TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Molekulare Ernährungsmedizin

Characterization of brown-like adipocytes differentiated in primary culture: thermogenic function, molecular basis of variation between strains and transcriptome analysis

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende:	Univ.-Prof. A. Schnieke, Ph.D.
Prüfer der Dissertation:	1. Univ.-Prof. Dr. M. Klingenspor
	2. Univ.-Prof. Dr. H. Daniel
	3. Univ.-Prof. Dr. S. Klaus, Universität Potsdam (schriftliche Beurteilung)
	Univ.-Prof. Dr. M. Schemann (mündliche Prüfung)

Die Dissertation wurde am 01.07.2015 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 07.10.2015 angenommen.

SUMMARY

Obesity and its associated metabolic disorders present a major public health challenge around the world. Current therapeutic strategies for correcting the obesity-causing energy imbalance are either by decreasing energy uptake or increasing energy expenditure. The discovery of active brown adipose tissue (BAT) in adult humans opens new avenues to increase energy expenditure since the major function of BAT is to dissipate energy as heat (thermogenesis) via uncoupling protein1 (UCP1) mediated leak respiration. Strenuous efforts are now being undertaken to learn how to expand or activate BAT in ways that might be therapeutic in humans. Recently, a new type of brown-like adipocytes, so-called brite (brown-in-white) or beige adipocyte, which exists predominantly in the white adipose tissue (WAT) and exists in both rodents and humans, was identified. However, little is known about its basic biology such as its thermogenic potential, molecular controls and modulators.

Aim of this PhD study was to study the thermogenic potential of murine brite adipocytes using in vitro primary cultures, to understand the molecular basis of strain-specific differences in brite adipocytes abundance and to identify putative novel determinants governing the potential of brite adipogenesis.

The results demonstrated brite adipocytes were functionally thermogenic, which was easily masked by an uncoupling artifact caused by free fatty acids released upon adrenergic stimulation at least in vitro cultures. We further demonstrated that differences in *Ucp1* expression both between depots and strains are maintained in primary cultures. Further detailed analyses demonstrated that the strain specific differences in *Ucp1* expression are caused by cell intrinsic trans-acting factors. To identify novel determinants governing the potential of brite adipogenesis, transcriptome analysis of primary cultured adipocytes from five inbred mouse strains with differential browning capacities were performed. A list of candidate genes associated with novel regulators, functional components and new markers of brite adipocytes was identified by bioinformatics analysis.

In summary, this PhD study provides new insights on the thermogenic function and molecular regulators of brite adipogenesis.

ZUSAMMENFASSUNG

Adipositas und damit zusammenhängende, metabolische Erkrankungen stellen eine große Herausforderung für die weltweiten Gesundheitssysteme dar. Die aktuellen therapeutischen Konzepte zielen darauf ab, das auslösende energetische Ungleichgewicht zu beheben, indem entweder die Energiezufuhr gesenkt oder der Energieumsatz gesteigert wird. Die Entdeckung von aktivem Braunen Fettgewebe in erwachsenen Menschen eröffnet eine neue Route, den Energieumsatz zu steigern, denn die Funktion dieses Gewebes ist es, Nahrungsenergie durch entkoppelte Atmung in Wärme umzuwandeln. Daher wird gegenwärtig mit hoher Intensität an Mitteln und Wegen geforscht, Braunes Fettgewebe auf therapeutische Art und Weise zu aktivieren und zu rekrutieren. In diesem Zusammenhang sind sogenannte beige oder brite (von engl. ‚brown in white') Adipozyten von Interesse, die eingestreut in ansonsten Weißem Fettgewebe vorliegen. Über die grundlegende Biologie dieser Zellen, wie ihr thermogenes Potential und ihre Modulatoren, ist allerdings bisher wenig bekannt.

In dieser Doktorarbeit wurde das thermogene Potential von brite Adipozyten verschiedener Mausstämme in primären Zellkulturen untersucht mit dem Ziel, die molekulare Basis von stammspezifischen Unterschieden in der Abundanz von brite Zellen zu verstehen und neue Regulatoren der brite Adipogenese zu identifizieren.

Die Ergebnisse belegen eine thermogene Funktion von brite Adipozyten. Dieser Befund ist in der Vergangenheit oft von einem Entkopplungsartefakt freier Fettsäuren maskiert worden, die zumindest in Zellkultur bei adrenerger Stimulation in großen Mengen frei werden. Wir konnten weiter zeigen, dass sowohl stamm-, als auch depotspezifische Unterschiede in der Expression des Entkopplerprotein 1 (Uncoupling protein1, UCP1) in Primärzellkultur erhalten bleiben und von zellintrinsischen, trans-agierenden Faktoren verursacht werden. Um neue Regulatoren der brite Adipogenese zu identifizieren, wurde das Transkriptom kultivierter Adipozyten von fünf Mausstämmen mit unterschiedlicher Neigung zur Rekrutierung von brite Adipozyten verglichen. Eine Kandidatenliste neuer Regulatoren, funktioneller Komponenten und neuer Marker für brite Zellen konnte bioinformatisch erarbeitet werden.

In der Gesamtschau belegt diese Doktorarbeit die thermogene Funktion von brite Zellen und beschreibt neue molekulare Regulatoren der brite Adipogenese.

PUBLICATIONS

CHAPTER 3 ‘‘White, brite and brown adipocytes: the evolution and function of a heater organ in mammals’’

Yongguo Li, David Lasar, Tobias Fromme and Martin Klingenspor

Can J Zool 92: 615–626, 2014

Personal contribution： Yongguo Li contributed 4 out of 7 sections of this review article.

CHAPTER 4 ‘‘Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes’’

Yongguo Li, Tobias Fromme, Sabine Schweizer, Theresa, Schöttl and Martin Klingenspor

EMBO Rep 15:1069–1076, 2014

Personal contribution： Yongguo Li performed the experiments, analyzed the data, prepaired the figures, drafted the manuscript and revised the final version.

CHAPTER 5 ‘‘Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains’’

Yongguo Li, Florian Bolze, Tobias Fromme and Martin Klingenspor

Biochim Biophys Acta **1841:** 1345-1352

Personal contribution： Yongguo Li performed the experiments, analyzed the data, prepaired the figures, drafted the manuscript and revised the final version.

Prof. Dr. Martin Klingenspor (Supervisor)

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CHAPTER 1

INTRODUCTION

1.1 **Different shades of fat: White, brown and brite/beige adipocytes**

Adipose tissue in mammals, for a long time, was categorized into two distinctly different types: white adipose tissue (WAT) and brown adipose tissue (BAT). The former stores energy as triglycerides, whereas the latter catabolizes lipids to produce heat (Gesta et al., 2007). The thermogenic ability of BAT is conferred by uncoupling protein 1 (UCP1), which is located in the inner membrane of mitochondria. Upon activation UCP1 acts as a proton carrier, dissipates proton motive force as heat, thus uncoupling substrate oxidation from ATP synthesis (Cannon and Nedergaard, 2004). Supporting its thermogenic function, BAT is densely innervated and vascularized and has a large number of mitochondria, which gives the tissue a reddish brown color. Interestingly, UCP1-expressing brown adipocyte-like cells, termed brite adipocytes (brown-in-white; also beige, inducible, or recruitable brown) develop in typical WAT depots in response to cold exposure, β3-adrenergic stimulation or naturally during post-natal development in a process called "browning" (Cousin et al., 1992; Guerra et al., 1998; Himms-Hagen et al., 2000; Xue et al., 2007; Young et al., 1984). Although this phenomenon has been known for decades, the developmental origin, transcriptional control and physiological function of this kind of cell are largely unknown. Recently, it has been suggested that brite cells represent a new type of adipocyte distinct from white and brown adipocytes in respect to their developmental lineage, molecular outfit and hormonal sensitivity (Petrovic et al., 2010; Seale et al., 2008; Wu et al., 2012). Large accumulation of brite cells can be found most readily in the subcutaneous inguinal adipose tissue, but is rather scarce in epididymal/perigonadal adipose tissue (Li et al., 2014a). Notably, the abundance of brites in WAT has been associated with obesity-resistance, enhanced fat oxidation and energy expenditure as well as improved systemic insulin sensitivity (Seale et al., 2011). Thus these cells represent putative therapeutic targets for the treatment of obesity and its associated comorbidities (Bartelt and Heeren, 2014; Harms and Seale, 2013; Pfeifer and Hoffmann, 2015). Understanding the molecular mechanisms underlying brite adipocyte recruitment is therefore of great scientific and medical interest. This scenario is further strengthened by the recent finding that metabolically active brown and brite adipocytes are present in healthy, adult humans (Cypess et al., 2009;

Cypess et al., 2013; Lidell et al., 2013; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

1.2 **The biology of brite adipocytes**

1.2.1 Brown or not brown: a new type of fat cell

Brown adipocyte-like multilocular cells, rich in mitochondria and expressing UCP1, were observed for the first time in white adipose tissue (WAT) depots of cold exposed mice (Young et al., 1984). Subsequent studies confirmed this and further showed that the phenotype change is reversible, i.e. when mice are returned to a warmer environment, UCP1 expression is reversed and with time becomes undetectable (Cousin et al., 1992; Lončar, 1991). These findings led to the definition of “the convertible adipocyte” or “the masked brown adipocyte” (Himms-Hagen et al., 2000) which has the phenotypical and biochemical properties of a white adipocyte with the potential to acquire phenotypical and biochemical characteristics typical of a brown adipocyte. These brown adipocyte-like cells can also appear in muscle tissue after chronic cold exposure or β-adrenergic stimulation (Almind et al., 2007). A lineage tracing experiment revealed that classical brown adipocytes, exemplified by the interscapular depots of rodents, are derived from a myogenic factor 5 (myf-5) positive muscle like cellular lineage, while ‘‘brown-like’’ cells within inguinal and epididymal fat depots are not (Seale et al., 2008). Furthermore, chronically treating primary cultures of epididymally-derived white adipocytes with the potent peroxisome proliferator-activated receptor gamma (PPARγ) ligand rosiglitazone resulted a subset of “white” adipocytes acquired brown-like phenotype but lacked expression of certain classical brown-adipocyte genes (Petrovic et al., 2010). To classify these unique fat cells, Petrovic et al., named them brite (brown in white) adipocytes, while Ishibashi et al., called them beige adipocytes (Ishibashi and Seale, 2010). To further characterize these cells, Spiegelman and colleagues established clonal cell lines from the mouse inguinal fat depot and found that some resemble white fat cells in having extremely low basal expression of *Ucp1*, but, like classical brown adipocytes, they respond to cyclic adenosine monophosphate (cAMP) stimulation with high *Ucp1* expression and respiration rates (Wu et al., 2012). In addition, these brite cells show a unique gene expression profile that can distinguish themselves from brown and white

cells (Wu et al., 2012). These data clearly demonstrated that brite adipocytes are similar to classical brown adipocytes but not exactly identical. Thus, they constitute a new type of fat cell.

1.2.2 Multiple origins

The developmental origin of brite cells is currently under debate. Two hypotheses for the recruitment of brite cells in WAT have been proposed. The first one is referred to as transdifferentiation of mature white adipocytes into brite adipocytes. This hypothesis is mainly based on the observations that (1) the total number of adipocytes (white plus brite) in a given WAT depot does not change after adrenergic stimulation (cold exposure), whereas the proportion of brite adipocytes significantly increases (Barbatelli et al., 2010); (2) the newly emerging brite adipocytes in WAT following β3-adrenergic stimulation are bromdeoxyuridine (BrdU, a pyrimidine analogue of thymidine, selectively incorporated into newly synthesized DNA) negative, indicating a low mitotic index (Himms-Hagen et al., 2000); (3) the process of brite cells recruitment under cold exposure and β3-adrenergic agonist treatment is really fast with increases in *Ucp1* mRNA in retroperitoneal fat evident as soon as 3h and with maximal levels occurring after 48h (Kozak and Koza, 2010). A recent lineage tracing study by employing two transgenic mouse lines allowing for either transient or permanent labeling of UCP1-expressing cells demonstrated that activated brite cells can reenter a white adipocyte-like mode (whitening) (Rosenwald et al., 2013). When the cold stimulation ceases, a large portion of whitened (former brite) adipocytes can be stimulated to undergo re-britening in a second round of cold stimulation (Rosenwald et al., 2013). This study provides the first direct evidence of a bidirectional interconversion process within white adipose tissue and corroborates the transdifferentiation hypothesis. It is tempting to speculate that those cells undergoing transdifferentiation might be pre-existing, unstimulated brite cells. The cells have a unilocular morphology in the basal state, but upon stimulation transform into multilocular cells. This phenotypic “transdifferentiation” may be one of the unique features of brite cells enabling energy storage at rest while upon stimulation energy is combusted.

The second hypothesis involves *de novo* differentiation from existing progenitor cells. For example, sorted precursors such as Sca-1+/CD45-/Mac1- (referred as ScaPCs; Schulz et al., 2011) and PDGFRa+/CD34+/Sca-1+ (referred as PDGFRa+ cells; Lee et al., 2012) have been suggested to possess brite adipogenic potential. This hypothesis has been further strengthened by recent studies of cloned progenitors derived from subcutaneous adipose tissue, which identified a subset of precursor cells that can give rise to brite cells with low basal and high inducible level of *Ucp1* expression similar to classical brown fat cells after stimulation (Wu et al., 2012; Wu et al., 2013).

In any case, anatomical location is of importance in the origin of brite cells, as brite cells in inguinal fat are not descendent of a myf5-expressing progenitor (Seale et al., 2008), while most of the brite cells in retroperitoneal WAT arise from the myf5-lineage, indicating divergent ontogeny of brite cells in different fat depots (Sanchez-Gurmaches et al., 2012).

Furthermore, even within a single depot, heterogeneity of brite cells may exist. For example, Long et al. identified a population of brite adipocytes in iWAT depot that are derived from cells positive for smooth muscle marker myosin heavy chain 11 (Myh11), indicating a smooth muscle-like origin (Long et al., 2014). Nevertheless, within the iWAT depot, not all of the UCP1 positive cells were Myh11 positive, demonstrating the heterogeneous origins of brite adipocytes even within a single depot.

In short, both transdifferentiation and de novo differentiation have been substantiated by experimental evidence. It remains to be clarified which mechanism is the dominant means of brite recruitment. Furthermore, the developmental lineages still need to be further characterized.

1.2.3 Abundance of brite cells is specific for fat depot and mouse strain

A large accumulation of brite cells can be found in the subcutaneous inguinal adipose tissue (iWAT), but is rather seldomly observed in epididymal (eWAT)/perigonadal adipose tissue. The rank order of brite cell abundance is iWAT>retroperitoneal WAT (rpWAT)>eWAT (Kozak and Koza, 2010). The propensity to accumulate brite cells differs not only among WAT depots but also among inbred mouse strains. Mice of some

strains, such as C57BL/6J (B6), are highly resistant to the induction of brite adipocytes by adrenergic stimulation, whereas others, such as A/J and 129/SvJ, are very sensitive in iWAT and rpWAT (Kozak and Koza, 2010). It is not clear whether the differences are caused by cell-extrinsic cues (such as innervation, hormones, cytokines, etc.) or by cell-intrinsic properties. A recent study describes that PRDM16 is selectively expressed in subcutaneous white adipocytes in comparison to other white fat depots in mice, suggesting that this transcription factor could act as a determinant of browning propensity among fat depots (Ohno et al., 2012; Seale et al., 2011). It is also possible that the presence and/or abundance of brite precursor cells vary among different white fat depots. For the depots more resistant to “browning” (such as eWAT), brite cell and/or precursor cells may be less abundant under basal conditions, precursor cells may have to go through a proliferation step before robust browning can take place (Wu et al., 2012). This notion is consistent with the observations that most UCP1 positive cells (UCP1+) in eWAT are derived by the induction of brown adipogenesis from progenitors, whereas the upregulation of UCP1 expression in iWAT involves the conversion or “transdifferentiation’’ of existing white adipocytes (or quiescent brite cells with a similar appearance) (Lee et al., 2012).

A similar scenario may be true for the strain-specific difference. For example, iWAT and rpWAT of mouse strains prone to browning such as A/J and 129/SvJ may have more brite progenitors compared to the depots of resistant mouse strains such as B6 (Chapter 5). From another point of view, the variations of *Ucp1* induction in white fat depots among inbred strains provide us a genetic system to identify genes critical for the induction of brite cells in white fat depots. Kozak and colleagues have already taken advantage of this genetic system and tried to identify genes controlling *Ucp1* induction in white fat (Koza et al., 2000; Xue et al., 2005). By analysis of quantitative trait loci (QTL) that control variation in *Ucp1* expression in the retroperitoneal white fat depot of A/J and B6 parental strains and selected AXB recombinant inbred strains, they show that four QTLs on chromosomes 2,3,8 and 19 have a strong linkage with levels of *Ucp1* mRNA (Koza et al., 2000). The levels of *Ucp1* mRNA in A/J and B6 mice after cold exposure for 7 days are determined by a minimum of nine QTLs on eight chromosomes (Xue et al., 2005). Nevertheless, the identity and function of the QTLs remain to be

determined, since the DNA regions identified as QTLs are still so large that they harbour more than one gene. Furthermore, given the multiple developmental origins of brite cells (most of the brite cells in rpWAT arise from a Myf5 positive lineage, while brite cells in iWAT are descendent from a Myf5 negative progenitor), we cannot assume that the QTLs found associated with the control of *Ucp1* mRNA levels in retroperitoneal fat are also controlling *Ucp1* in other depots, especially the iWAT and gWAT. Therefore the molecular basis of the strain-specific differences in *Ucp1* induction in white fat still needs to be further characterized by using state-of-art approaches such as next generation sequencing (RNA-seq) (Chapter 6).

1.2.4 Brite recruitment and molecular controls

1.2.4.1 Postnatal development

Brown and white fat are believed to originate from different developmental lineages and the transcriptional control of their development is an area of active research. During postnatal development of the retroperitoneal fat depot a majority of adipocytes acquire a multilocular and UCP1 positive phenotype between 10 and 30 days of age followed by the loss of multilocularity and reemergence of unilocular by 2 months of age (Xue et al., 2007). During this transient emergence, the magnitude of *Ucp1* expression in rpWAT is different between B6 and A/J mice while in iBAT it is not, suggesting that the developmental mechanisms for the two types of UCP1+ cells are fundamentally different. Up to now, neither the transcriptional control nor the physiological function of this postnatal brite cell recruitment is known. For the recruitment, it is of interest to test whether the transient induction is associated with hormonal regulators such as leptin or fibroblast growth factor 21 (Fgf21). Especially, it has been shown that there is a postnatal leptin surge, the time course of which is similar to the transient induction of brite cells (Ahima et al., 1998). Concerning the physiological function aspect, thermogenesis seems to be unlikely, since the appearance of brite cells in WAT does not coincide with the postpartum thermogenic requirements of the mouse when animals have already developed a coat of fur. This speculation could be further tested by raising animals under thermoneutral conditions, i.e. at a temperature at which an animal does not have to produce heat to maintain body temperature. Another physiological

significance of postnatal browning could be metabolic reprogramming, i.e. perturbations of energy balance during early development that cause cellular, molecular, and biochemical adaptations that affect physiology and metabolism in adulthood (Ozanne and Hales, 2004). Previous work from our lab demonstrates that postnatal browning attenuates WAT expansion locally (Lasar et al., 2013). A study by Kozak's group showed that the transient induction of brite cells could be strongly suppressed by under-nutrition from birth to weaning as determined by *Ucp1* expression analysis, however, this attenuation had no long-term effects on either diet-induced obesity or cold-induced thermogenesis in adult mice (Kozak et al., 2012). This study questioned the physiological importance of brite adipocytes induction during postnatal development. Nevertheless, the brite cells could develop into a masked mode without *Ucp1* expression. An optimal experimental model to determine the function of brite adipocytes in white fat depots would be to generate mice in which UCP1/brown adipocytes are selectively inactivated in white fat depots. At present, such a model has not been created.

1.2.4.2 Cold exposure and pharmacological β3-adrenergic stimulation

The appearance of small numbers of brown-like adipocytes in white fat depots has been observed in many species (mice, rats, dogs and cats) after cold exposure or treatment with β3-adrenergic agonists (Li et al., 2013). For example, cold exposure of female BALB/c mice results in expression of the brown adipocyte marker UCP1 in parametrial white adipose tissue. A similar phenomenon has been observed in mesenteric, epididymal, retroperitoneal, inguinal, periovarian, intermuscular adipose tissue depots upon exposure to cold or to treatment with a β-adrenoceptor (AR) agonist (Cousin et al., 1992). This kind of recruitment seems to be primarily mediated by the sympathetic nervous system through the β3-AR and is blunted in β3-AR knockout mice (Jimenez et al., 2003). Furthermore, it is reversible in mice returned to a warmer environment, in which UCP1 expression is reversed and with time becomes undetectable (Lončar, 1991). It is also under complex genetic control, as the β3-AR agonist-induced appearance of brown adipocytes in WAT varies considerably from one mouse strain to another (Kozak, 2011; Kozak and Koza, 2010). Mechanisms downstream of β3-AR

most likely involve cAMP-dependent protein kinase A (PKA) activation and phosphorylation of downstream PKA targets including p38 MAPK, resulting in the activation of specific transcription factors and cofactors, notably PGC-1α (Lowell and Spiegelman, 2000). Since β3-AR is ubiquitously expressed in adipocytes but not all adipocytes can express *Ucp1*, the mechanisms that selectively equip some white adipocytes with the capacity to express *Ucp1* in response to an adrenergic signal remain unknown.

1.2.4.3 Immune cells as determinants of brite adipocytes recruitment

The first hint of immune cells involved in regulating thermogenesis comes from observation by Ajay Chawla and colleagues (Nguyen et al., 2011). They demonstrate that macrophages in brown and white fat of cold-exposed mice undergo a clear shift towards alternative activation, which secrete catecholamine locally to amplify adrenergic tone to coordinate thermogenic response, while lacking of alternatively activated macrophages results in striking thermogenic defects (Nguyen et al., 2011). Upstream of this process, cold exposure stimulated activation of eosinophils that produce the type 2 cytokines interleukin 4 (IL-4) in adipose tissue, leads to alternative activation of macrophages (Qiu et al., 2014). Expression of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamine, and catecholamine production was increased in these macrophages, which finally led to browning of adipose tissue (Qiu et al., 2014). Similarly, but using a completely independent approach, Spiegelman and colleagues found that Meteorin-like (METRNL), a circulating hormone that is induced in muscle after exercise and in adipose tissue upon cold exposure, promotes browning through the same immune signaling pathways: METRNL stimulates an eosinophil-dependent increase in IL-4 expression and promotes alternative activation of adipose tissue macrophages (Rao et al., 2014). These two studies clearly highlight the important roles of eosinophils and alternatively activated macrophages in the induction of browning. In this scenario, it is highly probable that factors promoting and maintaining eosinophils and alternatively activated macrophages will also promote browning. This is exactly the case for group 2 innate lymphoid cells (ILC2s), which have been identified as a major innate producer of interleukin (IL)-13 and IL-5 to sustain eosinophils and

alternatively activated macrophages, in response to IL-25 and IL-33 stimulation. Very recently, two groups independently reported that treatment of recipient mice with IL-33 induces browning in an ILC2s dependent manner (Brestoff et al., 2014; Lee et al., 2015). Mechanistically, while one group showed that ILC2 activation results in the proliferation of bipotential adipocyte precursors (APs) and their subsequent commitment to the brite fat lineage via IL-4 receptor (Il4ra) signaling (Lee et al., 2015), the other group demonstrated that ILC2s seem to induce browning by producing methionine-enkephalin peptides that can act directly on adipocytes to upregulate *Ucp1* expression *in vitro* and that promote browning *in vivo*, independently of eosinophils or IL-4 receptor signaling (Brestoff et al., 2014). The reasons for these discrepancies are currently unknown, but it seems likely that both mechanisms are involved, since IL-33 can still induce a certain degree of browning in Il4ra−/− mice. Taken together, the development of brite adipose tissue is mediated by innate immune cells and molecules. Nevertheless, both the signaling cascades linking cold challenge and innate immunity activation and the cellular sources of IL-33 are still illusive.

1.2.4.4 PPARγ activation promotes brite adipocytes recruitment

The effects of peroxisome proliferator–activated receptor (PPAR) agonists on brown cells recruitment demonstrate the existence of a pathway that is not dependent on adrenergic stimulation (Petrovic et al., 2008). In fact, several studies have reported that exposure of white adipocytes in cell culture or *in vivo* to PPARγ agonists induce a “browning” of white cells (Ohno et al., 2012; Petrovic et al., 2010). PPARγ is considered to be a ligand-activated nuclear receptor with essential roles in adipogenesis, glucose and lipid homeostasis and inflammatory responses (Ahmadian et al., 2013). Upon ligand binding, PPARγ forms a heterodimer with the retinoic acid receptor (RXR) and controls the expression of genes that have PPAR response elements (PPRE) (Ahmadian et al., 2013). This transcription factor is further regulated by known coactivators such as CREB binding protein (CBP)/p300, the steroid receptor coactivator (SRC) family, thyroid hormone receptor-associated protein 220 (TRAP220), PGC-1α, PR domain containing 16 (PRDM16) and corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT), nuclear receptor corepressor (NCoR),

and receptor-interacting protein 140 (RIP140) (Powell et al., 2007). Although the browning effect of PPARγ agonists is well established, the molecular mechanisms involved are still not clear. Mechanisms such as stabilization of PRDM16 protein (Ohno et al., 2012), and posttranslational modifications of PPARγ (deacetylation, desumoylation) activity seem to be involved (Qiang et al., 2012). In addition, whether the antidiabetic effects of PPARγ agonist depends on the browning effects on adipose tissue are still not clear.

1.2.4.5 Transcriptional control

Many studies with transgenic and gene knockout (KO) mouse models have described the induction of brite adipocytes in white fat depots (for review, see Harms and Seale, 2013 and Wu et al., 2013). Given that the most notable physiological factor leading to browning is cold exposure, some of the browning results reported may be secondary to increased heat loss caused by the genetic modification (Nedergaard and Cannon, 2014).

Among the reported regulators, PRDM16, PPARγ and PGC-1α are considered to be the three main transcriptional regulators, since others exert their effects mainly through binding, interacting, activating or inhibiting these three. PRDM16 is regarded as the first transcriptional regulator that is absolutely required to promote the differentiation of brown/brite adipocytes, which has been demonstrated by both loss and gain of function studies. Expression of PRDM16 in undetermined fibroblasts or committed white preadipocytes drives a robust program of brown adipogenesis, while knocking down PRDM16 in preadipocytes isolated from brown adipose tissue leads to formation of myotubes in primary cell culture (Seale et al., 2008). An essential role of PPARγ and PGC-1α in brown/brite adipogenesis has been questioned. For example, overexpression of PPARγ leads to white adipogenesis (Sugii et al., 2009) and knockout of PGC-1α lacks effect on brown adipogenesis (Kleiner et al., 2012). A recent study describes that PRDM16 is selectively expressed in subcutaneous white adipocytes in comparison to other white fat depots in mice, suggesting that this transcription factor could act as a determinant of browning propensity among fat depots (Seale et al., 2011). Nevertheless, whether PRDM16 determines the "browning" capacity of white fat among inbred mouse strains is unknown.

Besides transcriptional factors and cofactors, noncoding RNAs emerge to play a role in brown and brite adipogenesis. Several microRNAs have been identified to regulate brown adipocyte determination, differentiation and uncoupling (Trajkovski and Lodish, 2013; Zhou and Li, 2014). Long noncoding RNAs (lncRNAs) also play a role in adipocyte differentiation (Sun et al., 2013). lncRNAs are a unique class of transcripts that share similarities with mRNA with regard to their transcriptional regulation and biogenesis but lack functional open reading frames and thus are not predicted to encode proteins (Geisler and Coller, 2013). Notably, a lncRNA named brown fat lncRNA 1 (Blnc1) recently was identified to promote brown and brite adipocyte differentiation and function (Zhao et al., 2014). Whether noncoding RNAs are crucial players in differential browning capacity of white fat between inbred mouse strains remains largely unexplored.

1.2.5 Functional significance of brite adipocytes

The functional significance of brite adipocytes is an urging question that has yet to be compellingly clarified even in rodents (Peirce et al., 2014). This is largely because it is difficult to experimentally distinguish the contribution of brite adipocytes from that of brown adipocytes. Many factors that recruit and activate brite adipocytes also recruit and activate brown adipocytes. Perhaps the most important unresolved issue is whether brite adipocytes are relevant for systemic thermogenic physiology and whether the metabolic benefits of brite adipocyte recruitment is contributed by its thermogenic function.

1.2.5.1 Thermogenic function of brite adipocytes

UCP1-based thermogenesis in brown adipose tissue was first described in small mammals and infants as an adaptation to defend against the cold (Cannon and Nedergaard, 2004). When a decreased body temperature triggered by cold exposure is sensed by the hypothalamus, catecholamines are released from the sympathetic nervous system, which activates adrenergic receptors of brown adipocytes and in turn stimulate the cAMP-dependent protein kinase PKA, leading to phosphorylation of hormone sensitive lipase (HSL) and thereby increased lipolysis. Free fatty acids (FFAs) released by lipolysis overcome the constitutively inhibitory effects of cytosolic purine

nucleotides (i.e. GDP, GTP, ADP and ATP) on UCP1. Upon activation, UCP1 short circuits the electrochemical proton gradient that drives ATP synthesis and thereby stimulates electron transport and enhances respiration. Heat is generated from the combustion of available substrates and is distributed to the rest of the body through the circulation (Klingenspor, 2003).

Since brite adipocytes also express UCP1, an obvious question is whether brite adipocytes have a thermogenic function. Recently, Schulz and coworkers generated a mouse model lacking expression of bone morphogenetic protein type 1A receptor (BMPR1A) in the cells of myf5 positive lineage, which led to a severe decrease in classical BAT mass (Schulz et al., 2013). This in turn increased the sympathetic tone to WAT, thereby promoting the formation of brite adipocytes within white fat depots. As a result of this compensatory “browning” of adipose tissue, myf5-BMPR1A-KO mice displayed normal thermogenic capacity and were resistant to diet-induced obesity (Schulz et al., 2013). It should be kept in mind that BMPR1A is also expressed in skeletal muscle and it is unknown whether a BMPR1A knockout could also affects thermogenic mechanisms in skeletal muscle. In any case, these findings provide the first hint that brite cells may possess similar capacities as classical brown cells for thermoregulation if maximally stimulated. Isolated mitochondria from inguinal fat depots of cold-exposed mice express substantial amounts of UCP1 protein also supporting a role for thermogenesis (Shabalina et al., 2013). However, there is a limited amount of data directly assessing bioenergetics and thermogenic capability of brite adipocytes (Chapter 4).

1.2.5.2 Metabolic benefits of brite adipocytes

In many published studies, the browning of WAT is highly correlated with anti-obesity effects (improved glucose tolerance, improved insulin sensitive) that happen without evidence for an increased BAT function. Whether the metabolic benefits of brite adipocyte recruitment are contributed by a thermogenic function is unknown. It is possible that brite adipocytes have other roles to control adiposity through nonthermogenic mechanisms, for example, through altering the secretome of adipose tissue (adipokines). Recently, a BAT-enriched secreted factor neuregulin 4 (Nrg4) was

identified, which binds to hepatic cells upon release and attenuates hepatic lipogenic signaling and preserves glucose and lipid homeostasis in obesity (Wang et al., 2014). Of note, this kind of metabolic benefits is independent of UCP1-mediated thermogenesis. This study strongly suggests that brown fat exerts metabolic benefits beyond its intrinsic thermogenic function. The same is likely true for brite adipocytes. In this scenario, a comprehensive analysis of the secretome of brown and brite adipocytes will be extremely informative.

1.2.5.3 Brite adipocytes in cancer cachexia

Stimulating the development of brite adipocytes in WAT holds metabolic benefits in the context of obesity and diabetes, but appears detrimental to cancer conditions (Kir et al., 2014; Petruzzelli et al., 2014). Cachexia is a wasting disorder of adipose and skeletal muscle tissues that frequently accompanies cancer. The molecular basis of cachexia is poorly understood. Recently, two research groups independently show that WAT browning represents an early and systemic event in cancer cachexia and contributes to the increased energy expenditure and lipid mobilization (Kir et al., 2014; Petruzzelli et al., 2014). Proinflammatory cytokine interleukin6 (IL-6) and tumour-derived parathyroid-hormone-related protein (PTHrP) are responsible for promoting WAT browning. Blocking of IL-6 and PTHrP signaling reduce WAT browning and ameliorate the severity of cachexia, respectively (Kir et al., 2014; Petruzzelli et al., 2014). Overall, these studies reveal the detrimental effects of WAT browning in the context of cancer cachexia. Inhibition of WAT browning represents a promising approach to ameliorate the severity of cachexia in cancer patients. From another perspective, IL6 and PTHrP may serve as powerful browning agents in humans, which warrant further investigation.

1.3 Brown and brite in humans

BAT has long been thought to be nonexistent or non-functional in the adult human. Its contribution to energy expenditure in adults was not considered relevant. However, recent studies using positron emission tomography (PET) scanning have revealed that adult humans have several discrete areas of metabolically active BAT (Cypess et al., 2009; Nedergaard et al., 2007; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). These depots were mainly observed in the supraclavicular, suprarenal,

paravertebral and neck regions. Regarding the effect of BAT on energy expenditure, Virtanen et al. estimate the size of the supraclavicular BAT depot (both sides included) to be 63 g. If this deposit is fully activated over a year, it would burn an energy equivalent to about 4.1 kg of adipose tissue and thus would contribute substantially to energy expenditure (Virtanen et al., 2009). In this regard, BAT may have a much more important role in human metabolism than it was previously thought, emerging as an important regulator of whole body energy homeostasis. The amount of BAT (detected by PET) in individuals is negatively correlated to age and obesity (Cypess et al., 2009; Virtanen et al., 2009). Acute (5-hour) but mild cold exposure in humans led up to a 12-fold increase in glucose uptake, an increase in energy expenditure, and improved insulin sensitivity (Chondronikola et al., 2014), while chronic mild cold acclimation resulted in significant recruitment of BAT, leading to an increase in cold induced thermogenesis and a decrease in body fat mass, which was proportional to BAT activity (van der Lans et al., 2013; Yoneshiro et al., 2013).These results support a physiologically significant role for BAT in glucose homeostasis and insulin sensitivity in humans and also highlight the notion that BAT may function as an antidiabetic tissue in humans (Sidossis and Kajimura, 2015). Even more exciting, although numbers of pharmacologic agents aiming to activate human BAT thermogenesis through the βAR pathway eventually failed in the past due to either a lack of efficacy or adverse effects (Sidossis and Kajimura, 2015), mirabegron, a β3-AR agonist currently approved to treat overactive bladder, is demonstrated to be able to acutely stimulate human BAT thermogenesis (Cypess et al., 2015), suggesting pharmacological strategies designed to activate human BAT thermogenesis and treat obesity are still promising.

Histological observations of BAT in humans show that brown adipocytes are mixed with white adipocytes (Cypess et al., 2009; Zingaretti et al., 2009). Even in the major supraclavicular depots the tissue consists of a mixture of brown and white cells (Virtanen et al., 2009). It is therefore of interest to determine whether these UCP1-positive adipocytes are classical brown adipocytes similar to interscapular BAT of rodents or they are brite adipocytes in nature. Further detailed molecular characterization of human BAT based on molecular markers identified in rodents reveals that human supraclavicular BAT consists of white, brown and brite adipocytes

(Jespersen et al., 2013). The classic brown adipocytes can be found deep in the neck, while brite adipocytes can be found in the fat depot between the superficial white fat and the deepest brown fat (Cypess et al., 2013). Currently it is unclear whether the BAT observed in the paravertebral region is brown or brite in nature. Taken together, since both brown and brite adipocytes exist in humans, the studies of both classical brown and brite adipocytes in mice could have important physiological and therapeutic implications in humans.

1.4 **Aims of the thesis**

The aim of this PhD thesis was to characterize brown-like adipocytes differentiated in primary culture, including thermogenic function analyses using microplate-based respirometry, to explore the molecular basis of strain-specific differences in brite adipocytes abundance and to perform transcriptome analysis of cultured brite adipocytes from five inbred mouse strains to identify putative novel determinants governing the potential of brite adipogenesis.

In chapter 3 (Li et al., 2014a) we reviewed brown and brite adipocytes with a distinct evolutionary perspective. While there are other reviews on the general topic, the comparative and evolutionary dimensions are largely overlooked by those with an essentially exclusive biomedical focus. Particularly, we propose pre-existing brite cells in adult mice camouflaged as white-like adipocytes, which go back to postnatal development (10-20 days of age). Camouflaged brite cells can be unmasked in response to cold exposure, β-3-adrenergic receptor stimulation, and PPARγ agonist treatment. Activated brite cells can reenter the camouflage mode when stimulation ceases.

In chapter 4 (Li et al., 2014b) we explored the thermogenic function of brite adipocytes using microplate-based respirometry. Of note, we have identified a major pitfall associated with established procedures generally applied for this purpose. Based on our insight, the functional data on brown adipocyte thermogenesis as published in many papers in the recent years must be revisited. By developing a new protocol, we reproducibly quantified the UCP1-mediated component of uncoupled respiration in both

brown and brite adipocytes as evidenced by comparison of respiration profiles of UCP1 wild-type and knock-out cells. Employing this protocol, for the first time we show that brite adipocytes display a similar thermogenic capacity as classical brown adipocytes.

In chapter 5 (Li et al., 2014c) we investigated whether strain-specific differences in browning capacity can be maintained in *in vitro* cultures, a situation where extrinsic cues such as innervation and circulating hormones are definitely excluded. To achieve this goal, we took advantage of the highly variable trait of induction of brite cells in WAT between mouse strains (C57BL/6J and 129S6sv/ev). By using the primary culture method combined with co-culture strategies and cell transfections, we demonstrated that differences in *Ucp1* expression both between depots and strains are maintained in primary cultures. Further detailed analyses demonstrated that the strain specific differences in *Ucp1* expression are caused by cell intrinsic trans-acting factors. Taken together, these data demonstrate that there are intrinsic differences between progenitors from different fat depots and mouse strains which contribute to the differential browning propensity of WAT.

Finally, in chapter 6 (unpublished data) we generated an unbiased quantification of transcriptomes by RNA-Seq in undifferentiated precursor cells cultured from the stromal-vascular fraction of murine WAT and in fully differentiated primary adipocytes (treated with rosiglitazone during differentiation to promote brite adipogenesis) from five inbred mouse strains (C57BL6/J (B6), 129S6sv/ev (129), A/J, AKR/J and SWR/J). The transcriptome analysis revealed pronounced differences in browning propensities across cultures from the five inbred mouse strains, which provides an excellent starting point to identify corresponding variation on the transcriptome level. Detailed bioinformatics analysis revealed potential transcriptional regulators, essential functional components and specific markers of brite adipocytes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents that used in this dissertation were listed below.

Name	Supplier	Catalogue Nr.
100 bp-DNA Ladder	Carl Roth	T833
1k bp-DNA Ladder	Carl Roth	Y014
3,3',5-Triiodo-L-thyronine sodium salt	Sigma-Aldrich	T6397
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	I5879
70% Ethanol, denatured	Carl Roth	T913
Aceton	Carl Roth	9372
Antimycin A	Sigma-Aldrich	A8674
Bovine serum albumin (BSA) fraction V	Carl Roth	8076
Bovine serum albumin (BSA) Fatty Acid Free	Sigma-Aldrich	A3803-100G
Chloroform p.a	Carl Roth	Y015.1
Collagenase A	Biochrom	C 1-22
Dexamethason	Sigma-Aldrich	D1159
Dulbecco's modified eagle medium (DMEM) high glucose	Sigma-Aldrich	D5796
DMSO	Carl Roth	4720
Donkey serum	Sigma-Aldrich	D9663-10ML
Ethanol 96%, denatured	Carl Roth	T171
Ethanol 99.8% p.a	Carl Roth	9065
FCCP	Sigma-Aldrich	C2920-10MG
Fetal bovine serum (FBS)	Biochrom	S0615
Fungizone (Amphotericin B)	Biochrom	A2612
Gentamycin	Biochrom	A2712
Glutamax	Life Technologies	35050-061
Glucose	Biochrom	HN06
Hank's balanced salt solution (HBSS) w/Mg;Ca	Invitrogen	14025-050
Indomethacin	Sigma-Aldrich	I7378
Insulin solution, human	Sigma-Aldrich	I9278-5ML
Isoproterenol	Sigma-Aldrich	I6504-100MG
Nuclease-free water	Qiagen	129114
Nitrocellulose	Li-Cor	926-31092
Oligomycin	Sigma-Aldrich	O4876-5mg
Penicillin/Streptomycin	Biochrom	A2212
Phosphate Buffered Saline (PBS) Tablets	Gibco	18912-014
Sodium pyruvate solution 100 mM	Gibco	11360-070
Rosiglitazone	Cayman Chemical	71740
Sodium Chloride	Carl Roth	3957
SensiMix SYBR no Rox	BioLine	QT650-20
TEMED	Carl Roth	2367
TRIsure	Bioline	BIO-38033
XF calibrant buffer	Seahorse Bioscience	100840-000

2.1.2 Equipment, devices and disposables

Name	Supplier	Catalogue Nr.
AxioVert 40 Microscope	Zeiss	451207-0000-000
Confocal microscope FluoView FV10i	Olympus	
Eppendorf Research plus 10-100, 8-Channel	Eppendorf	3122000035
Eppendorf Research plus 30-300, 8-Channel	Eppendorf	3122000051
Infinite M200 Microplate reader	Tecan	30016056
HeraCell 240	Heraeus (Thermo Scientific)	51026331
LI-COR Odyssey Infrared Imaging System	Li-Cor	Ody-2197
LightCycler 480 (384 well)	Roche Applied Science	5015243001
Nucleofector II	Amaxa (Lonza)	AAB-1001
Mini-PROTEAN Tetra Cell	BioRad	165-8000
Matix Electronic 384 Equalizer Pipette	Thermo Scientific	2139-11
Seahorse XF96 extracellular flux analyzer	Seahorse Bioscience	
Trans-Blot SD Semi-Dry Transfer Cell	BioRad	170-3940
Cellculture plates, 6 well	Sarstedt	83.3920
Cellculture plates, 12 well	Sarstedt	83.3921
Transwell cell culture inserts for 6 well plate	Corning	3450
Cell strainer, 40 µM	BD Biosciences	352340
384 well plates, for qPCR	4titude	4ti-0382

2.1.3 Antibodies

Name	**Species**	**Supplier**	**Catalogue Nr.**
Primary antibody			
Anti-Actin, cloneC4	Mouse	Millipore	MAB1501
PPARγ (H-100)	Rabbit	Santa Cruz	sc-7196
UCP1	Rabbit	Abcam	ab10983
HSP60	Goat	Santa Cruz	Sc-1052
Secondary antibody			
Rabbit anti goat IRDye 680	Rabbit	Li-Cor	926-32221
Donkey anti mouse IRDye 800	Donkey	Li-Cor	926-32212
Goat anti Rabbit Alexa Fluor 488	Goat	Molecular Probes	A-11008

2.1.4 Kits

Name	Supplier	Catalogue Nr.
Amaxa Cell Line Nucleofector Kit V	Amaxa (Lonza)	VCA-1003
Dual-Luciferase Reporter Assay System	Promega	E1960
Pierce BCA Protein Assay Kit	Pierce (Thermo Scientific)	PI-23225
Quantitect Reverse Transcription	Qiagen	205313
SV Total RNA Isolation System	Promega	Z3105
Seahorse XF96 fluxPak	Seahorse Bioscience	102310-001
VECTASHIELD mounting Media	Vector Labs	VEC-H-1200

2.1.5 Plasmids

The 129 3.2kb *Ucp1*-Luc pGL3 construct was kindly provided by Dr. Robert Koza (Maine Medical Center Research Institute). From B6/J BAC DNA (RP23-131G20) we amplified the 3.2kb *Ucp1*-promotor region by PCR utilizing the following primer pair: For: GGT ACC GTG CAC ACT GCC AAA TCA TCT CAA A, Rev: GAG CTC CTG CAG AGC CAC CTG GGC. To generate a corresponding B6/J reporter which cover exactly the same *Ucp1* promoter region as 129 we removed the 129 insert from the pGL3 construct by KpnI/SacI digest. The KpnI/SacI cut B6/J PCR product was ligated into the pGL3. Correctness of the construct was confirmed by DNA sequencing.

2.1.6 Primers

Primers were designed using Primer3web (version 4.0.0) to span exon-exon junctions to exclude gDNA amplification. All primers were synthesized by Eurofins Genomics, Germany and used at a final concentration of 400nM.

2.1.7 Animals

Male mice of the C57BL6/J, 129S6sv/ev, A/J, AKR/J, SWR/J and 129S1/SvImJ (UCP1-KO mice (Enerback et al., 1997) and wild-type littermates) strains bred at the animal facility of Technische Universität München in Weihenstephan, aged 5 to 6 weeks, were used to prepare primary cultures of brown and brite adipocytes. All mice were fed a regular chow diet (V1124-3 M-Z; ssniff Spezialdiäten GmbH, Germany) ad libitum and kept at a room temperature of 23°C ± 1 °C with a humidity of 55% and a 12 h light/dark cycle.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Primary cell isolation

Mice were killed by CO_2 exposure. After killing, multiple adipose tissue depots (interscapular brown adipose tissue, inguinal and epididymal white adipose tissue) were dissected and placed into a petri dish containing prewarmed phosphate buffered saline

(PBS) with 1% antibiotics mixture (2:2:1) (Gentamycin, Penicillin/Streptomycin and Fungizone). To improve cell yields, the same fat depots from 3-4 mice were pooled. The tissues were cut into small pieces and minced using surgical scissors and digested with 7ml digestion medium (Hank's Buffered Salt Solution (HBSS) containing 1mg/ml collagenase, 3.5%BSA and 0.55mM glucose) for 45min (30min for epididymal white adipose tissue) at 37°C in an orbital shaker incubator at 120 rpm. To further improve digestion efficiency, digestion tubes are vigorously shaken by hand every 10 min. After digestion, the homogenate was filtered through a sterilized-250 µm nylon mesh and centrifuged at 250g at room temperature to separate stromal vascular fraction (SVF) from mature adipocytes. To complete the separation of the stromal cells from the floating adipocytes, tubes were shaken vigorously to thoroughly disrupt the pellet. Cells were mixed and centrifuged again at 250g for 5 min and the supernatant was carefully removed without disturbing the cell pellet. In some cases, the floating fraction with mature adipocytes was collected for further molecular analysis. The SVF cell pellet was re-suspended, washed with 7 ml HBSS containing 3.5%BSA and further centrifuged at 500g for 5 min. Finally, the pellet was re-suspended in 1ml pre-warmed growth medium (see below), passed through a 40 µm cell strainer to obtain single cell suspensions, diluted in appropriated volume of culture medium and distributed into either 6-well or 12-well tissue culture plates or XF96 V3-PS cell culture microplates.

