14-3-3ζ over-expression improves tolerance to acute and chronic cold exposure in male mice via thermogenic-dependent and -independent mechanisms

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Abstract

Adaptive thermogenesis, which is partly mediated by sympathetic input on brown adipose tissue (BAT), is a mechanism of heat production in response to prolonged cold that relies upon the actions of Uncoupling Protein-1 (UCP1). Moreover, various stimuli, including norepinephrine and FGF-21, promote the conversion of inguinal white adipocytes to beige adipocytes, which represent a secondary cell type that can contribute to adaptive thermogenesis. Although we previously identified an essential role of the molecular scaffold 14-3-3ζ in adipogenesis, one of the earliest, identified functions of 14-3-3ζ is its regulatory effects on the activity of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of norepinephrine. This suggests that 14-3-3ζ could influence adaptive thermogenesis via actions on BAT activation or the beiging of white adipocytes. Herein, we report that transgenic over-expression of 14-3-3ζ (TAP) in male mice significantly improved tolerance to acute (3 hrs) and chronic (3 days) cold (4 ^{o}C) exposure. In response to cold,increased UCP1 expression in beige inguinal white tissue (iWAT) and BAT was observed in TAP mice following chronic cold exposure, in addition to significantly elevated body temperature. Of note was the paradoxical finding that cold-induced increases in body temperature were associated with significantly decreased energy expenditure in TAP mice. The marked improvements in cold tolerance were not due to changes in sensitivity to β-adrenergic stimulation; instead over-expression of 14-3-3ζ significantly decreased thermal conductance and heat loss in mice via increased peripheral vasoconstriction. Despite displaying elevations in cold-induced UCP1 expression in brown or beige adipocytes, these findings suggest alternative mechanisms that can be activated to mitigate heat loss during chronic cold exposure. Collectively, our results point to novel roles of 14-3-3ζ in regulating non-thermogenic mechanisms that regulate body temperature.

Introduction

Homeothermy is the maintenance of a stable, internal body temperature following changes in environmental temperature, and it is essential to the survival of endotherms [1]. To defend against hypothermia, mammals have evolved various thermogenic mechanisms, ranging from skeletal muscle shivering to non-shivering, adaptive thermogenesis [1-5]. Although short-lived, shivering is the fast contraction of skeletal muscles during which heat is released via the hydrolysis of ATP [6]. Another early mechanism to regulate body temperature is vasoconstriction, which helps to mitigate heat loss [7], but its duration and relative contribution to homeothermy, especially in the context of adaptive thermogenesis, is often underappreciated.

The sympathetic nervous system (SNS) is the primary regulator of adaptive thermogenesis, as the release of norepinephrine from sympathetic efferent nerves activates β3-adrenergic receptors on the surface of brown adipocytes to trigger heat production [8]. Long-term cold exposure can also induce the recruitment of brown-like adipocytes in inguinal white adipose tissue (iWAT) through a process known as beiging [9, 10]. Brown and beige adipocytes are distinct thermogenic fat cells rich in uncoupling protein-1 (UCP1), which uncouples the proton gradient from ATP synthesis to produce heat, during fatty acid oxidation [8, 9, 11]. Although the contributions of BAT to thermogenesis have been well-established, the relative contributions of beige adipocytes to thermogenesis are unclear [12], but in the context of severe adrenergic stress, such as severe burns, detectable increases in beige adipocyte thermogenic activity have been reported [13]. It has been postulated that both beige and brown adipocytes may have therapeutic potential in the treatment of obesity and diabetes [9, 14], but the mechanisms underlying brown and beige adipocytes development and function are still not completely understood.

14-3-3ζ is a member of the 14-3-3 scaffold protein family, which are highly conserved serine and threonine binding proteins present in all eukaryotes [15-18]. They bind to a diverse number of enzymes, signalling proteins, and transcription factors and have been implicated in the regulation of numerous cellular processes, including proliferation, protein trafficking, and apoptosis [16, 17]. Additionally, binding of 14-3-3 proteins to enzymes and kinases can influence their function. For example, interactions between 14-3-3 proteins and RAF-1 or PKA potentiate their kinase activity [19, 20] ; in contrast, interactions of DYRK1A with 14-3-3 proteins can attenuate kinase function [21].

Recently, we reported the contributions of 14-3-3ζ in whole-body metabolism, as it was found to be an important regulator of glucose metabolism and adipogenesis [22-24]. Interestingly, one of the first ascribed functions of 14-3-3 proteins is their regulation of tyrosine (TH) and tryptophan hydroxylase (TPH), both of which are rate limiting enzymes involved in the synthesis of norepinephrine and serotonin, respectively [25]. Both norepinephrine and serotonin have positive and negative effects, respectively, adaptive thermogenesis and beiging [1, 26-28]. Therefore, given the ability of 14-3-3ζ to regulate the activities of TH and TPH, we hypothesized that 14-3-3ζ may have critical roles in the development and function of beige and brown adipocytes, thereby influencing adaptive thermogenesis.

In present study, we report on the outcome of reducing or increasing 14-3-3ζ expression in the context of tolerance to acute and chronic cold stress. Despite increased body temperature in response to chronic cold, 14-3-3ζ over-expression was associated with a paradoxical decrease in energy expenditure. This phenomenon was associated with decreased thermal conductance, or heat loss, as a result of elevated vasoconstriction in 14-3-3ζ transgenic mice. These effects were not due to changes in the sensitivity of transgenic mice to β-adrenergic stimuli or sympathetic activity. Thus, our data demonstrate that 14-3-3ζ over-expression improves cold tolerance in mice via thermogenic-dependent and -independent mechanisms. Results from this study also raise the need to consider the importance of non-thermogenic mechanisms in the context of cold tolerance or adaptation.

Material and Methods

Animal studies

Wildtype (WT) and 14-3-3ζ heterozygous (HET) mice were on a C57Bl/6J background, whereas transgenic mice over-expressing a TAP-tagged human 14-3-3ζ (TAP) molecule were on a CD-1 background [24, 29]. Mice were housed in a temperature-controlled (22°C) animal facility on a 12-hour light/dark cycle in the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Mice had *ad libitum* access to water and standard chow (TD2918, Envigo, Huntingdon, United Kingdom), and all animal studies were performed in accordance to the Comité Institutionnel de Protection des Animaux (CIPA) of the CRCHUM. For acute cold challenges, 12 week-old mice were individually caged and fasted for 4 hours prior to and during a 3-hour challenge at 4°C with *ad libitum* access to water. Body temperature

was measured with a physio-suit rectal probe (Kent scientific, Torrington, CT, USA). For chronic cold challenges, mice were housed in Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments Columbus, OH, USA) metabolic cages or a cold room for 3 days at 4°C. The β3 adrenergic agonist CL316,243 (Sigma Aldrich, St Louis, MO, USA) was diluted in saline 0.9%, and mice received daily intraperitoneal injections of either saline 0.9% or CL316,243 (1mg/kg) for 7 days. Body composition (lean and fat mass) was determined on Day 1 and Day 7 using EchoMRI (EchoMRI™, Houston, TX, USA).

Cell culture

The immortalized UCP1-luciferase (UCP1-Luc) adipocyte cell line was kindly provided by Dr. Shingo Kajimura (Diabetes Center, University of California- San Francisco) [30]. Cells were grown in 25 mM glucose DMEM media (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum FBS (Thermo Fisher Scientific) and 1% streptomycin (Thermo Fisher Scientific), and grown at 37°C, 5% CO_2. UCP1-Luc cells were induced to differentiation into brown adipocytes with a cocktail containing 5µg/ml insulin (Sigma Aldrich), 0.5mM 3-Isobutyl-1-methylxanthine IBMX (Sigma Aldrich), 1µM dexamethasone (Sigma Aldrich), 0.125mM indomethacin (Sigma Aldrich), and 1nM 3,3′,5-Triiodo-L-thyronine T3 (Sigma Aldrich) for 2 days, followed by maintenance medium containing 5µg/ml insulin and 1nM T3 on day 3, and DMEM/FBS growth medium on day 5. Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA,USA), and Silencer Select siRNA against *Ywhaz* (gene encoding 14-3-3ζ) and a scrambled, control siRNA (Ambion, Austin, TX, USA) were used to knockdown 14-3-3ζ, as previously described [24].

