Université de Montréal

14-3-3ζ overexpression improves tolerance to acute and chronic cold exposure in male mice

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14-3-3ζ overexpression improves tolerance to acute and chronic cold exposure in male mice

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Résumé

La thermogenèse adaptative est un mécanisme de production de chaleur médié par les adipocytes bruns. En réponse au froid, ou à un stimulus adrénergique, les adipocytes blancs peuvent être convertis en adipocytes beiges lors d'un processus que l'on nomme le « beiging ». Contrairement aux adipocytes blancs, les adipocytes beiges et bruns expriment des taux élevés de la protéine de découplage 1 (UCP1) et dissipent l'énergie sous forme de chaleur grâce à l'oxydation des lipides. Il a été démontré chez les rongeurs que l'activation des adipocytes bruns et beiges entraîne une réduction significative du poids corporel et l'activation de ces adipocytes chez l'humain semble être un traitement prometteur contre l'obésité et le diabète. Nous avons précédemment identifié un rôle essentiel de la protéine d'échafaudage 14-3-3ζ dans l'adipogenèse, mais son rôle dans d'autres processus adipocytaires reste incertain. Une des premières fonctions identifiées de la 14-3-3ζ est sa capacité à réguler l'activité enzymatique de la tyrosine hydroxylase, indispensable à la production de norépinephrine pour la thermogenèse. Notre étude vise donc à déterminer si la 14-3-3ζ influence le développement et la fonction des adipocytes beiges et bruns.

Nos données montrent que la délétion d'un allèle du gène de la 14-3-3ζ n'affecte pas la tolérance au froid aiguë. Comparées aux souris de type sauvage (WT), les souris transgéniques mâles surexprimant la 14-3-3ζ (TAP) ont une meilleure tolérance au froid aiguë (3 heures, 4 °C) et chronique (3 jours, 4 °C). On observe chez les TAP une augmentation du beiging due à une élévation significative de l'ARNm et de la protéine UCP1 dans le tissu adipeux blanc inguinal (iWAT). Par ailleurs, les souris TAP présentent également une réduction significative de la conductance thermique lors d'exposition au froid leur permettant de mieux conserver la chaleur. Collectivement, nos résultats soulignent le rôle novateur de la 14-3-3ζ dans le beiging et nous permettent de mieux comprendre comment la thermogenèse adaptative est régulée.

Mots clés : 14-3-3ζ, protéines 14-3-3, beiging, browning, thermogenèse adaptative, adipocytes beiges, adipocytes bruns.

Abstract

Adaptive thermogenesis is a mechanism of heat production primarily mediated by brown fat. In some instances, cold exposure or adrenergic stimuli can convert white adipocytes into brown-like or beige adipocytes during a process termed “beiging”. Both beige and brown adipocytes express higher levels of uncoupling protein 1 (UCP1) and can release energy in the form of heat following lipid oxidation. The activation of these thermogenic adipocytes increases energy expenditure to reduce body weight in rodents, and it has been postulated to be a promising therapy for the treatment of obesity and diabetes. We previously identified an essential role of the molecular scaffold, 14-3-3ζ, in adipogenesis, but its roles in other adipocyte processes is uncertain. An early identified function of 14-3-3ζ was its ability to regulate the enzymatic activity of tyrosine hydroxylase, which is indispensable in the production of norepinephrine for thermogenesis. Thus, our study aims to investigate whether 14-3-3ζ influences the development and function of beige and brown adipocytes.

We report here that one allele deletion of the gene of 14-3-3ζ did not affect acute cold tolerance. On the other hand, transgenic overexpression of 14-3-3ζ in male mice (TAP) improves cold tolerance due to enhanced beiging with a remarkable increase in Ucp1 mRNA and protein in inguinal white adipose tissue (iWAT). Interestingly, beiging is increased in the TAP mice without any changes in sensitivity to beta-adrenergic stimuli, sympathetic innervation, or norepinephrine content being detected between WT and TAP mice. TAP mice also displayed significantly lower thermal conductance decreasing heat loss during the chronic cold challenge. Collectively, our results point to a novel role of 14-3-3ζ in beiging and increases our understanding of how adaptive thermogenesis is regulated.

Key words: 14-3-3ζ, 14-3-3 proteins, beiging, browning, adaptive thermogenesis, beige adipocytes, brown adipocytes

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List of abbreviations and acronyms

β-AR: β adrenergic receptor

ADRB3: adrenergic receptor beta 3

AS160: AKT substrate 160KD

ATP: adenosine triphosphate

BAT: brown adipose tissue

cAMP: cyclic adenosine monophosphate

CD137: cluster of differentiation 137

CD36: cluster of differentiation 36

CIDEA: cell death-inducing-like effector a

CITED1: Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1

FFA: free fatty acid

FGF21: fibroblast growth factor 21

FGFR1: fibroblast growth factor receptor 1

GLUT1/4: glucose transporter 1/4

gWAT: gonadal white adipose tissue

HSL: hormone sensitive lipase

iWAT: inguinal white adipose tissue

LPL: lipoprotein lipase

MAPK: mitogen activated protein kinase

NE: norepinephrine

PDK4: pyruvate dehydrogenase kinase, isoenzyme 4

PGC1α: peroxisome proliferator-activated receptor-gamma, coactivator 1, alpha

Pi: phosphate

PPARγ: peroxisome proliferator-activated receptor-gamma

PRDM16: PR domain-containing protein 16

scWAT: subcutaneous white adipose tissue

SERCA: sarco/endoplasmic reticulum Ca2+ ATP ase

SLN: sarcolipin

SNS : sympathetic nervous system

SVF : stromal vascular fraction

T3 : triiodothyronine

TBX1: T-box 1

TG: triglyceride

TH: tyrosine hydroxylase

TMEM26: transmembrane protein 26

UCP1: uncoupling protein 1

VEGF: vascular endothelial growth factor

vWAT: visceral white adipose tissue

WAT: white adipose tissue

YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

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Chapter1:

Introduction

Adipose tissue is a connective tissue mainly formed of adipocytes, which are cells specialized in storage of nutrients synthesized by our body or acquired from our diet [1, 2]. Mammals have several types of adipocytes that are yellow, white, brown and beige; these fat cells are dispersed under the skin and around organs and exert unique roles [3-5]. Over the last decades, the global pandemic of obesity and diabetes has renewed a particular interest in understanding white, brown and beige adipose tissue biology. Bone marrow fat also referred to as yellow fat is a type of adipose tissue located in the medullary canal of the long bones (tibia, femur and humerus) and can account for up to 70% of total bone marrow volume[6-8]. Very little is known concerning the origin of the bone marrow fat cells, but they are thought to be distinct from white, brown and beige adipocytes [9]. They contain a large unilocular lipid droplet, develop postnatally and accumulate with aging. Aberrant bone marrow adipocytes development and function has been associated with diseases such as cancer and osteoporosis [7]. This heterogenous adipose tissue is an active organ that exerts metabolic, endocrine and immune functions. Bone marrow fat has also been implicated in the regulation of hematopoiesis, bone marrow environment, and it serves as energy reservoir that can store up to 5% of total fat mass in adults [8-10]. Pink adipocytes are mammary gland alveolar epithelial cells that are formed during pregnancy and lactation [11, 12]. This fifth type of adipocyte is only found in females, and its primary function is to produce and secrete milk, although they can also secrete leptin that may help to prevent obesity in newborns. During pregnancy subcutaneous white adipocytes transdifferentiate into pink adipocytes, and in the post-lactation phase, they revert back to white adipocytes [11]. These alveolar mammary cells are characterized by abundant cytoplasmic lipids and appear pink in color during pregnancy [11, 12].

1. White adipose tissue (WAT)

1.1. Major characteristics of WAT

WAT is composed of mature white adipocytes that have triglycerides (TGs) packaged in large unilocular lipid droplets and the stromal vascular fraction (SVF), which contains immune cells, endothelial cells, preadipocytes, and progenitor cells [13]. WAT is localized to several depots that are anatomically, physiologically and patho-physiologically different; and their distribution varies among individuals, sexes and ages. They also differ in size, potential for replication and differentiation, developmental gene expression, and adipokine secretion [14, 15]. Each depot has unique properties and exerts different functions. As depicted in *figure 1*, mice have 2 major types of WAT: visceral WAT (vWAT) formed by perirenal, perigonadal and mesenteric depots, and the subcutaneous WAT (scWAT) formed by anterior and posterior depots. In humans, the major anatomical fat depots can be generally divided in 3 groups: the intra-abdominal fat that include the omental, mesenteric and pericardial depots, the upper body subcutaneous, and the lower body subcutaneous depots *(Figure 1).* In both mice and humans, there are also ectopic depots located in the liver, the muscles and the bone [15].

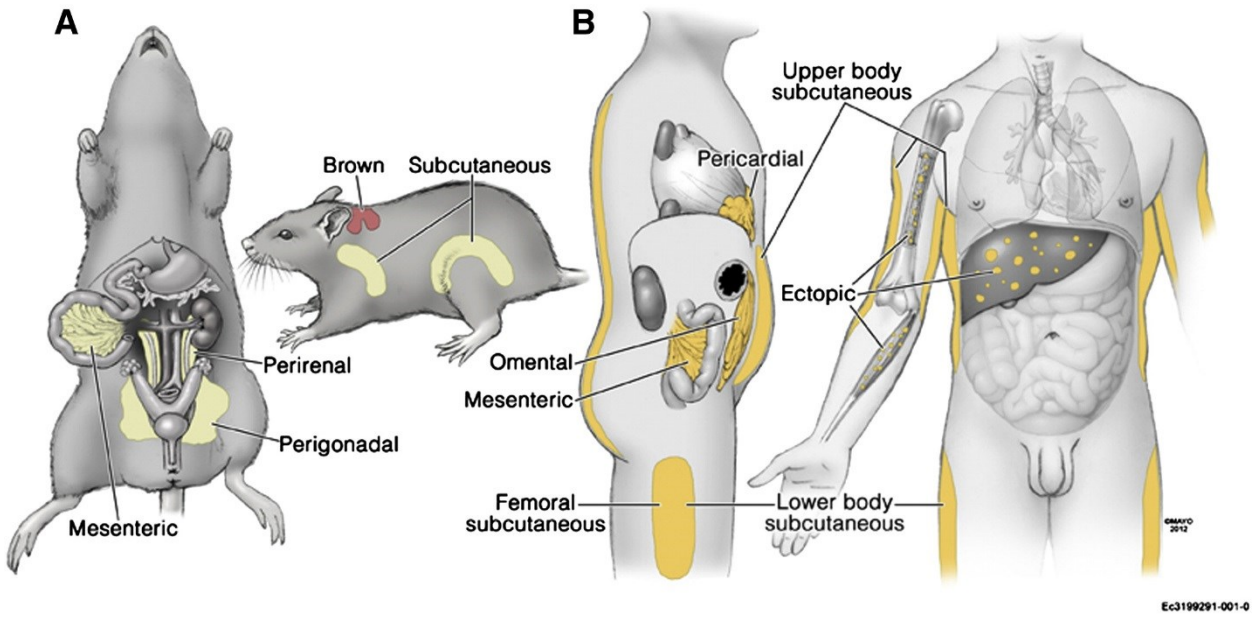

Figure 1: Anatomy of Major Fat Depots in Rodents and Humans. Several different names for particular fat depots in rodents (A) and humans (B) are used, as are different groupings of fat depots for physiological and clinical studies. Figure taken from [15].

WAT is formed in the mesoderm during embryogenesis and continues to develop postnatally [16]. Lineages studies show that white adipocytes originate from multipotent mesenchymal stem cells that express α-smooth muscle actin (αSMA) and platelet-derived growth factor receptors (PDGFRα and/or PDGFRβ) and may also be derived from endothelial cells and pericytes [17, 18]. During adipogenesis, progenitor cells and preadipocytes give rise to new fat cells in response to insulin, insulin like growth factor-1 (IGF-1), lipids, glucocorticoids and bone morphogenic proteins (BMPs) [19], while the WNT and Hedgehog signalling pathways are known suppressors of adipogenesis [20, 21]. Adipogenesis can be described as a two-step process: first, the commitment of mesenchymal precursor cells to the adipocyte lineage to form preadipocytes, and secondly, terminal differentiation of preadipocytes into mature adipocytes [22]. Commitment is stimulated by BMPs, notably BMP4 and BMP2, while terminal differentiation involves the master

adipogenic regulator, Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein alpha, beta and delta (C/EBPα, β, δ) [23]. These transcription factors act in concert with specific coactivators and corepressors to activate or repress adipocyte differentiation by regulating the expression of approximately 2500 genes including Krüppel-like transcription factors (KLFs), GATA binding proteins and activators of transcription, early B cell factors (EBFs), and interferon-regulatory factor families [23].

1.2. Physiological roles of the WAT

WAT is the major reservoir of lipids that stores and releases free fatty acids (FFAs) in response to energetic demands [24]. It is a dynamic organ that can expand through processes of hypertrophy (increase in size of adipocytes) and hyperplasia (increase in number of adipocytes) to allow lipid storage during sustained nutrient excess [22, 25]. During nutrient deprivation, hormones such as glucagon and norepinephrine (NE) stimulate lipolysis, which results in the breakdown of TGs and the subsequent release of FFAs and glycerol in circulation [26, 27]. FFAs can serve as fuel for the production of energy (ATP), heat, and they can also serve as signalling molecules that drive the release of cytokines or stimulate insulin secretion [27-29].

WAT is an important regulator of energy homeostasis and excessive fat accumulation, inflammation and fibrosis of WAT have been closely linked to many diseases, including obesity, type 2 diabetes, cardiovascular diseases, and cancer [2, 30, 31]. WAT also exerts immune, endocrine, thermal and mechanical functions. For example, it is an effector of glucose metabolism such that when blood glucose levels rise, insulin is secreted to suppress lipolysis and stimulate glucose uptake in the adipocytes [32]. Furthermore, adipose tissue is a mechanical barrier that increases insulation and thermogenesis to protect against cold [2], and it can protect delicate organs (the eye) and cushion body parts exposed to high levels of mechanical stress (the heels and toes)

[2]. WAT also releases hormones termed adipokines that act in an endocrine, paracrine and autocrine fashion to regulate systemic metabolism [28, 33-35]. These adipokines are mostly secreted by adipocytes, preadipocytes, and immune cells [33, 34]. For example, WAT releases pro-inflammatory and anti-inflammatory cytokines, such as Il-6 and TNF-α, which help fight bacterial infections and modulate insulin sensitivity [2, 15, 36]. Leptin acts centrally to increase satiety, decrease food intake, regulate body weight, and stimulate lipolysis [22]. Adiponectin sensitizes to insulin, reduces fibrosis and inflammation in peripheral organs, while FGF21 stimulates hepatic gluconeogenesis [22, 37, 38].

2. Thermogenic adipose tissues

2.1. Brown adipose tissue (BAT)

2.1.A. Anatomy and developmental origin

Brown adipocytes are phenotypically and functionally different from white adipocytes. They contain small, multilocular lipid droplets and are rich in mitochondria with abundant expression of uncoupling protein 1 (UCP1) [39]. In mice, they are found in the inter-scapular, cervical, axillary, retroperitoneal and peri-renal depots illustrated in *figure 2*. Brown adipocytes are formed during embryogenesis prior to WAT development to provide newborns with thermogenic capacities for protection against cold [40].

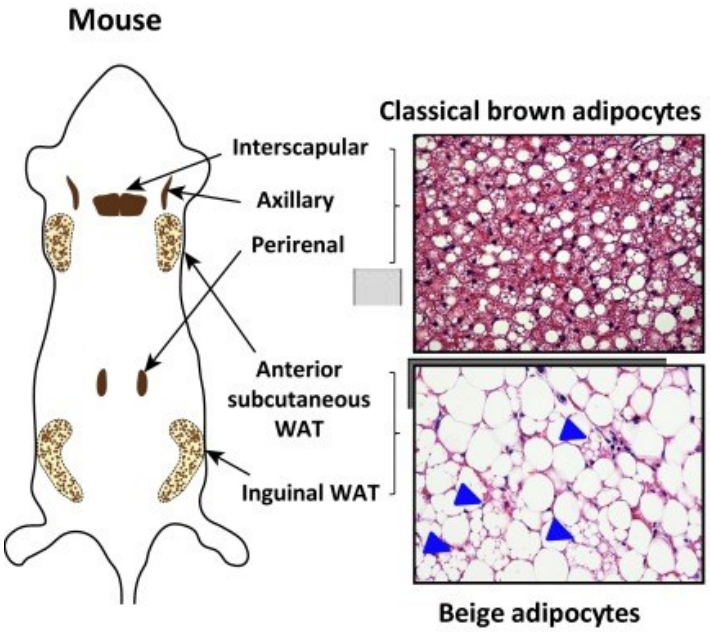

Figure 2: Anatomical locations of thermogenic fat in mice. Classical brown adipocytes reside in dedicated brown adipose tissue (BAT) depots, including interscapular, axillary, and perirenal BAT depots in mice and infants. Beige adipocytes sporadically reside in subcutaneous white adipose tissue (WAT) depots, such as the inguinal and anterior subcutaneous WAT in mice (arrowheads indicate the multilocular beige adipocytes). Figure taken from [41]

In terms of their developmental origin, brown adipocytes develop from the dermomyotomal precursor cells that express transcriptions factors Myogenic Factor 5 *(Myf5),* Paired-box protein 7 *(Pax7), Pax3,* and Engrailed 1 *(En1);* therefore, they are closely related to skeletal muscle, which also originates from *Myf5*$^{+}$ progenitor cells [42].

Brown fat development is regulated by the master regulator of adipogenesis PPARγ, which acts in concert with brown fat-specific transcription factors [43]. For example, the developmental switch between brown adipocytes and myocytes is regulated by the transcriptional regulator PR domain zinc finger protein 16 (PRDM16), which is enriched in brown fat compared to white adipocytes and skeletal muscle [42, 44]. PRDM16 is essential for brown fat development, as its overexpression in myoblasts results in the formation of brown adipocytes, whereas reduced expression of this protein in brown adipocyte precursors resulted in a loss of brown adipocyte characteristics and induction of muscle differentiation [42, 44]. The capacity for PRDM16 to repress muscle differentiation is completely dependent on EHMT1, a histone-N- methyltransferase co-regulator that physically interacts with PRDM16. Genetic loss of *Ehmt1* in *Myf5*$^{+}$ precursor cells impairs brown adipocyte differentiation and promotes muscle differentiation [45]. PRDM16 forms a transcriptional complex with PPARγ, C/EBP-β and early B cell factor 2 (EBF2) to stimulate expression of genes important for brown phenotype [46]. EBF2 functions as a marker of brown fat precursor cells and establishes brown fat characteristics of BAT by recruiting PPARγ to the brown-selective genes promoters [47]. Accordingly, EBF2-deficient mice lose brown-specific characteristics and thermogenic capacity of their BAT [47, 48]. Prior to differentiation, progenitor cells are committed to the brown adipocyte lineage, and this process is stimulated by BMP7, a member of the transforming

growth factor β family of proteins. BMP7 expression in brown adipocyte precursors is driven by the RNA binding protein EWS, which associates with the transcription factor YBX1 [49]. ZFP516, another PRDM16-interacting partner is also required to suppress the expression of muscle-specific genes during BAT development. Conversely, many transcription factors, including ZFP423, FOXO1, TWIST1, p107, LXRA, pRB, and RIP140, act as repressors of brown adipocyte differentiation [50]. *Figure 3* is a brief overview of the transcriptional regulation of brown adipogenesis.

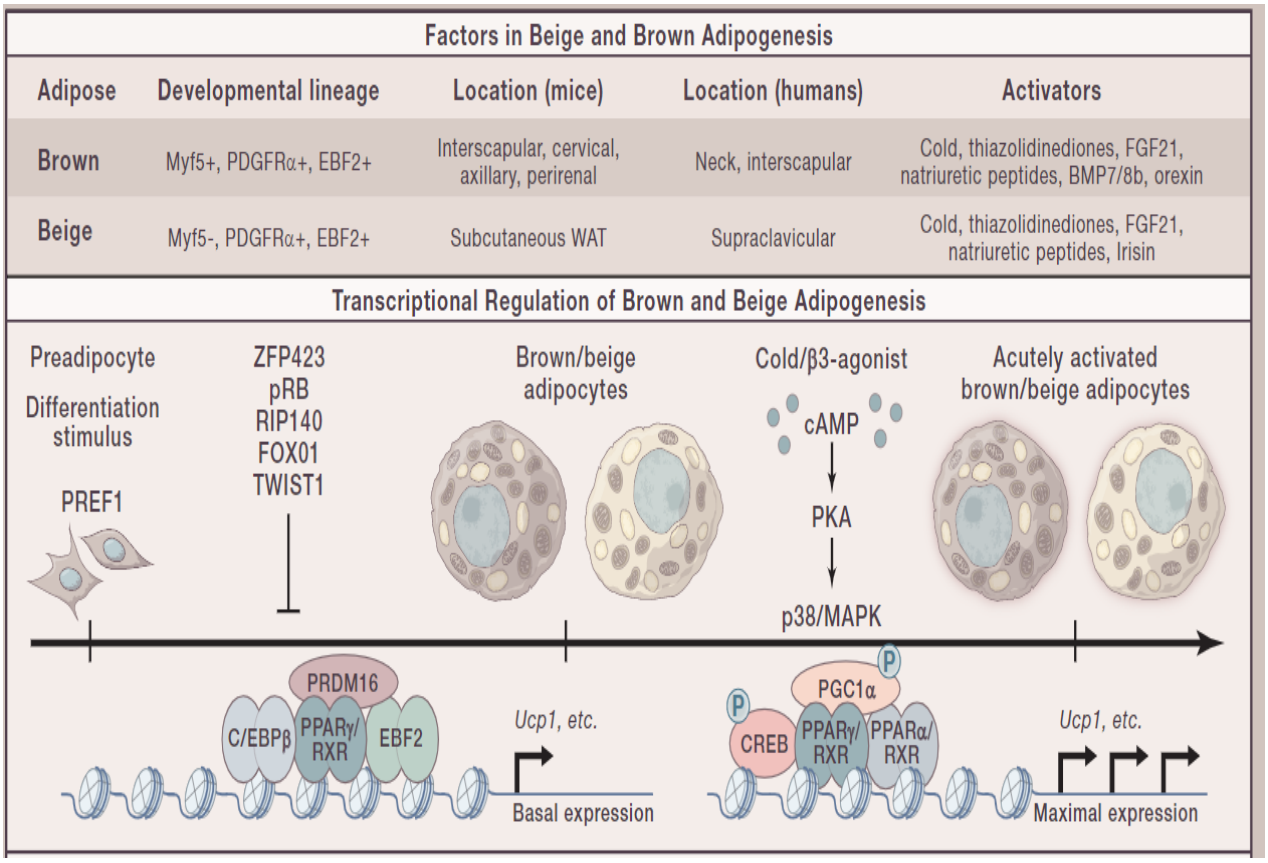

Figure 3: **Factors in beige and brown adipogenesis and transcriptional regulation of brown and beige adipogenesis.** Figure taken from [39]

BAT mass is dynamic and can undergo atrophy or hypertrophy in response to environmental and internal cues. The growth of BAT is principally stimulated by cold exposure and adrenergic stimuli, both of which promote proliferation and *de novo* differentiation of progenitor cells to increase thermogenic capacity [51]. β-Adrenergic signalling has been directly shown to induce the proliferation of EBF2 and platelet-derived growth factor receptor-α (PDGFRα) positive precursor cells [52]. Brown adipocytes express specific genes markers including *PRDM16, PGC1A, CIDEA, ZIC1 AND PDK4* [53].

