Brief Communication

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Intact vitamin A transport is critical for cold-mediated adipose tissue browning and thermogenesis

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ABSTRACT

Objective: Transformation of white into brown fat ("browning") reduces obesity in many preclinical models and holds great promise as a therapeutic concept in metabolic disease. Vitamin A metabolites (retinoids) have been linked to thermogenic programming of adipose tissue; however, the physiologic importance of systemic retinoid transport for adipose tissue browning and adaptive thermogenesis is unknown.

Methods: We performed cold exposure studies in mice and humans and used a genetic model of defective vitamin A transport, the retinol binding protein deficient ($Rbp^{-/-}$) mouse, to study the effects of cooling on systemic vitamin A and the relevance of intact retinoid transport on cold-induced adipose tissue browning.

Results: We show that cold stimulation in mice and humans leads to an increase in circulating retinol and its plasma transporter, Rbp. In $Rbp^{-/-}$ mice, thermogenic programming of adipocytes and oxidative mitochondrial function are dramatically impaired in subcutaneous white fat, which renders $Rbp^{-/-}$ mice more cold-sensitive. In contrast, retinol stimulation in primary human adipocytes promotes thermogenic gene expression and mitochondrial respiration. In humans, cold-mediated retinol increase is associated with a shift in oxidative substrate metabolism suggestive of higher lipid utilisation.

Conclusions: Systemic vitamin A levels are regulated by cold exposure in mice and humans, and intact retinoid transport is essential for cold-induced adipose tissue browning and adaptive thermogenesis.

1. INTRODUCTION

During obesity development, excess energy intake is mainly stored in white adipose tissue (WAT) in the form of triglycerides [1]. In contrast, brown adipose tissue (BAT) oxidises fatty acids and dissipates energy through uncoupled respiration and thermogenesis, a process mediated primarily by the key thermogenic factor uncoupling protein 1 (UCP1) [2,3]. Promoting brown fat function *in vivo* has consistently demonstrated a decrease in adiposity and related metabolic complications in multiple preclinical models [4—6]. In humans, BAT activity varies inversely with obesity and appears inducible by cold exposure. In addition to activating existing BAT, the induction of UCP1 expressing beige/brite adipocytes within white fat, a phenomenon termed *browning*, has been raised as another alternative increasing energy expenditure and promoting a lean phenotype [7—9]. Thus,

understanding the molecular mechanisms that lead to chronic BAT activation and/or browning of WAT represents a critical step in the development of novel therapeutic approaches for the treatment of obesity.

Retinoid metabolism (vitamin A and its derivatives) is known to be integral in regulating energy balance through actions in adipose tissue and the liver [10—12]. Retinoids are diverse signalling molecules that can modulate the activity of the nuclear receptors retinoic acid receptor (RAR) and retinoid X receptor (RXR) [13,14]. Physiologic retinoid concentrations depend on vitamin A intake, tissue storage and subsequent modification through a complex enzymatic network. Vitamin A must be obtained through dietary intake of either preformed vitamin A (retinol and/or retinyl esters) or provitamin A (carotenoids) [15]. Retinol is stored in the form of esters predominantly in hepatic stellate cells [16]. In plasma, retinol is transported to target tissues bound to retinol-

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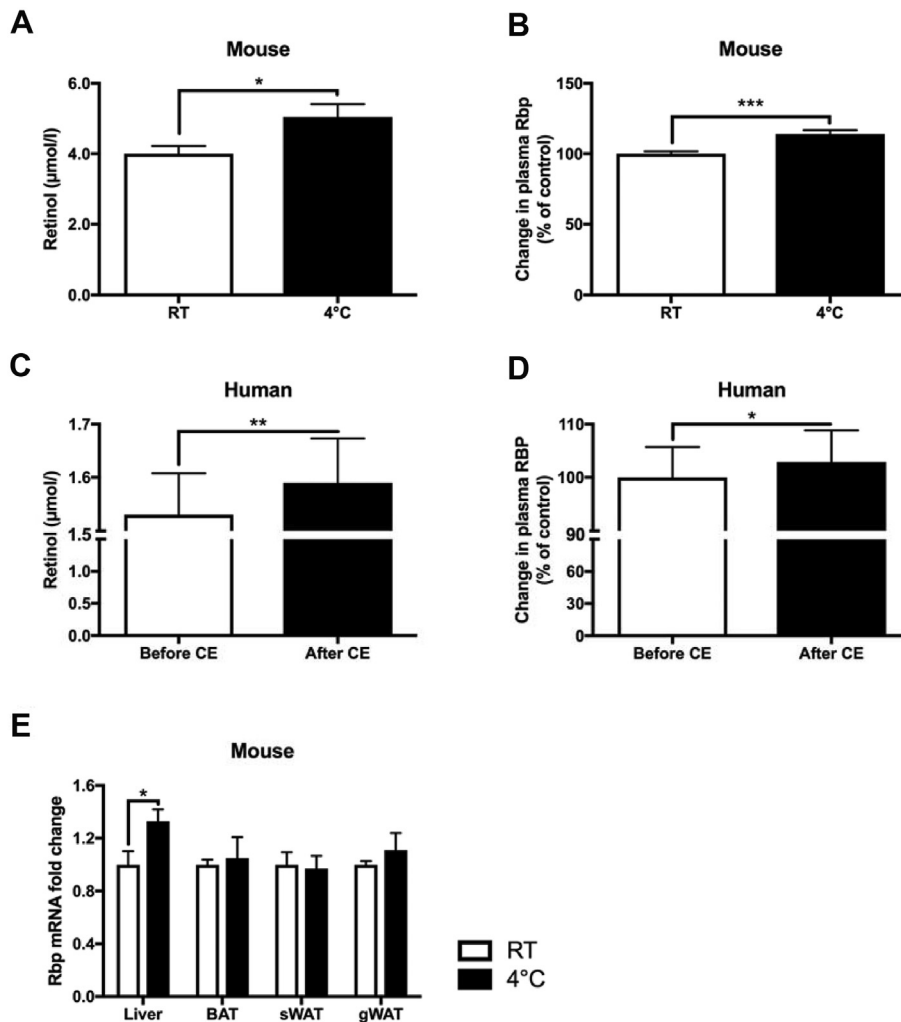
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Figure 1: Cold exposure regulates systemic retinoid levels in mice and humans. Circulating retinol and Rbp concentrations in mice (n = 9–10/group) housed at either room temperature or 4 °C (**A, B**). Plasma retinol and RBP concentrations in humans (n = 30) before and after cold exposure [48] for 2 h (**C, D**). Analysis of tissue retinol levels of mice (n = 7–9/group) housed at either room temperature or 4 °C (**E**). Rbp gene expression of WT mice (n = 4–5/group) housed at RT or 4 °C. Data are given as mean ± SEM. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