Immunoblotting

Inguinal (iWAT) and gonadal (gWAT) white adipose tissues and interscapular brown adipose tissue (BAT) were homogenized in RIPA lysis buffer (50 mM β glycerol phosphate, 10mM Hepes, pH=7.4, 70 mM NaCl, 1% Triton X-100, 2mM EGTA, 1mM Na_3VO_4, 1mM NaF), supplemented with protease and phosphatase inhibitors. Lysates were centrifuged at 13000 rpm for 15 minutes at 4°C, the supernatant was collected, and protein concentration was determined using Bio-Rad protein assay dye Reagent (Bio-Rad, Hercules, CA, USA). Protein samples were resolved by SDS-PAGE, transferred to PVDF membranes and blocked with I-block (Applied Bio-systems, Foster city, CA, USA) for 1 hour at room temperature, followed

by overnight incubation at 4 °C with primary antibodies against UCP1 (1:1000, R&D systems, Minneapolis, MN, USA), 14-3-3ζ (1:1000 Cell Signaling, Danvers, MA, USA), β-Actin (1:10000, Cell Signaling), β-Tubulin (1:1000, Cell Signaling) and Tyrosine hydroxylase (1:1000, Millipore, Bilerica, MA, USA). On the next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Cell Signaling) for 1 hour at room temperature. Immunoreactivity was detected by chemiluminescence with a ChemiDoc system (Bio-Rad). Information for each antibody can be seen in Supplemental Table 1.

Histology and Immunofluorescence

IWAT, gWAT, and BAT were excised and fixed in 4% PFA (Sigma Aldrich) for 7 days and stored in 70% ethanol prior to embedding in paraffin. Sections at 5 µm thickness were deparaffinized, re-hydrated and stained with Hematoxylin (Sigma Aldrich) and Eosin (Sigma Aldrich). Alternatively, slides were stained with a UCP1 antibody (1:250, Abcam, Cambridge, United Kingdom), followed by a HRP-conjugated secondary antibody for DAB labeling (Cell signaling). Images were taken at 20X (Nikon Eclipse Ti2, Nikon Instruments Inc, Melville, NY, USA).

For immunofluorescence, sections were stained for Perilipin (1:400, Cell Signaling). Antigen retrieval was performed with 10 mM Sodium Citrate buffer (Sigma Aldrich) at pH=6-6.2 for 15 min at 95°C. Sections were blocked 1 hour at room temperature with PBS-T (0.1% Triton, 5% normal donkey serum) and incubated overnight in PBS-T at 4°C with primary antibodies. Alexa Fluor 594-conjugated secondary antibodies (Jackson Immuno-research laboratories, Inc, West grove, PA, USA) were incubated for 1 hour at room temperature, and slides were mounted in Vectashield containing DAPI (Vector laboratories, Burlingame, CA, USA). Total adipocyte number and area was counted from 8-10 images per mouse, then measured using the Cell Profiler software (CellProfiler Analyst, Stable (2.2.1) [31]. All immunofluorescence pictures were acquired with an EVOS FL microscope (Thermo Fisher Scientific).

RNA isolation and quantitative PCR

Total RNA was isolated from cells and tissues using the RNeasy Mini kit or the RNeasy Plus Mini Kit (Qiagen, Montreal, Quebec, Canada respectively, and stored at -80°C. Reverse transcription was

performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or the Superscript VILO Kit (Invitrogen), in accordance with manufacturer's instructions. Gene expression was analysed by quantitative PCR (qPCR) with SYBR Green chemistry (PowerUp SYBR, ThermoFisher Scientific) on a QuantStudio 6 Real Time PCR machine (Applied Bio Systems, Life Technologies, Carlsbad, CA, USA). Relative gene expression was normalized to the reference gene, *Hprt,* as previously described [18, 24]. For a complete list of primers and their respective sequences, please see Supplemental Table 2.

Metabolic phenotyping

Male WT and TAP mice at 16 weeks of age received abdominal surgery to implant a temperature probe 10 days prior to their placement in CLAMS. Body weight and composition were measured before and after chronic cold exposure using EchoMRI on living, non-anesthetized mice. Mice were singly housed in CLAMS cages with *ad libitum* access to water and normal chow diet and were maintained on a 12-hour light/dark cycle on the following schedule: 24 hours at 22^{o}C for acclimatization, 24 hours at 22^{o}C for basal measurements, and 72 hours at 4^{o}C for the chronic cold challenge. Food intake, respiratory exchange ratio, locomotor activity (beam breaks), energy expenditure (heat), core body temperature (^{o}C) were measured in real-time every 15-20 mins. Following chronic cold exposure, blood and tissues were collected and snap frozen for subsequent use. Thermal conductance was calculated, as previously described [32].

Cohorts of mice were administered intraperitoneal glucose tolerance tests (IPGTTs) after 6 hours of fasting. IPGTTs were performed at 22^{o}C and after 3 days of 4^{o}C exposure. Mice were injected with glucose (2 mg/kg), and blood glucose was measured with a Contour Next glucose meter (Ascensia Diabetes Care, Basel, Switzerland)

Circulating free fatty acids were measured from plasma samples using the Wako NEFA-HR (2) assay kit (Wako Pure chemical Industries LTD, Osaka, Japan) and circulating glycerol was measured from plasma samples using the triglyceride and free glycerol reagents (Sigma Aldrich) as per to manufacturers' instructions. Circulating leptin (ALPCO, Salem, NH, USA) was measured from plasma samples, in accordance to manufacturers' protocols. Norepinephrine (Rocky Mountain Diagnostics, Colorado Springs, CO, USA) was measured from iWAT and BAT tissue extracts following manufacturers' instructions.

Laser doppler imaging

Laser doppler imaging was used to measure peripheral tail blood flow, as a surrogate for vasoconstriction [33]. In brief, female WT and TAP mice were anesthetized and tail perfusion was measured with a Laser Doppler Perfusion Imager (LDPI) System (Moor Instruments Ltd., Axminister, UK) [34, 35]. Consecutive measurements were obtained from anesthetized mice by scanning the base of the tail. Color images were acquired, and flux values were measured by calculating the average perfusion signal. All experiments were performed at 22^{o}C, and anesthetized mice were placed on heating pads until laser doppler measurements. Mice were sacrificed at the end of the study by exsanguination under anesthesia (isoflurane).

Statistical analysis

Data are presented as mean ± standard error. Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software) using Student's t-test or one- or two-way ANOVA, followed by appropriate *post-hoc* tests. Statistical significance was achieved when $p < 0.05$.

Results

Partial deletion of 14-3-3ζ does not affect acute cold tolerance

To understand the contributions of 14-3-3ζ to thermogenesis, we started by examining whether partial deletion of 14-3-3ζ would affect cold tolerance. We previously reported that homozygous 14-3-3ζ knockout mice weighed significantly less than WT mice due to reduced fat mass, which could affect their ability to tolerate cold [24, 36, 37]; thus, mice heterozygous (HET) for *Ywhaz*, the gene encoding 14-3-3ζ, were used. WT and HET mice were challenged with acute cold (4°C) for 3 hours. No differences in body weights were detected between WT and HET mice before and after the acute cold challenge (Figure 1A,B), and both groups displayed similar decreases in rectal temperature throughout the entire challenge (Figure 1C). Similar results were observed in female mice (Figures S1A,B). Acute cold exposure significantly increased *Ywhaz* and *Ucp1* mRNA levels only in brown adipose tissue (BAT) of WT and HET male mice in a similar fashion (Figure 1D, Figure S1C). In inguinal white adipose tissue (iWAT), levels of *Ywhaz* mRNA were significantly increased by cold exposure in WT and TAP mice, but no differences were observed in the expression of *Ucp1* and the beige selective gene *Tmem26* (Figure 1E, Figure S1D). To determine if 14-3-3ζ regulates the expression of *Ucp1* mRNA in brown adipocytes, we utilized the Ucp1-luciferase adipocyte cell line, an *in vitro* model of brown adipocytes [30]. Transient knockdown of *Ywhaz* by siRNA did not affect isoproterenol-induced *Ucp1* mRNA expression, which suggested that 14-3-3ζ does not directly regulate *Ucp1* expression (Figure 1F,G). Collectively, these data demonstrate that depletion of 14-3-3ζ does not affect cold tolerance or *Ucp1* gene expression.

14-3-3ζ over-expression provides tolerance to acute cold.

