harvested in TRIzol (Invitrogen Corporation, Carlsbad, CA) and 1x denaturing buffer to isolate RNA and protein. Some of the differentiated cells were also treated with 10 µM arterenol, 10 µM CL-316,243, 10 µM phenylephrine, or 10 µM forskolin without FBS for 4 h before collection (Altshuler-Keylin et al. 2016).

3.7. mRNA isolation, reverse transcription, and real-time quantitative RT-qPCR

Total RNA was isolated from preadipocytes and differentiated beige cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). The RNA concentration of the samples was determined with a NanoDrop spectrophotometer (NanoDrop, Erlangen, Germany). For reverse transcription (RT), samples were diluted to 100 ng / µl and stored at -20 ° C. Subsequently, using RNA as a template, cDNA was synthesized using a high-capacity cDNA RT kit as described by the manufacturer (Applied Biosystems, Foster City, CA). For samples obtained from preadipocytes and differentiated beige cells, 3 biological parallel measurements were performed, and three technical replicates were used for each sample. Gene expression levels were determined by RT-qPCR using SYBR Green (Applied Biosystems, Foster City, CA) or FAM-MGB-labeled TaqMan probes (Thermo Fisher Scientific, Waltham, MA) in a Roche Light Cycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Gene expression was determined by ΔΔCP method. The obtained values were normalized to the cyclophilin A (PPIA) housekeeping gene.

3.8. Western blot

Frozen samples were heat treated in 2x Laemmli buffer (125 mM TRIS / HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) (5 min, 100 ° C) and sonicated with a Branson Sonifier 450 (Branson Ultrasonics Corp, Danbury, CT) (2 min, maximum 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% SDS-PAGEs and blotted onto PVDF membrane. After blotting, the membranes were blocked with 4% non-fat dried milk in TTBS (150 mM NaCl, 25 mM Tris / HCl pH 7.5, 0.01% Tween 20) (1 hour, room temperature). The membranes were probed by primary antibodies overnight at 4 °C followed by incubation with horseradish-peroxidase (HRP)-conjugated mouse and anti-rabbit IgG secondary antibodies (Bio-Rad) for one hour at room temperature. After the incubation, we washed the membranes three times with TTBS (for 20 minutes), and the proteins were examined on an X-ray film (AGFA, Mortsel, Belgium) using the Pierce-ECL technique based on chemiluminescence, Films were developed using an automated Kodak Medical X-ray Processor (Carestream Health, Rochester, N.Y.). Densitometry was carried out using the Image J open-source software (version 1.51k, National Institutes of Health, Bethesda, MD), and the expression of proteins was normalized to β-actin expression.

3.9. Investigation of mitochondrial membrane potential by laser scanning cytometry

Preadipocytes and differentiated beige cells were cultured in 8-well Ibidi plates (Ibidi GmbH, Planegg / Martinsried, Germany) pre-coated with collagen (Sigma-Aldrich, St. Louis, MO). Nuclei were labeled with 2 μg / ml DAPI, and 10 μM antimycin (Sigma-Aldrich, St. Louis, MO) was added to the cells as a control. MitoTracker Deep Red dye (Thermo Scientific, Waltham, MA) at a final concentration of 300 nM was used to examine mitochondrial membrane potential. The excitation wavelengths were 405 nm and 633 nm, and the resulting fluorescent signals were collected using a 40 × (NA 0.75) objective into 4 detection channels (blue, Long Red, 570Sp, and Open channels). For fluorescence imaging, cells were imaged using an iCys Research Imaging Cytometer (Thorlabs Imaging Systems, Sterling, VA).

3.10. Measurement of oxygen consumption and extracellular acidification rate

The oxygen consumption rate (OCR) and pH changes of the cells, the so-called extracellular acidification rate (ECAR), were examined using an XF96 oximeter (Seahorse Biosciences,

North Billerica, MA). Cells were seeded and differentiated in 96-well Seahorse assay plates. During the measurements, the basal oxygen consumption of the cells for 30 minutes was determined. Subsequently, 2 μM oligomycin (Enzo Life Sciences, Farmingdale, N.Y.) was added to the cells to block ATP synthase activity for testing proton leak respiration. Finally, 10 μM antimycin A was added to the cells (Sigma-Aldrich, St. Louis, MO), which was responsible for inhibiting mitochondrial membrane potential, thus determining the noise level of the measurement independent of mitochondrial oxidation. Oxygen consumption rate was normalized to the amount of protein in each well as measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions.

3.11. Measurement of NADH dehydrogenase activity and ATP and NADH content of cells

The 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) assay was performed to determine the NADH dehydrogenase activity of the cells. Cells were cultured in 12-well plates (Corning Incorporated, Durham, NY) followed by incubation at 37°C for 120 minutes after the addition of 0.5 mg/ml MTT to the confluent culture. The supernatant was aspirated and DMSO was used to dissolve the formazan crystals. Photometric measurements were performed using a Synergy Multimode Microplate Reader (BioTek Instruments, Inc., Winooski, VT) at 540 nm.

Relative ATP content was determined using the ATP Bioluminescence Assay Kit II (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Relative NADH content was determined with NAD / NADH quantification kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. ATP and NADH levels were normalized to protein content using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

3.12. Immunocytochemistry

For fluorescence microscopic measurements, cells were grown in 96-well plates. As a control, cells were treated with a 10 μM antimycin electron transport inhibitor, and mitochondria were labeled with 300 nM MitoTracker Deep Red (Thermo Scientific, Waltham, MA). Cells were then washed in PBS and fixed with 4% paraformaldehyde for 15 minutes and then permeabilized in 1% Triton X-100 PBS for 10 minutes at room temperature. Cells were blocked in 1% BSA-PBS for 1 hour, and then TexasRed-X Phalloidin (1: 150 dilution, Thermo Fisher, Waltham, MA) was used to stain actin fibers for 1 hour at 4 ° C. Cell nuclei were visualized by

DAPI (1:10 dilution, Thermo Fischer Scientific, Rockford, IL) staining followed by washing twice with PBS for 10 minutes. Adipocytes were then analyzed using Harmony 4.6 software (Perkin-Elmer, Waltham MA), part of the Opera Phenix High Content Screening system.