binding protein (RBP or RBP4), the sole specific retinoid carrier [14,17]. Once taken up by target cells, retinol is reversibly oxidised to retinaldehyde by alcohol- and retinol dehydrogenases, followed by irreversible oxidation to retinoic acid by retinaldehyde dehydrogenases [9,15]. Most actions of the vitamin A pathway are considered to be mediated by the metabolite retinoic acid [14,17–25].

Previous reports in mice revealed an association between global vitamin A deficiency and obesity. Retinoids inhibit adipogenesis *in vitro*, while retinoid administration *in vivo* can limit obesity [12,26,27]. Retinoic acid stimulation of adipocytes induces expression of BAT marker genes, such as UCP1 [19,20,23]. We reported that raising retinaldehyde levels by retinaldehyde dehydrogenase 1 deficiency or antisense induced a WAT thermogenic programme increasing energy expenditure and protecting against obesity [10]. These findings suggest retinoids as potent regulators of a thermogenic programme in fat *in vitro* and *in vivo*. Given that RBP is the predominant retinol carrier in the blood transporting retinoids to target tissues, such as adipose tissue, we hypothesised that intact retinoid transport is important for cold adaption and thermogenic responses in adipocytes.

2. RESULTS

2.1. Cold exposure regulates systemic RBP and retinoid levels in mice and humans

To study the effects of cold exposure on systemic vitamin A transport, we first investigated cold-mediated regulation of retinol and RBP levels in mice and humans. Cold exposure (24 h at 4 °C) significantly increased circulating retinol and RBP concentrations in 129/Sv x C57BL/6J mice (Figure 1A,B). Next, we tested whether cold-induced alterations in vitamin A metabolism were also present in humans. Thirty healthy lean subjects (baseline characteristics Table S1) were exposed to moderate cold (14–17 °C) for 2.5 h using water-perfused cooling vests under a protocol which has been used successfully to activate BAT in humans [28–30]. Notably, only 2.5 h of moderate cold exposure increased circulating retinol and RBP concentrations significantly in these lean subjects (Figure 1C,D). In accordance with increased Rbp plasma concentrations, cold exposure induced *Rbp* mRNA expression in mouse liver, the predominant site for RBP production (Figure 1E). Other genes of the retinoid machinery such as

