



Genetic backgrounds determine brown remodeling of white fat in rodents

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ABSTRACT

Objective: Genetic background largely contributes to the complexity of metabolic responses and dysfunctions. Induction of brown adipose features in white fat, known as brown remodeling, has been appreciated as a promising strategy to offset the positive energy balance in obesity and further to improve metabolism. Here we address the effects of genetic background on this process.

Methods: We investigated browning remodeling in a depot-specific manner by comparing the response of C57BL/6J, 129/Sv and FVB/NJ mouse strains to cold.

Results: Surprisingly, 129/Sv and FVB/NJ mice showed distinct brown remodeling features despite their similar resistance to metabolic disorders in comparison to the obesity-prone C57BL/6J mice. FVB/NJ mice demonstrated a preference of brown remodeling in inguinal subcutaneous white adipose tissue (iWAT), whereas 129/Sv mice displayed robust brown remodeling in visceral epididymal fat (eWAT). We further compared gene expression in different depots by RNA-sequencing and identified *Hoxc10* as a novel “brake” of brown remodeling in iWAT.

Conclusion: Rodent genetic background determines the brown remodeling of different white fat depots. This study provides new insights into the role of genetic variation in fat remodeling in susceptibility to metabolic diseases.

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Keywords Genetic background; White adipose tissue; Brown remodeling; *Hoxc10*; Cold exposure; Browning brake

1. INTRODUCTION

Obesity is a worldwide public health problem [1–3] and a risk factor for many chronic diseases including type 2 diabetes, cardiovascular disorders, atherosclerosis, non-alcoholic fatty liver disease, and several forms of cancer [4,5]. Obesity arises as a consequence of excess fat storage and expansion of white adipose tissue (WAT) over energy expenditure. WAT functions primarily to store energy while brown adipose tissue (BAT) catabolizes free fatty acids and dissipates energy in the form of heat. Therefore, it is appealing to introduce BAT features into WAT, namely brown remodeling, to combat obesity and its co-morbidities.

With the discovery of brown remodeling, there has been growing interest in exploring this phenomenon to manage the consequences of obesity. Brown remodeling is characterized by the emergence of clusters of brown-like UCP1-positive multilocular cells (also referred to as beige or brite cells) interspersed within WAT. In rodents, brown remodeling can be induced by cold exposure or pharmacological treatments such as β -adrenergic receptor agonists and thiazolidinediones (PPAR γ agonists) [6–10]. Recently activated human brown fat has been shown to have a similar expression profile to beige fat, and beige adipocytes have been identified in human WAT [11–13]. The

origin of beige cells has been explained by either transdifferentiation, the direct conversion of white adipocytes into brown-like cells [7,14] or the differentiation of unique beige precursors [15,16]. Several studies in mouse models illustrate that brown remodeling contributes to an improved metabolic profile marked by protection against diet-induced obesity, improved glucose homeostasis and increased energy expenditure [6,17–21]. While brown remodeling of WAT offers a promising strategy to alleviate obesity, more knowledge is still needed to understand the genetic susceptibility of adipose plasticity to environmental cues.

The interaction of genetic and environmental factors plays a significant role in propagating obesity. It has been established that genetic background is a major contributing factor in the susceptibility to diet-induced obesity and type 2 diabetes. Among the commonly used inbred mouse strains, the C57BL/6J strain is more susceptible to obesity and type 2 diabetes while the 129/Sv strain is protected against these traits [22,23] as is the FVB/NJ strain, which is hyperactive and has higher body temperature [24–26].

Previous studies have integrated histological and quantitative trait loci analysis to identify potential browning genes and found that multiple loci on 8 different chromosomes function synergistically to increase *Ucp1* and *Pgc-1 α* expression in retroperitoneal WAT in the A/J mice

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Received August 5, 2016 • Revision received August 16, 2016 • Accepted August 18, 2016 • Available online 26 August 2016

<http://dx.doi.org/10.1016/j.molmet.2016.08.013>

but not in the C57BL/6J strain [27–29]. However, there was no significant difference in *Ucp1* expression in BAT and epididymal WAT between the two strains [17]. These results highlight the contribution of genetic variation to strain-specific differences together with depot-dependent browning potential of WAT. Given the pressing need to identify genetic mediators of adipose tissue remodeling, in this study, we examined the browning response in the 3 commonly used mouse strains, C57BL/6J, 129/Sv and FVB/NJ, coupled with RNA sequencing to identify genes that account for the adipose plasticity upon chronic cold exposure.

2. METHODS

2.1. Animal studies

Five-week old male C57BL/6J (000664), 129/Sv (002448), FVB/NJ (001800) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and exposed to 4 °C after two-week adaptation in the Columbia University animal barrier. Mice had ad libitum access to regular chow diet (PicoLab rodent diet 20 (LabDiet 5053)). Body temperature was measured by a subcutaneously implanted IPTT-300 transponder (DMDS). During chronic cold exposure, body temperature was measured in light phase at the same time on each day. Plasma glucose was measured with the One Touch Ultra II glucometer. The mice were gently handled by the same experienced animal technician to minimize stress-induced measuring variations. The Columbia University Animal Care and Utilization Committee approved all procedures.

2.2. RNA analysis

One entire side of each respective fat depot was used to isolate total RNA by using RNeasy Lipid Tissue kit (QIAGEN) following the manufacturer's instructions. 1 µg RNA was subjected to cDNA synthesis by using High-capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was performed on the CFX96 Real-Time PCR system (Bio-Rad) by using GoTaq qPCR Master Mix (Promega). The relative gene expression levels were calculated by ddCt method with TBP as the reference gene. qPCR primer sequences are available upon request.

2.3. RNA-seq analysis

mRNA was enriched by poly-A pull-down from 1 µg total RNA and used to generate proceed library with using Illumina TruSeq RNA prep kit. Libraries were then sequenced using Illumina HiSeq2000 at Columbia Genome Center. Sequencing reads were aligned to the mouse genome (UCSC/mm9) using Tophat [30] with 4 mismatches and 10 maximum multiple hits. Differential gene expression across strains and tissues was found using cuffdiff [31] with cutoff FPKM > 5, FDR < 0.05 and fold-change > 2. K-means clustering, heatmaps, and scatter plots were generated in R [32]. Genes coding for transcription factors were identified among other regulated genes using "Animal TFDB" database [33].