We next examined if increasing 14-3-3ζ expression could affect cold tolerance. 12-week-old transgenic mice over-expressing human 14-3-3ζ (TAP) were challenged with acute cold for 3 hours at 4°C (Figure S2A). In male mice, no differences in body weight were detected before or after cold exposure (Figure 1H); however, male TAP mice displayed a significant, rapid restoration of rectal temperature (Figure 1I), signifying improved tolerance to acute cold and changes in body temperature. A similar increase in body temperature was observed in female mice, but it was not statistically significant (Figure S2F). In BAT, acute cold exposure had no effect on the expression of *Ucp1*, *Pgc1a,* or *Cidea* (Figure 1J). In contrast,

significant increases in *Ucp1* mRNA levels in iWAT of both male and female TAP mice following acute cold were detected (Figure 1K, S2H). Taken together, these data demonstrate that only 14-3-3ζ over-expression influences the propensity of iWAT to undergo beiging, thereby contributing to acute cold tolerance.

14-3-3ζ over-expression improves tolerance to chronic cold.

Given the differences observed following acute cold challenge, male WT and TAP mice were subjected to a chronic cold challenge of 3 days at 4°C. Mice had similar body weights before cold exposure and lost comparable body weight after the challenge (Figure 2A,B). No differences in fat or lean mass were observed between the two groups (Figure 2C). Additionally, no visible differences in the pelage, or fur, of mice were observed (data not shown). Food intake was increased in both WT and TAP mice when temperature was lowered, which is consistent with the need to supply necessary fuel as compensation for energy dissipated during thermogenesis [38], but no differences were observed between TAP and WT mice (Figure 2E). Moreover, no differences in locomotor activity or RER were detected (Figure 2F,G). During the chronic cold challenge, TAP mice exhibited significantly higher body temperatures during the last 48 hours of cold exposure (Figure 2H,I), but this was associated with significantly decreased energy expenditure during the last 2 dark phases of the cold challenge (Figure 2J,K).

Circulating free fatty acids (FFAs) and glycerol following chronic cold exposure were similar between WT and TAP mice (Figure 3B). Analysis of adipocyte morphometry revealed no differences in adipocyte size in iWAT or gonadal WAT (gWAT) of WT and TAP mice (Figure 3A, Figure S2). However, after chronic cold exposure, distinct morphological differences were observed in BAT whereby smaller lipid droplets were visible in TAP mice (Figure 3A). Despite these differences in the size of lipid droplets in BAT of TAP mice, no difference in total triacylglycerol content in BAT was detected (Figure 3C).

Over-expression of 14-3-3ζ increases beiging of inguinal white adipocytes

To better understand how 14-3-3ζ could improve tolerance to chronic cold exposure, we examined whether increased signs of beiging or BAT activity occurred as a result of 14-3-3ζ over-expression. In BAT, *Ucp1* mRNA was not different between groups, and differences in the expression of various thermogenic genes, such as *Prdm16 and Pdk4,* could be detected (Figure 4A). In iWAT of chronic cold exposed animals,

a five-fold increase in *Ucp1* mRNA levels was detected in TAP mice, in addition to significantly higher levels of *Tbx1* mRNA (Figure 4B). Marked elevations in UCP1 protein abundance were also detected in iWAT and BAT of TAP mice when compared to littermate controls (Figure 4C,D), and increase *Fgf21* mRNA levels could be detected in cold-exposed iWAT (Figure 4E). Taken together, these findings indicate that 14-3-3ζ over-expression promotes the beiging of iWAT during chronic cold exposure, which may account for the increased body temperature and improved cold tolerance during the chronic cold challenge.

Over-expression of 14-3-3ζ does not alter sensitivity to β-adrenergic stimuli or sympathetic innervation

During long-term cold exposure, the sympathetic nervous system releases norepinephrine, which acts as the principal activator of beige and brown fat thermogenesis [8, 27, 28, 39-41]. Thus, to explore the possibility that altered sensitivity to β-adrenergic stimuli could account for differences in energy expenditure (Figure 2K,L), WT and TAP mice were chronically injected (7 days) with 0.9% saline or the β3-adrenergic receptor agonist CL-316,243 (CL, 1mg/kg) (Figure 5A). No differences in response to CL-mediated changes in total body weight, lean mass, or fat mass were observed between TAP and WT mice (Figure 5B,C). Levels of *Ucp1* mRNA were similarly increased by CL treatment in iWAT (Figure 5D) and BAT (Figure 5E) of both groups. Moreover, markers of brown, *Cidea* and *Pdk4*, and beige, *Tmem26* and *Tbx1*, adipocytes were not different between TAP mice and WT littermate controls (Figure 5D,E). Taken together, these data suggest that over-expression of 14-3-3ζ does not alter sensitivity to β-adrenergic stimuli.

The above *in vivo* studies demonstrate a role of 14-3-3ζ in cold adaptation that is not mediated by an increase in adrenergic sensitivity, and to further investigate how 14-3-3ζ could regulate cold tolerance, we looked at whether there were changes in sympathetic innervation or activity in iWAT or BAT. Tyrosine hydroxylase (TH) protein expression was not altered in iWAT or BAT (Figure 5F) of both groups following chronic cold exposure, and consistent with these observations, norepinephrine levels in iWAT and BAT were not different between WT and TAP mice (Figure 5G), nor were mRNA levels of *Adrb3* expression in iWAT and BAT (Figure 5H). Together, these findings suggest that sympathetic innervation or activity is not altered in TAP mice in response to chronic cold exposure.

14-3-3ζ does not influence glucose utilization during chronic cold exposure

Brown and beige adipocytes utilize triglycerides and glucose as sources of energy for heat production [9, 10], and given the differences in cold tolerance between WT and TAP mice, we examined whether glucose handling was altered by systemic 14-3-3ζ over-expression (Figure 6A). At 22ºC, TAP mice displayed significantly enhanced glucose tolerance following an intraperitoneal bolus of glucose, but this difference was lost following chronic cold exposure (Figure 6C), which suggests that no difference in glucose utilization between WT and TAP mice occur during cold exposure.

14-3-3ζ increases vasoconstriction to mitigate heat loss

During chronic cold exposure, the increase in body temperature and the paradoxical decrease in energy expenditure suggested that an alternative, adaptive mechanism of heat conservation was present in TAP transgenic mice. As a first step, thermal conductance, a measurement of the rate of heat dissipation to the environment [42], was determined in WT and TAP at 22ºC and 4ºC. In contrast to WT mice, TAP mice displayed significantly lower thermal conductance at room temperature and throughout the chronic cold exposure periods (Figure 6D), which suggests a separate mechanism that is active in TAP mice to mitigate heat loss during mild (22ºC) and severe cold (4ºC) stress. Leptin was previously found to have thermo-regulatory effects in *ob/ob* mice; however, circulating levels of leptin was not different between WT and TAP mice (Figure 2D) [32, 42, 43]. To explore whether 14-3-3ζ over-expression affected vasoconstriction *in vivo,* laser doppler imaging was used to measure superficial blood-flow as a surrogate measure of vessel diameter [33]. At 22ºC, blood flow was significantly reduced in TAP animals (Figure 6E), which suggests increase vasoconstriction. Taken together, these findings suggest that 14-3-3ζ can activate alternative mechanisms to conserve heat loss and maintain homeothermy.

Discussion

Substantial interest in understanding the roles of beige and brown adipocytes in the regulation of homeothermy and energy homeostasis has occurred over the past decade. Furthermore, due to their abilities to metabolize lipids via β-oxidation, this has sparked interest in understanding whether the activation of beige and brown adipocytes represents a therapeutic approach for the treatment of obesity

and diabetes. In the context of homeothermy, little emphasis has been placed on alternative, BAT-independent homeothermic mechanisms to influence cold tolerance. Herein, we demonstrate that 14-3-3ζ can influence thermogenesis and the adaptation to cold. Over-expression of 14-3-3ζ was found to significantly improve tolerance to both acute and chronic cold exposure by raising body temperature, while minimizing energy expenditure and restricting peripheral blood-flow to retain heat. By minimizing heat loss, less energy has to be expended to defend against hypothermia. It should be noted that 14-3-3ζ over-expression was not associated with changes in sympathetic innervation or sensitivity. Taken together, these findings demonstrate that while thermogenic mechanisms may occur by way of increased BAT activity or the beiging of white adipocytes, alternative mechanisms that promote heat conservation during cold adaptation should also be considered.

