



Brown adipose tissue (BAT) specific vaspin expression is increased after obesogenic diets and cold exposure and linked to acute changes in DNA-methylation

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ABSTRACT

Objective: Several studies have demonstrated anti-diabetic and anti-obesogenic properties of visceral adipose tissue-derived serine protease inhibitor (vaspin) and so evoked its potential use for treatment of obesity-related diseases. The aim of the study was to unravel physiological regulators of vaspin expression and secretion with a particular focus on its role in brown adipose tissue (BAT) biology.

Methods: We analyzed the effects of obesogenic diets and cold exposure on vaspin expression in liver and white and brown adipose tissue (AT) and plasma levels. Vaspin expression was analyzed in isolated white and brown adipocytes during adipogenesis and in response to adrenergic stimuli. DNA-methylation within the vaspin promoter was analyzed to investigate acute epigenetic changes after cold-exposure in BAT.

Results: Our results demonstrate a strong induction of vaspin mRNA and protein expression specifically in BAT of both cold-exposed and high-fat (HF) or high-sugar (HS) fed mice. While obesogenic diets also upregulated hepatic *vaspin* mRNA levels, cold exposure tended to increase *vaspin* gene expression of inguinal white adipose tissue (iWAT) depots. Concomitantly, vaspin plasma levels were decreased upon obesogenic or thermogenic triggers. Vaspin expression was increased during adipogenesis but unaffected by sympathetic activation in brown adipocytes. Analysis of vaspin promoter methylation in AT revealed lowest methylation levels in BAT, which were acutely reduced after cold exposure.

Conclusions: Our data demonstrate a novel BAT-specific regulation of *vaspin* gene expression upon physiological stimuli *in vivo* with acute epigenetic changes that may contribute to cold-induced expression in BAT. We conclude that these findings indicate functional relevance and potentially beneficial effects of vaspin in BAT function.

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Keywords Brown adipose tissue; Browning; Cold exposure; DNA methylation; High-fat diet; High-sucrose diet; SerpinA12; Thermogenesis; UCP1; Vaspin

1. INTRODUCTION

Adipose tissue (AT) secretes a multitude of hormones and bioactive molecules, termed adipokines, thereby regulating a plethora of processes such as energy homeostasis or inflammation. In the obese state, the adipose gene expression profile entails a switch from a healthy, insulin-sensitizing, anti-inflammatory and anti-atherogenic secretion pattern of adipokines towards a pro-inflammatory, insulin-resistance promoting, atherogenic, and, finally, systemic pathological state [1]. The adipokine vaspin (visceral adipose tissue-derived serine protease inhibitor; SERPINA12) was initially found to be upregulated in visceral adipose tissue of diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats [2]. In murine models, vaspin overexpression as well as its exogenous administration exerts encouraging therapeutic features on

metabolic parameters [3–5] while *vaspin* mRNA and serum levels correlate with obesity, BMI, and type II diabetes in humans [6,7]. Studies of central vaspin administration revealed a significant reduction of food intake in rats and mice [8,9], and, recently, a novel vaspin-mediated signaling axis between brain and liver, regulating hepatic glucose production and insulin signaling, was unraveled [10]. *In vitro* studies provide evidence of anti-inflammatory [11,12], anti-atherogenic [13–16], and anti-apoptotic [17–19] properties of vaspin in various endothelial cell lines (reviewed in [20]). Nonetheless, the underlying molecular mechanisms of vaspin action and signal transduction are not well understood. In mice, vaspin was reported to serve as a ligand for the 78-kDa glucose-regulated protein (GPR78)/MTJ-1 complex in the liver upon ER stress as well as a ligand for GPR78 and voltage-dependent anion channels in endothelial cells [3,19]. In own

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studies, we were able to identify an insulin-degrading serine protease kallikrein 7 (KLK7) as the first target protease of vaspin [5] and provided evidence of vaspin binding to heparin sulfates in the extracellular matrix, potentially influencing protease interaction or intracellular signal transduction [21]. Among others, vaspin was also found to be strongly upregulated in brown adipose tissue (BAT) after cold exposure in microarray studies exploring intrinsic differences in cold-induced gene expression of brown and white adipose tissues in mice [22]. This feature appeared to be of interest since an accumulating number of studies indicate the involvement of adipokines in the activation and recruitment of BAT as well as browning of white adipose tissue (WAT) [23–26]. Based on the current knowledge, increasing BAT thermogenesis and inducing browning of WAT may prove to be promising anti-obesity and anti-diabetes approaches (reviewed in [27–31]). Until now, vaspin was mainly specified in an anti-obesity and anti-inflammatory context while physiological regulation requires further clarification. This study investigates the effects of BAT activating physiological stimuli, such as high caloric diets or cold exposure, on vaspin gene expression and secretion in mice.

2. METHODS

2.1. Animal models

Female C57BL/6NTac mice were purchased from Taconic Bioscience (Taconic, Lille Skensved, Denmark) at an age of 10 weeks. Mice were allowed to acclimatize for 2 weeks in pathogen-free facilities at 22 °C with a 12:12 h dark–light cycle and free access to water. After randomization for body weight, mice were assigned to different experimental groups. For dietary analyses, mice were group housed at 22 °C and fed either a high fat (D12492, 60 kJ% fat, Ssniff Spezialdiäten GmbH), high sugar (D12450B, 10 kJ% fat, 63% sucrose, Ssniff) or low glycemic reference diet (D12450J, 10 kJ% fat, high amylose starch, Ssniff) for 12 weeks ($n = 8$ per group). See [Supplementary Table 1](#) for detailed nutritional composition of the matched diets.

Recent studies demonstrated that conventional housing temperatures of 22 °C already display a mild cold stress to mice [32]. Therefore, the effect of ambient temperature on *vaspin* mRNA expression and secretion was examined in mice housed at thermoneutrality (30 °C) or under cold stress (8 °C). Mice were single-housed and acclimatized for one week at thermoneutrality (30 °C) and thereafter housed at 30 °C or 8 °C for one week ($n = 6$ per group). Mice were fed the same low glycemic reference diet used for the diet cohort. Animals from both groups were sacrificed by CO₂ and samples from intrascapular brown (iBAT), gonadal white (gWAT), and inguinal white (iWAT) adipose tissue as well as livers were collected, snap frozen in liquid nitrogen, and stored at –80 °C until further use. All animal experiments were approved by the local authorities of the State of Saxony, Germany as recommended by the responsible local animal ethics review board (Regierungspräsidium Leipzig, TVV39/14, Germany).

2.2. Quantitative real-time-PCR (qPCR)

Total RNA was isolated from snap frozen tissue samples using RNeasy lipid tissue Mini kit (Qiagen, Hildesheim, Germany) and 1 µg RNA was reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen). RNA isolation from cultured cells was done with InviTrap Spin Tissue RNA Mini Kit (Strattec Biomedical, Birkenfeld, Germany) as specified by the manufacturer. For quantification of gene expression, qPCR was performed using the LightCycler System LC480 and LightCycler-DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) as previously described [33]. Gene expression was calculated by $\Delta\Delta$ CT method and

normalized with respect to 36B4 or NoNo levels in each sample expression [34,35]. Primer sequences are listed in [Supplementary Table 2](#).