2.4. In vitro cell culture analysis

The C3H/10T1/2 cell line was purchased from ATCC and cultured and differentiated as described [34]. Myc-tagged Human *HOXC10* cDNA was cloned into pTRIPZ inducible expression vector (Thermo Open Biosystems) [35] and used to generate stable cell line into C3H/10T1/2 cell under selection by 1 µg/mL puromycin. On Day 7 of differentiation, *HOXC10* overexpression was induced by adding 0.1 µg/mL doxycycline. Cells were treated with isoproterenol (10 µM) and CL-316,243 (1 µg/mL) for 4 h before harvesting for RNA analysis. *HOXC10*

expression was validated by probing with anti-*Hoxc10* antibody *HoxC10* (L-17) (Santa Cruz).

2.5. Morphological analysis

Fat tissues were fixed in 10% formalin, embedded in paraffin, and stained with Hematoxylin and Eosin (H&E). Lipid content in differentiated C3H/10T1/2 adipocytes was assessed by Oil Red O staining.

2.6. Statistical analysis

All values were presented as means ± standard error of means (SEM). Unpaired 2-tailed Student's *t* tests were used to evaluate statistical significance and *p* < 0.05 was declared as a statistically significant change.

3. RESULTS

3.1. Genetic background-dependent metabolic responses to cold challenge

To test whether genetic background impacts thermogenic response as well as physiological adaptation to cold, we compared the cold response among 129/Sv, C57BL/6J and FVB/NJ strains. During an 8-day cold challenge at 4 °C, both C57BL/6J and 129/Sv mice lost body weight (5.5% and 9.3%, respectively) while, surprisingly, FVB/NJ mice were able to maintain their body weight (Figure 1A–B). We also monitored their cold responses by measuring body temperature. 129/Sv and FVB/NJ mice were better to maintain their body temperature than C57BL/6J mice during both acute (Figure 1C) and prolonged (Figure 1D) cold challenge, indicating higher thermogenesis in these two strains. During cold challenge, body weight change is determined by the compromise between thermogenesis-induced fat consumption and food intake. The food intake of FVB/NJ mice was significantly higher than that of C57BL/6J and 129/Sv mice (Figure 1E), in support of their maintenance of body mass and preservation of body temperature. 129/Sv mice had less food intake compared to C57BL/6J mice, but also experienced elevated thermogenesis, accounting for their greater body weight loss during cold challenge. Lastly, we measured the blood glucose levels at the end of the cold challenge. The 129/Sv strain had much lower glucose levels than the other strains (Figure 1F). Taken together, these data indicate that genetic backgrounds determine physiological responses to cold exposure, consistent with previous work [17,27,36]

3.2. Distinct brown remodeling in subcutaneous WAT

Prolonged cold exposure is the classic method of inducing brown remodeling of subcutaneous WAT. Given their distinct cold responses, we hypothesized that the subcutaneous WAT from the three strains would have different extents of brown remodeling. Indeed, histological examination of their inguinal fat depots (iWAT) showed a strikingly higher prevalence of multilocular adipocytes, that is an increased browning morphology, in FVB/NJ mice than in C57BL/6J mice (Figure 2A). To our surprise, we observed fewer multilocular adipocytes in iWAT of 129/Sv mice than in C57BL/6J mice (Figure 2A) regardless of their superior defense of temperature loss in the cold (Figure 1D).

Consistent with the morphological analysis, the expression of representative brown genes and brown gene regulators was significantly higher in FVB/NJ mice as compared to C57BL/6J. Indeed, there was a significantly increased expression of brown markers (e.g., *Ucp1*, *Elovl3*, *Dio2*, *Cidea*), mitochondrial respiratory chain enzymes (e.g., *Cox7a1*, *Cox8b*), and genes involved in fatty acid oxidation pathway (e.g., *Cpt1b*) as well as regulatory genes involved

