Hairless promotes PPAR γ expression and is required for white adipogenesis

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Adipose tissue is the largest compartment in the mammalian body for storing energy as fat, providing an important reservoir of fuel for maintaining whole body energy homeostasis. Herein, we identify the transcriptional cofactor hairless (HR) to be required for white adipogenesis. Moreover, forced expression of HR in non-adipogenic precursor cells induces adipogenic gene expression and enhances adipocyte formation under permissive conditions. HR exerts its proadipogenic effects by regulating the expression of PPAR γ , one of the central adipogenic transcription factors. In conclusion, our data provide a new mechanism required for white adipogenesis.

Keywords: adipogenesis; hairless; JmjC; obesity; PPAR γ

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INTRODUCTION

Both excess and deficiency of adipose tissue result in severe metabolic disturbances [1]. A complex network of transcriptional events controls differentiation of adipocytes from preadipocytes of mesenchymal origin [2]. Peroxisome proliferator-activated receptor- γ (PPAR γ) and the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors represent core factors of the transcriptional machinery in this elaborate system. In brief,

C/EBP β and C/EBP δ mRNA and protein levels rise early in adipocyte differentiation and activate the expression of PPAR γ [3]. PPAR γ , considered to be the ‘master regulator’ of adipogenesis, in turn switches on transcription of C/EBP α . These two central regulators control the expression of numerous adipocyte-specific genes, as well as the transcription of each other [4,5].

The jumonji (JmjC) domain-containing proteins have recently emerged as important factors in transcriptional regulation of cellular differentiation [6]. The JmjC domain of most members of this family has been ascribed specific histone demethylase activity, thus providing a potential common mechanism underlying modulation of transcription. Interestingly, the JmjC domain-containing histone demethylase 2A (Jhdm2a) is critically involved in energy homeostasis and obesity in mice [7]. This phenotype seemed, however, not to be caused by changes in adipogenesis. Hence, specific roles of JmjC proteins in adipogenesis are unknown thus far.

Hairless (HR) is one of the best-studied members of the JmjC protein family, but has not been linked to adipogenesis so far. Several nuclear hormone receptors have been shown to interact with HR: the thyroid hormone receptor, the vitamin D receptor and the retinoic acid receptor-related orphan receptor- α [8]. HR is crucial for several aspects of hair formation, growth and regeneration [9]. Mice lacking functional *Hr* show normal initial hair growth. However, after shedding at ~ 3 weeks of age the hair is lost and does not regrow [10].

In this study, we identified HR as an important component of the transcriptional cascade governing white adipogenesis.

RESULTS AND DISCUSSION

Knockdown of HR impairs adipogenesis

Given the function of JmjC domain-containing proteins in cellular differentiation, we monitored mRNA expression of JmjC domain-containing genes during *in vitro* adipogenesis. Strikingly, hairless (*Hr*) was found to be the only regulated gene in differentiating 3T3-L1 preadipocytes (Fig 1A; supplementary Fig S1 online). *Hr* transcript

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levels peaked ~24 h and dropped back to almost basal levels 48 h after differentiation induction (Fig 1A). The highest amounts of HR protein were found after 24–48 h of differentiation (Fig 1B).