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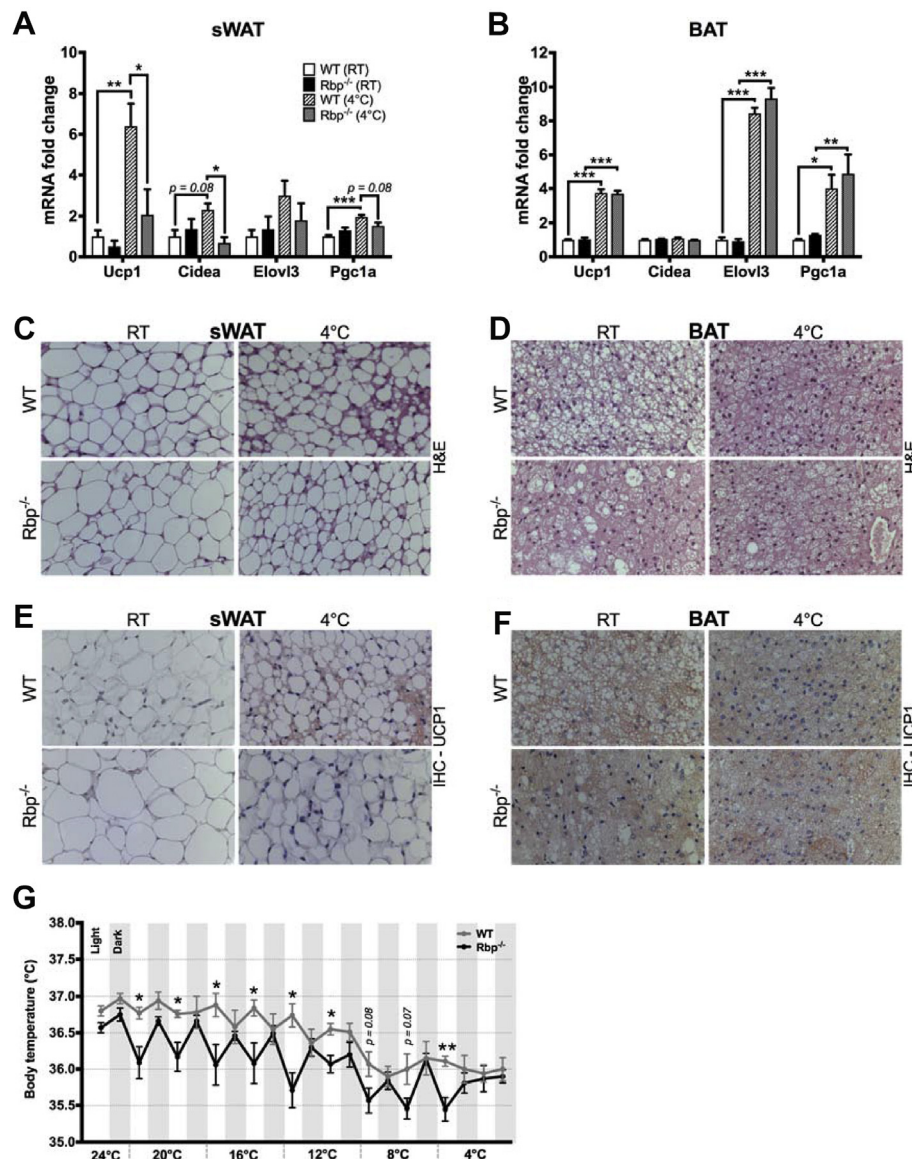
Figure 2: Intact retinol transport is vital for WAT browning. Expression of thermogenic genes in sWAT (**A**) and BAT (**B**) of WT and Rbp$^{-/-}$ mice. Representative images of haematoxylin and eosin staining (H&E) (**C, D**) as well as UCP1 immunohistochemistry (**E, F**) of sWAT and BAT from WT and Rbp$^{-/-}$- mice exposed to RT or 4 °C. Telemetric core body temperature measurements in WT and Rbp$^{-/-}$ mice during a stepwise cooling challenge (**G**). n = 5/group, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001, ns = not significant.

Cyp26a1, Rarβ and *Stra6* were unaltered by cold exposure in subcutaneous white adipose tissue (sWAT) and liver (Fig. S1a,b).

2.2. Intact retinol transport is vital for WAT browning and cold adaption

To study the functional effects of disturbed retinoid transport on cold-induced thermogenesis, we used Rbp-deficient (Rbp$^{-/-}$) mice, a model for impaired hepatic retinol mobilisation and defective retinol transport. Rbp$^{-/-}$ mice maintained on a vitamin A-rich diet do not manifest global vitamin A deficiency; however, mobilisation of hepatic retinoid stores and transport to target tissues are significantly impaired [31]. Both wild-type (WT) and Rbp$^{-/-}$ mice were maintained at room temperature (24 °C) or exposed to cold (4 °C) for 24 h. In line with perturbed systemic vitamin A transport, Rbp$^{-/-}$ mice had substantially lower circulating retinol levels than their WT littermates and the cold-induced retinol increase seen in WT mice was absent in the knockout animals

(Fig. S1c). Cold-stimulated expression of important thermogenic genes, including *Ucp1, Cidea, Elovl3, and Pgc1α,* was repressed in sWAT of Rbp$^{-/-}$ as compared to WT mice (Figure 2A), while Rbp deficiency had only modest or no effects on thermogenic gene expression in BAT (Figure 2B). A hallmark of WAT browning is the emergence of cold-induced UCP1-positive multilocular beige adipocytes. Whereas beige adipocytes were abundantly induced in sWAT of cold-exposed WT mice, adipocyte morphology as well as UCP1 protein expression in Rbp$^{-/-}$ sWAT were not affected by cold exposure (Figure 2C,E). Despite blunted sWAT browning, Rbp deficiency did not interfere with the thermogenic program in BAT (Figure 2B,D,F). Tissue norepinephrine (NE) concentrations did not differ between cold-challenged WT and Rbp$^{-/-}$ mice (Fig. S1d), suggesting that altered sympathetic output did not contribute to this phenotype. Although it cannot be completely excluded, we found no evidence that UCP-1-independent thermogenesis played a role given that the expression

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of UCP-1-independent creatinine cycle genes were unaltered between WT and Rbp−/− SWAT and BAT (Fig. S1e,f). To test the functional consequences of the observed molecular changes *in vivo*, we performed core body temperature measurements in cold-challenged WT and Rbp-deficient animals. In line with impaired sWAT browning, Rbp$^{-/-}$ mice were significantly more cold-sensitive as reflected by lower core body temperatures during cold stress (Figure 2G). These findings demonstrate that intact retinol transport is critical for the induction of a browning program in sWAT and cold adaption *in vivo*.