Images were taken from all three channels in 25 fields per well, resulting in a total of 2400 fields covered in the 96 well plates with a non-confocal fluorescent microscope. The raw images were then loaded into Ilastik (European Molecular Biology Laboratory, Heidelberg, Germany) for pixel classification and to segment the nuclei from the DAPI channel, the cytoplasms, and out-of-focus areas from the TexasRed Phalloidin channel. Cells were eligible for analysis only if they were not overlapping with an out-of-focus object. In the case of having an out-of-focus object inside the cell, all the previously segmented components of the cell - the related nucleus, cytoplasm, and mitochondrial classes - were discarded from the analysis. The resulting segmentation was exported to measure segmented objects in CellProfiler (Broad Institute, Cambridge, MA).

3.13. Global RNA- sequencing

To investigate global transcriptome data, high-throughput mRNA sequencing analysis was performed on the Illumina sequencing platform as described in the literature (Tóth et al. 2020). Genes related to mitochondrial function and the browning process were selected using BATLAS (http://green-l-12.ethz.ch:3838//BATLAS/) and PROFAT (http://ido.helmholtz-muenchen.de/profat/) tools.

3.14. Measurement of free triiodothyronine

The concentration of free triiodothyronine (fT3) in cell culture supernatants was measured in duplicate using Cobas ECLIA kit (Roche Diagnostics, Mannheim, Germany) with the Elecsys 2010 analyzer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations (Kazerouni et al. 2012).

3.15. Statistical analysis

Statistical significance was determined using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, Calofornia, USA) and Microsoft Excel 14.0. Statistical significance between two groups, treated and control, was determined by unpaired two-tailed Student's t test. To compare several groups, a one-way ANOVA was performed. Tukey's test was used for analysis of

variance. Results are expressed as mean ± SEM (mean sample error) or ± SD. Values of $p < 0.05$ were considered statistically significant with *, ** and *** corresponding to $p < 0.01$ and $p < 0.001$, respectively

4. RESULTS

4.1. $TG2^{-/-}$ mice have decreased tolerance to acute cold exposure and utilize a low amount of GONAT

Following the previous findings, the investigated $TG2^{+/+}$ and $TG2^{-/-}$ mice had similar body weights. They possessed the same amount of BAT, SCAT, and GONAT on a normal diet at 22 °C. However, we observed that the same amount of AT in mice could not support physiological functions similarly under stressful conditions induced by low temperature. The rectal body temperature of $TG2^{-/-}$ mice dropped to the ethically acceptable 28.8 ± 0.57 °C after 3 h spent at 4 °C. In contrast, $TG2^{+/+}$ animals could still maintain 31.8 ± 0.61 °C rectal body temperature and decreased from 38°C to about 30°C after 4 h of treatment. To explore how AT contributes to cold tolerance of $TG2^{-/-}$ mice, we isolated BAT, SCAT, and GONAT from animals after 3 h of acute cold exposure and compared their weights with tissues isolated from $TG2^{+/+}$ as well as untreated mice. While $TG2^{-/-}$ mice utilized their BAT and SCAT similarly to $TG2^{+/+}$ animals during the cold exposure, approximately half of the latter was lost after 3 hours of treatment. In contrast, $TG2^{-/-}$ animals lost significantly less of their gonadal adipose tissue than $TG2^{+/+}$ animals, for whom it has also been reduced by about half.

4.2. Browning of gonadal white adipose tissue is decreased in $TG2^{-/-}$ mice during exposure to cold

While SCAT browned similarly during 3 h cold exposure in the strains, GONAT of $TG2^{-/-}$ mice remained visibly lighter than the $TG2^{+/+}$ tissue after the treatment. Protein expression of UCP1 was markedly different in the GONAT of the two strains. It was detectable at 22°C in both strains with lower expression in $TG2^{-/-}$ GONAT compared to $TG2^{+/+}$. β-AR-deficient mice can enhance the thermogenic function of their white adipose tissue in response to cold exposure, suggesting the existence of other additional signaling pathways. Because high levels of noradrenaline were measured in serum samples from mice after cold treatment, we hypothesized that α1-AR activation may be such a mechanism. The possible relationship between α- and β-AR signaling has been previously described in the literature in other model systems in which TG2 as a possible G protein may play an important role. If this is the case,

activation of α1-AR should also reveal physiological differences between $TG2^{+/+}$ and $TG2^{-/-}$ mouse strains other than cold tolerance. Although the function of TG2 G-protein is known in many cell types (e.g., cardiac and smooth muscle cells, fibroblasts, endothelial cells, hepatocytes), its significance has not been extensively studied in vivo. To confirm the possible physiological role of TG2/Ghα, $TG2^{+/+}$ and $TG2^{-/-}$ mice were injected intraperitoneally with the specific α1-AR agonist phenylephrine and, as a control, with the specific β-AR agonist CL-316,243. Their various metabolic parameters (food and water intake, heat production, O_2 consumption, CO_2 production, respiration rate (RER)) were then examined by indirect calorimetry.

4.3. Respiratory exchange ratio was significantly lower in $TG2^{-/-}$ mice compared to $TG2^{+/+}$ animals after phenylephrine treatment

VO_2 values of the strains were the same both in the light and dark periods of the control day without any treatments. Phenylephrine induced a decrease in VO_2 levels after absorption in the case of both strains, which was more significant in $TG2^{-/-}$ animals. Nonetheless, $TG2^{+/+}$ animals set back the normal value within the light period after the treatment and recovered, but the VO_2 of $TG2^{-/-}$ mice remained lower. In contrast, CL treatment evoked a little increase of VO_2 of the strains, however, in a very similar way when they were compared to each other. We calculated the total VO_2 for the light and dark periods before and after the phenylephrine treatment and we found that the values of $TG2^{+/+}$ mice remained unchanged. Although the VO_2 values of $TG2^{-/-}$ animals were lower after the treatment both in the light and dark periods correlated to their corresponding control periods. There were no significant differences when we compared the values of $TG2^{-/-}$ mice to $TG2^{+/+}$ ones. Meanwhile, CL treatment did not cause any significant changes in the total VO_2 values of the strains. Similar to VO_2, the VCO_2 values of the strains were the same both in the light and dark control periods. Phenylephrine treatment caused a decline in VCO_2 levels of the strains equally, and then they started to increase gradually to the normal levels. However, the value of $TG2^{-/-}$mice remained a little lower after the treatment. Interestingly, the CL treatment did not affect the VCO_2 values of the strains, which remained the same. We also calculated the total VCO_2 for the light and dark periods before and after phenylephrine treatment and found that the values of $TG2^{+/+}$ mice remained unchanged. Though similarly to VO_2, the VCO_2 values of $TG2^{-/-}$ animals were lower both in the light and dark periods after phenylephrine treatment compared to their analogous control periods, there were no significant differences when we compared them to the values of $TG2^{+/+}$