This expression pattern prompted us to investigate the requirement of HR in adipogenesis. 3T3-L1 cells infected with lentivirus containing either a non-silencing sequence or short hairpin RNA directed against HR were subjected to *in vitro* adipogenic differentiation. mRNA expression of *Hr* was reduced by ~80% for one short hairpin (shHR1) and 60% for another short hairpin (shHR2; Fig 1C). Oil Red O staining visualized accumulation of lipids in non-silenced control cells indicating efficient differentiation into adipocytes. In contrast, cells expressing short hairpin RNA targeted against *Hr* showed markedly diminished Oil Red O staining and retained their fibroblastic morphology (Fig 1D). In line with silencing efficiency, depletion of HR using shHR1 resulted in a stronger inhibition of adipogenesis as compared with shHR2. Reduced adipogenesis is expected to be a result of changed expression of main transcriptional regulators. Expression of *C/EBPβ* and *C/EBPδ*, early regulators of adipocyte differentiation, were not affected in cells depleted of HR as compared with control cells (Fig 1E). However, induction of the two isoforms *PPARγ1* and *γ2*, generated by alternative splicing and promoter usage of *PPARγ*, was reduced in cells depleted of HR as compared with control cells (Fig 1F). Consequently, *PPARγ*-dependent mRNA induction of *C/EBPα*, fatty acid binding protein 4 (*Fabp4*) and adiponectin was significantly diminished (Fig 1G). Accordingly, induction of *PPARγ1* and *γ2*, *C/EBPα*, fatty acid synthase (FASN) and FABP4 proteins was markedly impaired (Fig 1H). Adipogenesis was unaffected when knock-down of HR was induced 2 or 4 days after differentiation induction (supplementary Fig S2A online), indicating that HR expression is required early during differentiation. Expression of shHr1 in cultured primary preadipocytes isolated from wild-type mice also resulted in efficient depletion of *Hr* mRNA (Fig 1I). These cells did not differentiate into adipocytes as compared with non-silenced control cells (Fig 1J).

3T3-L1 preadipocytes undergo approximately two rounds of mitotic clonal expansion preceding the adipogenic gene expression programme [11]. Knockdown of HR significantly, but only moderately, inhibited clonal expansion of 3T3-L1 cells as compared with control cells (supplementary Fig S2B online). HR might thus be partially required for mitotic clonal expansion, but in addition is likely to act on later transcriptional events.

The so-called ‘rhino’ mice carry a point mutation in the *Hr* gene, resulting in HR loss-of-function. Heterozygous animals showed no apparent skin or fur phenotype and were indistinguishable from wild-type mice. Heterozygous *Hr^{wt}/Hr^{rh}* mice were thus used as control mice in comparison to homozygous *Hr^{rh}/Hr^{rh}* mutant mice. To confirm a cell-autonomous defect, both primary preadipocytes from the stromal vascular fraction as well as primary fibroblasts were isolated from 10 weeks old mice. Almost no HR message was detected in primary preadipocytes and fibroblasts derived from *Hr^{rh}/Hr^{rh}* mice as compared with cells derived from heterozygous *Hr^{wt}/Hr^{rh}* control mice (supplementary Fig S3A online). Microscopic analysis showed that cells derived from *Hr^{rh}/Hr^{rh}* mice were unable to undergo adipogenesis to the extent of control cells. Lipid incorporation was markedly reduced in both primary *Hr^{rh}/Hr^{rh}* preadipocytes (Fig 2A) and primary *Hr^{rh}/Hr^{rh}* fibroblasts (Fig 2B) as compared

with control cells. To address whether observed reduction in differentiation was due to changes in the amount of preadipocytes, we determined their numbers in the stromal vascular fraction of mice by flow cytometry. This analysis revealed no differences between *Hr^{rh}/Hr^{rh}* and control mice (supplementary Fig S3B online). Quantitative reverse transcription–polymerase chain reaction (PCR) analysis showed markedly reduced mRNA levels of *PPARγ1* and *γ2*, *C/EBPα* as well as adiponectin, while expression of *C/EBPβ* and *C/EBPδ* was unchanged (Fig 2C,D). *C/EBPα* was highly expressed in mouse preadipocytes prior differentiation induction and was not enhanced on stimulation with adipogenic factors. Overall, our data provide strong evidence that HR is required for adipogenesis *in vitro*.