2.2.1.2 Primary cell cultivation

The day after seeding, culture medium was removed and cells were washed with prewarmed PBS 2-3 times to remove dead cells and debris. Attached cells were cultured in growth medium (high-glucose DMEM containing 20% fetal bovine serum and 1% antibiotics (2:2:1) (Gentamycin, Penicillin/Streptomycin and Fungizone)) until confluence. After reaching confluency, induction medium containing 10% fetal bovine serum (FBS), 0.5 mM isobutylmethylxanthine, 125 µM indomethacin, 1 µM dexamethasone, 850 nM insulin, 1 nM triiodothyronine (T3), 1 µM rosiglitazone was added. After 2 days of induction, cells were maintained in differentiation media (10% FBS, 850 nM insulin, 1 nM T3 and 1µM rosiglitazone). Media was changed every two

days. Cells were harvested after seven days of differentiation. Chronic treatment of primary adipocytes with rosiglitazone robustly induced UCP1 expression.

For conditioned medium and coculture experiments, detailed methods see Chapter 5-2.2.

2.2.1.3 Primary cell transfection and luciferase reporter assays

To transfect primary cells with an *Ucp1* promoter luciferase construct (*Ucp1*-Luc, 3.2Kb), an electroporation method using the Amaxa Nucleofector II electroporation system (program A-033 and nucleofector kit V, Lonza, Germany) was applied. Briefly, differentiating cells (after 1 day of differentiation) from 2 wells of a 6- well tissue culture plate were washed with prewarmed PBS (2ml/well) and trypsinized with 2 ml trypsin/EDTA solution (L 2163, Biochrome). Cells were pooled and resuspended in 4 ml high-glucose DMEM containing 10% FBS and pelleted by 3 min centrifugation at 300 g. Pelleted cells were resuspended in 100 µl electroporation buffer containing 82 µl of Nucleofector Solution, 18 µl of supplement and 2 µg DNA (1µg *Ucp1*-Luc plus 1µg pCMV-GL (Gaussia luciferase, co-transfected to normalize for transfection efficiencies and cell number), transferred into certified cuvette and electroporated. Following electroporation, 500 µl of pre-warmed differentiation medium without antibiotics was added immediately to the cuvette for recovery. Cells were transferred and re-plated onto 2 wells of 12-well tissue culture plate. After 24h, culture medium was changed to remove dead cells. Luciferase activity assay was performed at 4 days after transfection using the Dual-Luciferase Reporter Assay kit, according to the manufacturer's protocol (E1960, Promega).

Cells were lysed using 250µl 1x Passive Lysis Buffer (PLB) by shaking for 30 min on an orbital shaker at room temperature. After cells were lysed, 20 µL of the cell lysate was added to 50 µL LAR II reagent and placed in a luminometer (Sirius L single tube luminometer, Berthold Detection Systems) to take an initial reading. Then, 50 µL of Stop and Glo Reagent was added to stop firefly luciferase activity and a second reading was taken. The Firefly luciferase activity was divided by the Renilla luciferase activity to give relative luciferase activity.

2.2.2 Molecular biology

2.2.2.1 Isolation of genomic DNA (gDNA) for genotyping of the UCP1 knockout mice

Mouse tail tips were digested in 200 µl tail-lysis buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 0.45% Nonidet P-40, 0.45% Tween 20) freshly supplemented with 0.2mg/ml Proteinase K (Fermentas) at 65 °C and 1000rpm for at least 4h. After digestion, Proteinase K was heat inactivated at 95°C for 10min (Proteinase K digests DNA polymerase). After inactivation, the lysate is ready to be used in a PCR reaction (typically 1µl per reaction).

For PCR amplification, 1µl of each gDNA sample was added to a 25µl reaction containing 12.5µL of 2x Immomix Master Mix (Bioline), 1µL of 10µM forward and reverse primers, and 8.5µL nuclease-free water. Thermal cycling parameters were as follows: 95°C for 10 minutes; 35 cycles of: 97°C for 10 seconds, 53°C for 15 seconds, and 72°C for 20 seconds; 72°C for 3 minutes. PCR products were mixed with appropriate amount of 6X DNA loading buffer (10mM Tris pH 7.6, 60mM EDTA, 60% Glycerol, 0.2% Orange G) and separated on 1.5% agarose gels, checked for expected amplicon size. The estimated sizes were 371bp for wildtype and 198bp for mutant.

Primer sequences (5´-3´):

WT Forward: CCCCTGTCAGGTGGGAT

WT Reverse: CACCCACATTGTCCATGAAG

Neo Reverse: AGGGGAGGAGTAGAAGGTGG

2.2.2.2 RNA isolation, cDNA synthesis and qPCR

RNA isolation was performed with a commercial kit, according to the manufacturer´s instructions (SV Total RNA Isolation System, Promega) with modifications. Cell lysed in 1ml of TRIsure (Bioline) (For cells cultured in 12-well plates, 500µL/well of TRIsure were used) were further mixed with 200µl Chloroform (TRIsure: Chloroform 5:1), vortexed and incubated 2-3min at room temperature (RT). Further phase separation was achieved by centrifugation at 12000g at 4°C for 15 min. After phase separation, RNA

remains exclusively in the colorless upper aqueous phase, DNA and proteins are sequestered into the interphase phase and green colored phenol-chloroform organic phase. The aqueous phase was collected, mixed with 75% ethanol in diethylpyrocarbonate (DEPC, 0.1%) water and further purified with spin columns provided by the kit according to the manufacturer´s protocol. RNA was finally eluted in 40µl nuclease-free water. Eluted RNA was stored at -80°C.

Following isolation, RNA concentration was determined spectrophotometrically using the InfiniteM200 NanoQuant (Tecan). 500ng RNA were then subjected to cDNA synthesis in a final reaction volume of 10µl containing random hexamer and oligo-dT primers and reverse transcriptase using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer instructions. cDNA was further diluted 1:10 in nuclease-free water and stored at -20°C.

Quantitative PCR (qPCR) was performed using 1µL of cDNA in a 12.5µL reaction containing 6.25µL of 2x SensiMix SYBR Master Mix No-ROX (Bioline), 1µL of 10µM forward and reverse primers, and 3.25µL nuclease-free water, in 384 well plates (4titude). Thermal cycling and fluorescence detection were performed using Lightcycler 480 II (Roche). Cycling parameters were as follows: 95°C for 6 minutes; 45 cycles of 97°C for 10 seconds, 52°C for 15 seconds, 72°C for 20 seconds. Immediately after cycling, a melting curve protocol was run to verify that a single product was generated in each reaction. Reactions for cDNA samples were performed in triplicate, standard reactions were performed in duplicate. Standard reactions containing serial diluted pooled cDNA of all samples (Pure, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) as a template were used to establish a standard curve, from which gene expression levels of samples were calculated. The RNA abundance of each gene was normalized to a housekeeping gene.

2.2.2.3 Protein extraction, concentration determination and Western blotting

For immunological detection, cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris·HCl, 1mM EDTA, 1% NP-40, 0.25% Na-deoxycholat). Cell lysis was achieved by incubating the cells shaking at 4°C for 15 min. Solubilized proteins were separated from

cell nuclei and cellular debris by 15min centrifugation at 14,000*g* at 4°C. Supernatants were transferred to a new tube and used for concentration determination and western blotting.

Protein concentration was determined by the BCA (bicinchoninic acid) assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific). Assay was performed in 96well format (Nunc 96 well plates) according to the manufacturer´s instructions. Protein samples were diluted 1:10 to be within the detection range of the assay. BSA dissolved in RIPA-buffer (2000µg/ml, 1500µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml and 0µg/ml) were used to establish a standard curve. The absorbance at 562nm was recorded using Infinite 200 (Tecan) and the protein concentration was determined.

Lysates (30µg) were resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a PVDF membrane (Millipore) by using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) and probed with either rabbit anti-UCP1 antibody (1:10000) or rabbit anti-PPARγ antibody (1:1000), or mouse anti-Actin antibody (1:10000). Secondary antibodies conjugated to IRDye™ 680 or IRDye™ 800 (Licor Biosciences) were incubated at a dilution of 1:20000. Fluorescent images were captured by an Odyssey fluorescent imager (Licor Biosciences).

2.2.2.4 Immunocytochemistry

For detailed methods see Chapter 5-2.6.

2.2.2.5 RNA-seq

Transcriptome analysis by next generation sequencing (RNA-Seq) using Illumina HiSeq 2000 platform (Illumina) was performed by our collaborator Dr. Karol Szafranski of Matthias Platzer's group at the Fritz Lipman Institute for Age Research (Jena, Germany). RNA isolated as described in 2.3.2 from undifferentiated precursor cells cultured from the stromal-vascular fraction of murine WAT and fully differentiated primary adipocytes (treated with rosiglitazone during differentiation to promote brite adipogenesis) from five inbred mouse strains (C57BL/6J, 129S6sv/ev, A/J, AKR/J, and SWR/J) with different propensity for brite adipogenesis were used for RNA-seq. Sequencing libraries were prepared using the TruSeq RNA Sample Prep kit v2 (Illumina). Libraries from 4 samples

were pooled into one sequencing lane and sequenced using a 50-cycle TruSeq SBS Kit v3-HS (Illumina), resulting in a depth of >25 million reads/sample. Sequenced tags were aligned to the Ensembl 75 transcriptome annotation (NCBI38/mm10 mouse genome) using Bowtie 0.12.9 (parameters: '-a --best --strata -S -m 100 --chunkmbs 256 -p 8') after previous filtering using PRINSEQ-lite 0.20.4 (-custom_params 'A 70%; T 70%; G 70%; C 70%' -trim_tail_left 36 -trim_tail_right 36 -lc_method dust -lc_threshold 45 -min_gc 1 -out_format 3) and adaptor removal using cutadapat 1.5 (parameters -m36 -q20). Transcript quantification was performed using mmseq-1.0.8 (default). " All graphs and statistical tests were performed in R 3.0.2 and Bioconductor 2.13 using the packages pheatmap, gplots, topGO, Reactome and limma. Differential expression was assessed with the limma-voom pipeline, using a BH-adjusted p-value of 0.01 and a fold-change of 2 as cutoffs.

2.2.3 Respirometry

Oxygen consumption rate (OCR) was measured at 37°C using an XF96 extracellular flux analyzer (Seahorse Bioscience). Sensor cartridges were pre-incubated overnight with 200 µl of XF96 calibrant buffer at 37 °C in a room air incubator. At day 7 of differentiation, cells were washed once with prewarmed, unbuffered measurement solution (DMEM basal medium supplemented with 25 mM glucose, 2 mM sodium pyruvate, 31 mM NaCl, 2 mM GlutaMax and 15 mg/l phenol red, pH 7.4) (basal assay medium) and then the medium replaced with fresh assay medium with or without essentially fatty acid free bovine serum albumin (BSA), and incubated at 37°C in a room air incubator for 1 h. The drug injections ports of the sensor cartridges were loaded with the assay reagents at 10X in basal assay medium (no BSA). Basal respiration was measured in untreated cells. Coupled respiration was inhibited by oligomycin treatment (5 µM). UCP1 mediated uncoupled respiration was determined after isoproterenol (0.1-1 µM) stimulation. Maximum respiratory capacity was assessed after FCCP (Sigma-Aldrich) addition (1 µM). Finally, mitochondrial respiration was blocked by antimycin A (Sigma-Aldrich) (5 µM) treatment and the residual OCR was considered non-mitochondrial respiration. Oxygen consumption rates were automatically calculated by the Seahorse XF-96 software. Data were exported and reconstructed in GraphPad

Prism 6.0 software. After completion of an assay, the microplate was saved and protein was isolated for UCP1 phenotyping. Each experiment was repeated at least 3 times with similar results.

The following table was used to program the Mix, Wait, Measure and Injection protocol.

Protocol A for *general cell respiration*	Protocol B for *UCP1 activity*
1-Calibrate probes	1- Calibrate probes
2-Equilibrate 12min*	2-Equilibrate 12min*
3-Loop 3 times 4-(1-2-3)Mix 2min 5-(1-2-3)Measure 5 min	3-Loop 3 times 4-(1-2-3)Mix 2min 5- Time Delay of 2 min 6-(1-2-3)Measure 3 min
6-Loop end	7-Loop end
7-Inject A 20µl	8-Inject A 20µl
8-Loop 3 times 9-(4-5-6)Mix 2min 10-(4-5-6)Measure 5 min	9-Loop 3 times 10-(4-5-6)Mix 2min 11- Time Delay of 2 min 12-(4-5-6)Measure 3 min
11-Loop end	13-Loop end
12-Inject B 22µl	14-Inject B 22µl
13-Loop 3 times 14-(7-8-9)Mix 2min 15-(7-8-9)Measure 5 min	15-Loop 5 times 16-(7-8-9-10-11)Mix 2min 17- Time Delay of 2 min 18-(7-8-9-10-11)Measure 3 min
16-Loop end	19-Loop end
17-Inject C 24µl	20-Inject C 24µl
18-Loop 3 times 19-(10-11-12)Mix 2min 20-(10-11-12)Measure 5 min	21-Loop 3 times 22-(12-13-14)Mix 2min 23- Time Delay of 2 min 24-(12-13-14)Measure 3 min
21-loop end	25-Loop end
22-Program end	26-Inject D 26µl

	27-Loop 3 times 28-(15-16-17)Mix 2min 29- Time Delay of 2 min 30-(15-16-17)Measure 3 min
	31-Loop end
	32-Program end
*Default Equilibrate command consists of 2 min Mix, 2 min Wait repeated 3X	

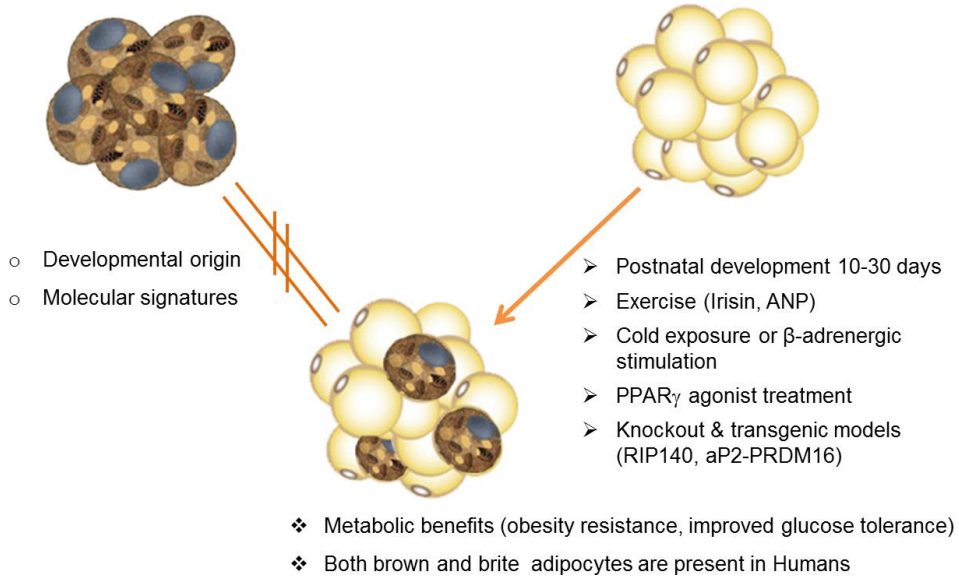
2.2.4 Statistics

All values are presented as means ± SEM. Significant differences were assessed by two-tailed Student's t test for single comparisons or Two-way ANOVA when two or more groups were compared (Prism 6.0 software). A p value < 0.05 was considered a statistically significant difference.

CHAPTER 3

White, brite, and brown adipocytes: the evolution and function of a heater organ in mammals

Can. J. Zool. 92(7): 615-626, 2014



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REVIEW

White, brite, and brown adipocytes: the evolution and function of a heater organ in mammals[1]

Yongguo Li, David Lasar, Tobias Fromme, and Martin Klingenspor

Abstract: Brown fat is a specialized heater organ in eutherian mammals. In contrast to the energy storage function of white adipocytes, brown adipocytes dissipate nutrient energy by uncoupling of mitochondrial oxidative phosphorylation, which depends on uncoupling protein 1 (UCP1). UCP1, as well as UCP2 and UCP3, belong to the family of mitochondrial carriers inserted into the inner mitochondrial membrane for metabolite trafficking between the matrix and the intermembrane space. UCP1 transports protons into the mitochondrial matrix when activated by a rise in free fatty acid levels in the cell. This UCP1-dependant proton leak drives high oxygen consumption rates in the absence of ATP synthesis and dissipates proton motive force as heat. The enormous heating capacity of brown fat is supported by dense vascularization, high rates of tissue perfusion, and high mitochondrial density in brown adipocytes.

It has been known for more than 50 years that nonshivering thermogenesis in brown fat serves to maintain body temperature of neonates and small mammals in cold environments, and is used by hibernators for arousal from torpor. It has been speculated that the development of brown fat as a new source for nonshivering thermogenesis provided mammals with a unique advantage for survival in the cold. Indeed brown fat and UCP1 is found in ancient groups of mammals, like the afrotherians and marsupials. In the latter, however, the thermogenic function of UCP1 and brown fat has not been demonstrated as of yet. Notably, orthologs of all three mammalian UCP genes are also present in the genomes of bony fishes and in amphibians. Molecular phylogeny reveals a striking increase in the substitution rate of UCP1 between marsupial and eutherian lineages. At present, it seems that UCP1 only gained thermogenic function in brown adipocytes of eutherian mammals, whereas the function of UCP1 and that of the other UCPs in ectotherms remains to be identified.

Evolution of thermogenic function required expression of UCP1 in a brown-adipocyte-like cell equipped with high mitochondrial density embedded in a well-vascularized tissue. Brown-adipocyte-like cells in white adipose tissue, called “brite” (brown-in-white) or “beige” adipocytes, emerge during adipogenesis and in response to cold exposure in anatomically distinct adipose tissue depots of juvenile and adult rodents. These brite adipocytes may resemble the archetypical brown adipocyte in vertebrate evolution. It is therefore of interest to elucidate the molecular mechanisms of brite adipocyte differentiation, study the bioenergetic properties of these cells, and search for the presence of related brown-adipocyte-like cells in nonmammalian vertebrates.

Key words: white, brite, brown, adipocytes, fat, *Ucp1*, evolution.

Résumé : Le tissu adipeux brun est un organe de production de chaleur spécialisé chez les mammifères euthériens. Contrairement aux adipocytes blancs, qui ont une fonction de stockage d’énergie, les adipocytes bruns dissipent l’énergie des aliments nutritifs en découplant la phosphorylation oxydative mitochondriale, un processus qui dépend de la protéine de découplage 1 (UCP1). Cette dernière, tout comme l’UCP2 et l’UCP3, appartient à la famille des transporteurs mitochondriaux insérés dans la membrane mitochondriale interne qui assurent la circulation de métabolites entre la matrice et l’espace intermembranaire. Quand elle est activée par l’augmentation des concentrations d’acides gras libres dans la cellule, l’UCP1 transporte des protons vers la matrice mitochondriale. Cette fuite de protons dépendante de l’UCP1 se traduit par des taux de consommation d’oxygène élevés en l’absence de synthèse d’ATP et dissipe sous forme de chaleur la force motrice des protons. Cette énorme capacité calorifique de la graisse brune est appuyée par une vascularisation dense, des vitesses de perfusion élevées et une forte densité mitochondriale d’adipocytes bruns.

Il est établi depuis plus de 50 ans que la thermogénèse sans frisson dans la graisse brune sert au maintien de la température du corps chez les nouveau-nés et les petits mammifères exposés au froid et aident les mammifères en hibernation à sortir de leur torpeur. Il a été postulé que le développement de la graisse brune comme nouvelle source de thermogénèse sans frisson fournissait aux mammifères un avantage unique leur permettant de survivre dans des conditions froides. Des tissus adipeux bruns et de l’UCP1 sont notamment présents chez des groupes de mammifères anciens, comme les afrothériens et les marsupiaux. Chez ces derniers, toutefois, la fonction thermogénique de l’UCP1 et de la graisse brune n’a pas encore été démontrée. Des orthologues des trois gènes d’UCP mammaliens sont notamment aussi présents dans les génomes de poissons osseux et

Received 8 July 2013. Accepted 18 October 2013.

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[1]This review is one of a series of invited papers arising from the symposium “Animal mitochondria: evolution, performance, and plasticity”, sponsored by Canadian Science Publishing and held during the Canadian Society of Zoologists’ Annual Meeting, University of Guelph, 13–17 May 2013.

Can. J. Zool. **92**: 615–626 (2014) dx.doi.org/10.1139/cjz-2013-0165

Published at www.nrcresearchpress.com/cjz on 21 October 2013.

d'amphibiens. La phylogénie moléculaire révèle une augmentation marquée du taux de substitution de l'UCP1 entre les lignées marsupiale et euthérienne. À l'heure actuelle, il semble que l'UCP1 ne présente une fonction thermogénique que dans les adipocytes bruns des mammifères euthériens, alors que la fonction de cette protéine et d'autres UCP chez les ectothermes demeure inconnue.

L'apparition de la fonction thermogénique a nécessité l'expression de l'UCP1 dans une cellule semblable aux adipocytes bruns et de forte densité mitochondriale, intégrée à un tissu bien vascularisé. Des cellules semblables à des adipocytes bruns présentes dans du tissu adipeux blanc, appelées adipocytes beiges, apparaissent durant l'adipogénèse et en réponse à l'exposition au froid dans des dépôts de tissus adipeux distincts de rongeurs juvéniles et adultes. Ces adipocytes beiges peuvent ressembler à l'adipocyte brun archétypique dans l'évolution des vertébrés. L'élucidation des mécanismes moléculaires de la différenciation des adipocytes beiges, l'étude des propriétés bioénergétiques de ces cellules et la recherche de cellules apparentées semblables à des adipocytes bruns chez les vertébrés non mammaliens sont donc d'un grand intérêt. [Traduit par la Rédaction]

Mots-clés : adipocytes, blanc, beige, brun, graisse, *Ucp1*, évolution.

Introduction on brown fat biology

In the course of evolution, the selection pressures to cope with periods of food shortage and cold spells led to multiple genetic and physiological adaptations in animals. Mammals are equipped with two types of adipose tissues, white adipose tissue (WAT) and brown adipose tissue (BAT), that enable them to face these energy-demanding situations. Whereas WAT buffers energy supply during periods of food shortage by lipolysis of triglycerides and release of fatty acids into the circulation, BAT is specialized for nonshivering thermogenesis to maintain body temperature against the cold. Brown adipocytes utilize a unique energy-dissipating mechanism catalyzed by the mitochondrial uncoupling protein 1 (UCP1), which upon activation short circuits mitochondrial oxidative phosphorylation (OXPHOS) and dissipates proton-motive force as heat (Klingenspor 2003). Both lipid mobilization in white adipocytes and nonshivering thermogenesis in brown adipocytes are triggered by the sympathetic innervation of these tissues (Klingenspor and Fromme 2012).

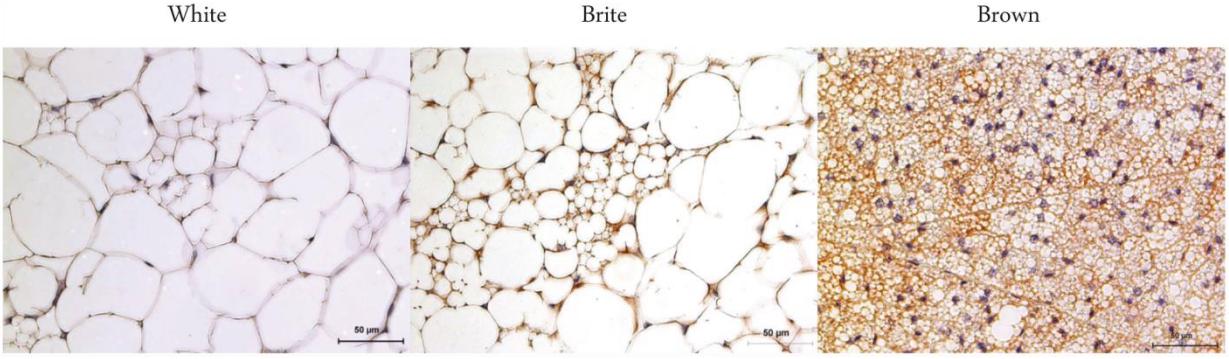
According to these distinct functions for energy storage and thermogenesis, WAT and BAT exhibit corresponding histological features. White adipocytes typically present a single large lipid droplet (unilocular), whereas brown adipocytes exhibit a larger abundance of small lipid droplets (multilocular) (Fig. 1). Brown adipocytes contain a very large number of mitochondria, which together with the dense vascularization of BAT, confers the eponymous brown color and allows for the enormous heating capacity to the tissue. Historically, BAT was first observed in the interscapular region of hibernating marmots in 1551 by the Swiss naturalist Konrad Gessner, who described it as "They have a lot of fat on their back, although the other parts of the body are lean. In truth it can be called neither fat nor flesh, but similar to the bovine mammary gland, it is something in between" (Gessner 1551). It was originally referred to as the "hibernation gland", implicating an endocrine function in the control of hypometabolism and arousal of hibernators. More than four centuries later, the thermogenic function of BAT was recognized based on pioneering research carried out in 1960s on small laboratory rodents and rabbits (genus *Oryctolagus* Lilljeborg, 1873) (reviewed in Smith and Horwitz 1969). Since then, research interest in BAT biology has steadily increased with some fluctuations, as judged by the publication record during the past 50 years (Klingenspor et al. 2012). The recent renaissance of research interest was stirred by the rediscovery of functional BAT in adult humans (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). Given the energy dissipation properties, the recruitment and activation of BAT appears as an attractive and potentially effective strategy for the prevention and treatment of obesity (Tseng et al. 2010). In most mammals, discrete classical BAT depots can be found in the interscapular, cervical, axillar, and perirenal regions. In a process casually termed "browning" of WAT, UCP1-expressing brown-like adipocytes now termed beige or "brite" (brown-in-white) can also develop in typical WAT of adult mice (*Mus musculus* L., 1758) in response to cold exposure, β_3-adrenergic receptor stimulation, and PPARγagonist treatment (Young et al. 1984; Cousin et al. 1992; Guerra et al. 1998; Fukui et al. 2000;Himms-Hagen et al. 2000; Xue et al. 2007). During murine postnatal development, these brite cells spontaneously emerge around weaning (Xue et al. 2007; Lasar et al. 2013). A similar phenomenon has also been observed in neonates from several other mammalian species such as the dog (*Canis lupus familiaris* L., 1758) (Ashwell et al. 1987), semi-domesticated reindeer (*Rangifer tarandus tarandus* L., 1758) (Soppela et al. 1991), and goat (*Capra hircus* L., 1758) (Trayhurn et al. 1993), as well as cold-acclimated rat (*Rattus norvegicus* Berkenhout, 1769) (Cousin et al. 1992), cat (*Felis catus* L., 1758) (Loncar et al. 1986), and ferret (*Mustela putorius furo* L., 1758) (Fuster et al. 2009). Recently, it has been suggested that brite cells represent a new class of adipocytes distinct from white and brown adipocytes with respect to their development, molecular outfit, and hormonal sensitivity (Petrovic et al. 2010; Wu et al. 2012). Notably, the abundance of brite cells in WAT has been associated with obesity resistance, enhanced fat oxidation, energy expenditure, and improved systemic insulin sensitivity (Guerra et al. 1998; Almind et al. 2007). Even more current evidence points to the presence of both classical brown and brite adipocytes in humans, both as babies and adults (Boström et al. 2012; Sharp et al. 2012; Cypess et al. 2013; Jespersen et al. 2013; Lidell et al. 2013). These findings are important because they suggest that the studies of both classical brown and brite adipocytes in mice could also have important physiological and therapeutic implications in humans. Thus, the physiological significance and regulatory mechanisms of brown versus brite adipocytes in both mice and humans clearly need to be determined. Perhaps the most relevant unresolved issue pertaining to brite adipocytes is whether these cells actually do contribute significantly to whole body thermogenesis. From an evolutionary perspective, the advantage of having a second type of UCP1-expressing adipocyte with thermogenic function in WAT is not evident.

In this review, we summarize the most relevant and recent knowledge on brown adipose tissue biology covering the molecular mechanism of UCP1-mediated uncoupling, the evolution of UCP1 and BAT, the molecular control of UCP1 expression, the markers and molecular mechanisms of brown and brite adipogenesis, and finally highlight the relationship between brite and brown cells from an evolutionary point of view.

Molecular mechanism of uncoupling OXPHOS

The unparalleled thermogenic capacity of BAT is conferred by uncoupling protein 1 (UCP1), a 33 kD BAT-specific transport protein located in the inner membrane of mitochondria. UCP1, as well as UCP2 and UCP3, belong to the family of nearly 50 mitochondrial carriers inserted into the inner mitochondrial membrane and employed in metabolite trafficking between matrix and intermembrane space. Within intact cells, UCP1 is constantly inhibited by cytosolic purine nucleotides (i.e., ADP, ATP, GDP, and GTP). This inhibition has to be overcome through a process that probably involves fatty acids interacting directly with UCP1 and which is initiated physiologically by noradrenergic stimulation.

Fig. 1. White, "brite" (brown-in-white), and brown adipocytes in murine adipose tissues. Immunohistochemical detection of uncoupling protein 1 (UCP1) positive adipocytes in epididymal and inguinal white adipose tissue and in interscapular brown adipose tissue of 129S6sv/ev mice. Nuclei were stained with hematoxylin. Magnification × 40; scale bars = 50 μm.



This process uncouples OXPHOS: activated UCP1 transports protons back into the mitochondrial matrix, which results in high oxygen consumption rates in the absence of ATP synthesis and dissipates proton-motive force as heat. The exact molecular mechanism of fatty acid inducible UCP1 activity remains elusive. Several mechanisms have been proposed (Ricquier and Bouillaud 2000; Cannon and Nedergaard 2004). The key discrepancy between these models is about whether fatty acids act as allosteric activators or cofactors of UCP1 or are directly transported by UCP1 across the inner mitochondrial membrane. Nevertheless, all models agree that fatty acids are absolutely required for the function of UCP1 (Klingenspor and Fromme 2012). Recently, by using direct patch-clamp recordings of UCP1 currents from the inner membrane of BAT mitochondria, Fedorenko and colleagues propose that UCP1 operates in a long-chain fatty acid shuttling mode where UCP1 is a fatty acid anion and H^+ symporter that simultaneously transports one fatty acid anion and one proton per transport cycle. Because of strong hydrophobic interactions, however, the long-chain fatty acid anions cannot dissociate from UCP1 thus returning to initiate another proton translocation cycle. In this way, UCP1 effectively operates as a proton carrier activated by long-chain fatty acids (Fedorenko et al. 2012).

Evolutionary origin of UCP1

Increased interest in the evolutionary history of uncoupling proteins emerged since the discovery of new paralogs in plants, mammals, fishes, and unicellular eukaryotes (Ricquier and Bouillaud 2000; Borecký et al. 2001; Hanák and Ježek 2001; Ledesma et al. 2002). Cold-induced expression of a novel UCP in potato (*Solanum tuberosum* L.) leaves implicated a function in plant thermogenesis (Vercesi et al. 1995; Laloi et al. 1997), which is now thought to be more dependent on alternative oxidase (Vercesi et al. 2006; Zhu et al. 2011). The discovery of UCP2 and UCP3 in zebrafish (*Danio rerio* (Hamilton, 1822)) and common carp (*Cyprinus carpio* L., 1758) suggested that these uncoupling proteins do not serve a thermogenic function, as ectothermic fish cannot maintain body temperature above water temperature by endogenous heat production (Stuart et al. 1999; Jastroch et al. 2005). The cloning of the only avian UCP gene, now identified as the ortholog of mammalian UCP3, revealed tissue-specific expression in skeletal muscle, and the cold-induced upregulation of avian UCP was supportive for a function in thermogenesis (Raimbault et al. 2001). UCP1, however, was always assumed to be unique to mammals with tissue-specific expression in brown adipose tissue being essential for nonshivering thermogenesis. The discovery of a gene orthologous to mammalian *Ucp1* in the genomes of zebrafish and pufferfish (*Fugu rubripes* (Temminck and Schlegel, 1850)) based on conserved synteny and phylogenetic inference was therefore unexpected (Jastroch et al. 2005). As for UCP2, a thermogenic function of this UCP1 ortholog in ectothermic fishes must be questioned. In the common carp, strongest expression of the *Ucp1* gene is observed in the liver with downregulation in response to cold acclimation (Jastroch et al. 2005). This physiological regulation does not support a role of UCP1 in thermogenesis. Nevertheless, proton leak measurements in liver mitochondria isolated from warm-acclimated carp showed fatty acid induced uncoupling not detectable after cold acclimation (Jastroch et al. 2007). These data suggest that fish UCP1 may share fatty acid induced activation as one characteristic feature with mammalian UCP1. In contrast to mammalian UCP1, however, purine nucleotides did not inhibit fatty acid induced uncoupling of carp mitochondria.

In the zebrafish, UCP1 shares 69% and 72% identity with UCP2 and UCP3, respectively, whereas the latter two share 74% identity (Jastroch et al. 2005). In contrast, human UCP1 shares less identity with UCP2 and UCP3 (54% and 57%, respectively). This observation was consolidated by phylogenetic inference, including 80 sequences from the animal kingdom (Jastroch et al. 2008). Uncoupling protein sequences from fishes, amphibians, monotremes, and marsupials could be unambiguously assigned as UCP1 orthologs because they grouped together with eutherian UCP1 in a monophyletic clade. Notably, the substitution rate turned out to be twofold higher between the marsupial and the eutherian lineage than between the amphibian and the marsupial lineage. This strongly suggests an accelerated evolution of UCP1 during the emergence of eutherian mammals, which mounted the development of the novel thermogenic function. To serve this novel function also required specific expression of UCP1 in a cell type with large respiratory capacity equipped to oxidize glucose and fatty acids at a high rate. The origin and evolution of the gene regulatory sequences resulting in strong and highly tissue-specific and cold-induced expression of UCP1 in brown adipocytes are poorly understood.

Transcriptional control of the *Ucp1* gene

Transcription of the *Ucp1* gene is restricted to brown or brite adipocytes and strongly induced upon cold exposure of the organism. Intriguingly, both the strict tissue specificity and the responsiveness to cold are conferred by a very short enhancer region upstream of the transcriptional start site. The murine *Ucp1* enhancer was initially identified as a DNase I hypersensitive region essential for expression (Boyer and Kozak 1991) and could soon be further pinpointed to 221 bp located more than 2 kb upstream of the start of transcription in the rat *Ucp1* gene (Cassard-Doulcier et al. 1993; Kozak et al. 1994).

This short enhancer region proved to be responsible for BAT specificity of *Ucp1* expression. In fact, it exerts sufficient control to

restrict the expression of a reporter gene driven by the viral thymidine kinase promoter to brown adipose tissue (Cassard-Doulcier et al. 1998). Thus, the *Ucp1* enhancer is both sufficient and necessary for the tissue-specific expression pattern of the gene, even in the absence of the endogenous core promoter.

Moreover, adaptive gene regulation is controlled by elements of the *Ucp1* enhancer. A thorough sequence analysis revealed several cAMP response elements (CRE) within this region, as well as other crucial elements without a known interacting transcription factor (brown adipocyte regulatory element, BRE) (Kozak et al. 1994). The second messenger cAMP is formed upon noradrenergic stimulation of brown adipocytes and directs gene expression via PKA-mediated activation of the transcription factor CREB (cAMP response element binding protein). Further signals integrated by the *Ucp1* enhancer include thyroid hormone, retinoic acid, and thiazolidinediones that act via binding of their respective nuclear receptors to characterized binding sites within less than 100 bp of the enhancer (Larose et al. 1996; Rabelo et al. 1996*a*, 1996*b*, 1997; Rim and Kozak 2002).

The principal functional properties of the murine and rat *Ucp1* enhancer are similar in the human gene (Del Mar Gonzalez-Barroso et al. 2000). Extending the comparison to further vertebrate species on the sequence level reveals a strong conservation within placental mammals including the ancient afrotherians, but the absence of such an identifiable region in marsupials, monotremes, frogs, and fish despite the presence of an *Ucp1* ortholog (Jastroch et al. 2005, 2007; Shore et al. 2010). Thus, the invention of the placental *Ucp1* enhancer seems to have occurred in parallel with the accelerated rate of *Ucp1* amino acid substitution during the emergence of the first placental mammals (Hughes et al. 2009).

In several marsupial species, *Ucp1* expression is limited to certain adipose tissue depots and increased in response to cold (Jastroch et al. 2008). Therefore, neither tissue-specific nor adaptive expression is an exclusive trait of the placental *Ucp1* enhancer. However, the responsible gene regulatory elements in marsupials have not been identified yet. A comparison of the 5′-upstream region in three marsupial *Ucp1* genes reveals a conserved region that resembles the placental *Ucp1* enhancer in size and location far upstream of the transcriptional start site (Figs. 2A, 2B). This putative marsupial *Ucp1* enhancer shares no significant sequence similarity with its placental counterpart. Bioinformatic analysis (DiAlign, Genomatix) does not reveal any conserved CREs or other elements typical for placental *Ucp1* regulation. So far no experimental data are available, but it is a hypothesis worth entertaining that the putative marsupial enhancer confers the tissue-specific and cold responsive *Ucp1* expression in marsupials. In that case, either marsupials use a different set of signaling pathways and transcription factors to achieve a similar expression pattern, or the well-characterized elements of the placental enhancer are in fact not responsible for this specificity.

Evolution of the *Ucp1* amino acid sequence implies a functional shift between marsupials and placentals, and it is tempting to assume that the basis of this change was the advent of brown adipose tissue. This is supported by the finding that *Ucp1* protein may not play a thermogenic role in marsupials (Klingenspor et al. 2008; Polymeropoulos et al. 2012*a*). In terms of *Ucp1* gene regulation, however, marsupials and placentals share many characteristics. Possibly, a rearrangement of the preexisting marsupial enhancer gave rise to the extremely high transcription rates that are necessary to equip placental brown adipose tissue with its vast amount of UCP1 protein.

Markers and molecular mechanisms of brown and brite adipogenesis

Despite their distinct physiological functions, WAT and BAT share many features and have long been assumed to have the same developmental origin. Lineage-tracing studies, however, revealed that classical brown adipocytes found in the interscapular depot of rodents are derived from a Myf5+ muscle-like cellular lineage, while white adipocytes are not (Seale et al. 2008). Interestingly, the same study has also demonstrated that brite adipocytes within inguinal and epididymal fat depots do not originate from Myf5+ precursors. In contrast, a recent study demonstrated that most brite adipocytes in retroperitoneal WAT arise from the Myf5+ lineage, indicating divergent ontogeny of brite cells in different fat depots (Sanchez-Gurmaches et al. 2012). This also raises the question whether brite adipocytes in different fat depots also exhibit functional divergence (Fig. 3). Even though they differ in developmental origin, white and brown adipocytes share several key transcription factors involved in adipogenesis (Rosen and MacDougald 2006). These adipogenic factors include the peroxisome proliferator activated receptor γ (PPARγ) and members of the CCAAT/enhancer binding protein family (C/EBP). Briefly, PPARγ is the master regulator of adipogenesis, and is both sufficient and required for the formation of brown and white adipocytes. C/EBPs coordinate this transcription cascade, with PPARγ to promote and maintain adipose differentiation. Although they are definitely necessary for brown adipocyte differentiation and the maintenance of other genes in the adipogenic program, the expression of PPARγ or C/EBPβ in stem cells leads to formation of white but not brown adipocytes (Wu et al. 1995; Kim et al. 2005), suggesting an additional set of transcriptional regulators seemed to be required to determine brown adipocyte fate. However, knowledge of those regulators is still very limited. Correspondingly, the molecular mechanisms of brown and brite adipogenesis are largely unknown.

Mouse models

Besides environmental and endocrine effectors of WAT browning, several studies demonstrated the appearance of brite adipocytes in WAT is subject to complex genetic control. The analysis of adipose tissue plasticity in genetically modified mouse models has largely advanced our understanding of the browning process in WAT. Several studies have identified potent endocrine and paracrine modulators of browning, mostly defined by the increased abundance of multilocular adipocytes expressing specific brown adipocyte marker genes.

The β_3-receptor mediated pathway

The β_3-receptor mediated pathway is the most dominant and best characterized pathway for the acute activation of uncoupled respiration as well as the recruitment of thermogenic capacity in BAT, and also leads to the browning of WAT upon chronic stimulation with β_3-adrenergic receptor agonists. The β_3-adrenergic receptor is coupled to a G_s-protein and G_i-protein (Chaudhry et al. 1994; Gerhardt et al. 1999; Bourová et al. 2000). In mature brown adipocytes, stimulation of the β_3-adrenergic receptor activates the formation of cAMP out of ATP by adenylate cyclase. Protein kinase A binds cAMP and activates the cAMP responsive element-binding protein (CREB) via phosphorylation (Thonberg et al. 2002).

The relevance of the β_3-receptor for adaptive thermogenesis has been questioned because β_3-adrenergic receptor knockout mice exhibit normal activation in the cold. These mice, however, do exhibit reduced cold-induced browning of WAT (Jimenez et al. 2003). Another signaling component that is phosphorylated by protein kinase A is p38 mitogen activated protein kinase (MAPK). Upon phosphorylation, p38 MAPK can activate via phosphorylation ATF2, a CREB family member that promotes the expression and phosphorylation of PGC1α—the main regulator of mitochondrial biogenesis and coactivator of UCP1 expression (Enslen et al. 1998; Cao et al. 2004; Robidoux et al. 2005). A knockout of PGC1α leads to the reduction of brite cell abundance in WAT, but BAT activity is unaltered probably due to unknown compensatory processes (Kleiner et al. 2012). Concurrently, ablation of RIP140, a negative

Fig. 2. Marsupial uncoupling protein 1 (*Ucp1*) genes exhibit a conserved promotor region. (A) Dot plot of 5000 bp 5′ upstream sequence of the gray short-tailed opossum (*Monodelphis domestica*) and tammar wallaby (*Macropus eugenii* (Desmarest, 1817)) *Ucp1* orthologs. The circle indicates the position of a conserved region approximately 1–2 kb upstream of the transcriptional start site. (B) Alignment of the conserved region of ~150 bp of opossum, wallaby, and Tasmanian devil (*Sarcophilus harrisii* (Boitard, 1841)).

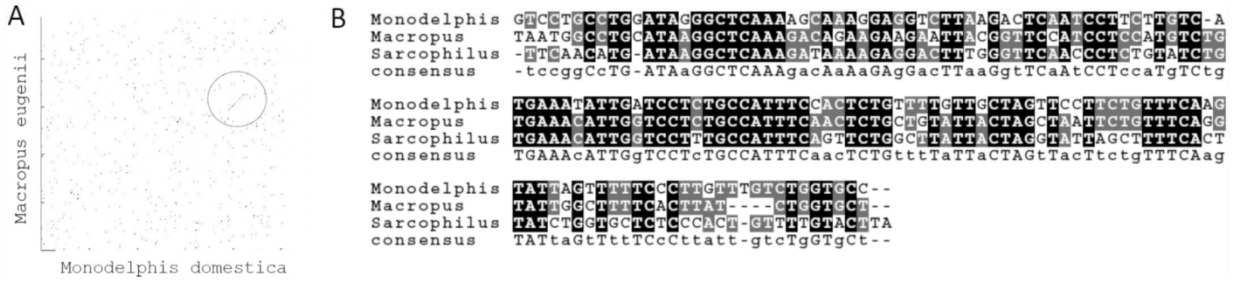
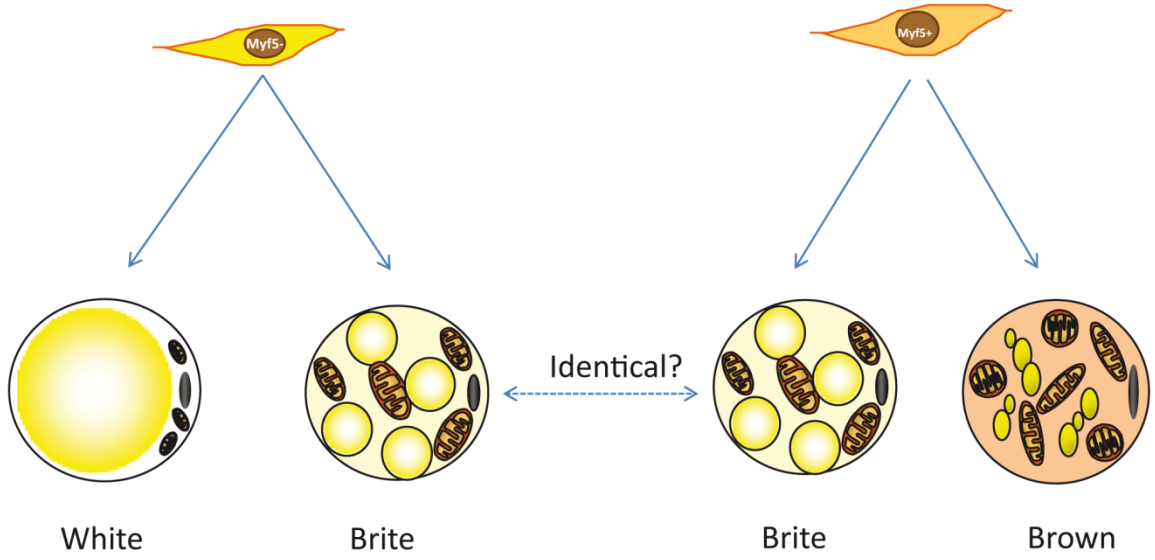


Fig. 3. Multiple developmental lineages of "brite" (brown-in-white) adipocytes. Lineage-tracing studies revealed that brite adipocytes within inguinal and epididymal fat depots originate from the Myf5– lineage, while most brite adipocytes (70%) in retroperitoneal white adipose tissue arise from the Myf5+ lineage.



regulator of PGC1α, stimulates the browning of WAT (Leonardsson et al. 2004).

One positive effector of browning acting downstream of the β_3-adrenergic receptor–cAMP–PKA pathway has been identified as cyclooxygenase 2 (COX2). The β_3-adrenergic activation of COX2 activity in white adipocytes increases the production of prostaglandin H_2 from arachidonic acid. The selective inhibition of COX2 with celecoxib or the ablation of COX2 also reduces browning of WAT in response to treatment with the β_3-adrenergic receptor agonist CL316,243 (Madsen et al. 2010; Vegiopoulos et al. 2010).

Transcriptional control of browning

Next to β_3-adrenergic receptor mediated browning, cell fate and developmental programming also influence WAT morphology. The main regulator determining the switch from a progenitor cell into a brown adipocyte is PRD1-BF-1-RIZ1 homologous domain containing protein-16 (PRDM16). Genetic ablation in mice is lethal; however, BAT in PRDM16 deficient mouse embryos at the age E17 exhibits an unusual morphology together with reduced expression of brown fat specific marker genes (Seale et al. 2008). Conversely, transgenic overexpression of PRDM16 in WAT leads to an increased browning by the formation of brite islets (Seale et al. 2007). Mechanistically, PRDM16 acts as a transcriptional co-regulator in concert with PPARγ and C/EBPs to induce the differentiation of progenitor cells into brown and brite adipocytes (Ohno et al. 2012). In embryonic fibroblasts, the complex of PRDM16 and C/EBPβ is sufficient to induce adipogenesis with the formation of mature adipocytes expressing brown fat marker genes (Kajimura et al. 2009).