mice. Like in the case of VO_2, the CL treatment did not cause any significant changes in the total VCO_2 values of the strains. RER values (VCO_2/VO_2) of $TG2^{-/-}$ and $TG2^{+/+}$ mice were very similar both in the light and the dark periods of the control day before phenylephrine treatment. After absorption of intraperitoneally injected 60 nM/g body weight phenylephrine, the RER values of both strains significantly decreased similarly in the first part of the light period on the treatment day demonstrating that the treatment affected. However, while the RER value of the $TG2^{+/+}$ strain started to increase constantly in the second part of the light period, and in the following dark period it reached the level detected before the treatment, RER remained still low in the case of $TG2^{-/-}$ mice. This resulted in a significantly lower RER value of $TG2^{-/-}$ animals compared to $TG2^{+/+}$ ones in the dark period on the measurement day. After absorption of intraperitoneally injected 60 nM/g body weight CL in the first part of the light period on the treatment day, the RER values of both strains promptly dropped down demonstrating the physiological effect of CL, and then started to increase equally in the second part of the light period. Consequently, while the RER values of both strains were significantly lower in the dark period on the measurement day compared to the dark period, there was no difference between the strains.

4.4. Phenylephrine treatment did not reduce tail blood pressure in $TG2^{-/-}$ mice

The tail blood pressure and heart rate of the animals were measured at the same time on the acclimatization day and the untreated control day. On the third day, mice were injected intraperitoneally with 60 nM / g phenylephrine. Comparing the tail blood pressure (TBP) and heart rate (HR) of $TG2^{+/+}$ and $TG2^{-/-}$ mice, we found that the values of both parameters were very similar on the control day before the treatment. Interestingly, at the first measurement time point of the experiment, which was 30 min after the injection of phenylephrine, we could not record both systolic and diastolic pressure of all the investigated $TG2^{+/+}$ mice in contrast to $TG2^{-/-}$ animals in which detection of TBP was successful.

Besides, while TBP values of the strains were similar in the light period, systolic pressure values and two of the three detected diastolic pressure values were significantly lower in $TG2^{+/+}$ animals compared to $TG2^{-/-}$ mice in the dark period. As a result, TBP values of $TG2^{-/-}$ mice remained unchanged after the phenylephrine treatment, while those of $TG2^{+/+}$ animals were lower in the dark period compared to the control day. Furthermore, we did not detect differences in HR values of the strains except the second measurement of the dark period after the treatment, when $TG2^{+/+}$ mice showed lower levels.

4.5. α1-adrenergic agonist causes lower tissue damage in $TG2^{-/-}$ mice

Serum samples of $TG2^{+/+}$ mice isolated 13 h after the phenylephrine treatment contained a significantly higher level of LDH compared to $TG2^{-/-}$ animals'. Electrophoretic separation and densitometry revealed that four out of the five isoenzymes were present in larger quantities in $TG2^{+/+}$ samples than in $TG2^{-/-}$ ones, namely LDH1, LDH2, LDH4, and LDH5. The KO/WT ratios of the isoenzymes LDH2, LDH4, and LDH5 were similar. However, the KO/WT ratio (the relative LDH activity) of LDH1, which may represent a cardiac-specific component, was lower. Because serum creatine kinase (CK-MB) activity increases significantly following myocardial injury, we examined its levels in samples of $TG2^{+/+}$ and $TG2^{-/-}$ mice. Significantly higher creatine kinase levels were measured in $TG2^{+/+}$ animals compared to $TG2^{-/-}$ animals, confirming that the α1-adrenergic agonist induces lower myocardial damage in $TG2^{-/-}$ mice than in the $TG2^{+/+}$ genotype.

4.6. Beige differentiation of preadipocytes isolated from $TG2^{-/-}$ and $TG2^{+/+}$ gonadal white adipose tissue was similar

To further investigate the lower cold tolerance of $TG2^{-/-}$ animals, stromal vascular fractions (SVF) were isolated from the GONAT of $TG2^{+/+}$ and $TG2^{-/-}$ mice and subjected to beige adipogenic cell differentiation. After the cells reached about 90% confluency, induction media were used for 2 days and then the preadipocytes were maintained in the differentiation media. In 14 days, cells accumulated multilocular lipid droplets and formed a characteristic phenotype of *in vitro* differentiated adipocytes from the fibroblast-like shape of preadipocytes. $TG2^{-/-}$ preadipocytes proliferate at the same rate as $TG2^{+/+}$ cells, and software analyses of microscopic images revealed the size of individual lipid droplets and the total fat content in $TG2^{-/-}$ beige adipocytes were the same as in $TG2^{+/+}$ cells. We assessed the rate of the effectiveness of the differentiation process by measuring the expression of marker genes in preadipocytes and beige cells. There was no significant difference in the expression of 'Preadipocyte factor 1' (Pref1), a preadipocyte marker, when comparing $TG2^{+/+}$ and $TG2^{-/-}$ preadipocytes, and its expression decreased equally with the progression of beige differentiation in both genotypes independent from adrenergic treatments. Beige markers Ucp1, Tbx1, Tnfrsf9, and Tmem26 significantly increased in both $TG2^{+/+}$ and $TG2^{-/-}$ cells after completing the differentiation process. Expression of Ucp1 and Tbx1 was significantly lower in the absence of TG2. Our results suggest that beige differentiation resulted in phenotypically similar cells in the presence and absence of TG2.

4.7. Protein expressions of UCP1 and mitochondrial protein complexes were lower in $TG2^{-/-}$ beige adipocytes independent from adrenergic treatments

To compare the mitochondria of $TG2^{+/+}$ and $TG2^{-/-}$ beige adipocytes, we examined the expression of UCP1 and the subunits of mitochondrial protein complexes. The expression of UCP1 at the protein level was significantly lower in $TG2^{-/-}$ beige adipocytes compared to $TG2^{+/+}$ cells. We found, that the levels of complex II-SDHB, III-UQCRC2, and V-ATPSA proteins were also lower in $TG2^{-/-}$ beige adipocytes compared to $TG2^{+/+}$ cells. These results raised the possibility that some of the expression differences between $TG2^{+/+}$ and $TG2^{-/-}$ beige cells existed already in the preadipocytes from which they started to differentiate. Therefore, we also examined the expression of UCP1 and mitochondrial protein complexes in preadipocytes. Surprisingly, UCP1 expression was also detected in preadipocytes, which was significantly lower in $TG2^{-/-}$ cells. The remarkable observation that preadipocytes may express a beige or brown differentiation marker is already known in the literature. We detected significantly lower expressions of UCP1 and III-UQCRC in $TG2^{-/-}$ preadipocytes while no significant difference was detected in the cases of II-SDHB and V-ATPSA compared to $TG2^{+/+}$ preadipocytes. We also examined the activity of mitochondrial dehydrogenases, which are indicators of the metabolic activity of functional mitochondria. Mitochondrial dehydrogenase activity was significantly lower in both preadipocytes and beige cells in the absence of TG2. Our results suggest that the function of mitochondrial electron transport in $TG2^{-/-}$ preadipocytes, and consequently in differentiated beige adipocytes, may be defective at several points. We examined the production of ATP and NADH in preadipocytes and beige adipocytes, and in both cases, we found significantly lower values in TG2-deficient phenotypes. Decreased ATP and NADH production suggest decreased energy production due to mitochondrial dysfunction.