2.1.B. Regulation of BAT function

BAT is highly vascularized and innervated by post ganglionic sympathetic nerves [54, 55], and it is activated in response to cold stimuli, beta adrenergic agonists, FGF21, and T3 [51, 56, 57]. Cold is sensed by peripheral tissues such as the skin, spinal cord, abdominal viscera and the brain itself. In general, the preoptic area (POA) is the most thermosensitive site of the brain and is located between the anterior commissure and optic chiasm. When cool temperatures are sensed in this brain region, it activates the sympathetic nervous system (SNS) and causes the release of NE from sympathetic nerve terminals in BAT [58]. NE then binds to β3 adrenergic receptors (β3-AR), triggering the activation of adenylate cyclase, cAMP production, and the activation of protein kinase A (PKA), which phosphorylates perilipin and hormone sensitive lipase (HSL) to increase lipolysis. The released FFAs activate UCP1 and are oxidized in mitochondria to serve as an energy source for thermogenesis *(see Figure 4)* [54, 59-61]. In parallel, PKA phosphorylates CREB and p38MAPK leading to the activation of PGC1α [59, 62]. This latter coordinates with PPARγ and PPARα to stimulate the transcription of *UCP1* [63, 64].

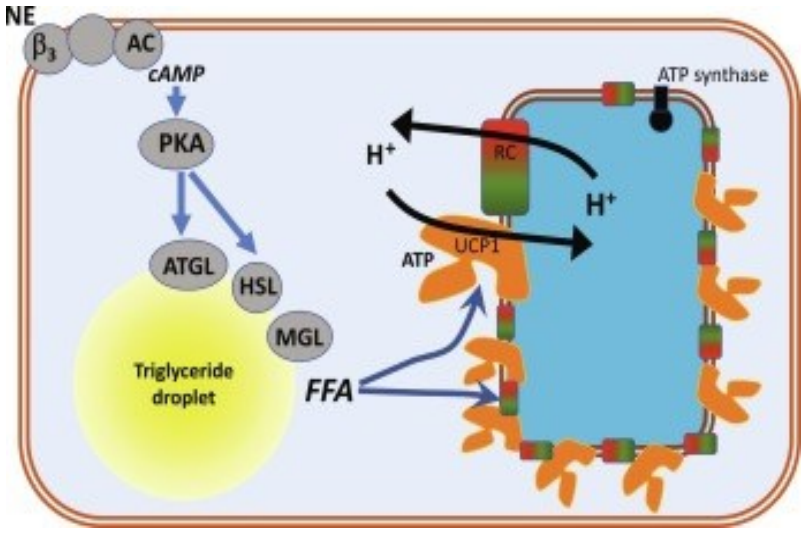

Figure 4: An overview of the heat-producing pathway in brown adipose tissue. Norepinephrine (NE) binds to β3-adrenergic receptors (β3) that stimulates adenylyl cyclase (AC) leading to cyclic adenosine monophosphate (cAMP) production. This activates Protein Kinase A (PKA) that will activate adipose tissue triglyceride lipase (ATGL) to break down triglycerides to diglycerides and free fatty acids (FFA). PKA will also activate hormone-sensitive lipase (HSL) that will break down the diglycerides to monoglycerides and more fatty acids; the last fatty acid will be released from the monoglycerides through monoglyceride lipase (MGL) activity. The fatty acids will counteract the inhibitory effect of cytosolic adenosine triphosphate (ATP) on Uncoupling Protein-1 (UCP1). This means that protons (H^+) that were pumped out of the mitochondria by the respiratory chain (RC) can re-enter the mitochondria and respiration can therefore proceed unhampered by the mitochondrial membrane potential. Figure taken from [51]

Thermogenesis rapidly depletes the limited brown fat stores, and a supplemental fuel is required to sustain adaptive thermogenesis. Thus, in addition to using its own lipid stores, brown adipocytes can take up fatty acids and glucose from the circulation to sustain thermogenesis [60, 65, 66]. NE released during cold also increases vascular endothelial growth factor (VEGF) [67] and lipoprotein lipase (LPL) expression [68, 69]. These two proteins, along with CD36, work

together to increase FFAs uptake in BAT. VEGF increases capillary permeability for plasma TGs, and LPL degrades TGs into FFAs transported through the plasma membrane by CD36, increasing FFAs availability for combustion by BAT. Glucose uptake in BAT after cold exposure, sympathetic nerve stimulation, and β-AR agonism has also been reported in several *in vivo* studies [60, 65, 66]. For example, NE stimulates the translocation of glucose transporters (GLUTs) from intracellular stores to the plasma membrane in mouse brown adipocytes, independent of insulin [70]. Both GLUT1 and GLUT4 are located on the plasma membrane of brown adipocytes, and GLUT4 expression is increased in BAT of rats fasted before chronic cold exposure to minimise any effect of insulin [71]. β3-adrenoceptors can also stimulate glucose uptake in BAT via cAMP-mediated increases in GLUT1 transcription and *de novo* synthesis and via mTORC2-stimulated translocation of GLUT1 [72]. Although glucose is an important contributor to BAT activity, FFA is the major substrate for thermogenesis [73], and it is required to maintain UCP1 function [74]. Succinate, another metabolite in the tricarboxylic cycle, also activates energy expenditure in the BAT, and in comparison to other fat cells, brown adipocytes largely uptake circulating succinate to serve as fuel for thermogenesis [75]. In addition to Glucose and FFAs, BAT can also use other nutrients such as branched-chain amino acids (BCAAs) to drive thermogenesis. In response to cold, BCAAs (leucine, isoleucine, valine) are transported and oxidized in the mitochondria to produce heat [76]. Increased circulating levels of BCAAs have been linked to obesity and diabetes and BCAA clearance by BAT may have implications in these disease states [76, 77]. In fact, impaired BCAA catabolism in BAT was shown to induce obesity and glucose intolerance [76] .

2.2. Beige fat

2.2.A. Induction and maintenance of beige fat

In mice kept at thermoneutrality (30 ^{o}C) or room temperature (22 ^{o}C), WAT is formed mostly of white adipocytes. However, when exposed to chronic cold or long-term treatment with β3-AR agonists, beige adipocytes appear in subcutaneous depots during a process termed browning or beiging [41, 78-80] illustrated in *Figures 2 and 3*. Beige adipocytes can be also formed in response to several other external or internal stimuli including caloric restriction or manipulation, exercise, cancer cachexia, bariatric surgery, tissue injury, and FGF21 [80]. Beige adipocytes phenotypically resemble brown adipocytes, but they are inducible thermogenic fat cells. They are rich in mitochondria and express high levels of UCP1, albeit at lower levels when compared to brown adipocytes [39]. Beige adipocytes express specific markers including *UCP1, PGC1A, PRDM16, CITED1, CD137, TMEM26, AND TBX1* [53]. Recent studies, however, suggest that there are at least 2 subtypes of beige adipocytes that are positive or negative for UCP1 [81].

Similar to BAT, the SNS is strongly involved in the development and function of beige fat. Beige adipocytes are recruited by NE [80]; however, other pathways independent of the SNS have been shown to stimulate beiging. For example, alternatively activated macrophages residing in adipose tissue synthesize and release catecholamines. These M2 anti-inflammatory macrophages are recruited to the scWAT and secrete NE to activate BAT and induce beige adipocyte development [82]; notwithstanding, data against this hypothesis has also been demonstrated [83].

Beige adipocytes are transient, and when mice are returned to thermoneutrality or room temperature, beige adipocytes gradually disappear from the WAT. Some studies have shown that

they can revert to unilocular white adipocytes within approximately 2 weeks following re-warming or withdrawal of the β3-AR agonist [78, 84, 85]. This process is thought to be mediated by an increase in mitochondrial degradation or mitophagy [41, 78, 86]. $UCP1^{+}$-adipocyte-specific deletion of *Atg5* or *Atg12* is essential for autophagosome formation and prevents beige adipocytes from reverting back to a white phenotype even after withdrawal of external stimuli [78]. In addition, Parkin is a mitochondrial E3 ubiquitin ligase regulated by the β3-AR and PKA signaling pathway, and its recruitment to the mitochondria is essential for the activation of mitophagy in beige fat and the maintenance of beige adipocyte thermogenesis *in vivo* [86].

2.2.B. Different origins of beige adipocytes

The cellular origins of beige adipocytes and the mechanism underlying their formation remain unclear and have been source of much debate. To date, some studies have reported *de novo* differentiation from resident precursor cells [87, 88], and several observations demonstrated a trans-differentiation model during which mature white adipocytes directly convert into functional beige adipocytes [89-91]. Altogether, the available data suggest that both *de novo* differentiation and trans-differentiation contribute to beige fat biogenesis [80]. Beige fat is heterogeneous, as it is formed by multiple subtypes of thermogenic adipocytes with distinct developmental origins and biological roles [41]. It has been proposed that beige adipocytes are related to vascular smooth muscle cells and mural cells [92]. Some lineage-tracing studies have demonstrated that some beige adipocytes are derived from progenitors expressing *Sma,* myosin heavy chain 11 *(Myh11), Pdgfra,* or *Pdgfrb (see Figure 3),* while some beige adipocytes residing in subcutaneous or retroperitoneal WAT depots are derived from progenitors expressing *Pax3* and/or *Myf5* [39]. In response to long term cold exposure, $Myh11^{+}$ muscle cells and mural cells, which express *Pdgfrb,* can differentiate

into beige adipocytes [92]. Similar to brown adipocytes, PRDM16 is also a key transcriptional regulator of beige adipocyte biogenesis and its expression in vascular smooth muscle cells promotes their differentiation into beige adipocytes [52]. Another transcription factor involved in beige adipogenesis is EBF2 whose expression in WAT or primary adipocyte cultures promotes the recruitment of beige adipocytes [47]. A recent study of Chen *et al.,* shows beige adipocytes in murine iWAT originate from a unique population of myoblast determination protein 1 (*Myod1*$^{+}$) progenitors, referred to as *Myod1*$^{+}$-derived beige fat. These *Myod1*$^{+}$-derived beige adipocytes accounted for about 15% of UCP1^{+} beige adipocytes in the iWAT, which supports the hypothesis of several progenitor cells responsible for beige adipocytes development. The *Myod*$^{+}$ derived beige fat is composed of glycolytic beige adipocytes that display enhanced glucose metabolism when β-AR signalling is blocked or during severe and prolonged cold. They are therefore distinct from classic beige adipocytes activated by β-AR signalling [93].

2.3. Human brown and beige adipocytes

The presence of brown adipocytes in mammals has been described since 1550, and their importance in heat production has been known for over 40 years [73]. In humans, inter-scapular BAT (iBAT) was known to be active in infants *(see figure 5)* and to regress with aging [94]. Research on BAT has re-initiated in recent years (2007-2009) when positron emission tomography-computerized tomography (PET-CT) scans in adult humans revealed the presence of several BAT depots displaying high uptake rates of the glucose analog 18F-fluoro-2-deoxy-glucose (^{18}F-FDG) [95-98]. BAT activity in humans is stimulated by cold, and no glucose uptake can be detected in study participants kept under warm conditions [98]. It has also been suggested that outdoor workers exposed to a cold environment have a higher amount of BAT than the general

population [99]. In humans, BAT and beige fat consist of small discrete depots principally located in the upper body including the neck area, the supraclavicular depots, suprarenal, paravertebral, and paraaortic depots depicted in *Figure 5* [100]. PET-CT imaging studies show women have greater quantities of BAT and higher expression of thermogenic genes than men, while young persons, in particular newborns, possess more BAT than adults [101, 102].

Human BAT depots are heterogeneous and are thought to resemble both murine classical BAT and inguinal beige adipocytes [53, 80]. For example, Wu *et al.,* have demonstrated that some brown fat in adult humans, notably the supraclavicular depot, has similar molecular characteristics to murine beige adipocytes, while infant iBAT and the deep neck regions in adult humans contain thermogenic fat that resembles classical brown fat in mice [53, 80]. Surprisingly, human and mice have opposing patterns of brown markers expression in vWAT versus scWAT [103]. In rodents, exercise increases beiging of scWAT and improves whole body metabolic homeostasis through the effects of the myokine irisin. However, exercise does not seem to affect human beige fat and is associated with a decreased concentration of circulating irisin [104]. Most knowledge of BAT and beige fat is from rodent studies; consequently, mechanisms underlying human thermogenic fat cells development and their significance to metabolism still remain to be elucidated.

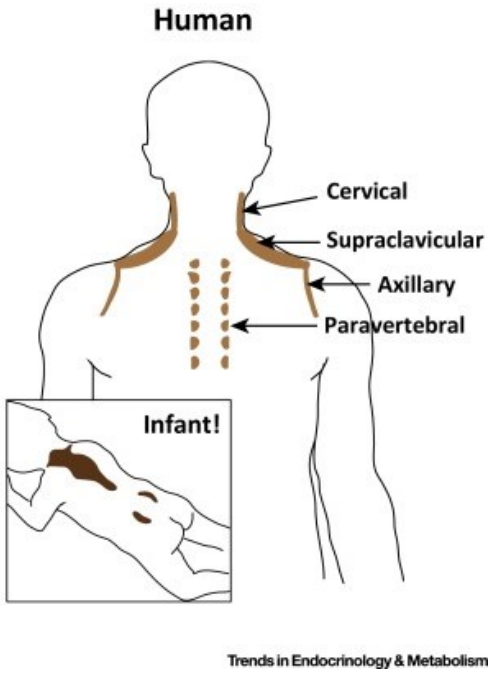

Figure 5: Anatomical locations of thermogenic fat in humans. In adult humans, BAT is present in multiple locations, including cervical, supraclavicular, axillary, paravertebral, and abdominal subcutaneous regions. UCP1-positive adipocytes from the supraclavicular region show a molecular signature resembling that of mouse beige adipocytes, whereas the deep neck regions contain thermogenic fat that resembles classical brown adipocytes in mice. Figure taken from [41]

2.4. Physiological role of brown and beige adipocytes

The principal role of brown adipocytes is to dissipate chemical energy in the form of heat. BAT is the principal site of non-shivering thermogenesis (NST), a mechanism that defends the body against hypothermia by maintaining core body temperature in the physiological range [51, 73, 105, 106]. Although BAT only represents a small portion of the total body mass in large mammals and adult humans, it is responsible for at least 60% of NST [39]. BAT also produces several "batokines" that exert autocrine, paracrine and endocrine functions and, thus, play important roles in regulating glucose homeostasis, bone and muscle function [138]. For example, BAT produces neuregulin 4 (NRG4) which reduces fat storage in the liver to improve peripheral

insulin sensitivity [107]. Brown and beige fat also secrete WNT10b and insulin-like growth factor-binding protein 2 (IGFBP2) to promote osteoblast activity and bone health [108]. Differentiated brown adipocytes or BAT from newborn and adult rodents exposed to cold for 1–2 days were found to express high levels of nerve growth factor (NGF). This growth factor can increase proliferative capacity of BAT by increasing mitosis of precursor cells in the SVF [109].

In mice, beige adipocytes represent a small percentage of iWAT and are considered to have a negligible role in whole-body energy expenditure [110, 111]. However, recent studies have shown that beige fat may play key roles in the regulation of whole-body energy homeostasis, as well as inflammation and fibrosis of WAT [126, 139]. Beige adipocytes support adaptive thermogenesis by generating heat in response to cold, and they can regulate glucose metabolism even in the absence of adrenergic stimuli [106] and improve adipose tissue function by decreasing fibrosis and inflammation [139]. Beige adipocytes secrete a PRDM16-regulated factor named Slit2, a member of the Slit extracellular protein family. Increased levels of Slit2 promote PKA activation and consequently augment the thermogenic activity of beige adipocytes and improve glucose homeostasis [140]. In humans, plasma SLIT2 is negatively correlated with serum glucose and HbA1c in diabetic individuals [141]. The study of beige fat is quite recent, and many characteristics of these adipocytes remain to be elucidated. One of the grey areas in beige fat biology is the determination of its mass, which is limited by current technology. In addition, beige adipocytes are transient fat cells that are recruited in response to external or internal stimuli. Unlike WAT and BAT which are homogenous and distinct adipose depots, beige fat is heterogenous and dispersed within scWAT [41, 43, 80]. In humans, some depots of beige fat are hardly distinguishable from classical BAT [53], and this has made it challenging to accurately determine beige fat mass. In a study by Blondin *et al.,* BAT mass is estimated to represent ~1% of total body

weight in adult humans [112]. However, this study could not discriminate between human brown fat that is similar to murine beige adipocytes or classical brown adipocytes. This suggests that in mice and humans, beige fat might represent less than 1% of total body weight [112].

3. Therapeutic potential of brown and beige fat

Obesity and diabetes are global pandemics that affect many social classes and demographics. The prevalence of obesity has remarkably increased over the last 50 years [113]. Currently, obesity affects over a third of the world's population, and it is expected by 2030 that an estimated 38% of the world's adult population will be overweight of which 20% will be obese [114, 115] . Changes in diet and physical activity are generally the first lines of treatment adopted to combat obesity; however, there are other therapeutic options available including weight loss medications and bariatric surgery [116, 117]. Bariatric surgery is effective, but it is expensive and cannot be afforded by most patients. In addition, it is a selective process that is generally performed on patients with morbid obesity, as defined by a body mass index (BMI) greater than 40 [117]. As of 2017, only 6 medications had been approved for the treatment of obesity [118]. These drugs include Orlistat, which reduces fat absorption by inhibiting gastrointestinal lipases, and Liraglutide, a GLP-1 agonist that decreases appetite and food consumption by slowing gastric emptying and increasing post prandial satiety. Phentermine and Lorcaserin, a serotonin receptor agonist, are also available for weight loss management [118-121].

To combat obesity, it is important to develop new pharmacological treatments accessible to more subjects with obesity. More reports of the potential power of brown and beige fat to combat metabolic disorders are culminating. Obesity results from an imbalance in energy homeostasis with energy expenditure being inferior to energy intake [101]. With their ability to regulate fat mass by increasing energy expenditure through lipid oxidation, brown and beige adipocytes

represent promising targets for reducing the excessive fat accumulation that defines obesity [122]. BAT has been also shown to positively impact whole-body metabolism. In addition to clearing stored triglycerides, these thermogenic adipocytes have a high capacity for glucose disposal allowing them to be potential targets in the treatment of diabetes [68]. It has also been demonstrated that the ability of BAT to efficiently dispose of stored energy allows a mouse exposed to cold to eat 3–4 times that of a mouse at thermoneutrality without becoming obese [56]. Although UCP1-deficient mice do not develop obesity under normal chow diet, they gain significantly more weight during a high fat diet (HFD) compared to WT mice [123]. Furthermore, mice with surgical removal or transgenic ablation of BAT become obese supporting a role of brown fat in the development of obesity [124, 125]. Additionally, inducing BAT angiogenesis in obese mice improves glucose metabolism [126, 127].

Recent studies in rodents have reported beneficial effects of beiging by conferring protection against obesity and diabetes. An elegant study by Seale *et al.* demonstrated improved glucose tolerance and insulin sensitivity in transgenic *Ap2-Prdm16* mice due to enhanced beige fat formation compared to littermate controls. The Ap2-*Prm16* mice also gain significantly less body weight and adiposity under HFD conditions. In contrast, beige fat-deficient mice, caused by the adipocyte-specific deletion of *Prdm16* or *Ehmt1*, develop obesity and systemic insulin resistance even under ambient temperatures [45, 128]. In adult humans, increased brown fat is correlated with low BMI and low adipose tissue content [129], and in contrast, subjects who are overweight or with obesity have decreased BAT activity compared to lean individuals [130, 131].

By lowering stored lipids and improving glucose metabolism, it is valid to think that increasing BAT activity and stimulating beiging in adult humans can represent potential treatments for obesity, diabetes and the metabolic syndrome [56]. To date selective β3-AR agonists that

activate adaptive thermogenesis have been trialed as a treatment for obesity but were unsuccessful. In fact, these agonists are potent activators of the cardiovascular system and increase the risk of developing higher blood pressure and cardiovascular diseases [132]. Consequently, identifying β-AR independent signalling pathways with minimal cardiovascular risks that activate BAT and promote beiging could lead to more effective therapeutic treatments of metabolic disorders.

4. Mechanisms of adaptation to cold

Adaptation to cold is a complex process that involves several physiological, behavioural and genetic responses [133]. As described previously, brown and beige adipocytes are fat cells specialized in heat production or thermogenesis and thus play a major role in adaptation to cold environments. Thermogenesis regulates body temperature through two involuntary, mostly autonomic, physiological processes that dissipate heat and include adaptive thermogenesis primarily mediated by brown and beige fat and skeletal muscle shivering [58, 101, 134, 135].

4.1.Non-shivering or adaptive thermogenesis

Adaptive thermogenesis, also known as non-shivering thermogenesis (NST) or facultative thermogenesis, is cold or diet-induced heat generation to defend against cold and to regulate energy balance after changes in diet.

The brain regulates adaptive thermogenesis by activating the SNS, which heavily innervates BAT and WAT [54]. Inhibiting the SNS with adrenergic blockers during cold exposure causes a drastic fall in body temperature; while mimicking its effects with administration of NE, L-DOPA, or isoproterenol increases energy expenditure and thermogenic protein expression in the iWAT and the BAT [136]. Furthermore, Dopamine β-hydroxylase knockout mice lacking noradrenaline and adrenaline are unable to induce thermogenesis in BAT when exposed to cold [136, 137]. The brain can also regulate adaptive thermogenesis by activating the hypothalamus-pituitary-thyroid axis [105, 138]. The mechanisms through which thyroid hormone controls thermogenesis and energy balance are unclear, but is likely via substrate and ion cycling and mitochondrial proton leaks [139]. During adaptive thermogenesis, adrenergic stimuli increase the expression of DIO2 that converts T4 into T3, the ligand for thyroid-hormone receptor TRβ. This

is consistent with the hypothesis of a thyroid-sympathetic axis synergism that potentiates adaptive thermogenesis [140, 141].

Several other molecules have been shown to activate adaptive thermogenesis in murine models [51, 142]. For example, PPARγ ligands such as rosiglitazone and FFAs can control adaptive thermogenesis. Some studies demonstrated that rosiglitazone can activate *Ucp1* expression in BAT and brown adipocyte cultures [143, 144]. Atrial natriuretic peptide (ANP) is released from the heart and can activate brown fat cells via its signaling receptor NPR-A, that activates guanyl cyclase and elevates cyclic guanosine monophosphate (cGMP). NPR-A has been shown to increase lipolysis via cGMP in human adipose tissue and isolated human adipocytes [145]. Furthermore, cardiac natriuretic peptides also act via p38MAPK to induce the brown fat thermogenic program in mouse and human adipocytes [146]. Another activator of NST is a peptide named fibroblast growth factor 21 (FGF21). The predominant source of FGF21 is the liver although both WAT and BAT release this growth factor in response to chronic cold exposure [147]. FGF21 is markedly increased in BAT of UCP1-ablated mice and is thought to increase glucose uptake and thermogenesis in brown and beige adipocytes via its receptor complex, consisting of FGFR1 and its obligatory co-receptor β-klotho [148]. Several BMPs have also been shown to play a role in brown fat development and activity. For example, BMP7 induces the differentiation of brown preadipocytes, while BMP8b seems to work together with NE to enhance lipolysis and thermogenic activity [149]. Finally, retinoic acid has also been shown to acutely activate UCP1 both in intact brown fat mitochondria and in isolated brown fat cells [51].