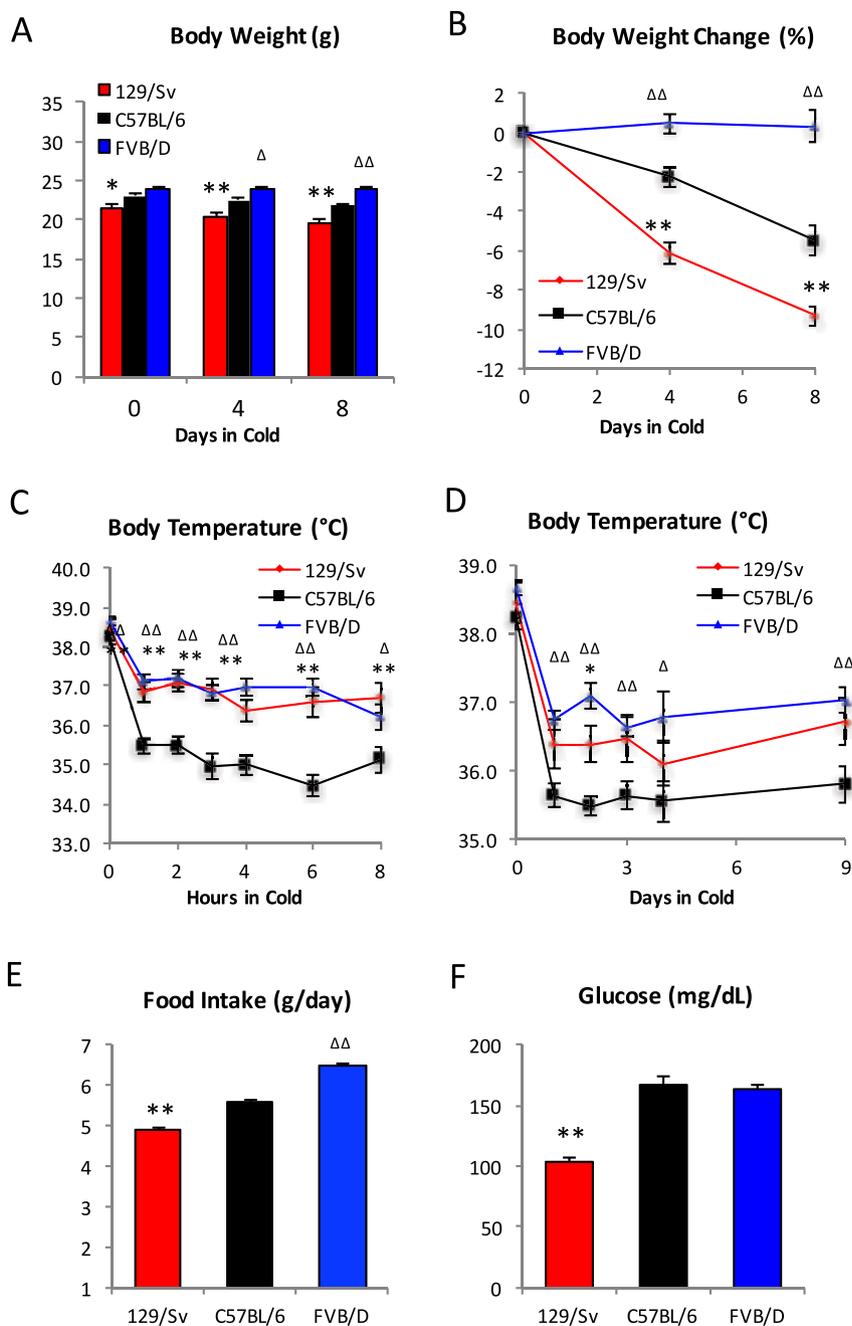


Figure 1: Metabolic response of three mouse strains in response to cold challenge. (A–B) Body weight (A) and body weight change (B) during cold challenge. (C–D) Core body temperature during acute (C) and chronic cold challenge (D). (E) Food intake during cold exposure. (F). Ad libitum plasma glucose levels in the cold. *: $p < 0.05$, **: $p < 0.01$ for 129/Sv vs. C57BL/6; Δ : $p < 0.05$, $\Delta\Delta$: $p < 0.01$ for FVB/D vs. C57BL/6, $n = 8, 8, 8$.

in brown adipocyte differentiation and activation (e.g., *C/ebp β* , *Pgc-1 α* , *Med1* and *Ppara α*) (Figure 2B,D). 129/Sv mice had relatively lower brown gene expression compared to C57BL/6J mice, in line with less angiogenesis as indicated by lower *Vegfa* expression (Figure 2C). On the other hand, the expression of representative white adipocyte genes (e.g., *Leptin*, *Adipsin*, *Angiotensinogen (Agnt)* and *Resistin*) was significantly lower in FVB/NJ mice compared to C57BL/6J mice (Figure 2C). Thus, FVB/NJ genetic background was predisposed to more brown remodeling in subcutaneous fat relative to the two other strains.

3.3. Brown remodeling of visceral WAT in 129/Sv mice

Visceral fat accumulation causes abdominal obesity, aggravating the risk for the metabolic syndrome, and it is much more resistant to browning compared to subcutaneous fat. Indeed, there was a notable absence of multilocular adipocytes in visceral epididymal fat (eWAT) across all three strains (Figure 3A). Surprisingly, in spite of their nearly identical eWAT morphology (Figure 3A), 129/Sv mice showed a much higher level of brown remodeling than C57BL/6J and FVB/NJ mice (Figure 3B). The representative brown adipocyte marker *Ucp1* was expressed ~ 400 -fold higher in 129/Sv mice than

