Published in final edited form as:
Aging Cell. 2012 December ; 11(6): . doi:10.1111/acel.12010.

Aging Leads to a Programmed Loss of Brown Adipocytes in Murine Subcutaneous White Adipose Tissue

Nicole H Rogers, **Alejandro Landa**, **Seongjoon Park**[*], and **Roy G Smith**
Department of Metabolism and Aging, Scripps Research Institute Florida, Jupiter, FL, 33458

Alejandro Landa: aland008@fiu.edu; Seongjoon Park: psj10262000@yahoo.co.kr

SUMMARY

Insulin sensitivity deteriorates with age, but mechanisms remain unclear. Age-related changes in the function of subcutaneous white adipose tissue (sWAT) are less characterized than those in visceral WAT. We hypothesized that metabolic alterations in sWAT, which in contrast to epididymal WAT, harbors a sub-population of energy dissipating UCP1+ brown adipocytes, promote age-dependent progression towards insulin resistance. Indeed, we show that a predominant consequence of aging in murine sWAT is loss of "browning." sWAT from young mice is histologically similar to brown adipose tissue (multilocular, UCP1+), but becomes morphologically white by 12 months of age. Correspondingly, sWAT expression of *ucp1* precipitously declines (~300-fold) between 3 and 12 months. Loss continues into old age (24 months), and is inversely correlated with the development of insulin resistance. Additional age-dependent changes in sWAT include lower expression of *adbr3* and higher expression of *maoa,* suggesting reduced local adrenergic tone as a potential mechanism. Indeed, treatment with a ®3-adrenergic agonist to compensate for reduced tone rescues the aged sWAT phenotype. Age-related changes in sWAT are not explained by differences in body weight; mice subjected to 40% caloric restriction for 12 months are of similar body weight to 3 month-old *ad lib* fed mice, but display sWAT resembling that of age-matched *ad lib* fed mice (devoid of brown adipose-like morphology). Overall, findings identify loss of "browning" in sWAT as a new aging phenomenon, and provide insight into the pathogenesis of age-associated metabolic disease by revealing novel molecular changes tied to systemic metabolic dysfunction.

Keywords

ucp1; cidea; caloric restriction; ghsr; ppara; klf15

Corresponding authors: Roy G Smith, PhD, 130 Scripps Way #3B3, Jupiter, FL 33458, Tel: 561-228-2950, Fax: 561-228-3059, rgsmith@scripps.edu. Nicole Rogers, PhD, 130 Scripps Way #3B3, Jupiter, FL 33458, Tel: 561-228-2973, Fax: 561-228-3059, nrogers@scripps.edu.
[*]Current address: Department of Investigative Pathology, Unit of Basic Medical Science, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki City 852-8523, Japan

AUTHOR CONTRIBUTIONS
NR performed experiments, with assistance from AL and SP. NR and RS designed experiments and wrote the manuscript.

SUPPORTING INFORMATION
The following are available through the online edition:
Supplementary experimental procedures
Supplementary table 1
Supplementary figures S1–S7

INTRODUCTION

Insulin resistance increases during aging. Although obesity often leads to insulin resistance, many under-weight older individuals develop diabetes; hence, qualitative changes in adipose tissue, which has considerable function outside of energy storage, are potentially important in the development of age-associated diabetes.

Adipose tissue can be broadly categorized as white (WAT) or brown (BAT). WAT serves as the primary reservoir for lipid storage, but is also an active endocrine organ, with deposits under the skin (subcutaneous, sWAT) and around internal organs (visceral). In contrast, BAT primarily functions to produce heat and is found predominantly between the scapulae of mice and in the supraclavicular/thoracic regions of adult humans (Cypess et al., 2009). The adipocytes that comprise WAT and BAT display several distinguishing features. White adipocytes contain a single ("unilocular") large lipid droplet, few mitochondria, and little cytosol, whereas brown adipocytes have numerous smaller ("multilocular") lipid droplets, abundant mitochondria, and normal cytosolic volume. Brown adipocytes are also defined by high expression of uncoupling protein-1 (UCP1), a mitochondrial protein that provides these cells with the unique ability to dissipate large quantities of energy as heat (thermogenesis) (Cannon and Nedergaard, 2004).

Importantly, UCP1+ adipocytes are found interspersed within WAT. These brown-like adipocytes, often described using terms such as "brite" (brown in white) (Petrovic et al., 2010) or "beige" (Ishibashi and Seale, 2010), can be induced with adrenergic stimuli or exposure to the cold, reportedly via β3 adrenergic receptor (β3AR)-mediated transdifferentiation of white adipocytes (Barbatelli et al., 2010; Himms-Hagen et al., 2000), although this remains a topic of debate (Petrovic et al., 2010). It has recently been reported that brown adipocytes residing within classic BAT depots share lineage with skeletal muscle, based on tracing studies that revealed a common precursor positive for the myogenic marker *myogenic factor 5* (*myf5*). In contrast, brown adipocytes residing in WAT do not display similar evidence of *myf5+* lineage (Timmons et al., 2007).

Accumulating evidence suggests UCP1 is physiologically relevant outside of thermoregulation. *Ucp1−/−* mice display metabolic abnormalities that include impaired diet-induced thermogenesis (Feldmann et al., 2009) and increased susceptibility to diet-induced obesity with age (Kontani et al., 2005), whereas transgenic mice overexpressing *ucp1* are leaner and more insulin sensitive than wild type mice (Kozak and Anunciado-Koza, 2008). Similarly in humans, a polymorphism that reduces *ucp1* expression is linked to obesity (Sramkova et al., 2007), and high expression of *ucp1* specifically in sWAT is associated with improved glucose tolerance in obese individuals (Timmons and Pedersen, 2009). *Ucp1* is thus an attractive therapeutic target, holding particular promise for the treatment of type 2 diabetes.

With advancing age, loss of BAT activity occurs in both rodents (Yamashita et al., 1999) and humans (Saito et al., 2009; Yoneshiro et al., 2011). Aging is also associated with increased production of pro-inflammatory signals in visceral WAT (Wu et al., 2007). However, little is known about consequences of aging specific to sWAT. The present studies demonstrate that a predominantly brown-like phenotype in sWAT from young adult mice is progressively lost during aging, and changes that occur later in life are linked to the development of age-related insulin resistance.

RESULTS

Loss of "browning" is a predominant effect of aging in murine sWAT

Sections of sWAT from six week (wk) old male C57Bl/6J mice stained with hematoxylin and eosin (H&E) reveal significant "brown-like" morphology, with prominent pink cytosolic staining surrounding multilocular adipocytes. In contrast, sWAT from mice at 12 months (mo) of age displays morphology that is uniformly white (Fig. 1A). Consistent with the brown-like morphology, brown-adipocyte specific gene expression is abundant in young sWAT, with transcript levels of *ucp1* and *cell death-inducing DFFA-like factor-a* (*cidea*) 133-fold and 3-fold lower, respectively, than that of interscapular brown adipose tissue (BAT) from the same animals (Fig. S1). UCP1 protein is also readily detected in sWAT from 6 wk old mice (Fig. 1B).

However, by 12 mo of age, a marked reduction in *ucp1* (276-fold) and *cidea* (42-fold) expression is observed in sWAT (Fig. 1C), but not BAT (Fig. S2). In contrast, sWAT levels of non-brown specific gene family members are either unchanged (*ucp2/3*), or modestly induced (1.5-fold, *cidec*) with age (Fig. 1C). Consistent with a reduction in oxidative brown adipocytes, sWAT from older mice displays lower expression of brown-specific oxidative enzymes *cytochrome c oxidase subunit VIIa polypeptide 1* (*cox7a1,* 17-fold, $p<0.01$) and *cytochrome c oxidase subunit VIIIb* (*cox8b,* 8-fold, $p<0.001$) (Fig. 1C).