2.3. Rbp deficiency perturbs the cold-induced TCA cycle and OXPHOS program in sWAT

Next, we studied the effects of Rbp deficiency on the genetic programme of cold-regulated mitochondrial energy metabolism. Rbp deficiency repressed cold-induced induction of canonical participants in the tricarboxylic acid (TCA) cycle (*Cs, Aco2, Dlat, Sdha*) as well as the mitochondrial electron transport chain (ETC, *Cox4, Cox7a1, Cox8b*) in sWAT (Figure 3A). In line with unaltered BAT thermogenesis, TCA and ETC gene expression was unaffected by Rbp deficiency in this depot (Fig. S2a). The transcriptional changes in Rbp-deficient sWAT were accompanied by lower protein levels of the mitochondrial ETC subunits I, II and V, the site for oxidative phosphorylation (OXPHOS) (Figure 3B). The reduction of the OXPHOS protein complexes was not associated with decreased mitochondrial content since expression of the mitochondrial marker protein TOM20 was similar in WT and Rbp$^{-/-}$ sWAT (Figure 3C).

To further characterise the molecular changes of impaired sWAT browning following cold exposure in the Rbp deficiency model, we performed unbiased mRNA sequencing (mRNAseq) analysis in sWAT. In WT mice, cold exposure significantly altered 390 genes, of which 318 were induced and 72 repressed (Figure 3D,E, Table S2). In Rbp$^{-/-}$ sWAT, 272 genes were significantly regulated, of which only 72 were induced and 200 were repressed (Figure 3D,E, Table S3). mRNAseq analysis also confirmed previous quantitative polymerase chain reaction (qPCR) results showing the top regulated genes in sWAT of cold-exposed WT mice were thermogenic markers *Dio2, Elovl3* and *Ucp1* among others (Figure 3F). In contrast, many thermogenic genes, including *Ucp1,* were not significantly induced in cold-exposed Rbp$^{-/-}$ mice, in line with our previous findings (Figures 2A and 3F). Gene set enrichment analysis (GSEA) of Gene Ontology (GO), Reactome and Hallmark gene sets revealed marked induction of browning pathways involving "*oxidative phosphorylation*", "*TCA cycle and respiratory electron transport*" and "*cellular respiration*" in cold-exposed WT versus Rbp $^{-/-}$ sWAT (Figure 3G, Table S4). We also identified a strong shift towards increased cold-induced lipid metabolism pathways in WT versus Rbp deficient sWAT, with significant gene set enrichment of "*fatty acid metabolism*", "*energy derivation by oxidation of organic compounds*" and "*cellular lipid catabolic processes*" (Figure 3H, Table S5). Indeed, hormone-sensitive lipase (HSL) activity, the rate-limiting step in lipolysis was significantly decreased in sWAT of cooled Rbp$^{-/-}$ compared to WT mice as evidenced by lower Ser660 phosphorylation of HSL (Figure 3I) in line with reduced cold-induced
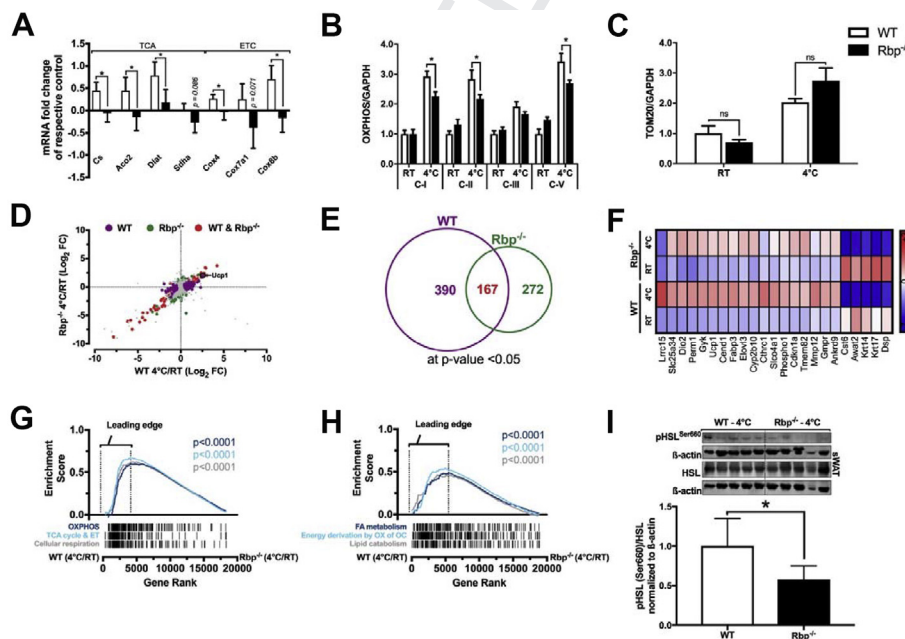
Figure 3: Defective retinol transport perturbs the cold-induced TCA cycle and OXPHOS program in sWAT. Gene expression of tricarboxylic acid (TCA) cycle genes and electron transport chain (ETC) genes, normalised to room temperature of the respective genotype (**A**). Protein expression of oxidative phosphorylation (OXPHOS) enzymes of the ETC (**B**) and TOM20 (**C**) in sWAT of WT and Rbp$^{-/-}$ mice. mRNA sequencing data showing cold-induced changes in gene expression in sWAT of WT and Rbp$^{-/-}$ mice. Purple, green and red spots represent genes significantly differentially expressed (p < 0.05; n = 5 mice per group). Grey dots indicate genes that are not statistically significantly regulated (**D**). Venn diagram illustrating the number of significantly cold-regulated genes in each genotype (**E**). Heatmap of the top 18 genes upregulated and the top 5 genes downregulated by cold exposure. Each column represents pooled data from sWAT of 5 mice (**F**). Gene set enrichment analysis (GSEA) of HALLMARK, GO and REACTOME gene sets in sWAT highlight browning pathways, including "oxidative phosphorylation", "TCA cycle and respiratory electron transport" and "cellular respiration", (**G**) as well as lipid metabolism, including "fatty acid metabolism", "energy derivation by oxidation of organic compounds" and "cellular lipid catabolic processes" (**H**). Western blot analysis of pHSL/HSL expression in sWAT (**I**). Gene expression is given as mRNA fold change as mean ± SEM. All data (n = 5/group). *p ≤ 0.05; ns = not significant. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