To defend against prolonged cold, adaptive mechanisms to maintain core body temperature are activated, which predominantly results in the production of heat via UCP1-dependent mechanisms in brown and beige adipocytes [1, 8]. However, in the present study, the improved tolerance of transgenic mice over-expressing 14-3-3ζ to prolonged cold was associated with decreased energy expenditure, despite the ability of transgenic mice to raise and maintain higher body temperatures. This suggests that thermogenic mechanisms are intact in TAP mice, as the expected increases in UCP1 expression in BAT and beige iWAT were indeed present in TAP mice, but alternative mechanisms to defend against changes in core body temperature that are independent of thermogenesis are active in mice over-expressing 14-3-3ζ.

Thermogenesis is largely viewed as primary mechanism for cold tolerance, but when this process is disrupted, as in the case with UCP1-defiicent mice, vasoconstriction becomes the predominant mechanism for cold adaptation [7]. The decrease in thermal conductance during mild (22ºC) and severe (4ºC) cold stress raised the possibility that 14-3-3ζ over-expression could activate processes, such as vasoconstriction, to mitigate heat loss from skin [42]. Indeed, we found that 14-3-3ζ over-expression was associated with reduced peripheral blood-flow in tails of TAP mice, which is indicative of vasoconstriction [7]. Leptin has been recently identified as having vasoconstrictive effects, but no differences in circulating leptin levels were detected between WT and TAP mice [32, 42, 43]. It is possible that the increase in vasoconstriction could be due elevated levels of norepinephrine, a potent vasoconstrictor, in the circulation, but it is also possible that 14-3-3ζ over-expression could have effects in endothelial cells. For example, 14-

3-3ζ has been found to interact with α2A-adrenergic receptors subtypes at the i3 loop, which blocks their interactions with β-arrestins to limit their internalization. This could potentiate α2A-adrenergic receptor-mediated effects on vasoconstriction [44, 45].

During prolonged cold exposure, increased glucose utilization in parallel with fatty acid oxidation occurs during BAT-dependent thermogenesis [46, 47]. Indeed, 14-3-3ζ and its related isoforms have been implicated in the regulation of glucose uptake, as they are known to sequester AS160, the AKT substrate that negatively regulates insulin-dependent GLUT4 translocation [48, 49]. Thus, 14-3-3ζ over-expression mice may increase AS160 inhibition, thereby permitting GLUT4 translocation to the plasma membrane and enhance BAT activity. Moreover, this suggests that TAP mice could have better cold tolerance due to improved glucose utilization in BAT or beige adipocytes. Surprisingly, intraperitoneal glucose tolerance tests revealed that TAP mice at room temperature displayed improved glucose tolerance, but no differences were detected following chronic cold exposure. More sensitive methods to detect glucose uptake in BAT or beige iWAT is needed to determine whether there are differences in glucose utilization between WT and TAP mice.

As over-expression of 14-3-3ζ was not associated with changes in food intake or locomotor activity during cold exposure, the observed decrease in energy expenditure could eventually lead to a long-term imbalance in energy homeostasis, thus increasing the propensity to development obesity [50, 51]. Indeed, we previously demonstrated that transgenic over-expression of 14-3-3ζ was sufficient to exacerbate age- and high-fat diet-induced weight gain [24]. Moreover, elevated expression of 14-3-3ζ has been detected in visceral adipose tissue from individuals with obesity [52, 53]. Whether the increase in adiposity due to elevations in 14-3-3ζ expression is due to changes in overall metabolism or cell-autonomous effects in adipocyte development require further in-depth investigation, and it is necessary to examine whether increasing 14-3-3ζ expression could have other effects on age-associated declines in metabolism and overall health [54].

In summary, we demonstrate in this study that over-expression of 14-3-3ζ in male mice is sufficient to increase tolerance to cold. Despite the activation of thermogenic mechanisms, we demonstrate that cold-induced increases in body temperature are paradoxically associated with decreased energy expenditure in mice over-expressing 14-3-3ζ and increased peripheral vasoconstriction to retain heat. This suggests that

alternative, non-thermogenic mechanisms can be activated to improve cold tolerance and raise the notion that other mechanisms for cold tolerance should be consider. Taken together, our results show an important role of 14-3-3ζ in adaptation to cold and add new insights to the regulation of key physiological processes by molecular scaffolds.

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Contributions

K.D. designed the studies, carried out the research, interpreted the results, and wrote the manuscript. A.F.L. provided 14-3-3ζ Het mice. S.D. and A.R designed and carried out laser doppler analysis of blood flow and revised the manuscript. G.E.L conceived the concept, designed the studies, interpreted the results, and wrote and revised the manuscript. G.E.L is the guarantor of this work.

Conflicts of interests

No conflicts of interest are reported.

Figure legends

Figure 1- Over-expression, but not reduction, of 14-3-3ζ enhances tolerance to acute cold in male mice. (A) Wildtype (WT), mice lacking one allele of *Ywhaz*, the gene encoding 14-3-3ζ (HET), and transgenic 14-3-3ζ over-expressing mice (TAP) were challenged with cold for 3 hours. Temperature was measured by rectal probes at each time point. **(B, C)** Body weights (B) and rectal temperatures (C) of WT and HET mice were obtained prior, during, and at the end of the 3 hour cold challenge (n=7 mice per group). **(D,E)** Expression of brown-selective (D) and beige-selective (E) genes from BAT and iWAT, respectively, at room temperature (22 ºC) and after 3 hours cold exposure (n=7 per group, *: p<0.05 when compared to 22 ºC). **(F,G)** Knock-down of 14-3-3ζ expression by siRNA (F) in Ucp1-Luc cells, a brown adipocyte cell line, does not affected isoproterenol (ISO, 10μM, 4 hours)-mediated induction of *Ucp1* expression (G) (n=6 per group, *: p<0.05 when compared to -ISO; #: p<0.05 when compared to siCon). **(H, I)** Body weights (H) and rectal temperatures (I) of WT and TAP mice were obtained prior, during, and at the end of the 3 hour cold challenge (n=7 mice per group). **(J,K)** Expression of brown-selective (J) and beige-selective (K) genes from BAT and iWAT, respectively, at room temperature (22 ºC) and after 3 hours cold exposure (n=7 per group, *: p<0.05 when compared to 22 ºC).

Figure 2- Over-expression of 14-3-3ζ improves tolerance to chronic cold exposure in male mice. **(A)** Wildtype (WT) or 14-3-3ζ over-expressing (TAP) mice were implanted with a temperature probe 1 week prior to placement in CLAMS metabolic cages for 3 days to stimulate chronic cold (4 ºC) exposure. **(B,C)** Body weights (B) and lean and fat mass measurements by echo-MRI (C) were obtained prior to and after the chronic cold challenge. **(D)** Plasma leptin was measured from blood samples obtained after the chronic cold challenge, **(E-G)** During the light and dark cycles at 22ºC and 4 ºC, food intake (G), locomotor activity (H), and RER were measured in WT and TAP mice. **(H-K)** Body temperature (H,I) and energy expenditure (J,K) were measured and are reported either as the average trace for all mice over the challenge (H,J) or as an average per light:dark cycle (I,K) (n=8 WT and 10 TAP mice; *: p<0.05 when compared to WT).

Figure 3- Adipocyte size is not affected by 14-3-3ζ over-expression following cold exposure. (A) Immunofluorescent staining was used to examine adipocyte morphology and size in gonadal white adipose

tissue (gWAT, a,b), inguinal WAT (c,d), or brown adipose tissue (BAT, e,f) of male WT and TAP mice. Enlarged regions bounded by the dashed boxes are shown in e' and f'. (Representative images of n=4-5 mice per group; scale bar for a-d= 200 μm, scale bar for e,f= 100 μm). **(B,C)** Circulating free fatty acids and triglycerides (B) and total triacylglycerols in gWAT, iWATm and BAT (C) of male WT and TAP mice were measured after 3 days of cold exposure (n= 4-5 per group).