Using PCR-arrays to simultaneously quantify expression of 84 genes related to white and/or brown adipogenesis in sWAT from old vs. young mice, we find the most pronounced effect of aging is reduced expression of *ucp1* (246-fold; Fig. 1D, Table 1). Additional genes significantly down-regulated with age include *fatty acid synthase* (*fasn,* 10-fold), *peroxisome proliferator-activated receptor alpha* (*ppara,* 8-fold), *krüppel-like factor 15 (klf15*, 8-fold), *angiotensinogen* (*agt,* 7-fold), *adipsin* (*cfd,* 5-fold), and *deiodinase iodothyronine, type II* (*dio2,* 4-fold), as well as adrenergic signaling modulators beta-2-adrenergic receptor (adrb2, 8-fold) and cAMP responsive element binding protein (creb1, 1.8-fold) (Fig. 1D, Table 1). Further, levels of CCAT/enhancer binding protein, beta (cebpb), vitamin d receptor (vdr), early growth response 2 (egr2), tsc22 domain family, member 3 (tsc22d3), and sirtuin 2 (sirt2) are modestly reduced (2–3-fold, $p < 0.05$) in sWAT from older animals (Fig. 1D, Table 1).

Consistent with depot expansion, as well as brown-to-white transitioning, genes induced with age include *leptin* (*lep*, 28-fold), *secreted frizzled-related protein 5* (*sfrp5*, 43-fold) and *fibroblast growth factor 1* (*fgf1*, 7-fold) (Fig. 1D, Table 1). A comprehensive list of PCR-array findings can be found in supplementary table 1, and QPCR confirmation of select genes produced very similar results (Fig. S3). Taken together, PCR-array findings indicate the most pronounced adipogenic change during aging is a decline in the expression of genes that favor brown-adipogenesis (Fig. 1D, Table 1).

As lower *adrb2* expression in sWAT from older mice suggests that loss of browning with age could be due to decreased adrenergic tone, we quantified expression of other *beta adrenergic receptors* in sWAT from young and old mice. Levels of *adrb3*, but not *adrb1*, similarly decline with age (4-fold; Fig. 1E). Additionally, we find that sWAT expression of *monoamine oxidase a* (*maoa*) is ~3-fold higher in the older animals (Fig. 1E). In contrast, *tyrosine hydroxylase* (*th*) levels are similar in sWAT from young and old mice (Fig. 1E), as are circulating concentrations of adrenaline and noradrenaline (Fig. S4).

Because sWAT expression of *dio2* declines with age, we investigated how aging impacts other components of the thyroid axis. Interestingly, we observe 42% higher triiodothyronine ($p < 0.01$) and 66% higher thyroxine ($p = 0.03$) levels in plasma from the older mice (Fig.

S4). Likewise, sWAT expression of *thyroid hormone receptor b* (*thrb*), but not *thyroid hormone receptor* (*thra*), is significantly increased (2-fold) with age (Fig. 1E).

Additional loss of ucp1 expression in sWAT between 1 and 2 years of age is inversely related to the development of age-associated hyperglycemia

A further 15-fold reduction in sWAT *ucp1* expression is evident from 1-to-2 yrs of age ($p < 0.05$; Fig. 2A). Consistent with the development of age-associated insulin resistance, 2 yr old mice also display significantly elevated blood glucose levels (Fig. 2B). Importantly, correlation analyses highlight a significant inverse association (Spearman $r = -0.83$; $p = 0.015$) between the loss of *ucp1* in sWAT and elevated blood glucose levels (Fig. 2C).

Age-related loss of ucp1 expression is first evident at 4 months of age and specific to sWAT

To identify more precisely when brown-to-white transitioning begins, we studied mice at 6, 9, 12, 16, 20, or 26 weeks (wk) of age. As expected, body weight increases steadily from 6-to- 26 wk (Fig. 3A). sWAT expression of *ucp1* is similar at 6, 9, and 12 wk, but lower at 16 (8-fold) and 26 wk (87-fold), compared to 6 wk. In addition, *cidea* (17-fold), *cox7a1* (10-fold) and *cox8b* (7-fold) levels are significantly lower at 26 wk (Fig. 3B). Accordingly, H&E stained sWAT sections reveal predominant brown adipose-like characteristics at 6 and 16 wk, features consistent with white adipocytes becoming more frequent at 20 wk, and white morphology that is largely exclusive by 26 wk (Fig. 3C).

From 6-to-20 wk of age, sWAT accumulation is indistinguishable from epididymal WAT (eWAT), with eWAT outgrowing sWAT by 26 wk (Fig. 4A). Consistent with this, production of white mediators (*lep*, *srfp5*) increases similarly with age in sWAT and eWAT (Fig. 4B). However, in contrast to sWAT, *ucp1* levels in eWAT are very low and do not change as a function of age (Fig. 4C). As a result, the > 1000:1 ratio of *ucp1* expression in sWAT: eWAT at 12 wk decreases to 6:1 by 26 wk (age x depot interaction 'p_i' = 0.006). Likewise, the depot differential for *cidea* is >100:1 at 6 wk, but diminishes to 6:1 by 26 wk ($p_i = 0.028$), with similar expression patterns evident for other brown-specific genes *cox7a1* and *cox8b* (Fig. 4C).

As both sWAT and eWAT display similar overall growth, but only the former is undergoing brown-to-white transitioning, we compared age-dependent variation in adipogenic gene expression between these depots. Pro-brown factor *klf15* displays an expression pattern most consistent with that of the brown-specific genes, with a significant age x depot interaction ($p_i = 0.03$). Levels are significantly higher in sWAT than eWAT at 6, 9, and 12 wk, and age- dependent loss is exclusive to sWAT at (26 vs 6 wk $p < 0.05$) (Fig. 4D). *Ppara* expression also displays a significant age x depot interaction ($p_i = 0.05$), with lower concentrations in sWAT at 9 and 26 wk (vs 6 wk), and significantly higher expression in sWAT than eWAT at 6 and 12 wk (Fig. 4D). In contrast, *pparg2* expression is similar in sWAT and eWAT, with age-related induction evident at 20 and 26 wk (vs respective 6 wk; Fig. 4D). *Ppargc1a,* notably more abundant in sWAT at 9 wk only, does not vary with age in either depot. Finally, *dio2* expression transiently increases in both depots at 9 wk (vs 6 wk), although this is statistically significant only in sWAT (Fig. 4D).

Loss of ucp1 expression in sWAT with age is independent of adiposity

To address a potential relationship between age-related loss of sWAT browning and associated increases in adiposity, we next studied mice displaying varying degrees of age-associated weight gain. Mice lacking the *growth hormone secretagogue receptor* (*ghsr −/−*) are indistinguishable from *ghsr +/+* mice at 6 wk, but gain significantly less weight by one year of age (Fig. 5A). Consistent with less adiposity, *lep* (4-fold) and *sfrp5* (2-fold) levels

are significantly lower in sWAT from the older *ghsr −/−* mice, compared to age-matched *ghsr +/+* littermates (Fig. 5B). Despite these differences in weight gain, age-induced loss of *ucp1* in *ghsr −/−* mice is similar to that of *ghsr +/+* mice (316- vs 276-fold; age x genotype interaction $p_i = 0.34$), with analogous findings for *cidea*, *cox7a1* and *cox8b* (Fig. 5C), suggesting weight gain is not a critical factor for age-dependent loss of brown adipocytes in sWAT.