lipolytic activity. In summary, these data suggest that the absence of a cold-induced retinol increase in sWAT mitigates the browning capacity while also causing a qualitative defect in mitochondrial respiratory function and concordant changes in lipid metabolism pathways.

2.4. Retinol enhances oxidative capacity in primary human adipocytes and is associated with altered substrate oxidation in humans

Given that Rbp's primary role is to carry retinol to target tissues, such as adipose tissue, we tested the effects of retinol on the thermogenic capacity of adipocytes in a cell autonomous manner. Human adipocyte precursor cells (hAPCs) isolated from abdominal subcutaneous fat specimens were differentiated for 5 days before stimulation with various retinol concentrations (1 nM—10 μM) or vehicle for 24 h. Furthermore, 1 μM retinol resulted in the most robust increase in UCP1 mRNA expression (Fig. S2b) and was therefore used for all other experiments. Retinol stimulation significantly increased thermogenic gene expression and UCP1 protein levels in differentiated hAPCs from four different donors (Figure 4A,B). Notably, retinol stimulation enhanced cellular respiration as demonstrated by increased maximum, mitochondrial and reserve capacity oxidative consumption rate using *Seahorse®* analysis (Figure 4C,D). Hence, these data suggest that retinol promotes browning in human adipocytes with coordinated increases in oxidative metabolism. In line with this finding, short-term cold exposure in 30 healthy lean subjects concomitantly increased circulating retinol concentrations (Figure 1C) with concurrent significant decreased respiratory quotients (RQ, defined as the ratio between CO_2 release and O_2 consumption) by 6.0% $\pm$ 1.87% (Figure 4E). A significant inverse correlation existed between the cold-mediated change in plasma retinol (delta retinol) and the change in the respiratory quotient (delta RQ) (Figure 4F), suggesting that enhanced cold-induced retinol levels are associated with a shift in substrate oxidation in humans possibly caused by an augmented lipid utilisation.
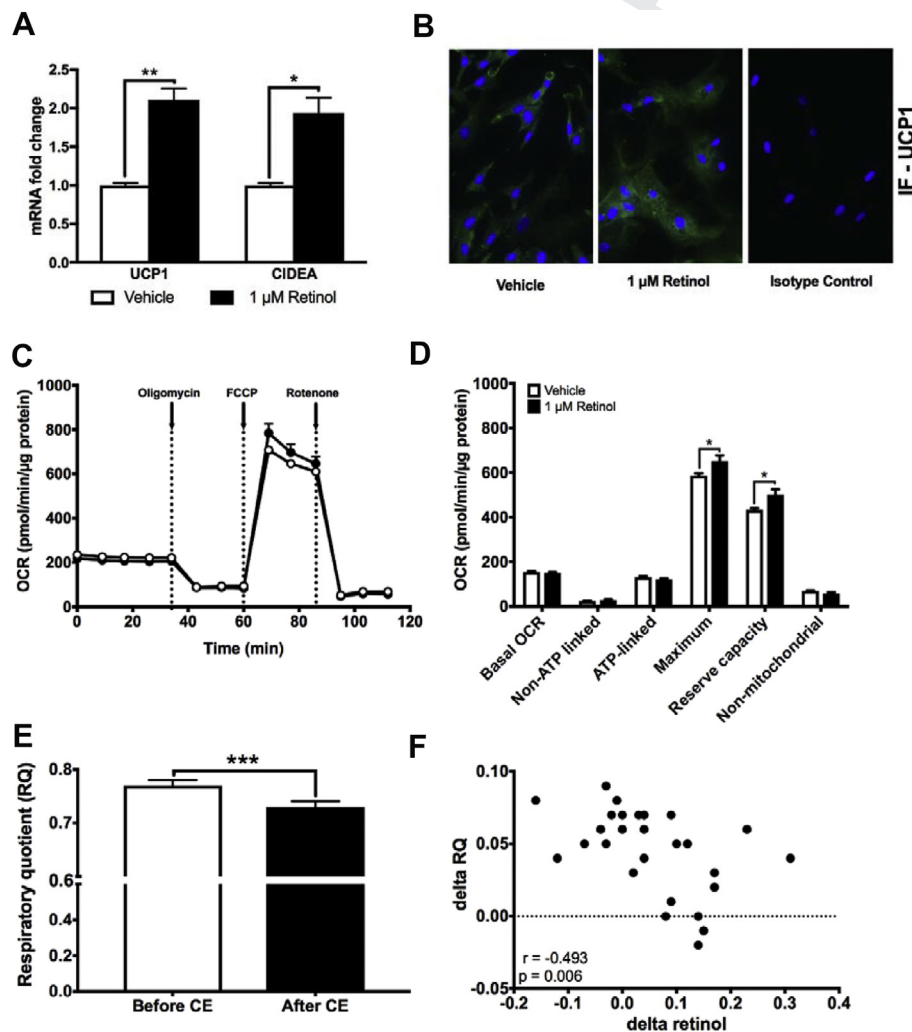
Figure 4: Retinol enhances oxidative capacity in primary human adipocytes. UCP1 gene expression (**A**) and immunofluorescence (**B**) in differentiated primary human adipocytes stimulated with retinol for 24 h (n = 5). Oxygen consumption rate (OCR) of primary human adipocytes treated with 1 μM of retinol for 24 h. Data are illustrated as real-time replicate readings (**C**) or as the group average of different respiratory phases (basal, non-ATP linked, ATP-linked, maximum, reserve capacity, non-mitochondrial and mitochondrial respiration) (**D**). Respiratory quotient (RQ) assessed by indirect calorimetry in 30 healthy lean subjects before and after 2.5 h of cold exposure (**E**). Spearman's correlation of cold-induced retinol changes with RQ changes in healthy lean humans (**F**). Values are given as mean $\pm$ SEM. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001.