2.3. Western blot analyses

Organs were collected and immediately snap frozen in liquid nitrogen. Frozen tissue samples were grounded in a mortar on liquid nitrogen together with protein extraction buffer (RIPA, 150 mM NaCl, 10 mM Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA) completed with protease- and phosphatase inhibitors (Roche), incubated at 6 °C for 30 min, and centrifuged (16,000 rpm, 4 °C, 15 min). Total protein concentration of lysate supernatants was determined using bicinchoninic acid (BCA) assay according to manufacturer's indications (Pierce, Thermo Fisher, Waltham, MA, USA). For immunoblotting, 20 µg of total protein were subjected to SDS polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane using tank blot method overnight. Membranes were blocked with 3% BSA for 1 h at room temperature followed by primary antibody incubation at 4 °C overnight. Specific HRP-coupled secondary antibodies were used and chemoluminescence signals were detected using a gel documentation system. Primary antibodies were the following: Vaspin (USC-PAA-706Mu01, Cloud Clone Corp., Houston, TX, USA), UCP1 (ab23841, Abcam, Cambridge, UK), β -actin (A2066, Sigma, Darmstadt, Germany), and anti-rabbit-HRP (CST#7074, Cell Signaling, Danvers, MA, USA).

2.4. ELISA analyses

For quantification of vaspin plasma levels, blood samples were taken from fasted mice by cardiac puncture at the end of experiment. To obtain clear lysates, blood-EDTA samples were centrifuged at 4 °C and 11,000 rpm for 10 min. ELISA analyses was performed according to manufactures instructions (NB-E20150; Novateinbioscience, Cambridge, MA, USA).

2.5. Cell culture experiments

Immortalized white (3T3-L1) and brown pre-adipocyte (BAT) cell lines [36] were used to analyze vaspin expression *in vitro*. Cells were cultured in Dulbecco's Modified Eagle (DMEM, Gibco) supplemented with 10% or 20% fetal bovine serum (FBS), respectively. At two days post confluence (day 2), adipogenesis was induced by addition of 0.5 mM IBMX, 1 µM insulin and 0.4 µg/ml dexamethasone to the culture medium. After 48 h, medium was changed to insulin containing DMEM for another two days and subsequently to DMEM-FBS until day 10. To determine vaspin gene expression in the course of adipogenesis, cells were either harvested when confluent (day 0) or at the end of differentiation (day 10) and subjected to RNA isolation and qPCR analyses. To evaluate the influence of the PPAR γ activator rosiglitazone on vaspin expression, cells were additionally treated with 1 µM rosiglitazone starting at day 0.

2.5.1. Stimulation assay

Fully differentiated brown adipocytes were serum-starved overnight (16 h) and stimulated with Saline, 1 µM norepinephrine (NE) or 1 µM CL316,243 for 2 h and subjected to RNA isolation and qPCR analysis.

2.5.2. DNA demethylation in BAT cells

To analyze the influence of DNA methylation status on vaspin expression in BAT cells, pre-adipocytes were treated with indicated concentrations of 5'-aza-2'-deoxycytidine for 48 h (medium was changed every other day) and subsequently differentiated. At day 10, cells were harvested and subjected to further analyses.

2.6. Methylation analyses

2.6.1. DNA extraction and bisulfite conversion

Genomic DNA was extracted from 20 to 40 ng tissue using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma—Aldrich, USA). 300 ng of extracted DNA was further modified using Qiagen EpiTect Fast DNA Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols in order to determine CpG methylation by pyrosequencing.

2.6.2. Analysis of CpG methylation and CpG-SNPs in the *vaspin* promoter region

For analysis of CpG methylation two CpG assays including two (assay_1) and four (assay_2) CpG sites located near or in transcription factor binding sites within the promoter region of *vaspin* (Supplementary Figure 1) were created by using PyroMark Assay Design Software 2.0 (Qiagen). Potential transcription factor binding sites were identified by LASAGNA-Search 2.0 [37]. Primer sequences are listed in (Supplementary Table 3). The amount of 20 ng bisulfite treated DNA per sample was PCR amplified and the subsequent pyrosequencing analysis was run on a PyroMark Q24. The obtained results were analyzed via PyroMark Q24 software, version 2.0.6 (Qiagen). Each sample was PCR amplified and analyzed twice on different plates for replication purposes. Two no-template controls per plate containing water as well as bisulfite treatment controls included in the CpG Assays were used for quality control. Only samples that reached “passed quality” during the pyrosequencing run were taken forward. The interday coefficients of variance (CVs) for replicates over all samples vary from 0.016 to 0.039 for all analyzed tissues and assays. Mean methylation of replicates per CpG site as well as mean methylation level across all six CpGs were calculated and used for statistical analyses.

2.7. Statistical analyses

Data are shown as mean \pm SEM. Statistical significance was determined by Student's *t*-test for comparison of two experimental groups or by one-way ANOVA for comparison of multiple conditions followed by Bonferroni's post hoc test. Before linear regression analysis of *vaspin* and *Ucp1* mRNA expression data, Shapiro—Wilk *W* test was performed to test for normal Gaussian distribution of parameters. Prior to linear regression analysis non-normally distributed parameters were logarithmically transformed to approximate normal distribution. Graph Pad Prism 7.0 software (GraphPad, San Diego, Ca, USA) was used for all analyses and final presentation. *p* Values \leq 0.05 were considered to be significant. Statistical analyses of methylation data were performed using SPSS version 20.0.1 (SPSS, Inc. Chicago, IL). DNA methylation levels were used as continuous variables. To analyze differences in methylation levels between certain adipose tissue depots, Wilcoxon-tests were applied. Mann-Whitney-tests were performed to test for differences between mice housed under different conditions.

3. RESULTS

3.1. Influence of different treatment regimens on body weight gain, body temperature and BAT *Ucp1* expression

We determined changes in body weight, body temperature, and activation of BAT in terms of the expression level of the key mitochondrial gene *Ucp1*. All mice within the study had an average starting weight of 22 g. After 12 weeks of feeding control diet (Ref) and high sugar diet (HSD), mice gained 6–8 g while the high fat diet (HFD) fed cohort

gained \sim 17 g (Figure 1A). There were no differences in body weight after one week of thermoneutrality or cold-exposure (Figure 1B). Body temperature was exclusively upregulated in HFD animals while no differences were found between the temperature groups at the end of the experiment (Figure 1C,D). As a surrogate of BAT activation, *Ucp1* gene expression was measured in iBAT. While *Ucp1* levels were only slightly upregulated after both obesogenic diets (Figure 1E), cold exposure resulted in a strong induction of *Ucp1* mRNA (19-fold) compared to thermoneutral controls (Figure 1F).