We next used a restrictive feeding paradigm to more fully eliminate weight gain as a causative factor in age-related changes in sWAT. Mice fed a 40% calorically restricted ('CR') diet for one year (initiated at 10 wk of age) were compared to ad libitum fed age-matched ('AM') littermates, as well as 12 wk old non-restricted mice with a mean body-weight (BW) matching that of the CR group (weight-matched, 'WM'). CR (22.7 g) and WM mice (22.7 g) are substantially smaller than AM mice (40.5 g), with ~75% less sWAT after normalizing for BW (Fig. 5D). Consistent with little change in adiposity, *lep* and *sfrp5* induction is > 90% precluded in the CR mice (Fig. 5E). Conversely, we observe only a very slight attenuation (< 1%) of age- associated changes in *ucp1* expression with CR (Fig. 5F), although loss of *cidea, cox7a1,* and *cox8b* expression is partially (~50%) attenuated (Fig. 5F). Again utilizing adipogenesis-targeted PCR arrays, we find CR also induces other genes we previously show decrease with age, including *ppara, fasn, acacb,* and *cfd* ($p < 0.05$ vs AM; Fig. S5).

Consistent with the *ucp1* expression data, H&E staining indicates sWAT from CR mice lacks the significant BAT-like areas we find characteristic of younger animals. Instead, CR tissue has little cytosolic staining and appears more similar to AM, but with smaller cells (Fig. 5G). However, in sWAT from CR mice only, we find occasional cells that appear multilocular (indicated by arrows in Fig. 5G). Similar multilocular cells are also observed in eWAT from CR mice (Fig. S6).

Older mice maintain the ability to induce brown adipocytes in response to adrenergic stimuli

Exogenous stimulation of the β3-adrenoreceptor (β3AR) is an appreciated means of inducing brown adipocytes within WAT (Barbatelli et al., 2010; Himms-Hagen et al., 2000). Because older mice exhibit substantially reduced brown-like characteristics, we wondered if the ability to induce *ucp1+* brown adipocytes in sWAT remains intact with age. To test whether older mice, maintain the ability to produce *ucp1+* brown adipocytes in response to adrenergic stimuli, we injected 13 mo old mice with the highly selective β3AR agonist CL 316,243 (CL) once daily (0.1 mg/kg) for seven days. Indeed, CL-treatment elicits the appearance of multilocular adipocytes and UCP1 immunoreactivity in sWAT from older mice (Fig. 6A–B). In addition, compared to those receiving the saline vehicle, mice receiving CL display significantly increased *ucp1, cidea, cox7a1, cox8b, dio2* and *ppara* mRNA expression in sWAT (Fig. 6C), with similar changes evident in eWAT (Fig. S7).

DISCUSSION

In the present study, we describe an inverse association between age and brown-like characteristics in WAT. Although it has been previously suggested that UCP1 expression is detectable only in WAT from very young mice (Rossmeisl et al., 2002), to our knowledge, we present the first detailed characterization of how aging affects the sWAT adipocyte phenotype. We demonstrate that with increasing age, there is a progressive loss of *ucp1+* brown adipocytes in murine sWAT, but not eWAT or interscapular BAT.

C57Bl/6J mice have completed maturational growth and are "mature adults" from 3 to 6 mo of age, with the human equivalent of 20–30 yrs, and middle-aged from 10–14 mo,

corresponding to 38–47 yr old humans (Flurkey K, 2007). We find that sWAT expression of *ucp1* decreases nearly 300-fold between the ages of 3 and 12 mo, followed by another 15-fold by 2 yrs of age. The pronounced changes we observe during early aging are important, as cellular events that link aging to associated metabolic disease are likely to begin prior to the development of systemic consequences. But additionally, and in further support of such a link, we observe a significant inverse relationship between the loss of *ucp1* that occurs later in life and associated increases in circulating glucose levels. Although it remains to be determined whether the phenotypic changes we have identified in sWAT are causal to the development of age-associated insulin resistance, these correlative findings highlight the relevance of sWAT to age-related metabolic disease, and provide an important foundation for subsequent mechanistic studies.

The complexities of brown adipogenesis are not well understood, but WAT contains a progenitor rich stroma (Hauner, 2005), including those that can be induced *in vitro* to differentiate into fully functional brown adipocytes (Elabd et al., 2009). Although an age-induced loss of appropriate precursors would preclude brown adipogenesis, older mice maintain the ability to produce brown-like cells in response to β3 agonism, arguing against this scenario. Unfortunately, whether brown adipocytes in sWAT arise from a unique progenitor or transdifferentiation of white adipocytes remains unclear, although the latter is supported for brown adipocytes induced in response to cold (Barbatelli et al., 2010). However, the source of basal brown adipocytes might differ from the ones that can be induced. In fact, an interesting question underscored by the present study is whether basal brown adipocytes present in young animals reflect a unique type of brown cell found within WAT, or instead, are inherently similar to the induced cells. A more detailed comparison of these two types of brown adipocytes within WAT is an important future study.

Promoter regions of *ucp1* (Barbera et al., 2001) and *cidea* (Viswakarma et al., 2007) contain PPAR-responsive elements, and both PPAR α and PPAR γ (Nedergaard et al., 2005) can promote brown adipogenesis (Villarroya et al., 2007). However, we find that expression of *ppara,* rather than *pparg,* declines in sWAT during aging, supporting a role for PPAR α in age- dependent shifts in sWAT. In addition to *ppara* and *ucp1,* genes reduced in sWAT from older mice include *cox7a1, cox8b,* and *dio2,* which intriguingly, are all regulated by adrenergic tone.

The sympathetic nervous system (SNS) acts through cell surface β adrenergic receptors to modulate cAMP production, which then bind cAMP responsive-elements within the *ucp1* promoter (reviewed in (Richard and Picard, 2011)). Although circulating catecholamine levels are unchanged, we observe attenuated sWAT expression of *adrb2/3*, as well as *creb1,* a down- stream modulator of adrenergic signaling. Additionally, expression of *maoa*, an enzyme that degrades endogenous catecholamines, is higher in sWAT from older mice. Collectively, these changes could promote relevant loss of adrenergic tone during aging.

Indeed, the sWAT aging phenotype is rescued by treating older mice with a β3AR agonist. Thus, by administering an exogenous agonist that is unlikely to be degraded by *maoa,* reduced adrenergic tone can be overcome. It is interesting that pharmacological activation of β3ARs induces browning in both sWAT and eWAT; however, this does not preclude endogenous depot differences that mediate selective WAT browning physiologically.

Acting synergistically with the SNS, the thyroid axis also modulates thermogenesis (Silva, 1995), with thyroid-hormone responsive elements likewise present in the *ucp1* promoter (Rabelo et al., 1995). Decreased expression of *dio2* in older animals suggests an age-dependent decline in local concentrations of the active thyroid hormone triiodothyronine. Counterintuitively, we find circulating levels of both triiodothyronine and thyroxine are

elevated in older animals, as are sWAT levels of *thrb*. Perhaps this reflects age-dependent development of peripheral thyroid hormone resistance, which has been hypothesized to occur in humans (Mooradian, 2008). While intriguing, determining if such resistance plays a causal role in age-dependent shifts in the phenotype of sWAT presently awaits future study.