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3. MATERIAL AND METHODS

3.1. Animals
Rbp knockout mice (Rbp$^{-/-}$) as well as their littermate controls (wildtype, WT) were kindly provided by Dr. Loredana Quadro (Rutgers University) and bred on a mixed background (129/Sv x C57BL/6J) [31]. Mice were kept on a standard chow diet with a vitamin A content of 25 IU g^{-1}. All experiments were approved by the local ethics committee for animal studies and the Federal Ministry of Science, Research and Economy (BMBWF-66.009/0124-V/3b/2018) and followed the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

3.2. Human cold exposure studies
Moderate cold exposure (14 °C—17 °C) for 2 h is usually sufficient to activate human BAT [28—30]. We applied this protocol in 30 young healthy lean subjects (age: 20—45 years, BMI: 18.5—24.9 kg/m^2), which was approved by the ethics committee of the Medical University of Vienna (EK-No. 1032/2013), registered at CinicalTrials.gov (NCT02381483) and performed in compliance with the Declaration of Helsinki and Good Clinical Practice. Details of the study protocol are described in the Supplementary Methods.

3.3. Cold exposure and body temperature measurements in mice
To determine the vitamin A abundance as a critical determinant of adaptive thermogenesis, 16- to 18-week-old male body weight matched WT and Rbp$^{-/-}$ mice were single housed, without cage enrichment, and cold exposed for 24 h in a climate chamber for keeping mice HPP750life (Memmert). In the stepwise cooling experiment, telemetric temperature probes (Anipill Capsule 0.1C Precision) were implanted intraperitoneally to control real-time body temperature. After a 1-week acclimation phase, ambient temperature was dropped by 4 °C every 48 h. Mice were fasted 2 h prior to killing, and ethylenediaminetetraacetic acid (EDTA) plasma was collected by heart puncture. The liver was perfused to exclude circulating retinol from the liver pool. Thus, 0.5 U of heparin was injected in the inferior vena cava, while the portal vein and the inferior vena cava were cannulated and perfused with 0.9% NaCl, and tissues were dissected and analysed.

3.4. Reverse transcription and gene expression
Total RNA was extracted using TRIzol (Invitrogen), treated with DNase (Thermo Scientific) and reverse transcribed to cDNA (Applied Biosystems) according to the manufacturer's instructions. Gene expression, normalised to 36B4, was analysed by quantitative real-time reverse transcription PCR (RT-PCR, SybrGreen, Roche or GoTaq, Promega) using a QuantStudio 6 cycler (Applied Biosystems). Primer sequences are shown in Table S6.

3.5. Immunoblotting
Tissue samples were homogenised in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined with the Pierce™ BCA protein assay kit (23225, Thermo Scientific). Standard western blotting was performed using mouse or rabbit polyclonal antibodies to OXPHOS (AB110413, Abcam, 1:500), TOM20 (HPA011562, Sigma, 1:1000), GAPDH (#2118, Cell Signalling, 1:3000), β-ACTIN (NB600-501, Novus Biologicals, 1:3000). Blots were visualised using either chemiluminescence (Roche) and quantified on Fusion Fx Vilber Lourmat (FusionFx, PeqLab) or using fluorescent secondary antibodies and visualisation with the Odyssey imaging system (Licor). Protein bands were quantified using densitometry in ImageJ.

3.6. Immunohistochemistry
Paraffin sections were prepared from mouse sWAT and BAT after fixation (4.5% (vol/vol) formaldehyde). Sections were stained using haematoxylin and eosin (H&E) and rabbit polyclonal antibody to UCP1 (U6382, Sigma, 1:1,000) and biotinylated secondary goat antibody to rabbit (Vector Laboratories Inc., 1:300). Control staining was performed on selected sections with isotype control. Counterstaining was performed with haemalaun for 1 min. Samples were analysed on an Axio Imager 2.

3.7. HPLC analysis
Plasma retinol was determined in 100 μl of EDTA-plasma. The detailed protocol is described in the Supplementary Methods.

3.8. Norepinephrine (NE) measurement
A commercially available ELISA Kit (BA E—52000, LND) was modified and established for the use of determining NE in tissue homogenates.