4.1.A. UCP1-dependent thermogenesis

UCP1, also known as thermogenin, is the primary protein that mediates heat generation during adaptive thermogenesis [51, 73]. In BAT, UCP1 is present at very high levels even during the non-stimulated state and resides in the inner mitochondrial membrane to uncouple the mitochondrial proton gradient from ATP synthesis *(Figure 4)* [73]. UCP1 functions as a H^+ transporter that requires FFAs. In this sense, FFAs are regarded as cofactors of UCP1 that allows H^+ transport by two distinct mechanisms, as illustrated in *figure 6*. In the first, FFAs function as H^+ donors to the UCP1 translocation channel *(model A,B,C of figure 6)* [74] and in the second, UCP1 does not transport H^+, but rather FFA anions outside the mitochondrial matrix. Once outside, FFA anions are re-protonated and flip flop back in the inner mitochondrial membrane transferring protons inside the mitochondrial matrix *(model D of figure 6)* [150].

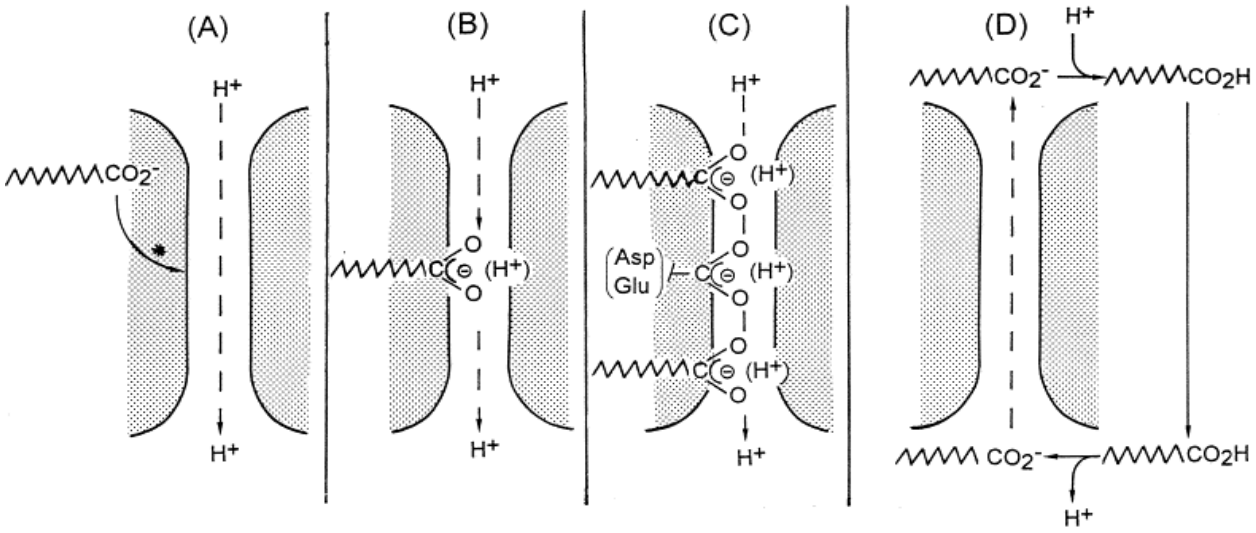

Figure 6: Variant models for the role of fatty acids (FAs) in H^+ transport by UCP1. (A) Conformational activation of the H^+ transport pathway by FA. (B) Cofactor role of FA by providing H^+ translocating groups. (C) Cofactor role of FA by providing H^+ translocating groups in addition to resident groups (Asp/Glu), where FAs fill gaps in the H^+ translocating path. (D) FA anion transport shuttle. FAs are translocated as anions by UCP1 and re-shuttle though the membrane as undissociated acids. Figure taken from [74]

Several studies have shown the prominent role of UCP1 in adaptive thermogenesis. UCP1-deficient mice have pronounced cold sensitivity and can only survive severe, long-term cold at 4 ºC through gradual reductions in environmental temperature [123]. Acute cold exposure of four hours or less is enough to increase UCP1 activity and mRNA expression, while chronic cold exposure of several hours or days will cause a significant elevation in the transcriptional coactivator PGC1α, which coordinates with PRDM16 to stimulate UCP1 protein and mitochondrial biogenesis [79, 106]. UCP2 and UCP3 are homologues of UCP1 expressed in BAT and other tissue types, such as the skeletal muscle [151, 152]. Although they have proton transport activity, their expression remains inconsistent in cold exposed rodents; therefore, their importance in adaptive thermogenesis remains unclear [153-155].

4.1.B. UCP1-independent thermogenesis

Recent data show that UCP1 is dispensable for adaptive thermogenesis as thermal homeostasis can be maintained without UCP1 [41]. In support of this claim, UCP1-deficient mice can be acclimated to cold by slowly decreasing the temperature [123]. In addition, discordance in the metabolic phenotypes between brown/beige fat-deficient mice and UCP1-deficient mice has suggested the existence of UCP1-independent mechanisms of adaptive thermogenesis [41]. For example, BAT-deficient mice, caused by the transgenic expression of diphtheria toxin (DTA), or beige fat deficient mice due to adipocyte-specific deletion of PRDM16 or EHMT1, exhibit obese and diabetic phenotypes even under ambient temperature [45, 128]. By contrast, UCP1-deficient mice are not diabetic and develop obesity only when they are kept at thermoneutrality [123, 156]. PRDM16 Tg-UCP1-/- mice (PRDM16 transgenic mice crossed with UCP1-/- mice) are resistant

to HFD induced weight gain and have improved glucose tolerance and insulin sensitivity when compared to UCP1 -/- control mice [41, 157]. This implies that the anti-obesity and anti-diabetic effects of beige fat are UCP1-independent.

To date, two thermogenic mechanisms have been described through which beige adipocytes can contribute to the regulation of thermal and energy homeostasis, independent of UCP1. These mechanisms are ATP-dependent and prominent in beige fat, however, they are not relevant to BAT function [81, 157-160]. Ikeda *et al.*, identified a thermogenic mechanism in beige adipocytes that involves ATP-dependent Ca^{2+} cycling between sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) and ryanodine receptor 2 (RyR2). Ca^{2+} cycling is a conserved mechanism present in both adult humans and mice. The mechanisms underlying Ca^{2+} cycling in beige adipocytes are not fully understood but is triggered by the binding of NE to α and β-AR, which in turn activates SERCA2b and RyR2 and leads to increased intracellular Ca^{2+} flux. Ca^{2+} is transported in the ER by SERCA2b and this non-canonical form of thermogenesis occurs when Ca^{2+} transport is uncoupled from ATP hydrolysis by SERCA2b.

A futile creatine cycle has also been identified as an alternative beige fat mechanism that results in increased heat production during cold exposure [81, 158, 159]. Creatine metabolism is an important part of adaptive thermogenesis that regulates energy expenditure in both brown and beige adipocytes. The creatine driven substrate cycle promotes adaptation to cold by stimulating mitochondrial respiration when ADP is limiting [81, 158, 159]. Indeed, creatine reduction diminishes oxidative properties of mouse and human brown adipocytes and, decreases core body temperature in UCP1-/- mice [158]. Furthermore, adipose tissue specific KO of glycine amidinotransferase (GATM), the first and rate-limiting enzyme of creatine biosynthesis increases body weight and fat mass during diet-induced obesity [158]. Another line of evidence supporting

this mechanism is that ablation of UCP1-dependent thermogenesis is compensated by an increase in genes involved in creatine metabolism [158]. In the model presented by Kazak *et al.,* creatine facilitates the regeneration of adenosine diphosphate (ADP) through the futile hydrolysis of phosphocreatine either by a phosphatase or via several phosphate-transfer reactions catalyzed by multiple enzymes depicted in *Figure 6*. Creatine-driven thermogenesis is present in beige adipocytes regardless of their UCP1 expression but is more prominent in epididymal beige adipocytes that do not express UCP1 [158]. This mechanism might co-exist in the beige adipocytes with UCP1-dependent thermogenesis, and Ca^{2+} cycling [81].

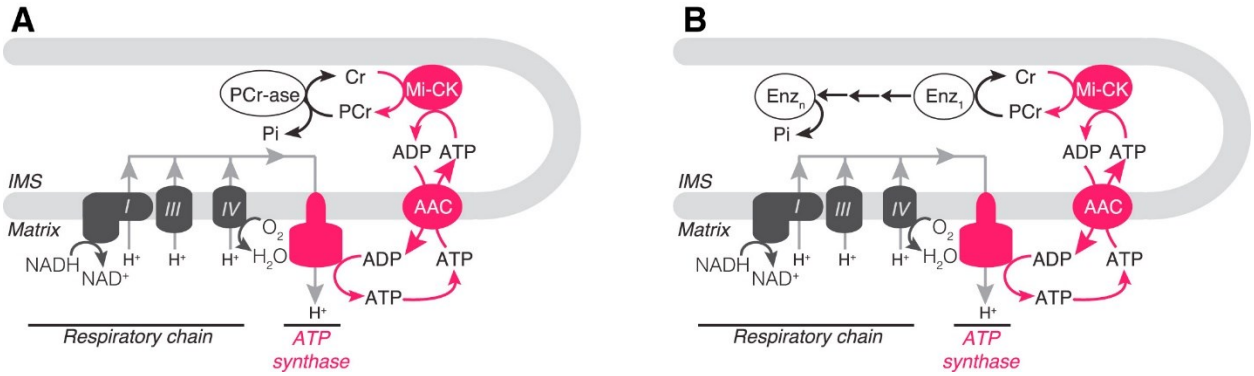

Figure 7: Models of Creatine-Driven Futile. Substrate Cycling. (A) Model of creatine-driven futile substrate cycling based on direct hydrolysis of phospho-creatine (PCr). (B) Model of creatine-driven futile substrate cycling based on multiple phospho-transfer events catalyzed by multiple enzymes (Enz). Creatine (CR), mitochondrial creatine kinase (miCK), ATP/ADP carrier (AAC), intermembrane space (IMS). Figure taken from [158]

4.1.C. Diet-induced adaptive thermogenesis

Diet-induced adaptive thermogenesis is activated in response to changes in diet, and it has been hypothesized to protect against intake of surplus energy [106]. In humans or rodents, a

positive-energy balance induced by excessive caloric intake will cause BAT activation to increase energy expenditure in order to limit weight gain [161]. Many mouse models of obesity and leptin-deficient *ob/ob* mice display decreased BAT mass and activity [162, 163]. In addition, mice with ablated or a reduced amount of BAT become obese, as well as diabetic and hyperlipidemic [124, 125]. Diet-induced adaptive thermogenesis is thought to be mediated by leptin which increases SNS activity to brown fat [164]. Central and peripheral administration of leptin have been shown to increase UCP1 mRNA and protein levels, and this effect is decreased when leptin levels fall due to starvation [165, 166].

4.2. Shivering Thermogenesis

Skeletal muscle (SKM) plays an important role as a thermogenic organ. SKM represents 40% of total body weight and plays an essential function in regulating the response to acute cold [102]. Within minutes of exposure to cold, heat is produced from the involuntary contractile activity of muscles, which is also known as shivering [5].

As pictured in *figure 8*, shivering is controlled by many brain regions including the lateral parabrachial nucleus (LPB), the preoptic area (POA), the dorsomedial hypothalamus (DMH), and the rostral raphe pallidus (rRPA) [6-9]. However, the circuitry that connects these brain structures and the precise pathway that leads to motor neuron activation are unclear. It has been proposed that thermal information is received and integrated into the POA and then transmitted to effectors through a descending pathway that exits the brain via the rostral medulla. These medullary output neurons then activate peripheral sympathetic neurons in the case of adaptive thermogenesis and somatic motor neurons in the case of shivering to mediate cold tolerance [167].

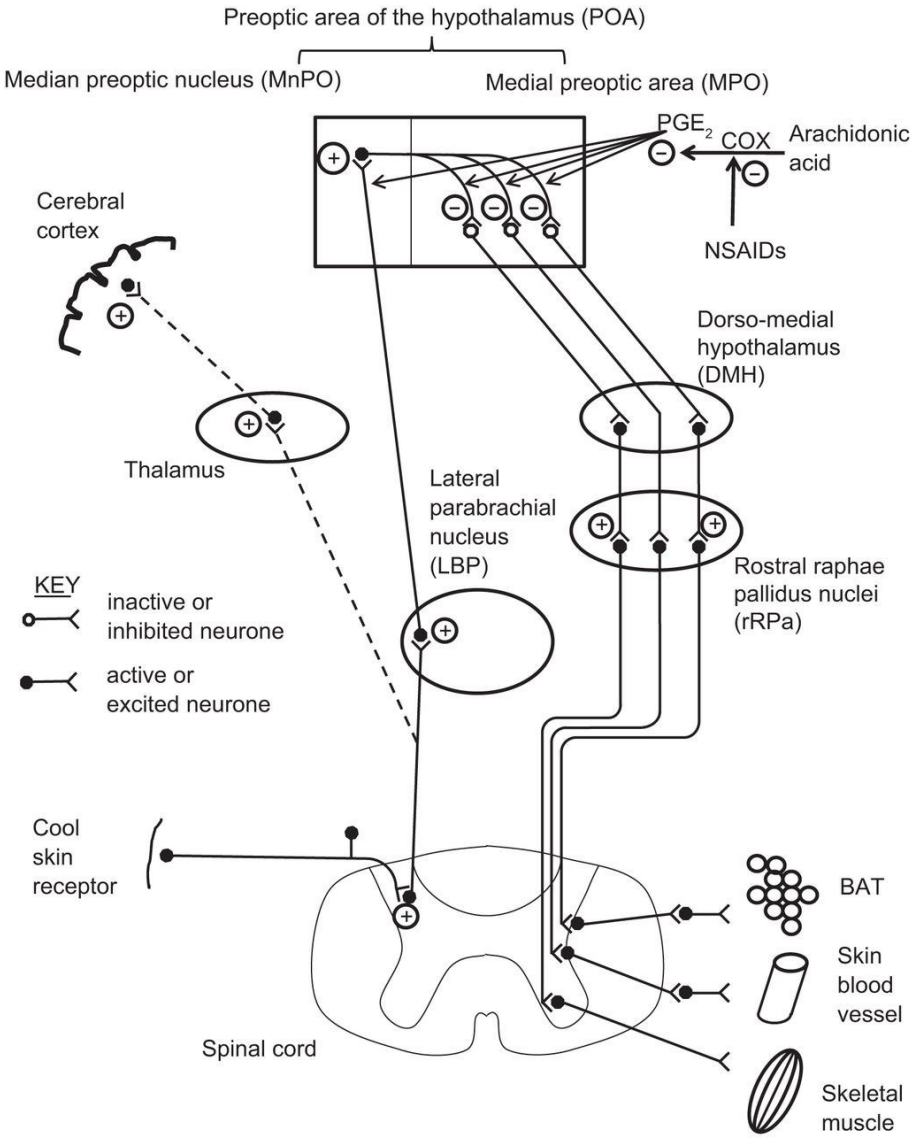

Figure 8: Central circuitry mediating the response to cold. POA, preoptic area of the hypothalamus; MnPO, median preoptic nucleus; MPO, medial preoptic area; DMH, dorsomedial hypothalamus; rRPa, rostral raphae pallidus nucleus; LPB, lateral parabrachial nucleus; BAT, brown adipose tissue. Figure taken from [133]

Shivering consists of fast, repetitive contraction-relaxation cycles of SKM that release heat from the exothermic reaction of ATP hydrolysis (ATP + H_2O= ADP + Pi + Heat) [10]. Myocytes have a great capacity for thermogenesis, and several pathways of heat production are present in these cells. Heat is primarily produced by enzymes hydrolysing ATP, including Na^+/K^+ ATPase, myosin ATPase, and SERCA [11]. When Ca^{2+} is released from the sarcoplasmic reticulum (SR) into the cytosol via the ryanodine receptor 1 (RYR1), it binds to myofilaments initiating contraction. Myosin ATPase hydrolyses ATP and uses the energy released to bind actin, resulting in contraction. When Ca^{2+} builds up in the cytosol, it triggers SERCA, which pumps Ca^{2+} back into the SR. In this process, SERCA utilises energy from ATP hydrolysis thereby causing relaxation, whereas Na^+/K^+ ATPase uses energy from ATP hydrolysis to reset resting ion gradients and membrane potential [10, 12, 13].

With long-term adaptation to cold, shivering decreases and is replaced by a more prominent increase in adaptive thermogenesis [20]. This is because NST protects the shivering muscles from severe damage due to defective Ca^{2+} handling [22]. In addition, shivering relies largely on muscle glycogen that can become limiting after few hours . However, recent evidence show SKM also contributes to thermogenesis via a mechanism of non-shivering thermogenesis (NST) mediated by a futile sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) activity [11, 14-17]. Muscle-based NST has been known for a long time in animals lacking BAT or functional UCP1 such as birds and fish, but its importance in mammals was described only recently through studies in rodents. NST in the SKM has been reported to be the earliest mechanism of NST in vertebrates preceding BAT-driven thermogenesis. This alternative mechanism of heat production is mediated by a transmembrane Ca^{2+}-ATPase (SERCA) located in the sarcoplasmic reticulum membrane and a peptide named sarcolipin (SLN). When SLN binds to SERCA, it allows ATP hydrolysis, but Ca^{2+}

slippage occurs decreasing the Ca^{2+} transported from the cytosol back into the SR. Consequently, more ATP needs to be hydrolyzed by SERCA to transport the released Ca^{2+} which leads to heat production [18]. Studies have shown that SLN protein levels are upregulated during diet overload and cold adaptation. In addition, overexpression of SLN in muscle protects against diet-induced obesity . Of note, muscles can produce myokines, such as FGF21, that stimulates BAT thermogenesis and beiging in iWAT, and as a result enhance thermogenesis in other tissues [19].

4.3.Vasoconstriction

Another physiologic response to cold exposure in mammals is the constriction of blood vessels or vasoconstriction [58, 168]. For the maintenance of body temperature to be efficient, it is important that heat generated through thermogenesis is retained, and this can be done by decreasing blood flow via vasoconstriction. Unlike thermogenesis, vasoconstriction does not generate heat, but prevents heat loss [58, 168]. Cold exposure activates vasoconstriction prior to shivering and BAT thermogenesis, and vasoconstriction is similarly regulated by NE through sympathetic neural stimulation [169, 170]. The importance of NE in controlling vasoconstriction, and therefore preventing heat loss, has been demonstrated in mice deficient in dopamine β-hydroxylase. These mice lack NE and epinephrine and consequently are unable to maintain their body temperature during cold exposure partly due to the failure of peripheral vasoconstriction [137]. In UCP1-deficient mice, reductions in heat loss from the tail through vasoconstriction was shown to be a part of the compensatory mechanism for maintaining homeothermy [168].

There is evidence that another hormone mediating heat conservation during cold exposure is leptin. In fact, leptin-deficient *ob/ob* mice are also characterized by mild hypothermia when housed

at ambient temperature and display profound hypothermia when exposed to cold [171, 172]. This indicates that a physiological role of leptin in thermoregulation consists of reducing thermal conductance, or heat loss, to maintain core body temperature under cold conditions. In addition, leptin treatment in *ob/ob* mice defends body temperature without increasing energy expenditure or BAT recruitment but by inducing vasoconstriction in the tail to reduce heat loss [173].

5. 14-3-3 proteins

5.1. A global characterization of 14-3-3 proteins

5.1.A. Structure and properties of 14-3-3 proteins

From plants to animals, 14-3-3 proteins are ubiquitously expressed in all eukaryotes [174]. 14-3-3 proteins which were named according to their electrophoretic mobility are found in all subcellular compartments and bind to a wide range of biosynthetic enzymes, signalling proteins, and transcriptions factors [175-177]. In mammals, the 14-3-3 protein family consists of seven isoforms, each encoded by a specific gene, and include β, ζ, ε, γ, η, σ, τ, with α, and δ being the phosphorylated forms of β and γ, respectively [175, 178]. As portrayed in *figure 9*, the 14-3-3 isoforms are 28-33 KDa acidic proteins that form hetero- or homo-dimers and function as adaptor proteins to stabilize their targets and facilitate interaction of their targets with other proteins [178-180]. Each 14-3-3 monomer comprises nine anti-parallel alpha helices, and the assembly of these monomers creates a negatively-charged amphipathic groove necessary for protein-protein interactions. Conserved and identical regions of each isoform are located in the inner core of this groove, while the variable residues are mostly located on the outer surface of the protein [181, 182]. Three phosphorylation dependent binding motifs of 14-3-3 proteins have been identified including RSXpS/TXP (mode 1) and RXXXpS/TXP (mode 2), where pS/T represents phosphorylated serine/threonine and X a generic amino acid [175, 183]. A third motif (mode 3) was later defined as when binding partners have a phosphorylated serine or threonine as the penultimate residue in the C-terminus [183]. Although 14-3-3 proteins were originally identified as phospho-serine and phospho-threonine binding protein, it is well established now that they bind

to unphosphorylated targets and proteins harboring the *O*-GlcNac post-translational modification [184, 185].

5.1.B. Mechanisms regulating 14-3-3 protein activity

14-3-3 protein activity is regulated by phosphorylation at specific residues, which have inhibitory effects on function. Phosphorylation induces dimer dissociation, and as a consequence, prevents binding to target proteins [179, 186, 187]. For example, phosphorylation at Ser58 at the dimerization interface of 14-3-3ζ by MAPKAPK-2 or SDK1 promotes dimer disassociation, thereby reducing client protein binding [188, 189]. JNK phosphorylates 14-3-3σ and ζ at Ser185 which causes the dissociation of BAX from 14-3-3 proteins and its translocation to mitochondria [190]. Two other important regulators of 14-3-3 proteins interaction with client proteins are protein phosphatases PP1, and PP2A. As shown in *figure 9*, these enzymes dephosphorylate serine and threonine residues on 14-3-3 docking sites of client proteins and reduces their interactions with 14-3-3 proteins [191]. Mechanisms of transcriptional and post translational regulation of 14-3-3 proteins through proteasomal degradation have also been identified, in particular for the 14-3-3σ isoform [192]. For example, in breast epithelial cells, the oestrogen-induced E3 ligase, EFP, initiates ubiquitination of 14-3-3σ, which results in its rapid degradation [193].