A number of genes significantly reduced in sWAT with age are PPAR γ target genes, including *fasn*, *klf15*, *agt*, and *cfd*. However, transcript levels of *peroxisome proliferator-activated receptor gamma 2* (*pparg2*) do not vary with age. Reduced expression of these genes could be explained by altered interactions of PPAR γ with transactivating partners. Indeed, over 300 cofactors have been identified that can associate with PPAR γ and modulate gene transactivation, and many have been implicated in brown adipogenesis. In eWAT, total PPAR γ levels are maintained upon aging, but expression of an important cofactor, *ncoa1* (*src-1),* decreases with age, resulting in impaired cofactor recruitment to PPAR γ and dissociation of adipogenic and insulin sensitizing gene regulation (Miard et al., 2009). However, in sWAT we find an increase, rather than decrease, in *src* expression with age. PPAR γ induced *ucp1* expression is also modulated by interactions with PGC1 α (Puigserver et al., 1998), and exogenously introducing PGC1a causes human white adipocytes to develop brown-like characteristics (Tiraby et al., 2003). However, we do not observe a significant decrease in *pgc1a* expression in sWAT during aging. Therefore, while we cannot preclude a role for PPAR γ in loss of sWAT browning with age, our data is more supportive of a role for PPAR α.

Recently, members of the krüppel-like zinc-finger (KLF) family of transcription factors have been highlighted as important regulators of adipogenesis. Of particular relevance, *klf15* can activate transcription of *ucp1* by directly interacting with the promoter (Yamamoto et al., 2010). Consistent with this, we find substantially down-regulated expression of *klf15,* but not *klf3* or *klf4,* in sWAT from older mice. In fact, *klf15* expression patterning is collectively quite analogous to that of *ucp1,* supporting a regulatory role for *klf15*, with expression significantly higher in sWAT than eWAT at 6, 9, and 12 wks, and not significantly modulated by CR. However, others have found that overexpression of *klf15* in white adipose tissue does not increase levels of *ucp1,* although increased oxygen consumption in adipocytes was observed (Nagare et al., 2011). It is possible that *klf15* can modulate an oxidative program in adipocytes, but additional factors are ultimately required for the full brown phenotype. Indeed, it has been suggested that small changes in the levels of different transcriptional components of the *ucp1* enhanceosome interact synergistically to achieve large differences in *ucp1* expression (Xue et al., 2005). Thus, the various changes in gene expression we observe in sWAT during aging might collectively be a strong enough impetus to cause the dramatic decline in *ucp1* expression. Further studies will be necessary to fully elucidate molecular mechanisms that promote age- dependent shifts in the phenotype of sWAT.

Interestingly, we find that age-related loss of *ucp1* expression in sWAT does not require any associated weight gain. It is peculiar that chronic CR, which is known to slow aging, does not more substantially attenuate loss of *ucp1.* However, expression is slightly elevated in CR vs AM mice, which could reflect a slower rate of aging, and if so, perhaps analysis performed after a shorter duration of restrictive feeding would reveal a more pronounced effect. It is also possible that other mechanisms inhibit expression of *ucp1* in the CR mice, as this protein promotes an energetically inefficient metabolism, which might be unfavorable when survival requires an exquisitely efficient use of fuel.

Despite low *ucp1* expression, we observe a few multilocular cells in sWAT from CR mice. Others have reported the appearance of similar infrequent multilocular cells in eWAT of mice subjected to long-term CR and suggested these cells could reflect a brown/white

intermediate (Higami et al., 2004). Indeed, in contrast to *ucp1,* changes in the genes less exclusive to brown adipocytes (*cidea, cox7a1, cox8b*) are considerably attenuated with CR. Moreover, the frequency of these 'intermediate' adipocytes is similar in sWAT and eWAT, further supporting a flexible phenotype of white adipocytes. Finally, sWAT from CR mice displays elevated expression of the regulatory factor *ppara*, as well as target genes *fasn, acacb* and *srebf1*. Although preferentially expressed in brown adipocytes, *ppara* is also found in white adipocytes and is required for oxidative gene induction in murine sWAT during white-to-brown transitioning in response to the cold (Li et al., 2005).

In summary, we demonstrate brown-to-white transitioning in murine sWAT during aging. Collectively, our data support loss of adrenergic tone as a potential mechanism, as evidenced by age-dependently reduced expression of adrenergic receptors and increased local production of *maoa* in sWAT, and reversal of the phenotype by administering exogenous β3AR agonist. This agonist is also an appreciated insulin sensitizer (Bloom et al., 1992; de Souza et al., 1997), but attributing a theoretical benefit in an aging setting to sWAT is complicated by the fact that β3ARs are present in other metabolic tissues, including BAT (Arch, 1989) and skeletal muscle (Chamberlain et al., 1999). Alternatively, our data suggest that therapeutic strategies specifically targeting the browning of sWAT might be useful in treating metabolic diseases of aging, as we observe a negative correlation between loss of *ucp1* specifically in sWAT and the development of age-associated insulin resistance.

Supporting the relevance of our findings to humans, *ucp1* expression in sWAT from obese individuals is higher in those without diabetes, which intriguingly suggests that *ucp1* expression in human sWAT may promote metabolic homeostasis (Timmons and Pedersen, 2009). In future studies, it would be interesting to determine if *ucp1* expression in sWAT from elderly individuals is similarly correlated with insulin sensitivity. Pharmaceutically increasing UCP1 activity presently holds promise for treating obesity-associated metabolic disease, and based on the current findings, perhaps also metabolic diseases of aging.

EXPERIMENTAL PROCEDURES

Animals

All experiments were approved by the Scripps Florida Institutional Animal Care and Use Committee. Male C57Bl/6J mice were housed in AAALAC-approved facilities maintained at 23°C with 12-h light/dark cycles, and unless otherwise noted, given free access to water and standard chow. Blood glucose was measured using a glucometer (OneTouchUltra, LifeScan Inc Milpitas, CA). After sacrifice with CO_2, blood was taken via cardiac puncture and bilateral (lower quadrant) inguinal subcutaneous white adipose tissue (lymph nodes removed), epididymal white adipose tissue, and interscapular brown adipose tissue (visible white fat trimmed away) were carefully collected. *Ghsr −/−* mice were generated as previously described (Sun et al., 2008).

Real-time RT-PCR

Real-time quantitative PCR was performed (7900HT, Applied Biosystems, Foster City, CA) using commercially available Taqman assays (Applied Biosystems, Foster City, CA). Fold changes were calculated as $2^{-\Delta\Delta CT}$ with *36B4* and/or *TATA-box binding protein* (*tbp*) used as endogenous controls.

PCR arrays

400 ng of total RNA used to perform 'adipogenesis' (#PAMM-049) PCR arrays (Qiagen, Valencia, CA) according to manufacturer's instructions without modification.

Histological analyses

Excised sWAT was fixed overnight with Z-fix (Anatech LTD, Battle Creek, MI). The Scripps Research Institute histology core embedded samples in paraffin, sectioned (3 uM) tissue, and performed H&E staining. For immunostaining, sections were blocked with 5% donkey serum in 0.2 % triton-X PBS (1 h), and incubated with rabbit polyclonal UCP1 antibody (Abcam, Cambridge, MA) overnight at 4°C (1:100), followed by DyLight 649-conjugated donkey anti- rabbit IgG (Jackson Immunoresearch, West Grove, PA) for 1 h (1:250). Hard-set medium with DAPI (Vector Laboratories Inc, Burlingame, CA) used to apply coverslips before imaging slides with laser scanning confocal microscopy (Olympus Fluoview FV1000, Center Valley, PA).