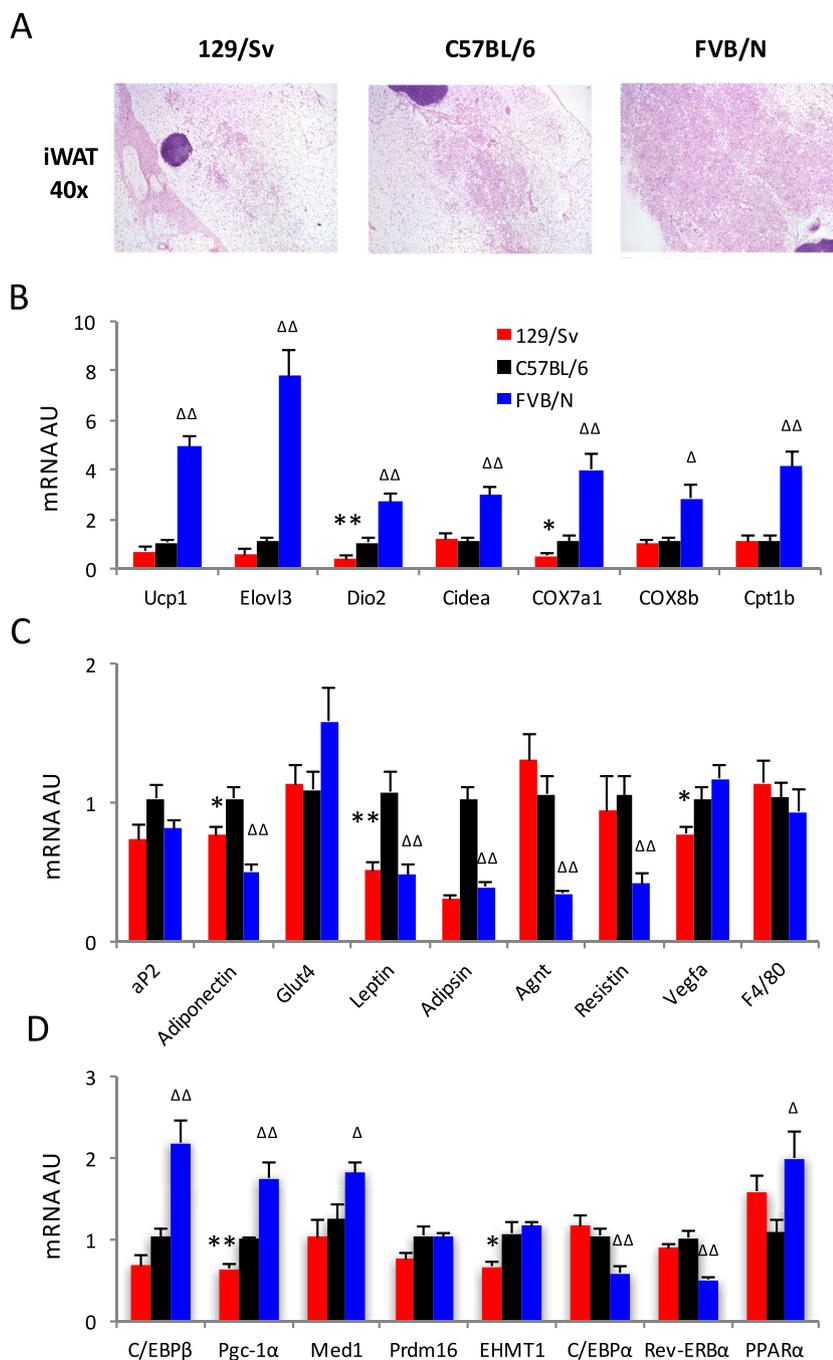


Figure 2: Comparisons of browning in subcutaneous white adipose tissue. (A) Histological analyses of inguinal WAT by H&E staining. (B) qPCR analyses of representative brown genes expression in iWAT (AU: relative gene expression arbitrary unit). (C) qPCR analyses of adipocyte markers and representative white genes expression in iWAT. (D) qPCR analyses of expression of browning regulators in iWAT. *: $p < 0.05$, **: $p < 0.01$ for 129/Sv vs. C57BL/6; Δ : $p < 0.05$, $\Delta\Delta$: $p < 0.01$ for FVB/D vs. C57BL/6, $n = 8, 8, 8$.

in C57BL/6J mice, as were other brown adipocyte markers, including *Elov13*, *Cidea*, *COX7a1* and *Cpt-1b* (Figure 3B). The pan-adipocyte genes *aP2* and *Glut4* were comparable between 129/Sv and C57BL/6J mice, but the white adipocyte genes *Leptin*, *Adipsin*, *Agnt*, and *Resistin* were all down-regulated in 129/Sv mice, in line with their more pronounced brown remodeling (Figure 3C). The enhanced brown remodeling in 129/Sv mice was unlikely to have been caused by the classic browning regulators since we failed to observe higher expressions of *C/EBP β* , *Pgc-1 α* , *Med1*, *Prdm16* and

PPAR α compared to C57BL/6J mice (Figure 3D). These data indicate that genetic backgrounds have differential impacts on brown remodeling of eWAT, and 129/Sv strain has a stronger brown remodeling in metabolically deleterious visceral fat.

3.4. Marginal impact of genetic backgrounds on BAT

Histological analyses of BAT in the three strains endorsed the preponderance of lipid contents in FVB/NJ mice, followed by C57BL/6J and lastly 129/Sv mice (Figure 4A). qPCR analyses of the expression of