Lipid droplet coating proteins and lipolytic pathway

Lipid droplets are cellular organelles that store triglycerides and cholesteryl esters in adipocytes. They are surrounded by a monolayer composed of phospholipids and lipid droplet coating proteins. These coating proteins contribute to the integrity and dynamics of lipid droplets and play a central role in the regulation of lipolytic rate. A rise in intracellular free fatty acid concentration as a result of high lipolytic rates leads to the activation of UCP1 and thermogenesis in brown adipocytes (Cannon and Nedergaard 2004). The most prominent fat-specific lipid droplet coating proteins involved in metabolic regulation are CideA, Fsp27, and perilipin.

Cell death inducing DNA fragmentation factor, alpha subunit like effector A (CIDEA) in mice is predominantly expressed in brown adipocytes (Nordström et al. 2005). It is located in the lipid droplet monolayer, but also found in mitochondria where it may

interact with UCP1 (Zhou et al. 2003; Puri et al. 2008). A knockout of CideA is associated with resistance to diet-induced obesity and the absence of multilocular adipocytes in WAT (Zhou et al. 2003). Multiple studies have utilized CideA expression as a robust marker for the appearance of brite adipocytes in WAT (Madsen et al. 2010; Meyer et al. 2010; Vegiopoulos et al. 2010; Fisher et al. 2012; Lasar et al. 2013).

Fsp27, another member of the cell death inducing DNA fragmentation factor family, results in the formation of unilocular adipocytes by promoting the fusion of small lipid droplets. Knockout of Fsp27 in mice is associated with the browning of WAT and the development of DIO resistance (Matsusue et al. 2008; Nishino et al. 2008).

The most abundant lipid droplet coating protein in white and brown adipocytes next to CideA and Fsp27 is perilipin (Greenberg et al. 1991; Greenberg et al. 1993). Perilipin is a PKA target and contains multiple consensus sites for the phosphorylation of serine residues (Brasaemle 2007). The ablation of perilipin results in browning of WAT with an increased rate of lipolysis also in BAT, and therefore perilipin seems to impede access of cellular lipases to the lipid droplet (Martinez-Botas et al. 2000). It was hypothesized that an overexpression of perilipin in mice leads to an increased lipid storage in WAT and therefore results in an obese phenotype (Sawada et al. 2010). Surprisingly, overexpression of perilipin in white adipose tissue during high fat diet feeding results in a diet-induced obesity resistance phenotype with brite adipocyte appearance in WAT accompanied by elevated brown fat markers like UCP1 and PGC1α (Miyoshi et al. 2010; Sawada et al. 2010). Upon stimulation of β_3-adrenergic receptors, perilipin and hormone sensitive lipase (HSL) are phosphorylated by PKA (Cahill et al. 1959; Leboeuf et al. 1959; Rizack 1964; Collins and Surwit 2001; Granneman et al. 2007). Phosphorylation leads to a translocation and docking of HSL to the surface of lipid droplets, where it hydrolyzes triacylglycerides and cholesteryl esters stored in the lipid droplet (Su et al. 2003; Wang et al. 2005). In HSL knockout mice, WAT mass is reduced with no signs of altered brite adipocyte abundance, but increased thermogenic activity in BAT (Haemmerle et al. 2002; Harada et al. 2003). The hydrolysis of triglycerides essentially requires adipose triglyceride lipase (ATGL) (Zimmermann et al. 2004; Haemmerle et al. 2006). Notably, adipocyte-specific ablation of ATGL promotes the conversion of BAT into WAT (Ahmadian et al. 2011).

Mitochondrial function

Mice with a loss of function of the *Ucp1* gene lack norepinephrine-induced nonshivering thermogenesis in BAT with partial monolocularization of brown adipocytes and exhibit reduced capacity for cold-induced thermogenesis (Enerbäck et al. 1997). However, *Ucp1* knockout mice when cold acclimated exhibit a stronger propensity for browning of WAT (Liu et al. 2003; Ukropec et al. 2006). It has been suggested that the formation of brite adipocytes in WAT may serve to compensate for the loss of norepinephrine-mediated nonshivering thermogenesis in BAT of these mice (Granneman et al. 2003; Ukropec et al. 2006; Meyer et al. 2010).

Other pathways involved in browning

Recent studies identified unexpected effectors of browning in WAT with myostatin as one prominent factor. Myostatin, a myokine secreted by skeletal muscle, was discovered in cattle as a negative regulator of muscle growth. A knockout of myostatin in mice leads to an exorbitant increase in muscle mass and reduced fat mass (McPherron et al. 1997; McPherron and Lee 1997). In myostatin-null mice, fatty acid oxidation is promoted with a simultaneous increase in browning of WAT and DIO resistance (Zhang et al. 2012). This appears to be an indirect effect as overexpression or tissue-specific knockdown of myostatin in WAT neither effects browning nor WAT mass (Feldman et al. 2006; Guo et al. 2009).

Recombinant inbred strains

Besides simple monogenetic manipulation, like knockout or overexpression mouse models, recombinant inbred strains will be instrumental to address the genetic network of the browning phenomenon in WAT. Kozak and colleagues utilized several A × B recombinant inbred strains generated from A/J and C57BL/6J at Jackson laboratories (Mu et al. 1992, 1993). The parental strains have a differential browning capability of WAT, with a low UCP1 expression in C57BL/6J corresponding to a low brite occurrence, and elevated UCP1 expression and brite occurrence in A/J. The F_1 offspring has an intermediate phenotype, but recombinant inbred strains established from the F_2 generation show large variation in UCP1 expression and brite occurrence in WAT. Some strains exhibit even higher brite abundance and UCP1 expression than the parental A/J strain pointing towards epistatic control (Guerra et al. 1998). These observations highlight the complex genetic control of WAT browning that is still elusive.

Thermogenic function of brite adipocytes

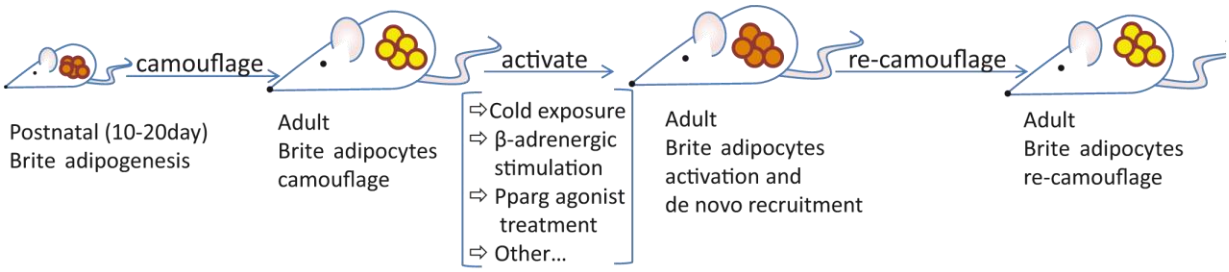
Perhaps the most urgent unresolved issue pertaining to brite adipocytes is whether these cells actually contribute significantly to thermogenesis. Recently, Schulz and coworkers generated a mouse model lacking expression of BMP type 1A receptor in cells of Myf5+ lineage, which led to a severe decrease in the mass of classical BAT. This in turn increased the sympathetic tone to WAT, thereby promoting the formation of brite adipocytes within white fat depots. As a result of this compensatory "browning" of adipose tissue, Myf5-BMPR1A-KO mice displayed normal thermogenic capacity and were resistant to diet-induced obesity (Schulz et al. 2013). These findings provide the first evidence that brite cells may possess similar capacities as classical brown cells for thermoregulation if maximally stimulated.

Notably, *Ucp1* knockout mice are capable of adaptive thermogenesis by recruiting thermogenic capacity in the cold, which is not responsive to sympathetic stimulation (Meyer et al. 2010). In the absence of functional BAT in these mice, browning of WAT with increased respiratory capacity can be observed (Liu et al. 2003; Ukropec et al. 2006; Meyer et al. 2010). Brite adipocytes may significantly contribute to adaptive thermogenesis during cold acclimation even in the absence of *Ucp1*. The underlying thermogenic mechanism may be related to futile calcium cycles (Ukropec et al. 2006).

Cell culture

The analysis of genetically engineered mouse models has led to the identification of several transcriptional modulators that strongly regulate the recruitment of brown and brite adipocytes. Nevertheless, understanding the molecular control of adipogenesis through in vivo studies is complicated by the fact that this process involves the proliferation of precursor cells, commitment towards a specific adipose lineage, and final differentiation into functional adipocytes. Moreover, adipocyte precursor cells differentiate relatively asynchronously and are difficult to detect in situ. From this aspect, the study of cellular models to unravel factors regulating expansion of precursor cells, commitment of either white, brite, or brown lineage, as well as differentiation and (or) activation of thermogenic adipocytes, is instrumental. It goes without saying that isolating the specific progenitor cells giving rise to either brown or brite adipocytes from adipose tissues followed by proliferation and differentiation in cell culture would be the most ideal model. Unfortunately, the identity of these progenitors is still unknown. Recent studies show that subpopulations of nonadipocyte cells sorted by cell surface markers from the stromal vascular cell fraction (SVF) of murine WAT (Lin-:CD29+:CD34+:Sca-1+:CD24+) and skeletal muscle (CD31-:CD45-:SM/C-2.6-:PDGFRa+) have a high adipogenic differentiation potential both under in vitro and in vivo conditions (Rodeheffer et al. 2008; Uezumi et al. 2010). Nevertheless, whether

Fig. 4. Schematic representation of postnatal development and the camouflage potential of "brite" (brown-in-white) adipocytes. In adult mice (*Mus musculus*), preexisting brite cells (orange circles in mice 1 and 3) camouflage as white-like adipocytes (yellow circles in mice 2 and 4), which are mainly derived from postnatal development (10–20 days of age). Camouflaged brite cells can be demasked in response to cold exposure, β_3-adrenergic receptor stimulation, and PPARγ agonist treatment. Activated brite cells can reenter the camouflage mode when stimulation ceases.



these progenitors have the potential for brite adipogenesis has not been tested. Similarly, based on cell surface markers, progenitors with brown or brite adipogenic potential (Sca-1+:CD45-:Mac1-; referred to as ScaPCs) have been identified in SVF of interscapular brown fat, subcutaneous white fat, and skeletal muscle (Schulz et al. 2011). On the other hand, using a lineage-tracing strategy, a bipotential adipogenic cell population (PDGFRa+:Sca-1+:CD34+; referred to as PDGFRa+ cells) has recently been characterized in mice (Lee et al. 2012). These PDGFRa+ cells gave rise to either brite or white adipocytes in response to β-adrenergic stimulation or high-fat diet feeding of mice, respectively. Apparently, brite and white adipocytes are closely related and may share a common precursor.

In principle, brite cells in WAT depots could derive from precursors in the tissue and (or) from direct transdifferentiation of mature white adiopocytes. Although the potential for transdifferentiation has not been definitely proven, significant advances have been made in the identification of brite precursors. Subcloning progenitors from subcutaneous adipose tissue of mice, Wu and coworkers found a subset of precursor cells that can specifically give rise to brite cells with a low basal level of *Ucp1* expression, whereas upon stimulation these cells achieve a high level of *Ucp1* expression comparable with classical brown fat cells (Wu et al. 2012). In this regard, it is tempting to speculate that preexisting brite cells with low *Ucp1* expression in response to stimulation undergo interconversion or transdifferentiation. Brite cells at basal state may operate in a camouflage mode appearing as white-like adipocytes with unilocular morphology, but upon stimulation convert into multilocular brown-like adipocytes positive for UCP1. This flexible "interconversion" phenotype may be one of the unique features of brite cells enabling energy storage at rest while upon stimulation energy is combusted. Employing two transgenic mouse lines allowing for either transient or permanent labeling of *Ucp1*-expressing cells, Rosenwald and coworkers recently demonstrated that activated brite cells can reenter the camouflage mode when cold stimulation ceases (Rosenwald et al. 2013). These experiments, however, did not exclude the possibility that the original cells undergoing interconversion are true white adipocytes with the capacity to transdifferentiate rather than preexisting brite cells hovering in the camouflage mode. In the latter case, the preexisting brite cells most likely emerge during a time window of postnatal adipose tissue development, from 10 to 20 days of age when brite adipocytes transiently appear. Possibly these brite cells enter camouflage in the juvenile and remain in this mode until reactivated by a proper stimulus (Fig. 4). In this scenario, the abundance of brite adipocytes during postnatal development even may program the browning capacity of different fat depots later in life. Further research is needed to reveal the factors controlling postnatal development of brite cells and to explore the cell biology of brite adipocyte interconversion. Pertaining to the transdifferentiation hypothesis, Cinti and colleagues propose that brown-like adipocytes appearing in WAT after cold exposure are derived from direct transformation of white adipocytes into brown adipocytes (white-to-brown transdifferentiation). This proposition is mainly based on their observations that most newly formed brown-like adipocytes do not display proliferation markers, the total number of adipocytes remains constant during this process, and white adipocytes in principal do have the capacity to transform as exemplified by their ability to convert into milk-secreting epithelial cells during lactation (Barbatelli et al. 2010). Nevertheless, it should be noticed that not all kinds of cells within WAT can attain brown characteristics, both in vivo and in vitro.

Compared with established cell lines, primary cells provide model systems more closely resembling the in vivo situation and thus have been widely used as cellular models to study white, brown, and brite adipogenesis. In fact, the brite cell was firstly recognized as a distinct type of adipocyte in primary cultures of white adipocytes (Petrovic et al. 2010). By treating primary cultures derived from epididymal WAT chronically with the potent PPARγ-ligand rosiglitazone, Petrovic and coworkers demonstrated that a subset of "white" adipocytes acquired a brown-like phenotype but lacked expression of certain classical brown-adipocyte genes (Petrovic et al. 2010). To classify these unique energy-dissipating fat cells, Petrovic and coworkers named them brite (brown in white) adipocytes, while Ishibashi and coworkers assigned them as beige adipocytes because their color was lighter than brown (Ishibashi and Seale 2010). Although PPARγ ligands promote brown adipogenesis and induce browning of white adipocytes, the underlying mechanisms remain unclear. Recent studies demonstrate that post-translational modification of PPARγ (deacetylation, desumoylation) and stabilization of PRDM16 may contribute to the white-to-brown fat conversion promoted by PPARγ agonists (Ohno et al. 2012; Qiang et al. 2012).

The ability to ectopically express or knockdown a specific gene provides a powerful approach for understanding its biological function. In this way, novel regulators of brown or brite adipocyte differentiation have been identified. For example, based on the observation that the transcription factor T-box 15 (Tbx15) is expressed predominantly in BAT and inguinal white adipose depot but not in classical white adipose depots such as the epididymal depot, Gburcik and colleagues sought to investigate the possible role of Tbx15 in brown and brite adipocyte differentiation in vitro. By analysis of gene function through short interfering RNAs (siRNA) mediated knockdown in cultured cells, an essential role of Tbx15 in the differentiation of brown and brite, but not white, adipocytes has recently been observed (Gburcik et al. 2012). Nevertheless, both the regulation of Tbx15 and the molecular

mechanisms through which it affects the browning program are elusive. Another transcription factor of interest, Tbx1, is expressed predominantly in the inguinal white adipose depot and has recently been suggested as a specific marker for brite adipocytes (Wu et al. 2012; Rosenwald et al. 2013). However, neither knockdown nor overexpression experiments have been performed to confirm its essential role in brite adipogenesis.

By taking advantage of primary cultures and cell lines, several extracellular factors contributing to brown and brite adipogenesis have been identified. Of all the known factors, norepinephrine is certainly one of the most important and best characterized. In addition to the activation of thermogenesis, norepinephrine also stimulates preadipocyte proliferation of brown fat cells in primary culture through a poorly understood mechanism that involves β_1-adrenoreceptors and cAMP signaling (Bronnikov et al. 1992). Interestingly, treatment of mice with a β_3-adrenoreceptor agonist (CL 316 243) increased the mitotic index of cells in both eWAT and iWAT but had no effect on cell proliferation in BAT (Lee et al. 2012). Thus, different factors or signaling pathways regulate proliferation of brown and brite precursor cells. Further studies are needed to test the direct effects of CL and NE on the expansion of brite precursor cells.

Another factor of particular interest is bone morphogenetic protein 7 (BMP7), which seems responsible for trigging commitment of progenitor cells specifically into the brown and brite adipogenic lineage. BMP7 pretreatment of either mesenchymal progenitor cells (C3H10T1/2 cells), brown preadipocytes, or ScaPCs isolated from skeletal muscle and subcutaneous fat during the proliferation phase (before hormonal induction of adipogenesis) promotes the differentiation of brown and brite adipocytes (Tseng et al. 2008; Schulz et al. 2011). Mechanistically, BMP7 treatment is associated with the induction of key brown adipogenic regulators such as PRDM16 and PGC-1α; however, the mechanisms mediating this effect are still unknown.

Similarly, treatment of preadipocytes with the neuropeptide orexin drives "BMP7-like" brown-fat programming. Orexin stimulates the differentiation of brown preadipocytes and multipotent C3H10T1/2 fibroblasts into mature brown adipocytes, in part through the induction of BMP7 and BMP signaling (Sellayah et al. 2011). Nevertheless, the secretory sources in vivo of both BMP7 and orexin are unknown. Moreover, whether orexin promotes brite adipogenesis from white preadipocytes has not been tested yet. There are also some factors that have been reported to act mainly during differentiation. Treatment of differentiated primary adipocytes from BAT and WAT with fibroblast growth factor 21 (FGF21) induces thermogenic gene expression (Fisher et al. 2012). Although FGF21 is originally identified as a liver-derived endocrine hormone, recent studies have shown that FGF21 is also produced in WAT and BAT under conditions like cold exposure, β-adrenergic stimulation, or treatment with PPARγ agonist. Nevertheless, adipose-derived FGF21, unlike liver-derived FGF21, appears to act in an autocrine or paracrine manner to induce thermogenic genes in adipose tissues (Dutchak et al. 2012). Mechanistically, on the one hand, FGF21 sensitizes adipocytes to the actions of PPARγ by inhibiting its conversion to an inactive, sumoylated form of PPARγ, while on the other hard, FGF21 increases PGC1α protein levels, thus inducing thermogenic gene expression (Dutchak et al. 2012; Fisher et al. 2012).

Apart from FGF21, a recent study revealed that thermogenic programs are induced by heart-derived hormone cardiac natriuretic peptides (NPs) in human primary adipocytes in vitro in a manner quite similar to the β-ARs agonist (Bordicchia et al. 2012). At a molecular level, NPs can bind to their respective receptors present on human adipocytes to stimulate lipolysis via cGMP-dependent signaling, a pathway known to control brown or brite fat cell differentiation and mitochondrial biogenesis (Mitschke et al. 2013). Based on the rapid advancements in the field, the discovery of further extracellular signals involved in browning can be expected.

Brown-like adipocytes in noneutherian vertebrates and invertebrates

The rationale for the evolutionary development of BAT in mammals is adaptive thermogenesis. However, the rationale behind the development of a second type of UCP1-expressing cells is puzzling. As the conservation of calories would probably provide an overall survival advantage, it seems paradoxical that mammals evolved a second type of UCP1-expressing cells that increase energy expenditure and protect against fat accumulation. Based on the facts that (*i*) UCP1 only gained thermogenic function in brown adipocytes of eutherian mammals, (*ii*) the evolution of thermogenic UCP1 function certainly required expression of UCP1 in a brown adipocyte-like cell equipped with high mitochondrial density in a well vascularized tissue, and (*iii*) brite adipocytes are indeed capable of demonstrating NE-mediated thermogenesis in vitro (though not evident in vivo), we propose that brite adipocytes may resemble the archetypical brown adipocyte in vertebrate evolution and UCP1 firstly gained uncoupling function in brite cells. To test this hypothesis, it is of interest to search for the presence of related brown adipocyte-like cells in noneutherian vertebrates where classical BAT has never been described.

In addition to *Ucp1* expression, another hallmark of brown adipocyte-like cells (brite cells) is the multilocular mode of triglyceride storage, though not all multilocular adipocytes are positive for UCP1. Actually, multilocular fat cells have been reported in some birds, including Muscovy duckling (*Cairina moschata* (L., 1758)), pigeon (*Columba livia* Gmelin, 1789), chicken, goose, Black-capped Chickadee (*Poecile atricapillus* (L., 1766)), and Great Tit (*Parus major* L., 1758) (Luckenbill and Cohen 1966; Lucas and Stettenheim 1972; Barre et al. 1986; Murphy et al. 1986; Olson et al. 1988; Saarela et al. 1989) and in some marsupials (Hayward and Lisson 1992). The appearance of those multilocular fat cells is even more prominent after cold acclimation. Although it has been proposed that those multilocular fat cells are unlike classical brown adipocytes in mammals and multilocularity probably facilitates the release of stored lipids by enlargement of surface area accessible to lipases, it is still of interest to determine the expression levels of necessary regulatory components for *Ucp1* expression such as PGC1α, PPARα, PRDM16, and specific markers for mammalian brown or brite adipocytes in those cells, since it has been recently reported that a stem cell like mesenchymal population from chicken embryos can be induced to differentiate into brown adipocyte like multilocular cells expressing virtually the complete mammalian-like brown adipocyte differentiation pathway and even are able to transactivate a mammalian *Ucp1* reporter vector introduced by transfection (Mezentseva et al. 2008). From this point, it seems that the transcriptional machinery driving *Ucp1* expression has an ancient history and is still present in those avian multilocular adipocytes, though birds have lost the *Ucp1* gene.

Even more interestingly, in some marsupials (gray short-tailed opossum, *Monodelphis domestica* (Wagner, 1842), and yellow-tailed antechinus, *Antechinus flavipes* (Waterhouse, 1838)), adipose tissue expressing *Ucp1* may be recruited transiently during early life stages and is lost in later life stages, whereas in the fat-tailed dunnart (*Sminthopsis crassicaudata* (Gould, 1844)), it is detected in adult life stages (Jastroch et al. 2008). Importantly, cold exposure could further elevate the oxidative capacity and UCP1 expression in the interscapular adipose depot of the fat-tailed dunnart. However, it was recently demonstrated that this does not contribute to classical nonshivering thermogenesis, as the thermogenic response to norepinephrine is similar in cold- and warm-acclimated animals (Polymeropoulos et al. 2012*b*). This finding was surprising as cold-induced molecular adaptations suggested recruitment of

interscapular BAT in this species, similar to eutherian rodents (Jastroch et al. 2008). Nevertheless, it is intriguing to investigate whether those UCP1-expressing adipocytes in marsupials are related to brite adipocytes in eutherian mammals. It is tempting to postulate that those adipocytes in marsupials are brite-like cells and resemble the archetypical brown adipocyte. More studies are needed to clarify this issue.

Pertaining to the evolution of BAT, afrotherian mammals are of particular interest because they emerged early during the radiation of eutherian mammals. Notably, in captive rock elephant shrews (*Elephantulus myurus* Thomas and Schwann, 1906), BAT exhibits UCP1 expression and significant capacity for nonshivering thermogenesis, although they seem incapable for cold-induced adaptive thermogenesis (Mzilikazi et al. 2007). In free-ranging western rock elephant shrews (*Elephantulus rupestris* (A. Smith, 1831)) and Hottentot golden mole (*Amblysomus hottentotus longiceps* (Broom, 1907)), seasonal adaptive thermogenesis has been observed but molecular and functional analysis of *Ucp1* is lacking (Scantlebury et al. 2008; Oelkrug et al. 2012). These observations suggest that the thermogenic features of afrotherian BAT may be primordial. In this context, the search and bioenergetic analysis of brite or brite-like cells in afrotherian species may shed light on the evolutionary relationship between brite and brown cells.

Acknowledgements

The Chair of Molecular Nutritional Medicine is funded by the Else Kröner-Fresenius Foundation. M.K. receives funding from the Deutsche Forschungsgemeinschaft and the EU FP7 project DIABAT (HEALTH-F2-2011-278373). Y.L. holds a fellowship from the Deutsche Akademische Austauschdienst (DAAD). D.L. is a Ph.D. fellow of the Z I E L – Ph.D. – School "Nutritional adaptation and epigenetic mechanisms" and the Faculty Graduate Center Weihenstephan of TUM Graduate School. The mouse model illustrated in Fig. 4 was kindly provided by N. Rink.

References

Ahmadian, M., Abbott, M.J., Tang, T., Hudak, C.S., Kim, Y., Bruss, M., Hellerstein, M.K., Lee, H.Y., Samuel, V.T., Shulman, G.I., Wang, Y., Duncan, R.E., Kang, C., and Sul, H.S. 2011. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. Cell Metab. **13**(6): 739–748. doi:10.1016/j.cmet.2011.05.002. PMID:21641555.

Almind, K., Manieri, M., Sivitz, W.I., Cinti, S., and Kahn, C.R. 2007. Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice. Proc. Natl. Acad. Sci. U.S.A. **104**(7): 2366–2371. doi:10.1073/pnas.0610416104. PMID:17283342.

Ashwell, M., Stirling, D., Freeman, S., and Holloway, B.R. 1987. Immunological, histological and biochemical assessment of brown adipose tissue activity in neonatal, control and beta-stimulant-treated adult dogs. Int. J. Obes. **11**(4): 357–365. PMID:2889691.

Barbatelli, G., Murano, I., Madsen, L., Hao, Q., Jimenez, M., Kristiansen, K., Giacobino, J.P., De Matteis, R., and Cinti, S. 2010. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. Am. J. Physiol. Endocrinol. Metab. **298**(6): E1244–E1253. doi:10.1152/ajpendo.00600.2009.

Barre, H., Cohen-Adad, F., Duchamp, C., and Rouanet, J.L. 1986. Multilocular adipocytes from muscovy ducklings differentiated in response to cold acclimation. J. Physiol. (Lond.), **375**: 27–38. PMID:3795059.

Bordicchia, M., Liu, D., Amri, E.-Z., Ailhaud, G., Dessì-Fulgheri, P., Zhang, C., Takahashi, N., Sarzani, R., and Collins, S. 2012. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. J. Clin. Invest. **122**(3): 1022–1036. doi:10.1172/JCI59701. PMID:22307324.

Borecký, J., Maia, I.G., and Arruda, P. 2001. Mitochondrial uncoupling proteins in mammals and plants. Biosci. Rep. **21**(2): 201–212. doi:10.1023/A:1013604526175. PMID:11725869.

Boström, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., Rasbach, K.A., Boström, E.A., Choi, J.H., Long, J.Z., Kajimura, S., Zingaretti, M.C., Vind, B.F., Tu, H., Cinti, S., Højlund, K., Gygi, S.P., and Spiegelman, B.M. 2012. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature, **481**(7382): 463–468. doi:10.1038/nature10777. PMID:22237023.

Bourová, L., Pesanová, Z., Novotný, J., Bengtsson, T., and Svoboda, P. 2000. Differentiation of cultured brown adipocytes is associated with a selective increase in the short variant of $G_s\alpha$ protein. Evidence for higher functional activity of $G_s\alpha$S. Mol. Cell. Endocrinol. **167**(1–2): 23–31. PMID:11000517.

Boyer, B.B., and Kozak, L.P. 1991. The mitochondrial uncoupling protein gene in brown fat: correlation between DNase I hypersensitivity and expression in transgenic mice. Mol. Cell. Biol. **11**(8): 4147–4156. PMID:1712903.

Brasaemle, D.L. 2007. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. J. Lipid Res. **48**(12): 2547–2559. doi:10.1194/jlr.R700014-JLR200. PMID:17878492.

Bronnikov, G., Houstěk, J., and Nedergaard, J. 1992. β-Adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via β_1 but not via β_3 adrenoceptors. J. Biol. Chem. **267**(3): 2006–2013. PMID:1346138.

Cahill, G.F., Jr., Leboeuf, B., and Renold, A.E. 1959. Studies on rat adipose tissue in vitro. III. Synthesis of glycogen and glyceride glycerol. J. Biol. Chem. **234**: 2540–2543. PMID:13806795.

Cannon, B., and Nedergaard, J. 2004. Brown adipose tissue: function and physiological significance. Physiol. Rev. **84**(1): 277–359. doi:10.1152/physrev.00015.2003. PMID:14715917.

Cao, W., Daniel, K.W., Robidoux, J., Puigserver, P., Medvedev, A.V., Bai, X., Floering, L.M., Spiegelman, B.M., and Collins, S. 2004. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol. Cell Biol. **24**(7): 3057–3067. doi:10.1128/MCB.24.7.3057-3067.2004. PMID:15024092.

Cassard-Doulcier, A.M., Gelly, C., Fox, N., Schrementi, J., Raimbault, S., Klaus, S., Forest, C., Bouillaud, F., and Ricquier, D. 1993. Tissue-specific and β-adrenergic regulation of the mitochondrial uncoupling protein gene: control by *cis*-acting elements in the 5′-flanking region. Mol. Endocrinol. **7**(4): 497–506. doi:10.1210/me.7.4.497. PMID:8388995.

Cassard-Doulcier, A.M., Gelly, C., Bouillaud, F., and Ricquier, D. 1998. A 211-bp enhancer of the rat uncoupling protein-1 (UCP-1) gene controls specific and regulated expression in brown adipose tissue. Biochem. J. **333**: 243–246. PMID:9657961.

Chaudhry, A., MacKenzie, R.G., Georgic, L.M., and Granneman, J.G. 1994. Differential interaction of β_1- and β_3-adrenergic receptors with G_i in rat adipocytes. Cell Signall. **6**(4): 457–465. doi:10.1016/0898-6568(94)90093-0. PMID:7946969.

Collins, S., and Surwit, R.S. 2001. The β-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. Recent Prog. Horm. Res. **56**: 309–328. doi:10.1210/rp.56.1.309. PMID:11237219.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Pénicaud, L., and Casteilla, L. 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. J. Cell Sci. **103**(4): 931–942. PMID:1362571.

Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y.-H., Doria, A., Kolodny, G.M., and Kahn, C.R. 2009. Identification and importance of brown adipose tissue in adult humans. N. Engl. J. Med. **360**(15): 1509–1517. doi:10.1056/NEJMoa0810780. PMID:19357406.

Cypess, A.M., White, A.P., Vernochet, C., Schulz, T.J., Xue, R., Sass, C.A., Huang, T.L., Roberts-Toler, C., Weiner, L.S., Sze, C., Chacko, A.T., Deschamps, L.N., Herder, L.M., Truchan, N., Glasgow, A.L., Holman, A.R., Gavrila, A., Hasselgren, P.-O., Mori, M.A., Molla, M., and Tseng, Y.-H. 2013. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. Nat. Med. **19**(5): 635–639. doi:10.1038/nm.3112. PMID:23603815.

Del Mar Gonzalez-Barroso, M., Ricquier, D., and Cassard-Doulcier, A.M. 2000. The human uncoupling protein-1 gene (UCP1): present status and perspectives in obesity research. Obes. Rev. **1**(2): 61–72. doi:10.1046/j.1467-789x.2000.00009.x. PMID:12119988.

Dutchak, P.A., Katafuchi, T., Bookout, A.L., Choi, J.H., Yu, R.T., Mangelsdorf, D.J., and Kliewer, S.A. 2012. Fibroblast growth factor-21 regulates PPARγ activity and the antidiabetic actions of thiazolidinediones. Cell, **148**(3): 556–567. doi:10.1016/j.cell.2011.11.062. PMID:22304921.

Enerbäck, S., Jacobsson, A., Simpson, E.M., Guerra, C., Yamashita, H., Harper, M.E., and Kozak, L.P. 1997. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature, **387**(6628): 90–94. doi:10.1038/387090a0. PMID:9139827.

Enslen, H., Raingeaud, J., and Davis, R.J. 1998. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J. Biol. Chem. **273**(3): 1741–1748. doi:10.1074/jbc.273.3.1741. PMID:9430721.

Fedorenko, A., Lishko, P.V., and Kirichok, Y. 2012. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. Cell, **151**(2): 400–413. doi:10.1016/j.cell.2012.09.010. PMID:23063128.

Feldman, B.J., Streeper, R.S., Farese, R.V., Jr., and Yamamoto, K.R. 2006. Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. Proc. Natl. Acad. Sci. U.S.A. **103**(42): 15675–15680. doi:10.1073/pnas.0607501103.

Fisher, F.M., Kleiner, S., Douris, N., Fox, E.C., Mepani, R.J., Verdeguer, F., Wu, J., Kharitonenkov, A., Flier, J.S., Maratos-Flier, E., and Spiegelman, B.M. 2012. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. Genes Dev. **26**(3): 271–281. doi:10.1101/gad.177857.111. PMID:22302939.

Fukui, Y., Masui, S., Osada, S., Umesono, K., and Motojima, K. 2000. A new thiazolidinedione, NC-2100, which is a weak PPAR-gamma activator, exhibits

potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAy obese mice. Diabetes, **49**(5): 759–767. doi:10.2337/diabetes.49.5.759. PMID:10905484.

Fuster, A., Oliver, P., Sanchez, J., Pico, C., and Palou, A. 2009. UCP1 and oxidative capacity of adipose tissue in adult ferrets (*Mustela putorius furo*). Comp. Biochem. Physiol. A, **153**(2): 106–112. doi:10.1016/j.cbpa.2009.01.007.

Gburcik, V., Cawthorn, W.P., Nedergaard, J., Timmons, J.A., and Cannon, B. 2012. An essential role for Tbx15 in the differentiation of brown and "brite" but not white adipocytes. Am. J. Physiol. Endocrinol. Metab. **303**(8): E1053–E1060. doi:10.1152/ajpendo.00104.2012. PMID:22912368.

Gerhardt, C.C., Gros, J., Strosberg, A.D., and Issad, T. 1999. Stimulation of the extracellular signal-regulated kinase 1/2 pathway by human *beta*-3 adrenergic receptor: new pharmacological profile and mechanism of activation. Mol. Pharmacol. **55**(2): 255–262. PMID:9927616.

Gessner, K. 1551. Conradi Gesneri medici Tigurini historiae animalium lib. I. de quadrupedibus viviparis. apud Christ. Froschoverum, Tiguri.

Granneman, J.G., Burnazi, M., Zhu, Z., and Schwamb, L.A. 2003. White adipose tissue contributes to UCP1-independent thermogenesis. Am. J. Physiol. Endocrinol. Metab. **285**(6): E1230–E1236. doi:10.1152/ajpendo.00197.2003. PMID:12954594.

Granneman, J.G., Moore, H.P., Granneman, R.L., Greenberg, A.S., Obin, M.S., and Zhu, Z. 2007. Analysis of lipolytic protein trafficking and interactions in adipocytes. J. Biol. Chem. **282**(8): 5726–5735. doi:10.1074/jbc.M610580200. PMID:17189257.

Greenberg, A.S., Egan, J.J., Wek, S.A., Garty, N.B., Blanchette-Mackie, E.J., and Londos, C. 1991. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. J. Biol. Chem. **266**(17): 11341–11346. PMID:2040638.

Greenberg, A.S., Egan, J.J., Wek, S.A., Moos, M.C., Jr., Londos, C., and Kimmel, A.R. 1993. Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. Proc. Natl. Acad. Sci. U.S.A. **90**(24): 12035–12039. doi:10.1073/pnas.90.24.12035. PMID:7505452.

Guerra, C., Koza, R.A., Yamashita, H., Walsh, K., and Kozak, L.P. 1998. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. J. Clin. Invest. **102**(2): 412–420. doi:10.1172/JCI3155.

Guo, T., Jou, W., Chanturiya, T., Portas, J., Gavrilova, O., and McPherron, A.C. 2009. Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. PLoS ONE, **4**(3): e4937. doi:10.1371/journal.pone.0004937.

Haemmerle, G., Zimmermann, R., Hayn, M., Theussl, C., Waeg, G., Wagner, E., Sattler, W., Magin, T.M., Wagner, E.F., and Zechner, R. 2002. Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. J. Biol. Chem. **277**(7): 4806–4815. doi:10.1074/jbc.M110355200. PMID:11717312.

Haemmerle, G., Lass, A., Zimmermann, R., Gorkiewicz, G., Meyer, C., Rozman, J., Heldmaier, G., Maier, R., Theussl, C., Eder, S., Kratky, D., Wagner, E.F., Klingenspor, M., Hoefler, G., and Zechner, R. 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science, **312**(5774): 734–737. doi:10.1126/science.1123965. PMID:16675698.

Hanák, P., and Ježek, P. 2001. Mitochondrial uncoupling proteins and phylogenesis—UCP4 as the ancestral uncoupling protein. FEBS Lett. **495**(3): 137–141. doi:10.1016/S0014-5793(01)02338-9. PMID:11334880.

Harada, K., Shen, W.J., Patel, S., Natu, V., Wang, J., Osuga, J., Ishibashi, S., and Kraemer, F.B. 2003. Resistance to high-fat diet-induced obesity and altered expression of adipose-specific genes in HSL-deficient mice. Am. J. Physiol. Endocrinol. Metab. **285**(6): E1182–E1195. PMID:12954598.

Hayward, J.S., and Lisson, P.A. 1992. Evolution of brown fat: its absence in marsupials and monotremes. Can. J. Zool. **70**(1): 171–179. doi:10.1139/z92-025.

Himms-Hagen, J., Melnyk, A., Zingaretti, M.C., Ceresi, E., Barbatelli, G., and Cinti, S. 2000. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. Am. J. Physiol. Cell. Physiol. **279**(3): C670–C681. PMID:10942717.

Hughes, D.A., Jastroch, M., Stoneking, M., and Klingenspor, M. 2009. Molecular evolution of UCP1 and the evolutionary history of mammalian non-shivering thermogenesis. BMC Evol. Biol. **9**: 4. doi:10.1186/1471-2148-9-4. PMID:19128480.

Ishibashi, J., and Seale, P. 2010. Medicine. Beige can be slimming. Science, **328**(5982): 1113–1114. doi:10.1126/science.1190816.

Jastroch, M., Wuertz, S., Kloas, W., and Klingenspor, M. 2005. Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian non-shivering thermogenesis. Physiol. Genomics, **22**(2): 150–156. doi:10.1152/physiolgenomics.00070.2005.

Jastroch, M., Buckingham, J.A., Helwig, M., Klingenspor, M., and Brand, M.D. 2007. Functional characterisation of UCP1 in the common carp: uncoupling activity in liver mitochondria and cold-induced expression in the brain. J. Comp. Physiol. B, **177**(7): 743–752. doi:10.1007/s00360-007-0171-6.

Jastroch, M., Withers, K.W., Taudien, S., Frappell, P.B., Helwig, M., Fromme, T., Hirschberg, V., Heldmaier, G., McAllan, B.M., Firth, B.T., Burmester, T., Platzer, M., and Klingenspor, M. 2008. Marsupial uncoupling protein 1 sheds light on the evolution of mammalian nonshivering thermogenesis. Physiol. Genomics, **32**(2): 161–169. doi:10.1152/physiolgenomics.00183.2007.

Jespersen, N.Z., Larsen, T.J., Peijs, L., Daugaard, S., Homøe, P., Loft, A., de Jong, J., Mathur, N., Cannon, B., Nedergaard, J., Pedersen, B.K., Møller, K., and Scheele, C. 2013. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. Cell Metab. **17**(5): 798–805. doi:10.1016/j.cmet.2013.04.011. PMID:23663743.

Jimenez, M., Barbatelli, G., Allevi, R., Cinti, S., Seydoux, J., Giacobino, J.-P., Muzzin, P., and Preitner, F. 2003. β_3-adrenoceptor knockout in C57BL/6J mice depresses the occurrence of brown adipocytes in white fat. Eur. J. Biochem. **270**(4): 699–705. doi:10.1046/j.1432-1033.2003.03422.x. PMID:12581209.

Kajimura, S., Seale, P., Kubota, K., Lunsford, E., Frangioni, J.V., Gygi, S.P., and Spiegelman, B.M. 2009. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-β transcriptional complex. Nature, **460**(7259): 1154–1158. doi:10.1038/nature08262. PMID:19641492.

Kim, S.W., Her, S.J., Kim, S.Y., and Shin, C.S. 2005. Ectopic overexpression of adipogenic transcription factors induces transdifferentiation of MC3T3-E1 osteoblasts. Biochem. Biophys. Res. Commun. **327**(3): 811–819. doi:10.1016/j.bbrc.2004.12.076. PMID:15649418.

Kleiner, S., Mepani, R.J., Laznik, D., Ye, L., Jurczak, M.J., Jornayvaz, F.R., Estall, J.L., Chatterjee Bhowmick, D., Shulman, G.I., and Spiegelman, B.M. 2012. Development of insulin resistance in mice lacking PGC-1α in adipose tissues. Proc. Natl. Acad. Sci. U.S.A. **109**(24): 9635–9640. doi:10.1073/pnas.1207287109. PMID:22645355.

Klingenspor, M. 2003. Cold-induced recruitment of brown adipose tissue thermogenesis. Exp. Physiol. **88**(1): 141–148. doi:10.1113/eph8802508. PMID:12525862.

Klingenspor, M., and Fromme, T. 2012. Brown adipose tissue. *In* Adipose tissue biology. *Edited by* M.E. Symonds. Springer-Verlag, New York. pp. 39–79.

Klingenspor, M., Fromme, T., Hughes, D.A., Jr., Manzke, L., Polymeropoulos, E., Riemann, T., Trzcionka, M., Hirschberg, V., and Jastroch, M. 2008. An ancient look at UCP1. Biochim. Biophys. Acta Bioenergetics, **1777**(7–8): 637–641. doi:10.1016/j.bbabio.2008.03.006.

Klingenspor, M., Herzig, S., and Pfeifer, A. 2012. Brown fat develops a *brite* future. Obes. Facts, **5**(6): 890–896. doi:10.1159/000346337.

Kozak, U.C., Kopecky, J., Teisinger, J., Enerbäck, S., Boyer, B., and Kozak, L.P. 1994. An upstream enhancer regulating brown-fat-specific expression of the mitochondrial uncoupling protein gene. Mol. Cell. Biol. **14**(1): 59–67. PMID:8264627.

Laloi, M., Klein, M., Riesmeier, J.W., Müller-Röber, B., Fleury, C., Bouillaud, F., and Ricquier, D. 1997. A plant cold-induced uncoupling protein. Nature, **389**(6647): 135–136. doi:10.1038/38156. PMID:9296489.

Larose, M., Cassard-Doulcier, A.-M., Fleury, C., Serra, F., Champigny, O., Bouillaud, F., and Ricquier, D. 1996. Essential *cis*-acting elements in rat uncoupling protein gene are in an enhancer containing a complex retinoic acid response domain. J. Biol. Chem. **271**(49): 31533–31542. doi:10.1074/jbc.271.49.31533. PMID:8940169.

Lasar, D., Julius, A., Fromme, T., and Klingenspor, M. 2013. Browning attenuates murine white adipose tissue expansion during postnatal development. Biochim. Biophys. Acta Mol. Cell Biol. Lipids, **1831**(5): 960–968. doi:10.1016/j.bbalip.2013.01.016.

Leboeuf, B., Flinn, R.B., and Cahill, G.F., Jr. 1959. Effect of epinephrine on glucose uptake and glycerol release by adipose tissue in vitro. Proc. Soc. Exp. Biol. Med. **102**: 527–529. doi:10.3181/00379727-102-25306. PMID:14415032.

Ledesma, A., de Lacoba, M.G., and Rial, E. 2002. The mitochondrial uncoupling proteins. Genome Biol. **3**(12): reviews 3015.1–3015.9. PMID:12537581.

Lee, Y.-H., Petkova, A.P., Mottillo, E.P., and Granneman, J.G. 2012. In vivo identification of bipotential adipocyte progenitors recruited by β_3-adrenoceptor activation and high-fat feeding. Cell Metab. **15**(4): 480–491. doi:10.1016/j.cmet.2012.03.009. PMID:22482730.

Leonardsson, G., Steel, J.H., Christian, M., Pocock, V., Milligan, S., Bell, J., So, P.W., Medina-Gomez, G., Vidal-Puig, A., White, R., and Parker, M.G. 2004. Nuclear receptor corepressor RIP140 regulates fat accumulation. Proc. Natl. Acad. Sci. U.S.A. **101**(22): 8437–8442. doi:10.1073/pnas.0401013101. PMID:15155905.

Lidell, M.E., Betz, M.J., Dahlqvist Leinhard, O.D., Heglind, M., Elander, L., Slawik, M., Mussack, T., Nilsson, D., Romu, T., Nuutila, P., Virtanen, K.A., Beuschlein, F., Persson, A., Borga, M., and Enerbäck, S. 2013. Evidence for two types of brown adipose tissue in humans. Nat. Med. **19**(5): 631–634. doi:10.1038/nm.3017. PMID:23603813.

Liu, X., Rossmeisl, M., McClaine, J., and Kozak, L.P. 2003. Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. J. Clin. Invest. **111**(3): 399–407. doi:10.1172/JCI200315737. PMID:12569166.

Loncar, D., Bedrica, L., Mayer, J., Cannon, B., Nedergaard, J., Afzelius, B.A., and Svajger, A. 1986. The effect of intermittent cold treatment on the adipose tissue of the cat. Apparent transformation from white to brown adipose tissue. J. Ultrastruct. Mol. Struct. Res. **97**(1–3): 119–129. PMID:3453365.

Lucas, A.M., and Stettenheim, P.R. 1972. Avian anatomy integument. US Government Printing Office, Washington, D.C.

Luckenbill, L.M., and Cohen, A.S. 1966. The association of lipid droplets with cytoplasmic filaments in avian subsynovial adipose tissue. J. Cell. Biol. **31**(1): 195–199. PMID:5971971.

Madsen, L., Pedersen, L.M., Lillefosse, H.H., Fjaere, E., Bronstad, I., Hao, Q., Petersen, R.K., Hallenborg, P., Ma, T., De Matteis, R., Araujo, P., Mercader, J., Bonet, M.L., Hansen, J.B., Cannon, B., Nedergaard, J., Wang, J., Cinti, S., Voshol, P., Døskeland, S.O., and Kristiansen, K. 2010. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on

cyclooxygenase activity. PLoS ONE, **5**(6): e11391. doi:10.1371/journal.pone.0011391. PMID:20613988.

Martinez-Botas, J., Anderson, J.B., Tessier, D., Lapillonne, A., Chang, B.H., Quast, M.J., Gorenstein, D., Chen, K.H., and Chan, L. 2000. Absence of perilipin results in leanness and reverses obesity in *Lepr*$^{db/db}$ mice. Nat. Genet. **26**(4): 474–479. doi:10.1038/82630. PMID:11101849.

Matsusue, K., Kusakabe, T., Noguchi, T., Takiguchi, S., Suzuki, T., Yamano, S., and Gonzalez, F.J. 2008. Hepatic steatosis in leptin-deficient mice is promoted by the PPARγ target gene *Fsp27*. Cell Metab. **7**(4): 302–311. doi:10.1016/j.cmet.2008.03.003. PMID:18396136.

McPherron, A.C., and Lee, S.-J. 1997. Double muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. U.S.A. **94**(23): 12457–12461. doi:10.1073/pnas.94.23.12457. PMID:9356471.

McPherron, A.C., Lawler, A.M., and Lee, S.-J. 1997. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature, **387**(6628): 83–90. doi:10.1038/387083a0. PMID:9139826.