Figure 4- Transgenic over-expression of 14-3-3ζ increases beiging of ingulnal white adipocytes from male mice. (A,B) Following 3 days of cold exposure, brown adipose tissue (BAT, A) and inguinal white adipose tissue (iWAT, B) were harvested for quantitative PCR to measure genes associated with thermogenesis and beiging (n=8 WT and 10 TAP; *: $p<0.05$). **(C, D)** Immunohistochemistry (C) and immunoblotting (D) were used to measure UCP1 protein localization and expression (representative images for n=8 WT and 10 TAP mice; scale bar= 50 μm). **(E)** *Fgf21* mRNA levels were measured from iWAT harvested from WT and TAP mice exposed for 3 days to (n=6 per group, *: $p<0.05$).

Figure 5- No differences in sympathetic innervation or sensitivity were detected in mice over-expressing 14-3-3ζ. (A) To examine if 14-3-3ζ over-expression confers increased sensitivity to β-adrenergic stimuli, wildtype (WT) and transgenic 14-3-3ζ over-expressing mice (TAP) were injected with the β3-adrenergic agonist CL,316,247 (CL, 1 mg/kg) for seven days. Lean and fat mass were measured before and after CL injections. **(B,C)** Body weights (B) and lead and fat mass (C) were measured before, during , and after CL-treatment. **(D, E)** Inguinal white adipose tissue (iWAT, D) and brown adipose tissue (BAT, E) were harvested from WT and TAP mice before and after CL exposiure to measure genes associated with thermogenesis. **(F-H)** Cold-exposed iWAT and BAT were harvested from WT and TAP mice to measure protein abundance of tyrosine hydroxylase by immunoblotting (F), norepinephrine by ELISA, and *Adrb3* mRNA levels by quantiative PCR (H). (n=5 WT and 7 TAP, *: $p<0.05$ when compared Day=0).

Figure 6: Increased heat retention is associated with 14-3-3ζ over-expression

(A-C) Wildtype (WT) or transgenic mice over-expressing 14-3-3ζ (TAP) housed at 22ºC or 4ºC did not display differences in body weight (A) or fasting blood glucose (B) prior to an intraperitoneal glucose tolerance test (C, 2 g/kg) (n=8 WT and 6 TAP; *: $p<0.05$). **(D)** Thermal conductance was calculated for WT and TAP mice housed at 22ºC or 4ºC during the chronic cold exposure CLAMS study (Figure 2). **(E)** Laser doppler imaging was used to measure blood flow in the base of the tail of WT and TAP mice housed at 22ºC. (n=16 WT and 22 TAP mice; *: $p<0.05$).

Supplemental Figure Legends

Figure S1- Reducing or over-expressing 14-3-3ζ in female mice does not impact acute cold tolerance

(A, B) Body weights (B) and rectal temperatures (C) of female WT and HET mice were obtained prior, during, and at the end of the 3 hour cold challenge (n=7 mice per group). **(C,D)** Expression of brown-selective (C) and beige-selective (D) genes from BAT and iWAT, respectively, at room temperature (22 ºC) and after 3 hours cold exposure (n=7 per group, *: $p<0.05$ when compared to 22 ºC). **(E,F)** Body weights (E) and rectal temperatures (F) of WT and TAP mice were obtained prior, during, and at the end of the 3 hour cold challenge (n=7 mice per group). **(G,H)** Expression of brown-selective (G) and beige-selective (H) genes from BAT and iWAT, respectively, at room temperature (22 ºC) and after 3 hours cold exposure (n=7 per group, *: $p<0.05$ when compared to 22 ºC).

Figure S2- Over-expression of 14-3-3ζ does not alter adipocyte size in cold-exposed male mice.

(A) Quantitative PCR was used to measure the mRNA for human *YWHAZ*, the gene encoding 14-3-3ζ, in gonadal (gWAT) and inguinal (iWAT) depots. (n=6 per group, *: $p<0.05$ when compared to iWAT). **(B-E)** Measurements of mean adipocyte area (B,D) and size distribution (C,E) were calculated from gonadal (gWAT) and inguinal (iWAT) white adipose tissue harvested from chronic cold exposure make wildtype (WT) and transgenic mice over-expressing 14-3-3ζ (TAP). (n=6 WT and 7 TAP).

Supplemental Table1: List of primers for qPCR

Gene	Forward	Reverse	Ref.
Adrb3	CCTTCAACCCGGTCATCTAC	GAAGATGGGGATCAAGCAAGC	1
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG	2
Fgf21	CTGGGGGTCTACCAAGCATA	CACCCAGGATTTGAATGACC	3
Hprt1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC	4
Pdk4	CCGCTTAGTGAACACTCCTTC	TCTACAAACTCTGACAGGGCTTT	2
Pgc1a	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG	2
Pparg2	GTTATGGGTGAAACTCTGGGAGAT	GGCCAGAATGGCATCTCTGTGTCAA	4
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG	2
Tbx1	GGCAGGCAGACGAATGTTC	TTGTCATCTACGGGCACAAAG	2
Tmem26	ACCCTGTCATCCCACAGAG	TGTTTGGTGGAGTCCTAAGGTC	2
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG	2
Ywhaz	CAGAAGACGGAAGGTGCTGAGA	CTTTCTGGTTGCGAAGCATTGGG	5
YWHAZ	ACCGTTACTTGGCTGAGGTTGC	CCCAGTCTGATAGGATGTGTTGG	5

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Supplemental Table 2: List of Antibodies

	Company	**Dilution**	**Product number**
Alexa Fluor 594	Jackson Immunoresearch	1:400	115-585-003
Anti-Mouse HRP IgG	Cell signaling	1:5000	7076S
β-Actin	Cell signaling	1:10000	3700S
β-Tubulin	Cell signaling	1:10000	86298S
GAPDH	Cell signaling	1:10000	5174S
Perilipin	Cell signaling	1:400	93495S
TH	Millipore	1:400	6A2911
TH	Millipore	1:400	6A2907
UCP1	ABCAM	1:1000	Ab10983
UCP1	R&D systems	1:400	MAB6158-SP

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Figure 1- Over-expression, but not reduction, of 14-3-3ζ enhances tolerance to acute cold in male mice