4.8. $TG2^{-/-}$ preadipocytes and beige cells generate significantly lower membrane potential in mitochondria compared to $TG2^{+/+}$ cells

We carried out laser scanning cytometry (LSC) measurements to investigate the mitochondrial membrane potential in preadipocytes and beige cells. Mitochondria were stained with Mitotracker Deep Red and nuclei were stained with DAPI. Mitochondrial retention of Mito Tracker Deep Red depends upon mitochondrial membrane potential and can accordingly be higher in cells with increased OXPHOS. To demonstrate that only intact cells take up Mito Tracker dye, we added the electron transport inhibitor antimycin A as a control. We found that

Mito Tracker Deep Red fluorescence intensity was significantly lower in the $TG2^{-/-}$ preadipocytes and beige adipocytes compared to the $TG2^{+/+}$ cells. Mitochondria play an important role in regulating the redox state of cells.

When the mitochondrial electron transport chain (ETC) generates the electrochemical proton gradient which drives ATP synthesis, it also induces the production of reactive oxygen species (ROS). The major sources of mitochondrial ROS production are the ubiquinone sites in complexes I and III. It is important to note that we observed differences in protein expression of these complexes. Thus, we measured the endogenous and total ROS productions of cells. Both endogenous and total ROS productions were lower in $TG2^{-/-}$ mitochondria, suggesting decreased mitochondrial activity compared to $TG2^{+/+}$ cells.

4.9. $TG2^{-/-}$ preadipocytes and beige cells are metabolically hypometabolic compared to $TG2^{+/+}$

Oxygen consumption rate (OCR) is an indicator of the ETC activity, therefore, we measured the mitochondrial OCR to test the functional capacity of preadipocytes and differentiated beige cells. We observed significantly lower basal respiration and proton leak respiration in the $TG2^{-/-}$ preadipocytes and beige adipocytes compared to the $TG2^{+/+}$ cells. In parallel, we detected significantly lower extracellular acidification rate (ECAR) both in $TG2^{-/-}$ preadipocytes and beige adipocytes compared to $TG2^{+/+}$ controls. In addition, OCR/ECAR ratio at the basal conditions was also lower in the TG2 deficient cells compared to control preadipocytes and beige adipocytes indicating that $TG2^{-/-}$ cells preferred oxidative phosphorylation less than glycolysis to produce energy. Analyses of the cell energy phenotypes revealed that $TG2^{+/+}$ cells were characterized by a pronounced glycolytic and oxidative metabolism, as indicated by high ECAR and OCR compared to the $TG2^{-/-}$cells. Therefore, we could define $TG^{+/+}$ cells as hypermetabolic cells. In contrast, our results strongly suggested that cells lacking TG2 were hypometabolic.

4.10. $TG2^{-/-}$ beige adipocytes have more fragmented mitochondria compared to $TG2^{+/+}$

Based on our results, we raised the question of what type of mitochondrial morphology is expected in $TG2^{-/-}$ beige adipocytes compared to $TG2^{+/+}$ cells. Because ultrathin sections obtained during TEM are not always suitable for determining the complexity of the mitochondrial network and only allowed the examination of preadipocytes in our experiments, we investigated the morphology of mitochondria and the structure of the mitochondrial network by fluorescence microscopy. Tubular mitochondrial morphology was dominant both in

preadipocytes and beige cells. Although the ratio of fragmented/tubular mitochondria was not different in preadipocytes, we found significantly more fragmented mitochondria in $TG2^{-/-}$ beige adipocytes compared to the $TG2^{+/+}$ beige cells.

4.11. Examination of mitochondrial fission and fusion proteins confirms that in the absence of TG2, cells contain significantly more fragmented mitochondria

Fragmentation observed by fluorescence microscopy was further examined at the protein level by measuring the expression of proteins responsible for mitochondrial fusion and fission. We did not see significant differences in fusion (MFN2, OPA1) protein levels in either preadipocytes or differentiated beige cells between the genotypes. However, after examination of mitochondrial fission proteins (DRP1, MFF), we found that expression of mitochondrial fission factor (MFF) was significantly increased in $TG2^{-/-}$ beige adipocytes compared to wild-type cells. Our Western blot results confirm the fluorescence microscopic results that mitochondrial fusion processes are more dominant in preadipocytes and beige cells in both genotypes.

In the study of mitochondrial fission-fusion processes in preadipocytes, we observed no differences either in immunocytochemical studies with fluorescence microscopy or in protein levels. However, we found significantly more fragmented mitochondria in TG2-deficient beige adipocytes compared to $TG2^{+/+}$ cells, supported by significantly higher expression of MFF protein. Overall, we detected smaller, more fragmented mitochondria and elevated mitochondrial fission processes in $TG2^{-/-}$ beige adipocytes compared to $TG2^{+/+}$ cells. These results suggest that loss of TG2 may alter mitochondrial morphology and functionality.

To further study whether fragmentation affects mitochondrial biogenesis, we examined the expression of PGC1α, a major regulator of energy homeostasis, and mitochondrial biogenesis. We found that PGC1α expression was significantly lower in $TG2^{-/-}$ beige cells. It is known from the literature that there is a strong association between the activation of AMPK and the induction of PGC-1a in adipocytes. We observed a decrease in AMPK phosphorylation in $TG2^{-/-}$ preadipocytes and differentiated beige adipocytes compared to $TG2^{+/+}$ control cells.

4.12. Expression of thyroid hormone-degrading DIO3 is higher in $TG2^{-/-}$ cells

To explore possible explanations for the obtained results, RNA-seq experiments were performed comparing the total gene expression profiles of $TG2^{-/-}$ and $TG2^{+/+}$ cells. Our analysis

revealed a total of 165 differentially expressed mitochondrial and browning genes in beige adipocytes, including 123 up-regulated genes and 42 down-regulated genes.