Meyer, C.W., Willershäuser, M., Jastroch, M., Rourke, B.C., Fromme, T., Oelkrug, R., Heldmaier, G., and Klingenspor, M. 2010. Adaptive thermogenesis and thermal conductance in wild-type and UCP_1-KO mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. **299**(5): R1396–R1406. doi:10.1152/ajpregu.00021.2009. PMID:20826705.

Mezentseva, N.V., Kumaratilake, J.S., and Newman, S.A. 2008. The brown adipocyte differentiation pathway in birds: an evolutionary road not taken. BMC Biol. **6**: 17. doi:10.1186/1741-7007-6-17. PMID:18426587.

Mitschke, M.M., Hoffmann, L.S., Gnad, T., Scholz, D., Kruithoff, K., Mayer, P., Haas, B., Sassmann, A., Pfeifer, A., and Kilić, A. 2013. Increased cGMP promotes healthy expansion and browning of white adipose tissue. FASEB J. doi:10.1096/fj.12-221580.

Miyoshi, H., Souza, S.C., Endo, M., 2nd, Sawada, T., Perfield, J.W., Shimizu, C., Stancheva, Z., Nagai, S., Strissel, K.J., Yoshioka, N., Obin, M.S., Koike, T., and Greenberg, A.S. 2010. Perilipin overexpression in mice protects against diet-induced obesity. J. Lipid. Res. **51**(5): 975–982. doi:10.1194/jlr.M002352. PMID:19797618.

Mu, J.-L., Cheah, Y.-C., and Paigen, B. 1992. Strain distribution pattern of 25 simple sequence length polymorphisms in the AXB and BXA recombinant inbred strains. Mamm. Genome, **3**(12): 705–708. doi:10.1007/BF00444366.

Mu, J.-L., Naggert, J.K., Nishina, P.M., Cheah, Y.-C., and Paigen, B. 1993. Strain distribution pattern in AXB and BXA recombinant inbred strains for loci on murine chromosomes 10, 13, 17, and 18. Mamm. Genome, **4**(3): 148–152. doi:10.1007/BF00352230.

Murphy, C.J., Bellhorn, R.W., and Buyukmihci, N.C. 1986. Subconjunctival hibernoma in a goose. J. Am. Vet. Med. Assoc. **189**(9): 1109–1110. PMID:3505942.

Mzilikazi, N., Jastroch, M., Meyer, C.W., and Klingenspor, M. 2007. The molecular and biochemical basis of nonshivering thermogenesis in an African endemic mammal, *Elephantulus myurus*. Am. J. Physiol. Regul. Integr. Comp. Physiol. **293**(5): R2120–R2127. doi:10.1152/ajpregu.00427.2007. PMID:17686883.

Nishino, N., Tamori, Y., Tateya, S., Kawaguchi, T., Shibakusa, T., Mizunoya, W., Inoue, K., Kitazawa, R., Kitazawa, S., Matsuki, Y., Hiramatsu, R., Masubuchi, S., Omachi, A., Kimura, K., Saito, M., Amo, T., Ohta, S., Yamaguchi, T., Osumi, T., Cheng, J., Fujimoto, T., Nakao, H., Nakao, K., Aiba, A., Okamura, H., Fushiki, T., and Kasuga, M. 2008. FSP_{27} contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. J. Clin. Invest. **118**(8): 2808–2821. doi:10.1172/JCI34090. PMID:18654663.

Nordström, E.A., Rydén, M., Backlund, E.C., Dahlman, I., Kaaman, M., Blomqvist, L., Cannon, B., Nedergaard, J., and Arner, P. 2005. A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-α)-like effector A (CIDEA) in adipocyte lipolysis and obesity. Diabetes, **54**(6): 1726–1734. doi:10.2337/diabetes.54.6.1726. PMID:15919794.

Oelkrug, R., Meyer, C.W., Heldmaier, G., and Mzilikazi, N. 2012. Seasonal changes in thermogenesis of a free-ranging afrotherian small mammal, the western rock elephant shrew (*Elephantulus rupestris*). J. Comp. Physiol. B, **182**(5): 715–727. doi:10.1007/s00360-012-0647-x.

Ohno, H., Shinoda, K., Spiegelman, B.M., and Kajimura, S. 2012. PPARγ agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell Metab. **15**(3): 395–404. doi:10.1016/j.cmet.2012.01.019. PMID:22405074.

Olson, J.M., Dawson, W.R., and Camilliere, J.J. 1988. Fat from Black-capped Chickadees: avian brown adipose tissue? Condor, **90**(3): 529–537. doi:10.2307/1368340.

Petrovic, N., Walden, T.B., Shabalina, I.G., Timmons, J.A., Cannon, B., and Nedergaard, J. 2010. Chronic peroxisome proliferator-activated receptor γ (PPARγ) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J. Biol. Chem. **285**(10): 7153–7164. doi:10.1074/jbc.M109.053942. PMID:20028987.

Polymeropoulos, E.T., Heldmaier, G., Frappell, P.B., McAllan, B.M., Withers, K.W., Klingenspor, M., White, C.R., and Jastroch, M. 2012*a*. Phylogenetic differences of mammalian basal metabolic rate are not explained by mitochondrial basal proton leak. Proc. R. Soc. B Biol. Sci. **279**(1726): 185–193. doi:10.1098/rspb.2011.0881. PMID:21632624.

Polymeropoulos, E.T., Jastroch, M., and Frappell, P.B. 2012*b*. Absence of adaptive nonshivering thermogenesis in a marsupial, the fat-tailed dunnart (*Sminthopsis crassicaudata*). J. Comp. Physiol. B, **182**(3): 393–401. doi:10.1007/s00360-011-0623-x.

Puri, V., Ranjit, S., Konda, S., Nicoloro, S.M., Straubhaar, J., Chawla, A., Chouinard, M., Lin, C., Burkart, A., Corvera, S., Perugini, R.A., and Czech, M.P. 2008. Cidea is associated with lipid droplets and insulin sensitivity in humans. Proc. Natl. Acad. Sci. U.S.A. **105**(22): 7833–7838. doi:10.1073/pnas.0802063105. PMID:18509062.

Qiang, L., Wang, L., Kon, N., Zhao, W., Lee, S., Zhang, Y., Rosenbaum, M., Zhao, Y., Gu, W., Farmer, S.R., and Accili, D. 2012. Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Pparγ. Cell, **150**(3): 620–632. doi:10.1016/j.cell.2012.06.027. PMID:22863012.

Rabelo, R., Reyes, C., Schifman, A., and Silva, J.E. 1996*a*. A complex retinoic acid response element in the uncoupling protein gene defines a novel role for retinoids in thermogenesis. Endocrinology, **137**(8): 3488–3496. doi:10.1210/en.137.8.3488. PMID:8754778.

Rabelo, R., Reyes, C., Schifman, A., and Silva, J.E. 1996*b*. Interactions among receptors, thyroid hormone response elements, and ligands in the regulation of the rat uncoupling protein gene expression by thyroid hormone. Endocrinology, **137**(8): 3478–3487. doi:10.1210/en.137.8.3478. PMID:8754777.

Rabelo, R., Camirand, A., and Silva, J.E. 1997. 3′,5′-cyclic adenosine monophosphate-response sequences of the uncoupling protein gene are sequentially recruited during darglitazone-induced brown adipocyte differentiation. Endocrinology, **138**(12): 5325–5332. doi:10.1210/en.138.12.5325. PMID:9389517.

Raimbault, S., Dridi, S., Denjean, F., Lachuer, J., Couplan, E., Bouillaud, F., Bordas, A., Duchamp, C., Taouis, M., and Ricquier, D. 2001. An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. Biochem. J. **353**: 441–444. doi:10.1042/0264-6021:3530441.

Ricquier, D., and Bouillaud, F. 2000. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. Biochem. J. **345**: 161–179. doi:10.1042/0264-6021:3450161.

Rim, J.S., and Kozak, L.P. 2002. Regulatory motifs for CREB-binding protein and Nfe2l2 transcription factors in the upstream enhancer of the mitochondrial uncoupling protein 1 gene. J. Biol. Chem. **277**(37): 34589–34600. doi:10.1074/jbc.M108866200. PMID:12084707.

Rizack, M.A. 1964. Activation of an epinephrine-sensitive lipolytic activity from adipose tissue by adenosine 3′,5′-phosphate. J. Biol. Chem. **239**: 392–395. PMID:14169136.

Robidoux, J., Cao, W., Quan, H., Daniel, K.W., Moukdar, F., Bai, X., Floering, L.M., and Collins, S. 2005. Selective activation of mitogen-activated protein (MAP) kinase kinase 3 and p38α MAP kinase is essential for cyclic AMP-dependent UCP1 expression in adipocytes. Mol. Cell. Biol. **25**(13): 5466–5479. doi:10.1128/MCB.25.13.5466-5479.2005. PMID:15964803.

Rodeheffer, M.S., Birsoy, K., and Friedman, J.M. 2008. Identification of white adipocyte progenitor cells in vivo. Cell, **135**(2): 240–249. doi:10.1016/j.cell.2008.09.036. PMID:18835024.

Rosen, E.D., and MacDougald, O.A. 2006. Adipocyte differentiation from the inside out. Nat. Rev. Mol. Cell Biol. **7**(12): 885–896. doi:10.1038/nrm2066. PMID:17139329.

Rosenwald, M., Perdikari, A., Rülicke, T., and Wolfrum, C. 2013. Bi-directional interconversion of brite and white adipocytes. Nat. Cell Biol. **15**(6): 659–667. doi:10.1038/ncb2740. PMID:23624403.

Saarela, S., Hissa, R., Pyörnilä, A., Harjula, R., Ojanen, M., and Orell, M. 1989. Do birds possess brown adipose tissue? Comp. Biochem. Physiol. Part A Physiol. **92**(2): 219–228. doi:10.1016/0300-9629(89)90157-6.

Sanchez-Gurmaches, J., Hung, C.-M., Sparks, C.A., Tang, Y., Li, H., and Guertin, D.A. 2012. *PTEN* loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors. Cell Metab. **16**(3): 348–362. doi:10.1016/j.cmet.2012.08.003.

Sawada, T., Miyoshi, H., Shimada, K., Suzuki, A., Okamatsu-Ogura, Y., Perfield, J.W., II, Kondo, T., Nagai, S., Shimizu, C., Yoshioka, N., Greenberg, A.S., Kimura, K., and Koike, T. 2010. Perilipin overexpression in white adipose tissue induces a brown fat-like phenotype. PLoS ONE, **5**(11): e14006. doi:10.1371/journal.pone.0014006. PMID:21103377.

Scantlebury, M., Lovegrove, B.G., Jackson, C.R., Bennett, N.C., and Lutermann, H. 2008. Hibernation and non-shivering thermogenesis in the Hottentot golden mole (*Amblysomus hottentottus longiceps*). J. Comp. Physiol. B, **178**(7): 887–897. doi:10.1007/s00360-008-0277-5.

Schulz, T.J., Huang, T.L., Tran, T.T., Zhang, H., Townsend, K.L., Shadrach, J.L., Cerletti, M., McDougall, L.E., Giorgadze, N., Tchkonia, T., Schrier, D., Falb, D., Kirkland, J.L., Wagers, A.J., and Tseng, Y.-H. 2011. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. Proc. Natl. Acad. Sci. U.S.A. **108**(1): 143–148. doi:10.1073/pnas.1010929108. PMID:21173238.

Schulz, T.J., Huang, P., Huang, T.L., Xue, R., McDougall, L.E., Townsend, K.L., Cypess, A.M., Mishina, Y., Gussoni, E., and Tseng, Y.-H. 2013. Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. Nature, **495**: 379–383. doi:10.1038/nature11943.

Seale, P., Kajimura, S., Yang, W., Chin, S., Rohas, L.M., Uldry, M., Tavernier, G., Langin, D., and Spiegelman, B.M. 2007. Transcriptional control of brown fat determination by PRDM16. Cell Metab. **6**(1): 38–54. doi:10.1016/j.cmet.2007.06.001. PMID:17618855.

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scimè, A., Devarakonda, S., Conroe, H.M., Erdjument-Bromage, H., Tempst, P.,

Rudnicki, M.A., Beier, D.R., and Spiegelman, B.M. 2008. PRDM16 controls a brown fat/skeletal muscle switch. Nature, **454**(7207): 961–967. doi:10.1038/nature07182. PMID:18719582.

Sellayah, D., Bharaj, P., and Sikder, D. 2011. Orexin is required for brown adipose tissue development, differentiation, and function. Cell Metab. **14**(4): 478–490. doi:10.1016/j.cmet.2011.08.010. PMID:21982708.

Sharp, L.Z., Shinoda, K., Ohno, H., Scheel, D.W., Tomoda, E., Ruiz, L., Hu, H., Wang, L., Pavlova, Z., Gilsanz, V., and Kajimura, S. 2012. Human BAT possesses molecular signatures that resemble beige/brite cells. PLoS ONE, **7**(11): e49452. doi:10.1371/journal.pone.0049452. PMID:23166672.

Shore, A., Karamitri, A., Kemp, P., Speakman, J.R., and Lomax, M.A. 2010. Role of *Ucp1* enhancer methylation and chromatin remodelling in the control of *Ucp1* expression in murine adipose tissue. Diabetologia, **53**(6): 1164–1173. doi:10.1007/s00125-010-1701-4. PMID:20238096.

Smith, R.E., and Horwitz, B.A. 1969. Brown fat and thermogenesis. Physiol. Rev. **49**(2): 330–425. PMID:4888392.

Soppela, P., Nieminen, M., Saarela, S., Keith, J.S., Morrison, J.N., Macfarlane, F., and Trayhurn, P. 1991. Brown fat-specific mitochondrial uncoupling protein in adipose tissues of newborn reindeer. Am. J. Physiol. **260**: R1229–R1234. PMID:2058749.

Stuart, J.A., Harper, J.A., Brindle, K.M., and Brand, M.D. 1999. Uncoupling protein 2 from carp and zebrafish, ectothermic vertebrates. Biochim. Biophys. Acta, **1413**(1): 50–54. doi:10.1016/S0005-2728(99)00081-X.

Su, C.-L., Sztalryd, C., Contreras, J.A., Holm, C., Kimmel, A.R., and Londos, C. 2003. Mutational analysis of the hormone-sensitive lipase translocation reaction in adipocytes. J. Biol. Chem. **278**(44): 43615–43619. doi:10.1074/jbc.M301809200. PMID:12832420.

Thonberg, H., Fredriksson, J.M., Nedergaard, J., and Cannon, B. 2002. A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation: mediation via α_1-adrenoceptors and protein kinase C activation. Biochem. J. **364**: 73–79. PMID:11988078.

Trayhurn, P., Thomas, M.E., and Keith, J.S. 1993. Postnatal development of uncoupling protein, uncoupling protein mRNA, and GLUT4 in adipose tissues of goats. Am. J. Physiol. **265**: R676–R682. PMID:8214163.

Tseng, Y.-H., Kokkotou, E., Schulz, T.J., Huang, T.L., Winnay, J.N., Taniguchi, C.M., Tran, T.T., Suzuki, R., Espinoza, D.O., Yamamoto, Y., Ahrens, M.J., Dudley, A.T., Norris, A.W., Kulkarni, R.N., and Kahn, C.R. 2008. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature, **454**(7207): 1000–1004. doi:10.1038/nature07221. PMID:18719589.

Tseng, Y.-H., Cypess, A.M., and Kahn, C.R. 2010. Cellular bioenergetics as a target for obesity therapy. Nat. Rev. Drug Discov. **9**(6): 465–482. doi:10.1038/nrd3138. PMID:20514071.

Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., and Tsuchida, K. 2010. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat. Cell. Biol. **12**(2): 143–152. doi:10.1038/ncb2014. PMID:20081842.

Ukropec, J., Anunciado, R.P., Ravussin, Y., Hulver, M.W., and Kozak, L.P. 2006. UCP1-independent thermogenesis in white adipose tissue of cold-acclimated *Ucp1*$^{-/-}$ mice. J. Biol. Chem. **281**(42): 31894–31908. doi:10.1074/jbc.M606114200. PMID:16914547.

van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M., Kemerink, G.J., Bouvy, N.D., Schrauwen, P., and Teule, G.J. 2009. Cold-activated brown adipose tissue in healthy men. N. Engl. J. Med. **360**(15): 1500–1508. doi:10.1056/NEJMoa0808718. PMID:19357405.

Vegiopoulos, A., Müller-Decker, K., Strzoda, D., Schmitt, I., Chichelnitskiy, E., Ostertag, A., Berriel Diaz, M., Rozman, J., Hrabe de Angelis, M., Nüsing, R.M., Meyer, C.W., Wahli, W., Klingenspor, M., and Herzig, S. 2010. Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. Science, **328**(5982): 1158–1161. doi:10.1126/science.1186034. PMID:20448152.

Vercesi, A.E., Martins, L.S., Silva, M.A.P., Leite, H.M.F., Cuccovia, I.M., and Chaimovich, H. 1995. PUMPing plants. Nature, **375**(6526): 24–24. doi:10.1038/375024a0.

Vercesi, A.E., Borecký, J., Maia Ide, G., Arruda, P., Cuccovia, I.M., and Chaimovich, H. 2006. Plant uncoupling mitochondrial proteins. Annu. Rev. Plant Biol. **57**: 383–404. doi:10.1146/annurev.arplant.57.032905.105335. PMID:16669767.

Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N.J., Enerbäck, S., and Nuutila, P. 2009. Functional brown adipose tissue in healthy adults. N. Engl. J. Med. **360**(15): 1518–1525. doi:10.1056/NEJMoa0808949. PMID:19357407.

Wang, J., Shen, W.-J., Patel, S., Harada, K., and Kraemer, F.B. 2005. Mutational analysis of the "regulatory module" of hormone-sensitive lipase. Biochemistry, **44**(6): 1953–1959. doi:10.1021/bi049206t. PMID:15697220.

Wu, J., Boström, P., Sparks, L.M., Ye, L., Choi, J.H., Giang, A.H., Khandekar, M., Virtanen, K.A., Nuutila, P., Schaart, G., Huang, K., Tu, H., van Marken Lichtenbelt, W.D., Hoeks, J., Enerbäck, S., Schrauwen, P., and Spiegelman, B.M. 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell, **150**(2): 366–376. doi:10.1016/j.cell.2012.05.016. PMID:22796012.

Wu, Z., Xie, Y., Bucher, N.L., and Farmer, S.R. 1995. Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. Genes Dev. **9**(19): 2350–2363. doi:10.1101/gad.9.19.2350. PMID:7557387.

Xue, B., Rim, J.-S., Hogan, J.C., Coulter, A.A., Koza, R.A., and Kozak, L.P. 2007. Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. J. Lipid Res. **48**(1): 41–51. doi:10.1194/jlr.M600287-JLR200. PMID:17041251.

Young, P., Arch, J.R.S., and Ashwell, M. 1984. Brown adipose tissue in the parametrial fat pad of the mouse. FEBS Lett. **167**(1): 10–14. doi:10.1016/0014-5793(84)80822-4. PMID:6698197.

Zhang, C., McFarlane, C., Lokireddy, S., Masuda, S., Ge, X., Gluckman, P.D., Sharma, M., and Kambadur, R. 2012. Inhibition of myostatin protects against diet-induced obesity by enhancing fatty acid oxidation and promoting a brown adipose phenotype in mice. Diabetologia, **55**(1): 183–193. doi:10.1007/s00125-011-2304-4. PMID:21927895.

Zhou, Z., Yon Toh, S., Chen, Z., Guo, K., Ng, C.P., Ponniah, S., Lin, S.-C., Hong, W., and Li, P. 2003. *Cidea*-deficient mice have lean phenotype and are resistant to obesity. Nat. Genet. **35**(1): 49–56. doi:10.1038/ng1225. PMID:12910269.

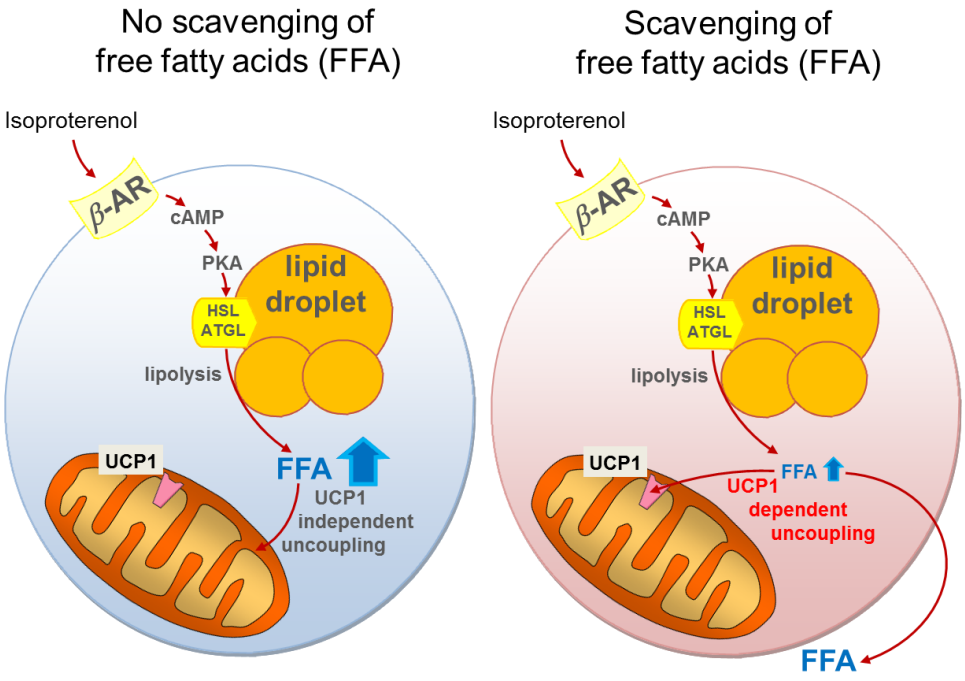
Zhu, Y., Lu, J., Wang, J., Chen, F., Leng, F., and Li, H. 2011. Regulation of thermogenesis in plants: the interaction of alternative oxidase and plant uncoupling mitochondrial protein. J. Integr. Plant Biol. **53**(1): 7–13. doi:10.1111/j.1744-7909.2010.01004.x. PMID:21205176.

Zimmermann, R., Strauss, J.G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., and Zechner, R. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science, **306**(5700): 1383–1386. doi:10.1126/science.1100747. PMID:15550674.

CHAPTER 4

Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes

EMBO Rep. 15:1069-1076, 2014



Published online: August 18, 2014

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Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes

Yongguo Li, Tobias Fromme, Sabine Schweizer, Theresa Schöttl & Martin Klingenspor*

Abstract

Thermogenesis in brown adipocytes, conferred by mitochondrial uncoupling protein 1 (UCP1), is receiving great attention because metabolically active brown adipose tissue may protect humans from metabolic diseases. In particular, the thermogenic function of brown-like adipocytes in white adipose tissue, known as brite (or beige) adipocytes, is currently of prime interest. A valid procedure to quantify the specific contribution of UCP1 to thermogenesis is thus of vital importance. Adrenergic stimulation of lipolysis is a common way to activate UCP1. We here report, however, that in this frequently applied setup, taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured brown and brite adipocytes. By the application of these findings, we demonstrate that UCP1 is functionally thermogenic in intact brite adipocytes and adrenergic UCP1 activation is largely dependent on adipose triglyceride lipase (ATGL) rather than hormone sensitive lipase (HSL).

Keywords brite adipocytes; brown adipocytes; thermogenesis; UCP1; uncoupled respiration

Subject Categories Metabolism; Membrane & Intracellular Transport

DOI 10.15252/embr.201438775 | Received 17 March 2014 | Revised 12 July 2014 | Accepted 23 July 2014 | Published online 18 August 2014

EMBO Reports (2014) 15: 1069–1076

Introduction

Mammalian adipose tissues are conventionally classified into two distinct types: white adipose tissue (WAT) and brown adipose tissue (BAT). The former stores energy as triglycerides, whereas the latter directly dissipates the chemical energy of fatty acids as heat through uncoupling protein 1 (UCP1) [1,2]. Interestingly, UCP1-expressing brown adipocyte-like cells (brite, "brown-in-white", also termed beige adipocytes) develop in typical WAT in response to cold exposure, β3-adrenergic receptor stimulation and PPARγ agonist treatment in a process termed "browning" of WAT [3–9]. Based on their energy dissipation property, brown and brite adipocytes represent putative therapeutic targets for the treatment of obesity and diabetes [10–12]. This scenario is further strengthened by the recent finding that brown and brite adipocytes are present in humans [13–17].

Non-shivering thermogenesis of brown adipocytes is conferred by mitochondrial uncoupling protein 1 (UCP1), which upon stimulation uncouples respiration from ATP synthesis and dissipates energy as heat [1,2]. UCP1 is constitutively inhibited by high concentrations of cytosolic purine nucleotides (i.e. GDP, GTP, ADP and ATP), but upon adrenergic stimulation of the cell, this inhibition is overcome by free fatty acids (FFAs) interacting directly with UCP1. Norepinephrine released from the sympathetic nervous system activates adrenergic receptors of brown adipocytes, which in turn stimulate the cAMP-dependent protein kinase PKA, leading to phosphorylation of hormone sensitive lipase (HSL) and thereby increased lipolysis. FFAs released by lipolysis serve both as activators of UCP1 and fuel for thermogenesis [18]. Adipose triglyceride lipase (ATGL) plays an essential role for the hydrolysis of triglycerides [19], and fatty acids released by phospholipases in the inner mitochondrial membrane have also been suggested to contribute to UCP1 activation [20], but the role of these lipases for the activation of UCP1 in brown and brite adipocytes has not been addressed.

Much effort has been invested into the development of assays quantifying UCP1-mediated uncoupled respiration in isolated mitochondria [21–24]. While this easily controllable system has many advantages, it is limited by artifacts associated with mitochondrial isolation, disruption of the intricate mitochondrial network integrity, lack of the native intracellular environment, and the large amount of cells or tissue needed for optimal yield and quality. A major goal of current bioenergetic research is thus the development and application of techniques to quantify mitochondrial function and cellular bioenergetics in cells [25]. One such technique, microplate-based respirometry, was developed to be used with cultured cells attached in a monolayer to a multi-well tissue culture plate and is now the preferred method to quantify UCP1-mediated leak respiration in cultured brown and brite cells [26–35]. From a historical

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Published online: August 18, 2014
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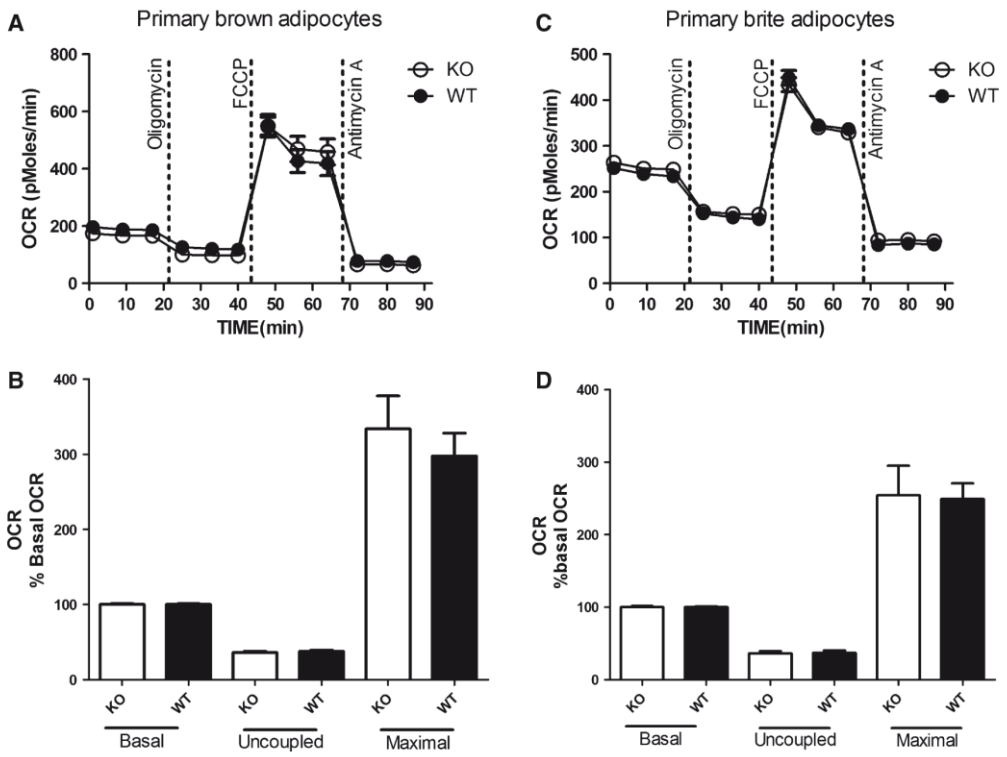

Figure 1. Activation of UCP1 is a prerequisite for quantifying UCP1-mediated leak respiration.

A, B Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 wild-type (WT) and knockout (KO) mice (A). Both genotypes display identical basal respiration, basal proton leak respiration (after oligomycin injection), maximal respiratory capacity (FCCP) and non-mitochondrial respiration (antimycin A). For comparison, mean OCR in these states was quantified (B).

C, D Representative time course of oxygen consumption rates (OCR) of primary brite adipocytes from UCP1 wild-type (WT) and knockout (KO) mice (C). Both genotypes display identical basal respiration, basal proton leak respiration (after oligomycin injection), maximal respiratory capacity (FCCP) and non-mitochondrial respiration (antimycin A). For comparison, mean OCR in these states was quantified (D).

Data information: All data presented are mean values ± SEM ($N = 3$).

perspective, albumin which acts as an acceptor of fatty acids must be used in the respiration medium for quantifying UCP1-mediated uncoupled respiration (for details see Supplementary Text S1). However, in the respiration buffer used by microplate-based respirometry, albumin is absent. Notably, no studies have been performed so far to validate this setup with cultured UCP1 knockout (KO) cells as the ultimate model to test the causality between uncoupled respiration and presence of UCP1. This relationship seems to have been taken for granted. It remains to be demonstrated that UCP1 is functionally thermogenic in intact brite adipocytes.

Here, we report that uncoupled respiration as measured in published protocols is not mediated by UCP1, since cultured primary adipocytes (both brown and brite) from UCP1 WT and KO have identical respiration profiles. We demonstrate that fatty acid-induced activation of UCP1 is a prerequisite for quantifying the UCP1-mediated leak respiration. In addition, when UCP1 is activated by stimulation of lipolysis, it is essential to take control over intracellular FFA levels to measure UCP1-mediated leak respiration in cells. Otherwise, an excessive rise of intracellular FFA levels released during lipolysis masks UCP1-mediated leak respiration through unspecific protonophoric action of FFAs and opening of the mitochondrial permeability transition pore (PTP) in both brown and brite adipocytes. Taken together, our studies provide critical guidelines for analyzing UCP1-mediated thermogenesis in intact brown and brite adipocytes.

Results and Discussion

Activation of UCP1 is a prerequisite for quantifying UCP1-mediated leak respiration in cultured primary adipocytes

To verify whether UCP1 is innately inactive within intact cultured primary brown and brite adipocytes, we compared the respiration profiles of UCP1 WT and KO cells without any (pre)treatment to activate UCP1. After determination of basal respiration, oligomycin, an inhibitor of adenosine triphosphate (ATP) synthase, was added to distinguish oxygen consumption used for ATP synthesis (coupled respiration) from proton leak (basal uncoupled respiration). Next, we employed the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy)

Published online: August 18, 2014

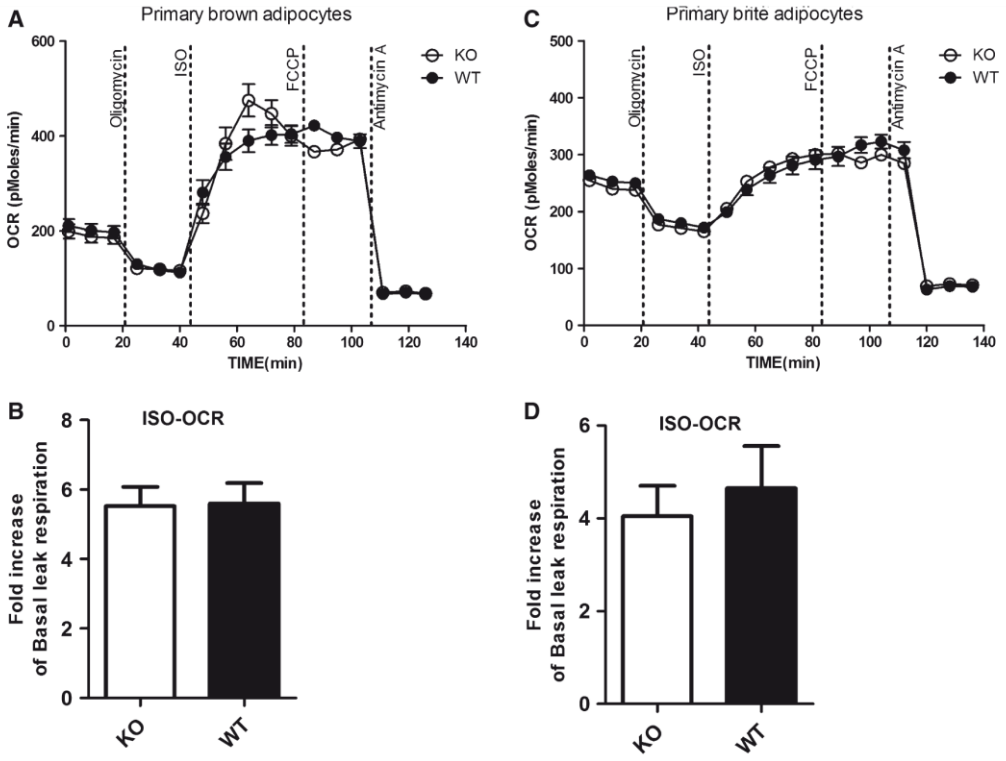

Figure 2. Identical isoproterenol (ISO)-induced leak respiration in primary brown and brite adipocytes from UCP1 WT and KO mice.

A, B Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 wild-type (WT) and knockout (KO) mice (A). Both genotypes display identical ISO-induced respiration, suggesting that ISO-induced leak respiration is independent of UCP1. For comparison, mean OCR in these states was quantified (B).

C, D Representative time course of oxygen consumption rates (OCR) of primary brite adipocytes from UCP1 wild-type (WT) and knockout (KO) mice (C). Both genotypes display identical ISO-induced respiration, suggesting that ISO-induced leak respiration is independent of UCP1. For comparison, mean OCR in these states was quantified (D).

Data information: All data presented are mean values ± SEM ($N = 3$).

phenylhydrazone (FCCP) to assess maximal respiratory capacity. Finally, antimycin A was added to block the electron transport chain, leaving only non-mitochondrial oxygen consumption (Fig 1A and C). We observed no differences in any of these four different respiration states between WT and KO in both brown and brite adipocytes, confirming UCP1 is inherently not leaky without stimulation (Fig 1B and D). Of note, this lack of differences was observed irrespective of the presence or absence of UCP1 in WT and KO cells, as confirmed by Western blotting (Supplementary Fig S1). Thus, without activation of UCP1, respiration measurements cannot reveal the consequences of absence or presence of UCP1 in a cell, although this setting is widely used in the literature, for example, to characterize white versus brown or brite adipocytes [26–29,35]. In these studies, the reported differential respiration between cell types is not due to UCP1 but rather reflects differences in mitochondrial content.

Free fatty acids mask UCP1-mediated leak respiration

A frequently employed method to activate UCP1 is to stimulate the intracellular signaling cascade controlling lipolysis. In our protocol, basal respiration was measured first succeeded by inhibition of coupled respiration (oligomycin) to determine basal leak respiration. Next, UCP1 was activated by FFAs released via β-adrenergic stimulation of lipolysis with isoproterenol (ISO), and the increase in UCP1-mediated leak respiration was measured. The maximum respiration rate was determined after adding the uncoupling agent FCCP. Finally, injection of antimycin A served to correct for non-mitochondrial oxygen consumption. Employing this protocol, ISO gradually increased respiration to a peak attained after 30 min (Supplementary Fig S2). Since 0.5 μM ISO was as potent as 1 μM, this concentration was chosen for further experiments.

We next evaluated our protocol with UCP1 KO primary brown and brite adipocytes. In contrast to the established model of UCP1 regulation, we found ISO-induced leak respiration to be identical in brown and brite adipocytes from both WT and KO mice (Fig 2). Thus, the ISO-induced leak respiration was completely independent of UCP1. Results were similar when we used a higher concentration of ISO (1 μM) or an alternative adrenergic agonist (CL-316,243, 1 μM) or dibutyryl cyclic AMP (db-cAMP, 0.5 and 1 mM) instead

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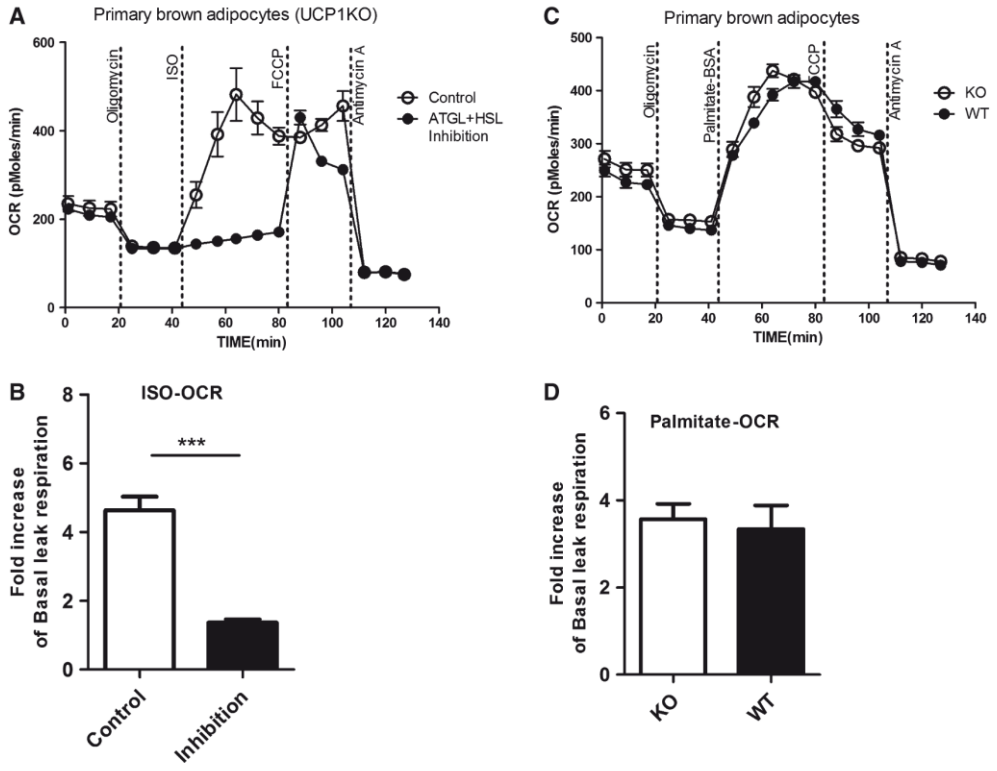

Figure 3. Uncoupled respiration in the absence of UCP1 is dependent on lipolysis and can be mimicked by addition of exogenous free fatty acids (FFA).

A, B Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 knockout (KO) mice pretreated with 40 μM Atglistatin (ATGL inhibitor) and 20 μM Hi76-0079 (HSL inhibitor) or vehicle (DMSO) for 1 h before bioenergetic profiling (A). Inhibition of lipolysis resulted in a significant suppression of ISO-induced leak respiration (B).

C, D Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 wild-type (WT) and knockout (KO) mice. Exogenous addition of free fatty acids by injection of a palmitate–BSA complex (0.2 mM) instead of ISO (C) mimics the full uncoupled respiration induced by ISO (D) in primary brown adipocytes from UCP1 WT and KO mice.

Data information: All data presented are mean values ± SEM ($N = 3$). $^{***}P < 0.001$.

(data not shown). Notably, the rate of UCP1-independent leak respiration could not be further increased by FCCP, indicating that mitochondria were already fully uncoupled.

In adipocytes, FFAs liberated by lipolysis are either released into the extracellular space or accumulate in the cell serving both as substrates of β-oxidation and as activators of UCP1. *In vivo*, extracellular FFAs are scavenged by albumin in the blood stream. *In vitro*, without any extracellular fatty acid acceptor available, FFAs accumulate to high concentrations in cells and may induce unspecific uncoupled respiration as evidenced in human adipocytes [36]. Using brown adipocytes from KO mice, we tested the hypothesis that ISO-stimulated UCP1-independent leak respiration is due to FFA liberated by lipolysis. We therefore inhibited adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) by pretreatment of cells with Atglistatin [37] and Hi 76-0079 [19], respectively. This combination of inhibitors, known to almost completely block (−95%) lipolysis [19], abolished ISO-induced UCP1-independent leak respiration (Fig 3A and B), confirming that this phenomenon depends on lipolysis. To further explore whether the released FFAs were responsible for unspecific mitochondrial uncoupling, we mimicked lipolysis by adding 0.2 mM palmitate–BSA to the medium. Indeed, exogenous fatty acids induced leak respiration independent of UCP1 to a similar extent as treatment with ISO (Fig 3C and D). Taken together, FFAs are the effectors of UCP1-independent uncoupling.

Opening of the mitochondrial permeability transition pore (PTP) participates in ISO-induced, UCP1-independent leak respiration

The ability of FFAs to uncouple mitochondrial respiration has been known for decades [38,39]. Besides their conventional protonophoric action based on flip-flop-mediated proton translocation by FFA inserted into the inner mitochondrial membrane [40], the involvement of PTP has been suggested [36]. The PTP is a high conductance channel spanning both mitochondrial membranes and opening leads to an increase of unspecific permeability which is a common feature of apoptosis. To determine whether the mitochondrial uncoupling mediated by PTP opening is also responsible for

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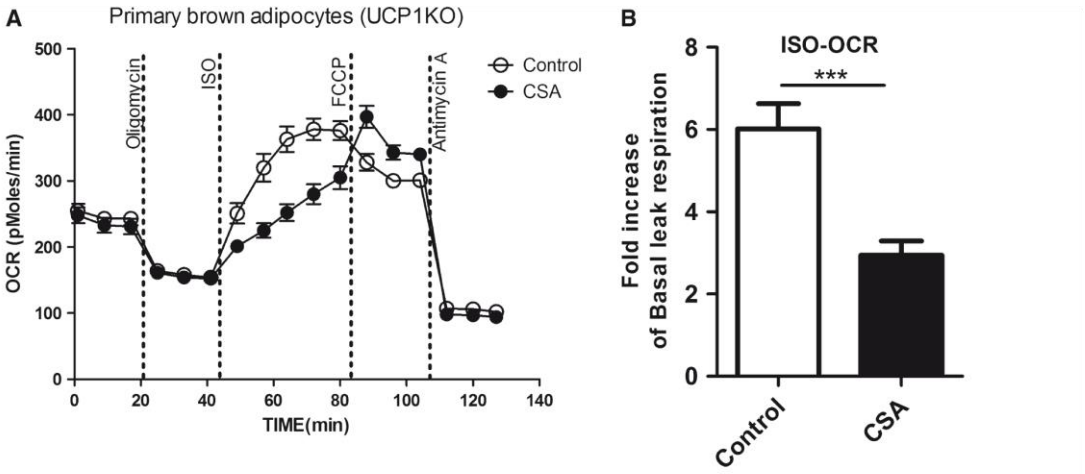

Figure 4. Uncoupled respiration in the absence of UCP1 is partially due to gating of the mitochondrial permeability transition pore (PTP).

A Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from knockout (KO) mice. Adipocytes were pretreated with the PTP inhibitor cyclosporine A (CSA) (5 μg/ml, 72 h) or vehicle prior to bioenergetic profiling.

B A significant portion of ISO-induced respiration is inhibited by CSA pretreatment.

Data information: All data presented are mean values ± SEM ($N = 3$). $^{***}P < 0.001$.

ISO-stimulated UCP1-independent leak respiration in brown and brite adipocytes, we pretreated primary brown cells with the specific PTP inhibitor cyclosporine A (CSA). We found that a significant portion of ISO-induced leak respiration was inhibited by CSA (Fig 4), demonstrating involvement of PTP opening. In any case, the functional data on brown adipocyte thermogenesis previously published must be revisited as they most likely do not reflect UCP1 activity but rather represent unspecific FFA-induced leak respiration [8,32–34,41–43].

Specific measurement of UCP1-mediated uncoupled respiration is enabled by scavenging of free fatty acids with bovine serum albumin (BSA)

To discriminate the specific contribution of UCP1 to brown adipocyte thermogenesis from other leak respiration mechanisms, we took control over the accumulation of FFAs by adding essentially fatty acid-free BSA to the respiration medium. It is known that the intracellular accumulation of FFAs can be controlled by varying the extracellular BSA concentration [44–48]. The ISO-stimulated leak respiration was significantly diminished when scavenging extracellular FFAs released by lipolysis with BSA in a dose-dependent manner in both primary brown and brite adipocytes (Supplementary Fig S3). Since 2% BSA was as effective as 3% in blocking the unspecific leak respiration induced by FFA, we conclude that these are optimal conditions to examine specific, UCP1-mediated leak respiration. Of note, the inhibitory effect of BSA on ISO-induced leak respiration is much more prominent in UCP1 KO cells, leading to significant lower levels of uncoupled respiration compared to WT cells in both brown and brite adipocytes (Fig 5). The difference in uncoupled respiration between WT and KO cells can be considered UCP1-mediated leak respiration.

We conclude that control over the intracellular fatty acid levels is essential for the analysis of thermogenic function in brown and brite/beige adipocytes and allows to specifically measure UCP1-mediated leak respiration. Employing this new protocol, we reproducibly quantified the UCP1-mediated component of uncoupled respiration in both brown and brite adipocytes. Importantly, brite adipocytes clearly resembled classical brown adipocytes in that they were thermogenically active in response to adrenergic stimulation. This uncoupled respiration was specific for the activity of UCP1. To our knowledge, this is the first report demonstrating that UCP1 is functionally thermogenic in intact cultured brite adipocytes. By applying this protocol, we recently revealed that strain differences in brite adipogenesis are associated with differential uncoupled respiration [49].

UCP1 activation largely depends on ATGL

It is generally accepted that lipolysis plays an important role in UCP1 activation although this concept has not been formally tested. ATGL and HSL are key enzymes involved in lipolysis. To determine the relative importance of these lipases for UCP1 activation, we compared the ISO-induced UCP1-mediated uncoupled respiration of brown adipocytes pretreated with either Atglistatin or Hi 76-0079 or both in the presence of 2% BSA. Lipase inhibition impaired ISO-induced respiration (Fig 6A). The effect of ATGL inhibition (80%), however, was much more pronounced compared with HSL inhibition (35%), demonstrating that UCP1 activity was largely dependent on ATGL rather than HSL (Fig 6B). Of note, combination of inhibitors led to an almost complete block (97%) of ISO-induced respiration, indicating a negligible contribution of phospholipases [20], previously implicated in UCP1 activation.