3.2. Tissue-specific *vaspin* gene expression

To obtain an overview of *vaspin* mRNA expression levels in various tissues, we first analyzed several murine tissues from control-fed mice at thermoneutrality (Figure 2). Consistent with previous findings from human studies [38], we detected highest expression of *vaspin* mRNA in liver and skin tissue. Robust expression was also found in all examined adipose tissue depots with the highest levels in the white gonadal (gWAT) depot and the lowest in iBAT. Detectable levels were also measured in brain, heart, and spleen, and very low levels were detected in bone marrow; Ct-values in muscle and kidney remained below the threshold of our analyses (Figure 2). Subsequent analyses of dietary and cold exposure effects on *vaspin* gene expression were exclusively performed in iBAT, gWAT, iWAT, and liver tissue.

3.3. Obesogenic diets increase BAT *vaspin* mRNA and protein expression

After 12 weeks of HFD or HSD, *vaspin* mRNA expression was reduced in inguinal (iWAT) and gonadal WAT depots compared to controls (Figure 3A,B). In contrast, analysis of liver and iBAT tissue revealed a significant induction of *vaspin* mRNA expression by both high caloric diets and despite the differences in body weight gain (Figure 3C,D). The BAT-specific increase was particularly pronounced (\sim 10-fold) with the final *vaspin* expression in iBAT exceeding WAT levels (Supplementary Figure 2). In iBAT, *vaspin* protein levels were increased especially in HSD-fed animals (Figure 3E).

3.4. Cold-exposure induces *vaspin* gene and protein expression in BAT

In rodents, the most commonly used model of activating and recruiting BAT as well as inducing the browning of white adipose tissue is a sustained exposure to cold [39]. When comparing *vaspin* gene expression in gWAT and liver of mice housed under thermoneutral or cold, we observed no difference in expression (Figure 4A,D). Expression was increased in iWAT of cold-exposed animals (Figure 4B). Again, cold-induced changes were significant specifically in iBAT, where *vaspin* expression increased 5-fold (Figure 4C) and reached similar expression levels as gWAT (Supplementary Figure 3). In accordance with mRNA levels, *vaspin* protein expression was increased in iBAT of cold exposed mice, accompanied by a significant induction of UCP1 protein (Figure 4E).

3.5. BAT *vaspin* expression is associated with *Ucp1* gene expression

Gene expression analyses revealed a significant induction of *Ucp1* and *vaspin* mRNA in iBAT after obesogenic diets and cold exposure. Linear regression of expression data from all animals of the study demonstrated a significant association of *vaspin* and *Ucp1* mRNA expression in iBAT (standardized $\beta = 0.473$; $p = 0.004$, Supplementary Figure 4A).

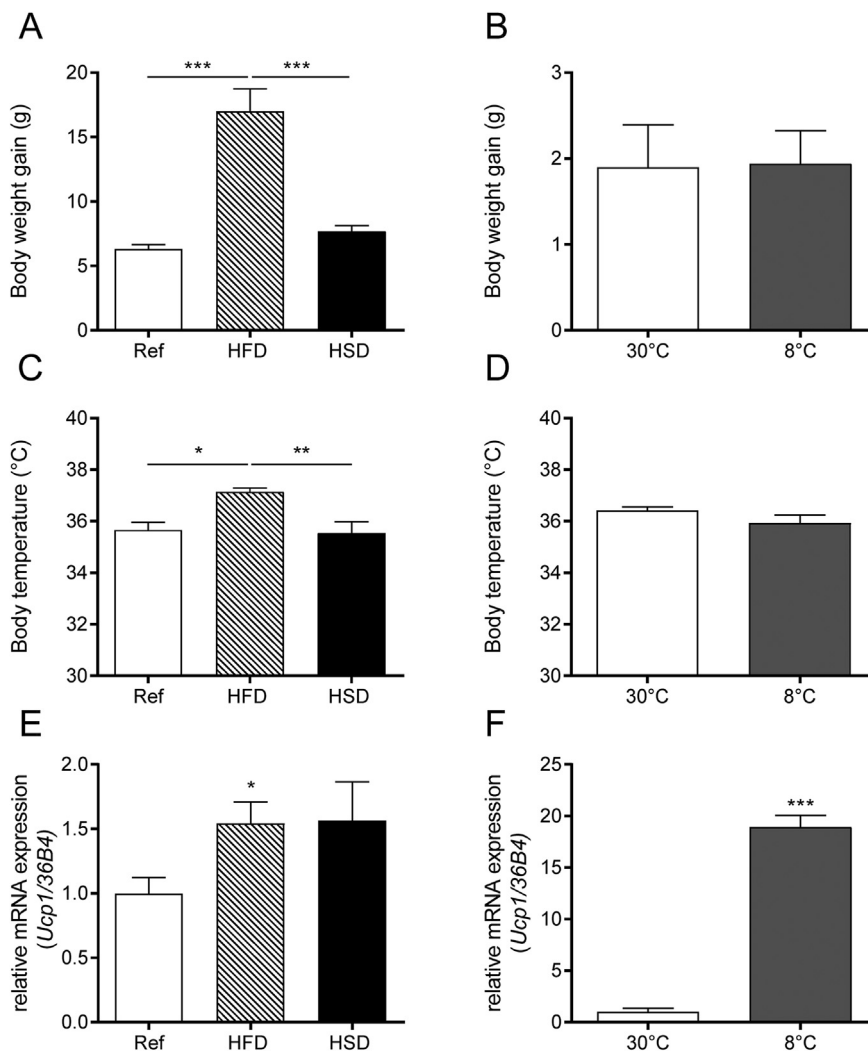


Figure 1: Dietary and thermogenic influences on body weight, body temperature and iBAT *Ucp1* expression. End point body weights and body temperatures after feeding the reference (Ref), high-fat (HFD) or high-sugar (HSD) diet for 12 weeks (A, C) and dependent on housing temperature (B, D). *Ucp1* gene expression in intrascapular brown adipose tissue (iBAT) of dietary (E) and thermogenic mice (F) was slightly increased after obesogenic diets and markedly after cold exposure. mRNA data are calculated as relative values to Ref diet or thermoneutral (30 °C) groups, respectively. Results are normalized to *36B4* and presented as mean \pm SEM (* p < 0.05; ** p < 0.01, *** p < 0.001), n = 8 (diets) or 6 (cold exposure) per group.

3.6. High caloric diets and cold-exposure decrease vaspin plasma levels

Despite increased expression in iBAT and liver after high caloric diets, overall vaspin plasma concentrations were decreased after feeding a HFD and more pronounced in HSD fed mice after 12 weeks (Figure 5A). Likewise, vaspin plasma levels were also decreased in cold-exposed animals compared to the thermoneutral cohort, despite higher expression levels in iBAT and iWAT (Figure 5B).

3.7. *Vaspin* gene expression is increased during differentiation in BAT cells

Based on previous results indicating a BAT-specific regulation of vaspin, we analyzed vaspin gene expression in cell culture models of white (3T3-L1) and brown (BAT) adipocytes (Figure 6A). Comparing *vaspin* gene expression in undifferentiated 3T3-L1 and BAT pre-adipocytes, we found no significant differences. However, during adipogenesis *vaspin* gene expression was significantly increased in BAT cells. We did not observe differential expression in 3T3-L1

adipocytes. In addition, we chronically treated BAT cells with the PPAR γ specific agonist rosiglitazone. At the end of differentiation (day 10), rosiglitazone treatment enhanced vaspin mRNA expression three-fold (Figure 6B) and slightly increased *Ppar γ* gene expression (Figure 6C) within BAT cultures.