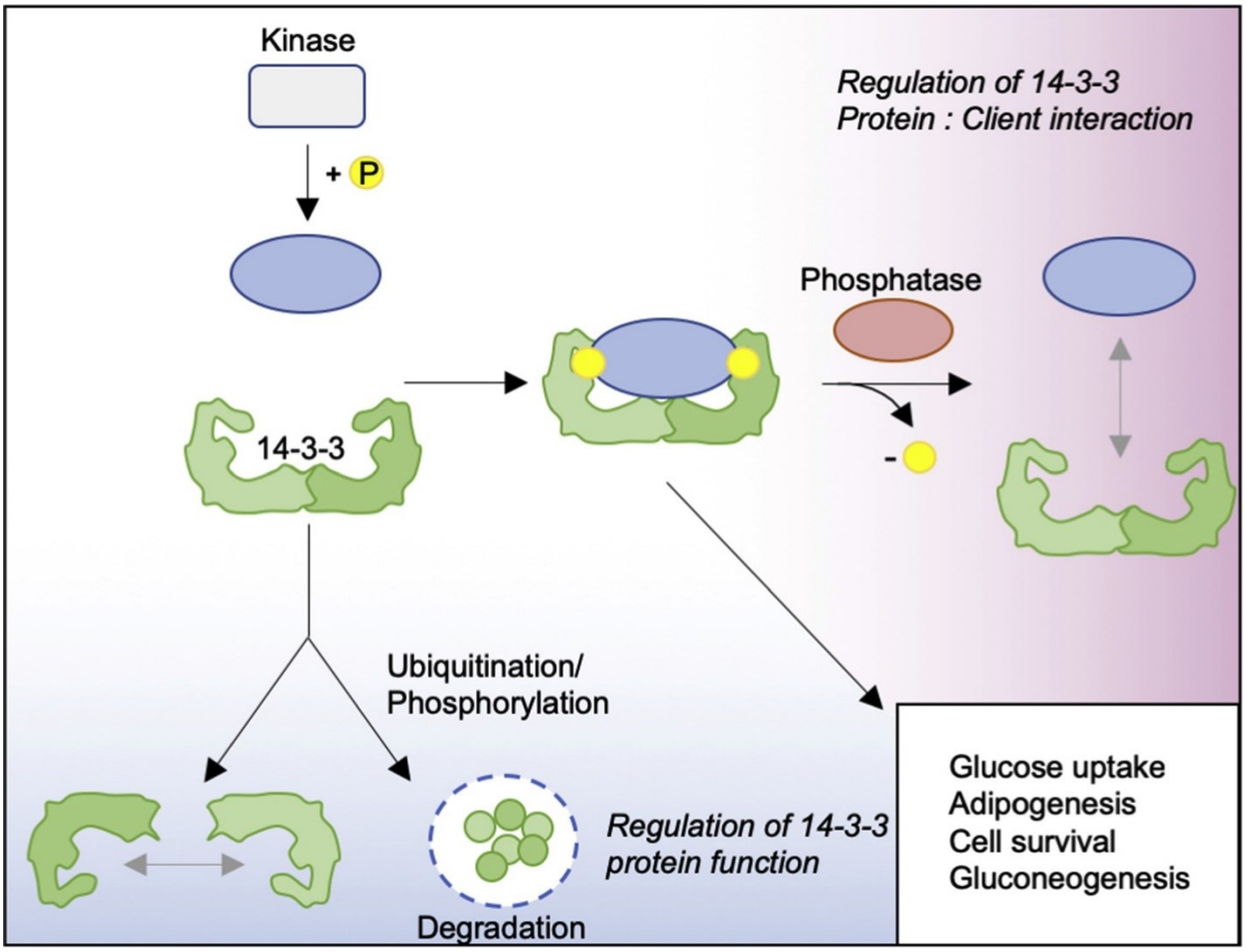

Figure 9: Regulation of 14-3-3 protein function. In most cases, 14-3-3 proteins interact with target client proteins at high-affinity phosphorylation binding motifs created by serine and/or threonine residues. Removal of phosphate groups on target proteins by phosphatases results in the loss of 14-3-3 binding sites and subsequent 14-3-3 protein dissociation from target proteins. Regulation of 14-3-3 protein activity is facilitated by their phosphorylation or ubiquitination. These post-translational modifications either promote the dissociation of 14-3-3 protein dimers or target them for degradation. Figure taken from [194]

5.2. A brief glimpse at 14-3-3 proteins function

Since their discovery by Moore and Perez in 1967 [175, 179], the number of identified binding partners of 14-3-3 proteins have been escalating, reaching more than 1000 identified binding partners [175-177]. This has increased our knowledge of the processes regulated by these

molecular scaffolds including cell signalling, apoptosis, metabolism, cell cycle, transcription, and DNA repair [177, 180, 195-197]. One of the first assigned roles to 14-3-3 proteins was their regulation of tyrosine and tryptophan hydroxylase, rate-limiting enzymes involved in the synthesis of catecholamines and serotonin, respectively. These functions gave rise to their gene name tyrosine- and tryptophan hydroxylase activators (YWHAs) [176]. Another defining feature of 14-3-3 proteins is their ability to mediate protein-protein interactions. 14-3-3 proteins function as adaptor proteins that can induce conformational changes in their target proteins revealing an active site, a ligand–binding region, or a region that interacts with another protein [198]. For example, 14-3-3β binding to the serine/threonine rich B box in the kinase domain in BCR promotes the formation of a RAF-1/BCR complex [199]. 14-3-3 proteins β and θ can facilitate the coupling of PKCζ to RAF-1 [200]. 14-3-3 proteins can also promote steric hindrance or stabilize enzyme substrate complexes. For example, binding of 14-3-3ζ to Serotonin–*N*-acetyltransferase (AANAT) stabilizes the enzyme active conformation which increases its catalytic rate and its interactions with substrates [201].

14-3-3 proteins also regulate protein trafficking into several cellular compartments, often resulting in the inhibition of their target protein [179, 202]. 14-3-3 isoforms can also sequester client proteins or mask import and export sites on their interacting partners [203, 204]. For example, 14-3-3ε binding to the pro-apoptotic transcription factor FOXO1 causes structural changes that unmask two nuclear export sequences (NESs), leading to its cytoplasmic translocation [205]. Several reports demonstrated an important role for 14-3-3 proteins in the regulation of the cell cycle. These molecular scaffolds can mediate the progression from G1, S, or G2 phases and ensure proper timing of mitosis by regulating the activity and/or localization of several cyclins [198, 203, 206]. For example, 14-3-3 proteins bind to and activate WEE1, a tyrosine kinase, during

interphase to inhibit CDC2, a key player in the progression to mitosis [206]. 14-3-3 proteins also sequester CDC25A and CDC25B, 2 phosphatases that activate cyclin-dependent kinases such as CDC2 [207]. Recently, our host laboratory reported a novel role for 14-3-3ζ in the regulation of the mitotic clonal expansion of adipocyte precursor cells. Depletion of this isoform reduced mitotic clonal expansion increasing the transcriptional activity of Gli3, which resulted in the up-regulation of Cdkn1b/p27^{Kip1} expression and cell cycle arrest [208].

5.3. Metabolic functions of 14-3-3 proteins and their relevance to metabolic diseases

5.3.A. From glucose metabolism to adipogenesis, metabolic roles of 14-3-3 proteins are diverse

An important role of 14-3-3 proteins in cellular and whole-body metabolism has been defined over the years. 14-3-3 proteins have been found to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which couples the conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate via the reduction of NADP+ to NADPH [209, 210], as well as the cardiac isoform phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), involved in gluconeogenesis and glycolysis [211, 212]. 14-3-3 proteins also play a role in GLUT4-facilitated glucose uptake. They can bind to and inhibit the inhibitory activity of AS160, allowing the transport of GLUT4 to the plasma membrane [213, 214]. Through the use of systemic 14-3-3ζ knockout mice and 14-3-3ζ overexpressing mice, our host laboratory has identified unprecedented roles of 14-3-3ζ in glucose homeostasis [195, 215]. It has also reported strong evidence of this isoform in the regulation of adipogenesis. 14-3-3ζ KO mice are strikingly lean with reduced visceral fat mass, but not scWAT or BAT. On the contrary, transgenic 14-3-3ζ overexpressing

mice gain significantly more weight and fat mass when fed a high fat diet. Interestingly, this increase in fat mass was not associated with metabolic dysfunction, and no impairments in glucose tolerance or insulin sensitivity was observed in these mice [208] .

5.3.B. The emerging importance of 14-3-3 proteins in metabolic diseases.

Aberrant 14-3-3 proteins expression has been linked to the pathogenesis of several human diseases, such as cancer, neurological disorders and metabolic diseases [203, 216-220]. 14-3-3 proteins were first identified in bovine brain homogenates, where they are widely expressed. In parallel, changes in the expression of 14-3-3 proteins in the nervous system have been associated with several neurological disorders including Alzheimer, Parkinson and Creutzfeldt-Jakob disease [218, 220, 221]. 14-3-3 proteins overexpression has long been associated with several types of cancer, specifically those of the liver, colon, lung, vulva, and breast [222-228]. A number of 14-3-3 isoforms have been linked to the development of metabolic diseases however their impacts in the pathogenesis of these diseases remain to be elucidated. Both 14-3-3β and 14-3-3γ were linked to the development of non-alcoholic fatty liver disease (NAFLD). 14-3-3γ and 14-3-3ζ have been reported to be elevated in subcutaneous and visceral adipose tissue of subjects with obesity [216], and in a separate study, evaluation of visceral adipose tissue from female subjects with obesity revealed an upregulation of these two isoforms when compared to female without obesity [219]. A SNP associated with *YWHAZ,* the gene encoding 14-3-3ζ, was recently associated with weight gain, although its significance has yet to be determined [167]. Recently, our host laboratory has demonstrated an importance of 14-3-3ζ in adiposity and diet-induced obesity, as 14-3-3ζ overexpression causes significant increase in body weight and fat mass under normal or high-fat diets [208] . mRNA transcript and protein levels of 14-3-3 proteins are differentially modulated

in various tissues of murine models of type 1 diabetes mellitus [229, 230], and in a mouse model of streptozotocin induced diabetes, specific expression of a dominant-negative 14-3-3 mutant protein in the myocardium increased cardiac hypertrophy, fibrosis, and inflammation [230].

Given their numerous metabolic functions and links to several metabolic diseases, 14-3-3 proteins may serve as potential targets for the treatment of obesity and diabetes [194], but as a result of their ubiquitous expression and involvement in numerous cellular processes, targeting specific 14-3-3 isoforms for therapeutic intervention can be challenging. There are systemic pan-inhibitors available; however, given the high degree of homology shared among all isoforms, it may be difficult to design isoform-specific inhibitors [194, 231]. Inhibitors of 14-3-3-target protein interactions, such as R18, [232, 233] and BV02 [234], and stabilizers of 14-3-3-ligand interactions, including cotylenin A [235] and fusicoccin A [236], have been developed, but work is required to assess their potential as therapeutic agents. Furthermore, targeting components of the interactome of a 14-3-3 isoform relevant to a certain disease might also yield novel therapeutic avenues for the treatment of cancer, neurological and metabolic diseases.

6. Hypothesis and objectives

Tyrosine hydroxylase (TH) is the first and rate limiting enzyme in the biosynthesis of catecholamines such as dopamine, norepinephrine and epinephrine [237]. 14-3-3 proteins regulate TH activity by binding and directly activating the enzyme following its phosphorylation at Ser19 and Ser 40 (pS19, pS40). Located in the regulatory domain of TH, these sites can be phosphorylated by CaM kinase II, p38 regulated/activated kinase (PRAK) and, mitogen and stress-activated protein kinase 1 (MSK1) [238, 239]. Both hetero- and homodimers of 14-3-3 proteins strongly inhibit dephosphorylation of TH at pS19 and pS40 resulting in TH activation and stabilization [239, 240].

NE is the major activator of brown and beige fat biogenesis and function in response to cold [51, 73, 82, 169]. The involvement of 14-3-3ζ in the crucial step of NE synthesis may suggest a potential role of the molecular scaffold in adaptive thermogenesis. Further adding to this idea, 14-3-3 proteins also bind tryptophan hydroxylase 1 and 2 (TPH1, TPH2), two enzymes that catalyse the rate-limiting step in the synthesis of serotonin following its phosphorylation at Ser19 by CaM kinase II and PKA [241]. Serotonin diminishes β-adrenergic induction of the thermogenic program in brown and beige adipocytes resulting in decreased energy expenditure, and inhibition of peripheral serotonin synthesis reduces obesity by promoting BAT thermogenesis [242].

Given the ability of 14-3-3ζ to regulate the activities of TH and TPH, both of which are required for norepinephrine and serotonin synthesis, respectively [241, 243], I hypothesized that 14-3-3ζ is essential for the development and function of beige and brown adipocytes (*see figure 10).* Therefore, I aimed to determine whether 14-3-3ζ represents a novel regulator of adaptive thermogenesis. The objectives of this study were to:

1. Determine whether modulating the levels of 14-3-3ζ influences the conversion of white adipocytes into beige adipocytes.
2. Examine if 14-3-3ζ directly influences the browning of white adipocytes.
3. Investigate how 14-3-3ζ affects tolerance to acute and chronic cold in mice.

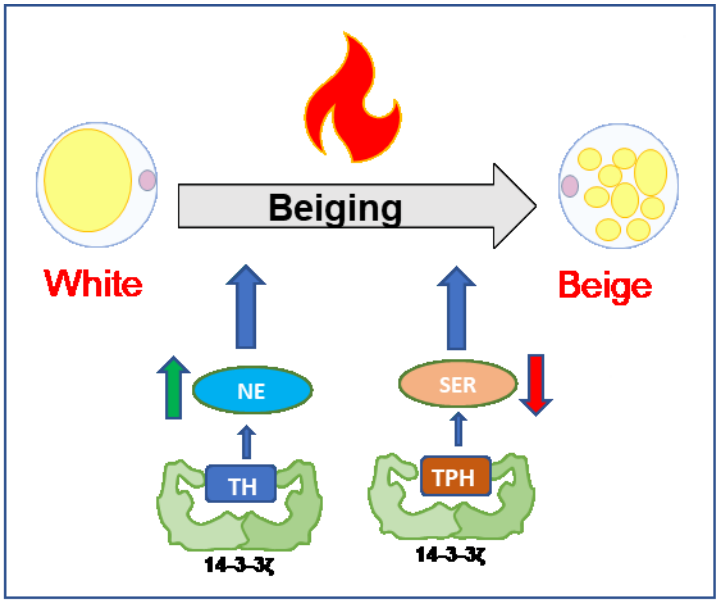

Figure 10: Hypothesis. 14-3-3ζ regulates the activity of TH and TPH which are essential to the production of norepinephrine (NE) and serotonin (SER), key modulators of BAT thermogenesis and beiging of iWAT. Tyrosine hydroxylase (TH), Tryptophan hydroxylase (TPH).

Chapter 2:

Article

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14-3-3ζ overexpression improves tolerance to acute and chronic cold exposure in male mice

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Running title: 14-3-3ζ overexpression improves tolerance to cold exposure in male mice

Key words: 14-3-3 proteins, 14-3-3ζ, beiging, browning, adaptive thermogenesis, beige adipocytes, brown adipocytes.

1. Abstract

Adaptive thermogenesis is a mechanism of heat production primarily mediated by brown fat. In some instances, cold exposure or adrenergic stimuli can convert white adipocytes into brown-like or beige adipocytes during a process termed “beiging”. Both beige and brown adipocytes express higher levels of uncoupling protein 1 (UCP1) and can release energy in the form of heat following lipid oxidation. The activation of these thermogenic adipocytes increases energy expenditure to reduce body weight in rodents, and it has been postulated to be a promising therapy for the treatment of obesity and diabetes. We previously identified an essential role of the molecular scaffold, 14-3-3ζ, in adipogenesis, but its contributions to other adipocyte processes is uncertain. An early identified function of 14-3-3ζ was its ability to regulate the enzymatic activity of tyrosine hydroxylase, which is indispensable in the production of norepinephrine for thermogenesis. Thus, our study aims to investigate whether 14-3-3ζ influences the development and function of beige and brown adipocytes. We report here that transgenic overexpression of 14-3-3ζ in male mice (TAP) improves adaptation to cold due to enhanced beiging in inguinal white adipose tissue (iWAT), as defined by significantly elevated levels of Ucp1 mRNA and protein. Interestingly, beiging is increased in the TAP mice without any changes in sensitivity to beta-adrenergic stimuli, sympathetic innervation, or norepinephrine content being detected between WT and TAP mice. TAP mice also displayed significantly lower thermal conductance, representing decreased heat loss during chronic cold exposure. Collectively, our results point to a novel role of 14-3-3ζ in beiging and increases our understanding of how adaptive thermogenesis is regulated.

Key words: 14-3-3ζ, 14-3-3 proteins, beiging, browning, adaptive thermogenesis, beige adipocytes, brown adipocytes

2. Introduction

Homeothermy is the maintenance of a stable internal body temperature regardless of changes in environmental temperature, and it is essential to the survival of endotherms [133, 244]. To achieve homeothermy in cold environments, mammals have evolved thermogenesis, a mechanism of heat generation that mainly involves skeletal muscle shivering and non-shivering thermogenesis (NST), or adaptive thermogenesis [58, 101, 134, 135]. Shivering is the fast contraction of skeletal muscles during which heat is released from the hydrolysis of ATP by myosin ATPase, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and NA^{+}/K^{+} ATPase [55].

Vasoconstriction is a heat-saving process that consist of constriction of blood vessels to lower blood flow to mitigate heat loss [58, 168]. During long-term cold exposure, adaptive thermogenesis and vasoconstriction regulate thermal homeostasis and protect against hypothermia [133, 244, 245]. Vasoconstriction is partially mediated by leptin, and adaptive thermogenesis requires the activation of the brown adipose tissue (BAT) by the sympathetic nervous system (SNS) releasing norepinephrine (NE) [54, 73, 136, 169, 246] . This catecholamine binds to its β3-adrenergic receptors at the surface of brown adipocytes, leading to an increase in cAMP and the subsequent activation of PKA, which ultimately triggers several pathways to promote thermogenesis [51, 59, 73]. Additionally, recent data have shown that long-term cold exposure induces the recruitment of brown-like adipocytes in the inguinal white adipose tissue (iWAT) of mice, a phenomenon known as beiging [39, 48, 52, 80]. Brown and beige adipocytes are distinct thermogenic fat cells and are rich in uncoupling protein-1 (UCP1), a mitochondrial inner membrane protein that uncouples the proton gradient from ATP synthesis to produce heat from fatty acid oxidation. By dissipating chemical energy in form of heat, brown and beige adipocytes

increase energy expenditure and actively regulate energy homeostasis [41, 53, 73, 80, 89]. Usage of PET-CT scan has led to the identification of both brown and beige adipocytes in adult humans [95-98], and several studies in rodents show their therapeutic potential in the treatment of obesity and diabetes [45, 68, 127, 128]. Despite these advances, the mechanisms underlying brown and beige adipocytes development and function are not completely understood.

14-3-3ζ is a member of the 14-3-3 protein family of a highly conserved, serine and threonine binding proteins, present in all eukaryotes [175, 179, 180, 202]. 14-3-3 proteins function as adaptor proteins that bind to a broad number of enzymes, signalling proteins and transcription factors. Hence, 14-3-3 proteins have been implicated in the regulation of several cellular processes, including signal transduction, cell cycle, protein trafficking, and apoptosis [195, 196, 198, 202]. 14-3-3 proteins have been also linked to the pathogenesis of several human diseases including cancer, Alzheimer, Parkinson disease, obesity and diabetes [203, 218, 225, 226, 228, 229, 247]. Recently, we reported 14-3-3ζ's importance in metabolism and have shown this isoform to be a novel regulator of glucose metabolism and adipogenesis [194, 195, 215]. One of the first ascribed function of 14-3-3 proteins is their regulation of tyrosine (TH) and tryptophan hydroxylase (TPH); 2 rate limiting enzymes involved in the synthesis of NE and serotonin respectively [176]. Moreover, both NE and serotonin are known to influence adaptive thermogenesis and beiging [54, 73, 136, 169, 242, 244].

Given the ability of 14-3-3ζ to regulate the activities of TH and TPH, we hypothesized that 14-3-3ζ could contribute to the development and function of beige and brown adipocytes, thereby influencing adaptive thermogenesis. In this study we explored the contribution of 14-3-3ζ using loss-of-function and gain-of-function mouse models under acute and chronic cold stress. Together, our data demonstrate that 14-3-3ζ overexpression improves tolerance to acute and chronic cold

exposure in male mice. This is through enhanced beiging of iWAT and decreased thermal conductance during chronic cold exposure, which collectively demonstrate a combination of increased heat production and decreased heat loss to improve adaptation to cold.

3. Material and Methods

Animal studies

Wildtype (WT) and 14-3-3ζ heterozygous (HET) mice were on a C57Bl/6J background, whereas whole body transgenic mice overexpressing 14-3-3ζ mice (TAP) were on a CD-1 background [208, 248]. The TAP mice express the Ubi-NTAP-14-3-3ζ transgene in all tissues. The human ubiquitin C promoter is weak in activity and drives a modest overexpression of YWHAZ, which is roughly two-fold higher in adipose tissues of TAP mice in comparison to WT [208]. Mice were housed in the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) animal facility, which is temperature-controlled at 22°C on a 12-hour light/dark cycle. Mice had *ad libitum* access to water and standard chow (TD2918, Envigo, Huntingdon, United Kingdom). All animal studies were performed in accordance to the Comité Institutionnel de Protection des Animaux (CIPA) of the CRCHUM. Genotyping was performed by PCR on DNA extracted from ear notch biopsies using primers previously described . 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 ºC with *ad libitum* access to water. Body temperature was measured with a physio-suit rectal probe (Kent scientific, Torrington, CT, USA). For chronic cold challenge, mice were housed in Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments Columbus, OH, USA) metabolic cages for 3 days at 4 ºC. 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^{TM}, Houston, TX, USA)

Cell culture

Immortalized UCP1-luciferase adipocyte cell line were kindly provided by Dr. Shingo Kajimura (Diabetes Center, University of California- San Francisco) [249]. Cells were cultured 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 split with 0.025% trypsin (Thermo Fisher Scientific) when they reached 80-90% confluency. Cells were incubated at 37°C, 5% CO_2. For experiments, cells were plated in 12-well plates and differentiated 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. On day 3, maintenance medium containing 5µg/ml insulin and 1nM T3 was added to the cells and on day 5, cells received only growth medium. Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA,USA) and 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 . Following transfection, cells were either treated with differentiation cocktail or treated with isoproterenol 10µM (Sigma Aldrich) for 4 hours, and RNA was then isolated for qPCR analysis.

Immunoblotting

Inguinal and gonadal white adipose tissues and interscapular brown adipose tissue 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 ^oC, 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 ºC with primary antibodies against UCP1 (1:1000, R&D systems, Minneapolis, MN, USA), 14-3-3ζ (1:1000 Cell Signaling, Danver, 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). See Table 2 for complete list of antibody information.

Histology and Immunofluorescence

Inguinal and gonadal white adipose tissues and interscapular brown adipose tissue 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 deparaffinised, 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 conjugated to a DAB chromogen (Cell signaling). Images were taken at 10-20X with a microscope (Nikon Eclipse Ti2, Nikon Instruments Inc, Melville, NY, USA).