In summary, we report that meaningful respirometric measurements of cultured brown and brite adipocytes imperatively require

Published online: August 18, 2014
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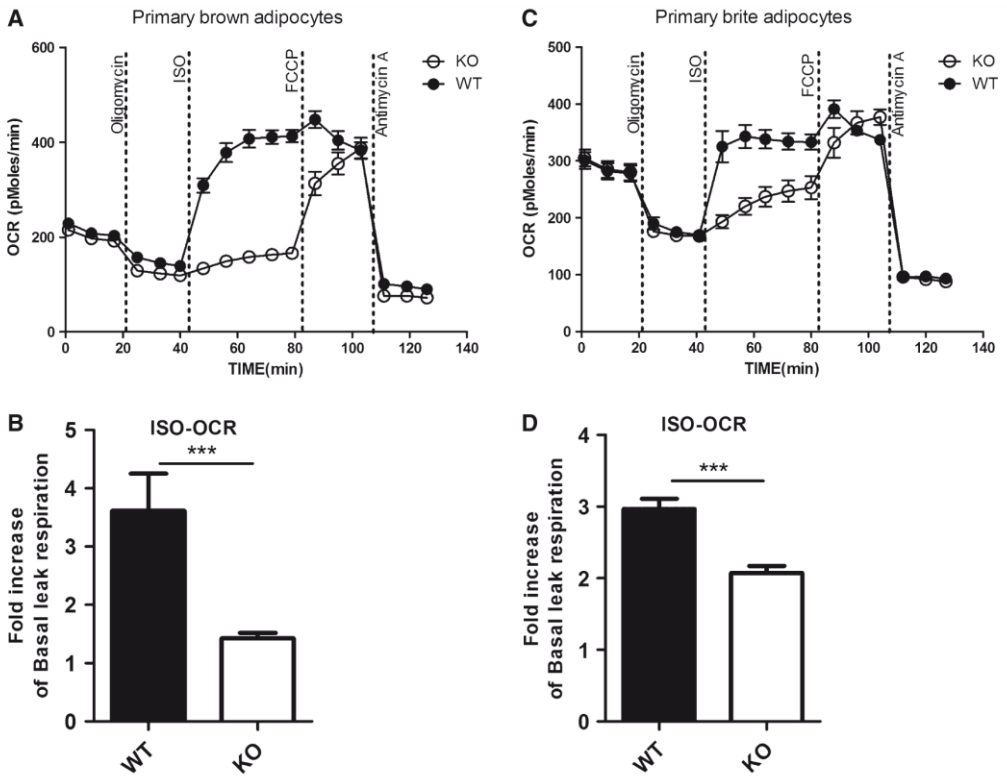


Figure 5. Scavenging extracellular free fatty acids with 2% BSA allows to specifically measure UCP1-mediated leak respiration in primary brown and brite adipocytes.

A, B Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 wild-type (WT) and knockout (KO) mice in the presence of 2% BSA. Comparison between WT and KO cells reveals ISO-induced leak respiration to be specific for UCP1 in brown adipocytes (B).

C, D Representative time course of oxygen consumption rates (OCR) of primary brite adipocytes from UCP1 wild-type (WT) and knockout (KO) mice in the presence of 2% BSA. Comparison between WT and KO cells reveals ISO-induced leak respiration to be specific for UCP1 in brite adipocytes (D).

Data information: All data presented are mean values ± SEM ($N = 3$). $^{***}P < 0.001$.

(i) activation of UCP1 and (ii) control over free fatty acid levels. By application of these findings, we demonstrate that UCP1 in brite adipocytes is thermogenically functional in a similar fashion as in brown adipocytes, with activation being largely dependent on adipose triglyceride lipase (ATGL).

Materials and Methods

Detailed methods can be found in Supplementary Materials and Methods.

Animals and primary cell culture

Male 129S1/SvImJ mice (UCP1-KO mice and wild-type littermates), aged 5–6 weeks, were used to prepare primary cultures of brown and brite adipocytes. Adipocyte differentiation was induced by treating confluent cells in DMEM medium (D-glucose, 25 mM) containing 10% fetal bovine serum (FBS), 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 mM dexamethasone, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone. After 2 days of induction, cells were maintained in differentiation media (10% FBS, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone).

Respirometry

Oxygen consumption rate (OCR) was measured at 37°C using microplate-based respirometry (XF96 extracellular flux analyzer, Seahorse Bioscience). For detailed experimental procedures, see Supplementary Materials and Methods. After the completion of an assay, the microplate was saved and protein was isolated for UCP1 phenotyping. All data presented are mean values ± SEM of three independent experiments with 8–12 replicate wells each.

Statistical analysis

Significant differences between two groups were assessed by two-tailed Student's *t*-test (Prism 6.0 software). A P-value < 0.05 was considered a statistically significant difference.

Published online: August 18, 2014

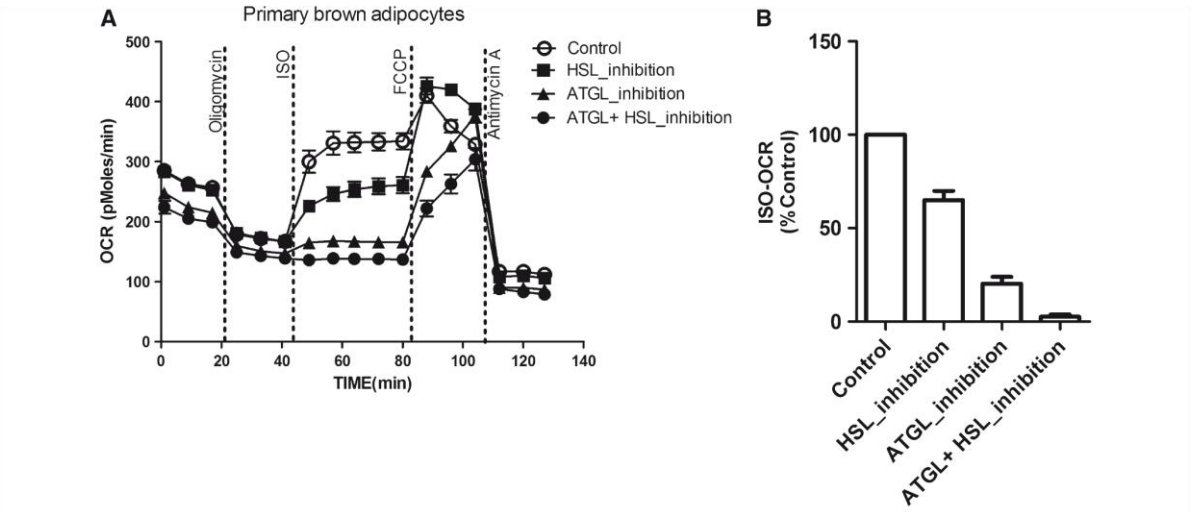


Figure 6. UCP1 activation largely depends on ATGL.

A Representative time course of oxygen consumption rates (OCR) of primary brown adipocytes from UCP1 wild-type (WT) mice in the presence of 2% BSA. Adipocytes were pretreated with 40 μM Atglistatin (ATGL inhibitor), 40 μM Hi76-0079 (HSL inhibitor) or both before bioenergetic profiling.

B The effect of ATGL inhibition on ISO-induced respiration was much larger compared to the effect of HSL inhibition.

Data information: All data presented are mean values ± SEM ($N = 3$).

Supplementary information for this article is available online: http://embor.embopress.org

Acknowledgements

The Chair of Molecular Nutritional Medicine received financial support from by the Else Kröner-Fresenius Foundation. YL holds a fellowship from the Deutsche Akademische Austauschdienst (DAAD).

Funding

EU FP7 project DIABAT (HEALTH-F2-2011-278373), the Competence Network Obesity (BMBF Seed Money 01GI1325) and DFG grant KL 973/11-1 to MK.

Author contributions

YL, TF, and MK conceived the study; TS helped to establish the seahorse measurements; YL performed experiments; SS helped with the lipase inhibition experiment and Western blot analysis; YL, TF, and MK analyzed the data; YL, TF, and MK wrote the manuscript. All authors commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Klingenspor M, Herzig S, Pfeifer A (2012) Brown fat develops a brite future. *Obes Facts* 5: 890 – 896
2. Li Y, Lasar D, Fromme T, Klingenspor M (2014) White, brite and brown adipocytes: the evolution and function of a heater organ in mammals. *Can J Zool* 92: 615 – 626
3. Guerra C, Koza RA, Yamashita H, Walsh K, Kozak LP (1998) Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. *J Clin Invest* 102: 412 – 420
4. Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S (2000) Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol* 279: C670 – C681
5. Young P, Arch JRS, Ashwell M (1984) Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett* 167: 10 – 14
6. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, Casteilla L (1992) Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci* 103: 931 – 942
7. Xue B, Rim JS, Hogan JC, Coulter AA, Koza RA, Kozak LP (2007) Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. *J Lipid Res* 48: 41 – 51
8. Ohno H, Shinoda K, Spiegelman BM, Kajimura S (2012) PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* 15: 395 – 404
9. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J (2010) Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J Biol Chem* 285: 7153 – 7164
10. Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. *Nat Med* 19: 1252 – 1263
11. Bartelt A, Heeren J (2014) Adipose tissue browning and metabolic health. *Nat Rev Endocrinol* 10: 24 – 36
12. Tseng YH, Cypess AM, Kahn CR (2010) Cellular bioenergetics as a target for obesity therapy. *Nat Rev Drug Discov* 9: 465 – 482
13. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, Huang TL, Roberts-Toler C, Weiner LS, Sze C *et al* (2013) Anatomical localization,

Published online: August 18, 2014
EMBO reports Measuring UCP1-mediated leak respiration in cells *Yongguo Li et al*

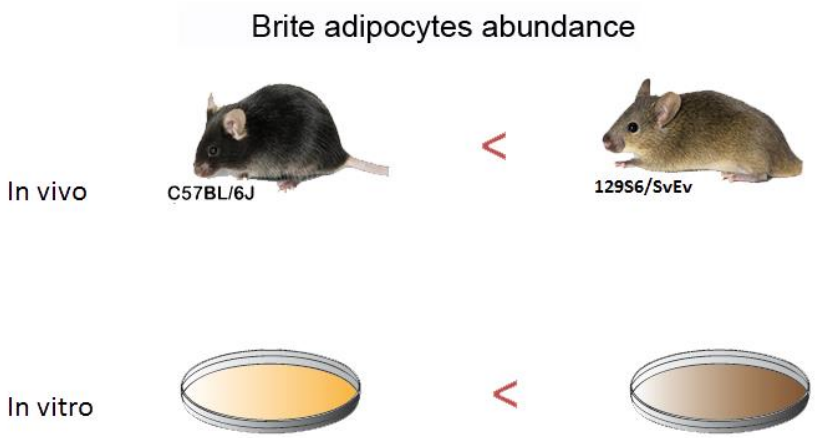
gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* 19: 635 – 639

14. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, Mussack T, Nilsson D, Romu T, Nuutila P *et al* (2013) Evidence for two types of brown adipose tissue in humans. *Nat Med* 19: 631 – 634
15. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A *et al* (2009) Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* 360: 1509 – 1517
16. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJ (2009) Cold-activated brown adipose tissue in healthy men. *N Engl J Med* 360: 1500 – 1508
17. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerbäck S *et al* (2009) Functional brown adipose tissue in healthy adults. *N Engl J Med* 360: 1518 – 1525
18. Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277 – 359
19. Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, Tornqvist H, Zechner R, Zimmermann R (2006) Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *J Biol Chem* 281: 40236 – 40241
20. Fedorenko A, Lishko PV, Kirichok Y (2012) Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* 151: 400 – 413
21. Shabalina IG, Ost M, Petrovic N, Vrbacky M, Nedergaard J, Cannon B (2010) Uncoupling protein-1 is not leaky. *Biochim Biophys Acta* 1797: 773 – 784
22. Shabalina IG, Petrovic N, de Jong JM, Kalinovich AV, Cannon B, Nedergaard J (2013) UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep* 5: 1196 – 1203
23. Parker N, Crichton PG, Vidal-Puig AJ, Brand MD (2009) Uncoupling protein-1 (UCP1) contributes to the basal proton conductance of brown adipose tissue mitochondria. *J Bioenerg Biomembr* 41: 335 – 342
24. Hirschberg V, Fromme T, Klingenspor M (2011) Test systems to study the structure and function of uncoupling protein 1: a critical overview. *Front Endocrinol (Lausanne)* 2: 63
25. Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochem J* 435: 297 – 312
26. Qiang L, Wang L, Kon N, Zhao W, Lee S, Zhang Y, Rosenbaum M, Zhao Y, Gu W, Farmer SR *et al* (2012) Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Pparγ. *Cell* 150: 620 – 632
27. Trajkovski M, Ahmed K, Esau CC, Stoffel M (2012) MyomiR-133 regulates brown fat differentiation through Prdm16. *Nat Cell Biol* 14: 1330 – 1335
28. Sun L, Xie H, Mori MA, Alexander R, Yuan B, Hattangadi SM, Liu Q, Kahn CR, Lodish HF (2011) Mir193b-365 is essential for brown fat differentiation. *Nat Cell Biol* 13: 958 – 965
29. Ahfeldt T, Schinzel RT, Lee YK, Hendrickson D, Kaplan A, Lum DH, Camahort R, Xia F, Shay J, Rhee EP *et al* (2012) Programming human pluripotent stem cells into white and brown adipocytes. *Nat Cell Biol* 14: 209 – 219
30. Roberts LD, Boström P, O'Sullivan JF, Schinzel RT, Lewis GD, Dejam A, Lee YK, Palma MJ, Calhoun S, Georgiadi A *et al* (2014) beta-Aminoisobutyric acid induces browning of white fat and hepatic beta-oxidation and is inversely correlated with Cardiometabolic risk factors. *Cell Metab* 19: 96 – 108
31. Wang J, Liu R, Wang F, Hong J, Li X, Chen M, Ke Y, Zhang X, Ma Q, Wang R *et al* (2013) Ablation of LGR4 promotes energy expenditure by driving white-to-brown fat switch. *Nat Cell Biol* 15: 1455 – 1463
32. Bordicchia M, Liu D, Amri E-Z, Ailhaud G, Dessì-Fulgheri P, Zhang C, Takahashi N, Sarzani R, Collins S (2012) Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest* 122: 1022 – 1036
33. Keipert S, Jastroch M (2014) Brite/beige fat and UCP1 - is it thermogenesis? *Biochim Biophys Acta* 1837: 1075 – 1082
34. Lee P, Werner CD, Kebebew E, Celi FS (2014) Functional thermogenic beige adipogenesis is inducible in human neck fat. *Int J Obes* 38: 170 – 176
35. Sellayah D, Bharaj P, Sikder D (2011) Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab* 14: 478 – 490
36. Yehuda-Shnaidman E, Buehrer B, Pi J, Kumar N, Collins S (2010) Acute stimulation of white adipocyte respiration by PKA-induced lipolysis. *Diabetes* 59: 2474 – 2483
37. Mayer N, Schweiger M, Romauch M, Grabner GF, Eichmann TO, Fuchs E, Ivkovic J, Heier C, Mrak I, Lass A *et al* (2013) Development of small-molecule inhibitors targeting adipose triglyceride lipase. *Nat Chem Biol* 9: 785 – 787
38. Di Paola M, Lorusso M (2006) Interaction of free fatty acids with mitochondria: coupling, uncoupling and permeability transition. *Biochim Biophys Acta* 1757: 1330 – 1337
39. Wojtczak L, Lehninger AL (1961) Formation and disappearance of an endogenous uncoupling factor during swelling and contraction of mitochondria. *Biochim Biophys Acta* 51: 442 – 456
40. Wojtczak L, Schonfeld P (1993) Effect of fatty acids on energy coupling processes in mitochondria. *Biochim Biophys Acta* 1183: 41 – 57
41. Lee P, Linderman JD, Smith S, Brychta RJ, Wang J, Idelson C, Perron RM, Werner CD, Phan GQ, Kammula US *et al* (2014) Irisin and FGF21 Are Cold-Induced Endocrine Activators of Brown Fat Function in Humans. *Cell Metab* 19: 302 – 309
42. Wu J, Boström P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G *et al* (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* 150: 366 – 376
43. Tews D, Fischer-Posovszky P, Fromme T, Klingenspor M, Fischer J, Rüther U, Marienfeld R, Barth TF, Möller P, Debatin KM *et al* (2013) FTO deficiency induces UCP-1 expression and mitochondrial uncoupling in adipocytes. *Endocrinology* 154: 3141 – 3151
44. Vallano ML, Lee MY, Sonenberg M (1983) Hormones modulate adipocyte membrane potential ATP and lipolysis via free fatty acids. *Am J Physiol* 245: E266 – E272
45. Kampf JP, Kleinfeld AM (2004) Fatty acid transport in adipocytes monitored by imaging intracellular free fatty acid levels. *J Biol Chem* 279: 35775 – 35780
46. Angel A, Desai KS, Halperin ML (1971) Reduction in adipocyte ATP by lipolytic agents: relation to intracellular free fatty acid accumulation. *J Lipid Res* 12: 203 – 213
47. Cushman SW, Heindel JJ, Jeanrenaud B (1973) Cell-associated nonesterified fatty acid levels and their alteration during lipolysis in the isolated mouse adipose cell. *J Lipid Res* 14: 632 – 642
48. Civelek VN, Hamilton JA, Tornheim K, Kelly KL, Corkey BE (1996) Intracellular pH in adipocytes: effects of free fatty acid diffusion across the plasma membrane, lipolytic agonists, and insulin. *Proc Natl Acad Sci USA* 93: 10139 – 10144
49. Li Y, Bolze F, Fromme T, Klingenspor M (2014) Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains. *Biochim Biophys Acta* 1841: 1345 – 1352.

CHAPTER 5

Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains

Biochim Biophys Acta. 1841(9):1345-52, 2014



Biochimica et Biophysica Acta 1841 (2014) 1345–1352

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains

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ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 16 May 2014
Accepted 8 June 2014
Available online 19 June 2014

Keywords:
Nonshivering thermogenesis
Brown adipose tissue
Mitochondria
Uncoupling protein

ABSTRACT

BRITE (brown-in-white) cells are brown adipocyte-like cells found in white adipose tissue (WAT) of rodents and/or humans. The recruitment of BRITE adipocytes, referred to as the browning of WAT, is hallmarked by the expression of UCP1 and exerts beneficial metabolic effects. Here we address whether beyond systemic cues depot- and strain-specific variation in BRITE recruitment is determined by a cellular program intrinsic to progenitors. Therefore we compared the browning capacity of serum and investigated brown and BRITE adipogenesis in primary cultures of stromal-vascular cells isolated from interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) in two inbred mouse strains C57BL/6J (B6, a strain with low browning propensity) and 129/S6SvEv (129, a strain with high browning propensity). Paradoxically, serum collected from B6 mice was more potent in the promotion of browning than serum collected from 129 mice. Nevertheless, we demonstrate that depot- and strain-specific differences observed in vivo are phenocopied in primary cultures in vitro, as judged by UCP1 expression and by functional analysis. Notably, primary adipocytes from 129 mice had a higher capacity for isoproterenol-induced uncoupled respiration than B6. We conclude that cues intrinsic to the progenitor cells contribute to differential BRITE adipogenesis. Further analyses demonstrate that these cues are independent of autocrine/paracrine mechanisms, BRITE progenitor abundance and genetic variation in the gene regulatory region of Ucp1 but rather depend on trans-acting factors. These results provide new insights on the molecular basis of strain and depot-specific differences in BRITE adipogenesis.

1. Introduction

In mammals, two types of adipose tissues are present, white (WAT) and brown (BAT). The former stores energy as triglycerides, whereas the latter catabolizes lipids to produce heat through uncoupling protein 1 (UCP1)-mediated thermogenesis. BAT was originally identified in infants and rodents, but recent studies have also found functional BAT in adult humans [1]. Interestingly, UCP1-expressing brown adipocyte-like cells (BRITE, brown-in-white, also termed 'beige') can also develop in typical WAT depots in response to cold exposure, beta3-adrenergic receptor stimulation and PPARγ agonist treatment, a process termed "browning" of WAT [2–7]. During post-natal development these BRITE cells spontaneously emerge around weaning [6,8]. Although the browning phenomenon has been first described in 1984, the developmental origin, transcriptional control and physiological function of BRITEs are largely unknown. It has been suggested that BRITE cells represent a newly identified type of adipocytes distinct from white and brown adipocytes in respect to their development, molecular outfit and hormonal-sensitivity [9–11]. Notably, the abundance of BRITEs in WAT has been associated with obesity-resistance, enhanced fat oxidation and energy expenditure as well as improved systemic insulin sensitivity [2,12]. Even more, current evidence points to the presence of both classical brown and BRITE adipocytes in humans, both as babies and adults [10,13–16]. Thus, increasing the numbers of BRITE adipocytes within WAT depots may represent an innovative approach to treat obesity and associated metabolic conditions [17–19]. Understanding the molecular mechanisms underlying BRITE adipocyte recruitment is therefore of great scientific and medical interest [1].

In mice a large accumulation of BRITE cells can be found most readily in the subcutaneous inguinal adipose tissue, but is rather scarce in epididymal/perigonadal adipose tissue [12]. The propensity to accumulate BRITE cells, however, differs not only between WAT depots but also among inbred mouse strains [8,12,20]. Mice of some strains, such as C57BL/6J, upon adrenergic stimulation exhibit only minor induction of BRITE adipocytes, whereas others, such as A/J and 129, are very responsive to this stimulus [21,22]. The density of sympathetic fibers in the parenchyma of adipose tissues increases during cold acclimation and positively correlates with the number of BRITE adipocytes in mice of the B6 and 129 strains, thus suggesting that strain differences in the sympathetic tone may be the cause for different browning propensity

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http://dx.doi.org/10.1016/j.bbalip.2014.06.003
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between strains [23]. It is not clear, however, whether different browning propensities are caused by cell-extrinsic cues only (such as innervation, angiogenesis, hormones, cytokines, etc.) or also by cell-intrinsic properties (such as progenitor abundance, cis-elements and trans-acting factors, etc.). Elucidating the underlying mechanisms will not only help us to understand the development of BRITE cells but also may help us to enrich these cells in the attempt to treat obesity.

Primary cultures represent invaluable tools to characterize cell autonomous or non-autonomous traits. Previous studies have shown that white and brown fat precursor cells in culture proliferate and develop into adipocytes which on the molecular level have distinct inherent characteristics resembling white and brown adipocytes differentiated in vivo [9]. Moreover, an induction of Ucp1 gene expression has been observed in cultured primary adipocytes upon chronic treatment with peroxisome proliferator-activated receptor- (PPAR) ligands [9]. In fact, several recent studies have reported that exposure of white adipocytes in culture or in vivo to potent PPARγ agonists induces browning [7,9,24]. The molecular basis of browning induced by PPARγ agonists has not been finally resolved. Nevertheless, mechanisms such as induction of peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) expression, stabilization of PRD1-BF-1-RIZ1 homologous domain containing-16 protein (PRDM16), increment of fibroblast growth factor 21 (FGF21) secretion, and posttranslational modifications of PPARγ (deacetylation, desumoylation) activity seem to be involved [7,25,26].

Pertaining to the transcriptional control of brown and BRITE adipogenesis, in addition to PPARγ, other core transcriptional regulators of brown and BRITE adipogenesis such as PGC-1α and PRDM16 have been suggested. Among those factors, PRMD16 is considered the first transcriptional regulator that is absolutely required to promote the differentiation of brown/BRITE adipocytes. A recent study describes that PRDM16 is selectively expressed in subcutaneous white adipocytes in comparison to other white fat depots in mice, suggesting that this transcription factor could act as a determinant of browning propensity among fat depots [7,27]. Nevertheless, whether PRDM16 determines the differential browning capacity of white fat between inbred mouse strains is unknown.

By taking advantage of the highly variable trait of induction of BRITE cells in WAT between mouse strains (C57BL/6J and 129S6/SvEv) and using the primary culture method combined with co-culture strategies and cell transfection, we demonstrate that differences in Ucp1 expression both between depots and strains are maintained in primary cultures, a condition definitely excluding extrinsic cues such as innervation, angiogenesis and blood-borne hormones. In support of this point, we demonstrate that the B6 serum is paradoxically more potent in Ucp1 induction compared to 129, thus questioning a major hormonal contribution to the strain difference in Ucp1 expression. Furthermore, gene profiling and co-culture experiments verify that this cell-autonomous trait is contributed neither by differences in progenitor abundance nor by autocrine or paracrine factors. Importantly, cell transfection experiments confirm that the strain specific differences in Ucp1 expression are caused by cell intrinsic trans-acting factors. Taken together, these data demonstrate that there are intrinsic differences between progenitors from different fat depots and mouse strains which contribute to the differential browning propensity of WAT.

2. Materials and methods

2.1. Animals, serum collection, cell isolation, and cell culture

Male C57BL/6J and 129S6/SvEv mice bred at the animal facility of Technische Universität München (Weihenstephan), aged 5 to 6 weeks were used for the collection of serum and preparation of primary cultures of brown and white adipocytes. Briefly, adipose tissues were dissected and carefully minced and treated with collagenase for 30–45 min. The primary cells were filtered through 250 μm nylon mesh and centrifuged at 500 *g* to collect the stromal vascular fraction (SVF). The SVF cell pellets were rinsed and seeded in 6-well plates. When the cells were confluent, the induction media containing 10% fetal bovine serum (FBS), 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 mM dexamethasone, 850 nM insulin, 1 nM T3, and 1 μM rosiglitazone was added. After 2 days of induction, cells were maintained in differentiation media (10% FBS, 850 nM insulin and 1 nM T3) with 1 μM rosiglitazone. Media was changed every 2 days. Cells were harvested on day 7 of differentiation.

For serum collection, blood was centrifuged at 2500 *g* for 5 min at 4 °C. Serum was sterilized by filtration through a 0.22 μ membrane filter and stored at −20 °C until used.

2.2. Conditioned medium and co-culture experiments

For conditioned media experiments, cells were cultured as described above but medium was changed every 3 days during differentiation. On the 3rd and 6th day of differentiation, media conditioned by B6 cells was collected and treated to 129 cells and vice versa. Cells were harvested at 7th day of differentiation.

For co-culture experiment, co-culture was performed in 2 different ways as follows.

1. In the transwell system, cells were co-cultured by using transwell inserts with a 0.4-μm porous membrane (Corning) to separate B6 adipocytes from 129 adipocytes. After differentiation, the cells in the lower well were harvested and analyzed.
2. In the contact system (direct co-culture), B6 primary cells were transfected with an Ucp1 promoter luciferase construct (Ucp1-Luc) by electroporation 1 day after inducing differentiation (see below). pCMV-GL (Gaussia luciferase) was co-transfected to normalize for transfection efficiencies and cell number. Transfected cells were mixed with untransfected B6 and 129 cells with 1:1 ratio and re-plated onto cell culture plates. After 4 days of differentiation, Luciferase activity was measured.

2.3. RNA isolation and qPCR analysis

Total RNA was isolated from cells using Trisure (Bioline). Reverse transcriptase reactions were performed using QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative real-time PCR (qPCR) was performed with SYBR green fluorescent dye using Roche LightCycler 480. Transcription factor IIB (TFIIB) served as an internal control. Primer sequences are provided in Supplementary Table.

2.4. Cell transfection and luciferase reporter assays

Primary cells were transfected with an Ucp1 promoter luciferase construct (Ucp1-Luc, 3.2Kb) by electroporation method using Nucleofector II device (Amaxa) 1 day after inducing differentiation. pCMV-GL (Gaussia luciferase) was co-transfected to normalize for transfection efficiencies and cell number. Luciferase activity was performed at 4 days after transfection using the Dual Luciferase assay kit, according to the manufacturer's protocol (Promega).

2.5. Western blot analysis

For western blot analysis, cells were lysed in RIPA buffer. Lysates were resolved by SDS-PAGE, transferred to Odyssey® nitrocellulose membrane (926-31092, LI-COR), and probed with anti-UCP1 (ab10983, Abcam), anti-Actin (MAB1501, Millipore) and anti-PPARγ (sc-7196, Santa Cruz). Fluorescent images were captured by Odyssey infrared imaging system (Licor Biosciences).

2.6. Immunocytochemistry

Primary inguinal adipocytes were cultured as described above by using 8-well chamber slides (flexiPERM® slide). After 7 days of differentiation, cells were washed with PBS and fixed with cooled 95% ethanol and 5% glacial acetic acid for 10 min at −20 °C. Cells were washed three times with PBS and permeabilized with 50%, 100% and 50% aceton each for 3 min at −20 °C. Then the cells were washed three times with PBS and blocked with 5% donkey serum plus 20% FBS in PBS for 2 h at room temperature. Cells were washed three times with PBS and incubated with 1:500 diluted anti-UCP1 antibody in 0.25% donkey serum in PBS for 2 h at room temperature. Cells were washed three times with PBS and incubated with anti-rabbit-Alexa Fluor 488-labeled secondary antibodies (Molecular Probes), diluted 1:500 in 0.25% donkey serum in PBS for 1.5 h at room temperature. Cells were then washed three times with PBS. Nuclei were stained with DAPI for 3 min. Finally, coverslips were mounted on microscope slides with VECTASHIELD mounting media (Vector Labs). Microscopic pictures were acquired at a magnification of 600-fold using the confocal microscope FluoView FV10i (Olympus, Hamburg, Germany). Images in each figure were processed equally.

2.7. Seahorse XF-96 metabolic flux analysis

Oxygen consumption rate (OCR) was measured at 37 °C using an XF-96 extracellular analyzer (Seahorse Bioscience). Briefly, primary cells were isolated, seeded into Seahorse XF-96 cell culture microplates, and differentiated into mature adipocytes. At day 7 of differentiation, the medium was replaced with prewarmed unbuffered DMEM (DMEM basal medium supplemented with 25 mM glucose, 2 mM sodium pyruvate, 31 mM NaCl, 2 mM GlutaMax and 15 mg/l phenol red, 2% essentially fatty acid free bovine serum albumin (BSA), pH 7.4) and incubated at 37 °C in a non-CO_2 incubator for 1 h. Basal respiration was measured in untreated cells. Coupled respiration was inhibited by oligomycin treatment (5 μM). Ucp1 mediated uncoupling respiration was determined after isoproterenol (0.5 μM) stimulation. Maximum respiratory capacity was assessed after FCCP (Sigma) stimulation (1 μM). Finally, mitochondrial respiration was blocked by antimycin A (Sigma) (5 μM) and the residual OCR being non-mitochondrial. Oxygen consumption rates were automatically calculated by the Seahorse XF-96 software. Every point represents an average of 10–12 different wells.

2.8. Statistical analysis

All values are presented as means ± SEM. Significant differences between two groups were assessed by two-tailed Student's *t* test by SigmaPlot 12.0 software. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. Paradoxical higher browning capacity of serum from C57BL/6J mice compared to serum from 129S6/SvEv mice

It has been shown that the browning potential of white fat differs not only between fat depots but also among different strains of inbred mice [12]. Since blood-borne hormones such as fibroblast growth factor 21 (FGF21), thyroid hormone T_4 and cardiac natriuretic peptides (NPs) contribute significantly in the browning of white fat (18), we reason that differences in blood-borne hormone levels may contribute to the strain differences in Ucp1 expression. To test this possibility, we collected serum from B6 and 129 mice and treated fully differentiated primary adipocytes isolated from inguinal fat depot (iWAT). After 24 h treatment, serum from both strains had a much higher potency to promote browning than fetal bovine serum (FBS) (Fig. 1A). Paradoxically, serum collected from B6 mice was more potent than serum collected from 129 mice as evidenced by the induction of Ucp1 expression in B6 primary adipocytes. A similar trend was observed in 129 cells (Fig. 1A). Of note, primary adipocytes of 129 background have higher Ucp1 expression compared to primary adipocytes of B6 background under both serum conditions, demonstrating minor hormonal contribution to the strain difference in Ucp1 expression.

3.2. Fat depot and strain-specific differences in Ucp1 expression are maintained in primary cultures in vitro

To further systematically determine whether differences in browning potential were cell autonomous and could be maintained after differentiation in vitro, cells from the stromal-vascular fraction (SVF) isolated from three fat depots (iBAT, iWAT, and eWAT) of the two different mouse strains (C57BL/6 J and 129S6/SvEv) were subjected to in vitro proliferation and differentiation. To stimulate Ucp1 expression, cells were treated with rosiglitazone during differentiation. After 7 days of differentiation, cells were harvested and gene expression analysis was performed using qPCR. The difference in Ucp1 mRNA levels between strains (Fig. 1B) and among fat depots (Fig. 1C) was maintained after differentiation in vitro. Among fat depots, Ucp1 mRNA level is high in iBAT-derived adipocytes, moderate in iWAT-derived adipocytes, and lowest in eWAT-derived adipocytes (Fig. 1C). Between strains, primary cells from iWAT of 129 mice have significantly high levels of Ucp1 expression compared to cells from B6 mice (Fig. 1B). The strain difference was further confirmed at the protein level (Fig. 1D). These lines of evidence strongly suggest that the strain-specific differences in Ucp1 expression at least partly are contributed by intrinsic differences of progenitors.

3.3. Primary cultures of 129 mice develop more BRITE cells and exhibit higher UCP1 mediated uncoupling respiration compared with B6 mice

In principle, the strain differences in Ucp1 expression can be the result of differences in either BRITE cell abundance or Ucp1 expression levels per cell, or both. Notably, in our study we found that the differential Ucp1 expression between strains is independent of Cidea and Cox7a1 (Fig. 1B), two widely used specific markers for brown adipocytes. Thus differences in Ucp1 expression could be contributed by differences in Ucp1 expression levels per cell, rather than the number of beige cells. To clarify this issue, we performed UCP1 immunocytochemistry staining in primary cultures. Primary cultures of 129 mice had more UCP1-positive cells compared with B6 mice (Fig. 1E). Correspondingly, at the functional level, 129 cells also showed higher levels of uncoupling respiration when activating UCP1 by isoproterenol treatment (Fig. 1F), while there were no differences in basal respiration, coupled respiration, maximum cell respiration and non-mitochondrial OCR between strains (Fig. 1F).

3.4. The expression of progenitor markers differs between fat depots but not between mouse strains

To explore the underlying mechanisms of the cell intrinsic differences in BRITE adipogenesis, we hypothesized that the abundance of BRITE precursor cells in various white fat depots of inbred mice may differ. Although it remains to be determined which population of progenitors gives rise to BRITE cells, some progenitors, such as Sca-1+/CD45-/Mac1- (referred to as ScaPCs; [28]), PDGFRα +/CD34+/Sca-1+ (referred to as PDGFR α + cells; [29]) and CD137+ or Tmem26+ [10] have been suggested to have BRITE adipogenic potential. To test our hypothesis, we quantified the expression of these progenitor markers in undifferentiated SVF isolated from iWAT and gWAT of both strains. No statistically significant differences in any of these markers were observed between strains, suggesting that the progenitor abundance between strains is similar (Fig. 2). Between fat depots, however, the iWAT depot has more PDGFRα/Sca-1 positive BRITE progenitors

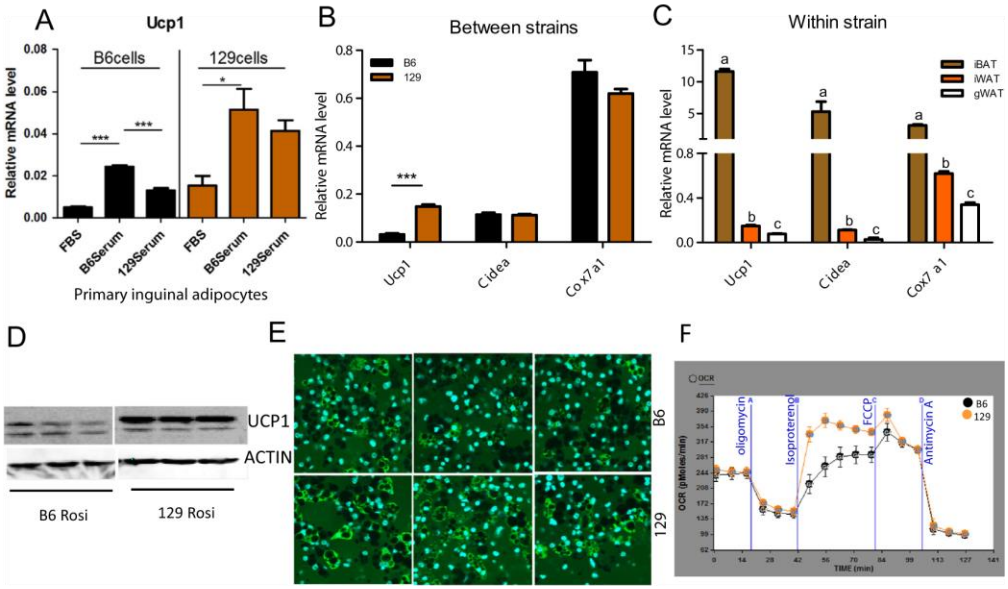


Fig. 1. Strain and fat depots specific differences in browning potential of primary adipocytes. (A) Differential browning capacity of serum collected from B6 and 129 mice. Differentiated primary inguinal adipocytes isolated from B6 and 129 mouse strains were treated with serum collected from either B6 or 129 mice for 24 h. *P < 0.05. ***P < 0.001 (B) Expression levels of Ucp-1, Cidea and Cox7a1 in primary inguinal adipocytes isolated from B6 and 129 mouse strains. ***P< 0.001 (C) Expression levels of Ucp-1, Cidea and Cox7a1 genes in primary adipocytes isolated from interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT) and epididymal white adipose tissue (eWAT) of 129 mouse strain. Different letters above the bars indicate statistically significant differences. (D) Western blot analysis of UCP1 and ACTIN in primary inguinal adipocytes isolated from B6 and 129 mouse strains. (E) Immunocytochemistry of UCP1 in primary cultured inguinal adipocytes isolated from B6 and 129 mouse strains. After 7 days of differentiation, as described in Materials and methods, cells were analyzed by immunofluorescence cell staining using antibody against UCP1 (green). (F) Oxygen consumption rates (OCRs) in fully differentiated B6 and 129 cells (day 7) were assessed using a Seahorse XF-96 extracellular flux analyzer. First, basal respiration is measured, and then oligomycin (oligo) is added, which inhibits adenosine triphosphate (ATP) synthase and lowers oxygen consumption rate (OCR). Ucp1 mediated uncoupling respiration was determined after isoproterenol (0.5 μM) injection. Next, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone [FCCP], an uncoupler that allows assessment of maximal respiratory capacity, is added. Finally, antimycin A is added to block complex 3 of the electron transport chain, leaving only non-mitochondrial OCR to be measured. All data points are the average of 8–10 wells, and error bars are means of standard deviation.

compared to the gWAT, suggesting that the progenitor pool between depots is different (Fig. 2).

3.5. Conditioned media from 129 cells have no browning effect on B6 cells

To further explore the underlying mechanisms, we next considered the possibility that autocrine or paracrine factors from the primary adipocyte during differentiation may account for the strain-specific differences in browning potential, as some secretory factors such as fibroblast growth factor 21 (FGF21) and prostaglandins indeed promote browning in an autocrine/paracrine manner [30,31]. In addition, macrophages in adipose tissues, when alternatively activated (M2), produce catecholamines to enhance and sustain the thermogenic response [32]. To test these possibilities, we first analyzed the gene expression levels of FGF21 and cyclooxygenase 2 (COX2) and the rate-limiting enzyme in prostaglandin synthesis and Arginase 1 (Arg1) as a M2-macrophage marker in primary inguinal adipocytes from both strains. FGF21, COX2 and Arg1 mRNA levels were significantly higher in cells from 129 compared to cells from B6 (Fig. 3A). It is tempting to speculate that such differences may contribute to the differential browning propensity. In this case we would expect that conditioned media from 129 cells should enhance the Ucp1 expression of B6 cells. However, by using conditioned media transfer (Fig. 3B) and transwell indirect co-culture devices (Fig. 3C), we found no evidence for autocrine/paracrine effectors of browning. One explanation could be the lack of direct cell–cell contact and short half-life of paracrine mediators. To address this possibility, we transfected primary B6 cells with an Ucp1 promoter reporter (Ucp1-Luc, B6 background) construct and performed direct co-culture

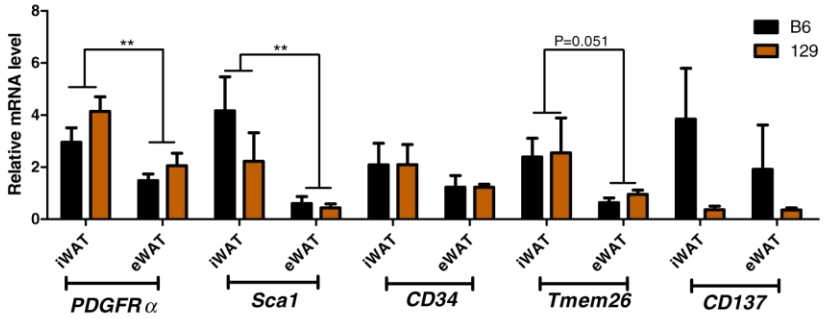


Fig. 2. BRITE progenitor pool differs between fat depots but not between mouse strains. Gene expression analysis of potential BRITE progenitor markers in stromal vascular cells isolated from iWAT and gWAT of B6 and 129 mouse strains after 3–4 days of culture in growth medium (means ± SEM, N = 4). **P < 0.01.

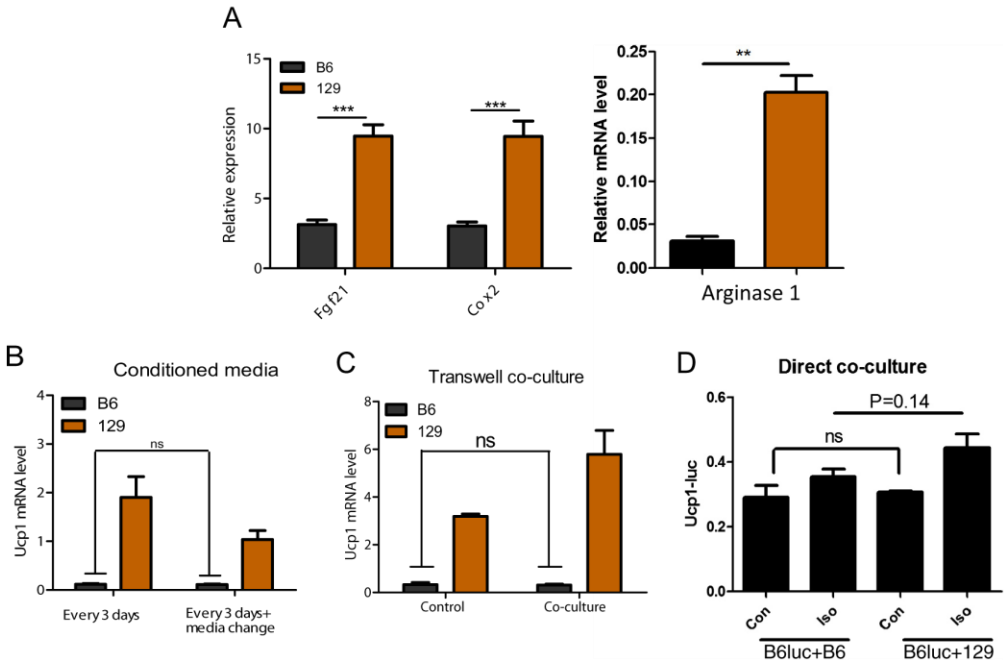


Fig. 3. Strain specific differences in paracrine secreted factors and its contribution to differences in Ucp1 expression of primary adipocytes between strains. (A) mRNA levels of Fgf21, Cox2 and Arg1 in primary inguinal adipocytes from B6 and 129 mouse strains.(B) Conditioned media from 129 cells have no significant effect on the Ucp1 expression of B6 cells. Culture media was changed every 3 days and conditioned media exchange was performed one day before culture media change. Ucp1 expression was determined by qPCR. (C) Indirect-contact co-culture of primary inguinal adipocytes from 129 and B6 mice in transwell plates from seeding to harvesting. Ucp1 expression was determined by qPCR. (D) Direct-contact co-culture of primary inguinal adipocytes from 129 and B6 mice. Ucp1-Luc transfected B6 primary cells ($B6^{luc}$) were directly co-cultured with either untransfected B6 ($B6^{luc}$ + B6) or 129 ($B6^{luc}$ + 129) cells during differentiation. After 4 days of co-culture, Luciferase activity was measured. Values are means ± S.E.M. (N = 3). ns = Not statistically significant. **P < 0.01. ***P < 0.001.

with either primary B6 cells or primary 129 cells. Nevertheless, direct co-culture with 129 cells did not increase the Ucp1 promoter activity in B6 cells (Fig. 3D).

3.6. Strain-specific differences in Ucp1 expression maintain in Ucp1-Luc transfected cells

Since extracellular cues could not explain the cell-autonomous characteristics, we next focused on the cis-regulatory elements of Ucp1 and its trans-acting factors. We transfected differentiated primary adipocytes with an Ucp1 promoter reporter (Ucp1-Luc) construct by electroporation method using Nucleofector II device (Amaxa). Data from luciferase assay revealed that the strain-specific difference in Ucp1 transcriptional activity could be maintained even after transfected with Ucp1 vector of either B6 background (Fig. 4A), or 129 background (Fig. 4B). These results strongly suggest that the strain differences in Ucp1 expression in primary culture are governed by trans-acting factors.

3.7. PRDM16 does not determine the strain differences in Ucp1 expression

The transcriptional mechanisms that ultimately control BRITE cell enrichment are not known, but transcription factors such as PPARγ, PGC-1α, receptor interacting protein 140 (RIP140) and PRDM16 seem to play key roles in regulation [33–35]. We asked whether these transcription factors could be responsible for the strain-specific differences

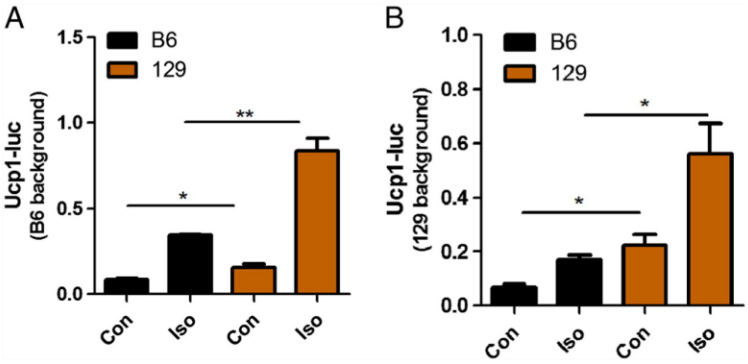


Fig. 4. Strain-specific differences in UCP1 promoter activity are maintained in Ucp1-Luc transfected primary inguinal adipocytes. Cells were transfected with a mouse 3.2-kb Ucp1 promoter luciferase construct (Ucp1-Luc) of either B6 (A) or 129 (B) background and subsequently stimulated (16 h) with isoproterenol (Iso, 1 μM) or PBS (Con). Normalized luciferase activities are shown. *P < 0.05. **P < 0.01.

in Ucp1 expression. We first determined the mRNA levels of PPARγ, Pgc-1α, Prdm16, Rip140 in primary 129 and B6 adipocytes by using qPCR. No differences were found for PPARγ, Pgc-1α and Rip140 mRNA levels between strains (Fig. 5A). Consistent with the mRNA level, we also found no significant differences at PPARγ protein level between stains (Fig. 5B). A striking finding was that although B6 cells had significantly higher Prdm16 mRNA expression (Fig. 5C) this did not result in higher levels of Ucp1 expression. To further test whether the BRITE fate determination role of Prdm16 may operate at other time windows rather than late differentiation phase, we conducted a time course analysis of Prdm16 expression levels covering proliferation, induction and differentiation. At all-time points, we found that Prdm16 levels were higher in B6 cells compared to 129 cells (Fig. 5 D).