3.8. β -adrenergic stimuli do not increase *vaspin* mRNA expression *in vitro*

As *vaspin* mRNA expression was increased in differentiated BAT cells and in BAT of cold exposed mice, we asked if β -adrenergic signaling as a classical BAT activator may be involved in the regulation of vaspin gene expression. Fully differentiated, immortalized BAT cells were stimulated with norepinephrine (NE) or the β_3 -specific adrenoceptor agonist CL316,243 [40], and *Ucp1* expression served as a surrogate for thermogenic gene activation. As expected, *Ucp1* mRNA levels increased significantly under both treatment regimens (Figure 7A,B). However, we found no effect of NE or CL316,243 on *vaspin* mRNA expression in our set up,

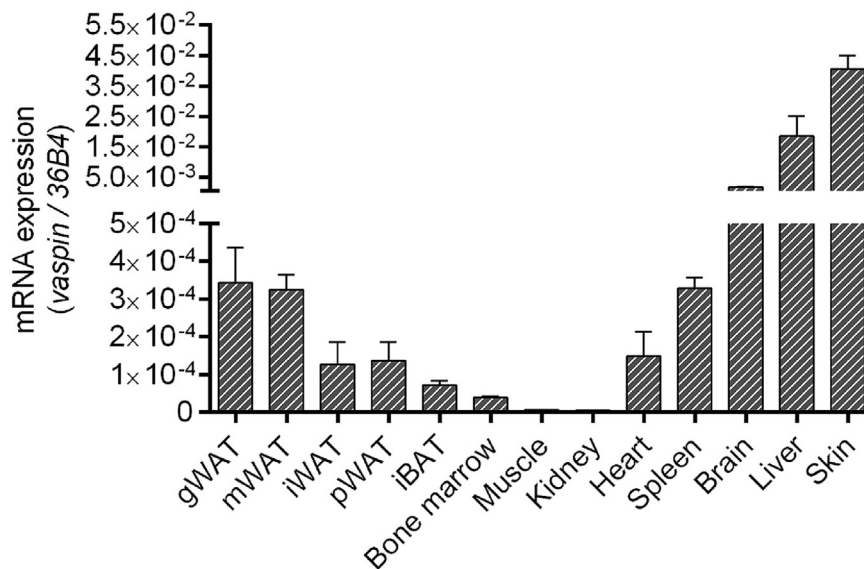


Figure 2: Tissue-specific vaspin gene expression at thermoneutrality. Absolute *vaspin* mRNA expression levels in various tissues of female C57BL/6NTac mice housed at thermoneutrality for two weeks. Data are presented as mean \pm SEM and gene expression was normalized to *36B4*. gWAT, mWAT, iWAT, pWAT and iBAT indicate gonadal, mesenteric, inguinal, perirenal white, and intrascapular brown adipose tissues (n = 5 per group).

indicating the lack of direct norepinephrine or β_3 -specific effects (Figure 7C,D).

3.9. Methylation of the vaspin promoter region in AT

Given the induction of *vaspin* gene expression especially in iBAT of cold exposed mice, further analyses were carried out to elucidate potential mechanisms regulating *vaspin* expression. Thus, we focused on DNA methylation, a reversible epigenetic process enabling the adaption to environmental changes, which has already been shown to affect expression of other important adipokines such as leptin and adiponectin [41–43]. Since a decrease in promoter DNA methylation is mostly accompanied by amended binding of transcription factors (TF) and increased mRNA expression, we focused on analyzing promoter DNA methylation of the *vaspin* gene at binding sites for PPAR γ :RXR α and TCF7L2 — both TFs involved in adipogenesis (Supplementary Figure 1). In summary, we observed very high DNA methylation within the analyzed vaspin promoter regions, which was over 88% in all ATs (Figure 8A). Importantly, in iBAT of cold-exposed mice, mean methylation (over all sites) was significantly lower compared to iWAT and gWAT from cold exposed groups (Figure 8B). Interestingly, exclusively iBAT methylation levels decreased after cold exposure (Figure 8C). This seems to be particularly driven by CpG-525 (Figure 8D; Supplementary Table 4), since methylation at this site associated significantly with the overall mean methylation in iBAT ($p = 0.009$; standardized $\beta = 0.714$, Supplementary Figure 4B). The CpG-525 site is directly located proximal to a potential PPAR γ :RXR α binding site (Supplementary Figure 1). To investigate the impact of methylation status on *vaspin* gene expression *in vitro*, BAT pre-adipocytes were exposed to the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) for 48 h and subsequently differentiated until day 10. Supporting the *in vivo* findings, vaspin mRNA expression was markedly enhanced after 5-aza-dC treatment of BAT cells (Figure 8E).

4. DISCUSSION

Little is known about physiological stimuli influencing vaspin expression and secretion. Using different rat models, Gonzales et al. found

regulation of *vaspin* gene expression by age, gender, and fed state in gWAT. gWAT expression was also influenced by thyroid hormone status with higher *vaspin* mRNA levels in hypothyroid rats [44]. Treatment with the insulin-sensitizing agent metformin increased *vaspin* mRNA levels as leptin did under fasting conditions in normal-weight rats [44]. Notably, most conditions were accompanied by significant changes in body weight, which may additionally influence or regulate vaspin expression. Also noteworthy, vaspin came up in a global expression analyses evaluating genes enriched in WAT and BAT of cold exposed mice, ranging under the top five upregulated BAT-specific gene probes [22]. This is of particular interest as the activation and recruitment of BAT may represent new strategies to combat obesity and associated diseases such as type-2 diabetes [29,30] and thus stresses the importance in identifying novel regulators of BAT activity.

To gain further insight into regulatory mechanisms, we focused on physiological factors known to influence BAT metabolism and activity. Sustained cold exposure, i.e. sympathetic activation, especially provokes non-shivering thermogenesis, enabling maintenance of body temperature through a muscle-independent mechanism [32,45]. Calorie-dense diets provoke a similar effect on BAT activity termed diet-induced thermogenesis [46]. Mentionable, dietary carbohydrates and fatty acids are known to differentially affect body composition, metabolic pathways, or gene expression [47–49]. To delineate nutritional effects of fat and sugar we used a high fat, a high sugar diet or a matched, low glycemic reference diet to evaluate the effects of high caloric diets and source of calories on *vaspin* expression. In accordance with others, *Ucp1* mRNA expression was upregulated upon HFD [50], after HSD and markedly after cold exposure. Previous studies mainly focused on vaspin expression in WAT depots [4,44] while BAT-specific expression of vaspin has not been further examined. Our data reveal that vaspin is specifically regulated in BAT by various physiological stimuli. Both obesogenic diets significantly enhanced expression in iBAT but also liver tissue accompanied by a slight reduction of *vaspin* mRNA levels in gWAT and iWAT. The reduction in WAT depots did not reach statistical significance but is in line with previous reports on vaspin expression in periepididymal WAT