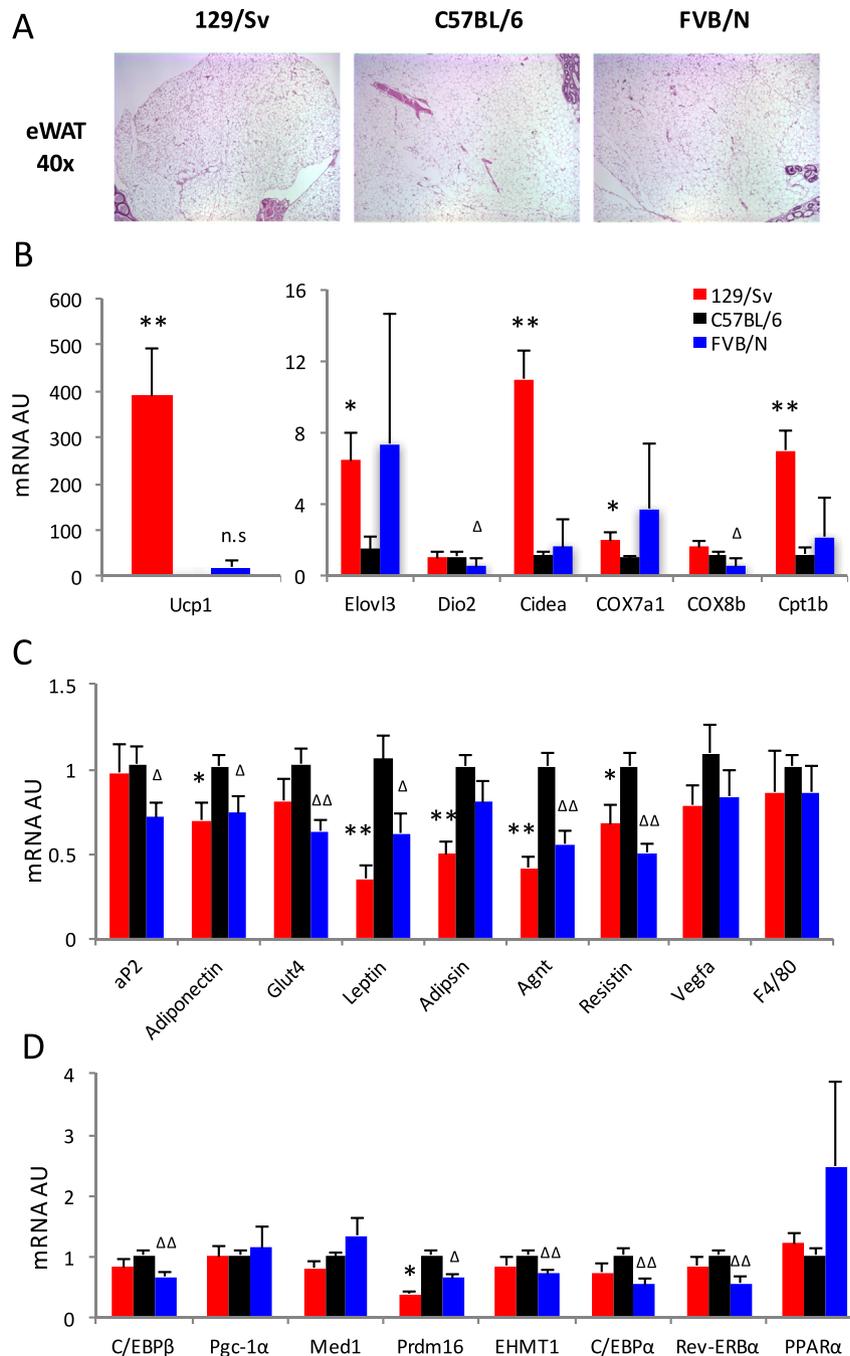


Figure 3: Comparisons of browning in visceral white adipose tissue. (A) Histological analyses of epididymal WAT (eWAT) by H&E staining. (B) qPCR analyses of representative brown genes expression in eWAT. (C) qPCR analyses of adipocyte markers and representative white genes expression in eWAT. (D) qPCR analyses of expression of browning regulators in eWAT. *: $p < 0.05$, **: $p < 0.01$ for 129/Sv vs. C57BL/6; Δ: $p < 0.05$, ΔΔ: $p < 0.01$ for FVB/D vs. C57BL/6, $n = 8, 8, 8$.

representative and regulatory brown genes demonstrated a weaker BAT activation in 129/Sv than in FVB/NJ and C57BL/6J mice (Figure 4B,D). No statistically relevant difference was found regarding adipocyte markers and representative white genes expression in BAT (Figure 4C). These data together with analyses in iWAT and eWAT suggest that the physiological responses to cold exposure are unlikely caused by the variations of BAT activation but rather by brown remodeling in white fat.

3.5. Gene expression profiling of WAT by RNA-seq analysis

To understand the distinct effects of genetic background on brown remodeling patterns, we compared WAT gene expression profiles across the three strains by performing RNA sequencing. The eWAT of 129/Sv mice showed a distinct expression from that of C57BL/6J and FVB/NJ mice with a large number of brown markers up-regulated (Figure 5A), consistent with the qPCR analysis (Figure 3). In iWAT, FVB/NJ mice demonstrated a consistently higher

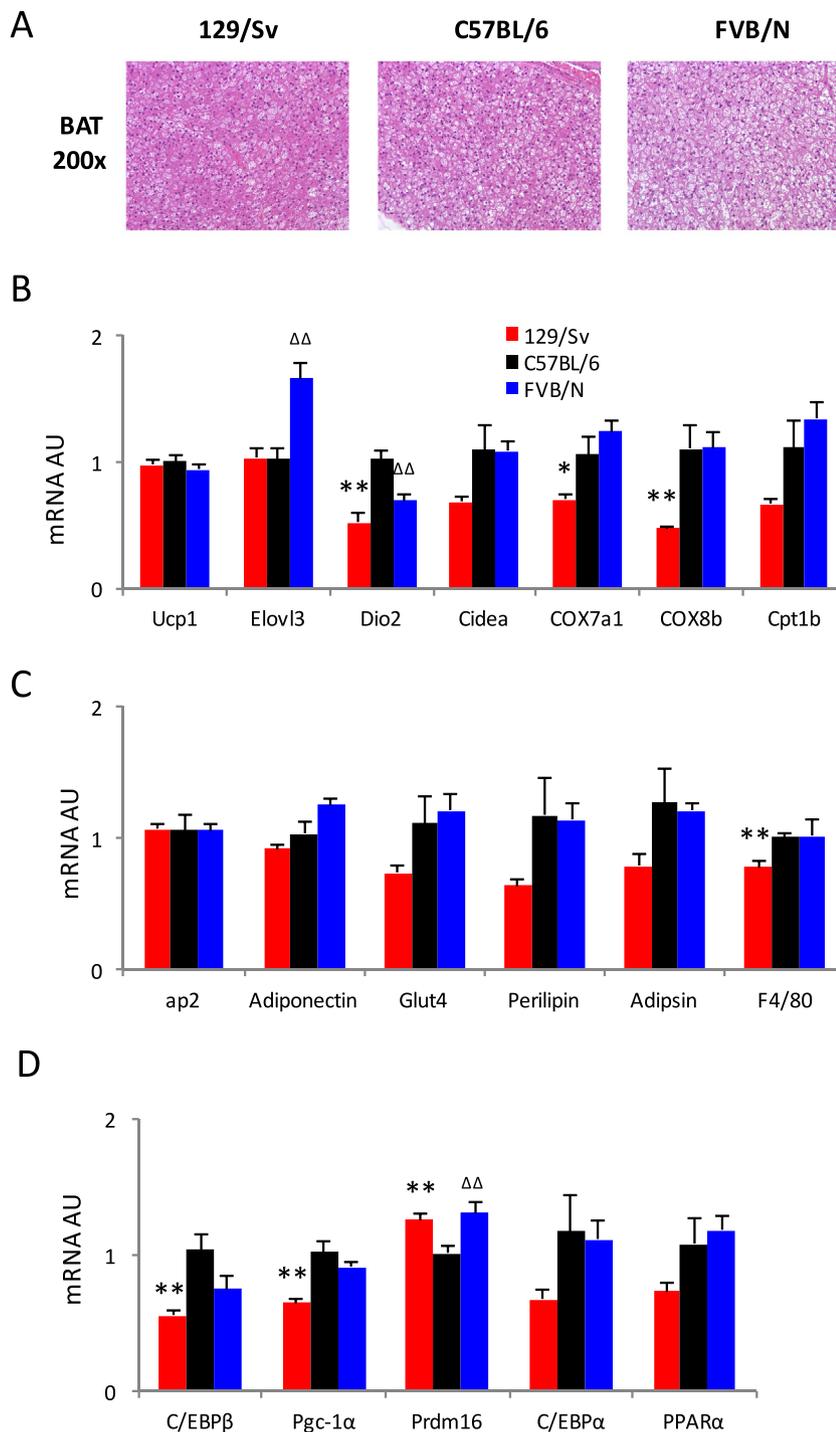


Figure 4: Comparisons of brown adipose tissue (BAT) of three strains during cold challenge. (A) Histological analyses of BAT by H&E staining. (B) qPCR analyses of representative brown genes expression in BAT. (C) qPCR analyses of adipocyte markers and representative white genes expression in BAT. (D) qPCR analyses of expression of browning regulators in BAT. *: $p < 0.05$, **: $p < 0.01$ for 129/Sv vs. C57BL/6; Δ : $p < 0.05$, $\Delta\Delta$: $p < 0.01$ for FVB/D vs. C57BL/6, $n = 8, 8, 8$.