Statistical analyses

All data are presented as means + standard error of the mean. T-tests or ANOVA (Newman-Keuls procedure for post hoc testing) were used as appropriate with $p \leq 0.05$ considered significant (GraphPad Prism v5, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Scripps ARC for excellent animal care and Donny Strosberg, PhD for kindly providing CL 316,243. We gratefully acknowledge funding support by the NIA: R01AG29740 and R01AG19230.

References

Arch JR. The brown adipocyte beta-adrenoceptor. Proc Nutr Soc. 1989; 48:215–223. [PubMed: 2552450]

Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, Giacobino JP, De Matteis R, Cinti S. 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. 2010; 298:E1244–1253. [PubMed: 20354155]

Barbera MJ, Schluter A, Pedraza N, Iglesias R, Villarroya F, Giralt M. Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. The Journal of biological chemistry. 2001; 276:1486– 1493. [PubMed: 11050084]

Bloom JD, Dutia MD, Johnson BD, Wissner A, Burns MG, Largis EE, Dolan JA, Claus TH. Disodium (R, R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino] propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A potent beta-adrenergic agonist virtually specific for beta 3 receptors. A promising antidiabetic and antiobesity agent. Journal of medicinal chemistry. 1992; 35:3081–3084. [PubMed: 1354264]

Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004; 84:277–359. [PubMed: 14715917]

Chamberlain PD, Jennings KH, Paul F, Cordell J, Berry A, Holmes SD, Park J, Chambers J, Sennitt MV, Stock MJ, et al. The tissue distribution of the human beta3-adrenoceptor studied using a monoclonal antibody: direct evidence of the beta3- adrenoceptor in human adipose tissue, atrium and skeletal muscle. International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity. 1999; 23:1057–1065. [PubMed: 10557026]

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009; 360:1509–1517. [PubMed: 19357406]

de Souza CJ, Hirshman MF, Horton ES. CL-316,243, a beta3-specific adrenoceptor agonist, enhances insulin-stimulated glucose disposal in nonobese rats. Diabetes. 1997; 46:1257–1263. [PubMed: 9231648]

Elabd C, Chiellini C, Carmona M, Galitzky J, Cochet O, Petersen R, Penicaud L, Kristiansen K, Bouloumie A, Casteilla L, et al. Human multipotent adipose- derived stem cells differentiate into functional brown adipocytes. Stem Cells. 2009; 27:2753–2760. [PubMed: 19697348]

Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009; 9:203–209. [PubMed: 19187776]

Flurkey, K.; CJ; Harrison, DE. The mouse in aging research. In: Fox, BSJG.; Davisson, MT.; Newcomer, CE.; Quimby, FW.; Smith, AL., editors. The Mouse in Biomedical Research. Burlington, MA: Elsevier Academic Press; 2007. p. 637-672.

Hauner H. Secretory factors from human adipose tissue and their functional role. Proc Nutr Soc. 2005; 64:163–169. [PubMed: 15960861]

Higami Y, Pugh TD, Page GP, Allison DB, Prolla TA, Weindruch R. Adipose tissue energy metabolism: altered gene expression profile of mice subjected to long-term caloric restriction. The FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2004; 18:415–417.

Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. Am J Physiol Cell Physiol. 2000; 279:C670–681. [PubMed: 10942717]

Ishibashi J, Seale P. Medicine. Beige can be slimming. Science. 2010; 328:1113–1114. [PubMed: 20448151]

Kontani Y, Wang Y, Kimura K, Inokuma KI, Saito M, Suzuki-Miura T, Wang Z, Sato Y, Mori N, Yamashita H. UCP1 deficiency increases susceptibility to diet-induced obesity with age. Aging Cell. 2005; 4:147–155. [PubMed: 15924571]

Kozak LP, Anunciado-Koza R. UCP1: its involvement and utility in obesity. Int J Obes (Lond). 2008; 32(Suppl 7):S32–38. [PubMed: 19136989]

Li P, Zhu Z, Lu Y, Granneman JG. Metabolic and cellular plasticity in white adipose tissue II: role of peroxisome proliferator-activated receptor-alpha. American journal of physiology Endocrinology and metabolism. 2005; 289:E617–626. [PubMed: 15941786]

Miard S, Dombrowski L, Carter S, Boivin L, Picard F. Aging alters PPARgamma in rodent and human adipose tissue by modulating the balance in steroid receptor coactivator-1. Aging Cell. 2009; 8:449–459. [PubMed: 19485965]

Mooradian AD. Asymptomatic hyperthyroidism in older adults: is it a distinct clinical and laboratory entity? Drugs Aging. 2008; 25:371–380. [PubMed: 18447402]

Nagare T, Sakaue H, Matsumoto M, Cao Y, Inagaki K, Sakai M, Takashima Y, Nakamura K, Mori T, Okada Y, et al. Overexpression of KLF15 Transcription Factor in Adipocytes of Mice Results in Down-regulation of SCD1 Protein Expression in Adipocytes and Consequent Enhancement of Glucose-induced Insulin Secretion. The Journal of biological chemistry. 2011; 286:37458–37469. [PubMed: 21862590]

Nedergaard J, Petrovic N, Lindgren EM, Jacobsson A, Cannon B. PPARgamma in the control of brown adipocyte differentiation. Biochimica et biophysica acta. 2005; 1740:293–304. [PubMed: 15949696]

Petrovic N, Walden TB, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. 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. 2010; 285:7153–7164. [PubMed: 20028987]

Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998; 92:829–839. [PubMed: 9529258]

Rabelo R, Schifman A, Rubio A, Sheng X, Silva JE. Delineation of thyroid hormone-responsive sequences within a critical enhancer in the rat uncoupling protein gene. Endocrinology. 1995; 136:1003–1013. [PubMed: 7867554]

Richard D, Picard F. Brown fat biology and thermogenesis. Front Biosci. 2011; 16:1233–1260.

Rossmeisl M, Barbatelli G, Flachs P, Brauner P, Zingaretti MC, Marelli M, Janovska P, Horakova M, Syrovy I, Cinti S, et al. Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. Eur J Biochem. 2002; 269:19–28. [PubMed: 11784294]

Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, Iwanaga T, Miyagawa M, Kameya T, Nakada K, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes. 2009; 58:1526–1531. [PubMed: 19401428]

Silva JE. Thyroid hormone control of thermogenesis and energy balance. Thyroid. 1995; 5:481–492. [PubMed: 8808101]

Sramkova D, Krejbichova S, Vcelak J, Vankova M, Samalikova P, Hill M, Kvasnickova H, Dvorakova K, Vondra K, Hainer V, et al. The UCP1 gene polymorphism A- 3826G in relation to DM2 and body composition in Czech population. Exp Clin Endocrinol Diabetes. 2007; 115:303–307. [PubMed: 17516293]

Sun Y, Butte NF, Garcia JM, Smith RG. Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology. 2008; 149:843–850. [PubMed: 18006636]

Timmons JA, Pedersen BK. The importance of brown adipose tissue. N Engl J Med. 2009; 361:415–416. author reply 418–421. [PubMed: 19625723]

Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proc Natl Acad Sci U S A. 2007; 104:4401–4406. [PubMed: 17360536]

Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, Langin D. Acquirement of brown fat cell features by human white adipocytes. J Biol Chem. 2003; 278:33370–33376. [PubMed: 12807871]

Villarroya F, Iglesias R, Giralt M. PPARs in the Control of Uncoupling Proteins Gene Expression. PPAR Res. 2007; 2007:74364. [PubMed: 17389766]