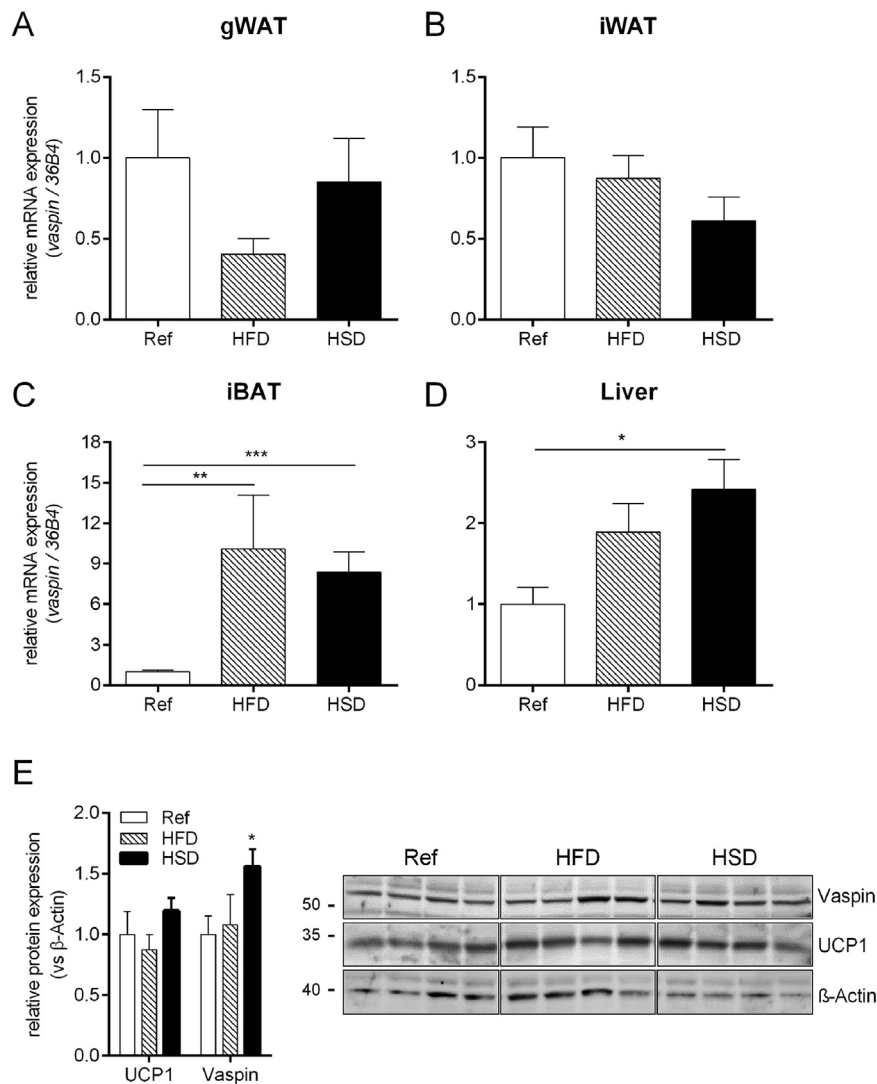


Figure 3: Effects of obesogenic diets on vaspin gene expression and protein levels. Female C57BL/6NTac mice were subjected to HFD, HSD, or reference (Ref) diet for 12 weeks and *vaspin* mRNA expression in gonadal white (gWAT) (A), inguinal white (iWAT) (B), intrascapular brown (iBAT) (C), and liver tissue (D) was measured. Obesogenic diets increased *vaspin* expression in iBAT and liver. Relative values were calculated using Ref-fed animals as control group. *Vaspin* gene expression was normalized to *36B4*, and data are presented as mean \pm SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), $n = 8$ per group. (E) Western blot analysis of vaspin protein levels in iBAT indicated higher vaspin protein expression in HSD fed mice compared to controls. UCP1 protein expression was not significantly increased. Protein expression of β -actin served as reference ($n = 4$ per group).

depots after HFD in rats [51]. In WAT of the type-2 diabetes OLETF rat, vaspin expression increased with body weight gain and progressing insulin resistance before declining with further progression of the diabetic phenotype [4]. While body weight or body weight gain may contribute to differential expression of vaspin in tissues of HFD-fed mice, the similar vaspin expression changes induced by the HSD were independent of body weight. Furthermore, cold acclimation had no effect on gWAT and liver vaspin expression but solely increased vaspin levels in iBAT and iWAT which is known to have a high susceptibility to undergo a white to brown transdifferentiation [52]. Together, these expression data support functional relevance of vaspin in BAT activation or activity.

With respect to expression in AT, autocrine functions of vaspin have been reported in adipocytes, as vaspin reduced expression of inflammatory genes in WAT of transgenic mice and after intraperitoneal application [3,4]. Also, it was shown to promote adipocyte differentiation by increasing the expression of adipocyte-specific markers (C/EBP α ,

PPAR γ , FABP4) in a dose-dependent manner in 3T3-L1 cells [53]. Moreover, lipid droplet size was also markedly decreased in vaspin treated 3T3-L1 cells, which may indicate a more brown-like phenotype [53]. When we analyzed vaspin expression in 3T3-L1 before and after differentiation (day 0 vs. day 10), we did not observe significant changes in *vaspin* expression after full differentiation. This is in line with a previous study reporting unchanged vaspin protein expression and secretion in 3T3-L1 cells over the course of differentiation [51]. Others though found significant increases of both vaspin mRNA and protein expression detected during differentiation of 3T3-L1 cells, with a peak of 4-fold at day 4 and a moderate 2-fold increase in fully differentiated adipocytes [54]. In immortalized BAT cells, however, we observed enhanced expression of *vaspin* mRNA with induction of adipogenesis and significantly higher expression upon full differentiation. Together, these data indicate beneficial effects of vaspin on BAT activation and function. Counterintuitively, enhanced *vaspin* gene expression was accompanied by decreased vaspin plasma levels in both high-caloric-diet fed