3.9. Gene expression profiling and GSEA
Sequencing libraries were prepared using the NEBnext Ultra II directional RNA library prep kit for Illumina according to the manufacturer's protocols (NEB) and sequenced in the 75bp single-read mode on the Illumina NextSeq 500 (Illumina) at the Genomics Core Facility, Medical University of Vienna. For the heatmap generation, genes that passed the t.statistics criteria of applied tests were included and first sorted by the Benjamini-Hochberg corrected p-value and then by the mean foldchange 4 °C/RT (log2). Datasets include pooled data from 5 mice per group. The detailed protocol, including GSEA are described in the Supplementary Methods.

3.10. Isolation of human adipocyte precursor cells (hAPCs) and *in vitro* adipocyte differentiation
Cells were obtained from five healthy female donors, aged 31—45 years undergoing abdominoplasty. The detailed protocol is provided in the Supplementary Methods.

3.11. Immunofluorescence
Cells cultured in chamber slides were fixed in 2% (vol/vol) paraformaldehyde, permeabilised in 1% Triton X (30 min) and blocked in 3% bovine serum albumin-phosphate-buffered saline (BSA-PBS). UCP1 staining was performed overnight with a rabbit polyclonal antibody against UCP1 (U6382 Sigma Aldrich, 1:1,000). UCP1 was visualised with a fluorescence secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes, Inc.). Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min. UCP1 staining was analysed on an Axio Imager 2 microscope (Zeiss). As a negative control, staining was performed on selected sections with isotype control.

3.12. Measurement of oxygen consumption rate
Cellular respiration was measured using the Seahorse XF24 Analyser (Agilent) in four healthy females, aged 35—45 years donors. Details are described in the Supplementary Methods.

3.13. Statistics
The sample size is stated in Figure and Table legends. Sample size calculation for mouse studies was performed by Java Applets for

Q2 Power and Sample Size [Computer software]. Retrieved Mai 15, 2013, from http://www.stat.uiowa.edu/~rlenth/Power]. To evaluate differences of 50% with a standard deviation of 30%, a significance level of 5% and a power of 75% was established. The human data presented here were part of a cross-sectional clinical study with the correlation between systemic retinol concentrations and cold-induced BAT activity as the primary outcome parameter. The approximate power to detect a given correlation at a level of 0.05 was calculated based on Fisher's z transformation. In case of a true correlation of 0.4, a sample size of 30 was required for a power of 80%.

Statistical analysis was conducted using explorative data analysis and descriptive statistics. Results are given as mean $\pm$ standard error of the mean (SEM). Differences were analysed using unpaired two-tailed Student's t-test for two-group comparisons and with one-way analysis of variance (ANOVA) followed by Holm-Sidak's *post-hoc* test for multiple group comparisons, respectively. Data were tested for Gaussian distribution using column statistics in GraphPad Prism 7. A p-value of ≤ 0.05 was considered statistically significant.

4. DISCUSSION

Thermogenesis is an essential and at times adaptive biologic function required for survival among endothermic species that must generate heat internally. BAT, by uncoupling electron generation and storage, offers a source for this energy production [2]. Given excess energy intake as a significant contributor to the epidemic obesity, the prospect of inducing WAT to take on the energy-wasting characteristics of BAT has received considerable attention [7—9]. Extensive prior work establishes retinoid signalling as a key regulator of adipogenesis, UCP1 expression and overall energy balance [10—12]. Despite this, specific connections between systemic retinoid transport and thermogenic programming of adipocytes are lacking. We establish here that systemic vitamin A levels are regulated by cold exposure and that Rbp, the sole specific circulating retinoid carrier, controls WAT browning, oxidative metabolism and adaption to cold. We are aware that retinoid plasma concentrations per se may not necessarily have a dramatic biological impact. However it has been demonstrated that very subtle changes in the tissue retinoid content can have substantial functional effects, particularly on the transcriptional level [32]. It is also possible that longer cold challenges may have even more pronounced effects on retinoid metabolism and biological outcomes, such as WAT browning. Rbp is well established as a key protein in retinoid metabolism [33,34], mediating retinol secretion and extrahepatic transport preferentially towards adipose tissue as previously demonstrated in Rbp$^{-/-}$ mice challenged with dietary conjugated linoleic acid supplementation or chronic alcohol consumption [35,36]. Both studies showed that Rbp facilitates hepatic retinol redistribution towards fat depots, although some residual adipose tissue retinol uptake may persist in Rbp deficiency [36]. Impaired cold-induced WAT browning and thermogenic capacity in Rbp$^{-/-}$ mice suggest that coordinated retinol delivery to target tissues, such as adipose tissue, is a crucial process during cold adaption. It is possible that cold exposure stimulates mobilisation of hepatic retinoid stores via Rbp [37,38], thus shuttling retinol towards extrahepatic tissues. However, to prove this assumption *in vivo,* retinoid tracing studies would be necessary, which are technically challenging and have not been previously reported. In contrast to WAT, cold-mediated changes were not abrogated in Rbp-deficient BAT. These fat depot-specific differences may stem from an alternative retinol uptake mechanism in BAT. The postprandial tissue retinol supply is enabled by lipoprotein lipase (LPL)-dependent hydrolysis of retinol-containing chylomicrons released by enterocytes after dietary intake [39,40]. Retinoid provision to BAT during cold challenge via this mechanism, even in the absence of Rbp, would be consistent with cold-mediated induction of LPL expression in both WT and Rbp$^{-/-}$ BAT (data not shown). In general, the rapid changes of the thermogenic program of white adipocytes suggest a functional effect rather than a differentiation effect. Additional evidence for this hypothesis comes from the retinol stimulation experiments in fully differentiated adipocytes.