Viswakarma N, Yu S, Naik S, Kashireddy P, Matsumoto K, Sarkar J, Surapureddi S, Jia Y, Rao MS, Reddy JK. Transcriptional regulation of Cidea, mitochondrial cell death-inducing DNA fragmentation factor alpha-like effector A, in mouse liver by peroxisome proliferator-activated receptor alpha and gamma. J Biol Chem. 2007; 282:18613– 18624. [PubMed: 17462989]

Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, Meydani SN. Aging up- regulates expression of inflammatory mediators in mouse adipose tissue. J Immunol. 2007; 179:4829–4839. [PubMed: 17878382]

Xue B, Coulter A, Rim JS, Koza RA, Kozak LP. Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. Molecular and cellular biology. 2005; 25:8311–8322. [PubMed: 16135818]

Yamamoto K, Sakaguchi M, Medina RJ, Niida A, Sakaguchi Y, Miyazaki M, Kataoka K, Huh NH. Transcriptional regulation of a brown adipocyte-specific gene, UCP1, by KLF11 and KLF15. Biochem Biophys Res Commun. 2010; 400:175–180. [PubMed: 20709022]

Yamashita H, Sato Y, Mori N. Difference in induction of uncoupling protein genes in adipose tissues between young and old rats during cold exposure. FEBS Lett. 1999; 458:157–161. [PubMed: 10481056]

Yoneshiro T, Aita S, Matsushita M, Okamatsu-Ogura Y, Kameya T, Kawai Y, Miyagawa M, Tsujisaki M, Saito M. Age-Related Decrease in Cold-Activated Brown Adipose Tissue and Accumulation of Body Fat in Healthy Humans. Obesity (Silver Spring). 2011

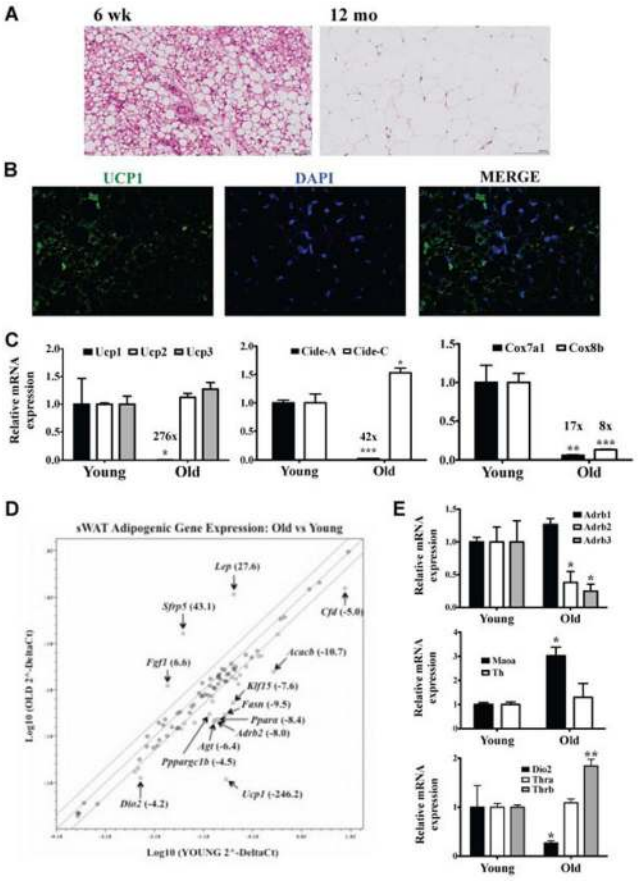

Figure 1. Brown-to-white transitioning in murine subcutaneous white adipose tissue (sWAT) with age

(A) Representative hematoxylin and eosin staining of sWAT from young (six weeks) and old (one year) mice. **(B)** Representative staining of sWAT sections from young mice with UCP1-specific antibodies. UCP1 antigen is stained green on the left, nuclei are counterstained blue with dapi in the middle, and a merged image is on the right. Relative mRNA expression of **(C)** uncoupling proteins (left), *cidea* and *cidec* (middle), and oxidative markers (right) in sWAT from young and old mice (QPCR; endogenous control = *tbp*). *p < 0.05, **p < 0.01 ***p < 0.001 vs young, with fold-change values indicated. **(D)** Scatterplot illustrating changes in adipogenic gene expression, as determined using targeted PCR-arrays, in sWAT from young and old mice. Genes altered > 2-fold (old vs young) are above (increased) or below (decreased) the three diagonal lines; genes altered > 4-fold are identified by name, with fold change values in parentheses. **(E)** Corresponding expression of *beta adrenergic receptors* (top), *maoa* and *th* (middle), and *dio2*, *thra*, and *thrb* (bottom) in sWAT (QPCR; endogenous control = *tbp*). *p < 0.05, **p < 0.01, ***p < 0.001 vs young; n = 3–4.

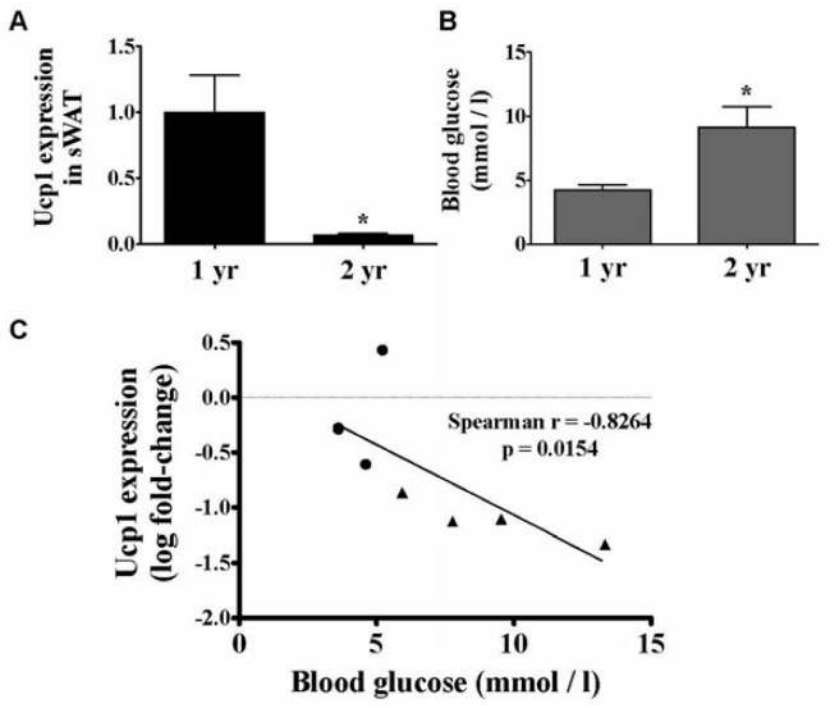

Figure 2. Loss of *ucp1* expression in sWAT with age is inversely correlated with development of age-associated hyperglycemia

(A) sWAT expression of *ucp1* and **(B)** blood glucose levels in one and two year old mice. *p < 0.05; n = 4–8. **(C)** sWAT expression of *ucp1* (log fold-change, y-axis) versus circulating glucose concentrations (x-axis), in one (circles) and two (triangles) year old animals.