For immunofluorescence, sections were stained for TH (1:400, Millipore) and 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 95ºC. 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 4ºC 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 [250] (CellProfiler Analyst, Stable (2.2.1)). All immunofluorescence pictures were acquired with the EVOS microscope (Thermo Fisher Scientific) at 20X.

RNA isolation and real time PCR

Total RNA was isolated from cells and tissues using the RNeasy Mini kit or the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), respectively, and stored at -80ºC. 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 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*. For a complete list of primers and their respective sequences, please see Supplemental Table 1.

Enzyme-linked immunosorbent assay (ELISA) and other biochemical assays

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.

Metabolic phenotyping

16-17 weeks old male mice 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 22ºC for acclimatization, 24 hours at 22ºC for basal measurements, and 72 hours at 4ºC for the chronic cold challenge. Food intake, respiratory exchange ratio, locomotor activity (beam breaks), energy expenditure (heat), core body temperature (ºC) 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 [172].

Intraperitoneal glucose-tolerance test (IPGTT)

Cohorts of mice were administered IPGTTs after 6 hours of fasting. IPGTTs were performed at 22ºC and after 3 days of 4ºC exposure. Mice were injected with glucose (1mg/kg), and blood glucose was measured with a Contour Next glucose meter (Ascensia Diabetes Care, Basel, Switzerland)

Statistical analysis

Data are presented as mean and standard error. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software) using Student's t-test and one- or two-way ANOVA, when appropriate. Statistical significance was achieved when $p < 0.05$.

4. Results

A 50% deletion of 14-3-3ζ does not affect acute cold tolerance

To understand the role of 14-3-3ζ in adaptation to cold, we started by examining how 14-3-3ζ deletion affects acute cold tolerance. Hence, wildtype (WT) and heterozygous (HET) 14-3-3ζKO mice were challenged with an acute cold challenge of 3 hours at 4ºC. We decided to measure the effect of deleting one allele of *Ywhaz*, as HET mice have similar body weights, size and adiposity in comparison to WT mice [208]. We did not measure the effect of both allele deletion of 14-3-3ζ as the 14-3-3ζKO mice weigh significantly less than WT and HET mice due to reduced fat mass that may affect their ability to adapt to cold [251, 252]. Mice had similar body weights before the acute cold challenge, and no changes in body weights were detected after the challenge (Figure 1A). Within the first hour of cold exposure, WT and HET displayed a decrease in their body temperature, but no differences in acute cold tolerance were detected between groups throughout the entire challenge (Figure 1B). A similar trend was observed in female mice, as no differences in body weight (figure S1A) and body temperature (Figure S1B) were observed. Interestingly, acute cold exposure remarkably increased *Ywhaz* and *Ucp1* gene expression in the BAT (Figure 1C) of WT and HET mice in a similar fashion. However, transcript levels of the brown marker *Ucp1* was the same in BAT of both male and female HET mice compared to control littermates (Figure 1C, S1C). Although *Ywhaz* expression in the iWAT was considerably increased by cold exposure, no significant differences were observed in the expression of *Ucp1* and the beige selective gene *Tmem26* between the WT and the HET mice (Figure 1D, S1D).

To determine if 14-3-3ζ exerts cell autonomous roles in brown adipocyte function, we utilized the Ucp1-luciferase adipocyte cell line, which is *in vitro* model of brown adipocytes [249]. Treatment with isoproterenol increased *Ywhaz* and *Ucp1* similar to what we observed *in vivo*. A 50% depletion of *Ywhaz* mRNA did not alter transcript levels of *Ucp1* and *Pparg2* when compared to control under basal or stimulated state with isoproterenol (Figure 2 A-C).

14-3-3ζ overexpression improves tolerance to acute cold.

Next, we examined if increasing 14-3-3ζ expression would have any effect on cold tolerance. 12 weeks-old transgenic mice overexpressing 14-3-3ζ (TAP) were challenged with acute cold of 3 hours at 4ºC. Male TAP mice displayed a significant restoration of body temperature (Figure 3B), signifying improved tolerance to acute cold when compared to WT mice. A similar increase in body temperature was observed in female mice but was not statistically significant (Figure S2B). This increase in body temperature was not associated with changes in body weight (Figure 3A and S2A), which remained the same between WT and TAP mice before and after the cold challenge. In the BAT, acute cold exposure had no effect in the expression of brown markers *Ucp1*, *Pgc1a* or *Cidea* (Figure 3C). However, there was a significant increase in *Ucp1* (Figure 3D, S2D) expression in the iWAT of both male and female TAP mice following acute cold. No differences were observed in the expression of beige selective genes, *Tmem26* and *Tbx1* (Figure 3D, S2D).

14-3-3ζ overexpression 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 4ºC. Mice had similar body weights before cold exposure and lost comparable weight after the challenge (Figure 4A). No differences in fat

mass loss were observed between the two groups (Figure S3D). Additionally, no visible differences in the pelage 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 meet metabolic demands and supply the body with fuel to compensate for the considerable amount of energy dissipated during thermogenesis [68]. Overall, no differences in food intake were observed between TAP and WT mice (Figure 4B and Figure S3C).

During the chronic cold challenge, TAP mice significantly maintained higher body temperatures during the last 48 hours of cold (Figure 4C). Surprisingly, energy expenditure was significantly decreased in TAP mice during the last 2 dark phases of the cold challenge (Figure 4D). No changes were observed in lean mass (Figure S3E) nor were there differences in Respiratory exchange ratio (RER) and locomotor activity (Figure S3A and B).

No differences in circulating FFAs and glycerol following chronic cold exposure were observed between WT and TAP mice (Figure S4A and B). Furthermore, immunofluorescent staining for perilipin revealed no differences in adipocyte size (Figure S4C and D) and distribution (Figure S4E and F) in the inguinal or gonadal depots of WT and TAP mice after chronic cold exposure.

Transgenic TAP mice overexpressing 14-3-3ζ are not more sensitive to adrenergic stimuli.

During long-term cold exposure, the SNS releases NE, which acts as the principal activator of beige and brown fat thermogenesis [54, 73, 83, 136, 137, 169]. Therefore, to explore if there were differences in sensitivity to adrenergic stimuli, WT and TAP mice were chronically injected (7 days) with 0.9% saline or the β3-adrenergic receptor agonist CL316,243 (CL, 1mg/kg). No differences in response to CL-mediated changes in total body weight (Figure 5A), lean mass

(Figure 5B) or fat mass (Figure 5C) were observed between TAP and WT mice. *Ucp1* gene expression was similarly increased by CL treatment in iWAT (Figure 5D) and BAT (Figure 5G) of both groups. Moreover, markers of brown, *Cidea* and *Pdk4*, and beige, *Tmem26* and *Tbx1*, selective genes were not different between TAP mice and littermate controls (Figure 5E,F,H,I). Taken together, these data suggest that TAP mice are not more sensitive to adrenergic stimuli than WT.

14-3-3ζ overexpression does not affect adrenergic innervation or norepinephrine content in BAT and iWAT.

The *in vivo* studies described above demonstrate a role of 14-3-3ζ in adaptation to cold that is not mediated by an increase in adrenergic sensitivity. To further investigate the mechanisms by which 14-3-3ζ may regulate cold tolerance, we looked at alterations in adrenergic innervation or activity in beige and brown fat. Tyrosine hydroxylase (TH) protein expression was not altered in iWAT (Figure 6A) or BAT (Figure 6B) of both groups following chronic cold exposure. In addition, immunofluorescence imaging showed similar density of TH positive neurons in iWAT of TAP and WT mice (Figure S5). Consistent with these observations, norepinephrine levels in iWAT (Figure 6C) and BAT (Figure 6D) were not different between WT and TAP mice. *Adrb3* expression in the iWAT (Figure 6E) and BAT (Figure 6F) of TAP mice was also not changed compared to littermate controls. Together, these findings suggest that sympathetic innervation or activity is not altered in TAP mice in response to chronic cold exposure.

Adaptation to chronic cold is associated with increased heat production and retention in TAP mice

The paradoxical increase in body temperature and decrease in energy expenditure observed during the chronic cold challenge prompted us to investigate thermal conductance, a measurement of the rate of heat dissipation to the environment [171]. In contrast to WT mice, TAP mice displayed significantly lower thermal conductance at room temperature and throughout the chronic cold exposure periods (Figure 7A), suggesting an ability of TAP mice to reduce heat loss under mild or severe cold stress. Interestingly, circulating levels of leptin which is known to decrease thermal conductance by stimulating vasoconstriction was not changed between WT and TAP (Figure 7B) [171-173].

Improved tolerance to chronic cold in the TAP mice was associated with increased beiging of iWAT. Compared to WT mice, TAP mice had a five-fold increase in *Ucp1* mRNA expression (Figure 7E) and presented significantly higher levels of *Fgf21* (Figure 7F) and *Tbx1* (Figure 7G) in iWAT. *Ucp1* and other thermogenic genes mRNA expression in the BAT were similar between WT and TAP mice, although *Prdm16* expression was significantly increased in the TAP mice (Figure S7). Consistent with these observations, Ucp1 protein levels were also increased in iWAT and BAT of TAP mice compared to littermate controls (Figure 7C, 7D). Additionally, we did not observe any changes in the expression of genes involved in lipolysis or de novo lipogenesis in the iWAT (Figure S6). Taken together, these data indicate that 14-3-3ζ overexpression upregulates Ucp1 expression to enhance beiging and decreases thermal conductance to improve chronic cold tolerance.

Both brown and beige adipocytes use triglycerides and glucose as energy fuel for heat production. We first performed an IPGTT at ambient temperature and following chronic cold exposure (3 days, 4 ^{o}C) to detect any changes in glucose clearance. During chronic cold stress, fasting glucose levels were significantly higher in TAP mice compared to WT (Figure 8A), and

unlike what was observed at ambient temperature, TAP mice displayed higher blood glucose levels following the glucose bolus at the end of the chronic cold challenge (Figure 8B, C).

5. Discussion

Over the last decade, beige and brown adipocytes have gained a lot of research interest since their identification in adult humans. In contrast to white adipocytes, which store excess energy, brown and beige adipocytes dissipate energy in form of heat. Due to their important roles in regulating thermal and energy homeostasis, brown fat and beige fat activation has been regarded as a potential therapeutic for the treatment of obesity and diabetes. In this study, we show a prominent role of 14-3-3ζ in the regulation of adaptation to cold. A 50% deletion of 14-3-3ζ did not affect brown adipocyte function *in vitro* or the ability to tolerate cold *in vivo*. On the other hand, 14-3-3ζ overexpression significantly improves tolerance to both acute and chronic cold exposure. Our data indicate that 14-3-3ζ overexpression considerably raises body temperature while minimizing energy spent to defend against hypothermia. Interestingly, adaptation to cold is enhanced without any alterations in innervation or sensitivity to the SNS. Our studies suggest that 14-3-3ζ overexpression increases beiging in the iWAT and decreases thermal conductance to significantly improve adaptation to cold.

A potential role for vasoconstriction

In most studies, adaptive thermogenesis is associated with an increase in energy expenditure. Body temperature and energy expenditure are raised in a similar pattern as a result of increased heat production through lipid oxidation [73, 244, 253, 254]. However, some studies have challenged this canonical view and reported an uncoupling of body temperature and energy

expenditure [171-173]. In this present study, TAP mice displayed decreased energy expenditure, while maintaining higher body temperature during chronic cold exposure. This observation challenges the current dogma of how body temperature and energy expenditure are correlated. We interpreted this uncoupling of energy expenditure and body temperature as a consequence of TAP mice having decreased thermal conductance, which is the ease with which heat escapes from the body core to the environment [171]. Indeed, TAP mice maintained a lower thermal conductance during all the three days they were kept under cold conditions, and they were able to increase energy expenditure in response to cold, which indicates functional mechanisms of thermogenesis. In fact, energy expenditure is likely decreased because heat loss is reduced while body temperature is raised because more heat is being produced and conserved inside the mice. This phenomenon of decreased heat loss is likely due to increased vasoconstriction in the tail of TAP mice during cold exposure by vasoconstrictors such as leptin and/or NE [171-173]. Although there were no differences in circulating leptin or NE, it is possible that vasoconstriction in this case could be mediated by other hormones or mechanisms.

Is there increased glucose utilisation in the BAT of TAP mice?

During cold exposure, the SNS activates glucose utilization in parallel with fatty acid oxidation in the BAT thermogenesis [60, 65, 66]. This suggests that TAP mice may have better tolerance to cold due to improved glucose uptake in BAT. 14-3-3ζ has been implicated in the regulation of glucose uptake, as it is known to sequester AS160, the AKT substrate regulating GLUT4 translocation [213, 214]. GLUT4 is expressed in brown and white adipocytes and mediates insulin-stimulated glucose uptake in the BAT and the WAT. Consequently, 14-3-3ζ overexpression in the TAP mice may increase inhibition of AS160, causing an increase in GLUT4 translocation to the plasma membrane. 14-3-3ζ overexpression would translate in an increase in

glucose uptake and enhancement of brown fat activity. Contrary to this line of reasoning, we measured glucose tolerance in our TAP mice during chronic cold exposure, and they displayed higher blood glucose levels. These data suggest 3 possible scenarios. Firstly, the higher blood glucose levels of the TAP mice before glucose bolus suggest that these mice mobilize more glucose during chronic cold stress to respond to the metabolic demands of brown and beige fat to produce heat. This is necessary to provide fuel for thermogenesis and may partly explain why their blood glucose levels remain high during the IPGTT. Secondly, TAP mice may be more efficient at utilizing lipids and/or glucose to produce heat and therefore do not use as much fuel as the WT to sustain brown and beige fat thermogenesis. This is consistent with their higher fasting blood glucose levels showing they do not require glucose for fuel as much as control mice which have lower blood glucose levels because they actively uptake glucose to supply fuel for thermogenesis. Lastly, TAP mice have decreased thermal conductance at ambient temperature and also during cold stress. These mice have reduced heat loss and are more efficient than WT mice at conserving the heat. Consequently, TAP mice need less heat production to maintain their body temperature and accordingly uptake less glucose during the IPGTT. It is likely that these three scenarios are not mutually exclusive. For example, it is possible that TAP mice are more efficient at making heat from glucose and/or FFAs and at the same time they do not need to produce more heat than the WT due to their reduced thermal conductance. Better insights in glucose metabolism during the chronic cold challenge can be obtained by performing ^{18}F-FDG and ^{11}C acetoacetate PET-CT imaging to examine BAT-dependent glucose uptake and metabolic rates, as discussed below.

Is the increased tolerance to cold observed in TAP mice also due to shivering or skeletal muscle NST?

Muscle shivering is recruited as the first line of defense during acute exposure to cold and might have a contribution in improving acute cold tolerance in the TAP mice. However, studies have shown that shivering is not required to maintain body temperature during both acute and chronic cold exposure, and it is progressively replaced by NST during long term adaptation to cold [106, 135, 255]. This is consistent with the increased mRNA expression of *Ucp1* in the iWAT during acute cold, and the increase in protein and transcript levels of Ucp1 in iWAT and BAT during chronic cold observed herein. Furthermore, there were no differences in lean mass or locomotor activity observed between the WT and the TAP mice. To summarize, shivering may contribute to increasing body temperature of TAP mice during acute cold; however, it cannot account for the enhanced cold tolerance observed in TAP mice during the chronic cold exposure.

NST primarily takes place in the brown and beige fat, but some studies have reported NST in SKM. This alternative thermogenic mechanism is mediated by a transmembrane Ca^{2+}-ATPase (SERCA) located in the sarco/endoplasmic reticulum and by sarcolipin. Sarcolipin is a micro-peptide that uncouples SERCA to increase ATP hydrolysis and generate heat [135, 256, 257]. Despite this knowledge, skeletal NST's importance in thermoregulation of mice remains to be elucidated. It is possible that skeletal NST may impact thermogenesis in male TAP mice and allow them to have better tolerance to cold. However, it is unlikely to have a more profound effect in mice than BAT thermogenesis or beiging. In fact, small placental mammals like our TAP mice, while being capable of skeletal NST, have evolved to heavily employ BAT thermogenesis to adapt to cold [55]. Furthermore, although SLN is found in high levels in SKM of larger mammals such as pigs and rabbits, its levels in smaller mammals like mice and rats is often undetectable [258, 259]. Data available suggest that SLN-mediated NST in the maintenance of core temperature is central in animals that have reduced BAT content or in which functional BAT is absent (birds and

pigs) [256]. This suggests that skeletal NST may play a role in larger mammals, whereas in smaller mammals as used in our study, its role in cold adaptation may be negligible.

Is it due to UCP1-independent thermogenesis?

Recent data show that UCP1 is dispensable for adaptive thermogenesis, as thermal homeostasis can be maintained without this protein. Two thermogenic UCP1-independent mechanisms have been identified in beige adipocytes. They involve ATP-dependent Ca^{2+} cycling by SERCA2b and RyR2 and a futile creatine cycling [81, 157-159]. These alternative mechanisms result in increased heat production and energy expenditure during cold challenge. In this present study, TAP mice displayed increased beiging in the iWAT associated with increased Ucp1 mRNA and protein. Nonetheless, recent data show that both UCP1-dependent and UCP1-independent mechanisms of adaptive thermogenesis can be present simultaneously in the scWAT [81, 93]. Consequently, UCP1-independent thermogenic mechanisms, although they were not investigated in TAP mice, may have potentially contributed in the regulation of their thermal and energy homeostasis during chronic cold stress.

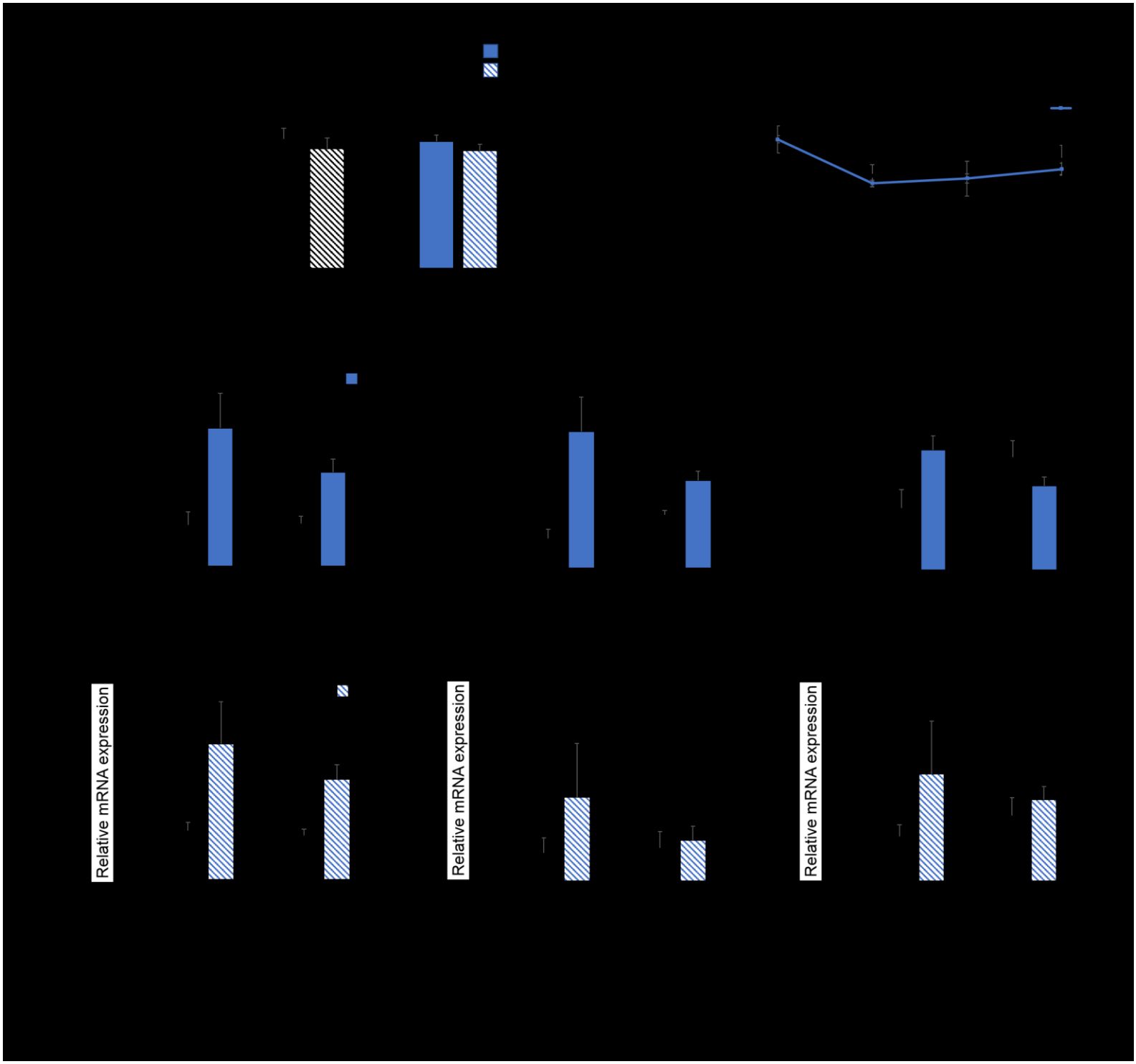
In summary, we demonstrate in this study that deletion of one allele for 14-3-3ζ does not influence tolerance to acute cold. We also demonstrate that overexpression of 14-3-3ζ in male mice leads to improved tolerance to acute and chronic cold via increased heat production through increased beiging in the iWAT and decreased heat loss through decreased thermal conductance. 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.

6. Acknowledgements

Funding for this study was provided by a Canadian Institute of Health Research (CIHR) Project Grant (PJT-153144). GEL is supported by the Canada Research Chairs Program and is the Canada Research Chair in Adipocyte Development.