thermogenic gene expression than the other two strains (Figure 5C). In an effort to identify potential browning regulators, we focused on the transcriptional factors that showed significant changes of their expression among the three strains (Figure 5B,D). Among them, *C/ebp β* expression was the highest while *Rev-erb α* (*Nr1d1*) was the lowest in iWAT of FVB/NJ mice (Figure 5D). It's known that *C/ebp β* promotes brown adipogenesis (7) and *Rev-erb α* has been demonstrated as a repressor of thermogenic response (8). Thus the

comparison of differential brown remodeling on different genetic backgrounds could provide a useful tool to discover novel factors regulating this process.

3.6. Identification of *Hoxc10* as a novel “brake” for brown remodeling

A search for transcription factors sharing the same expression patterns in iWAT and eWAT across all three strains identified *Ppar α* and *Hoxc10*

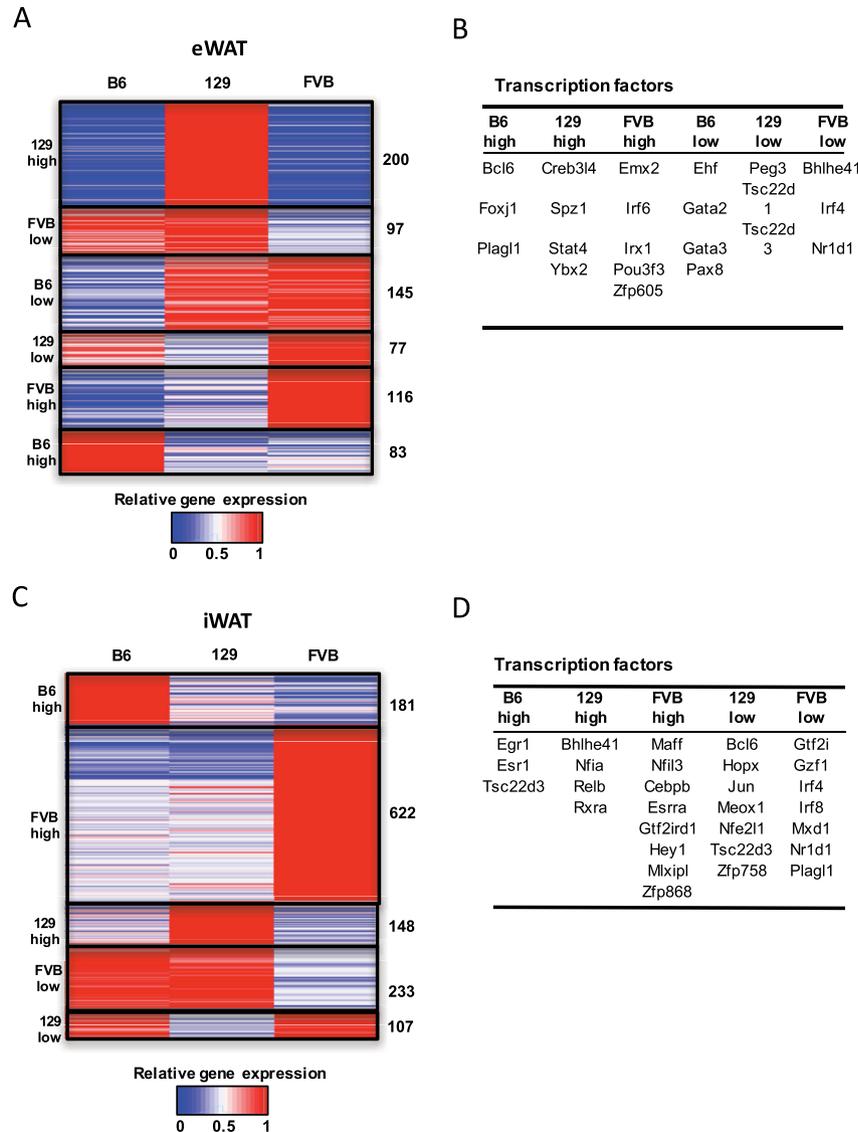


Figure 5: WAT gene expression profiling by RNA-seq analysis. Heatmaps showing genes expressed differentially across three strains (B6, 129 and FVB) in eWAT (A–B) and iWAT (C–D). Genes with FPKM > 5, FDR < 0.05 and fold-change > 2 are represented in the heatmaps using relative expression across three tissues. The genes were k-means clustered with name and size of each cluster labeled on the left and right, respectively. Each heatmap is accompanied by a list of transcription factors found in genes with high or low expression in B6, 129 or FVB.

as the only two transcription factors expressed at higher level in subcutaneous fat in all three strains (Figure 6). *Ppar α* is well known to regulate lipid oxidation and brown adipocyte activity, and *Hoxc10* has been found to be enriched in subcutaneous fat compared with visceral fat in humans [37], raising the possibility of *Hoxc10* in regulating adipose tissue remodeling.

We then set out to investigate *Hoxc10*'s function in adipocytes. We first confirmed the expression of *Hoxc10* in iWAT but at background levels in BAT and eWAT (Figure 7A). *HOXC10* is expressed at higher level in stromal vascular fraction (SVF) cells than in adipocytes [37], and we did observe a down-regulation of *Hoxc10* during adipogenesis (data not shown). To avoid any possible involvement of *Hoxc10* in adipogenesis, we then employed doxycycline-inducible *HOXC10* over-expression in C3H/10T1/2 cells after differentiation (Figure 7B). After the induction of *HOXC10* for two days, we observed little to no effects on lipid contents in C3H/10T1/2 cells (Figure 7C), neither on pan-

adipocyte gene expression (Figure 7D). Strikingly, brown adipocyte regulators and markers represented by *Ppar α* , *Pgc-1 α* , *Ucp1*, *Cidea*, and *Elovl3* were significantly repressed. On the other hand, the white adipocyte gene *Adipsin* was up-regulated to above four-fold. Taken together, we have identified *Hoxc10* as a specific repressor for brown adipocyte gene expression that does not affect basic adipocyte activity.