Concerning the upregulated genes in the $TG2^{-/-}$ beige samples, gene ontology (GO: Reactome pathway) analysis showed that these genes were significantly enriched for mitochondrial biogenesis and especially the function of the thyroid hormone. Given that the regulation of thyroid hormone activity was one of the most significant Reactome pathways, we attempted to identify which differentially expressed genes are regulated by the thyroid hormone receptor α (THRA). From the resulting thyroid metabolism-related genes, we selected up-and down-regulated genes that could support our previous results on browning and mitochondrial function based on the literature. For up-regulated genes in $TG2^{-/-}$ beige samples, we validated the Bnip3 gene, which is involved in the positive regulation of mitochondrial fission, which we previously observed in $TG2^{-/-}$ beige adipocytes. The Cxcl1 gene, which is also associated with obesity, and the Dio3 gene, which is responsible for the inactivation of thyroid hormones, were also analyzed by quantitative real-time PCR. Trpv1 and Slc25a45 genes associated with mitochondrial function were selected for validation from down-regulated genes in $TG2^{-/-}$ beige cells. Experimental validation of RNA-seq results was successful for all genes, confirming the hypothesis that differentially expressed genes involved in thyroid metabolism may contribute to lower mitochondrial function in the absence of TG2. It is important to note that based on our results, the Dio3 gene, which reduces the effect of thyroid hormones that play an important role in energy metabolism, is expressed at significantly higher levels in $TG2^{-/-}$ beige adipocytes.

DIO3 catalyzes the inactivation of the thyroid hormone by inner ring deiodination both of the prohormone thyroxine (T4) forming inactive reverse triiodothyronine (rT3) and the bioactive hormone T3 producing inactive T2. 3,5,3′-Triiodo-L-thyronine, also known as T3, plays a crucial role in various processes related to development, differentiation, and metabolism. In addition, T3 is found in both nuclei and mitochondria, promoting mitochondrial biogenesis. The concentration of free T3 (fT3) in the cell supernatant of beige adipocytes was measured. It is important to note that T3 is used at 0.2 nM final concentration for beige differentiation. We found that fT3 concentration was significantly lower in the supernatant of $TG2^{-/-}$ beige cells compared to $TG2^{+/+}$ control samples, suggesting that higher-expressed DIO3 degrades some of it in this cell type.

This idea is also supported by our gene and protein expression results, where we found that DIO3 expression was significantly higher in $TG2^{-/-}$ beige adipocytes compared to $TG2^{+/+}$ control cells. The association between higher DIO3 expression and lower fT3 concentration

raises the possibility that this enzyme may contribute to lower mitochondrial functions of the TG2$^{-/-}$ beige genotype.

4.13. In the absence of TG2, the transport of fatty acids, amino acids, and coenzyme A into the mitochondrial matrix is reduced

Gene ontology analysis showed that the down-regulated genes in the TG2$^{-/-}$ beige samples were significantly enriched for the metabolism and transport of amino acids and derivatives and biological oxidation. From our RNA sequencing data, we identified those differentially expressed genes that are responsible for mitochondrial transport processes. We found mitochondrial 'solute carrier' (SLC) proteins transporting amino acids, and interestingly, these down-regulated genes also play a role in fatty acid metabolism through the transport of acylcarnitine and coenzyme A. The Slc25a45 transporter may be one of the most relevant, as we observed significantly lower expression in TG2$^{-/-}$ preadipocytes. This carrier plays an important role in the transport of acylcarnitine, ATP, and amino acids across the inner mitochondrial membrane to the mitochondrial matrix. It is important to note that this transporter is also included in genes involved in thyroid metabolism as a differentially expressed, down-regulated gene in TG2$^{-/-}$ beige cells. The relationship between Slc25a45 expression and thyroid metabolism has been described in the literature when a putative thyroid response element was found in the promoter region of the Slc25a45 gene. Validation of RNA sequencing data was performed by quantitative real-time PCR and Western blot analysis, which showed that both Slc25a45 gene and protein levels were significantly lower in TG2$^{-/-}$ beige adipocytes compared to wild-type control cells. An important orthologous gene of Slc25a45 is Slc25a47, which is also down-regulated in TG2$^{-/-}$ adipocytes, and lower expression was also detected in Slc25a42, which is responsible for the transport of coenzyme A and ADP to the mitochondria, and thus, plays an important role in energy production and utilization.

5. DISCUSSION

Mouse models resistant to weight gain through increased brown and beige fat activity have indicated that activation of thermogenesis can be a powerful strategy to improve metabolic health and prevent weight gain.

Alteration in UCP1 expression has pathological consequences: ablation of UCP1 positive cells in transgenic mice causes an increased propensity to obesity and diabetes, and conversely, UCP1 KO mice develop obesity under thermoneutral conditions when fed a high-fat diet.

Previous studies have shown up-regulation of TG2 in human brown adipose tissue compared to white adipose tissue, 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 and utilize a low amount of GONAT during cold treatment compared to $TG2^{+/+}$ animals.

According to our results, normal BAT and SCAT functions could support $TG2^{-/-}$ animals to a certain degree at unusually low temperatures; however, limited utilization of GONAT led to their increased sensitivity to cold. The browning process was also curbed in GONAT of $TG2^{-/-}$ mice during cold exposure, whereas no difference was observed in the other abundant adipose depot, the subcutaneous adipose tissue, in the absence of TG2.

The reduced fatty acid oxidation in $TG2^{-/-}$ GONAT, together with significantly elevated noradrenaline levels and possible association of signaling pathways, raises the possibility that TG2 acts as a G protein in this fat depot. To investigate this phenomenon, we first studied the physiological effects of α1 and β-AR agonists by measuring the metabolic parameters of mice. Phenylephrine is a sympathomimetic amine with a chemical structure similar to noradrenaline and therefore binds and activates selectively to α1-ARs. Adrenergic receptors have three subtypes, which are approximately 75% homologous: α1A, α1B, and α1D. Phenylephrine appears to act similarly on all three receptor subtypes. However, it is well-recognized that TG2 may act as a Ghα protein binding to α1B-ARs or α 1D-ARs, but not α 1A-ARs.

Comparing $TG2^{+/+}$ and $TG2^{-/-}$ mice, we found striking differences in RER (ratio of CO_2 production to O_2 consumption) values, especially in the dark period, a few hours after phenylephrine treatment. In the case of $TG2^{+/+}$ animals, RER dropped, then started to increase gradually from about 0.8 to the level close to 1, which was detected as a control value before injection of both strains. Meanwhile, the RER of $TG2^{-/-}$ mice did not start to increase, after dropping and remained low even in the dark period after the treatment.