4. Discussion

Enrichment of BRITE cells within WAT depots (so called "browning" of white fat) is now regarded as a promising approach to treat obesity and associated metabolic conditions [17]. In this attempt it must be taken into account that the browning propensity of adipose tissues varies substantially among depots and depends on genetic background as reflected by pronounced inter-strain differences in laboratory mice [12,20]. Elucidating the underlying mechanisms will not only help us to understand the development of BRITE cells but also devise routes to enrich these cells in the treatment of obesity. Mechanisms already known to promote the browning of WAT involve systemic effectors such as sympathetic innervation, angiogenesis and hormone levels, local paracrine/autocrine stimuli as well as cellular pathways intrinsic to (pre)adipocytes [18,36]. Beyond the systemic cues we here addressed whether cell autonomous mechanisms contribute to differential browning propensity as reflected in inbred mouse strains and by anatomical sites. Along this line we expanded the stromal vascular cell population isolated from adipose tissues in primary cell culture and induced differentiation to investigate local signaling and cell intrinsic properties influencing BRITE adipogenesis, while excluding systemic cues. In the present study, taking advantage of high variation between B6 and 129 mouse strains, we demonstrate a major contribution of cell intrinsic trans-acting factors in BRITE adipogenesis.

As a hallmark of BRITE adipogenesis, high levels of UCP1 can be induced in distinct WAT depots (such as inguinal and retroperitoneal WAT) of A/J and 129 mice by cold exposure or treatment with β3-adrenergic receptor agonists, whereas B6 mice are much less responsive to these external cues [20]. In principle, this strain difference could be contributed either by differential microenvironments within fat depots or by differential intrinsic properties of (pre)adipocytes. In the former case, the microenvironment could be primed by the sympathetic tone, angiogenesis and blood-borne hormones. Conversely, our analysis revealed that primary cultures from 129 compared to B6 develop a higher abundance of Ucp1-positive BRITE adipocytes associated with an increased capacity of 129 adipocytes for uncoupled respiration. Thus, the strain difference in browning propensity observed in vivo is pheno-copied in vitro in primary cell culture, in the absence of aforementioned systemic cues. Further favoring a role for non-systemic effectors, serum collected from B6 compared to 129 mice unexpectedly has a much higher browning capacity. These two lines of evidence strongly suggest that the strain differences in BRITE adipogenesis, as judged by the number of Ucp1-positive BRITE adipocytes, must largely be determined by cell intrinsic properties, such as progenitor abundance, secretion of paracrine or autocrine factors, cis-regulatory elements and/or trans-acting factors acting directly or indirectly on Ucp1.

Within strain comparisons of WAT depots revealed that primary cultures from iWAT, a depot exhibiting high browning propensity, develop much higher expression levels of BRITE/brown progenitor marker genes compared to eWAT, characterized by low browning propensity. The lower abundance of BRITE precursor cells in eWAT may explain why this depot is resistant to "browning" as suggested previously [10].

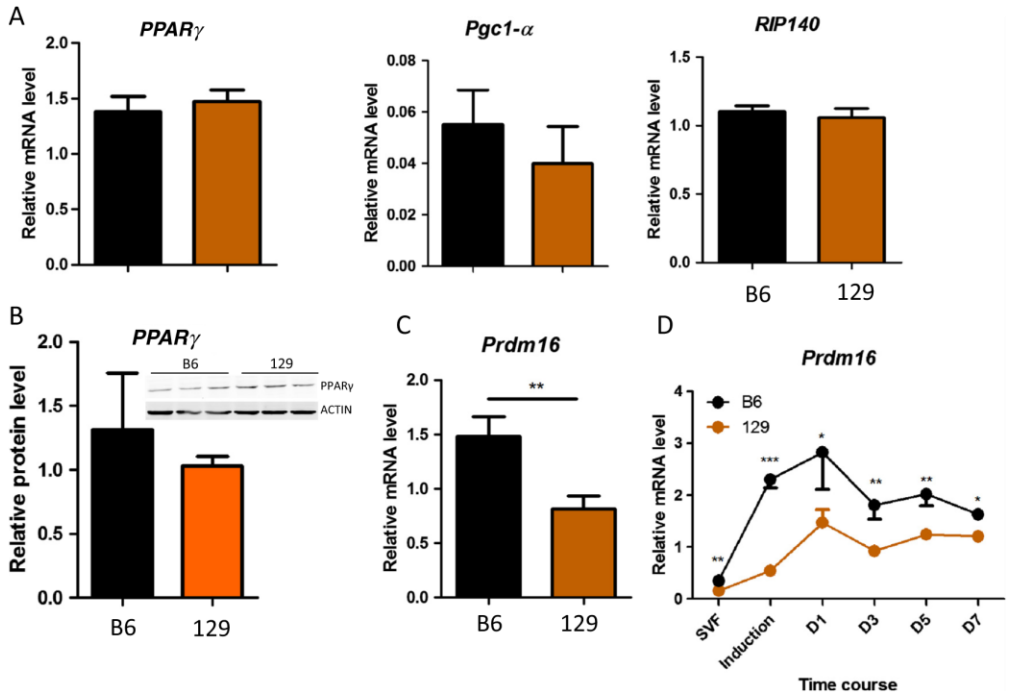


Fig. 5. Gene expression analysis of regulators involved in BRITE adipogenesis. (A) mRNA levels of PPARγ, PGC-1α and RIP140 in primary inguinal adipocytes from B6 and 129 mouse strains. (B) Protein level of PPARγ in primary inguinal adipocytes from B6 and 129 mouse strains. (C) mRNA levels of Prdm16 in primary inguinal adipocytes from B6 and 129 mouse strains. (D) Time course analysis of Prdm16 expression levels covering proliferation (SVF), induction (after 2 days of induction) and differentiation (1 day (D1), 3 days (D3), 5 days (D5) and 7 days (D7) of differentiation) in primary inguinal adipocytes from B6 and 129 mouse strains. Values are means ± S.E.M. (N = 3). *P < 0.05. **P < 0.01. ***P < 0.001.

When stimulated, these precursor cells may have to undergo a proliferation phase before robust browning can take place [10]. This proposition is consistent with in vivo observations that most UCP1 positive cells in eWAT are derived from de novo BRITE adipogenesis, whereas the upregulation of UCP1 expression in iWAT involves either the transdifferentiation of white into BRITE adipocytes [29] or reactivation of camouflaged BRITE adipocytes [37].

However, our strain comparisons within WAT depot revealed no differences in expression levels of BRITE progenitor marker genes, suggesting that the abundance of BRITE progenitors are similar in WAT depots of 129 and B6 mice. Therefore, differential progenitor abundance does not explain the strain difference in BRITE adipogenesis in primary cultures. Although this conclusion may require further validation by fluorescence-activated cell sorting (FACS) analysis, consistent with our results, Schulz and colleagues also found that C57BL/6N and 129-S1 mice had comparable abundance of Sca-1 + cells as revealed by FACS analysis [28]. Alternatively, mouse strains may differ in respect to the propensity of white adipocytes to convert into BRITE adipocytes.

As we found that the strain difference in Ucp1 expression is maintained in primary cultures, we hypothesize that autocrine or paracrine factors derived from adipocytes or M2 macrophages (possible source of catecholamines) may be responsible for the strain differences. It had been demonstrated that adipose-derived FGF21 and COX2-derived prostaglandins such as PGE2 and PGI2 act in an autocrine/paracrine manner to increase expression of Ucp1 and other brown adipocyte marker genes in adipose tissues [30,31]. Macrophages in adipose tissues, when alternatively activated (M2), produce catecholamines to enhance and sustain the thermogenic response [32]. In line with these observations, primary cultures from 129 compared to B6 showed higher expression levels of Fgf21, Cox2 and Arg1 as a M2-macrophage marker. We therefore applied three complementary experimental approaches to test for the bioactivity of secreted factors or direct cell–cell communication in BRITE adipogenesis. Unexpectedly, neither conditioned media and trans-well co-culture, nor direct co-culture of 129 and B6 cells had any significant effect on BRITE adipogenesis. Of note, the direct co-culture model should allow even short-lived autocrine and paracrine mediators, such as prostaglandins, to efficiently exert their biological activities. Based on these observations, the observed difference between strains cannot be explained by autocrine/paracrine influences.

The upstream gene-regulatory regions of the Ucp1 alleles in 129 and B6 mice harbor multiple polymorphisms, SNPs and insertions/deletions. The transcriptional regulation of the Ucp1 gene is controlled through a critical enhancer region, which locates around 2.5 kb upstream of the transcription start site (TSS), contains one peroxisome proliferator-activated receptor (PPAR)-responsive element (PPRE) and two putative cAMP-responsive elements (CREs), with various candidate transcription factors being proposed to regulate this enhancer region, including nuclear receptors, nuclear receptor coactivators and co-repressors [38–43]. In addition, the methylation state of CpG dinucleotides in the Ucp1 enhancer has also been suggested to have functional importance in the regulation of gene expression [44]. We reasoned that these sequence variations may contribute to the strain differences in Ucp1 expression. Nevertheless, our Ucp1-Luc transfection experiments clearly demonstrate that strain-specific differences in Ucp1 gene transcription are independent of the sequence variations between strains. Strain differences in Ucp1 gene expression must therefore be mainly governed by trans-acting factors. The obvious candidates, such as PPARγ, PGC-1α and PRDM16, which are well-known as potent transcriptional regulators of Ucp1, did not reveal strain differences in gene expression coinciding with the differential in Ucp1 expression. However, this does not exclude their roles in the strain-specific regulation of Ucp1 expression, as the recruitment of these transcription factors to UCP-1 promoter may differ between strains.

Taken together, our data demonstrate that the natural variation in BRITE cell abundance that occurs among inbred mouse strains is maintained in vitro cultures and the cell-autonomous differences in BRITE adipogenesis depends on trans-acting factors. In addition, the present study also reveals a unique cell experimental model to identify genes responsible for the BRITE cell recruitment. Given the recent characterization of classical brown as well as BRITE adipocytes in humans, studies on the development and physiological significance of these thermogenic adipocytes in mice have the potential to elucidate physiological and therapeutic implications for humans. From this aspect, the strain comparisons provide a promising system to identify genes critical for the induction of BRITE adipocytes in white fat depots. By taking advantage of the cell intrinsic phenotype documented in our present work, the molecular mechanisms of BRITE adipogenesis may be explored by subjecting primary cell culture models to unbiased “Omics”-based analyses.

Acknowledgments

We are grateful to all the members of Molecular Nutritional Medicine research team for their helpful discussions. We would also like to thank all three anonymous referees for their constructive comments. The present work at the Chair of Molecular Nutritional Medicine was funded by the Else Kröner-Fresenius Foundation and the EU FP7 project DIABAT (HEALTH-F2-2011-278373). YL holds a fellowship from the Deutsche Akademische Austauschdienst (DAAD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.06.003.

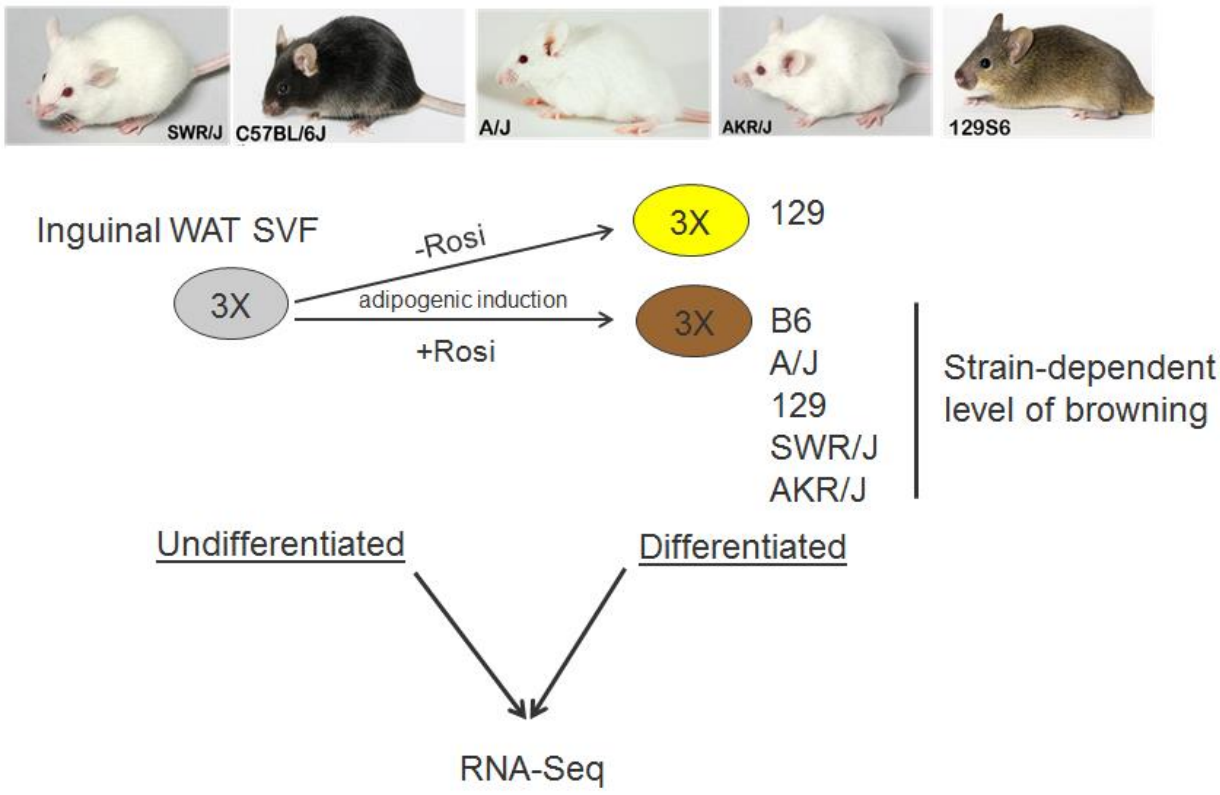
References

[1] M. Klingenspor, S. Herzig, A. Pfeifer, Brown fat develops a brite future, Obes. Facts 5 (2012) 890–896.
[2] C. Guerra, R.A. Koza, H. Yamashita, K. Walsh, L.P. Kozak, Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity, J. Clin. Investig. 102 (1998) 412.
[3] J. Himms-Hagen, A. Melnyk, M.C. Zingaretti, E. Ceresi, G. Barbatelli, S. Cinti, Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes, Am. J. Physiol. Cell Physiol. 279 (2000) C670–C681.
[4] P. Young, J.R.S. Arch, M. Ashwell, Brown adipose tissue in the parametrial fat pad of the mouse, FEBS Lett. 167 (1984) 10–14.
[5] B. Cousin, S. Cinti, M. Morroni, S. Raimbault, D. Ricquier, L. Penicaud, L. Casteilla, Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization, J. Cell Sci. 103 (1992) 931.
[6] B. Xue, J.S. Rim, J.C. Hogan, A.A. Coulter, R.A. Koza, L.P. Kozak, Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat, J. Lipid Res. 48 (2007) 41.
[7] H. Ohno, K. Shinoda, B.M. Spiegelman, S. Kajimura, PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein, Cell Metab. 15 (2012) 395–404.
[8] D. Lasar, A. Julius, T. Fromme, M. Klingenspor, Browning attenuates murine white adipose tissue expansion during postnatal development, Biochim. Biophys. Acta 1831 (2013) 960–968.
[9] N. Petrovic, T.B. Walden, I.G. Shabalina, J.A. Timmons, B. Cannon, J. Nedergaard, Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes, J. Biol. Chem. 285 (2010) 7153–7164.
[10] J. Wu, P. Bostrom, L.M. Sparks, L. Ye, J.H. Choi, A.H. Giang, M. Khandekar, K.A. Virtanen, P. Nuutila, G. Schaart, K. Huang, H. Tu, W.D. van Marken Lichtenbelt, J. Hoeks, S. Enerback, P. Schrauwen, B.M. Spiegelman, Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human, Cell 150 (2012) 366–376.
[11] P. Seale, B. Bjork, W. Yang, S. Kajimura, S. Chin, S. Kuang, A. Scime, S. Devarakonda, H. M. Conroe, H. Erdjument-Bromage, P. Tempst, M.A. Rudnicki, D.R. Beier, B.M. Spiegelman, PRDM16 controls a brown fat/skeletal muscle switch, Nature 454 (2008) 961–967.
[12] K. Almind, M. Manieri, W.I. Sivitz, S. Cinti, C.R. Kahn, Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 2366–2371.
[13] M.E. Lidell, M.J. Betz, O.D. Leinhard, M. Heglind, L. Elander, M. Slawik, T. Mussack, D. Nilsson, T. Romu, P. Nuutila, K.A. Virtanen, F. Beuschlein, A. Persson, M. Borga, S. Enerback, Evidence for two types of brown adipose tissue in humans, Nat. Med. 19 (2013) 631–634.
[14] N.Z. Jespersen, T.J. Larsen, L. Peijs, S. Daugaard, P. Homoe, A. Loft, J. de Jong, N. Mathur, B. Cannon, J. Nedergaard, B.K. Pedersen, K. Moller, C. Scheele, A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans, Cell Metab. 17 (2013) 798–805.

[15] A.M. Cypess, A.P. White, C. Vernochet, T.J. Schulz, R. Xue, C.A. Sass, T.L. Huang, C. Roberts-Toler, L.S. Weiner, C. Sze, A.T. Chacko, L.N. Deschamps, L.M. Herder, N. Truchan, A.L. Glasgow, A.R. Holman, A. Gavrila, P.O. Hasselgren, M.A. Mori, M. Molla, Y.H. Tseng, Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat, Nat. Med. 19 (2013) 635–639.
[16] L.Z. Sharp, K. Shinoda, H. Ohno, D.W. Scheel, E. Tomoda, L. Ruiz, H. Hu, L. Wang, Z. Pavlova, V. Gilsanz, Human BAT possesses molecular signatures that resemble beige/brite cells, PLoS ONE 7 (2012) e49452.
[17] Y.H. Tseng, A.M. Cypess, C.R. Kahn, Cellular bioenergetics as a target for obesity therapy, Nat. Rev. Drug Discov. 9 (2010) 465–482.
[18] M. Harms, P. Seale, Brown and beige fat: development, function and therapeutic potential, Nat. Med. 19 (2013) 1252–1263.
[19] A. Bartelt, J. Heeren, Adipose tissue browning and metabolic health, Nat. Rev. Endocrinol. 10 (2014) 24–36.
[20] L.P. Kozak, R.A. Koza, The genetics of brown adipose tissue, Prog. Mol. Biol. Transl. Sci. 94 (2010) 75–123.
[21] L.P. Kozak, R.A. Koza, R. Anunciado-Koza, Brown fat thermogenesis and body weight regulation in mice: relevance to humans, Int. J. Obes. 34 (2010) S23–S27.
[22] S. Collins, K.W. Daniel, A.E. Petro, R.S. Surwit, Strain-specific response to β 3-adrenergic receptor agonist treatment of diet-induced obesity in mice 1, Endocrinology 138 (1997) 405–413.
[23] A. Vitali, I. Murano, M.C. Zingaretti, A. Frontini, D. Ricquier, S. Cinti, The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes, J. Lipid Res. 53 (2012) 619–629.
[24] C. Vernochet, S.B. Peres, K.E. Davis, M.E. McDonald, L. Qiang, H. Wang, P.E. Scherer, S. R. Farmer, C/EBPalpha and the corepressors CtBP1 and CtBP2 regulate repression of select visceral white adipose genes during induction of the brown phenotype in white adipocytes by peroxisome proliferator-activated receptor gamma agonists, Mol. Cell. Biol. 29 (2009) 4714–4728.
[25] P.A. Dutchak, T. Katafuchi, A.L. Bookout, J.H. Choi, R.T. Yu, D.J. Mangelsdorf, S.A. Kliewer, Fibroblast growth factor-21 regulates PPARgamma activity and the antidiabetic actions of thiazolidinediones, Cell 148 (2012) 556–567.
[26] L. Qiang, L. Wang, N. Kon, W. Zhao, S. Lee, Y. Zhang, M. Rosenbaum, Y. Zhao, W. Gu, S. R. Farmer, D. Accili, Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma, Cell 150 (2012) 620–632.
[27] P. Seale, H.M. Conroe, J. Estall, S. Kajimura, A. Frontini, J. Ishibashi, P. Cohen, S. Cinti, B.M. Spiegelman, Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice, J. Clin. Invest. 121 (2011) 96–105.
[28] T.J. Schulz, T.L. Huang, T.T. Tran, H. Zhang, K.L. Townsend, J.L. Shadrach, M. Cerletti, L. E. McDougall, N. Giorgadze, T. Tchkonia, D. Schrier, D. Falb, J.L. Kirkland, A.J. Wagers, Y.H. Tseng, Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 143–148.
[29] Y.H. Lee, A.P. Petkova, E.P. Mottillo, J.G. Granneman, In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding, Cell Metab. 15 (2012) 480–491.
[30] F.M. Fisher, S. Kleiner, N. Douris, E.C. Fox, R.J. Mepani, F. Verdeguer, J. Wu, A. Kharitonenkov, J.S. Flier, E. Maratos-Flier, B.M. Spiegelman, FGF21 regulates PGC-1alpha and browning of white adipose tissues in adaptive thermogenesis, Genes Dev. 26 (2012) 271–281.
[31] A. Vegiopoulos, K. Muller-Decker, D. Strzoda, I. Schmitt, E. Chichelnitskiy, A. Ostertag, M. Berriel Diaz, J. Rozman, M. Hrabe de Angelis, R.M. Nusing, C.W. Meyer, W. Wahli, M. Klingenspor, S. Herzig, Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes, Science 328 (2010) 1158–1161.
[32] K.D. Nguyen, Y. Qiu, X. Cui, Y.P. Goh, J. Mwangi, T. David, L. Mukundan, F. Brombacher, R.M. Locksley, A. Chawla, Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis, Nature 480 (2011) 104–108.
[33] P. Seale, Transcriptional control of brown adipocyte development and thermogenesis, Int. J. Obes. 34 (2010) S17–S22.
[34] S. Kajimura, P. Seale, B.M. Spiegelman, Transcriptional control of brown fat development, Cell Metab. 11 (2010) 257–262.
[35] P. Seale, S. Kajimura, B.M. Spiegelman, Transcriptional control of brown adipocyte development and physiological function -of mice and men, Genes Dev. 23 (2009) 788.
[36] Y. Cao, Angiogenesis and vascular functions in modulation of obesity, adipose metabolism, and insulin sensitivity, Cell Metab. 18 (2013) 478–489.
[37] Y. Li, D. Lasar, T. Fromme, M. Klingenspor, White, brite and brown adipocytes: the evolution and function of a heater organ in mammals, Can. J. Zool. 92 (2014) 615–626.
[38] B. Cannon, J. Nedergaard, Brown adipose tissue: function and physiological significance, Physiol. Rev. 84 (2004) 277–359.
[39] S. Collins, E. Yehuda-Shnaidman, H. Wang, Positive and negative control of Ucp1 gene transcription and the role of [beta]-adrenergic signaling networks, Int. J. Obes. 34 (2010) S28–S33.
[40] U.C. Kozak, J. Kopecky, J. Teisinger, S. Enerback, B. Boyer, L.P. Kozak, An upstream enhancer regulating brown-fat-specific expression of the mitochondrial uncoupling protein gene, Mol. Cell. Biol. 14 (1994) 59–67.
[41] A.M. Cassard-Doulcier, C. Gelly, N. Fox, J. Schrementi, S. Raimbault, S. Klaus, C. Forest, F. Bouillaud, D. Ricquier, Tissue-specific and beta-adrenergic regulation of the mitochondrial uncoupling protein gene: control by cis-acting elements in the 5′-flanking region, Mol. Endocrinol. 7 (1993) 497–506.
[42] B.B. Boyer, L.P. Kozak, The mitochondrial uncoupling protein gene in brown fat: correlation between DNase I hypersensitivity and expression in transgenic mice, Mol. Cell. Biol. 11 (1991) 4147–4156.
[43] T. Kanzleiter, T. Schneider, I. Walter, F. Bolze, C. Eickhorst, G. Heldmaier, S. Klaus, M. Klingenspor, Evidence for Nr4a1 as a cold-induced effector of brown fat thermogenesis, Physiol. Genomics 24 (2006) 37–44.
[44] A. Shore, A. Karamitri, P. Kemp, J.R. Speakman, M.A. Lomax, Role of Ucp1 enhancer methylation and chromatin remodelling in the control of Ucp1 expression in murine adipose tissue, Diabetologia 53 (2010) 1164–1173.

CHAPTER 6

Transcriptome analysis (RNA-Seq) of primary cultured adipocytes from five inbred mouse strains



6.1 Introduction

In chapter 5, by taking advantage of the comparison between two inbred strains (129S6sv/ev vs C57BL/6J), we reported that the strain- and depot-specific differences in brite adipogenesis observed *in vivo* were pheno-copied in primary cultures *in vitro*. It was further concluded that, beyond systemic cues such as innervation and hormone levels, cues intrinsic to the progenitor cells contribute to differential brite adipogenesis. Detailed analyses demonstrated that these cues are independent of autocrine/paracrine mechanisms and genetic variation in the *Ucp1* gene but rather depend on trans-acting factors. Transcript levels of obvious candidates, such as PPARγ, PGC-1α and PRDM16, which are well-known transcriptional regulators of *Ucp1*, did not reveal strain differences in gene expression coinciding with the differential *Ucp1* expression, indicating other undefined determinants must play critical roles in governing the potential of brite adipogenesis (Li et al., 2014c). Therefore, the intrinsic strain-specific difference in brite adipogenesis provides an excellent starting point to identify corresponding variations at the transcriptome level.

To achieve this goal, the transcriptomes of undifferentiated precursor cells cultured from the stromal-vascular fraction of murine inguinal WAT and of fully differentiated primary adipocytes (treated with rosiglitazone during differentiation to promote brite adipogenesis) were probed in five inbred mouse strains (C57BL/6J, 129S6sv/ev, A/J, AKR/J, and SWR/J) (Table 1).

Table 1: List of 33 biological samples subjected to RNA Sequencing (RNA-Seq) analysis. RNA was extracted from primary preadipocytes (preconfluent and undifferentiated) and adipocytes (fully differentiated) grown from the stromal vascular fraction of murine WAT. From each condition three RNA samples were subjected to RNA-seq.

Sample No.	Inbred strain	RNA Source	Experimental condition	Treatment
1-3	C57BL/6J	primary cells	undifferentiated (B6-undiff-R1-3)	none
4-6	C57BL/6J	primary cells	fully differentiated (B6-diff-R1-3)	rosiglitazone
7-9	A/J	primary cells	undifferentiated (AJ-undiff-R1-3)	none
10-12	A/J	primary cells	fully differentiated (AJ-diff-R1-3)	rosiglitazone
19-21	SWR/J	primary cells	undifferentiated (SWRJ-undiff-R1-3)	none

22-24	SWR/J	primary cells	fully differentiated (SWRJ-diff-R1-3)	rosiglitazone
25-27	AKR/J	primary cells	undifferentiated (AKRJ-undiff-R1-3)	none
28-30	AKR/J	primary cells	fully differentiated (AKTJ-diff-R1-3)	rosiglitazone
13-15	129S6	primary cells	undifferentiated (129SV-undiff-R1-3)	none
31-33	129S6	primary cells	fully differentiated (129SV-diff-R1-3)	none
16-18	129S6	primary cells	fully differentiated (129SV-diffnorosi-R1-3)	rosiglitazone

6.2 Quality analysis of RNA-Seq data

After sequencing quality control of the sequence reads was performed. All our samples delivered more than 20 million raw reads, even after filtering based on quality and adaptor contamination (Figure 1A and B). Among all the reads of each sample, more than 80% were aligned to the reference transcriptome (Figure 2A and B). Non-specific matches (reads mapped more than 100 loci) were less than 0.021% (Figure 2A and B). The correlation coefficients (Spearman's rho) of gene expression between biological replicates were above 0.90, indicating that the RNA-Seq data are highly reproducible (Figure 3). This was further confirmed by the similarity of the overall distributions of gene expression values (measured as log Fragments Per Kilobase of transcript per million fragments mapped (FPKM)) (Figure 4A) and the number of expressed genes (~ 13,000 at a >= 1 FPKM cutoff) per sample (Figure 4B). Together, these results clearly show that the obtained RNA-Seq data are of high quality and form a firm basis for further analysis.

6.3 Transcriptome analysis (RNA-Seq) reveals pronounced differences in browning propensities across cultures from the five inbred mouse strains

The RNA-Seq data revealed pronounced differences in browning propensities across cultures from the five inbred mouse strains, as judged by normalized transcript abundance of *Ucp1* and validated by qPCR analysis (Figure 5). Surprisingly, we found that primary inguinal adipocyte cultures from the obesity-resistant SWR/J mouse strain had the lowest *Ucp1* expression, while primary cultures from the obesity-prone AKR/J

mouse strain have an expression level comparable to cultures from other obesity-resistant mouse strains such as A/J and 129. Since the browning phenomenon in SWR/J and AKR/J mice *in vivo* are less studied, it is currently not clear whether the *in vitro* data reflect the *in vivo* situation. Regardless, the phenotypic variation among mouse strains provides a genetic system to identify corresponding variations on the transcriptome level. In this transcriptome data set we started to seek for gene transcripts associated with browning across five different genomic backgrounds.

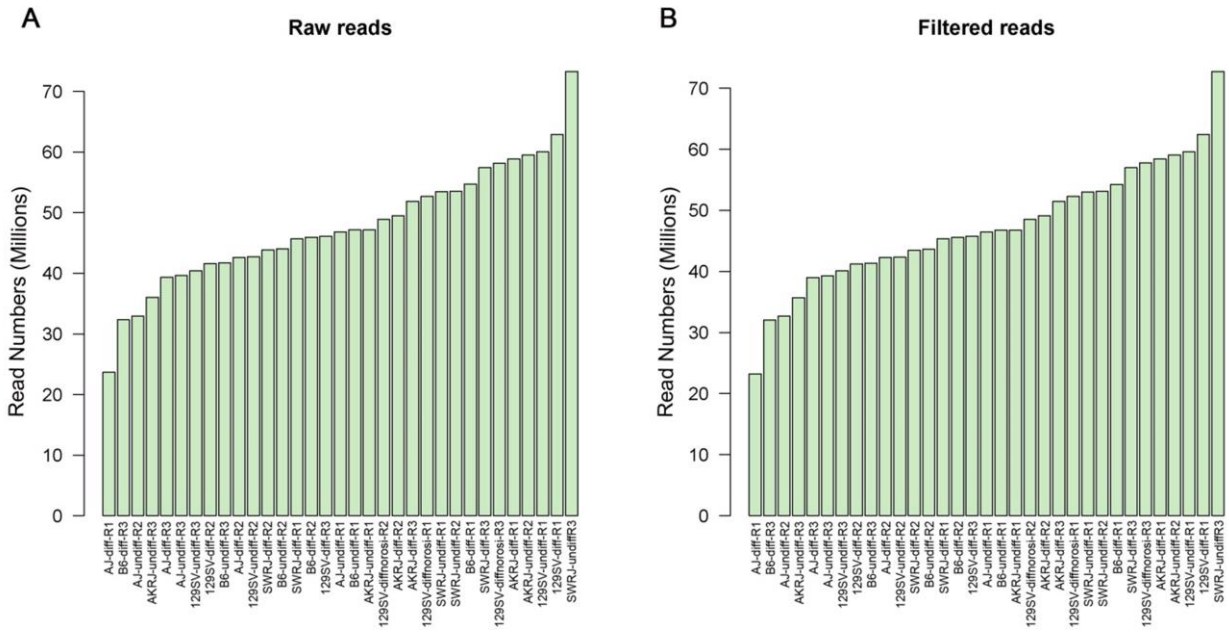


Figure 1. Total number of reads obtained from the sequencer in all samples before (A) or after filtering (B). All samples have total raw reads number above 20 million, even after filtering.

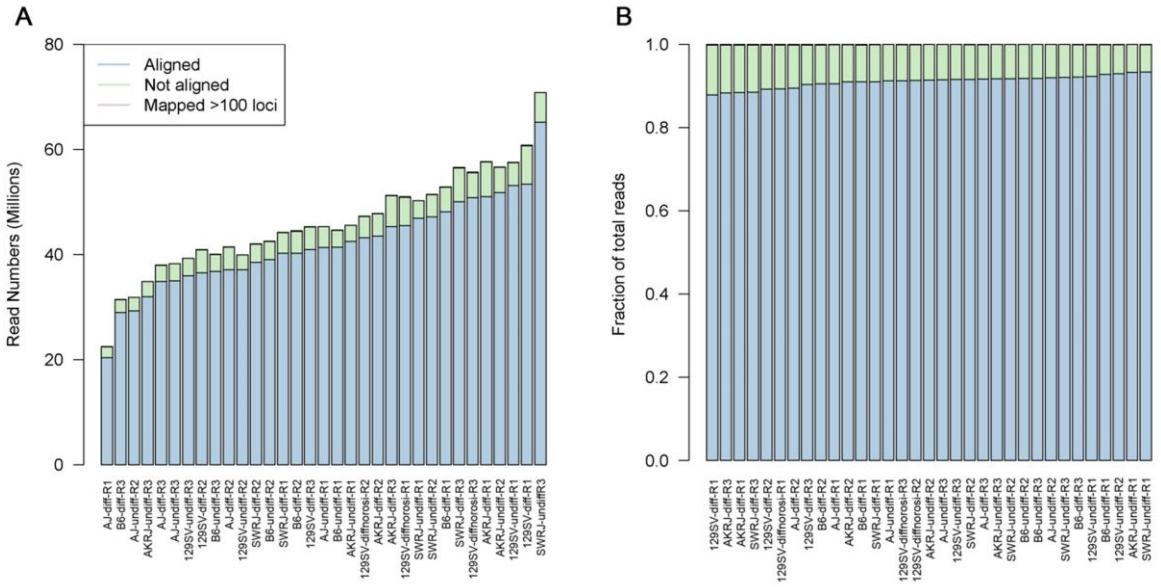


Figure 2. Total number of reads mapped to the reference transcriptome (A) and their relative fraction compared to total reads (B).

6.4 Identification of genes associated with browning

We have used two basic strategies for the identification of genes that may play a role in the induction of brite adipogenesis. The first strategy is based on our observation that primary cultures derived from A/J, AKR/J and 129Sv mice have high browning propensities, thus genes significantly up or down regulated upon differentiation across all three mouse strains should be relevant to browning. To probe these genes, we first screened all the differentially expressed genes upon adipogenesis using a false discovery rate (FDR, Benjamini-Hochberg adjusted P value) =<0.01 and a fold change (FC) >=2 across three mouse strains (A/J, AKR/J and 129Sv) (Figure 6). As a result, we found that ~1,680 genes were up regulated, while ~1,223 genes were down regulated. Notably, in differentiated samples, 129 cells without rosiglitazone treatment during differentiation formed a cluster with SWR/J cells corresponding to their low level of browning (Figure 6).

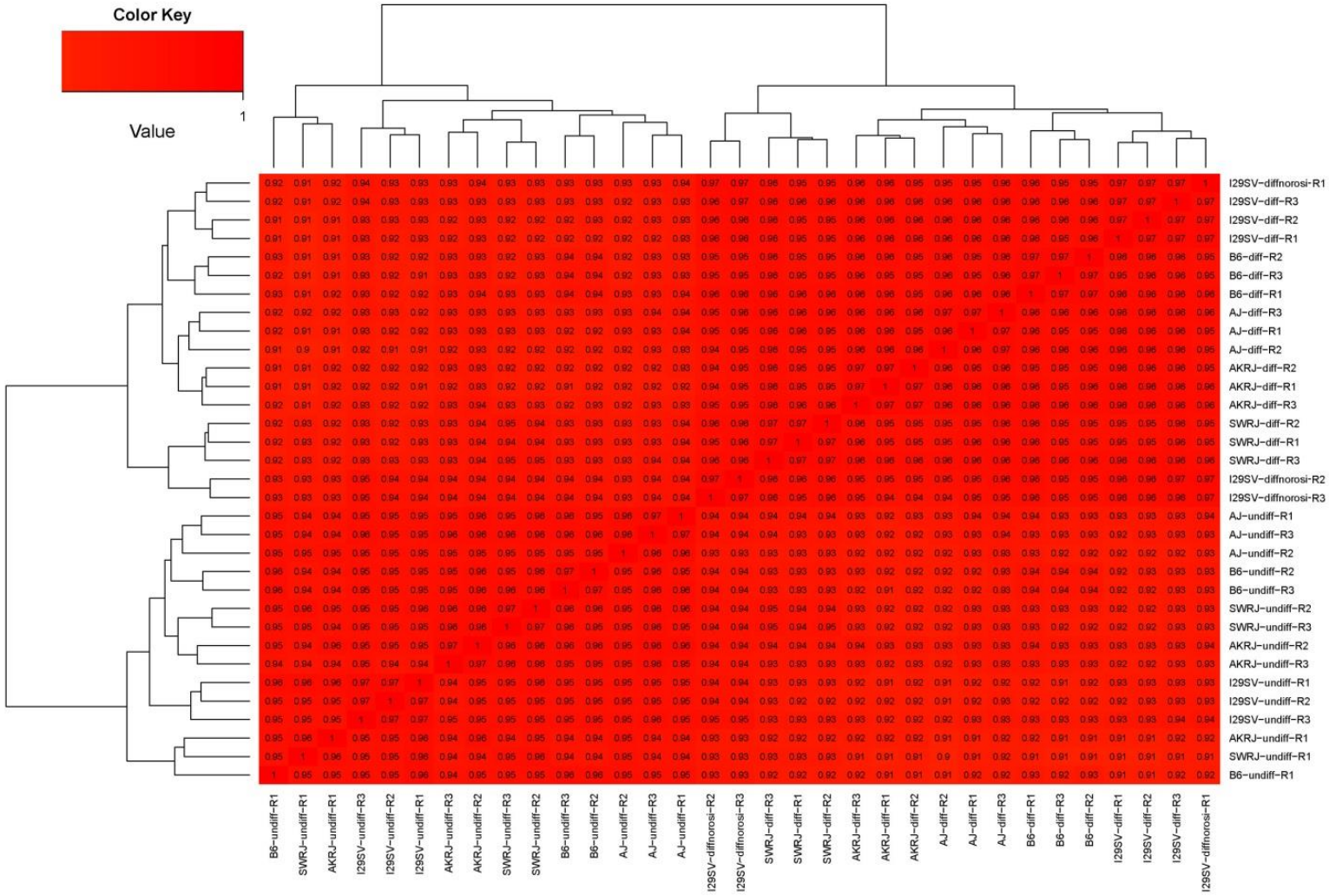


Figure 3. Hierarchically clustered heatmap based gene expression similarities of all analyzed samples (Spearman's rho of log FPKM values). Biological replicates cluster together and show particularly high correlation (>=0.94) in the differentiated states. Differentiated and undifferentiated samples form separate clusters, irrespective of the strains, suggesting a strong effect of the differentiation on the transcriptome.

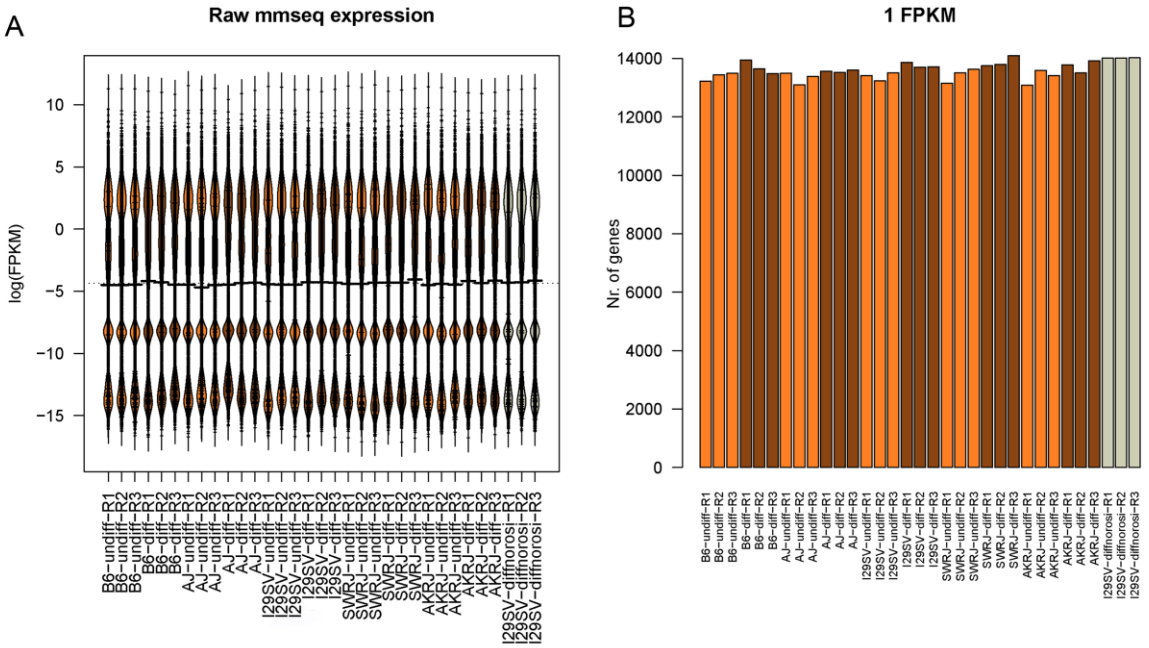


Figure 4. Boxplot distribution of the logFPKM expression values (A) and numbers (Nr.) of genes with FPKM value above 1.0 (B) in all 33 samples.

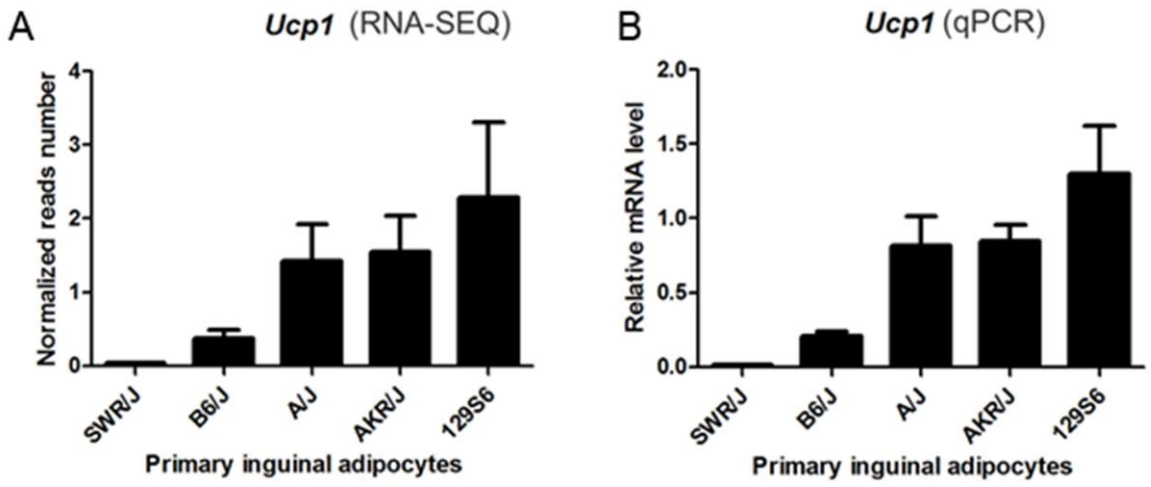


Figure 5. Transcriptome analysis (RNA-Seq) of primary cultured adipocytes from five inbred mouse strains revealed differential browning propensity as judged by *Ucp1* expression (N=3). (A) pronounced differences in browning propensities across cultures from the five inbred mouse strains as judged from normalized transcript abundance of *Ucp1*, which was further verified by qPCR analysis (B). The transcriptome profiles provide an excellent resource to identify transcripts associated with the inter-strain variation of *Ucp1* expression.

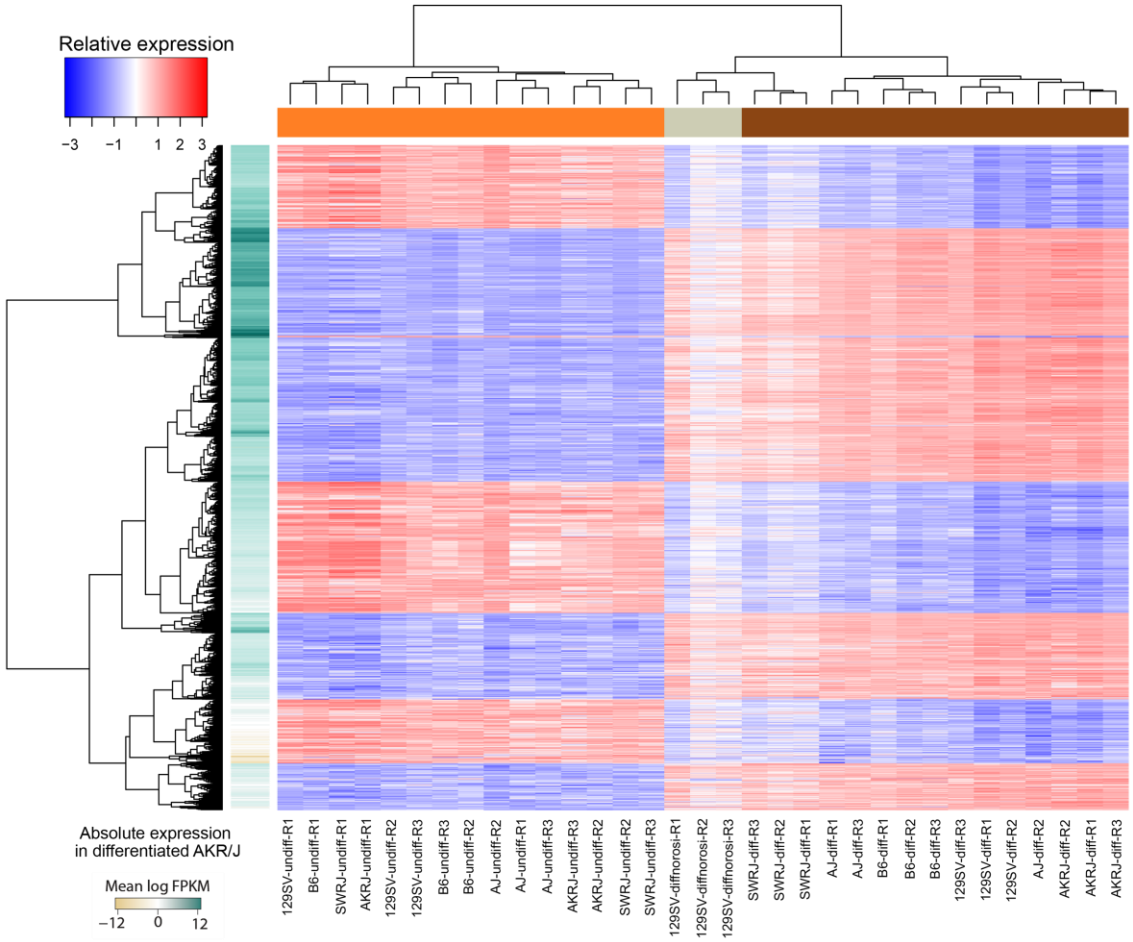


Figure 6. Heatmap showing expression levels of genes significantly regulated upon differentiation across three mouse strains with high browning propensities (A/J, AKR/J and 129Sv). In total, ~1,680 genes were significantly up regulated, while ~1,223 genes were down regulated. The top panel shows a hierarchical clustering dendrogram of samples. For clusters (top), orange branches represent undiffentiated cells (nondiff), grey branches represent differentiated cells without rosiglitazone treatment (diffnorosi) and brown branches represent differentiated cells treated with rosiglitazone (diffrosi).