4. DISCUSSION

A number of studies in rodents have shown that brown remodeling of WAT confers metabolic benefits by protecting against diet-induced obesity and type 2 diabetes [18,38–40]. In order to fully harness the therapeutic potential of brown remodeling, we need to better understand the genetic factors and mechanisms that regulate adipose plasticity, particularly, the recruitment of brown-like cells in WAT. To this end, we compared the physiological response and browning

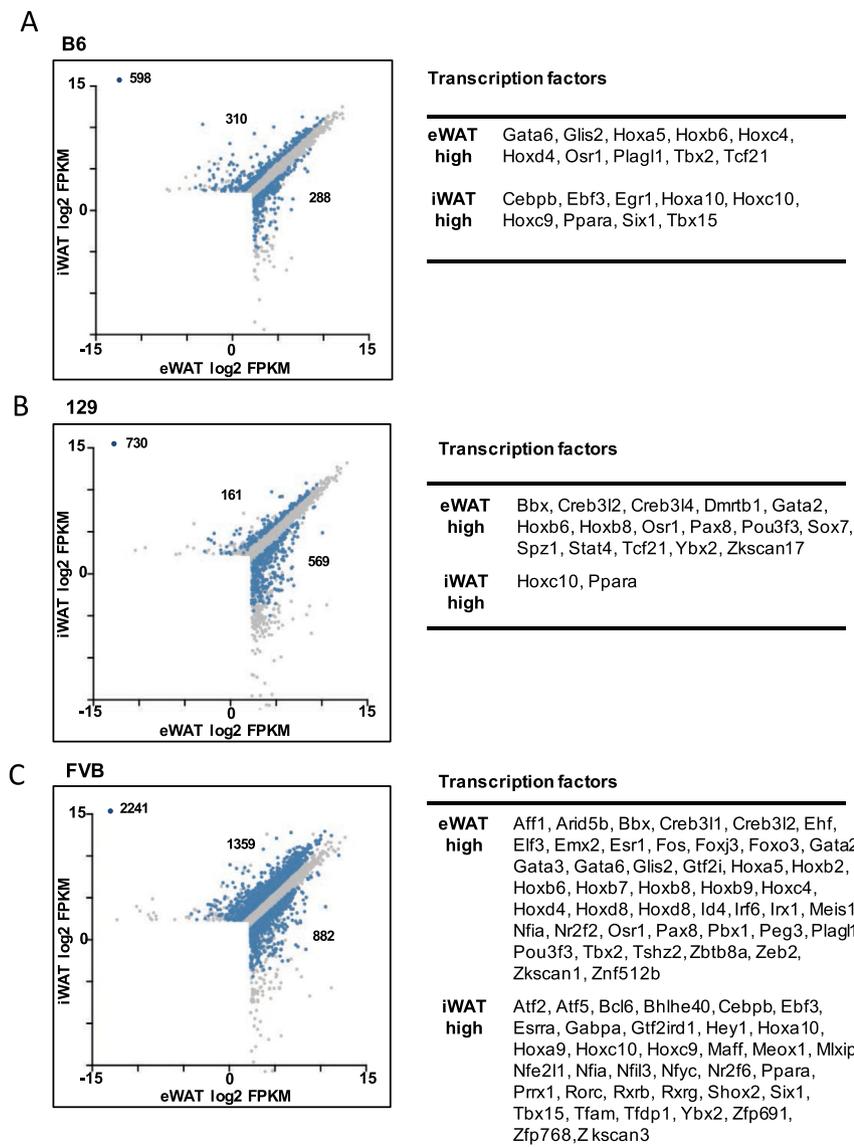


Figure 6: Subcutaneous and visceral fat gene expression profiling in three strains. Scatter plots showing genes expressed differentially across two tissues (eWAT and iWAT) in three strains, B6 (A), 129 (B) and FVB (C). Genes with FPKM > 5 and q-value < 0.05 are represented in the plots. The blue dots represent genes changing more than two-fold. Each plot is accompanied by a list of transcription factors found in the genes with higher expression in eWAT or iWAT.

capacity of different WAT depots in C57BL6/J, FVB/NJ, and 129/Sv mice upon cold exposure. We found a surprising depot-specific browning preference in these commonly used mouse strains. Brown remodeling has been shown to predominantly occur in the subcutaneous depot [7,38,41,42]. Our results were in overall agreement with this notion but specified that brown remodeling occurs in a strain-dependent manner.

Relative to the C57BL6/J mice, the 129/Sv strain showed preferential brown remodeling in the visceral depot whereas the FVB/NJ strain showed brown remodeling predominantly in the subcutaneous depot. Other studies have reported the C57BL6/J mice to have lower browning potential of the retroperitoneal WAT and subcutaneous depot when compared to the A/J and the 129 strains, respectively [28,29,36,43], and this distinction may contribute to the latter's obesity-resistance phenotype [10,42,44]. Despite the similar obesity-resistance and better defense of body temperature loss in 129/Sv and FVB/NJ strains (Figure 1), our study demonstrates that genetic

background determines the preference of brown remodeling in specific WAT depots during cold stimulation, which could be achieved through SNPs in the regulatory regions of downstream genes [45] and/or differential expression of regulatory factors.