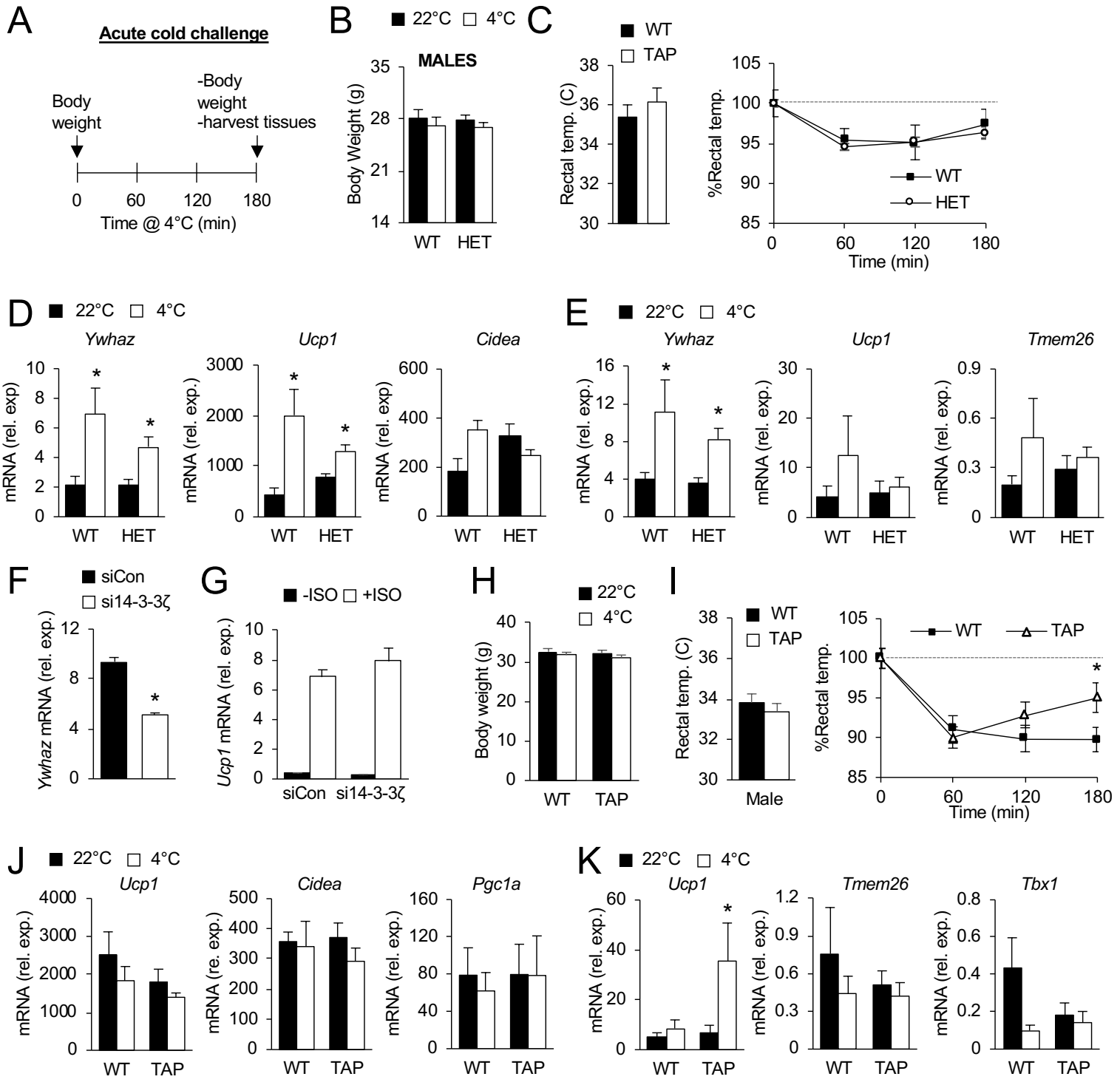

Figure 2- Over-expression of 14-3-3ε improves cold tolerance to chronic cold exposure in male mice

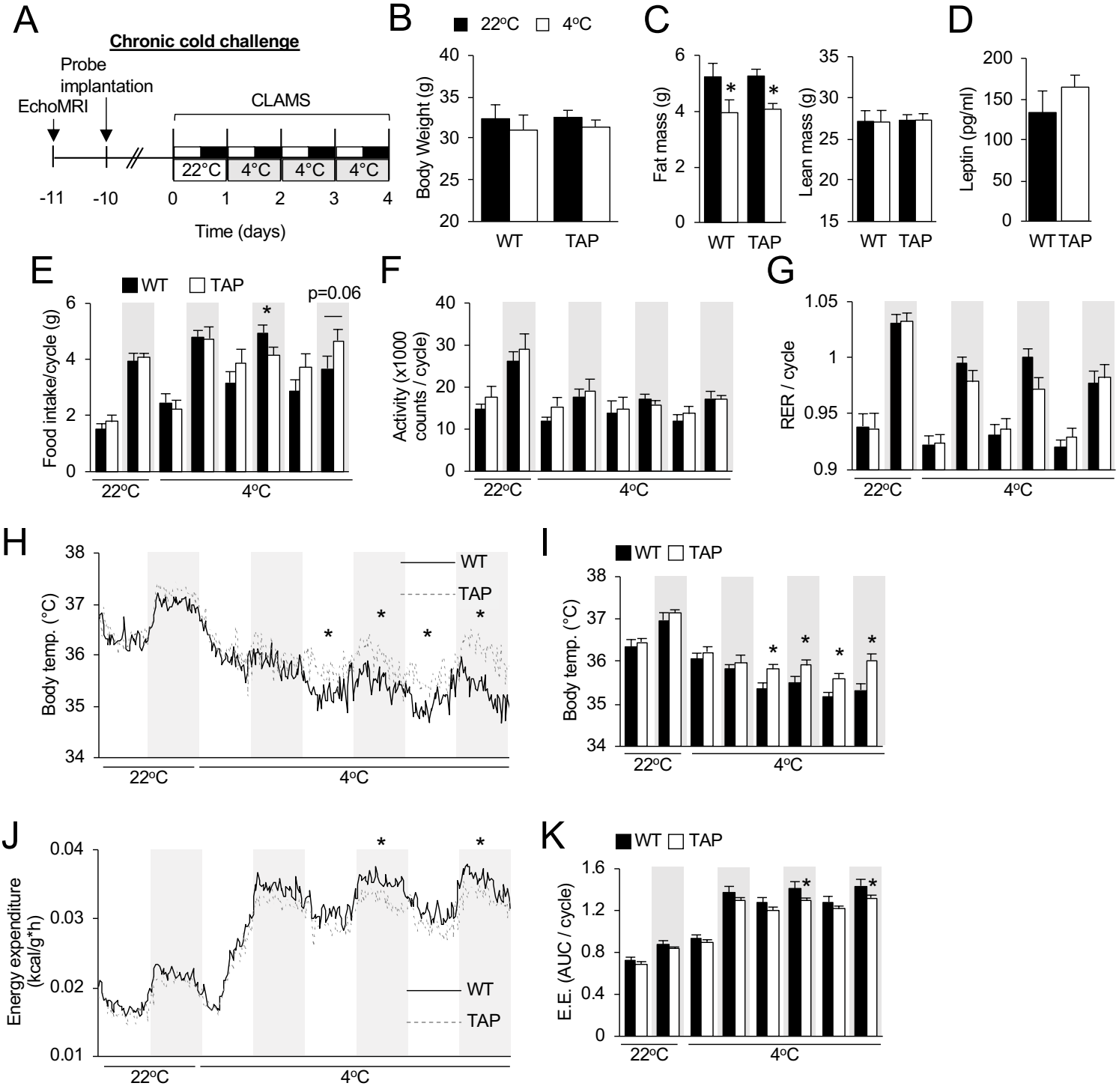

Figure 3- Adipocyte size is not affected by 14-3-3ζ over-expression following cold exposure

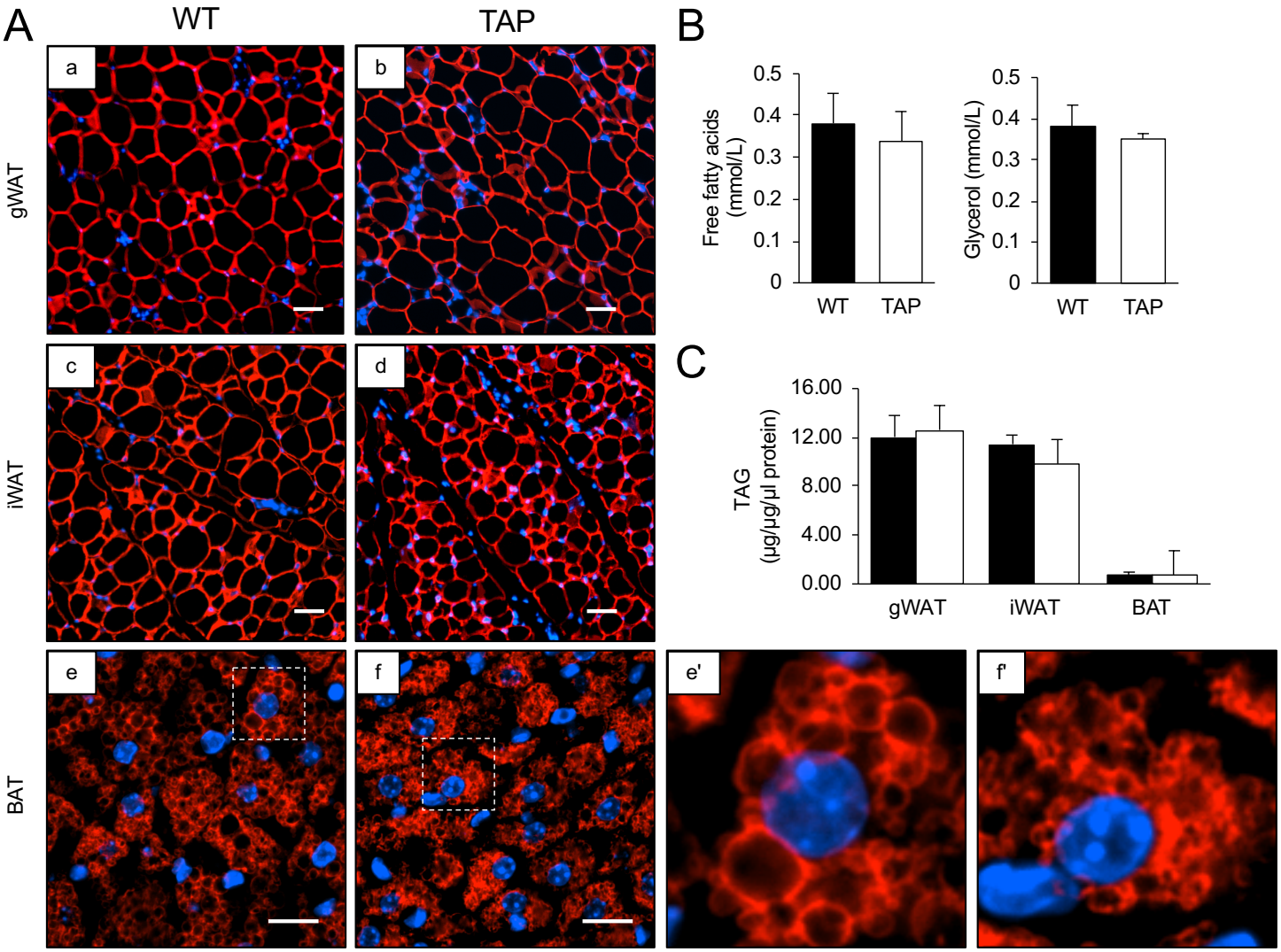

Figure 4- Transgenic over-expression of 14-3-3ζ increases beiging of inguinal white adipocytes from male mice