The dark period is normally the active phase of mice, but the development of this deviation is unrelated to this as there was no difference in the physical activity of the animals, which decreased and remained significantly lower for both strains even in the dark period after the injection. It is generally accepted that RER indicates which type of fuel is being preferably metabolized. It ranges from 1.0 where pure glucose is oxidized to 0.7 where pure FFAs are oxidized. Accordingly, $TG2^{+/+}$ and $TG2^{-/-}$ mice gained energy mainly from the degradation of carbohydrates before the phenylephrine injection, but after the treatment, they immediately started to utilize FFAs in our experiments. Interestingly, however, $TG2^{-/-}$ animals continued to break down FFAs for a much longer time while $TG2^{+/+}$ mice started to utilize carbohydrates increasingly. Higher lactate levels in the plasma of $TG2^{+/+}$ mice probably indicated that they utilized more glucose than $TG2^{-/-}$ animals. The observed approximately 0.1 difference in the RER values after the phenylephrine treatment on a maximum 0.3 scale (from 0.7 to 1.0) reflects a quite different metabolism of the whole organism kept on the same diet and is a surprising finding worth investigating further. These results indicate that TG2 functioning as a Ghα protein in adrenergic receptor signaling may play a role in determining the balance between carbohydrate and fatty acid oxidation. We applied CL, a specific β3-AR agonist as a control for the α1-AR agonist phenylephrine. At this time, the metabolic parameters of the strains were very similar, suggesting that TG2 has no direct role in β3-AR-regulated signaling.

Phenylephrine is a potent vasoconstrictor that increases cardiac preload without any important direct effect on cardiac myocytes. It can increase blood pressure keeping a slow heart rate through stimulation of vascular baroreceptors. Thus, phenylephrine is generally administered for patients with normal cardiac function but with clinically significant hypotension caused by vasodilation. Phenylephrine can cause pronounced vascular adverse effects, including increases in both systolic and diastolic BP, therefore we measured TBP of the mice. We found that both systolic and diastolic BP of $TG2^{-/-}$ animals were similar to $TG2^{+/+}$ values on the control day before the treatment. It is important to note that phenylephrine is known to cause contraction of tail caudal arteries making impossible the detection of TBP until the compound is degraded. Indeed, we could not detect TBP of $TG2^{+/+}$ mice at the first time point 30 min after the phenylephrine injection. However, TBP could be measured in all investigated $TG2^{-/-}$ animals, and their TBP values were comparable with those found on the control day suggesting absence or just a low level of contraction in their caudal arteries at the first time point of the measurement. Because of its vasoconstrictive effect, phenylephrine can cause severe necrosis in tissues. LDH is a soluble cytoplasmic enzyme that is present in almost all cells and released into extracellular space when the plasma membrane is damaged during necrosis. Quantifying

the LDH activity in the serum samples indicates the degree of tissue damages, therefore, we measured the LDH activity in serum samples of the strains to compare the level of necrosis caused by phenylephrine treatment. Total LDH activity was pathologically high in both strains compared to normal values 13 h after the injection, but it was significantly higher in $TG2^{+/+}$ samples compared to $TG2^{-/-}$ sera. The tetrameric LDH is composed of two types of subunits, the LDH-M, and the LDH-H proteins. These two subunits can form five possible isoenzymes (LDH1-5) that are enzymatically similar but show different tissue distribution. Consequently, the pathological appearance of different isoenzymes in serum can indicate in which tissue or organ the damage occurred. Electrophoretic separation and quantitation of LDH isoenzymes revealed that phenylephrine treatment caused damages in several organs, but these were higher in $TG2^{+/+}$ mice. The level of LDH1 characteristic for damage in cardiac muscle and kidney, LDH2 for kidney, LDH4 for brain and lung, and LDH5 for liver and skeletal muscle were higher in $TG2^{+/+}$ mice-derived samples compared to $TG2^{-/-}$ ones, respectively. When we calculated the relative LDH isoenzyme activity values in serum samples of mice, we found that compared to the other isoenzymes, the elevation of LDH1 level was the most prominent in $TG2^{+/+}$ samples suggesting that the highest difference in organ damages is in the heart. To confirm that the heart was less damaged in $TG2^{-/-}$ animals, we also checked CK-MB activity in sera which is a much more specific marker for the heart. We found that it was also significantly lower in $TG2^{-/-}$ animals compared to $TG2^{+/+}$ mice. Although our data indicate that the heart was damaged more seriously in $TG2^{+/+}$ animals after phenylephrine treatment, it is important to note that there was no difference in the HR values of the mice, except at a single time point of the experiments in the dark period, which suggest that heart damages did not cause functional disorders in the investigated period. Overall, it should be emphasized, that the role of TG2 as a G protein in the maintenance of normal cardiovascular function has not been clarified yet. Our results raise the possibility that TG2 may function as a G protein in vascular smooth muscle cells, but we have not demonstrated its similar function in adipose tissue.

Recently, we have identified TG2 as a regulator in the browning process of gonadal white adipose tissue of mice in a tissue-specific manner. Based on our cold-tolerance results, it has been suggested that regulatory processes related to TG2 function may be among the potential targets for pharmacological interventions in obesity and metabolic disorders.

Previous data in the literature have reported that TG2 regulates adipocyte differentiation, and thus, may also affect beige cell formation as well. Several studies have described the role of TG2 in mitochondrial homeostasis. Besides its role in stabilizing the mitochondrial respiratory chain, it can modulate the transcription of critical genes, including PGC-1α and cytochrome C,

which play important role in the function of mitochondria and biogenesis. There is further evidence that TG2 may play a role in the metabolism of some cells, such as in the respiratory functions of mitochondria in myocardial cells, as $TG2^{-/-}$ mice showed a severe defect in ATP production.

Together with these findings and the evidence linking TG2 to mitochondrial homeostasis, we decided to investigate the role of TG2 in the adrenergic response of beige adipocytes of gonadal white adipose tissue focusing on the effect of the enzyme in the role of adipocytes metabolism. For this purpose, we isolated preadipocytes from the GONAT of $TG2^{-/-}$ and $TG2^{+/+}$ mice and differentiated them, and then performed comparative studies.