Hairless is required for adipocyte differentiation *in vivo*

We next aimed at exploring adipose mass in HR mutant mice. They show a skin phenotype characterized by hair loss starting at ~18 days of age. Once the hair is lost, the skin becomes gradually thickened and wrinkled [10]. At 10 weeks of age, *Hr^{rh}/Hr^{rh}* mice exhibited significantly reduced adipose mass, albeit significantly enhanced body weight, as compared with control mice (supplementary Fig S4 online). Expansion of skin is likely to account for the overall increased body weight despite reduced fat mass. To uncouple adipose tissue mass from compromised insulation due to loss of fur and altered skin, pups at 18 days of age were assessed. Subcutaneous adipose tissue in the region of the scapulae as well as brown adipose tissue (BAT) were dissected and weighted. *Hr^{rh}/Hr^{rh}* pups showed a significant but moderate reduction in body weight. While BAT weights were unchanged, a marked reduction in subcutaneous white adipose tissue (WAT) was observed in *Hr^{rh}/Hr^{rh}* pups (Fig 3A). In control mice, histology revealed a distinct subcutaneous layer of white adipose tissue. However, in *Hr^{rh}/Hr^{rh}* mice, this layer was markedly diminished (Fig 3B). mRNA expression of two BAT-specific genes, lim homeobox 8 (*Lhx8*) and uncoupling protein 1 (*Ucp1*), were not changed in BAT (Fig 3C) confirming that BAT was not affected in *Hr^{rh}/Hr^{rh}* pups. Expression of these genes in WAT was also unaltered (Fig 3C), excluding increased conversion of WAT into BAT and thus increased BAT activity. Altogether, our data indicate that HR is crucial in white adipose tissue formation *in vivo* independent of differences in thermoregulation.

Hairless overexpression increases adipogenic potential

Very few genes were attributed enough potency to render non-adipogenic cell lines susceptible to induction of adipogenesis. 3T3-L1 preadipocytes show very high adipogenic potential in response to the standard hormonal cocktail, whereas NIH3T3 fibroblasts are largely insensitive to induction of adipocyte differentiation [12]. When comparing endogenous *Hr* expression levels in (undifferentiated) 3T3-L1 with those seen in NIH3T3 cells, significantly higher mRNA levels were found in 3T3-L1 cells (Fig 4A). To determine whether forced expression of *Hr* might induce adipogenesis in non-adipogenic cell lines, we expressed green fluorescent protein (GFP)-tagged HR and GFP alone in NIH3T3 fibroblasts (Fig 4B) and C2C12 myoblasts (Fig 4C). On HR expression, *PPARγ1* and *γ2*, *C/EBPα* as well as adiponectin were induced in NIH3T3 prior induction of adipogenesis (Fig 4D), while HR had no effect on basal expression of adipogenic and myogenic genes in C2C12 cells (Fig 4E). Expression of *C/EBPβ*

and *C/EBPδ* were not affected in both cell types (Fig 4D,E). Adipogenesis as well as induction of adipogenic genes were markedly enhanced in HR-expressing NIH3T3 and C2C12 cells, as compared with GFP-expressing cells under adipogenic conditions (Fig 4F–I). Myogenic markers, however, were unaltered in

C2C12 cells overexpressing HR, as compared with control cells on myogenic differentiation (Fig 4J), indicating that HR is unable to change muscle fate once conditions for muscle differentiation are set in place. As shown above, the amount of preadipocytes in *Hr* mutant mice is not affected, challenging its requirement in