7. Conflicts of interests

No conflicts of interest are reported.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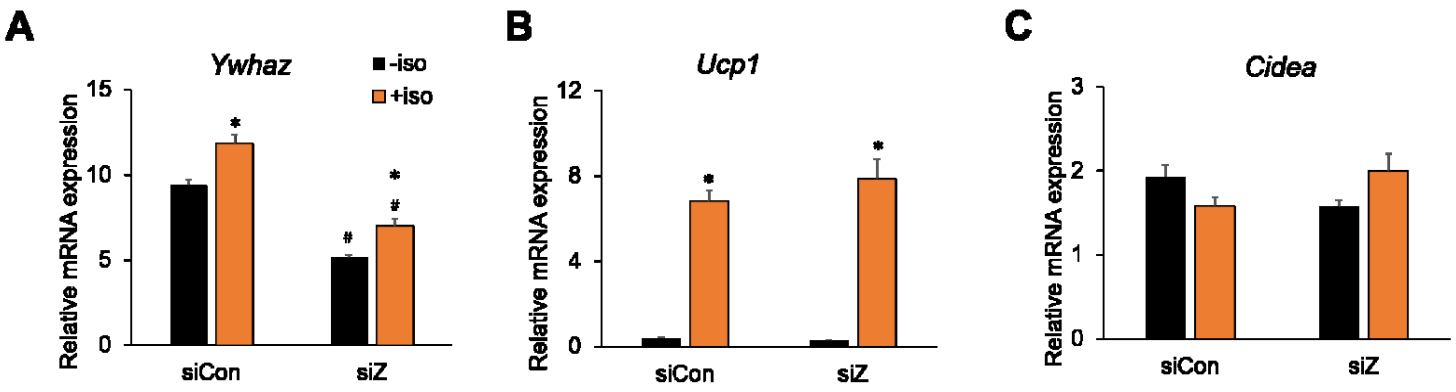


Figure 2: Depletion of 14-3-3ζ in Ucp1-Luciferase cells does not affect the induction of Ucp1 by isoproterenol. Expression of Ywhaz (A), Ucp1 (B), and Cidea (C) mRNA in differentiated Ucp1-luciferase brown adipocytes following knockdown of 14-3-3ζ. Cells were differentiated and transfected with si14-3-3ζ, and following transfection, cells were treated with isoproterenol (10μM, 4 hours), followed by isolation of total RNA quantitative PCR. (n=6 per group; *: $p<0.05$ when compared to -iso; #: $p<0.05$ when compared to siCon)

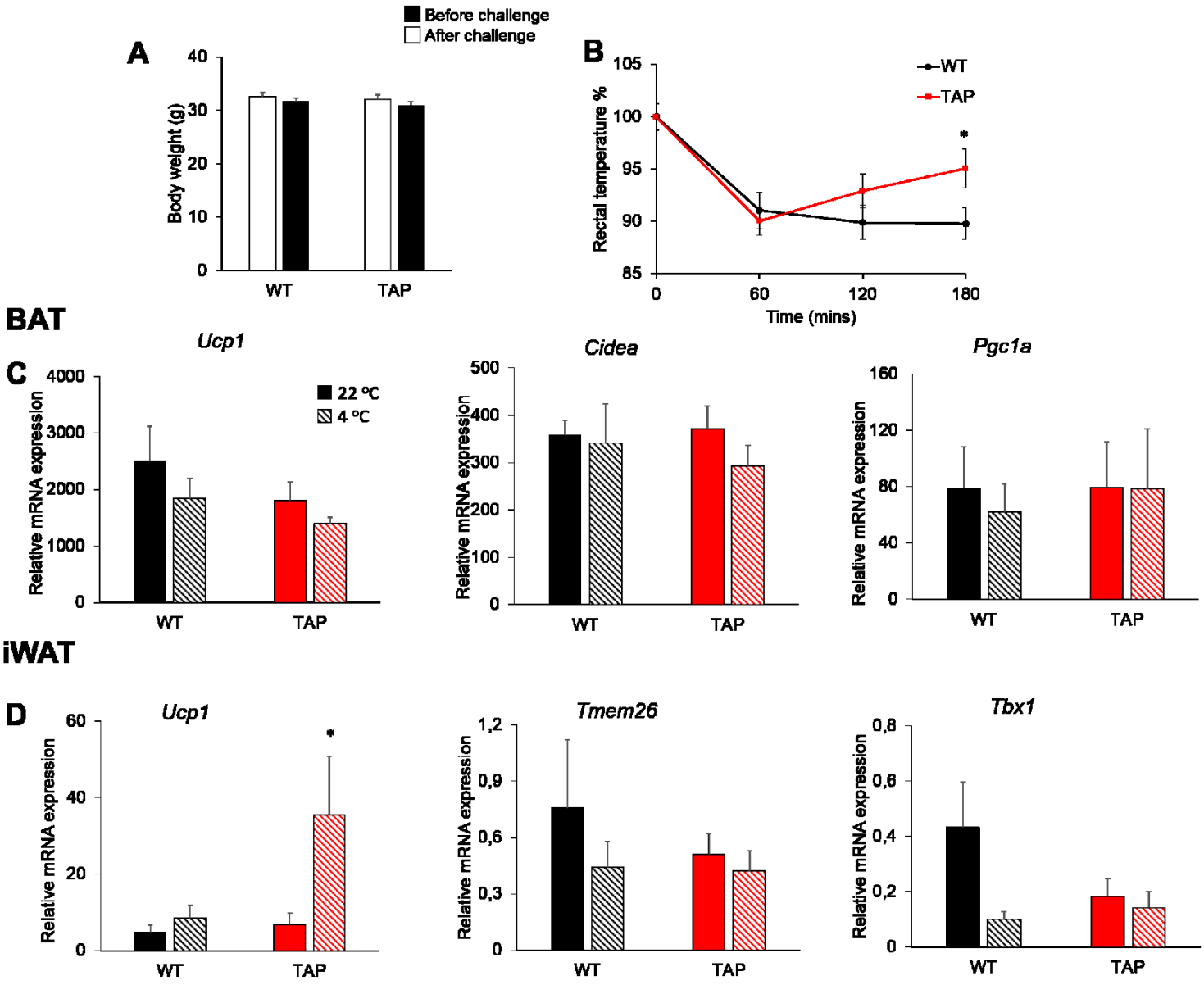


Figure 3: Effect of 14-3-3ζ overexpression in the response to acute cold exposure. (A) Body weights and (B) rectal temperatures of male WT and TAP mice during acute cold challenge (4 °C, 3hrs). (C,D) Gene expression of brown selective markers (C) and beige selective markers (D) of WT and TAP mice housed at 22 °C or subjected to an acute cold challenge at 4 °C. Total RNA was isolated from BAT and iWAT, and mRNA expression was analysed by quantitative PCR. (n=7 mice per group; *: p<0.05 when compared to 22 °C)

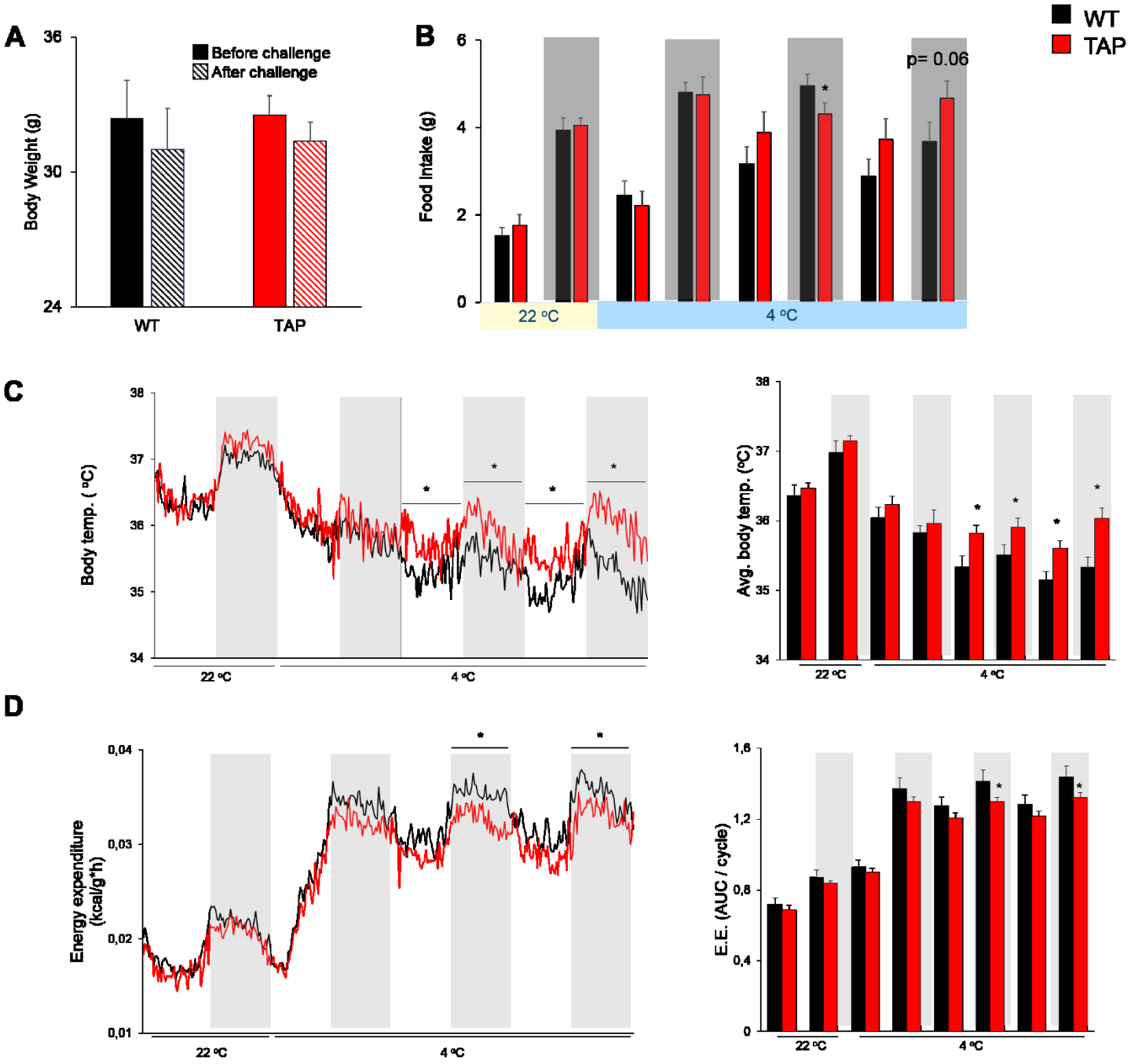


Figure 4: 14-3-3ζ overexpression increases body temperature but not energy expenditure during chronic cold exposure. (A-D) WT and TAP male mice were singly housed in metabolic cages (CLAMS) for 1 day at 22 °C, followed by 3 days at 4 °C with full access to food and water. (A) Body weight (A), food intake (B), body temperature (°C), energy expenditure (D) were measured every 15 mins during light and dark cycles. Values are expressed as mean ± SEM. (WT=8, TAP=10; *: p<0.05 when compared to WT)

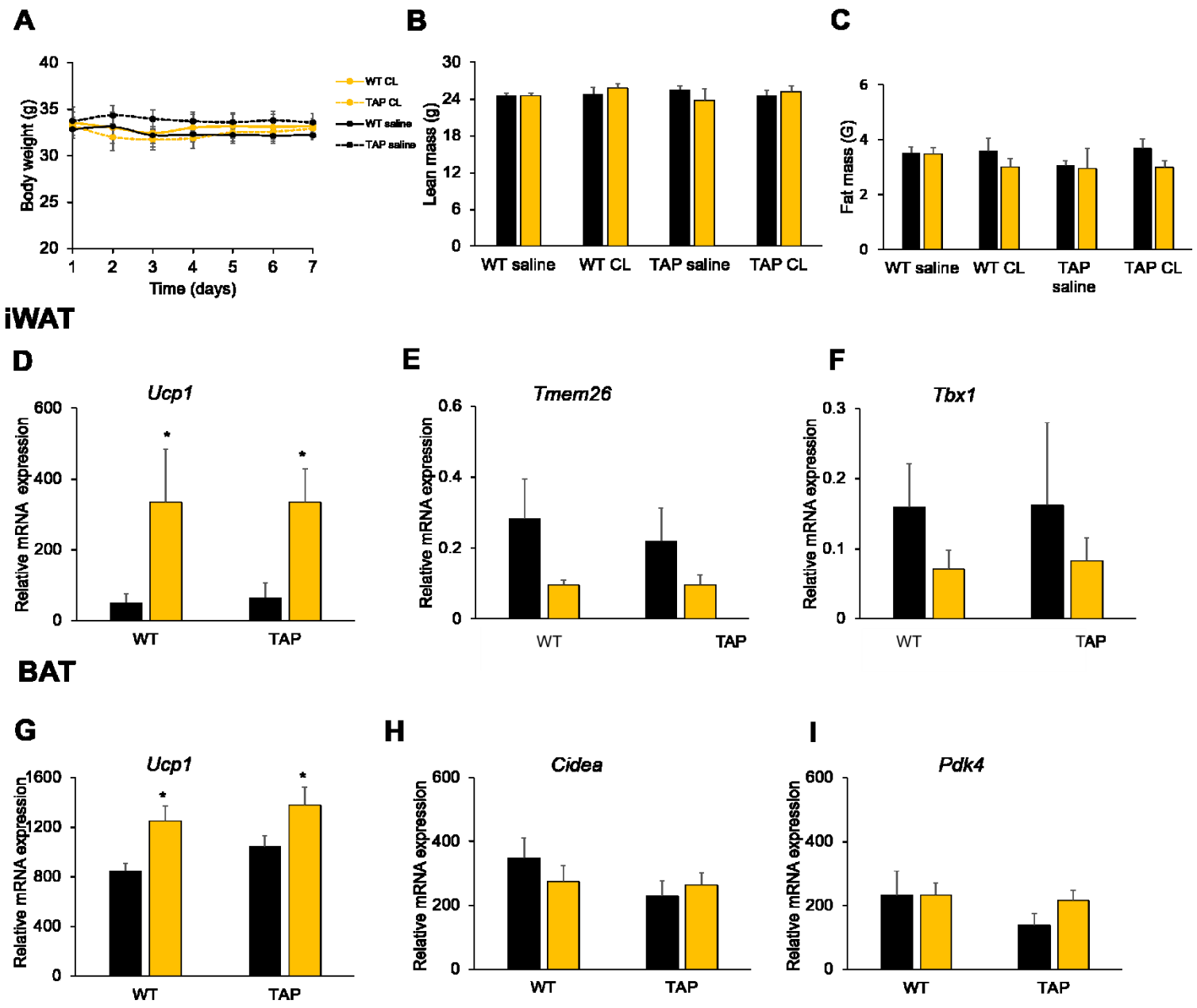


Figure 5: Chronic CL treatment does not improve thermogenic capacity of TAP mice. Male WT and TAP mice received daily intraperitoneal injections of CL316, 247 (1 mg/kg; CL), a β3 adrenergic receptor agonist, or 0.9% saline for 7 days. Body weights were measured daily (A), and lean mass (B) and fat mass (C) were measured by EchoMRI before the first injection and after the last injection. (D-F) Analysis of mRNA levels for *Ucp1* (D) and beige adipocyte markers (E,F) in iWAT of WT and TAP mice treated with CL or saline. (G-I), Analysis of mRNA levels for *Ucp1* (D) and brown adipocyte markers (H,I) in iWAT of WT and TAP mice treated with CL or saline . Total RNA was isolated on Day 7 of injection, and mRNA expression was analysed by quantitative PCR. (n=6 mice per group; *: p<0.05 when compared to -CL)

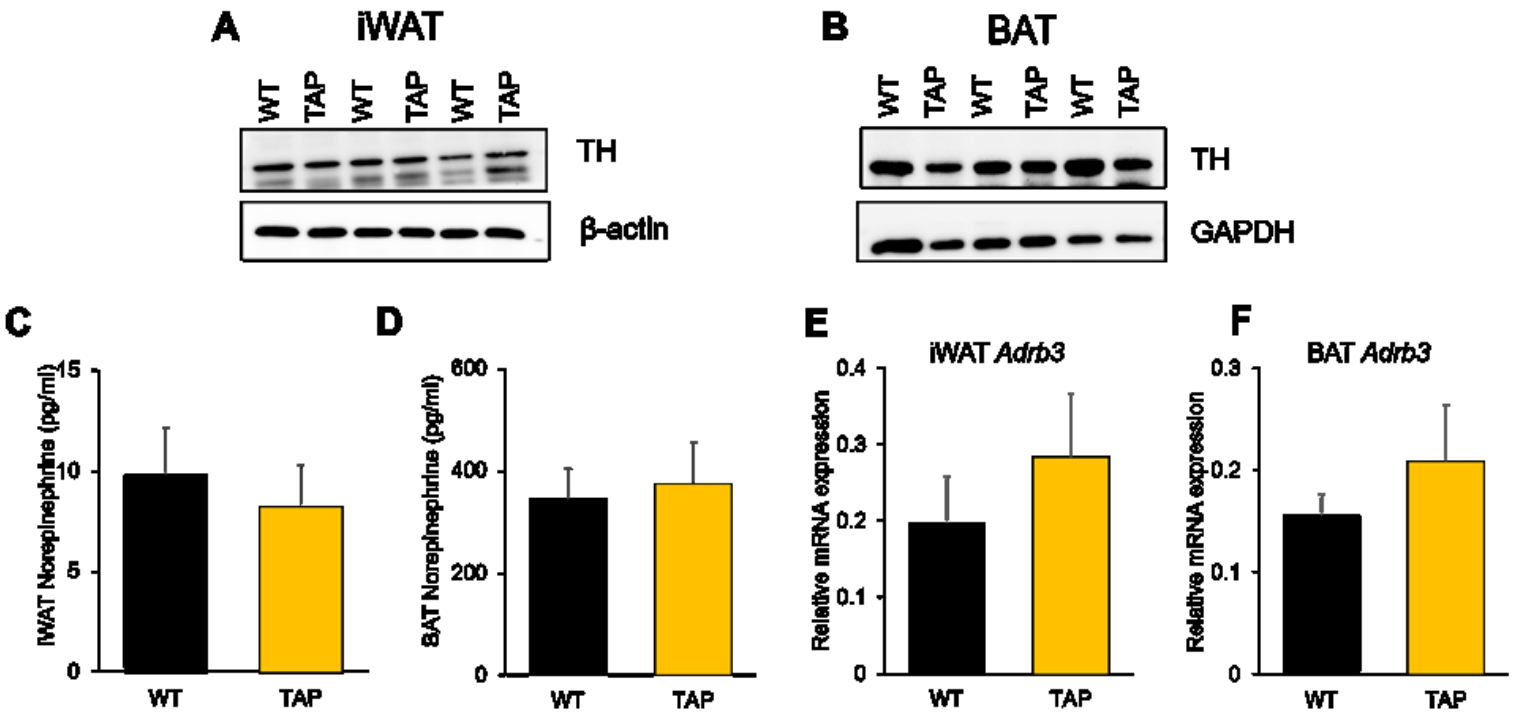


Figure 6: 14-3-3ζ overexpression does not change adrenergic content or TH expression in TAP mice during cold adaptation. (A,B) Analysis of *Adrb3* mRNA in iWAT (A) and BAT (B) was performed by quantitative PCR. (C,D) Norepinephrine content in iWAT (C) and BAT (D) of WT and TAP mice chronically challenged (4C, 3 days) was measured by ELISA. (E,F) Tyrosine hydroxylase (TH) protein expression in iWAT (E) and BAT (F) was measured by immunoblotting. Values are expressed as mean ± SEM. (n=5-7 mice per group; *: $p<0.05$ when compared to WT)

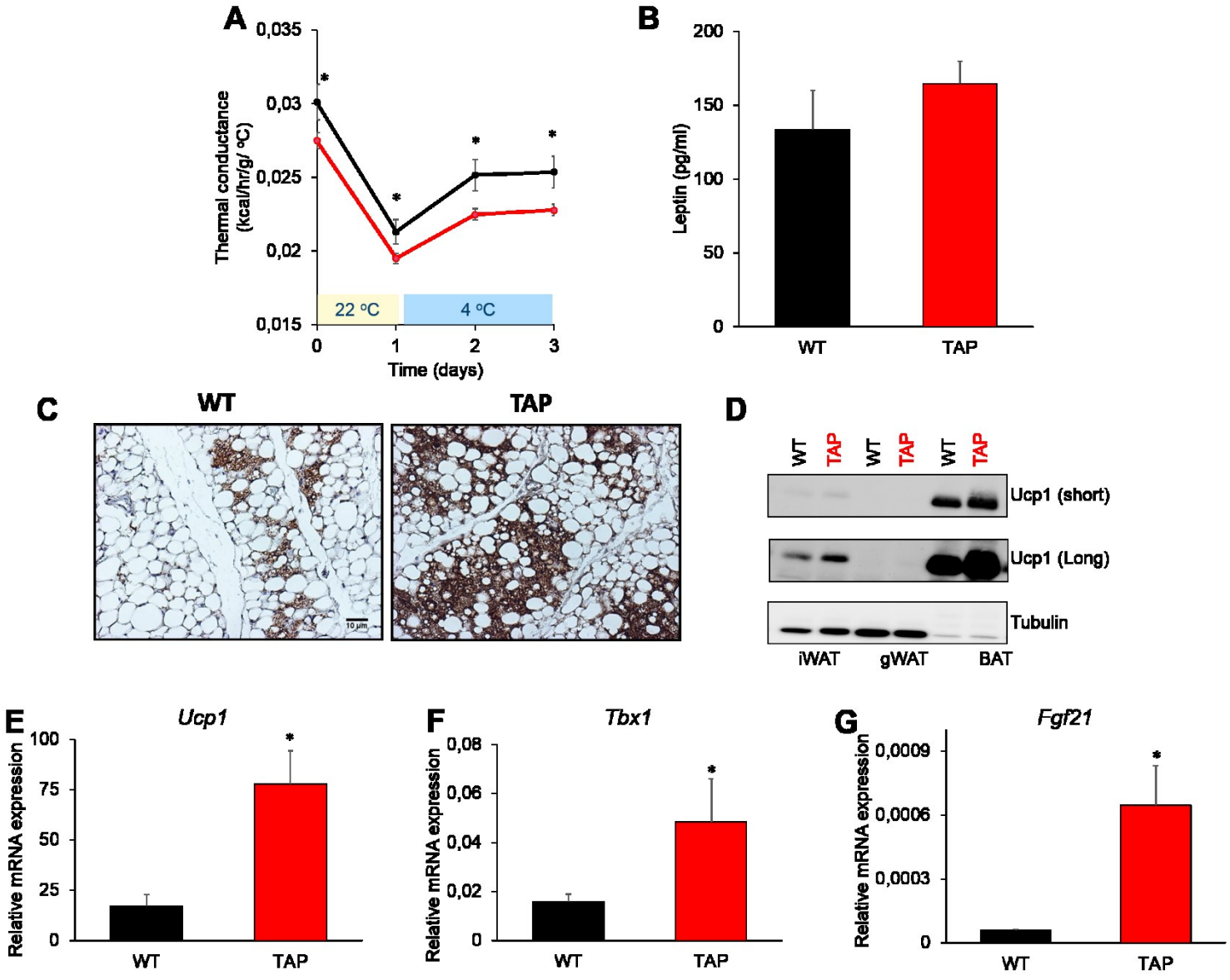


Figure 7: Reduced thermal conductance and enhanced beiging in TAP mice following chronic cold. (A) Thermal conductance was calculated per 24-hour intervals at 22 °C or 4 °C. (B) Circulating levels of Leptin as measured by ELISA. (C) Immunohistological detection of Ucp1 protein in paraffin-embedded iWAT sections. Images are shown at 20X magnification and scale bar =10µm. (D) immunoblotting for Ucp1 and β-tubulin in iWAT, gWAT, and BAT. (E-G) Analysis of gene expression of *Ucp1* (E), *Tbx1* (F), and *Fgf21* (G) in iWAT. Tissues were isolated and snap frozen following chronic cold challenge. Total RNA was isolated from BAT, gWAT and iWAT tissues, and mRNA expression was analysed by quantitative PCR. Values are expressed as mean±SEM. (WT=8, TAP=10; *: $p<0.05$)