Based on our results the proliferation capacity of $TG2^{+/+}$ and $TG2^{-/-}$ preadipocytes were the same. In addition, differentiated beige adipocytes contained the same amount of lipid with similar droplet sizes. There were no differences in the expression profile of beige marker genes between the two genotypes, except the TBX1, which was lower in the $TG2^{-/-}$ differentiated cells. TBX1 is a transcription factor, but its exact role in beige cells is still unknown. We also treated the obtained cells by adding different adrenergic drugs, and interestingly, those that are β-AR pathway activators increased the UCP1 expression only in $TG2^{+/+}$ cells, but not in $TG2^{-/-}$ ones. However, the adrenergic drugs did not have any significant effects on other beige marker genes. These results revealed the $TG2^{+/+}$ and $TG2^{-/-}$ preadipocytes were able to differentiate to beige direction similarly, but their UCP1 expression was not reactive to β-AR stimulation.

Mitochondria are crucial in the generation of heat *via* the inner membrane protein UCP1 which uncouples respiration from ATP synthesis, and therefore, provokes energy dissipation in the form of heat while also stimulating high levels of fatty acid oxidation. The notable observation that a preadipocyte expresses a terminal beige differentiation marker like UCP1 is already known from the literature. The protein expression of UCP1 was lower in $TG2^{-/-}$ beige cells and preadipocytes suggesting that they have significantly lower heat-producing capacity compared to $TG2^{+/+}$. Indeed, proton leak respiration which reflects UCP1-dependent heat production was significantly reduced in $TG2^{-/-}$ beige cells as well as preadipocytes.

Reduced energy production capacity was confirmed by detecting significantly lower NADH and ATP levels both in $TG2^{-/-}$ preadipocytes and beige cells. We also demonstrated that the lack of TG2 led to lower mitochondrial membrane potential in preadipocytes and beige cells. Interestingly, while the altered structure and function of ETC could be expected to increase the ROS levels, we found the opposite, a significant decrease of ROS production in the $TG2^{-/-}$ cells suggesting lower mitochondrial metabolism.

It has been shown in other cell types that TG2 is implicated in maintaining the homeostasis of ETC and energy production. Deletion of TG2 in mice caused significant deregulation of the respiratory complexes I and II and a reduction of ATP production in mouse embryonic fibroblasts (MEFs). Previously it was shown that PDI activity of TG2 is important for the formation of disulfide bridges in the ATP synthase complex and other key components of the ETC. However, our results on lower NADH and ROS levels suggest that reduced mitochondrial membrane potential and ATP production are also due to low levels of available fuels in $TG2^{-/-}$ mitochondria. Indeed, analysis of the cell energy phenotypes revealed that $TG2^{+/+}$ preadipocytes and beige cells were characterized by pronounced oxidative and glycolytic metabolism indicated by high OCR levels and ECAR compared with the corresponding $TG2^{-/-}$ cells and therefore, were defined as the "energetic" cells. In contrast, our results strongly suggest that preadipocytes and beige cells are hypometabolic in the lack of TG2.

Mitochondria continuously fuse and divide through the processes known as fusion and fission forming dynamic networks. The metabolism of mitochondria depends on the balance of fusion and fission processes. Fusion leads to the formation of tubular, while fission yields fragmented structures of mitochondria. Mitochondrial fragmentation is primarily required to isolate and eliminate dysfunctional mitochondria. It is known from the literature that fragmentation of mitochondria has been observed in the presence of TG2 in mouse embryonic fibroblast cells. Our HCS results showed that the tubular mitochondrial morphology is the most characteristic of both preadipocytes and beige cells, however, there are significantly higher fractions of fragmented mitochondria in the $TG2^{-/-}$ beige cells compared to $TG2^{+/+}$ controls. We were able to demonstrate an important molecular factor behind this phenomenon, as we detected significantly higher expression of MFF in $TG2^{-/-}$ beige cells. MFF is an outer mitochondrial membrane protein that forms a complex with the GTPase DRP1 and promotes mitochondrial fragmentation. Mitochondrial fragmentation is known to associate with depolarization, significantly decreased capacity of respiration, and reduced ATP production. Interestingly, altered mitochondrial morphology and functionality have also been shown in $TG2^{-/-}$ MEFs containing more fragmented and depolarized mitochondria.

To learn more about the possible explanations of the obtained results, we have carried out RNA-seq experiments comparing gene expression profiles of $TG2^{+/+}$ and $TG2^{-/-}$ beige cells. A similar investigation was performed in MEFs demonstrating that most of the differentially expressed genes were enriched in clusters related to cytoskeleton, actin regulation, and extracellular matrix regulation.

In our study, we found differential expression of thyroid hormone-regulated pathways and identified up-regulation of DIO3 in $TG2^{-/-}$ beige cells, which inactivates T3 added to the differentiation media. We found that the free bioactive (fT3) concentration was significantly lower in the differentiation media of $TG2^{-/-}$ beige cells compared to $TG2^{+/+}$ controls in line with the higher expression of DIO3 in this cell type. T3 plays a crucial role in the activation of the browning process and differentiation of beige adipocytes, and it is a standard component of the differentiation medium. It is important to note that we did not add T4 to the culture media. The association of higher DIO3 expression with lower fT3 concentration raises the possibility that this enzyme may contribute to the lower mitochondrial functions of the $TG2^{-/-}$ beige genotype.

We also identified 3 down-regulated mitochondrial solute transporters. The most important is the SLC25A45 transporting acylcarnitine, ATP, and amino acids across the inner mitochondrial membrane into the mitochondrial matrix for further processes. The expression of SLC25A45 was significantly lower both in $TG2^{-/-}$ preadipocytes and beige cells which probably has an essential role in the development of the hypometabolic phenotype of these cells. Importantly, the expression of SLC25A45 can be activated by T3 as a putative thyroid response element was found in the promoter region of its gene, that is DIO3 mediated T3 inactivation can contribute to its observed down-regulation. An important paralog of SLC25A45 is SLC25A47, which was also found down-regulated in $TG2^{-/-}$ beige adipocytes. Furthermore, we also demonstrated lower expression of SLC25A42 in TG2-deficient cells that is responsible for the transport of CoA and ADP into mitochondria, which may also contribute to their hypometabolic characterization. The importance of our results is further enhanced by the fact that SLC25A42 has been described as a browning marker contributing to thermogenesis in mice.

Our data suggest that TG2 plays a critical role in controlling the development and thermogenic capacity of beige adipocytes. The differences between $TG2^{+/+}$ and $TG2^{-/-}$ cells cannot be explained by different responses to adrenergic agonists, as they already existed in preadipocytes. Lack of TG2 in preadipocytes results in impaired mitochondrial functions that cannot be compensated by decreased thyroid sensitivity during the differentiation process. The result is a beige cell in the GONAT in which the mitochondria cannot reach the threshold required for normal thermogenic functions. We hypothesize that this may play a key role in the development of the reduced cold tolerance shown by $TG2^{-/-}$ mice. Clarification of the role of TG2 in the development of mitochondrial dysfunction in beige adipocytes may offer potential opportunities in the development of interventions to prevent and treat obesity and other metabolic disorders.