Fig 1 | Hairless is required for adipocyte differentiation *in vitro*. (A) Relative hairless (HR) expression in 3T3-L1 cells at indicated time points of differentiation using quantitative reverse transcription–polymerase chain reaction (RT–PCR). (B) HR expression in 3T3-L1 cells at indicated time points of differentiation using western blotting. GCN5 was used as a loading control for nuclear extracts. (C) Determination of efficiency of HR knockdown in non-stimulated HR-depleted (shHr1 and shHr2) and non-silenced (ns) 3T3-L1 cells using quantitative RT–PCR. (D) Visualization of lipid incorporation by Oil Red O stains (left panels) and phase-contrast microscopy (right panels) in 3T3-L1 cells 8 days after differentiation induction. Scale bars, 50 μ m. (E) Relative expression of CCAAT/enhancer-binding protein- β (C/EBP β) and C/EBP δ in 3T3-L1 cells before (0 h) and 8 h after stimulation of adipogenesis using quantitative RT–PCR. (F) Relative expression of peroxisome proliferator-activated receptor- γ 1 (*PPAR* γ 1) and *PPAR* γ 2 in 3T3-L1 cells at indicated time points of differentiation using quantitative RT–PCR. (G) Relative expression of C/EBP α , fatty acid binding protein 4 (*FABP*4) and adiponectin in 3T3-L1 cells before (0 h) and 8 days after stimulation of adipogenesis using quantitative RT–PCR. (H) Expression of HR, *PPAR* γ 1, *PPAR* γ 2, C/EBP α , fatty acid synthase (*FASN*) and *FABP*4 in 3T3 cells at indicated time points of differentiation using western blotting. The histone acetyl transferase GCN5 was used as a loading control for nuclear extracts. Tubulin was used as a loading control for total extracts. (I) Determination of efficiency of HR knockdown in non-stimulated HR-depleted (shHr1 and shHr2) or non-silenced (ns) primary mouse preadipocytes using quantitative RT–PCR. (J) Visualization of lipid incorporation by phase-contrast microscopy in primary mouse preadipocytes 8 days after differentiation induction. Scale bars, 50 μ m. Error bars indicate standard deviations. * P < 0.05; ** P < 0.01; *** P < 0.001 (n = 3 for B,C,E,F,G,I; n = 4 for A, Student's t -test). NS, nonsignificant; sh, short hairpin.

adipocyte lineage commitment *in vivo*. Nevertheless, changes in HR expression in precursors might still be important in lineage specification as experiments in non-adipogenic cell lines hint at such a function. Importantly, the adipogenic genes were induced by ectopic expression of HR in NIH3T3 cells even under non-permissive conditions. It was shown that white and brown adipocytes do not share a direct common precursor, but that brown adipocytes are developmentally closer to myocytes [13]. Measuring expression levels of the two brown adipocyte marker genes *Ucp1* and *Lhx8* in C2C12 cells expressing HR showed no significantly higher induction of these factors as compared with control cells (supplementary Fig S5 online), corroborating a role of HR specifically in white adipogenesis.

Altogether, these results indicate that HR has a pivotal role in white adipocyte differentiation and that HR regulation might modulate adipogenic potential.

Hr promotes *PPAR* γ transcription

To test whether the HR's JmjC domain has an important function during HR-dependent adipogenesis, we expressed GFP alone, full-length HR–GFP and truncated HR lacking the JmjC domain in NIH3T3 cells (Fig 5A). Both cells expressing full-length HR and HR lacking the C-terminal JmjC domain showed key features of adult adipocytes on induction of differentiation (Fig 5B). This suggests that the JmjC domain within HR is not required to potentiate adipocyte differentiation.

Forced expression of HR in non-adipogenic cell lines induced transcription of *PPAR* γ 1 and γ 2 in NIH3T3 cells even in the absence of an adipogenic stimulus. We therefore investigated a possible regulatory mechanism of HR on *PPAR* γ expression. Even in the presence of rosiglitazone, a potent *PPAR* γ agonist that directly binds to its ligand-binding domain, 3T3-L1 cells depleted of HR were not able to undergo differentiation (Fig 5C). This result hints at a direct modulation of *PPAR* γ expression by HR. To investigate this assumption, luciferase promoter assays were performed. HR stably induced reporter gene expression under the control of the *PPAR* γ 1 promoter, but failed to activate the *PPAR* γ 2 promoter (Fig 5D). Importantly however, our loss- and gain-of-function experiments mainly revealed regulation of transcription of *PPAR* γ 2 and to a lesser extend of *PPAR* γ 1. This discrepancy might be explained by a deficit in the ability of the *PPAR* γ 2 reporter to recapitulate endogenous gene expression.