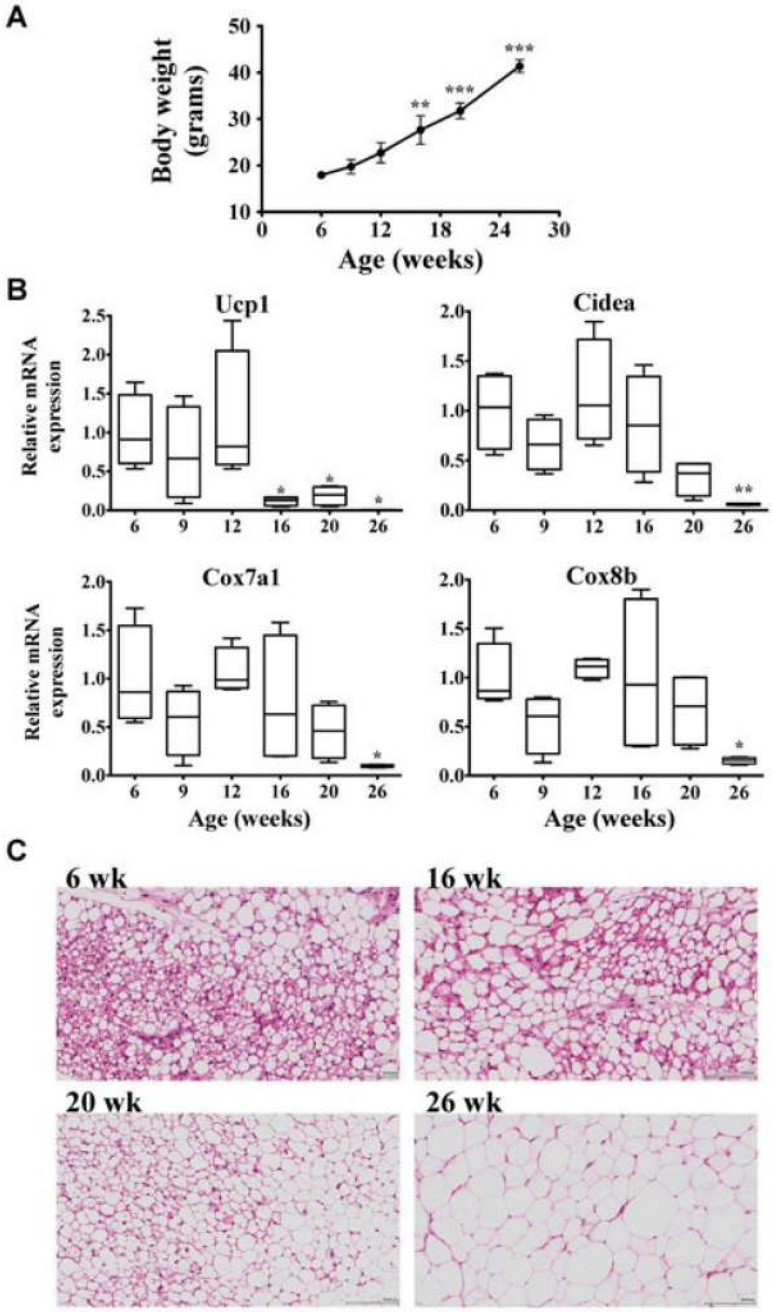

Figure 3. Age-related loss of browning in sWAT is first evident at four months of age

(A) Body weight and **(B)** sWAT expression of brown adipocyte-specific genes (QPCR; endogenous control = *36B4*) in mice at 6, 9, 12, 16, 20, and 26 weeks of age. *p < 0.05, **p < 0.01 vs 6 wk, (1-way ANOVA); n = 4–5. **(C)** Representative hematoxylin and eosin staining of sWAT at 6, 16, 20, and 26 weeks.

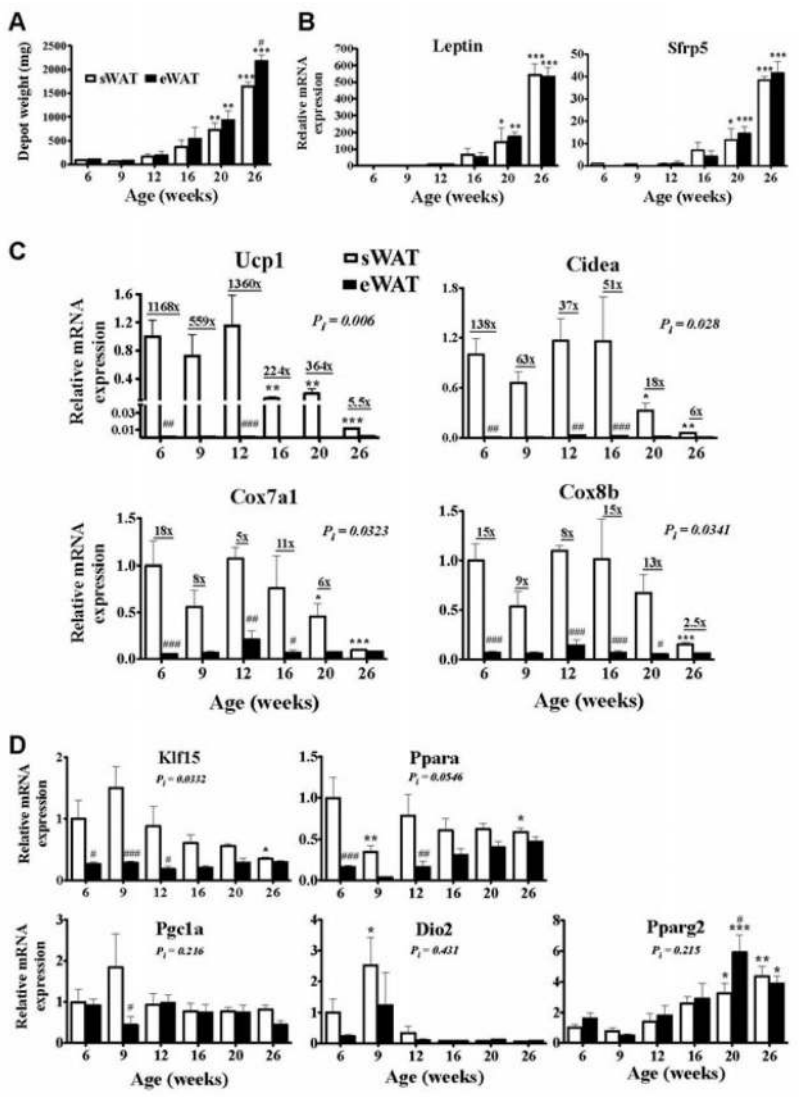

Figure 4. Subcutaneous (sWAT) and visceral (epididymal, eWAT) white adipose tissue depots grow similarly with age, but loss of browning is specific to sWAT

sWAT (white bars) and eWAT (black bars) **(A)** depot weight (milligrams) and **(B)** expression of white- indicator genes at 6, 9, 12, 16, 20, and 26 weeks (QPCR; endogenous control = *36B4*); corresponding expression of **(C)** brown-indicator or **(D)** brown-regulatory genes, with age x depot interaction p-values (Pi) indicated. *p < 0.05, **p < 0.01, ***p < 0.001 vs 6 wk; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs sWAT; n = 4–5.