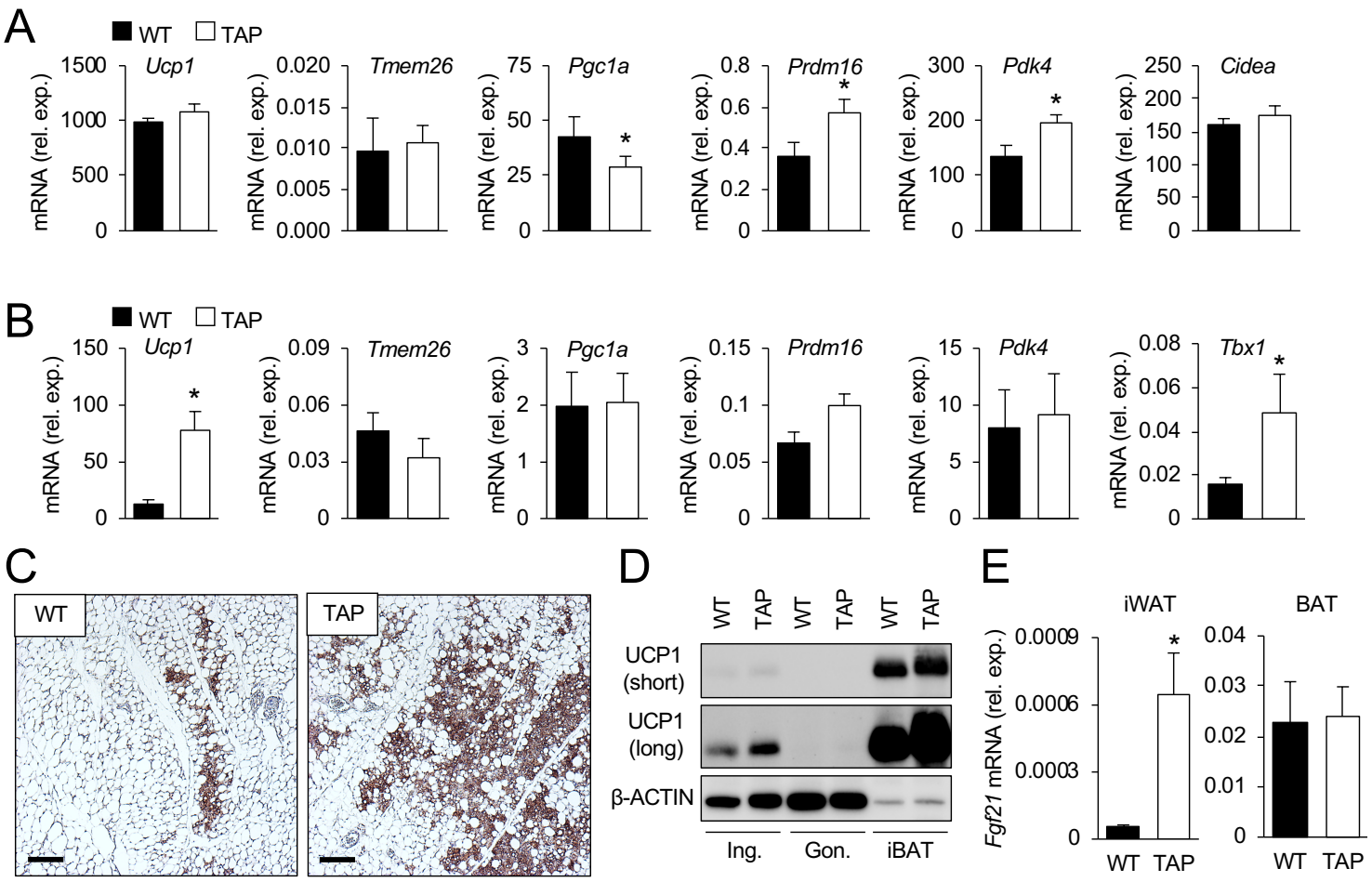

Figure 5- No differences in sympathetic innervation or sensitivity were detected in mice over-expressing 14-3-3ζ