Rbp is a known player in energy metabolism, including diabetes. Prior studies found an association between increased circulating Rbp levels and insulin resistance in various genetic and diet-induced models of obesity [41,42]. Human studies have been more controversial with variable relationships between Rbp concentrations and different cohorts with obesity, insulin resistance or type 2 diabetes [43—45], which could reflect dietary or genetic differences. DNA-arrays in the insulin-resistant adipose-specific glucose transporter type 4 (GLUT4) knockout mice revealed elevated Rbp expression levels in adipose tissue but unaltered Rbp expression in the liver [41]. Thus, elevated circulating Rbp levels in insulin-resistant states were attributed to increased expression and secretion by adipose tissue [41]. In our study, cold exposure increased Rbp mRNA expression in the liver accompanied with elevated circulating Rbp concentrations with no changes in adipose depots. In general, cold exposure improves insulin sensitivity and glucose homeostasis in mice and humans [46—48], suggesting that increased circulating Rbp levels after cold stimulation do not cause insulin resistance. Recent evidence suggests that the Rbp tissue source may determine insulin resistance. Mice with hepatocyte-specific deletion of Rbp (LRKO) exhibit no detectable plasma Rbp levels on chow or high-fat diet, despite intact adipose Rbp production, establishing hepatocytes as the principal source of circulating Rbp. More importantly, LRKO mice are not protected from diet-induced obesity or insulin resistance consistent with adipocyte-secreted Rbp, which is confined to autocrine or paracrine actions within adipose tissue, even in an insulin-resistant state [49,50]. In support of this notion, adipocyte-specific Rbp overexpression increases adipose tissue inflammation, lipolysis and circulating fatty acid (FA) levels [51]. These findings may also explain why cold-mediated increases in systemic Rbp levels do not cause insulin resistance. Q3

Control over thermogenesis involves regulation of sources of energy substrates through lipolysis and FA release as well as combustion of those resources through beta oxidation. In keeping with this, blunted WAT browning in Rbp deficiency is coupled to repressed expression of lipid catabolism and FA oxidation genes, with decreased mitochondrial oxidative phosphorylation capacity. Here, we show that the cold-induced increase of circulating retinol occurs in conjunction with a decreased respiratory quotient in humans (Figure 4F), suggesting that enhanced retinol supply is associated with higher lipid oxidation. Indeed, retinol stimulation in primary human adipocytes augmented the mitochondrial oxidative capacity (Figure 4C,D), suggesting that the retinol effects are cell-autonomous. Given that retinol is intracellularly converted to retinoic acid, which activates the nuclear receptors RAR and RXR, increased retinoic acid signalling may contribute to the observed thermogenic effects (Figure 4A) as widely reported [12,18—21,24,25]. Accordingly, retinoic acid target gene expression was markedly induced in retinol-stimulated human adipocytes (Fig. S2d). We are aware that this brief communication has some limitations, including comparing the thermogenic phenotype between mice and humans. Housing mice at 20—22 °C ambient temperature already confers a moderate thermogenic stress with the occurrence of some UCP1-positive cells, mainly in subcutaneous WAT depots, whereas humans mainly live in a thermoneutral environment, which would be

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approximately 30 °C for mice. It is possible that the changes in retinoid metabolism would be even more pronounced when exposing mice to cold temperatures after rearing them at thermoneutrality. However, the goal of our cold exposure experiment was to achieve optimal WAT browning; therefore, we decided to perform a stepwise cooling protocol. When maintaining mice at 30 °C, any pre-existing WAT browning typically gets lost. However, the *in vitro* retinol experiments in differentiated human adipocytes suggest that the proposed retinol effects also occur in a cell-autonomous manner in human fat cells.

The study is also limited by the lack of direct retinoid flux analyses, demonstrating if and how cold exposure alters retinoid shuttling towards distinct tissues and how the lack of Rbp may interfere with this process. Such tracing studies are technically challenging and will be the subject of our future investigations. Nonetheless, we demonstrate here that cold exposure raises systemic vitamin A levels in mice and humans. We show that the action of the circulating retinol transport protein Rbp is indispensable for cold-induced thermogenic responses in adipose tissue and for normal adaption to cold by enabling a transcriptional thermogenic program. Hence, our data reveal Rbp as a novel player in regulating cold responses *in vivo* and thus add to the growing body of evidence that retinoid metabolism plays a pivotal role in exerting control over energy balance and thermogenesis with potential for new insights into obesity and its treatment.

AUTHOR CONTRIBUTIONS

A.F. and F.W.K. designed the study; A.F., O.C.K, K.S., G.M., R.M., M.B., S.B.P, J.P., L.Q. and F.W.K. researched data; A.F. and F.W.K. wrote the manuscript; O.C.H, K.S., G.M., R.M., M.B., S.B.P, A.K.W, J.P. and L.Q. reviewed the manuscript.

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CONFLICT OF INTERESTS

No competing interests declared.

APPENDIX A. SUPPLEMENTARY DATA

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