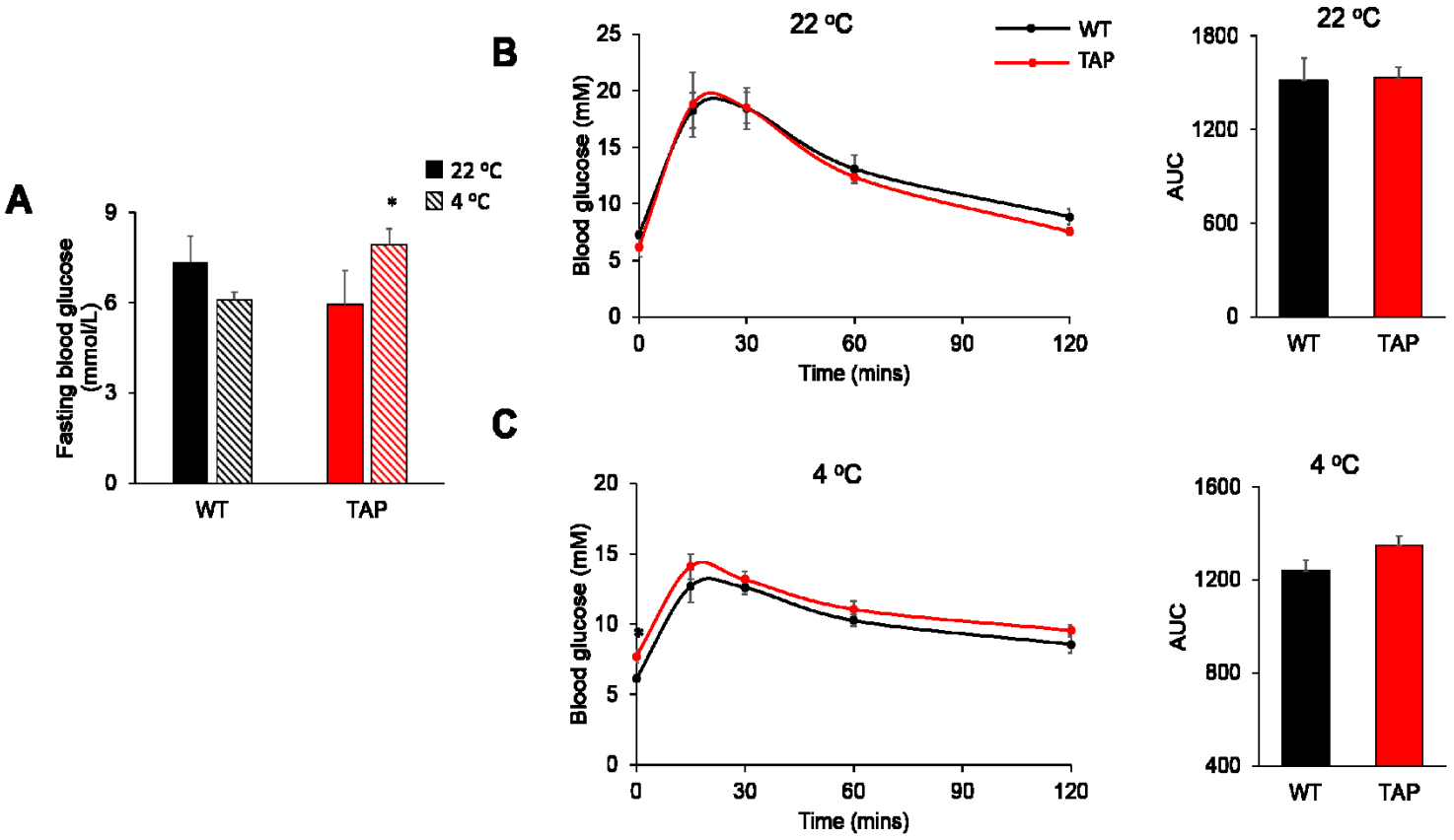
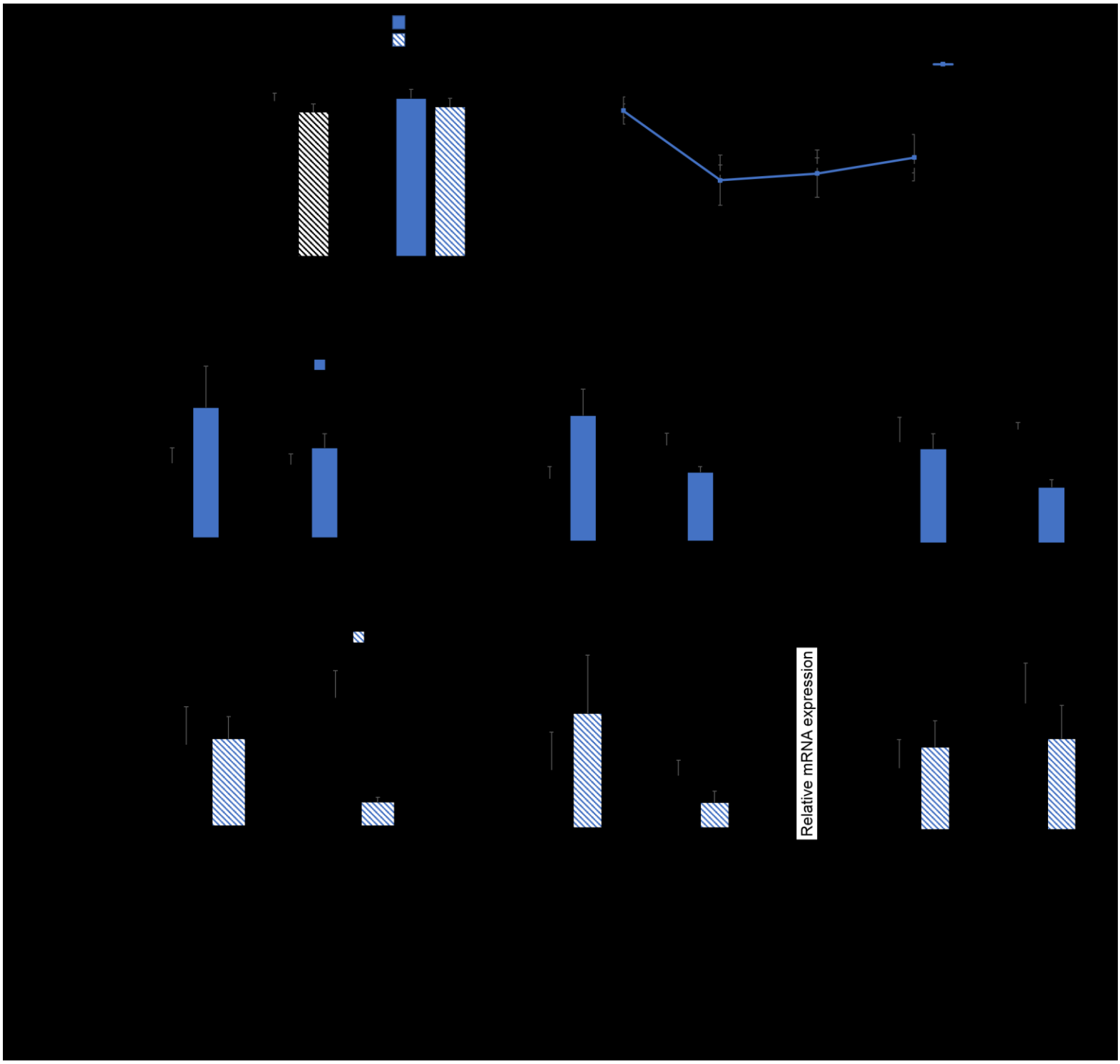


Figure 8: Effect of 14-3-3ζ overexpression on glucose tolerance during cold adaption. (A) Fasting blood glucose levels of WT and TAP mice at ambient and cold temperatures. (B, C) IPGTT (2 g/kg) were performed on 16-week old male WT and TAP mice at 22 °C (B) and following a chronic cold challenge of 3 days at 4 °C (C). Mice were fasted for 6 hours prior to the IPGTT. Values are expressed as mean ± SEM. (n=4 per group; *: $p<0.05$ when compared to WT)


Relative mRNA expression

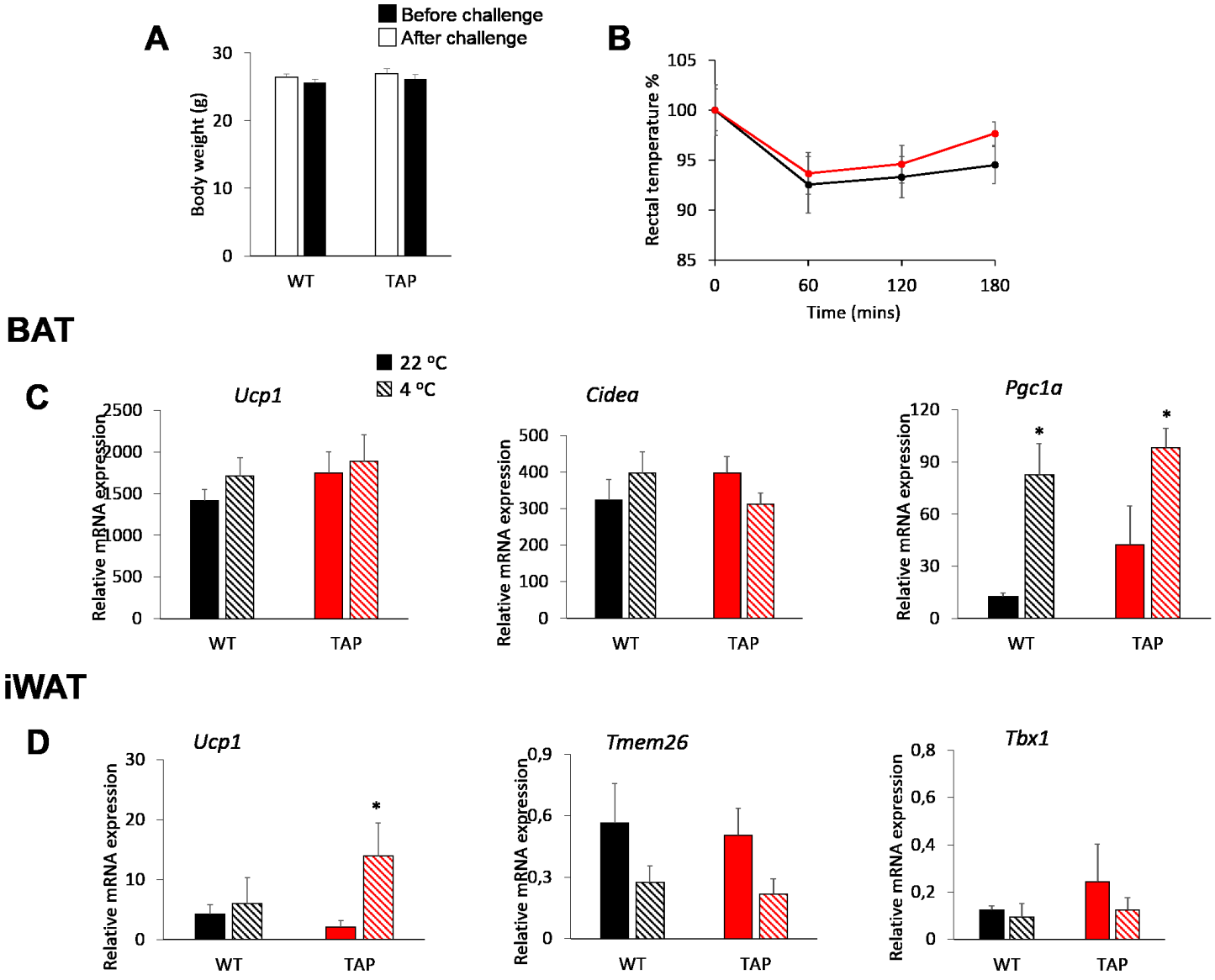


Figure S2: Effect of 14-3-3ζ overexpression in the response to acute cold exposure. (A) Body weights and (B) rectal temperatures of female WT and TAP mice during acute cold challenge (4 °C, 3hrs). (C,D) Gene expression of brown selective markers (C) and beige selective markers (D) of WT and TAP mice housed at 22 °C or subjected to an acute cold challenge at 4 °C. Total RNA was isolated from BAT and iWAT, and mRNA expression was analysed by quantitative PCR. (n=7 mice per group; *: $p<0.05$ when compared to 22 °C)

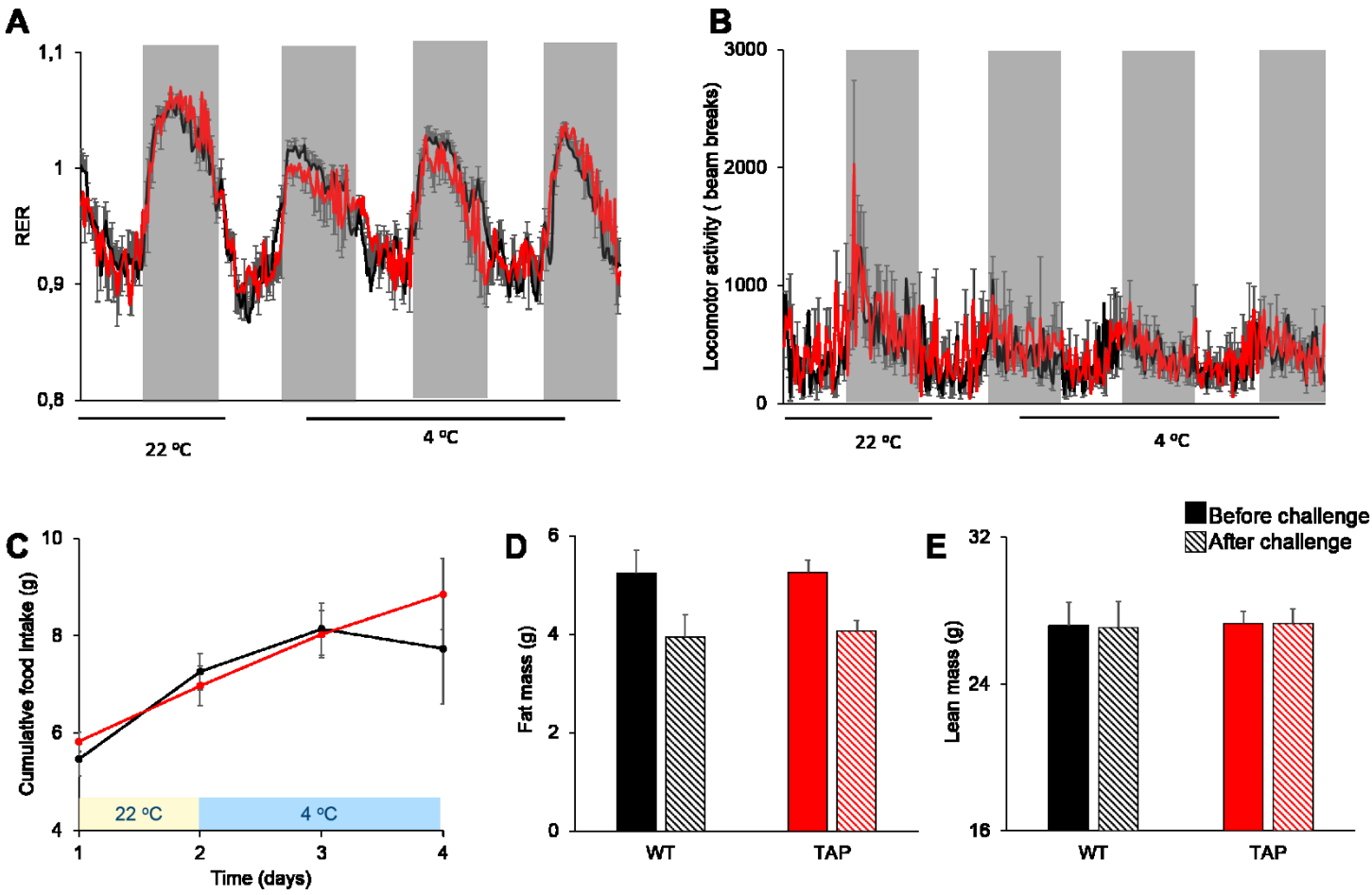


Figure S3: 14-3-3ζ overexpression does not affect energy intake or body composition following chronic cold exposure. (A-C) Respiratory exchange ratio (A), locomotor activity (B), cumulative daily food intake (C) were measured in WT and TAP mice during a chronic cold challenge by CLAMS. (D,E) Fat mass (D) and lean mass (E) of WT and TAP mice before and after chronic cold challenge were measured by echoMRI. Values are expressed as mean ± SEM. (n=6-7 mice per group)

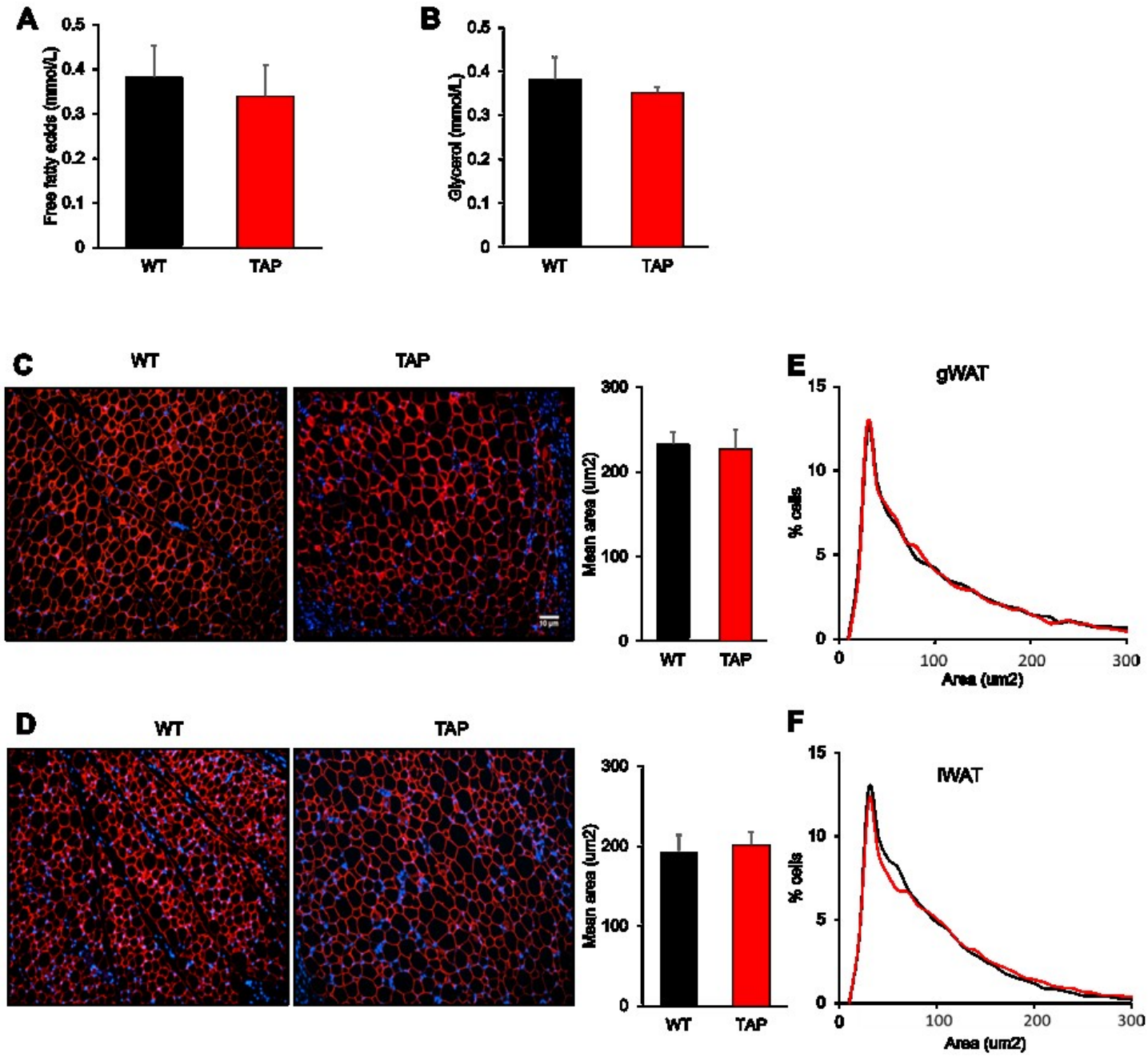


Figure S4: Immunofluorescent detection of perilipin in paraffin-embedded gWAT and iWAT sections. (A,B) Determination of circulating free fatty acids (A) and glycerol (B) of WT and TAP mice following a chronic cold challenge. (C-F) Analysis of white adipocyte area (C,D) and size distribution of adipocytes (E,F) in gWAT and IWAT sections, as assessed by perilipin staining, from 16-week old WT and TAP mice following chronic cold exposure. Values are expressed as mean ± SEM. (n=6-7 mice per group).

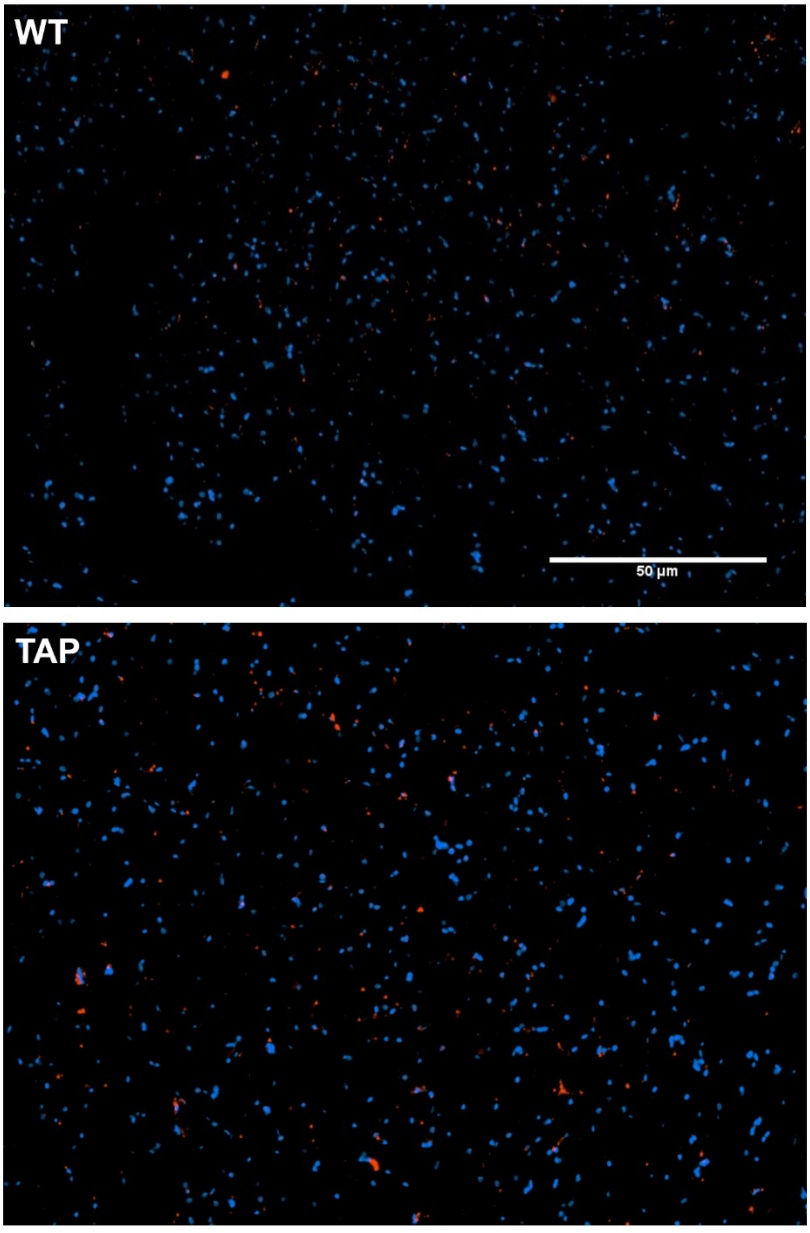


Figure S5: Immunofluorescent detection of TH in iWAT sections. TH density (red) was assessed in paraffin-embedded iWAT sections from 16 weeks old WT and TAP mice following chronic cold exposure (3 days, 4C). DAPI (blue) was used to visualize nuclei. Images are shown at 20X magnification.