6. SUMMARY

In our work, we have studied the role of TG2 in thermogenesis in a mouse model. We have shown that tissue $TG2^{-/-}$ mice have reduced cold tolerance and are less efficient in mobilizing their gonadal fat after cold exposure compared to their $TG2^{+/+}$ littermates. The lower mobilization of the gonadal adipose tissue was most likely caused by the inhibited browning process of the tissue in $TG2^{-/-}$ mice with lower expression of browning markers, including UCP1.

We have investigated the role of TG2 in the possible interaction of α- and β-adrenergic signaling pathways by treatment of adrenergic agonists using indirect calorimetry. Phenylephrine, the α1 adrenergic agonist, has induced a significantly lower respiration rate (RER) in $TG2^{-/-}$ animals compared to $TG2^{+/+}$ mice, suggesting significantly different metabolic processes in them. Our results suggest that TG2, which may act as a G protein in the adrenergic receptor signaling pathway, might play a role in determining the balance between carbohydrate and fatty acid oxidation. We have shown that the vasoconstrictive effect of phenylephrine is weaker in $TG2^{-/-}$ animals, which in turn induced lower levels of tissue necrosis.

A further aim of our research program was to compare the biochemical mechanisms by which TG2 is involved in mitochondrial function and thus, in the activation of thermogenesis by comparing $TG2^{+/+}$ and $TG2^{-/-}$ preadipocytes and beige cells isolated from gonadal adipose tissue. We have demonstrated that beige differentiation of preadipocytes was successful for both genotypes. We have found that the expression of subunits of some mitochondrial protein complexes and UCP1is significantly lower in $TG2^{-/-}$ preadipocytes and beige adipocytes compared to $TG2^{+/+}$ cells, suggesting that the function of mitochondrial electron transport is defective at several points in $TG2^{-/-}$ preadipocytes and differentiated beige cells. In our work, we have demonstrated that TG2 deficiency leads to lower mitochondrial membrane potential and lower ATP and NADH content, in parallel with lower oxygen consumption compared to $TG2^{+/+}$ control cells. In addition, ROS production is lower in $TG2^{-/-}$ cells, suggesting lower activity of their mitochondria. By examining the energetic phenotypes of the cells, we have shown that $TG2^{+/+}$ preadipocytes and beige cells are strongly characterized by glycolytic and oxidative metabolism, as indicated by high extracellular acidification (ECAR) and oxygen consumption (OCR) compared to $TG2^{-/-}$ cells, and therefore we can define them as energetic cells. In contrast, our results strongly suggest that cells are hypometabolic in the absence of TG2.

Based on our immunocytochemical and protein-level studies of mitochondrial fission and fusion proteins, we have described that tubular mitochondrial morphology is more characteristic of both cell types; however, the fractions of fragmented mitochondria in $TG2^{-/-}$ beige adipocytes are significantly higher than in $TG2^{+/+}$ cells. We have also found that the expression of the well-known coactivator PGC1α, which regulates mitochondrial biogenesis, and with it the phosphorylated AMP-activated protein kinase protein, was significantly lower in $TG2^{-/-}$ beige cells. Comparative global transcriptome analysis of preadipocytes and differentiated beige cells has identified potential genes to explain mitochondrial differences caused by TG2 deficiency. We have demonstrated that the DIO3 protein may play a key role in the decreased mitochondrial function of $TG2^{-/-}$ cells. In addition, it has been described that in the absence of TG2, the transport of fatty acids, amino acids, and coenzyme A into the mitochondrial matrix is less efficient, which may contribute to lower energy production by mitochondria. Based on our results, TG2 plays an essential role in the function of beige cells of gonadal origin and the process of thermogenesis. The importance of our work is further enhanced by the fact that similar systematic studies have not been performed on $TG2^{-/-}$ cell types yet. The data we presented supplemented the list of possible targets for obesity and related metabolic disorders with TG2-related regulatory processes.

7. PUBLICATIONS

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/512/2021.PL
Subject: PhD Publication List

Candidate: Kinga Fedor-Lénárt
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Lénárt, K.**, Pap, A., Pórszász, R., Oláh, A., Fésüs, L., Mádi, A.: Transglutaminase 2 Has Metabolic and Vascular Regulatory Functions Revealed by In Vivo Activation of Alpha1-Adrenergic Receptor.
   Int. J. Mol. Sci. 21 (11), 1-18, 2020.
   DOI: http://dx.doi.org/10.3390/ijms21113865
   IF: 5.923

2. Mádi, A., Cuaranta-Monroy, I., **Lénárt, K.**, Pap, A., Mezei, Z. A., Kristóf, E., Oláh, A., Vámosi, G., Bacsó, Z., Bai, P., Fésüs, L.: Browning deficiency and low mobilization of fatty acids in gonadal white adipose tissue leads to decreased cold-tolerance of transglutaminase 2 knock-out mice.
   Biochim. Biophys. Acta. Mol. Cell Biol. Lipids. 1862 (12), 1575-1586, 2017.
   DOI: http://dx.doi.org/10.1016/j.bbalip.2017.07.014
   IF: 4.966

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

3. Sebestyén, F., Póliska, S., Rácz, R., Bereczki, J., **Lénárt, K.**, Barta, Z., Lendvai, Á. Z., Tökölyi, J.: Insulin/IGF Signaling and Life History Traits in Response to Food Availability and Perceived Density in the Cnidarian Hydra vulgaris.
Zool. Sci. 34 (4), 318-325, 2017.
DOI: http://dx.doi.org/10.2108/zs160171
IF: 0.906

4. Tökölyi, J., Bradács, F., Hóka, N., Kozma, N., Máté, M., Mucza, O., **Lénárt, K.**, Ősz, Z., Sebestyén, F., Barta, Z.: Effects of food availability on asexual reproduction and stress tolerance along the fast-slow life history continuum in freshwater hydra (Cnidaria: Hydrozoa).
Hydrobiologia. 766 (1), 121-133, 2015.
DOI: http://dx.doi.org/10.1007/s10750-015-2449-0
IF: 2.051

Total IF of journals (all publications): 13,846
Total IF of journals (publications related to the dissertation): 10,889

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

26 November, 2021