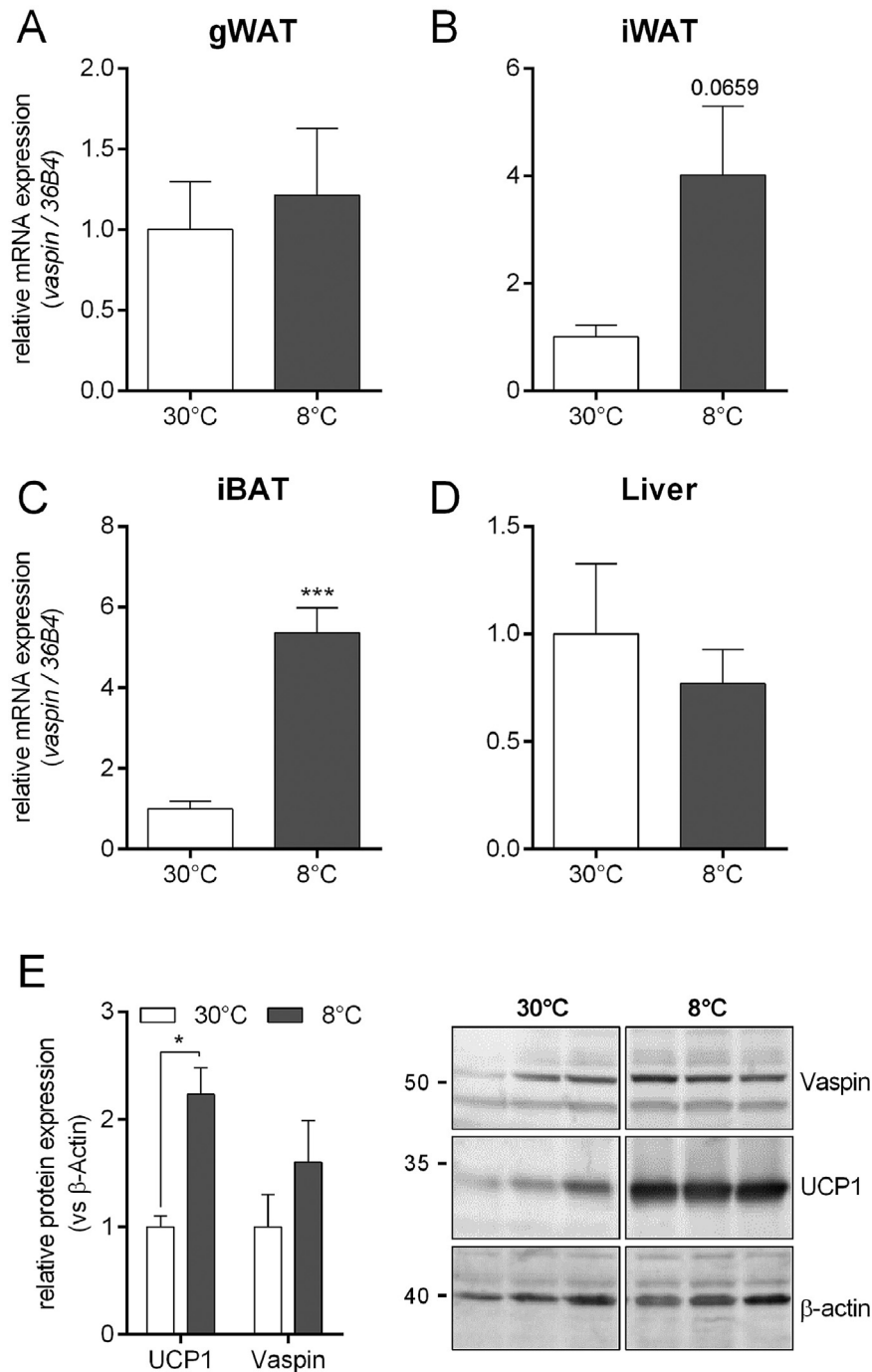


Figure 4: Effects of cold acclimatization on *vaspin* gene expression and BAT-specific protein levels. Mice were adapted to thermoneutrality (30 °C) or cold (8 °C) for one week and *vaspin* mRNA expression was determined. Analyses of gonadal white (gWAT) (A), inguinal white (iWAT) (B), intrascapular brown (iBAT) (C), and liver tissue (D) revealed strong induction of *vaspin* gene expression in iBAT and a mild increase in iWAT. Relative values were calculated using the thermoneutral mice as control. *Vaspin* gene expression was normalized to *36B4* and data are presented as mean \pm SEM (* $p < 0.05$;*** $p < 0.001$), $n = 6$ per group. (E) Western blot analysis revealed enhanced vaspin and UCP1 protein levels upon cold exposure in iBAT, β -actin served as reference ($n = 3$ per group).

and cold exposed animals. Previous studies also have reported a reduction of vaspin serum levels after HFD in rats [51]. For the obesogenic cohort, it may be assumed that the increased expression in iBAT and liver may not prove enough to compensate for the blunted expression in iWAT and gWAT. Yet after cold exposure, vaspin plasma levels were decreased although mRNA expression was increased or

unchanged in all investigated tissues. With respect to endocrine functions of vaspin as an inhibitory serpin, increased expression of target proteases by obesogenic diets or cold-exposure may, in turn, increase vaspin consumption and result in lower detectable levels in the circulation. Furthermore, it remains unclear which organs contribute to circulating vaspin levels and to what extent, especially as

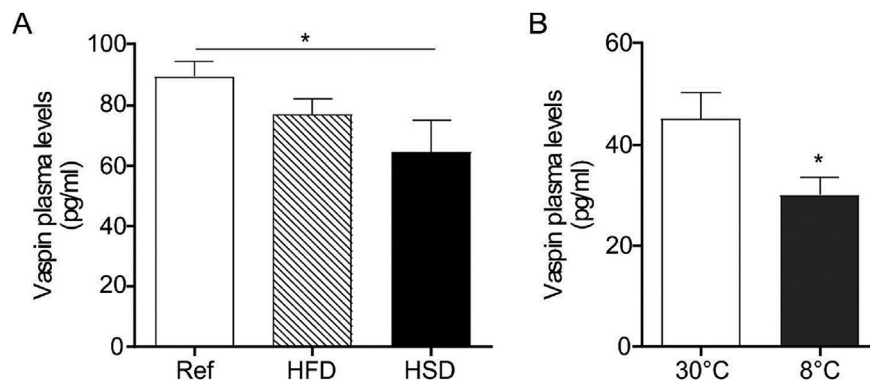


Figure 5: Influence of obesogenic diets and cold exposure on vaspin plasma levels. Mice were exposed to obesogenic and thermogenic stimuli, and plasma was obtained at the end of the experiment. Vaspin plasma levels were decreased after feeding obesogenic diets (A) and after cold exposure (B). Data are presented as mean \pm SEM (* p < 0.05), n = 6–8 per group.

we and others observed highest expression levels in liver and skin. Together, these data indicate that serum vaspin levels are not a good surrogate for AT vaspin expression. In transgenic mice overexpressing vaspin in AT, it has been shown that vaspin may act in an endocrine manner and ameliorates HFD-induced hepatic steatosis and oxidative

stress in the liver via interaction with the endoplasmic reticulum chaperone GRP78 [3]. Also, vaspin expressed in the liver may act locally or in an autocrine fashion by interacting with GRP78 on hepatocytes. It remains elusive whether these signaling mechanisms are activated under the conditions investigated in this study and to what extent they may contribute to clearance of vaspin from the circulation. As mentioned above, both cold exposure and also high caloric diets induce BAT activation via sympathetic stimulation by NE. However, exogenous stimulation of BAT cells with NE or CL316,243 did not induce vaspin expression *in vitro* implying that vaspin is not directly regulated by beta adrenergic agonists. In isolated white and beige