A key addition from our study is that we used an unbiased approach to identify novel regulators of brown remodeling (Figure 5). Beyond elaborating the specific expression of representative brown and white genes among the three strains, we also investigated potential mechanisms that differentially drive WAT remodeling in visceral and subcutaneous fat. For example, we found that *C/ebpβ*, *Pgc1-α*, and *Med1* positively correlated with brown gene expression in inguinal WAT, and they have been identified as positive regulators of brown remodeling [46,47]. In contrast, *Rev-ERBα* negatively correlates with the brown remodeling in this depot by suppressing the expression of brown genes [48]. Herein, analyses of brown remodeling on different genetic backgrounds, as we employed in the current study, can be informative to understand the mechanisms

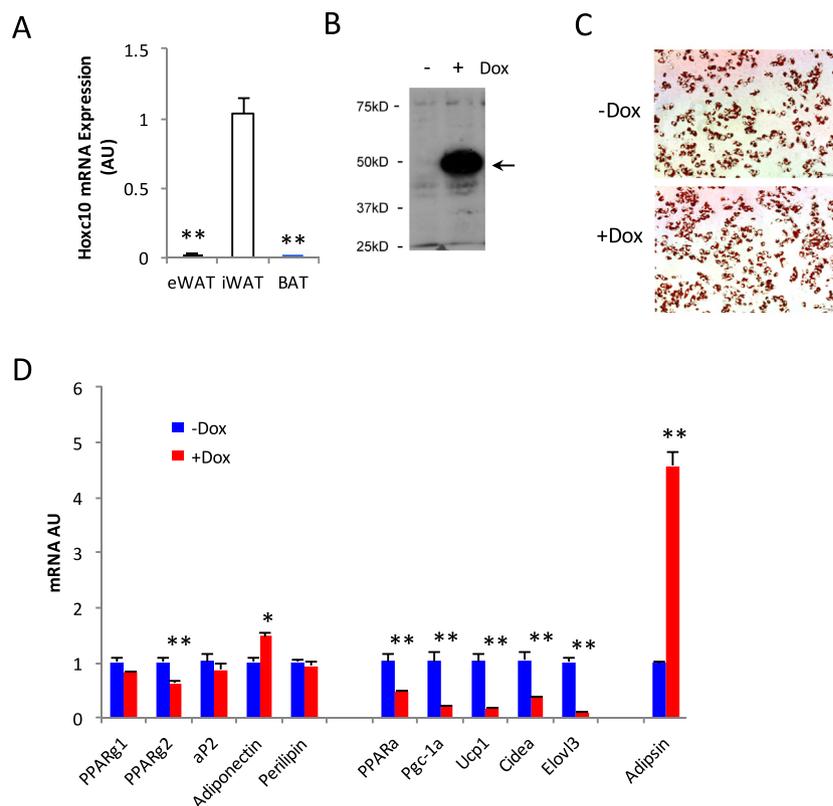


Figure 7: Identification of Hoxc10 as a novel repressor for browning. (A) Hoxc10 expression in 3 adipose tissues from C57BL/6 mice, **: $p < 0.01$ vs. iWAT ($n = 8, 8, 8$). (B) Overexpression of HOXC10 in C3H/10T1/2 cells. Doxycycline was added on D7 of differentiation for two days. Arrow indicates the Myc-tagged HOXC10. (C) Oil Red O staining of differentiated C3H/10T1/2 adipocyte after two days' doxycycline treatment (D7-D9). (D) qPCR analysis of gene expression in fully differentiated C3H/10T1/2 cells after induction of HOXC10 expression for two days. *: $p < 0.05$, **: $p < 0.01$ for -Dox vs. +Dox ($n = 3-4$).

involved in regulating adipose plasticity and, to take a step further, targeting a specific fat depot. Moreover, besides the intrinsic regulations within adipocytes, it will be of interest to investigate the behavioral differences associated with different genetic backgrounds exposed to cold. For example, we observed that 129/Sv mice were less active and FVB/NJ showed higher anxiety during cold challenge. These behavioral differences together with intrinsic regulations within adipocytes largely determine their corresponding responses to the cold.

In order to overcome the limitation of studying brown remodeling in one strain, we compared iWAT with eWAT across the three strains (Figure 6). The rationale was that if we identified regulators, here with a focus on transcription factors, that share the same expression pattern (iWAT vs. eWAT) among the three strains, they are more likely to be involved in brown remodeling. Indeed, only four such factors were identified: *Hoxb6* and *Osr1* were abundantly expressed in eWAT, and *Ppar α* and *Hoxc10* were highly expressed in iWAT. We chose to test whether Hoxc10 is a novel browning factor. To our surprise, we discovered that it had minimal effects on adipocyte housekeeping gene expression but markedly repressed brown markers *Ucp1*, *Cidea*, and *Elovl3*, possibly through repressing brown regulators *Ppar α* and *Pgc-1 α* (Figure 7). To date, many transcriptional regulators of fat remodeling have been identified (comprehensively reviewed in [49,50]) and among them, only a few are repressors of brown remodeling in subcutaneous fat depot, including *Foxa3* [51], *p107* [52], *RIP140* [53,54], and *Rev-erb α* [48].

Our *in vitro* studies suggest that Hoxc10 likely represses brown remodeling in iWAT *in vivo*. Since thermogenic activity is catabolic,

which acts against the native energy-storing function of white adipocyte, brown remodeling in white fat should be tightly controlled. The energy wasting character of beige adipocytes is evolutionarily disadvantageous unless it is necessary to defend body temperature or to adapt to environmental stimulations. From this stand point, Hoxc10 is important to be quickly turned on by activators sensitive to the corresponding stimuli, but it should also be equally important to be turned off when not necessary. Therefore, a ready-to-use brake system is desired. Given its abundance in brown-prone iWAT, but not in brown-resistant eWAT, neither in thermogenic BAT, Hoxc10 is an ideal candidate to function as a “browning brake” in iWAT. Further investigation is required on the detailed repression mechanism of Hoxc10 on brown remodeling as well as the regulation of Hoxc10 in cold response and in the pathogenesis of obesity.

In summary, our study has revealed that genetic background is a significant factor in regulating brown remodeling of WAT in a strain-specific and depot-specific manner. We have identified novel candidates in regulating adipose tissue plasticity, particularly Hoxc10 as a bona fide “browning brake” in iWAT. Our expectation is that the coordination between activators and repressors in brown remodeling determines the physiological consequences of adipose tissue and thus may provide novel insights for obesity treatment and in a depot-specific manner.

ACKNOWLEDGMENTS

We would like to thank Thomas Kolar for technical assistance. This work was supported by National Institutes of Health grants R00DK97455 to L. Q., Pilot and

Feasibility funding from the Diabetes Research Center to L.Q. (P30 DK063608), R01DK49780 to M. L. and by the JPB Foundation.

CONFLICT OF INTEREST

None declared.

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