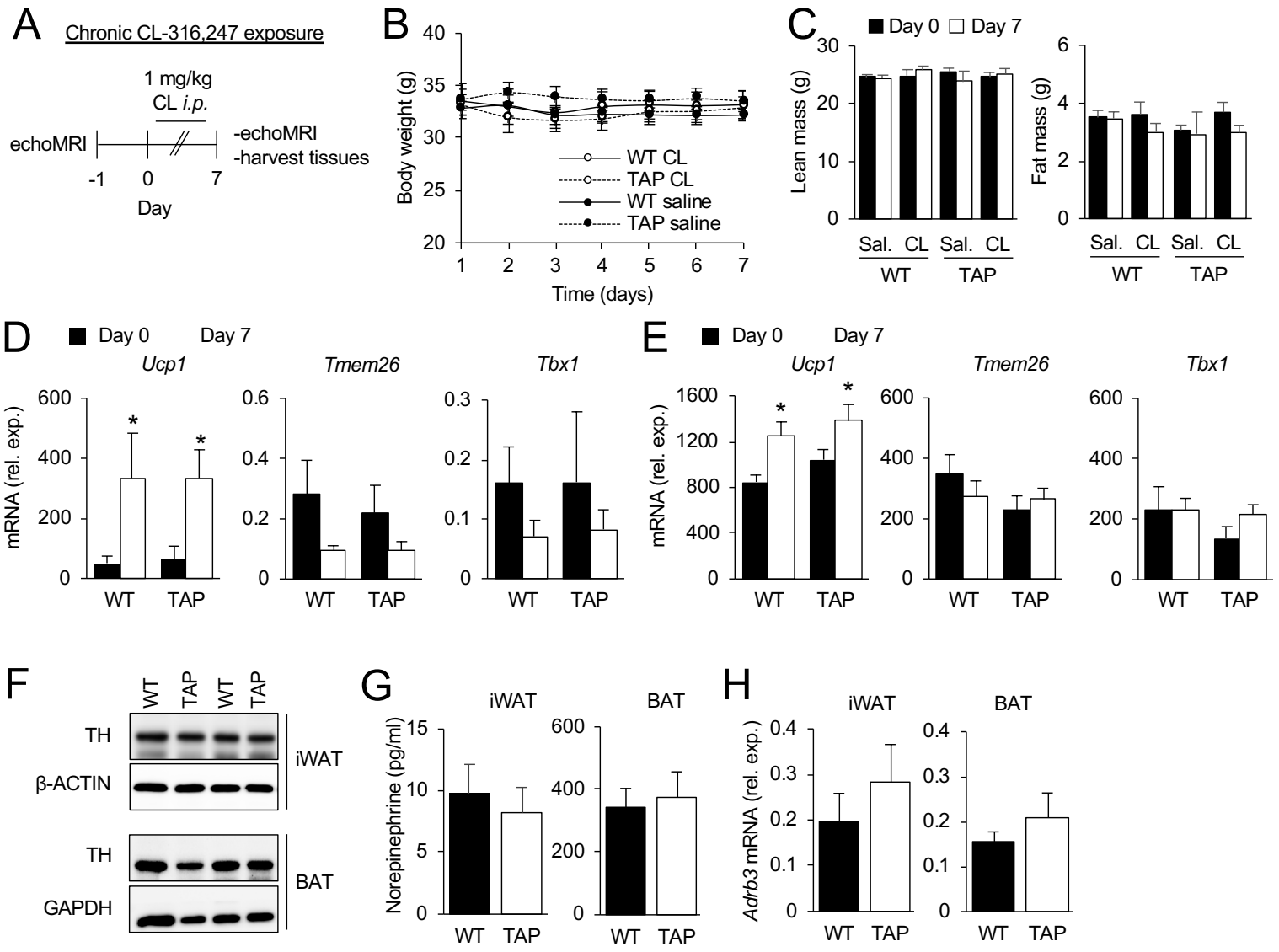

Figure 6: Increased heat retention is associated with 14-3-3ζ over-expression

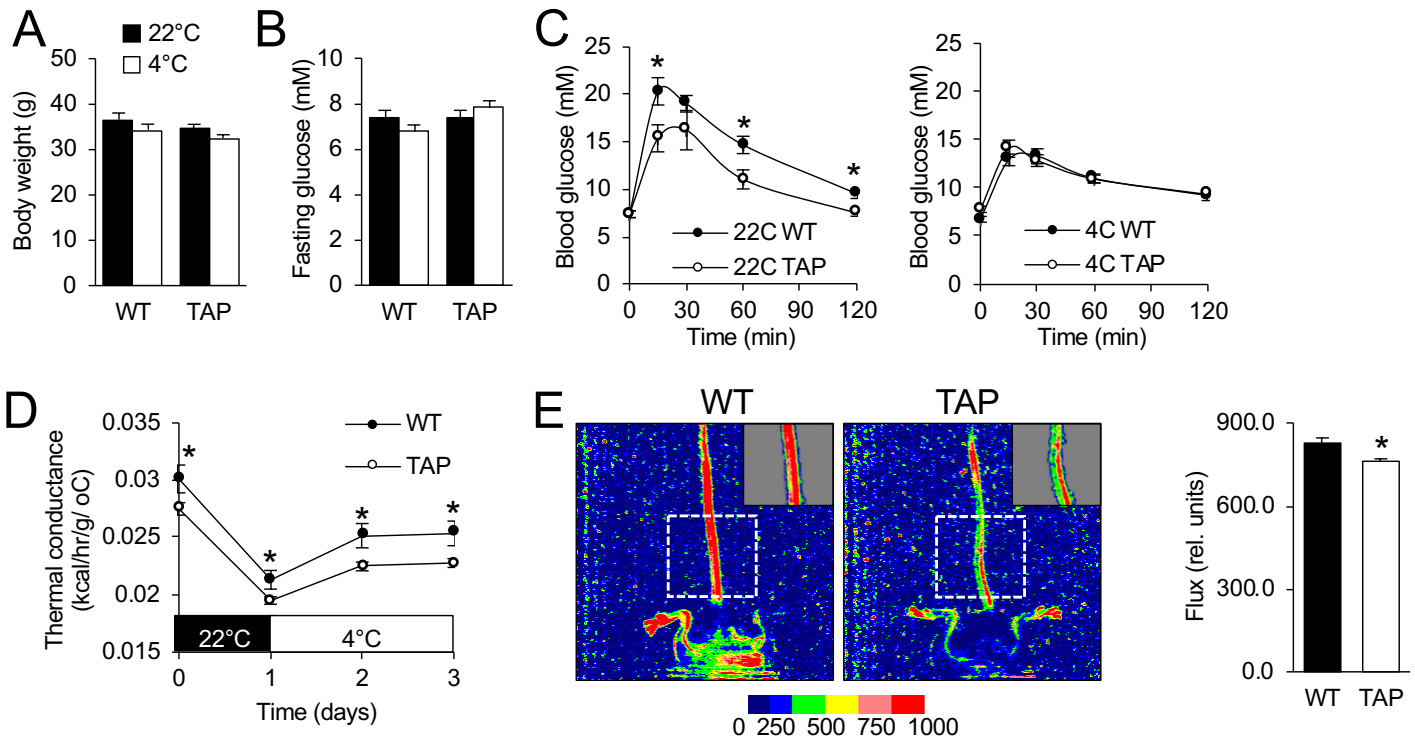

Figure S1- Reducing or over-expressing 14-3-3ζ in female mice does not impact acute cold tolerance

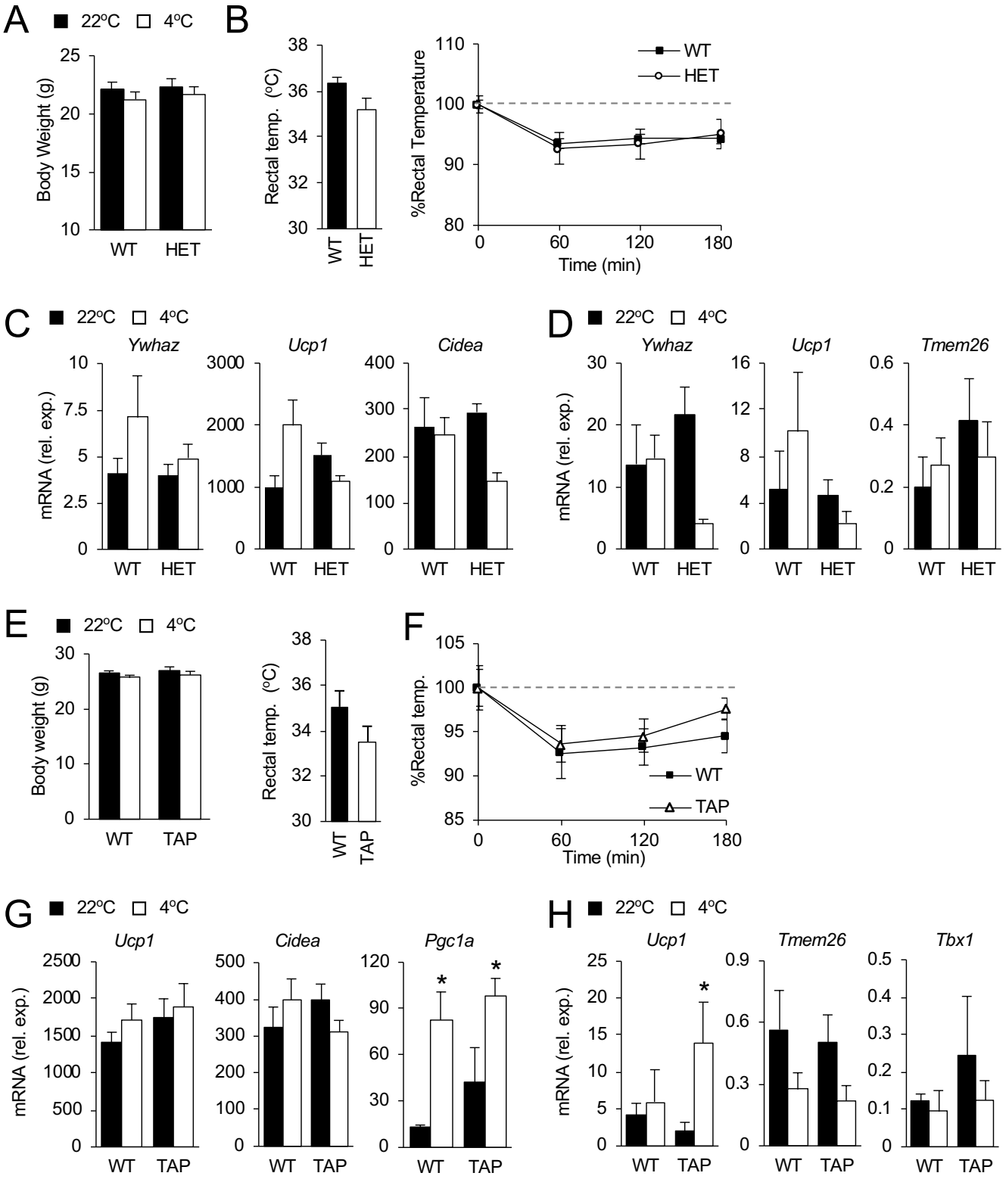

Figure S2-Over-expression of 14-3-3ζ does not alter adipocyte size in cold-exposed male mice.