Our second strategy focused on gene transcripts for which differences in transcript levels across strains correlated with the respective difference in *Ucp1* expression. In total, we found ~1,977 genes that showed a significant positive and ~2,200 genes a significant negative correlation with *Ucp1*, at Spearman's rank correlation coefficients (rho) >=0.8 and p=<0.01 (Figure 7).

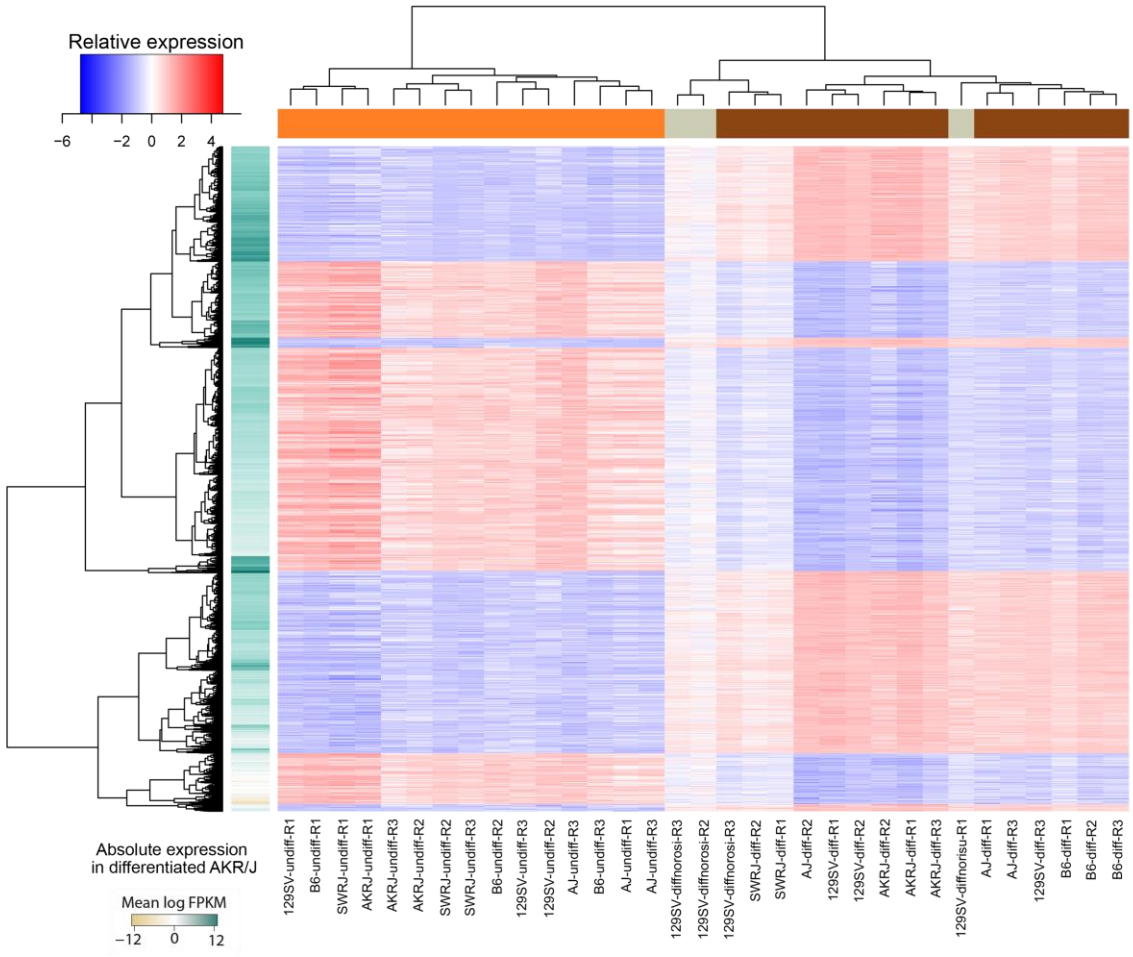


Figure 7. Heatmap showing expression levels of genes highly correlated with *Ucp1* across all datasets. In total, ~1,977 genes were positively and ~2,200 genes were negatively correlated with *Ucp1* expression (Spearman's rank correlation coefficient rho >=0.8, p=<0.01). The top panel shows a hierarchical clustering dendrogram of samples. For clusters (top), orange branches represent undiffentiated cells (nondiff), grey branches represent differentiated cells without rosiglitazone treatment (diffnorosi) and brown branches represent differentiated cells treated with rosiglitazone (diffrosi).

Of note, considerable overlap existed between genes of interest identified by our two strategies (Figure 8), indicating that both strategies are promising. Among the overlapped candidate genes there are well established brown specific marker genes such as *Cidea, Cox7a1, Ppargc1a, Cox8b* and *Elovl3*, the recently identified brown specific surface maker *Slc36a2* (Ussar et al., 2014), the brown fat–enriched secreted factor *Nrg4* (Wang et al., 2014), genes important for brown adipocytes function such as *Cd36* (Anderson et al., 2015), as well as established transcriptional factors such as Zinc

finger and BTB domain containing 16 (*Zbtb16*) (Plaisier et al., 2012) and Myocardin-related transcription factor A (*Mkl1*) (McDonald et al., 2015). A summary of genes that have already been demonstrated to be of functional relevance is presented in Table 2. The identified candidate transcripts could either encode for a) transcriptional regulators of *Ucp1*, b) essential functional components of brite adipocytes, or c) specific markers of brite adipocytes. In total, we have identified ~46 transcription factors and co-regulators, ~320 mitochondrial proteins, ~49 surface markers and ~80 lncRNAs which were positively associated brite adipogenesis.

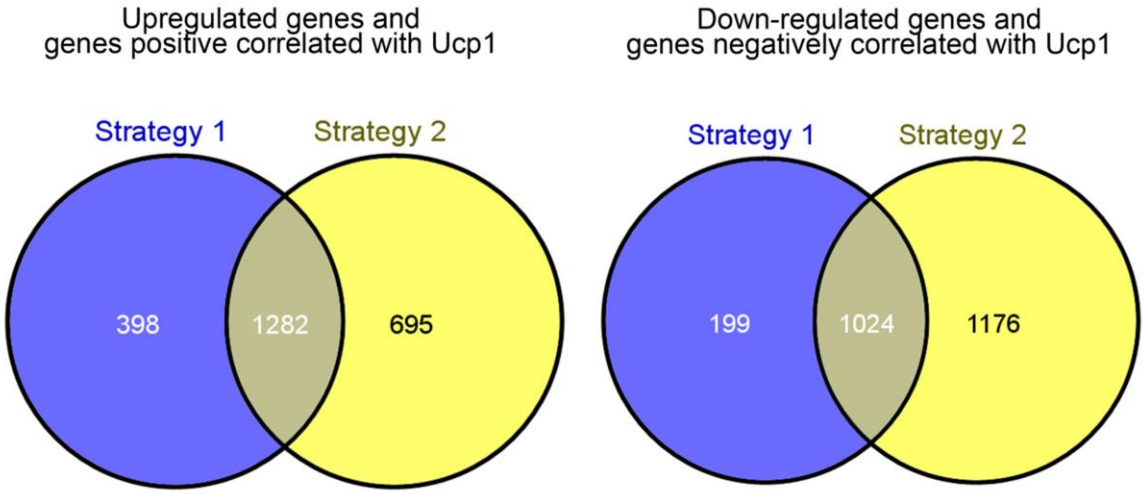


Figure 8. Venn diagram of genes identified by strategy 1 compared with genes regulated identified by strategy 2. Considerable overlap existed between genes of interest identified by two strategies.

Table 2: Examples of genes identified in our present RNA-Seq data set based on our two strategies as described. The Spearman correlation coefficient in our database and related references are provided.

Gene symbol	Gene name	Spearman correlation coeff.	References
Transcriptional regulators of Ucp1			
Zbtb16	Zinc finger and BTB domain containing 16	0.91	Plaisier et al., 2012
Mkl1	Myocardin-related transcription factor A, also known as MRTF-A	-0.91	McDonald et al., 2015
Essential functional components of brite adipocytes			
Agpat2	1-acylglycerol-3-phosphate-O-acyltransferase 2	0.92	Cortes et al., 2009
CD36	Scavenger receptor CD36	0.88	Anderson et al., 2015

Kcnk3	Potassium channel K3	0.91	Shinoda et al., 2015
Nrg4	Neuregulin 4	0.88	Wang et al., 2014
Mtus1	Mitochondrial tumor suppressor 1	0.87	Shinoda et al., 2015
Specific markers of brite adipocytes			
Slc36a2	Solute carrier family 36, member 2, also known as PAT2	0.92	Ussar et al., 2014
long non-coding RNAs			
Blnc1	Brown fat long non-coding RNA1	0.72	Zhao et al., 2014
3930402G23Rik	RIKEN cDNA 3930402G23 gene	0.86	Zhao et al., 2014

A number of marker genes selectively expressed in white, brite and brown adipose tissue and adipocytes have been reported (Sharp et al., 2012; Walden et al., 2012; Wu et al., 2012). These marker genes are not identified consistently between studies. Moreover, some markers have been identified from gene expression profiling of different fat depots, whereby depot identity but not cell specificity might be a significant contribution. Thus, our model using primary cultures of the same fat depot from different mouse strains with distinct browning capacities is more informative regarding the validation of the proposed markers and identification of novel markers. An ideal marker would correlate positively with *Ucp1* expression levels. Nevertheless, we found most of the suggested markers to be non-informative. For example, Hoxc9, Hoxc8, Tbx1, Tmem26 and CD137, were not regulated in a brite specific manner in our primary adipocyte cultures. Instead, we have identified ~49 putative surface markers for brite adipocytes (data not shown).

Of note, our RNA-Seq database can also be used to identify polyadenylated long non-coding RNAs (lncRNAs) that potentially regulate brite adipogenesis. lncRNAs are a unique class of transcripts that share similarities with mRNA with regard to their transcriptional regulation and biogenesis but lack functional open reading frames and thus are not predicted to encode proteins (Geisler and Coller, 2013). lncRNAs have emerged as a novel class of functional RNAs that impinge on gene regulation by a broad spectrum of mechanisms such as the recruitment of epigenetic modifier proteins, control of mRNA decay and DNA sequestration of transcription factors (Hu et al., 2012). Recent studies demonstrated that lncRNAs play a role in white, brown and brite

adipocyte differentiation (Sun et al., 2013; Zhao et al., 2014). Of note, brown fat lncRNA 1 (*Blnc1*), the first and only reported lncRNA which has been shown to drive brown and brite differentiation, is also a potential candidate in our database (Table 2). Based on this report, it is thus of pertinent interest to conduct functional analyses of other top candidate lncRNAs identified in our database, such as lncRNA 3930402G23Rik (Table 2), which was also identified by Zhao et al., but has not yet been validated.

6.6 Identified candidate genes underpin the browning effects of rosiglitazone

Our transcriptome data sets could also be used to identify potential mechanisms responsible for the browning effects of rosiglitazone by focusing the transcriptome comparison of 129 cells in undifferentiated and differentiated state (Figure 9). On a global level, we found that 1) a large amount of transcriptional changes (~2,500 genes) (FDR=<0.01 and FC>=2) were shared between the rosiglitazone and no rosiglitazone differentiation protocols; 2) more transcriptional changes (~2,000 genes) (FDR<0.01 and FC>2) were induced when rosiglitazone was added compared to the absence of rosiglitazone; 3) the transcriptional differences between 129SV_diffrosi and 129SV_diffnorosi were minor, only ~500 genes show significant differences between them (FDR<0.01 and FC>2) (Figure 9). In particular, we observed a cluster of genes responding to differentiation in both 129SV_diffrosi and 129SV_diffnorosi samples but in different extent, associated with brown fat cell differentiation including *Ucp1, Cidea, Cox7a1, Elovl3* and *Otop1* (Figure 10).

Interestingly, gene ontology (GO) analysis of genes significantly down regulated by rosiglitazone treatment during differentiation revealed that genes associated with innate immunity system and extracellular matrix remodeling as top categories, indicating down-regulation of genes associated with inflammation and remodeling of extracellular matrix were associated with the brite adipogenesis driven rosiglitazone (Table 3). Importantly, we found that most of these genes were also high expressed in browning-resistant cells of SWR/J background (data not shown). We also observed that Toll-like receptor (TLR) cascades as top category (Table 3), it is thus likely TLR4-mediated innate immunity activation can inhibit browning.

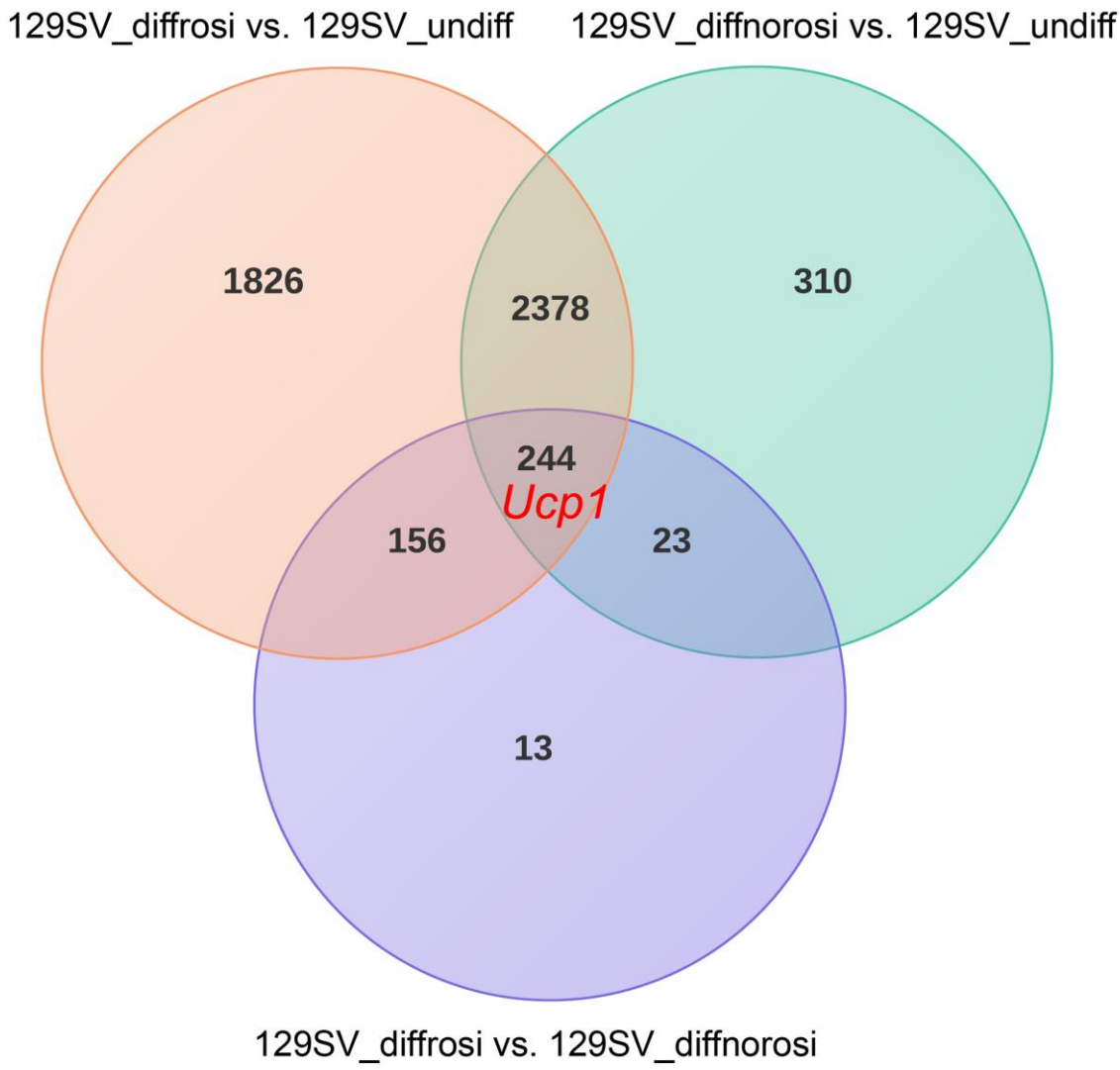


Figure 9. Venn diagram of genes significantly regulated upon differentiation with rosiglitazone treatment (light orange), without rosiglitazone treatment (light green) and enriched in rosiglitazone treatment versus non-rosiglitazone treatment (light blue). A large amount of transcriptional changes (~2,500 genes) were shared between the rosiglitazone and no rosiglitazone differentiation protocols, only ~500 genes showed significant differences between them.

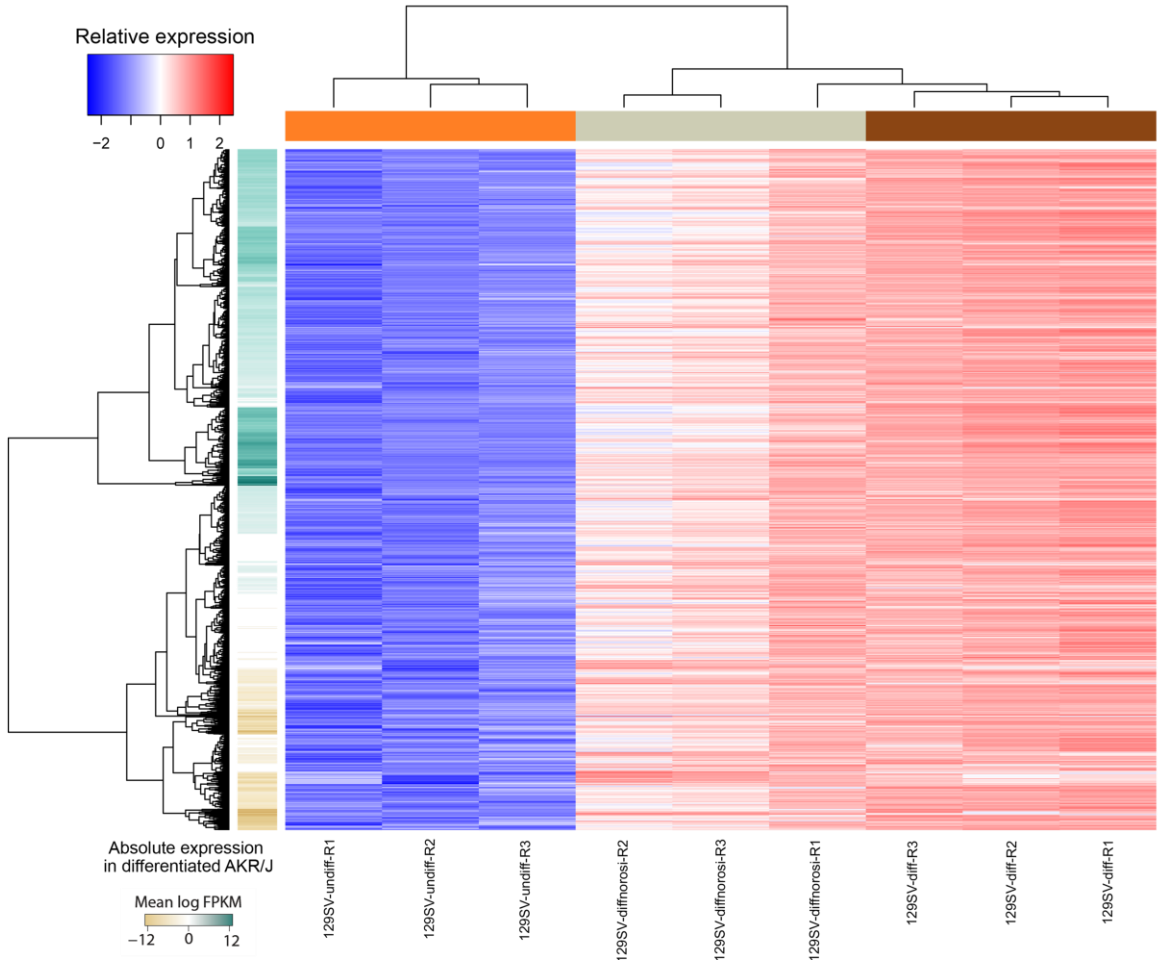


Figure 10. Heatmap showing expression levels of genes up-regulated upon differentiation in both rosiglitazone and non-rosiglitazone treated conditions but in different extent. The top panel shows a hierarchical clustering dendrogram of samples. For clusters (top), orange branches represent undiffentiated cells (nondiff), grey branches represent differentiated cells without rosiglitazone treatment (diffnorosi) and brown branches represent differentiated cells treated with rosiglitazone (diffrosi).

Table 3. Summary of gene ontology (GO) categories of genes significantly down regulated by rosiglitazone treatment during differentiation.

ID	Description	pvalue
4810015	Innate Immune System	3.560789e−11
4810378	Complement cascade	4.935258e−09
4810016	Immune System	3.175320e−08
4810377	Initial triggering of complement	4.592627e−08
4810248	Cell surface interactions at the vascular wall	4.923527e−07
4810246	Extracellular matrix organization	9.529447e−05
4810154	Toll−Like Receptors Cascades	1.585563e−03
4810243	Hemostasis	4.587242e−03

To conclude, we have presented a detailed global transcriptome analysis of brite adipogenesis by using primary inguinal fat cultures from five inbred mouse strains. Our results showed that there were pronounced differences in browning propensities across cultures with different genomic backgrounds. We were able to identify several transcriptional regulators that are likely to drive these variations. In addition, we have revealed novel functional components and new markers of brite adipocytes. Clearly, validation of their relevance in brite adipogenesis will require dedicated functional studies.

CHAPTER 7

Discussion and outlook

Obesity is the result of energy imbalance (energy intake exceeds energy expenditure) and has been considered as a global pandemic. It poses a tremendous threat to human health (Ng et al., 2014). Current therapeutic strategies for restoring the obesity-causing energy imbalance are either by decreasing energy uptake or increasing energy expenditure. In the latter scenario, brown and brite adipocytes, due to their energy dissipation property, are receiving great attention (Tseng et al., 2010). The development of successful brown and brite-based therapeutic strategies to treat obesity associated metabolic syndrome relies on a good understanding of their basic biology. In this dissertation, the thermogenic function of *in vitro* differentiated brown and brite adipocytes were characterized using microplate-based respirometry. The molecular basis of brite adipocyte abundance variation between strains was investigated. Lastly, transcriptome analysis of cultured brite adipocytes from five inbred mouse strains with different browning capacities was performed. The results of this PhD thesis provide crucial insights into the functional analysis of both cultured brown and brite adipocytes *in vitro*, the molecular basis of strain-specific differences in brite adipogenesis and outline a strategy to identify potential novel regulators, functional components and markers of brite adipocytes.

7.1 UCP1 in cultured brite/beige adipocytes is functionally thermogenic

The functional significance of brite adipocytes is still not settled even in rodents. Briefly, there are two major questions: 1) whether brite thermogenesis is relevant for systemic thermoregulation; 2) whether the metabolic benefits of brite adipocyte recruitment is contributed by a thermogenic function. A recent study demonstrated that recruitment of brite adipocytes is increased to compensate for decreased BAT thermogenesis, providing the first evidence that brite alike brown adipocytes may contribute to thermogenesis if maximally stimulated (Schulz et al., 2013). Isolated mitochondria from inguinal fat depots of cold-exposed mice express substantial amounts of UCP1 protein also supporting a role for thermogenesis (Shabalina et al., 2013). However, there is a limited amount of data directly assessing bioenergetics and thermogenic capability of brite adipocytes. In addition, no studies have been performed so far by comparing bioenergetic profiles of brite cells from UCP1 wildtype and knockout mice to test the

causality between uncoupled respiration and presence of UCP1. Based on our findings (Chapter 4), meaningful respirometric measurements of cultured brown and brite adipocytes imperatively require (1) activation of UCP1 and (2) control over free fatty acid levels, while previous established procedures fail to meet these criteria (Li et al., 2014b). Thus we have identified a major pitfall associated with established procedures generally applied for this purpose. The functional data on brown adipocyte thermogenesis as published in many papers in the recent years must be revisited. By developing a new protocol, we reproducibly quantified the UCP1-mediated component of uncoupled respiration in both brown and brite adipocytes evidenced by the comparison of respiration profiles between UCP1 wild-type and knock-out cells. Employing this protocol, we for the first time demonstrate that brite adipocytes display a similar thermogenic capacity as classical brown adipocytes and reveal that strain differences in brite adipogenesis are associated with differential uncoupled respiration (Chapter 4, Figure 5 and Chapter 5, Figure 1F). This novel assay system even allows studying the functional relevance of candidate genes in brite adipocytes, for example the candidate genes identified in our transcriptome analysis (Chapter 6).

Based on our results, it is tempting to speculate that brown adipocytes do not strictly require UCP1 to perform uncoupled respiration but alternatively increase fatty acids flux, even *in vivo*. However, this seems unlikely, since UCP1 knockout mice are cold intolerant (Enerback et al., 1997). One possible explanation for this discrepancy between *in vitro* and *in vivo* could be that the albumin in blood serves as a fatty acid sink for brown adipocytes *in vivo*. Another possible explanation is that cultured adipocytes *in vitro* lose their ability to control the intracellular level of free fatty acid. Indeed, some fatty acid binding proteins (FABPs) such as FABP3 are not expressed in cultured brown and brite adipocytes, but highly expressed *in vivo*. Whether the lack of some fatty acid binding proteins renders cultured adipocytes less efficient in controlling intracellular FFA levels warrants further investigation.

A limitation of this study is that the intracellular free fatty acid level is not determined. Nevertheless, UCP1 activity is extremely sensitive to fatty acids. It has been demonstrated that the unbound free fatty acid concentrations needed to increase the

proton conductance of brown fat mitochondria is in the nanomolar (nM) range (Cunningham et al., 1986). Measuring the intracellular free fatty acids level with or without extracellular BSA complexing will be informative.

7.2 Intrinsic, trans-acting factors control brite adipogenesis in primary adipocytes from different mouse strains

The natural variation in *Ucp1* expression in WAT that occurs among inbred strains of mice not only provides a model to explore the relationship between induction of *Ucp1* and resistance to both genetic and diet-induced obesity, but also provides a system to identify genes critical for the induction of brite adipocytes in white adipose tissue. In a first step toward understanding the strain specific differences in brite adipogenesis, we found that, beyond systemic cues such as innervation and hormone levels, mouse strain differences in brite adipogenesis are sustained in cultured primary adipocytes (Chapter 5). We concluded that cues intrinsic to the progenitor cells contribute to differential brite adipogenesis. Detailed analyses demonstrated that these cues are independent of autocrine/paracrine mechanisms, brite progenitor abundance and genetic variation in the *Ucp1* gene but rather depend on trans-acting factors. Transcript levels of obvious candidates, such as PPARγ, PGC-1α and PRDM16, which are well-known transcriptional regulators of *Ucp1*, did not reveal strain differences in gene expression coinciding with the differential *Ucp1* expression, indicating other undefined determinants must play critical roles in governing the potential of brite adipogenesis. Partially consistent with our results, Kozak and colleagues reported that the most upstream sites of regulation for *Ucp1* in retroperitoneal fat that differed between A/J and B6 were the phosphorylation of p38 mitogen-activated protein kinase and CREB and then followed by downstream changes in levels of mRNA for PPARγ, PPARα, PGC-1α, and DIO2, these transcriptional factors and signaling molecules may interact synergistically to maximize the expression of *Ucp1* (Kozak, 2011; Kozak and Koza, 2010). Taken together, the intrinsic differences in brite adipogenesis of primary adipocytes from different mouse strains provide a unique opportunity to identify novel transcriptional regulators.

In primary cultures, brite adipogenesis was achieved by treatment of PPARγ agonist such as rosiglitazone. One appealing hypothesis is that the strain-specific differences in PPARγ binding may be responsible for strain-specific differences in brite adipogenesis. The fat depot-selective binding of PPARγ links to depot-specific gene expression in mice (Siersbæk et al. 2012; Rajakumari et al. 2013), which indicates that PPARγ, in addition to its role as a general activator of adipogenesis, plays a prominent role in the induction of genes characteristic of different adipocyte lineages. In fact, PPARγ plays an active role in the formation of super-enhancers (Loft et al., 2015), which are large enhancer regions with a high density of transcription factor binding sites, characterized by very high levels of Mediator subunit 1 (MED1) binding and seem to play key roles in the control of cell type-specific identity genes (Whyte et al., 2013). It is therefore plausible that differential PPARγ binding sites between mouse strains leads to the formation of distinct super-enhancers. The super-enhancers together with the super-enhancer driven genes, which may cooperate with super-enhancers in a feed-forward manner, are robust activators of brite adipogenesis. Future studies will be needed to assess this hypothesis.

7.3 Transcriptome analysis (RNA-Seq) of primary cultured adipocytes from five inbred mouse strains reveals novel genes potentially involved in brite adipogenesis

Brite adipogenesis in mice is under genetic control (Guerra et al., 1998 and Kozak, 2011). To identify novel determinants governing the potential of brite adipogenesis, transcriptome analysis of primary cultured adipocytes from five inbred mouse strains with differential browning capacities were performed. In this transcriptome data set we started to seek for gene transcripts associated with browning across five different genomic backgrounds. A list of candidate genes was identified by bioinformatic analysis, which provides a valuable source for more in-depth investigation. Further functional studies based on genetic perturbations need to be conducted to validate these findings. Genetic perturbations can be broadly classified as either loss-of-function or gain-of-function on the basis of their mode of action. RNA interference (RNAi) is a robust gene-silencing mechanism to knockdown genes in functional studies. Delivery of siRNA into

primary cultures of preadipocytes and newly differentiated adipocytes for gene silencing can be achieved by electroporation or using commercially available lipid-based transfection reagents. For lipid-mediated siRNA delivery, based on transfection approach, forward (also known as conventional transfection) and reverse transfection strategies can be taken. Since siRNA-mediated knockdown is limited to cells capable of transfection and is primarily utilized during transient *in vitro* studies, for long-term knockdown of the targeted gene, viral vectors such as adeno-associated virus (AAV) or lentivirus or retrovirus mediated small-hairpin RNA (shRNA) gene transduction can be performed. Regarding gain-of-function assays, a cDNA overexpression system can be applied.

Genome editing through the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 system offers the opportunity to manipulate specific genomic loci in mammalian cell lines with relative ease (Hsu et al., 2014; Sander and Joung, 2014). As promising alternative method both gene knockdown and overexpression can be achieved by using the CRISPR/Cas9 system (Shalem et al., 2015).

UCP1 mediated uncoupled respiration is a hallmark function of brown and brite adipocytes. If a candidate gene is essential for brite adipogenesis or thermogenic function, manipulating the expression of this gene should affect the bioenergetic function of brite adipocytes. To study the metabolic consequences of gene manipulation, bioenergetic function analysis of cells with either loss or gain of gene function can be performed by microplate respirometry (Seahorse extracellular flux analyzer XF96) using the method established in chapter 4.

7.4 Outlook

This study demonstrates that UCP1 in cultured brite adipocytes is functionally thermogenic, which is consistent with the conclusion based on bioenergetics data of isolated mitochondria from inguinal fat depots of cold-exposed mice (Shabalina et al., 2013). However, direct assessment of thermogenic contribution of brite adipocytes *in vivo* is still missing. This is largely due to a lack of approaches to distinguish the contribution of brite adipocytes from that of brown adipocytes, since many factors that

recruit and activate brite adipocytes also recruit and activate brown adipocytes. Recently it has been reported that the Cre recombinase activity of Prx1-Cre is restricted to adipose precursors of iWAT, much less in gWAT, and little to no recombination in BAT (Krueger et al., 2014). The specificity of Prx1-Cre activity could enable deletion of *Ucp1* in inguinal fat by crossing Prx1-Cre line to *Ucp1* flox/flox mice (*Ucp1* gene is flanked by two LoxP sites to allow for its subsequent removal by cre-mediated recombination), which then allows quantifying the relative thermogenic contribution of brite adipocytes compared to brown adipocytes. Furthermore, this mouse model could also be used to study the metabolic consequences of brite adipocyte ablation and to test whether the metabolic benefits of brite adipocyte recruitment are contributed by its thermogenic function. On other hand, based on the observation that brown adipocytes derive from a myf-5 lineage, while brite adipocytes within inguinal and epididymal fat depots are not (Seale et al., 2008), the myf5 promoter driving Cre recombinase expression can be used to specifically delete *Ucp1* in brown adipocytes. Nevertheless, it should be kept in mind that the brite cells in retroperitoneal WAT also arise from myf5-lineage (Sanchez-Gurmaches et al., 2012). This could lead to under or overestimation of the functional importance of brite adipocytes.

Another intriguing yet enigmatic issue is the cellular plasticity of brite adipocytes. Several studies indicate that mature white adipocytes may directly convert into beige adipocytes (i.e., transdifferentiation) *in vivo* (Barbatelli et al., 2010; Himms-Hagen et al., 2000 and Rosenwald et al., 2013). Since evidence supporting direct lineage reprogramming of post-mitotic mature white adipocytes into brown adipocytes is lacking, we speculated that these so called ‘mature white adipocytes’ are actually pre-existing brite adipocytes (or called ‘dormant’ brite adipocytes), which appear to be white yet can rapidly reinstate the brite phenotype upon adrenergic activation (Chapter 3). Recently, by taking advantage of genetic labeling of adipocytes at the time of weaning, Granneman and colleagues found that the vast majority of UCP1-positive cells were pre-labeled adipocytes when challenged with beta adrenergic agonist 5 weeks later (Contreras et al., 2014). This study provides the first line of evidence supporting our hypothesis. Factors that maintain phenotypic flexibility of brite adipocytes are largely unexplored, further work in this direction is warranted.

Lastly, the idea of direct lineage reprogramming (transdifferentiation) of post-mitotic mature white adipocytes into brite or brown adipocytes is still attractive, since white-to-brown adipocyte transdifferentiation could offer new therapeutic prospects for obesity and related disorders (Cinti, 2012). With the rapid progress in the field of cellular reprogramming methodology during the recent years, direct conversion of white into brown holds great promise. Enforced expression of one or several lineage specific transcription factors has been extensively used to induce lineage conversion in many studies (for review, see Xu et al., 2015). However, little effort has been made in direct reprogramming white adipocytes into brown. Future studies should consider a combination of minimal number of factors in addition to focusing on a single factor, similar to the strategy of Yamanaka to induce pluripotent stem cells (Takahashi and Yamanaka, 2006). In addition, epigenetic regulators which may facilitate lineage conversion through reactivation of an epigenetically repressed state of the target cell type-specific master genes can also be taken into account.

In conclusion, the findings presented in this thesis support a thermogenic function of UCP1 in cultured brite adipocytes. The method established here provides essential guidelines to specifically assess the thermogenic capability of cultured brown and brite adipocytes. The work towards understanding the strain specific differences in brite adipogenesis delivers new insights into the intrinsic cellular programming of browning potential. The transcriptome analysis of primary cultured adipocytes from five inbred mouse strains reveals putative novel regulators, functional components and new markers of brite adipocytes. The experiments proposed above warrant further research to validate the identified genes potentially involved in brite adipogenesis and define the functional significance of brite adipocytes *in vivo*. I am convinced that these findings contribute to the understanding of the biology of brite adipocytes. Identification of novel regulators and markers of brite adipocytes may provide potential strategies for combating the obesity epidemic affecting our society.

APPENDIX

Appendix I-Approval letter from publisher for Chapter 3

Li, Yongguo

From: pubs@nrcresearchpress.com
Sent: Montag, 20. April 2015 19:35
To: Li, Yongguo
Subject: RE: Permissions enquiry

Dear Dr. Yongguo Li,

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From: Li, Yongguo [mailto:yongguo.li@tum.de]
Sent: April-16-15 5:11 AM
To: pubs@nrcresearchpress.com
Subject: Permissions enquiry

Dear Sir or Madam,
I am the author of the following work published by Canadian Science Publishing (NRC Research Press):
Li Y, Lasar D, Fromme T, KlingensporM. White, brite, and brown adipocytes: the evolution and function of a heater organ in mammals. **Canadian Journal of Zoology** 2014; 92: 615-626.

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Yours sincerely,
Yongguo Li

Appendix I-Approval letter from publisher for Chapter 4

Li, Yongguo

From: Susanne Hofner-Harris <hofner@embo.org>
Sent: Freitag, 17. April 2015 10:30
To: Li, Yongguo
Subject: Re: Permissions enquiry

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Kind regards,
Susanne

Susanne Hofner-Harris
Editorial Assistant
EMBO reports

Susanne Hofner-Harris
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From: "Li, Yongguo" <yongguo.li@tum.de>
Subject: Permissions enquiry
Date: April 16, 2015 11:12:48 AM GMT+02:00
To: "publishing@embo.org"
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Dear Sir or Madam,
I am the author of the following work published by EMBOpress:
Li Y, Fromme T, Schweizer S, Schöttl T, Klingenspor M. Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes. **EMBO Rep** 2014; 15:1069-1076
This work is based on my PhD thesis. I wish to include this work within the electronic version of my thesis, which I am required to deposit in the Technische Universität München's online repository, mediaTUM (https://mediatum.ub.tum.de/). MediaTUM is a non-commercial facility which is freely and openly available to all.
I would be grateful if you could advise if this will be acceptable.
Yours sincerely,
Yongguo Li

Yongguo LI
Chair for Molecular Nutritional Medicine
Technische Universität München
Else Kröner-Fresenius Center for Nutritional Medicine &
Z I E L - Research Center for Nutrition and Food Sciences
Gregor-Mendel-Str. 2

Appendix I-Approval letter from publisher for Chapter 5

Li, Yongguo

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From: Li, Yongguo [mailto:yongguo.li@tum.de]
Sent: Thursday, April 16, 2015 5:14 AM
To: Permissions Helpdesk
Subject: Permissions enquiry

Dear Sir or Madam,
I am the author of the following work published by Elsevier:
Li Y, Bolze F, Fromme T, Klingenspor M. Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains.
BBA – Mol Cell Biol Lipids. 2014; 1841(9):1345-52.

This work is based on my PhD thesis. I wish to include this work within the electronic version of my thesis, which I am required to deposit in the Technische Universität München's online repository, mediaTUM (https://mediatum.ub.tum.de/). MediaTUM is a non-commercial facility which is freely and openly available to all. I would be grateful if you could advise if this will be acceptable.
Yours sincerely,
Yongguo Li

Appendix II-Supplements of Chapter 4

A-Supplemental Data

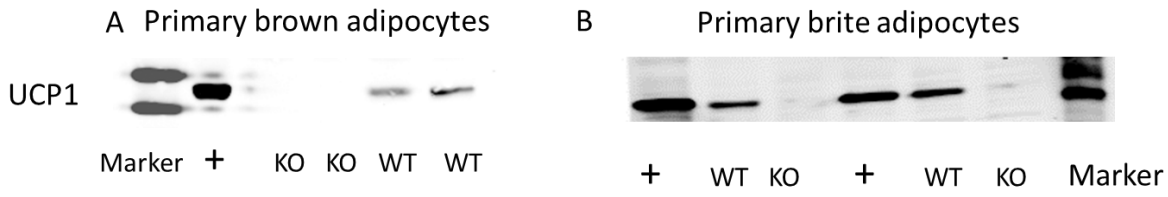

Figure S1. Western blotting analysis confirming the phenotype of primary brown and brite adipocytes from UCP1 wildtype (WT) and knockout (KO) mice. Primary brown (A) and brite adipocytes (B) from UCP1 KO mice cultured in XF96 V3-PS cell culture microplate are UCP1 negative, while primary adipocytes from UCP1 WT mice are UCP1 positive. Primary brown adipocytes cultured in 6-well plates serve as positive control (+).

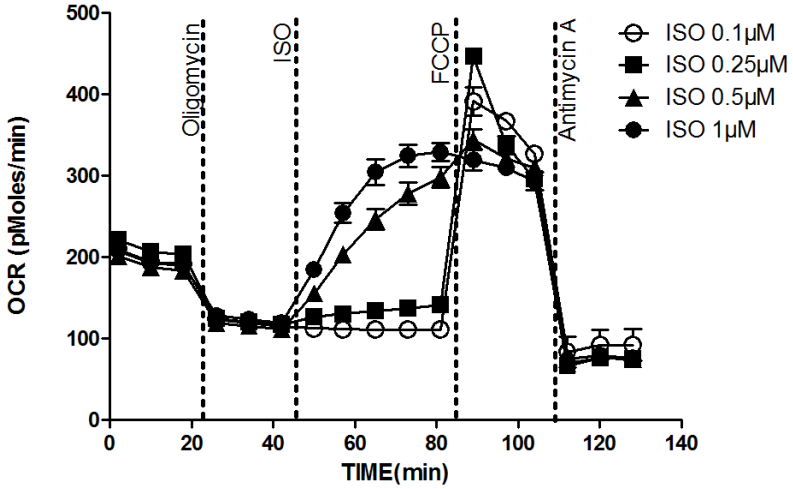

Figure S2. Isoproterenol (ISO) increases leak respiration in a dose dependent manner. Time course of oxygen consumption rates (OCR) of primary brite adipocytes from wildtype mice. Leak respiration is stimulated by ISO induced lipolysis. All data presented are mean values ± SEM with an average of 10–12 different wells.

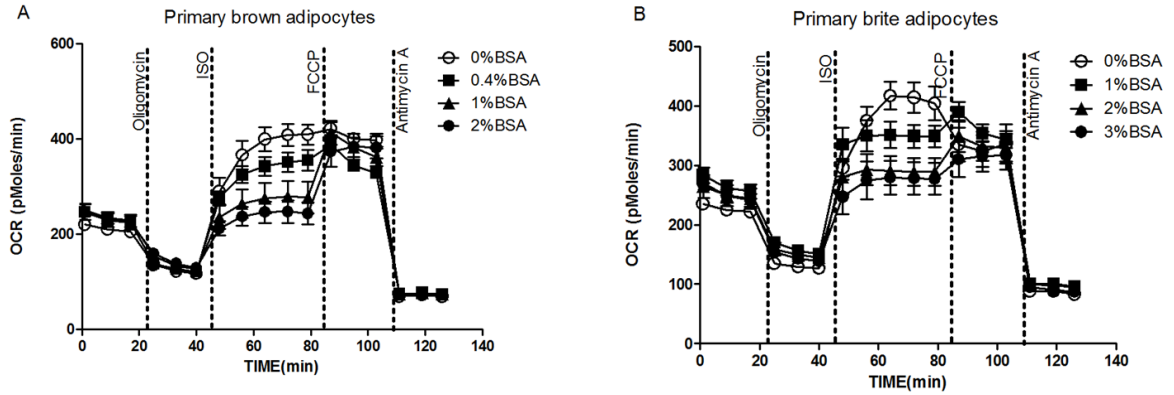

Figure S3. Effects of various concentrations of BSA on ISO-induced respiration in primary brown and brite adipocytes. Time course of oxygen consumption rate (OCR) of primary brown (A) and brite (B) adipocytes from UCP1 wildtype (WT) mice in the presence of different BSA concentrations. All data presented are mean values ± SEM with an average of 10–12 different wells.

B-Supplementary Historical perspective

The functions of isolated brown adipocyte mitochondria have been intensely studied for decades. It is clear from these early studies that freshly isolated brown fat mitochondria contains significant amounts of free fatty acids which are partially responsible for the uncoupled state[1, 2]. The high sensitivity of brown fat mitochondria to uncoupling by fatty acids suggested a physiological regulator of uncoupling, from which the concept of 'fatty acid uncoupling' was developed and later confirmed and widely accepted[3]. In fact, albumin which acts as an acceptor of fatty acids must be used in isolation and respiration medium for coupled respiration to be observed. Thus, bovine serum albumin (BSA) was generally used in the respiration buffer of isolated brown adipocyte mitochondria. Perhaps due to this valuable knowledge, in early publications on oxygen consumption measurements in isolated brown adipocytes following norepinephrine stimulation, 4 % bovine serum albumin (0.6mM, equate to the concentration found in blood) was also used in the respiration medium [4-7]. In 1979, based on the rationales that (1) brown fat when activated release fatty acids and (2) in vivo those released fatty acids are bound by albumin in blood, Nedergaard and Lindberg investigated the effect of albumin on the metabolism of isolated brown fat cells and found that the addition of albumin increases norepinephrine-induced fatty acid release and induces a more stable norepinephrine-stimulated respiration rate[8]. This study provides direct evidence that addition of albumin has beneficial effects on brown adipocytes metabolism. By using this setup, in 2000, Matthias et al. reported marked difference in oxygen consumption between isolated brown adipocytes from wild-type and UCP1 knock-out mice, both following NE stimulation and stimulation with oleate[9]. This study also showed that there was no difference in basal respiration between the two preparations demonstrating UCP1 was not active without stimulation. These observations were further confirmed by Shabalina et al. when studying the bioenergetics of wild-type and UCP1 knock-out brown-fat mitochondria[10]. In addition, investigations with trypsinized primary cultures of brown and brite cells by Petrovic et al. in 2008 and 2010, respectively, showed a correlation between UCP1 expression (as induced by rosiglitazone) and NE-stimulated oxygen consumption[11, 12]. In all these studies, UCP1-mediated leak respiration was observed when albumin was present in respiration medium using Warburg apparatus or Clark-type oxygen electrode systems. Nowadays, microplate-based respirometry has become a mainstream method for measuring UCP1-mediated leak respiration in cultured brown and brite cells. However, in the respiration buffer defined by the respirometry manufacturer albumin is absence. Notably, no studies have been performed so far to validate this setup with cultured UCP1 knock-out (KO) cells as the ultimate model to test the causality between uncoupled respiration and presence of UCP1. This relationship seems to have been taken for granted.