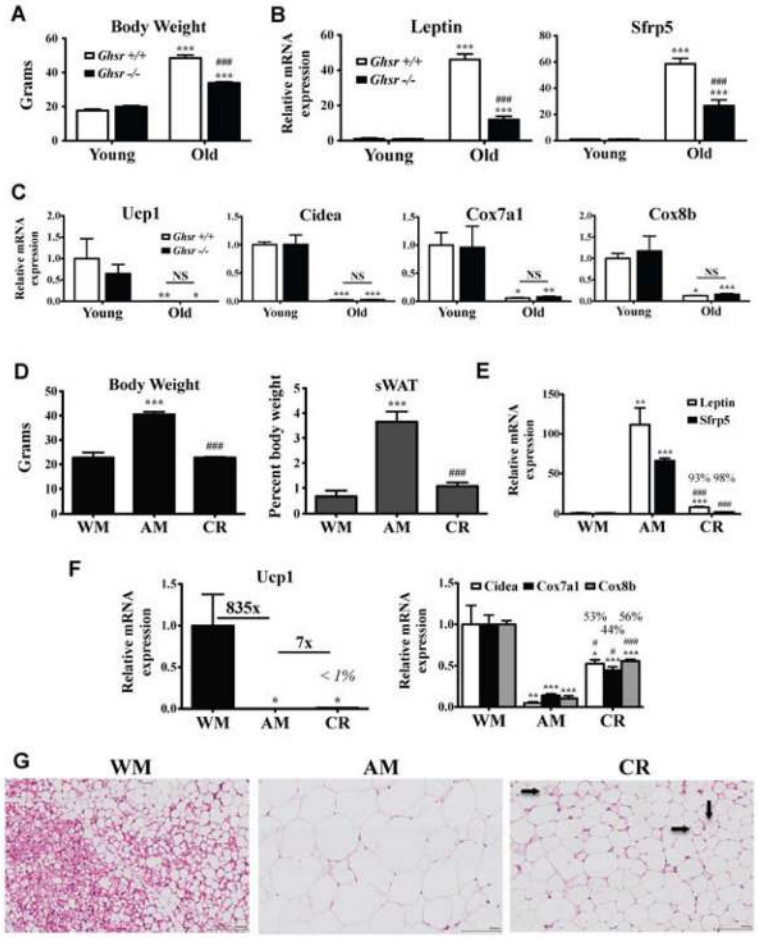

Figure 5. Age-dependent loss of browning is evident when mice gain less weight while aging

(A–C) Young (six weeks) and old (one year) *growth hormone secretagogue receptor* (*ghsr*) *–/–* mice (black bars) are compared to *ghsr +/+* (white bars). **(A)** Body weight and sWAT expression of **(B)** white-specific or **(C)** brown-specific genes (QPCR; endogenous control = *tbp*).

*p < 0.05, **p < 0.01, ***p < 0.001 vs young; ###p<0.001 vs *ghsr +/+* (2-way ANOVA); n = 3- 8. **(D–G)** Mice calorically restricted for one year (CR) are compared to age-matched ad libitum fed mice (AM) and weight-matched ad libitum fed 12 week old mice (WM). **(D)** Body weight (left) and sWAT accumulation (right), and sWAT expression of **(E)** white-specific or **(F)** brown- specific genes (QPCR; endogenous control = *36B4*). *p < 0.05, **p < 0.01, ***p < 0.001 vs WM; #p < 0.05, ###p < 0.001 vs AM (2-way ANOVA); n = 4. Percent values indicate the degree to which the effect of aging (AM vs WM) is attenuated with CR. **(G)** Representative hematoxylin and eosin staining of sWAT from WM, AM, and CR (arrows point to multilocular cells) mice.

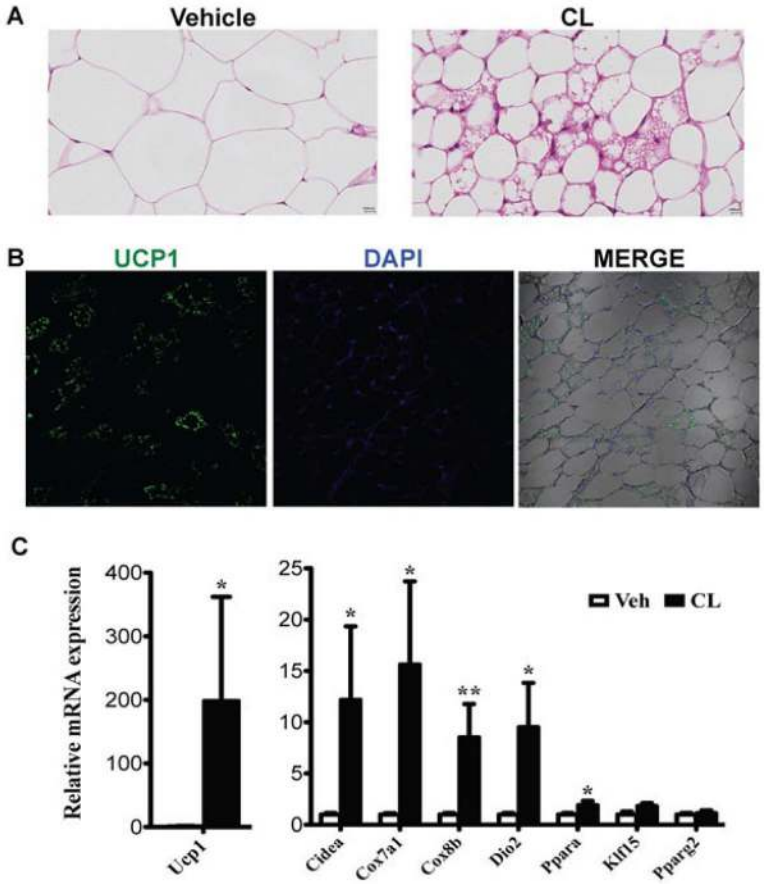

Figure 6. Exogenous β3-adrenergic receptor activation induces brown adipocytes in sWAT of older mice

Old mice (13 mo) were injected with vehicle (saline) or the β3-adrenoreceptor agonist CL 316,243 (CL, 0.1 mg/kg) once daily for seven days. **(A)** Representative hematoxylin and eosin staining of sWAT from control (left) and CL-treated (right) mice. **(B)** Representative staining of sWAT from CL-treated mice with UCP1-specific antibodies; UCP1 antigen is stained green on the left, nuclei are counterstained blue with dapi in the middle, and a merged image is on the right (with translucent light to illustrate morphology). **(C)** Corresponding sWAT expression of brown-adipocyte related genes in control (white bars) and CL-treated (black bars) mice (QPCR; endogenous control = *36B4*). *p< 0.05, **p < 0.01 vs Veh; n=3–6.

Table 1

Adipogenic gene expression in subcutaneous white adipose tissue is altered during aging.

UP-REGULATED			DOWN-REGULATED		
Gene Symbol	**Fold-change (old vs young)**	**p-value**	**Gene Symbol**	**Fold-change (old vs young)**	**p-value**
Lep	27.60	0.036	Ucp1	−246.20	0.053
Sfrp5	43.11	0.001	Fasn	−9.46	0.019
Fgf1	6.60	0.037	Ppara	−8.35	0.051
Src	1.60	0.035	Adrb2	−8.07	0.015
			Klf15	−7.55	0.043
			Agt	−7.45	0.049
			Cfd	−4.98	0.028
			Dio2	−4.21	0.019
			Retn	−3.37	0.020
			Cebpb	−2.49	0.017
			Vdr	−2.29	0.001
			Egr2	−1.99	0.041
			Tsc22d3	−1.94	0.037
			Creb1	−1.76	0.022
			Sirt2	−1.62	0.001

PCR arrays (Qiagen, #PAMM-049) were used to determine expression of 84 ‘adipogenic’ genes in subcutaneous white adipose tissue isolated from mice at six weeks (young) or one year (old) of age. The genes significantly up-regulated (left) or down-regulated (right) in old versus young mice ($p < 0.05$) mice are listed; $n = 3$.

Aging Cell. Author manuscript; available in PMC 2013 November 25.