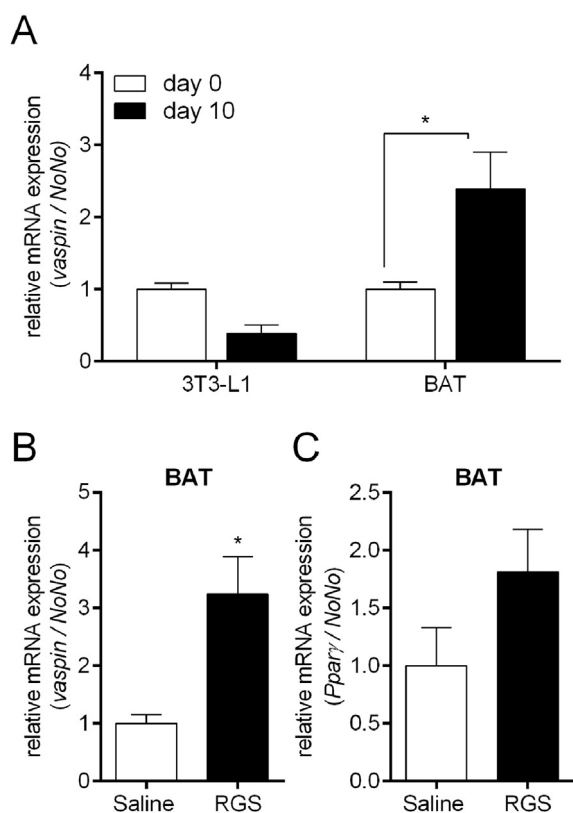


Figure 6: Vaspin mRNA expression pre- and post-differentiation of 3T3-L1 and BAT cells and after rosiglitazone (RGS) treatment. (A) BAT cells and 3T3-L1 adipocytes were harvested as pre-adipocytes (day 0) or after full differentiation (day 10) and *vaspin* gene expression was determined. *Vaspin* mRNA was upregulated in BAT cells but not in 3T3-L1 adipocytes after adipogenic differentiation. (B) Rosiglitazone (RGS) treatment significantly increased *vaspin* mRNA expression and slightly upregulated *Ppar γ* gene expression in BAT cells. Gene expression was normalized to *NoNo*. Data are presented as mean \pm SEM (* p < 0.05) from at least three experiments with two biological replicates.

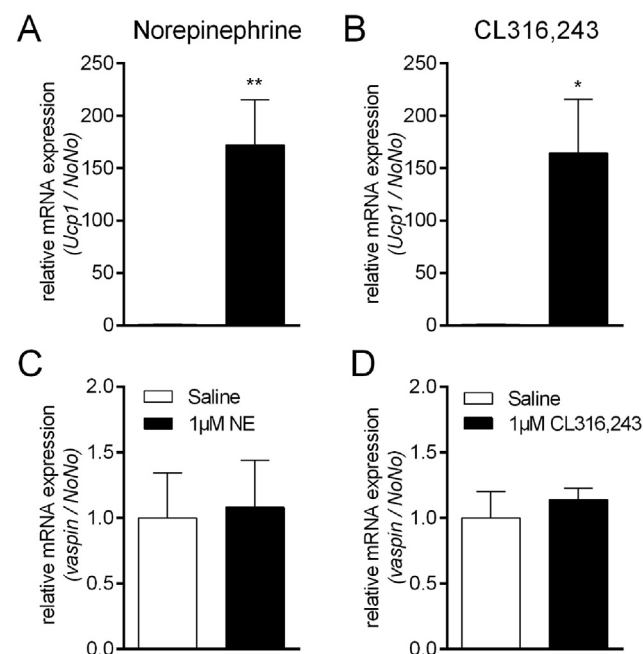


Figure 7: Regulation of *vaspin* gene expression in BAT cell cultures stimulated with NE or CL316,243. Fully differentiated BAT cells were exposed to (A, C) 1 μ M norepinephrine (NE) or (B, D) 1 μ M CL316,243 for 2 h and *Ucp1* (A, B) and *vaspin* (C, D) gene expression was subsequently analyzed. Both stimuli markedly induced *Ucp1* expression but did not affect *vaspin* mRNA levels. Gene expression was normalized to *NoNo*. Data are shown as mean \pm SEM and relative values were calculated using saline treated cells as control. (* p < 0.05; ** p < 0.01), n = at least three experiments with two biological replicates.