1. Drahota Z, Honova E, Hahn P (1968) The effect of ATP and carnitine on the endogenous respiration of mitochondria from brown adipose tissue. *Experientia* **24:** 431-432
2. Hittelman KJ, Lindberg O, Cannon B (1969) Oxidative phosphorylation and compartmentation of fatty acid metabolism in brown fat mitochondria. *Eur J Biochem* **11:** 183-192

3. Nicholls DG, Locke RM (1984) Thermogenic mechanisms in brown fat. *Physiol Rev* **64:** 1-64
4. Fain JN, Reed N, Saperstein R (1967) The isolation and metabolism of brown fat cells. *J Biol Chem* **242:** 1887-1894
5. Reed N, Fain JN (1968) Stimulation of respiration in brown fat cells by epinephrine, dibutyryl-3',5'-adenosine monophosphate, and m-chloro(carbonyl cyanide)phenylhydrazone. *J Biol Chem* **243:** 2843-2848
6. Prusiner SB, Cannon B, Lindberg O (1968) Oxidative metabolism in cells isolated from brown adipose tissue. 1. Catecholamine and fatty acid stimulation of respiration. *Eur J Biochem* **6:** 15-22
7. Prusiner SB, Cannon B, Ching TM, Lindberg O (1968) Oxidative metabolism in cells isolated from brown adipose tissue. 2. Catecholamine regulated respiratory control. *Eur J Biochem* **7:** 51-57
8. Nedergaard J, Lindberg O (1979) Norepinephrine-stimulated fatty-acid release and oxygen consumption in isolated hamster brown-fat cells. Influence of buffers, albumin, insulin and mitochondrial inhibitors. *Eur J Biochem* **95:** 139-145
9. Matthias A, Ohlson KB, Fredriksson JM, Jacobsson A, Nedergaard J, Cannon B (2000) Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty scid-induced thermogenesis. *J Biol Chem* **275:** 25073-25081
10. Shabalina IG, Ost M, Petrovic N, Vrbacky M, Nedergaard J, Cannon B (2010) Uncoupling protein-1 is not leaky. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1797:** 773-784
11. Petrovic N, Shabalina IG, Timmons JA, Cannon B, Nedergaard J (2008) Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARgamma agonist. *Am J Physiol Endocrinol Metab* **295:** E287-296
12. Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J (2010) Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. *J Biol Chem* **285:** 7153-7164

Appendix III-Primer sequences used in quantitative RT-PCR

Gene		Sequences
Ucp1	Forward	GTACACCAAGGAAGGACCGA
	Reverse	TTTATTCGTGGTCTCCCAGC
Cidea	Forward	TGCTCTTCTGTATCGCCCAGT
	Reverse	GCCGTGTTAAGGAATCTGCTG
Cox7a1	Forward	TGTCTCCGTGTGTGAGTGGT
	Reverse	ATATGCTGAGGTCCCCCTT T
TFIIB	Forward	TGGAGATTTGTCCACCATGA
	Reverse	GAATTGCCAAACTCATCAAAACT
Pdgfra	Forward	ACGTTCAAGACCAGCGAGTT
	Reverse	CTCCAGGGTAAGTCCACTGC
Sca1	Forward	CTGATTCTTCTTGTGGCCCTA
	Reverse	CAATAACTGCTGCCTCCTGA
CD34	Forward	TTGGGCACCACTGGTTATTT
	Reverse	TTTTCTTCCCAACAGCCATC
Tmem26	Forward	ACCCTGTCATCCCACAGAG
	Reverse	TGTTTGGTGGAGTCCTAAGGTC
CD137	Forward	CGTGCAGAACTCCTGTGATAAC
	Reverse	GTCCACCTATGCTGGAGAAGG
Fgf21	Forward	AGATCAGGGAGGATGGAACA
	Reverse	TCAAAGTGAGGCGATCCATA
Cox2	Forward	GACTGGGCCATGGAGTGG
	Reverse	CACCTCTCCACCAATGACC
Arginase1	Forward	TGGCTTGCGAGACGTAGAC
	Reverse	GCTCAGGTGAATCGGCCTTTT
PPARg	Forward	TCAGCTCTGTGGACCTCTCC
	Reverse	ACCCTTGCATCCTTCACAAG
PGC-1α	Forward	GGACGGAAGCAATTTTTCAA
	Reverse	GAGTCTTGGGAAAGGACACG
Rip140	Forward	ATGGTGTTGTCCCTTCCTC
	Reverse	AACTGCTCGCTCTCTCGTTC
Prdm16	Forward	CAGCACGGTGAAGCCATTC
	Reverse	GCGTGCATCCGCTTGTG

Appendix IV Sequence alignment: Ucp1-Promoter 3.2 kb 129SvJ vs. B6/J

Multiple sequence alignment using CLUSTAL W (1.81)

Consensus key

* -Single, fully conserved residue
  - No consensus
  - Enhancer region

```
Ucp1-Promoter_3.2_kb_B6          GGTACCGTGCACACTGCCAAATCATCTCAAAATCAGCATGCCAATTTATA
Ucp1-Promoter_3.2_kb_129         GGTACCGTGCACACTGCCAAATCATCTCAAAATCAGCATGCCAATTTATA
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          GTGCCGTCACTAACAGTACTGATACTTTAACATGCTAAGTTTAAAGTGTG
Ucp1-Promoter_3.2_kb_129         GTGCCGTCACTAACAGTACTGATACTTTAACATGCTAAGTTTAAAGTGTG
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          TGCTATATTAATTGTAAGATTGGTGAAGAGAGGTGTTATCAGATGGAAGC
Ucp1-Promoter_3.2_kb_129         TGCTATATTAATTGTAAGATTGGTGGAGAGAGGTGTTATCAGATGGAAGC
                                 ************************ ************************

Ucp1-Promoter_3.2_kb_B6          TGCACATTTCTGGATTAATGTGGTTAAATGTATCTTCTCCTGTGATTACT
Ucp1-Promoter_3.2_kb_129         TGCACATTTCTGGATTAATGTGGTTAAATGTATCTTCTCCTGTGATTACT
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Ucp1-Promoter_3.2_kb_B6          GTCTTTATTTCTTCTTTTAAAATATTGTCATTTGGACATCTATCTGTATA
Ucp1-Promoter_3.2_kb_129         GTCTTTATTTCTTCTTTTAAAATATTGTCATTTGGACATCTATCTGTATA
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          GCTACGCCCTGACACGTCCTCCTGGAGACAGATAAGAAGTTACGACGGGA
Ucp1-Promoter_3.2_kb_129         GCTACGCCCTGACATGTCCTCCTGGAGACAGATAAGAAGTTACGACGGGA
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Ucp1-Promoter_3.2_kb_B6          GGA-GCAGATGGAGGCAAAGCGCTGTGATGCTTTTGTGGTTTGAGTGCAC
Ucp1-Promoter_3.2_kb_129         GGGTGCAGATGGAGGCAAAGCGCTGTGATGCTTTTGTGGTTTGAGTGCAC
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Ucp1-Promoter_3.2_kb_B6          ACATTTGTTCAGTGATTCTGTGAAATGAGTGAGCAAATGGTGACCGGGTG
Ucp1-Promoter_3.2_kb_129         ACATTTGTTCAGTGATTCTGTGAAATGAGTGAGCAAATGGTGACCGGGTG
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Ucp1-Promoter_3.2_kb_B6          CCCTGTAAATGGTGTTCTACATCTTAAGAGAAGAACACGGACACTAGGTA
Ucp1-Promoter_3.2_kb_129         CCCTGTAAATGGCGTTCTACATCTTAAGAGAAGAACACGGACACTAGGTA
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Ucp1-Promoter_3.2_kb_B6          AGTGAAGCTTGCTGTCACTCCTCTACAGCGTCACAGAGGGTCAGTCACCC
Ucp1-Promoter_3.2_kb_129         AGTGAAGCTTGCTGTCACTCCTCTACAGCGTCACAGAGGGTCAGTCACCC
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          TTGACCACACTGAACTAGTCGTCACCTTTCCACTCTTCCTGCCAGAAGAG
Ucp1-Promoter_3.2_kb_129         TTGACCACACTGAACTAGTCGTCACCTTTCCACTCTTCCTGCCAGAAGAG
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          CAGAAATCAGACTCTCTGGGGATATCAGCCTCACCCCTACTGCTCTCTCC
Ucp1-Promoter_3.2_kb_129         CAGAAATCAGACTCTCTGGGGATATCAGCCTCACCCCTACTGCTCTCTCC
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Ucp1-Promoter_3.2_kb_B6          ATTATGAGGCAAACTTTCTTTCACTTCCCAGAGGCTCTGGGGGGCAGCAAG
Ucp1-Promoter_3.2_kb_129         ATTATGAGGCAAACTTTCTTTCACTTCCCAGAGGCTCTGGGGGGCAGCAAG
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          GTCAACCCTTTCCTCAGACTCTAGAACCACTCCCTGCCTTGAGTTGACAT
Ucp1-Promoter_3.2_kb_129         GTCAACCCTTTCCTCAGACTCTAGAACCACTCCCTGCCTTGAGTTGACAT
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          CACGGGGCCTCCTTGAGCGAGAATTTCATTCTGCCATATGCCACATTTTG
Ucp1-Promoter_3.2_kb_129         CACGGG-CCTCCTTGAGCGAGAATTTCATTCTGCCATATCCCACATTTTG
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Ucp1-Promoter_3.2_kb_B6          GGCTCAGGAGTTCAGGAAAGCCTTTGCTGGGTAATAATTCTTTCTATTCA
Ucp1-Promoter_3.2_kb_129         GGCTCAGGAGTTCAGGAAAGCCTTTGCTGGGTGATAATTCTTTCTATTTA
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Ucp1-Promoter_3.2_kb_B6          CATCACAGAAATGACTTGATGTGTGGAGCTGAGTAGCCGAAGGGTTCAGG
Ucp1-Promoter_3.2_kb_129         CATCACAGAAATGACTTGATGTGTGGAGCTGAGTAGCCGAAGGGTTCAGG
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Ucp1-Promoter_3.2_kb_B6          GTGGTCGGGACCTTGGTGAGAAACAACAGAAGCTCCTATCTGGTCCCTCT
Ucp1-Promoter_3.2_kb_129         GTGGTCGGGACCTTGGTGAGGAACAGCAGAAGCTCCTATCTGGTCCCTCT
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Ucp1-Promoter_3.2_kb_B6          CAAGCATACCATCCTCAAGGGCATTTGATTTTTTTATGCCGTGGCCCAGG
Ucp1-Promoter_3.2_kb_129         CAAGCATACCATCCTCAAGGGCATTTGATTTTTTTATGCCATGGCCCAGG
                                 ****************************************** *********

Ucp1-Promoter_3.2_kb_B6          GCTCCGAGTGCCACTCCTCTTAGACCATAGCTGTGGTTCCCAGGGCTCCG
Ucp1-Promoter_3.2_kb_129         GCTCCGAGCACCACTCCTCTTAGACCATAGCTGTGGTTCCCAGGGCT---
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Ucp1-Promoter_3.2_kb_B6          AGTGCCACTCCTCTTAGACCATAGCTGTGGTTCCCAGGGCTCCGAGTGCC
Ucp1-Promoter_3.2_kb_129         ----------------------------------------CCGAGTGCC
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Ucp1-Promoter_3.2_kb_B6          ATTCCTCTAAGACCATAGCTTGGTTCCTCATGCTTTGTTCCTGTCTCCTT
Ucp1-Promoter_3.2_kb_129         ATTCCTCTAAGACCATAGCTTGGTTCCTTATGCTTTGTTCCTGTTTCCTT
                                 **************************** *************** *****

Ucp1-Promoter_3.2_kb_B6          TCAATCCGGCTGTGCTCATTCCCACAGAAAGTTACCAGTTCCTTCACTAC
Ucp1-Promoter_3.2_kb_129         TCAATCCGGCTGTGCTCATTCCCACAGAAAGTTACCAGTTCCTTCACTAC
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          CCTACTAACCACCTCCGATACCTCTCAGCTTTGATTCTCTGATGCAAGTA
Ucp1-Promoter_3.2_kb_129         CCTACTAACCACCTCCGATACCTCTCAGCTTTGATTCTCTGATGCAAGTA
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          GCATGACGCTTCCTATGGGATCATGATGCAAGCAGCATGATGTTTTAATG
Ucp1-Promoter_3.2_kb_129         GCATGACGCTTCCTATGGGAGCATGATGCAAGCAGCATGATGTTTTAATG
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Ucp1-Promoter_3.2_kb_B6          AAAACATCAATGCCATTTTCTCCTAATAATCCTAAAACGGAAAGTTGCCA
Ucp1-Promoter_3.2_kb_129         AAAACATCAATGCCATTTTCTCCTAATAATCCTAAAACGGAAAGTTGCCA
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Ucp1-Promoter_3.2_kb_B6          TCTTATAAAGAGTATTTACTTGCCAACTGCTTTGTGACAATCAAGTGGAA
Ucp1-Promoter_3.2_kb_129         TCTTATAAAGAGTATTTACTTGCCAACTGCTTTGTGACAATCAAGTGGAA
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Ucp1-Promoter_3.2_kb_B6          ACATTGCCAAGACTGCGGCCATCTTCTCAAGTGTAGAGTCATTTTCTAAT
Ucp1-Promoter_3.2_kb_129         ACATTGCCAAGACTGCGGCCATCTTCTCAAGTGTAGAGTCATTTTCTAAT
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Ucp1-Promoter_3.2_kb_B6          AATCTCTGACTCCATAGTAACCCTTTCTTAAGCTGTATTATCTGGTTAGA
Ucp1-Promoter_3.2_kb_129         AATCTCTGACTCCATAGTAACCCTTTCTTAAGCTGTATTATCTGGTTAGA
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Ucp1-Promoter_3.2_kb_B6          TGTGTCATTCATGTGAGCACAGTTTACGATCTACCAAGCCCTGCACAACA
Ucp1-Promoter_3.2_kb_129         TGTGTCATTCATGTGAGCACAGTTTACGATCTACCAAGCCCTGCACAACA
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          TATAAGATGAACTCACATGCAGAAAGGATGCTCACAGACTGCCGTTTATT
Ucp1-Promoter_3.2_kb_129         TATAAGATGAACTCACATGCAGAAAGGATGCTCACAGACTGCCGTTTATT
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          ATACTGTTGTTGCTGCTGCTGTTTGGGGGTTTATTCTAGCCTCGGCAGCC
Ucp1-Promoter_3.2_kb_129         ATACTGTTGTTGCTGCTGCTGTTTGGGGGTTTATTCTAGCCTCTGTAGCC
                                 ****************************************** * ****

Ucp1-Promoter_3.2_kb_B6          CTGGCTGTCCTAGAACTCACTCTGTAGACCAGGCTGGCCTCAAACTCACA
Ucp1-Promoter_3.2_kb_129         CTGGCTGTCCTAGAACTCACTCTGTAGACCAGGCTGGCCTCAAACTCACA
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Ucp1-Promoter_3.2_kb_B6          AAGACCCCTTGCTGCTTTGGCCTCTGCCTCCCTGGTGCTGGGATTAAAGG
Ucp1-Promoter_3.2_kb_129         AAGATCCCTTGCTGCTTTGGCCTCTGCCTCCCTGGTGCTGGGATTAAAGG
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Ucp1-Promoter_3.2_kb_B6          TGTGTGTCACCACCACTAGGCTGCTTATTACACTTTTCATCCATAGGTCT
Ucp1-Promoter_3.2_kb_129         TGTGTGTCATCACCACTAGGCTGTTTATTACACTTTTCATCCATAGGTCT
                                 ********* ************* ************************

Ucp1-Promoter_3.2_kb_B6          TGCAGCTTCCTGCCCCTGCCCCTCTTTTCTCCCCGCCCCCCATTCAGTTT
Ucp1-Promoter_3.2_kb_129         TGCAGCTTCCTGCCCCTGCCCCTCTTTTCTCCCCGCCCCCCATTCAGTTT
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          CCCAGCCCCCAGGCATTCTGCTCCCCTCCATATCATTGTCTTCACACACA
Ucp1-Promoter_3.2_kb_129         CCCAGCCCCCAGGCATTCTGCTCCCCTCCATATCATTGTCTTCACACACA
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          CCACTGACTGCTCATCACTGCTGTTCTGGCTCAAGTTCCAGGTGTGTATT
Ucp1-Promoter_3.2_kb_129         CCACTGACTGCTCATCACTGCTGTTCTGGCTCAAGTTCCAGGTGTGTATT
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          TTATTGTGGAAGCTCCCAGGACTAGGTTCTTGCTTAGTGTGCACGCTTGC
Ucp1-Promoter_3.2_kb_129         TTATTGTGGAAGCTCCCAGGACTAGGTTCTTGCTTAGTGTGCACACTTAC
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Ucp1-Promoter_3.2_kb_B6          CCCTTTAAATGTGGTGTCCAGGGGTGTTGTCTCTTACATGCCCCAATCCA
Ucp1-Promoter_3.2_kb_129         CCCTTTAAATGTGGTGTCCAGGGGTGTTGTCTCTTACATGCCCCACTCCA
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Ucp1-Promoter_3.2_kb_B6          GGAGATGAGTCCCACCTCCTTCACTCTCCTGGGACCACGCTGAGTAGCAG
Ucp1-Promoter_3.2_kb_129         GGAGATGAGTCCCACCTCCTTCACTCTCCTGGGACCACGCTGAGTAGCAG
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Ucp1-Promoter_3.2_kb_B6          AACCTGGCCAACCAGGGAGGATACAGAGAACAGGCATCGGCTCCAGCCTG
Ucp1-Promoter_3.2_kb_129         AACCTGGCCAACCAGGGAGGATACAGAGAACAGGCATCGGCTCCAGCCTG
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Ucp1-Promoter_3.2_kb_B6          CTCTCTTTCTCACCATTTCTTACGTAGTATTTTTTTT-AAAGCTTTTCAA
Ucp1-Promoter_3.2_kb_129         CTCTCTTTCTCACCATTTCTTACGTAGTATTTTTTTTTAAAGCTTTTCAA
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Ucp1-Promoter_3.2_kb_B6          ATACAAATCTTTGCTCAAAGATAGAAGTAAGAAACACCCCCCCCCACAC
Ucp1-Promoter_3.2_kb_129         ATACAAATCTTTGCTCAAAGATAGAAGTAAGAAACACCCCCCACACACAC
                                 ****************************************** * *****

Ucp1-Promoter_3.2_kb_B6          ACACACACACCAACAAACCACCTTACAATGTCTGAACTGGCACTAGGGAT
Ucp1-Promoter_3.2_kb_129         ACACACACACCAACAAACCACCTTACAATGTCTGAACTGGCACTAGGGAT
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Ucp1-Promoter_3.2_kb_B6          AACAAGAGTTCAAATCTGGGACTATATTTGACACAATCACAAACCAAAGC
Ucp1-Promoter_3.2_kb_129         AACAGGAGTTCAAATCTGGGACTATATTTGACACAATCACAAACCAAAGC
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Ucp1-Promoter_3.2_kb_B6          TAGAGGACTTTCTCAGGGTCACGAAGCTGGTCAGATGGCTTGGTTGCATC
Ucp1-Promoter_3.2_kb_129        TAGAGGACTTTCTCAGGGTCACGAAGCTGGTCAGATGGCTTGGTTGCATC
                                 **************************************************

Ucp1-Promoter_3.2_kb_B6          TGCAGCCAACCAACCCTCTGTCCTTCCAGGGCTCCTGCTAAGAACTATTC
Ucp1-Promoter_3.2_kb_129         TGCAGCCAACCAACCCTCTGTCCTTCCAGGGCTCCTGCTAAGAACTATTC
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Ucp1-Promoter_3.2_kb_B6          AAAGGAGCAAGGGCTGGC--------------------------------
Ucp1-Promoter_3.2_kb_129         AAAGGAGCAAGGGCTGGCATTCCCTGCAGAGATCCACAGGCTTGCTAGCG
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Ucp1-Promoter_3.2_kb_B6          ---TTATGAGCTTAGTTGATCTCGCCCAACTAAAAGTGACCACACGATGC
Ucp1-Promoter_3.2_kb_129         TGGCTATGAGCTTAGTTGATCTCGCCCAACTAAAAGTGACCACACGATGC
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Ucp1-Promoter_3.2_kb_B6          ACTCACTTTCTCGAAAAAGTTTAGGGCGTTATTAGTTTTTGCATGGATTC
Ucp1-Promoter_3.2_kb_129         ACTCACTTTCTCGAAAAAGTTTAGGGCGTTATTAGTTTTTGCATGGATTC
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Ucp1-Promoter_3.2_kb_B6                       CCTCCCCTCCCACATCCTCATCCCCACCCCATCCAGTCACCCAAATCTGA
Ucp1-Promoter_3.2_kb_129                      CCTCCCCTCCCACATCCTCATCCCCACCCCATCCAGTCACCCGAATCTGA
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Ucp1-Promoter_3.2_kb_B6                       AGGTGACATTTGAAAAGGAATGTAAAGAAAGGGACATTCAGAATTGAAAG
Ucp1-Promoter_3.2_kb_129                      AGGTGACATTTGAAAAGGAATGTAAAGAAAGGGACATTCAGAATTGAAAG
                                              **************************************************

Ucp1-Promoter_3.2_kb_B6                       GAGAAGAATCAGTAGCCAAGTAGATGCATGGGAAGGGGGCCCGGGACTGG
Ucp1-Promoter_3.2_kb_129                      GAGAAGAATCAGTAGCCAAGTAGATGCATGGGAAGGGGGCCCGGGACTGG
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Ucp1-Promoter_3.2_kb_B6                       GACGTTCATCCTACAGCTATTCTAGCTGTGGAACTTTTCAGCAAAATCTC
Ucp1-Promoter_3.2_kb_129                      GA-GTTCATCCTACAGCTATTCTAGCTGTGGAACTTTTCAGCAAAATCTC
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Ucp1-Promoter_3.2_kb_B6                       GGAGGAGATCAGATCGCGCTTATTCAAGGGAACCAGCCCCTGCTCTGCGC
Ucp1-Promoter_3.2_kb_129                      GGAGGAGATCAGATCGCGCTTATTCAAGGGAGCCAGTCCCTGCTCTGCGC
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Ucp1-Promoter_3.2_kb_B6                       CCTGGTCCAAGGCT-----GTTGAAGAGTGACAAAAGGCACCACGCTGCG
Ucp1-Promoter_3.2_kb_129                      CCTGGTCCAAGGCTCCGCGGTTGAAGAGTGACAAAAGGCACCACGCTGCG
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Ucp1-Promoter_3.2_kb_B6                       GGGACGCGGGTGAAGCCCCTCTGTGTGTCCTCTGGGCATAATCAGGAACT
Ucp1-Promoter_3.2_kb_129                      GGAACGCGGGTGAAGCCCCTCTGTGTGTCCTCTGGGCATAATCAGGAACT
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Ucp1-Promoter_3.2_kb_B6                       GGTGCCAAATCAGAGGTGATGTGGCCAGGGCTTTGGGAGTGACGCGCGGC
Ucp1-Promoter_3.2_kb_129                      GGTGCCAAATCAGAGGTGATGTGGCCAGGGCTTTGGGAGTGACGCGCGGC
                                              **************************************************

Ucp1-Promoter_3.2_kb_B6                       TGGGAGGCTTGCGCACCCAAGGCACGCCCCTGCCAAGTCCCACTAGCAGC
Ucp1-Promoter_3.2_kb_129                      TGGGAGGCTTGCGCACCCAAGGCACGCCCCTGCCAAGTCCCACTAGCAGC
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Ucp1-Promoter_3.2_kb_B6                       TCTTTGGAGACCTGGGCCGGCTCAGCCACTTCCCCCAGTCCCTCCTCCGG
Ucp1-Promoter_3.2_kb_129                      TCTTTGGAGACCTGGGCCGGCTCAGCCACTTCCCCCAGTCCCTCCTCCAG
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Ucp1-Promoter_3.2_kb_B6                       CAAGGGGCTATATAGATCTCCCAGGTCAGGGCGCAGAAGTGCCGGGCAAT
Ucp1-Promoter_3.2_kb_129                      CAAGGGGCTATATAGATCTCCCAGGTCAGGGCGCAGAAGTGCCGGGCAAT
                                              **************************************************

Ucp1-Promoter_3.2_kb_B6                       CTGGGCTTAACGGGTCCTCCCTGCCCGAGCAAGAGGAAGGGACGCTCACC
Ucp1-Promoter_3.2_kb_129                      CTGGGCTTAACGGGTCCTCCCTGCCCGAGCAAGAGGAAGGGACGCTCACC
                                              **************************************************

Ucp1-Promoter_3.2_kb_B6                       TTTGAGCTGCTCCACAGCGCCGCCTCTGCACTGGCACTACCTAGCCCAGG
Ucp1-Promoter_3.2_kb_129                      TTTGAGCTGCTCCACAGCGCCGCCTCTGCACTGGCACTACCTAGCCCAGG
                                              **************************************************

Ucp1-Promoter_3.2_kb_B6                       TGGCTCTGCAGGAGCTC
Ucp1-Promoter_3.2_kb_129                      TGGCTCTGCAGGAGCTC
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REFERENCES

Ahima, R.S., Prabakaran, D., and Flier, J.S. (1998). Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. The Journal of clinical investigation *101*, 1020-1027.

Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., and Evans, R.M. (2013). PPARgamma signaling and metabolism: the good, the bad and the future. Nature medicine *19*, 557-566.

Almind, K., Manieri, M., Sivitz, W.I., Cinti, S., and Kahn, C.R. (2007). Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice. Proceedings of the National Academy of Sciences of the United States of America *104*, 2366-2371.

Anderson, C.M., Kazantzis, M., Wang, J., Venkatraman, S., Goncalves, R.L., Quinlan, C.L., Ng, R., Jastroch, M., Benjamin, D.I., Nie, B., et al. (2015). Dependence of Brown Adipose Tissue Function on CD36-Mediated Coenzyme Q Uptake. Cell reports *10*, 505-515.

Barbatelli, G., Murano, I., Madsen, L., Hao, Q., Jimenez, M., Kristiansen, K., Giacobino, J.P., De Matteis, R., and Cinti, S. (2010). The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. American journal of physiology. Endocrinology and metabolism *298*, E1244-1253.

Bartelt, A., and Heeren, J. (2014). Adipose tissue browning and metabolic health. Nature reviews. Endocrinology *10*, 24-36.

Brestoff, J.R., Kim, B.S., Saenz, S.A., Stine, R.R., Monticelli, L.A., Sonnenberg, G.F., Thome, J.J., Farber, D.L., Lutfy, K., Seale, P., et al. (2014). Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. Nature *519*, 242–246

Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. Physiological Reviews. *84*, 277-359.

Chondronikola, M., Volpi, E., Borsheim, E., Porter, C., Annamalai, P., Enerback, S., Lidell, M.E., Saraf, M.K., Labbe, S.M., Hurren, N.M., et al. (2014). Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. Diabetes *63*, 4089-4099.

Cinti, S. (2012). The adipose organ at a glance. Disease Models & Mechanisms *5*, 588-594.

Contreras, G.A., Lee, Y.H., Mottillo, E.P., and Granneman, J.G. (2014). Inducible brown adipocytes in subcutaneous inguinal white fat: the role of continuous sympathetic stimulation. American journal of physiology. Endocrinology and metabolism *307*, E793-799.

Cortes, V.A., Curtis, D.E., Sukumaran, S., Shao, X., Parameswara, V., Rashid, S., Smith, A.R., Ren, J., Esser, V., Hammer, R.E., et al. (2009). Molecular mechanisms of hepatic steatosis and insulin resistance in the AGPAT2-deficient mouse model of congenital generalized lipodystrophy. Cell metabolism *9*, 165-176.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Penicaud, L., and Casteilla, L. (1992). Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. Journal of Cell Science *103*, 931.

Cunningham, S.A., Wiesinger, H., and Nicholls, D.G. (1986). Quantification of fatty acid activation of the uncoupling protein in brown adipocytes and mitochondria from the guinea-pig. European journal of biochemistry / FEBS *157*, 415-420.

Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y.H., Doria, A., et al. (2009). Identification and importance of brown adipose tissue in adult humans. The New England Journal of Medicine *360*, 1509-1517.

Cypess, A.M., Weiner, L.S., Roberts-Toler, C., Elia, E.F., Kessler, S.H., Kahn, P.A., English, J., Chatman, K., Trauger, S.A., Doria, A., et al. (2015). Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. Cell metabolism *21*, 33-38.

Cypess, A.M., White, A.P., Vernochet, C., Schulz, T.J., Xue, R., Sass, C.A., Huang, T.L., Roberts-Toler, C., Weiner, L.S., Sze, C., et al. (2013). Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. Nature medicine *19*, 635-639.

Enerback, S., Jacobsson, A., Simpson, E.M., Guerra, C., Yamashita, H., Harper, M.E., and Kozak, L.P. (1997). Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature *387*, 90-94.

Geisler, S., and Coller, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nature reviews. Molecular cell biology *14*, 699-712.

Gesta, S., Tseng, Y.H., and Kahn, C.R. (2007). Developmental origin of fat: tracking obesity to its source. Cell *131*, 242-256.

Guerra, C., Koza, R.A., Yamashita, H., Walsh, K., and Kozak, L.P. (1998). Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. Journal of Clinical Investigation *102*, 412.

Harms, M., and Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. Nature medicine *19*, 1252-1263.

Himms-Hagen, J., Melnyk, A., Zingaretti, M.C., Ceresi, E., Barbatelli, G., and Cinti, S. (2000). Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. American Journal of Physiology - Cell Physiology *279*, C670-681.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell *157*, 1262-1278.

Hu, W., Alvarez-Dominguez, J.R., and Lodish, H.F. (2012). Regulation of mammalian cell differentiation by long non-coding RNAs. EMBO reports *13*, 971-983.

Ishibashi, J., and Seale, P. (2010). Medicine. Beige can be slimming. Science *328*, 1113-1114.

Jespersen, N.Z., Larsen, T.J., Peijs, L., Daugaard, S., Homoe, P., Loft, A., de Jong, J., Mathur, N., Cannon, B., Nedergaard, J., et al. (2013). A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. Cell metabolism *17*, 798-805.

Jimenez, M., Barbatelli, G., Allevi, R., Cinti, S., Seydoux, J., Giacobino, J.P., Muzzin, P., and Preitner, F. (2003). Beta 3-adrenoceptor knockout in C57BL/6J mice depresses the occurrence of brown adipocytes in white fat. European journal of biochemistry / FEBS *270*, 699-705.

Kir, S., White, J.P., Kleiner, S., Kazak, L., Cohen, P., Baracos, V.E., and Spiegelman, B.M. (2014). Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia. Nature *513*, 100-104.

Kleiner, S., Mepani, R.J., Laznik, D., Ye, L., Jurczak, M.J., Jornayvaz, F.R., Estall, J.L., Chatterjee Bhowmick, D., Shulman, G.I., and Spiegelman, B.M. (2012). Development of insulin resistance in mice lacking PGC-1alpha in adipose tissues. Proceedings of the National Academy of Sciences of the United States of America *109*, 9635-9640.

Klingenspor, M. (2003). Cold-induced recruitment of brown adipose tissue thermogenesis. Experimental Physiology *88*, 141-148.

Koza, R.A., Hohmann, S.M., Guerra, C., Rossmeisl, M., and Kozak, L.P. (2000). Synergistic gene interactions control the induction of the mitochondrial uncoupling protein (Ucp1) gene in white fat tissue. The Journal of biological chemistry *275*, 34486-34492.

Kozak, L.P. (2011). The Genetics of Brown Adipocyte Induction in White Fat Depots. Frontiers in Endocrinology *2*.64.

Kozak, L.P., and Koza, R.A. (2010). The genetics of brown adipose tissue. Progress in molecular biology and translational science *94*, 75-123.

Kozak, L.P., Koza, R.A., Anunciado-Koza, R., Mendoza, T., and Newman, S. (2012). Inherent plasticity of brown adipogenesis in white fat of mice allows for recovery from effects of post-natal malnutrition. PLoS One *7*, e30392.

Krueger, K.C., Costa, M.J., Du, H., and Feldman, B.J. (2014). Characterization of Cre recombinase activity for in vivo targeting of adipocyte precursor cells. Stem cell reports *3*, 1147-1158.

Lasar, D., Julius, A., Fromme, T., and Klingenspor, M. (2013). Browning attenuates murine white adipose tissue expansion during postnatal development. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids *1831*, 960-968.

Lee, M.W., Odegaard, J.I., Mukundan, L., Qiu, Y., Molofsky, A.B., Nussbaum, J.C., Yun, K., Locksley, R.M., and Chawla, A. (2015). Activated type 2 innate lymphoid cells regulate beige fat biogenesis. Cell *160*, 74-87.

Lee, Y.H., Petkova, A.P., Mottillo, E.P., and Granneman, J.G. (2012). In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. Cell metabolism *15*, 480-491.

Li, Y., Lasar, D., Fromme, T., and Klingenspor, M. (2014a). White, brite, and brown adipocytes: the evolution and function of a heater organ in mammals. Canadian Journal of Zoology *92*, 615-626.

Li, Y., Fromme, T., Schweizer, S., Schottl, T., and Klingenspor, M. (2014b). Taking control over intracellular fatty acid levels is essential for the analysis of thermogenic function in cultured primary brown and brite/beige adipocytes. EMBO reports *15:*1069–1076

Li Y, Bolze F, Fromme T, Klingenspor M (2014c) Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains. Biochim Biophys Acta *1841*: 1345-1352

Lidell, M.E., Betz, M.J., Dahlqvist Leinhard, O., Heglind, M., Elander, L., Slawik, M., Mussack, T., Nilsson, D., Romu, T., Nuutila, P., et al. (2013). Evidence for two types of brown adipose tissue in humans. Nature medicine *19*, 631-634.

Loft, A., Forss, I., Siersbaek, M.S., Schmidt, S.F., Larsen, A.S., Madsen, J.G., Pisani, D.F., Nielsen, R., Aagaard, M.M., Mathison, A., et al. (2015). Browning of human adipocytes requires KLF11 and reprogramming of PPARgamma superenhancers. Genes & development *29*, 7-22.

Lončar, D. (1991). Convertible adipose tissue in mice. Cell and tissue research *266*, 149-161.

Long, J.Z., Svensson, K.J., Tsai, L., Zeng, X., Roh, H.C., Kong, X., Rao, R.R., Lou, J., Lokurkar, I., Baur, W., et al. (2014). A smooth muscle-like origin for beige adipocytes. Cell metabolism *19*, 810-820.

Lowell, B.B., and Spiegelman, B.M. (2000). Towards a molecular understanding of adaptive thermogenesis. Nature *404*, 652-660.

McDonald, M.E., Li, C., Bian, H., Smith, B.D., Layne, M.D., and Farmer, S.R. (2015). Myocardin-related transcription factor a regulates conversion of progenitors to beige adipocytes. Cell *160*, 105-118.

Nedergaard, J., Bengtsson, T., and Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. American journal of physiology. Endocrinology and metabolism *293*, E444-452.

Nedergaard, J., and Cannon, B. (2014). The browning of white adipose tissue: some burning issues. Cell metabolism *20*, 396-407.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F., et al. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet *384*, 766-781.

Nguyen, K.D., Qiu, Y., Cui, X., Goh, Y.P., Mwangi, J., David, T., Mukundan, L., Brombacher, F., Locksley, R.M., and Chawla, A. (2011). Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. Nature *480*, 104-108.

Ohno, H., Shinoda, K., Spiegelman, B.M., and Kajimura, S. (2012). PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell metabolism *15*, 395-404.

Ozanne, S.E., and Hales, C.N. (2004). Lifespan: catch-up growth and obesity in male mice. Nature *427*, 411-412.

Peirce, V., Carobbio, S., and Vidal-Puig, A. (2014). The different shades of fat. Nature *510*, 76-83.

Petrovic, N., Shabalina, I.G., Timmons, J.A., Cannon, B., and Nedergaard, J. (2008). Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARgamma agonist. American journal of physiology. Endocrinology and metabolism *295*, E287-296.

Petrovic, N., Walden, T.B., Shabalina, I.G., Timmons, J.A., Cannon, B., and Nedergaard, J. (2010). Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of

thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. The Journal of biological chemistry *285*, 7153-7164.

Petruzzelli, M., Schweiger, M., Schreiber, R., Campos-Olivas, R., Tsoli, M., Allen, J., Swarbrick, M., Rose-John, S., Rincon, M., Robertson, G., et al. (2014). A switch from white to brown fat increases energy expenditure in cancer-associated cachexia. Cell metabolism *20*, 433-447.

Pfeifer, A., and Hoffmann, L.S. (2015). Brown, Beige, and White: The New Color Code of Fat and Its Pharmacological Implications. Annual review of pharmacology and toxicology *55*, 207–227

Plaisier, C.L., Bennett, B.J., He, A., Guan, B., Lusis, A.J., Reue, K., and Vergnes, L. (2012). Zbtb16 has a role in brown adipocyte bioenergetics. Nutrition and Diabetes *2*, e46.

Powell, E., Kuhn, P., and Xu, W. (2007). Nuclear Receptor Cofactors in PPARgamma-Mediated Adipogenesis and Adipocyte Energy Metabolism. PPAR research *2007*, 53843.

Qiang, L., Wang, L., Kon, N., Zhao, W., Lee, S., Zhang, Y., Rosenbaum, M., Zhao, Y., Gu, W., Farmer, S.R., et al. (2012). Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma. Cell *150*, 620-632.

Qiu, Y., Nguyen, K.D., Odegaard, J.I., Cui, X., Tian, X., Locksley, R.M., Palmiter, R.D., and Chawla, A. (2014). Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. Cell *157*, 1292-1308.

Rao, R.R., Long, J.Z., White, J.P., Svensson, K.J., Lou, J., Lokurkar, I., Jedrychowski, M.P., Ruas, J.L., Wrann, C.D., Lo, J.C., et al. (2014). Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. Cell *157*, 1279-1291.

Rosenwald, M., Perdikari, A., Rulicke, T., and Wolfrum, C. (2013). Bi-directional interconversion of brite and white adipocytes. Nature cell biology *15*, 659-667.

Sanchez-Gurmaches, J., Hung, C.M., Sparks, C.A., Tang, Y., Li, H., and Guertin, D.A. (2012). PTEN loss in the Myf5 lineage redistributes body fat and reveals subsets of white adipocytes that arise from Myf5 precursors. Cell metabolism *16*, 348-362.

Sander, J.D., and Joung, J.K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nature biotechnology *32*, 347-355.

Schulz, T.J., Huang, P., Huang, T.L., Xue, R., McDougall, L.E., Townsend, K.L., Cypess, A.M., Mishina, Y., Gussoni, E., and Tseng, Y.-H. (2013). Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. Nature *495*, 379-383.

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H.M., Erdjument-Bromage, H., et al. (2008). PRDM16 controls a brown fat/skeletal muscle switch. Nature *454*, 961-967.

Seale, P., Conroe, H.M., Estall, J., Kajimura, S., Frontini, A., Ishibashi, J., Cohen, P., Cinti, S., and Spiegelman, B.M. (2011). Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. The Journal of clinical investigation *121*, 96-105.

Shabalina, I.G., Petrovic, N., de Jong, J.M., Kalinovich, A.V., Cannon, B., and Nedergaard, J. (2013). UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. Cell reports *5*, 1196-1203.

Shalem, O., Sanjana, N.E., and Zhang, F. (2015). High-throughput functional genomics using CRISPR-Cas9. Nature Reviews Genetics *16*, 299-311.

Sharp, L.Z., Shinoda, K., Ohno, H., Scheel, D.W., Tomoda, E., Ruiz, L., Hu, H., Wang, L., Pavlova, Z., and Gilsanz, V. (2012). Human BAT Possesses Molecular Signatures That Resemble Beige/Brite Cells. PLoS One *7*, e49452.

Sidossis, L., and Kajimura, S. (2015). Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. Journal of Clinical Investigation *125*, 478-486.

Sugii, S., Olson, P., Sears, D.D., Saberi, M., Atkins, A.R., Barish, G.D., Hong, S.-H., Castro, G.L., Yin, Y.-Q., Nelson, M.C., et al. (2009). PPARγ activation in adipocytes is sufficient for systemic insulin sensitization. Proceedings of the National Academy of Sciences *106*, 22504-22509.

Sun, L., Goff, L.A., Trapnell, C., Alexander, R., Lo, K.A., Hacisuleyman, E., Sauvageau, M., Tazon-Vega, B., Kelley, D.R., Hendrickson, D.G., et al. (2013). Long noncoding RNAs regulate adipogenesis. Proceedings of the National Academy of Sciences of the United States of America *110*, 3387-3392.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Trajkovski, M., and Lodish, H. (2013). MicroRNA networks regulate development of brown adipocytes. Trends in Endocrinology and Metabolism *24*, 442-450.

Tseng, Y.H., Cypess, A.M., and Kahn, C.R. (2010). Cellular bioenergetics as a target for obesity therapy. Nature Reviews Drug Discovery *9*, 465-482.

Ussar, S., Lee, K.Y., Dankel, S.N., Boucher, J., Haering, M.F., Kleinridders, A., Thomou, T., Xue, R., Macotela, Y., Cypess, A.M., et al. (2014). ASC-1, PAT2, and P2RX5 are cell surface

markers for white, beige, and brown adipocytes. Science Translational Medicine *6*, 247ra103.

van der Lans, A.A., Hoeks, J., Brans, B., Vijgen, G.H., Visser, M.G., Vosselman, M.J., Hansen, J., Jorgensen, J.A., Wu, J., Mottaghy, F.M., et al. (2013). Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. Journal of Clinical Investigation *123*, 3395-3403.

van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M., Kemerink, G.J., Bouvy, N.D., Schrauwen, P., and Teule, G.J. (2009). Cold-activated brown adipose tissue in healthy men. New England Journal of Medicine *360*, 1500-1508.

Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N.J., Enerback, S., et al. (2009). Functional brown adipose tissue in healthy adults. New England Journal of Medicine *360*, 1518-1525.

Walden, T.B., Hansen, I.R., Timmons, J.A., Cannon, B., and Nedergaard, J. (2012). Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. American journal of physiology. Endocrinology and metabolism *302*, E19-31.

Wang, G.X., Zhao, X.Y., Meng, Z.X., Kern, M., Dietrich, A., Chen, Z., Cozacov, Z., Zhou, D., Okunade, A.L., Su, X., et al. (2014). The brown fat-enriched secreted factor Nrg4 preserves metabolic homeostasis through attenuation of hepatic lipogenesis. Nature Medicine *20*,1436–1443

Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell *153*, 307-319.

Wu, J., Bostrom, P., Sparks, L.M., Ye, L., Choi, J.H., Giang, A.H., Khandekar, M., Virtanen, K.A., Nuutila, P., Schaart, G., et al. (2012). Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell *150*, 366-376.

Wu, J., Cohen, P., and Spiegelman, B.M. (2013). Adaptive thermogenesis in adipocytes: is beige the new brown? Genes & development *27*, 234-250.

Xu, J., Du, Y., and Deng, H. (2015). Direct lineage reprogramming: strategies, mechanisms, and applications. Cell stem cell *16*, 119-134.

Xue, B., Coulter, A., Rim, J.S., Koza, R.A., and Kozak, L.P. (2005). Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. Molecular and cellular biology *25*, 8311-8322.

Xue, B., Rim, J.S., Hogan, J.C., Coulter, A.A., Koza, R.A., and Kozak, L.P. (2007). Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. Journal of Lipid Research *48*, 41.

Yoneshiro, T., Aita, S., Matsushita, M., Kayahara, T., Kameya, T., Kawai, Y., Iwanaga, T., and Saito, M. (2013). Recruited brown adipose tissue as an antiobesity agent in humans. Journal of Clinical Investigation *123*, 3404-3408.

Young, P., Arch, J.R.S., and Ashwell, M. (1984). Brown adipose tissue in the parametrial fat pad of the mouse. FEBS letters *167*, 10-14.

Zhao, X.Y., Li, S., Wang, G.X., Yu, Q., and Lin, J.D. (2014). A long noncoding RNA transcriptional regulatory circuit drives thermogenic adipocyte differentiation. Molecular cell *55*, 372-382.

Zhou, J.Y., and Li, L. (2014). MicroRNAs are key regulators of brown adipogenesis. Biochim Biophys Acta-Molecular and Cell Biology of Lipids. *1841*, 1590–1595

Zingaretti, M.C., Crosta, F., Vitali, A., Guerrieri, M., Frontini, A., Cannon, B., Nedergaard, J., and Cinti, S. (2009). The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. FASEB journal *23*, 3113-3120.

ACKNOWLEDGEMENTS

This study was performed in the lab of Chair for Molecular Nutritional Medicine, at the Center of Life and Food Sciences Weihenstephan (Technical University of Munich, Germany). I was lucky to have encountered amazing supervisors, colleagues and friends. All people have not only made this PhD study possible but also fill it with energy, happiness and wisdom. I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this dissertation work possible.

First of all, I would like to thank my supervisor Prof. Dr. Martin Klingenspor for providing me this opportunity and sharing your wisdom in science with all the valuable and inspiring discussions. I still remember how thrilled I was when you accepted me as your student. I am also impressed by how much you care about your students, not only in research but also in life.

My special thanks go to Dr. Tobias Fromme, Dr. Florian Bolze and Dr. Monja Willershäuser for their constant support and constructive suggestions, which definitely improved the quality of the work presented in this thesis. Their knowledge, enthusiasm and brilliant mind made them wonderful persons to work with and to learn from.

I am in debt to all members of the Klingenspor lab, past and present, for making the lab a wonderful place to work. Dr. Kathrin Seyfarth, Dr. Christoph Hoffmann, Dr. David Lasar and Patrice Mengue help me a lot when I first came to the lab. Thanks to Nadine Rink, Stefanie Maurer, Sarah-Madeleine Gabler, Caroline Kleß, Krisitina Hüttinger, Theresa Schöttl, Sabine Schweizer and Hui Wang for being great labmates, who are always willing to help. I also want to thank our technicians Anika Zimmerman and Sabine Mocek, not only for their technical assistance, but also for their efforts in keeping the lab running smoothly.

I would like to thank Dr. Petra Catalina Schwalie in Prof. Dr. Bart Deplancke's Lab in Lausanne (Switzerland) for being great collaborator. Her professional bioinformatics data analysis and proofreading of Chapter 6 are sincerely appreciated.

I acknowledge the financial support from Deutscher Akademischer Austauschdienst (DAAD) for my fellowship (2011-2014).

Last, but not least, I thank my family: my parents for unconditional support and encouragement to pursue my interests, even when the interests went beyond boundaries of language and geography; my old sister for taking care of our parents when I was far away from home; my wife and lovely son, for sharing their love, for accompanying me and for understanding when science demanded all my time, energy and focus.

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2. **Li Y,** Lasar D, Fromme T, Klingenspor M (2014). White, brite and brown adipocytes: the evolution and function of a heater organ in mammals**.** *Can J Zool* **92:** 615–626
3. **Li Y**, Bolze F, Fromme T, Klingenspor M (2014) Intrinsic differences in BRITE adipogenesis of primary adipocytes from two different mouse strains. *Biochim Biophys Acta* **1841:** 1345-1352
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EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Characterization of brown-like adipocytes differentiated in primary culture: thermogenic function, molecular basis of variation between strains and transcriptome analysis

am Lehrstuhl für Molekulare Ernährungsmedizin unter der Anleitung und Betreuung durch Univ.-Prof. Dr. Martin Klingenspor ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Die vollständige Dissertation wurde noch nicht veröffentlicht.

Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich einverstanden.

Freising, den 26.06.2015 Unterschrift Kongguo Li