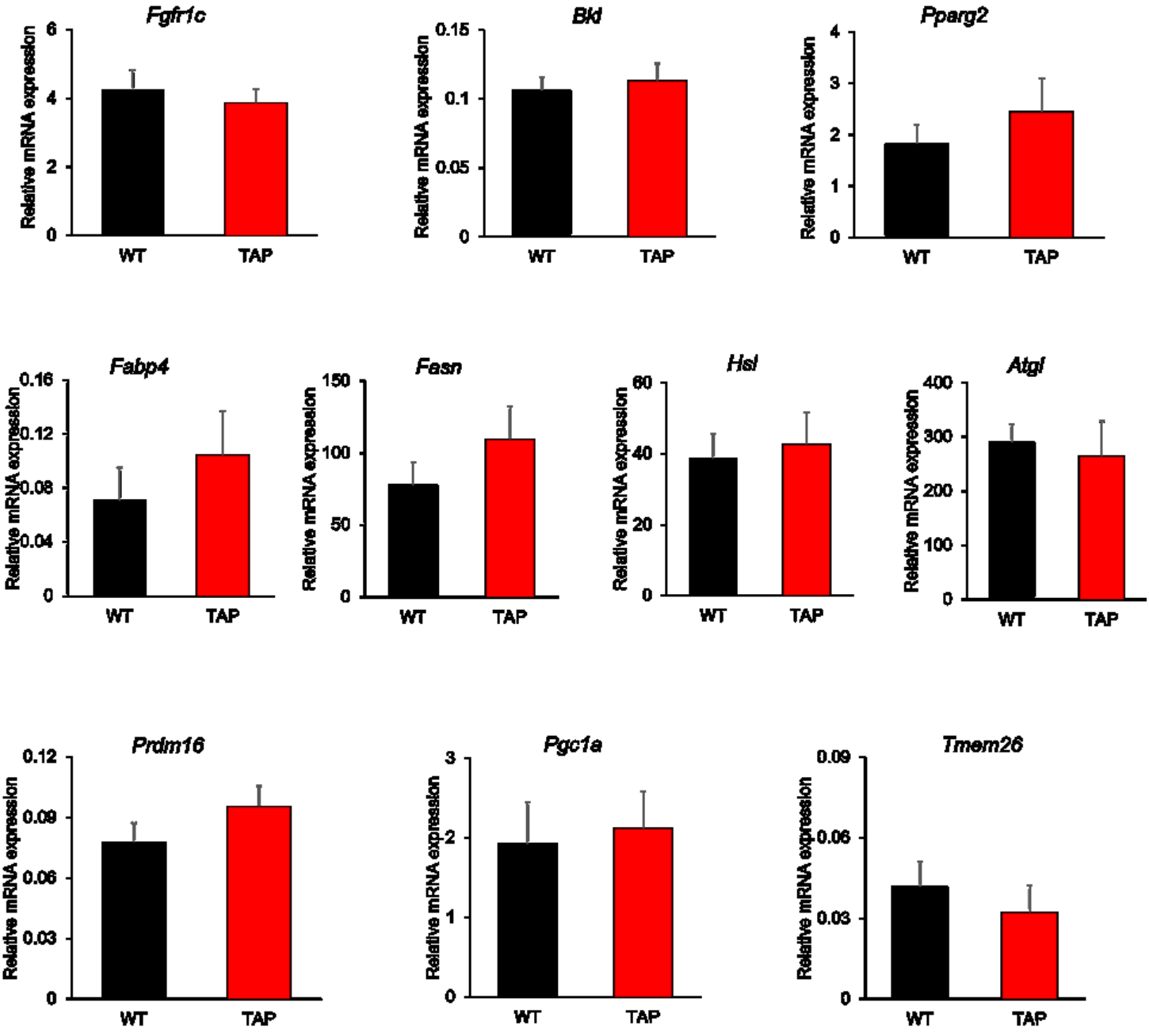


Figure S6: Relative mRNA levels of adipogenic and thermogenic genes (as indicated) expression in iWAT following chronic cold challenge. 16-17-week old male WT and TAP mice were subjected to chronic cold exposure (3 days, 4C). Total RNA was isolated from iWAT, and mRNA expression was analyzed by quantitative PCR. Values are expressed as mean ± SEM. (n=6-7 mice per group)

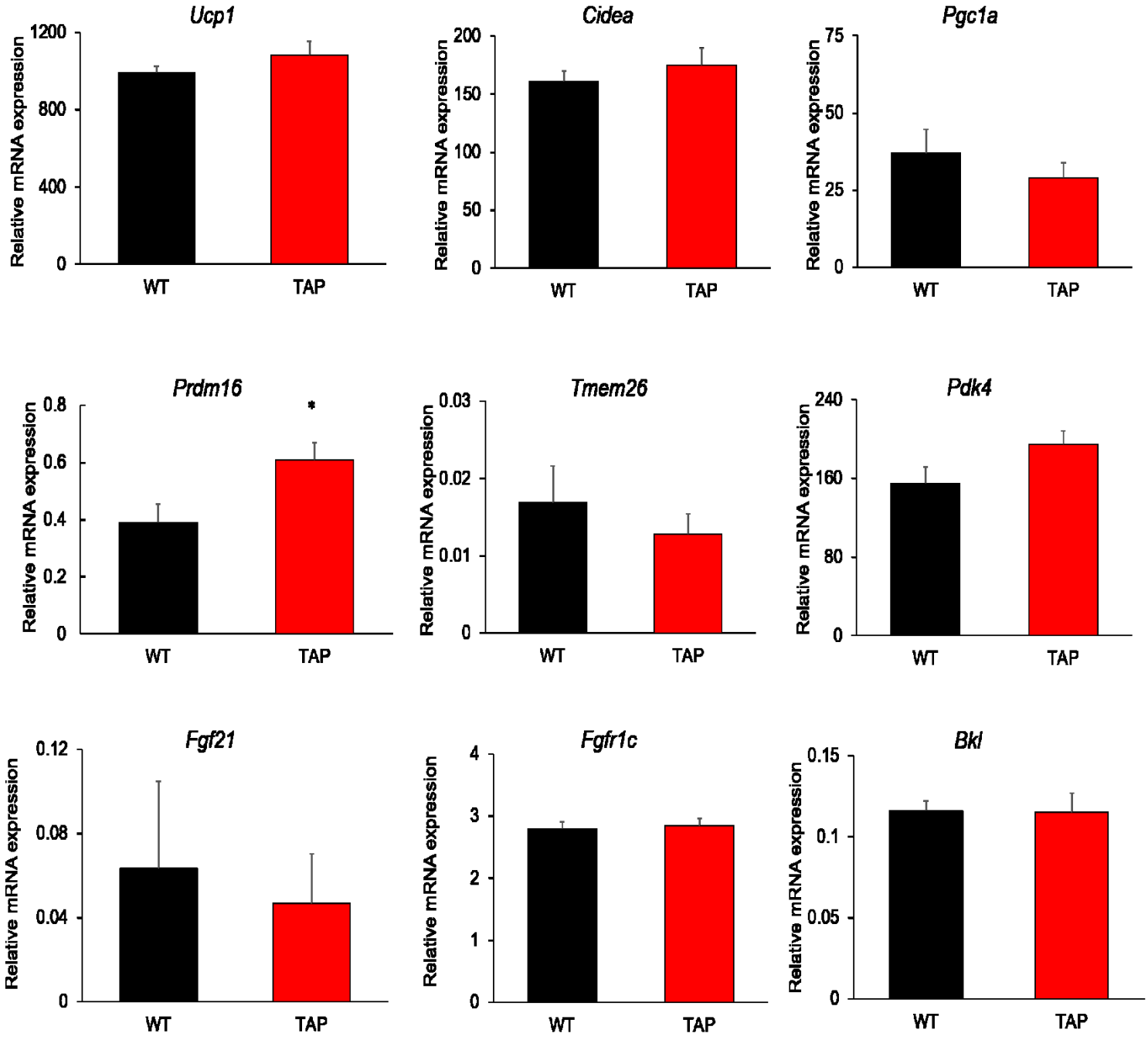


Figure S7: Relative mRNA levels of thermogenic genes (as indicated) in the BAT following chronic cold exposure. 16-17-week old male WT and TAP mice were subjected to chronic cold exposure (3 days, 4C). Total RNA was isolated from BAT, and mRNA expression was analyzed by quantitative PCR. Values are expressed as mean ± SEM. (n=6-7 mice per group)

Table1: List of primers for qPCR

Primer name	SEQUENCE
mAdrb3-F	CCTTCAACCCGGTCATCTAC
mAdrb3-R	GAAGATGGGGATCAAGCAAGC
mAtgl-F	AAC ACC AGC ATC CAG TTC AA
mAtgl-R	GGT TCA GTA GGC CAT TCC TC
mCidea-F	TGCTCTTCTGTATCGCCCAGT
mCideaR	GCCGTGTTAAGGAATCTGCTG
mFabp4/Ap2-F	AGTACTCTCTGACCGGATGG
mFabp4/Ap2-R	GGAAGCTTGTCTCCAGTGAA
mFasn-F	TGG GTT CTA GCC AGC AGA GT
mFasn-R	ACC ACC AGA GAC CGT TAT GC
mFgf21-F	CTG GGG GTC TAC CAA GCA TA
mFgf21-R	CAC CCA GGA TTT GAA TGA CC
mFgfr1c-F	GCCAGACAACTTGCCGTATG
mFgfr1c-R	ATTTCCTTGTCGGTGGTATTAACTC
mHprt1-F	TCC TCC TCA GAC CGC TTT T
mHprt1-R	CCT GGT TCA TCA TCG CTA ATC
mHsl-F	ACC GAG ACA GGC CTC AGT GTG
mHsl-R	GAA TCG GCC ACC GGT AAA GAG
mKlb-F	GATGAAGAATTTCCTAAACCAGGTT
mKlb-R	AACCAAACACGCGGATTTC
mPdk4-F	CCGCTTAGTGAACACTCCTTC
mPdk4-R	TCTACAAACTCTGACAGGGCTTT
mPgc1a-F	AGCCGTGACCACTGACAACGAG
mPgc1a-R	GCTGCATGGTTCTGAGTGCTAAG
mPparg2-R	GGCCAGAATGGCATCTCTGTGTCAA
mPparg2-F	GTTATGGGTGAAACTCTGGGAGAT
mPRDM16-F	CAGCACGGTGAAGCCATTC
mPRDM16-R	GCGTGCATCCGCTTGTG

mTbx1-F	GGCAGGCAGACGAATGTTC
mTbx1-R	TTGTCATCTACGGGCACAAAG
mTmem26-F	ACCCTGTCATCCCACAGAG
mTmem26-R	TGTTTGGTGGAGTCCTAAGGTC
mUcp1-F	ACTGCCACACCTCCAGTCATT
mUcp1-R	CTTTGCCTCACTCAGGATTGG
mYwhaz-F	CAG AAG ACG GAA GGT GCT GAG A
mYwhaz-R	CTT TCT GGT TGC GAA GCA TTG GG

Table 2: List of Antibodies

Protein	**Company**	**Product number**
Alexa Fluor 594	Jackson Immuno research	115-585-003
Anti Mouse HRP IgG	Cell signaling	7076S
BETA actin	Cell signaling	3700S
Beta tubulin	Cell signaling	86298S
GAPDH	Cell signaling	5174S
Perilipin	Cell signaling	93495S
TH	Millipore	6A2911
TH	Millipore	6A2907
UCP1	ABCAM	Ab10983
UCP1	R&D systems	MAB6158-SP

Chapter 3: General discussion

Chapter 3: General discussion

In the course of evolution, adaptation to cold has required various species to develop several mechanisms of thermogenesis to maintain endothermy [260]. These mechanisms involve SKM shivering and NST, which is mediated in part by brown and beige adipocytes [51, 73, 255, 260]. Recently, it has been demonstrated that beige adipocytes can support thermogenesis through UCP1-dependent or UCP1-independent mechanisms of heat production [81, 157-159]. With all these pathways of heat generation being capable of working independently and simultaneously to regulate thermal homeostasis, it is difficult to identify a sole mechanism as being responsible for cold adaptation, especially in the case of TAP mice. Furthermore, it is not known how 14-3-3ζ over-expression can affect these processes. Adaptive thermogenesis mediated by brown and beige adipocytes has been shown repeatedly to be crucial and sufficient in mediating long term adaptation to cold in rodents [124, 125, 128, 156] and is likely the major mechanism of thermogenesis responsible for the improved cold tolerance in the TAP mice.

Is the uncoupling of body temperature and energy expenditure due to insulation?

In this work, we found that the decreased energy expenditure in TAP mice is associated with a reduction in thermal conductance. However, it is not known if this is entirely due to vasoconstriction. Insulation is another physiological mechanism that retains heat during cold adaptation in which the adipose tissue function as a thermal insulating barrier that decreases heat loss from the organism [261]. In addition to fat, fur was found to be a significant protector against heat loss, responsible for roughly half of a mouse's insulation [261].

Regardless, insulation decreases the amount of energy needed to keep an organism warm, thereby decreasing energy expenditure while allowing the maintenance of body temperature like

we observed in the TAP mice. However, in the current study, WT and TAP mice have the same amount of fat mass, and no differences in fur were observed, which makes insulation unlikely to be causing the improved tolerance to cold observed in the TAP mice.

Are other activators of beiging and brown fat activity playing a role?

As discussed in the introduction, BAT activity and beiging are stimulated by an extensive list of external and internal factors including cold, β3 adrenergic agonists, T3, FGF21, PPARγ ligands, and leptin [41, 48, 52, 80]. In the present study, no differences in leptin levels were observed between WT and TAP mice. Chronic treatment of mice with the β3-AR agonist, CL-316,243 did not lead to differences in their response. This experiment suggests that there were no differences in sensitivity to adrenergic stimuli. Furthermore, there were also no differences in NE content in the BAT and iWAT of these mice, thereby implying no changes in sympathetic innervation. Nevertheless, there is a possibility that the increase in beiging observed in TAP mice is stimulated by a yet unidentified or unmeasured factor, such as T3 or ANPs. In addition, we have not assessed the effect of PPARγ ligands, which may play a role in enhancing beige adipocyte development and function that we observed in the TAP mice.

Can we target 14-3-3ζ for the treatment of obesity?

During chronic cold, male TAP mice lose body weight to a similar degree as WT mice, and both groups display about a 25% decrease in their fat mass at the end of the cold challenge. In the context of fat mobilization, it is likely that WT and TAP mice are oxidizing similar amounts of lipids, as supported by equal levels of circulating FFAs and glycerol. Moreover, there are no changes in inguinal and gonadal adipocyte morphometry between the two genotypes. From the metabolic caging studies, TAP mice have lower energy expenditure during chronic cold exposure.

As energy intake is the same between the WT and the TAP mice, this suggests that TAP mice are in a state of positive energy balance compared to the WT, which may lead to increased weight gain. This is consistent with the observation that TAP mice gain significantly more body weight following HFD exposure or during aging [208].

Several studies have reported positive roles of beige fat in improving glucose metabolism and insulin sensitivity [45, 93, 128, 262]. We have found that enhanced beiging of scWAT does not necessarily correlate with improved glucose metabolism. Fasting blood glucose levels of TAP mice were significantly higher than WT mice following chronic cold exposure, despite having enhanced beiging in their iWAT and increased Ucp1 protein in BAT.

To summarize, although systemic 14-3-3ζ overexpression improves adaptation to cold by enhancing beiging of iWAT, the potential for weight gain as a result of decreased energy expenditure suggest that attempting to upregulate 14-3-3ζ expression in all tissues may not represent a viable target for the treatment of obesity.

Limitations of the study

NE acts as the principal sympathetic neurotransmitter and is often used as an index of sympathetic activity [136, 137]. Two common approaches to measure NE are either radioenzymatic or enzyme immunoassay methods including enzyme-linked immunosorbent assay (ELISA) [263, 264]. While these methods are well-validated, they can require significant experimental considerations (e.g. use and disposal of radioisotopes) and present wide variation [264]. In this study, we used ELISA to measure NE and leptin; however, some studies have reported beneficial effects of using high performance liquid chromatography with electrochemical detection (HPLC-ED) or mass spectrometry (HPLC-MS) to separate and quantify NE. In addition, 3,4-

dihydroxyphenylglycol (DHPG), the principal metabolite of NE can be measured using the same techniques to gain a better sense of NE dynamics and turnover [265]. Using these techniques in future studies may reveal more accurate and reliable insights into NE content in iWAT and BAT of TAP mice.

Contrary to white adipocytes that have established *in vitro* models such as the 3T3-L1 cell line, brown and beige adipocytes still lack reliable cell lines that can be used to study the mechanisms underlying the development and function of these cell types. I was not able to find reliable cells lines that differentiate into beige adipocytes, and I encountered problems differentiating D12 cells, which are a proposed model of beige adipocytes [53]. This has made it hard to investigate if there are any cell autonomous roles of 14-3-3ζ in beige adipocytes. I also report the effect of reducing the gene dosage of 14-3-3ζ in the context of acute cold, but it remains to be determined how complete deletion of 14-3-3ζ affects adaptation to chronic cold. In comparison to WT and HET mice, homozygous 14-3-3ζ knockout mice display significant reductions in body weight, size, and adiposity. These confounding variables make it difficult to confidently assess how well they would adapt to chronic cold, as their decreased fat mass can affect cold tolerance [68, 251, 252]. Given that vasoconstriction is a known mechanism of adaptation to cold, it would be interesting to measure this physiological response in mice exposed to chronic cold. Arterial vasoconstriction can be assessed by isolating arteries in mice and treating them with vasoconstrictor such as norepinephrine to measure vascular tension [266]. The organ bath system is a common method used to investigate vascular contractility while the vessels length remains constant [266]. Traditionally, the bathing media is maintained at 37 ºC, but we could asses arterial vasoconstriction in cold bathing media at 4 ºC although this *ex vivo* experiment would not be the best representation of our chronic cold challenge at 4 ºC. In addition, this experiment is

limited by the loss of innervation of the blood vessels by the sympathetic nervous system (SNS) and consequently the loss of the effects of vasoconstrictors released by the SNS. When vasoconstriction is assessed ex vivo, there is also a lack of exposure to other vasoconstrictors that act independently of the SNS such angiotensin II and endothelin I [267]. Therefore, it is impossible to know if the physiological responses observed in TAP mice result directly from the effects of vasoconstrictors or increased innervation by the SNS. Systolic blood pressure can be a good indicator of increased vasoconstriction. However, blood pressure is known to be highly variable, and there are many factors that affect it, such as cardiac output, blood volume and viscosity, and elasticity of blood vessels walls [268, 269]. Therefore, a higher blood pressure in the TAP mice would not be necessarily indicative of an increased vasoconstriction. Systolic blood pressure and heart rate can be measured with a computerized tail-cuff system that determines systolic blood pressure using a photoelectric sensor as described [270].

Future perspectives

In future experiments, acute and chronic cold challenges with adipocyte-specific 14-3-3ζ knockout mice (*Adipoq*-CreERT2:14-3-3ζfl/fl) will help to determine if 14-3-3ζ's role in adaptation to cold is cell autonomous. Some helpful models would include transgenic mice (*Adipoq-rtTA:TRE-Ywhaz*) as well as (*Prdm16-rtTA:TRE-Ywhaz*) in which 14-3-3ζ overexpression is specific to mature adipocytes and mature brown and beige adipocytes respectively. In these double transgenic mice, doxycycline supplemented in water or chow diet induces the expression of Ywhaz in mature adipocytes using the 7X-tetracycline-responsive element (TRE) system. This transgenic mouse line is currently being validated by our research group and was not available for use for this project.. These models can help determine with

confidence if the improvement in cold tolerance observed in the TAP mice is due to 14-3-3ζ overexpression in the different adipose tissues.

Another future experiment could consist in measuring glucose uptake in the TAP mice to determine if they have increased glucose utilisation during chronic cold exposure. WT and TAP mice would be subjected to ^{18}F-FDG and ^{11}C-acetoacetate PET-CT imaging during chronic exposure to cold (3 days, 4ºC). PET-CT is a sensitive technology that allows the imaging, quantification and anatomical localization of glucose or acetoacetate uptake in the whole body to assess any differences in oxidative metabolism during chronic cold exposure [271]. The ^{18}F-FDG radiotracer is a glucose analog commonly used to determine tissue glucose uptake and for determining cancer metastases [272]. ^{11}C-acetoacetate is a ketone body radiotracer that allows the measurement of oxidative metabolism. Acetoacetate is converted into acetoacetyl-CoA by succynyl-CoA-3-ketoacid transferase and then into acetyl-CoA by acetoacetyl-CoA thiolase. In the mitochondria, acetyl-CoA can serve as a substrate for the Krebs cycle or be incorporated in the *de novo* lipogenesis pathway in the cytosol [112, 273]. During cold exposure, mice would be given a bolus of ^{18}F-FDG and ^{11}C acetoacetate followed by a whole-body CT scan and a static whole-body PET acquisition to determine whole body ^{18}F-FDG and ^{11}C acetoacetate organ distribution. This experiment will allow us to determine glucose uptake in the muscles to assess whole body shivering activity and glucose uptake in the brown and beige fat to determine non-shivering thermogenesis. We can also quantify and compare the total volume of the BAT and skeletal muscles in WT and TAP mice based on activity [112, 274].

Chapter 4: Conclusion

Conclusion

Adaptive thermogenesis is essential for the survival and normal physiological functions of mammals evolving in a cold environment. The present study is unique in demonstrating how molecular scaffolds, such as 14-3-3ζ, are an important contributor to thermoregulation. My data indicates that 14-3-3ζ overexpression play an important role in improving adaptation to cold by increasing heat generation and by decreasing heat loss. Partial deletion of 14-3-3ζ during acute cold exposure significantly decreases the expression of Ucp1 in the BAT without affecting tolerance to cold, probably due to other active mechanisms of thermogenesis, such as shivering. 14-3-3ζ overexpression significantly raises body temperature, with a paradoxical decrease in energy expenditure, due to a reduction in thermal conductance. The mechanism underlying how 14-3-3ζ overexpression improves cold tolerance is not known, and this presents novel avenues for future research to fully understand the physiological mechanisms underlying this phenomenon.

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