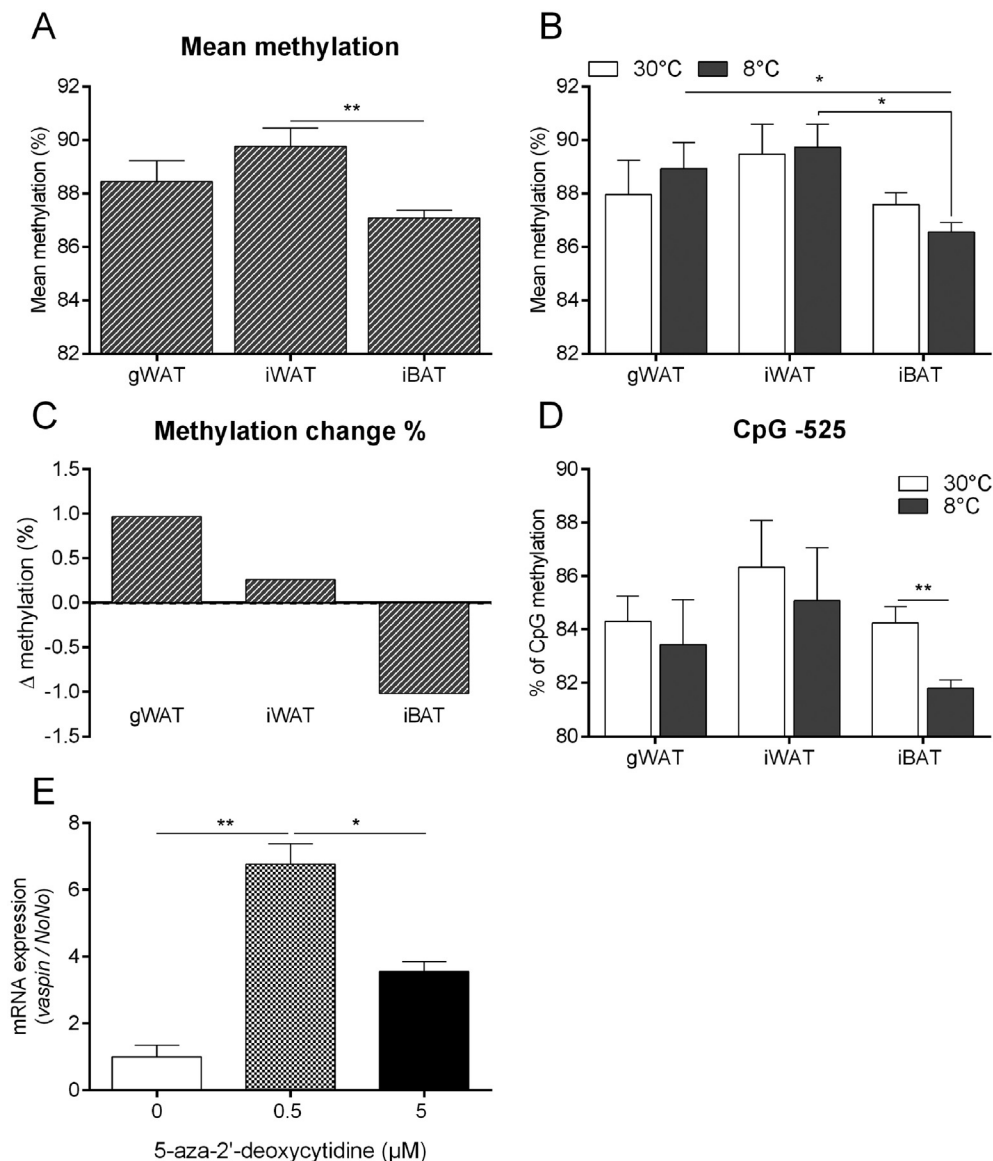


Figure 8: Effects of cold exposure on vaspin promoter methylation in adipose tissues and influence of DNA demethylation on vaspin mRNA expression in BAT cells. Mice were housed at indicated temperatures (30 °C vs 8 °C) for one week, genomic DNA was extracted, bisulfite converted, and promoter methylation was determined. (A) Mean promoter methylation of gonadal white (gWAT), inguinal white (iWAT) and intrascapular brown (iBAT) AT revealed high general methylation levels but lowest methylation in iBAT. (B, C) Percentage of vaspin promoter methylation after cold exposure was decreased exclusively in iBAT. (D) Promoter methylation at CpG-525 proximal to the putative PPAR γ :RXR α binding site was significantly reduced in iBAT of cold exposed mice. $n = 6$ per group. (E) Treatment of BAT pre-adipocytes with 5-aza-2'-deoxycytidine results in increased vaspin mRNA levels after adipogenic differentiation. $n =$ two experiments with two biological replicates. Data are presented as mean \pm SEM. (* $p < 0.05$; ** $p < 0.01$).

adipocytes, it has been shown that cold-exposure can induce thermogenic genes in a cell autonomous manner independent of the NE/cAMP/PKA/CREB pathway [55]. Though the exact mechanism of this regulatory process is unclear, it may also be relevant in the regulation of *vaspin* gene expression in WAT in response to cold. Importantly, external cold stimulus did not induce activation of isolated brown adipocytes [55].

Thus, we asked for other molecular mechanisms contributing to the induction of *vaspin* gene expression observed in BAT. Previous studies have demonstrated whole-genome epigenetic changes induced by feeding a HFD [56,57]. Epigenetic regulations via DNA methylation in AT and obesity have been already shown for GLUT4 [58] and prominent adipokines such as leptin and adiponectin [41,43,59]. Leptin gene expression depends on methylation-status prior to adipogenesis in

3T3-L1 cells [42] and is regulated by variations in promoter methylation and acetylation in AT of diet-induced obese mice [59,60]. Recent studies also demonstrated that the target protease of vaspin, KLK7, is regulated by epigenetic mechanisms. Treatment with the histone deacetylase inhibitor trichostatin A increased KLK7 expression in pancreatic (Panc-1) and cervical (HeLa) cancer cell lines [61]. Importantly, HFD-induced changes seem to be readily reversible after weight-loss, as reported for hepatic epigenetic changes induced after 7 weeks of HFD in mice [62], and HFD-induced DNA-hypermethylation could also be suppressed via exercise in mice [57]. DNA methylation in the promoter regions of the *vaspin* gene in WAT and BAT has not been investigated previously. Methylation analyses of AT from cold-exposed mice revealed a high basal vaspin promoter methylation of at least 87% independent of depot or treatment. Thereby, iBAT exhibited

significantly lower methylation levels compared to white AT depots. Most interestingly, cold exposure exclusively downregulated methylation of the vaspin promoter in iBAT and may contribute to the increase in *vaspin* gene expression in iBAT. Also for *Ucp1*, tissue-specific methylation levels of *Ucp1* enhancer regions have been found in AT of cold-exposed mice and *Ucp1* gene expression seemed to be influenced by demethylation but also chromatin remodeling [63]. Supporting the role of epigenetic regulation of *vaspin* in BAT, we found significantly increased *vaspin* expression in isolated BAT cells after DNA demethylation using the DNA methyltransferase inhibitor 5-azadC. In BAT of cold-exposed mice, especially the reduction in methylation at CpG -525 proximal to the putative PPAR γ :RXR α binding site implies physiological relevance. PPAR γ is known as a key regulator of adipogenesis and is involved in brown adipocyte development, regulation of the thermogenic program and treatment with PPAR γ agonists promotes browning of WAT (reviewed in [64]). Activation of PPAR γ in 3T3-L1 cells using pioglitazone significantly induced vaspin protein expression and secretion [51]. *In vivo*, chronic pioglitazone treatment increased vaspin serum levels and mRNA expression in subcutaneous AT but decreased visceral AT expression in rats [4]. Also, activation of PPAR α using fenofibrate increased *vaspin* mRNA expression in 3T3-L1 cells, visceral AT depots in rats, as well as serum levels in rats and humans [54]. Using the PPAR γ selective agonist rosiglitazone, we also found enhanced *vaspin* expression in chronically treated BAT cells. *Ppar γ* expression was only marginally increased by rosiglitazone in BAT cells, which is in line with *in vivo* data in BAT of rats, where chronic PPAR γ activation did not induce expression of *Ppar γ* , *Pgcl α* or *C/EBP α* [65]. *Ucp1* expression was significantly upregulated, both in rats with innervated and denervated BAT [65]. Thus, rosiglitazone seems to induce *vaspin* expression via a rosiglitazone/PPAR γ pathway independent of BAT adipocyte differentiation.

In addition, free fatty acids (FFA) as endogenous activators of PPAR γ can activate PPAR γ target gene expression (reviewed [66]). In line with this hypothesis, *vaspin* expression in WAT is repressed by fasting and significantly increased upon refeeding [44]. Increased FFA levels after HFD and HSD but also after cold-exposure [67] as well as increased FFA synthesis especially in BAT after cold-exposure [68] may activate PPAR γ -regulated gene and *vaspin* expression in parallel to the NE/cAMP/PKA/CREB axis regulating *UCP1* expression in mice. The pronounced effects of *vaspin* regulation in BAT may also further be aided by the ANGPTL4 mediated shuttling of FFAs to BAT during cold-exposure [69]. Furthermore, BAT-specific methylation of the specific CpG -525 site significantly correlated with *Ucp1* gene expression and food intake. Together, these data show for the first time a differential expression of vaspin in BAT which is in part regulated by epigenetic changes at least after cold-exposure. It is important to note, that we did not investigate the full promoter region, but focused on selected sites with respect to potential physiological relevance. Thus, likely more sites are to be found, that contribute to differential transcription of the *vaspin* gene via acute changes in DNA-methylation.

In conclusion, we demonstrate a novel BAT-specific regulation of *vaspin* gene expression upon BAT activating physiological stimuli *in vivo* with contribution of epigenetic changes. Our data suggest that vaspin gene expression is specifically increased in activated BAT, indicating functional relevance and potentially beneficial effects of vaspin on BAT function.

AUTHOR CONTRIBUTIONS

JW and JTH conceived the study, designed and conducted experiments, analyzed data, and wrote the paper. KR and YB performed the

gene methylation analysis. KK and KZ conducted experiments and analyzed data. NK performed mouse experiments. SK contributed the BAT cell line and cell culture experiments. PK, MS and MB interpreted and analyzed data and critically edited the manuscript. All authors discussed results and edited and commented on the manuscript. JTH supervised the project.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2017.03.004>.

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