Overall, our data indicate that HR might control *PPAR* γ transcription during white adipogenesis.

Concluding remarks

A tightly controlled transcriptional network regulates the process of adipocyte differentiation. Using cell culture experiments and mouse models, we showed that HR is required during both *in vitro* as well as *in vivo* white fat cell development. Furthermore, forced expression of HR in non-adipogenic cell lines increased their potential to undergo adipocyte differentiation.

HR exerts its proadipogenic function, at least partially, by promoting *PPAR* γ transcription. The exact mechanisms of *PPAR* γ regulation need to be addressed in the future. HR has several independent transcriptional repressor domains supporting its function as a transcriptional repressor [14]. Given positive regulation of *PPAR* γ transcription, HR might repress the function of a negative regulator of *PPAR* γ transcription. However, HR might also act as a transcriptional coactivator. Indeed, HR also contains motifs that have previously been shown to mediate binding of coactivators to nuclear receptors [15].

Interestingly, hair growth and cycling have recently been shown to be controlled by the subcutaneous fat tissue [16]. It will thus be interesting to investigate whether a crosstalk between subcutaneous adipocytes and hair follicle stem cells, both of which seem to be affected in *Hr* mutant mice, is dependent on HR. Overall, this study establishes HR as a key component in the transcriptional control of white adipocyte differentiation.

METHODS

Mouse experiments. Breeding and maintenance of mice and isolation of primary cells are described in supplementary information online.

Cell culture and cell differentiation. 3T3-L1 preadipocytes, NIH3T3 fibroblasts and C2C12 myoblasts (American Type Culture Collection) were cultured in DMEM (Sigma) with 10% fetal bovine serum at 37 °C and 5% CO₂. We thank A. Christiano for sharing HR overexpression constructs with us. Protocols for differentiation of cells, transfection and infection are provided in the supplementary information online.

Quantitative real-time PCR. RNA isolation, reverse transcription and PCR procedures are described in supplementary information online. Expression levels of genes in relation to the reference

Fig 3 | Subcutaneous white adipose tissue is reduced in *Hr* mutant mouse pups. (A) Body weight, weight of brown adipose tissue (BAT), weight of subcutaneous white adipose tissue (WAT) and WAT mass to body weight ratios of 18-days-old heterozygous control (Hr^{wt}/Hr^{rh}) and homozygous mutant (Hr^{rh}/Hr^{rh}) pups. (B) Transverse histological sections (haematoxylin-eosin (H&E, left) and Oil Red O (right)) at the level of the scapula from Hr^{wt}/Hr^{rh} and Hr^{rh}/Hr^{rh} pups 4 days after birth. Black arrows indicate subcutaneous adipose tissue. Scale bars, 1mm (upper panels) and 250 μ m (lower panels). (C) Relative expression of LIM homeobox 8 (*Lhx8*) and uncoupling protein 1 (*Ucp1*) in BAT and WAT of 10-week-old heterozygous control (Hr^{wt}/Hr^{rh}) and homozygous (Hr^{rh}/Hr^{rh}) mutant mice using quantitative reverse transcription-polymerase chain reaction. Error bars indicate standard deviations. * $P < 0.05$; *** $P < 0.001$ ($n = 4$, Student's *t*-test). HR, hairless; NS, nonsignificant.

gene 18S were normalized to time point 0 (Figs 1A,E,F,G and 2C,D), to expression in non-silencing control cells (Fig 1C,I), to expression in heterozygous mice (Fig 3C) and to expression in GFP-expressing cells (Fig 4D,E,G,I,J).

Western blot analysis. Cells were collected and proteins were enriched into nuclear and cytosolic fractions as previously described [17]. Antibodies and western blotting procedures are described in the supplementary information online.

Histological analysis. Specimens were embedded in Tissue-Tek OCT Compound (Sakura) and frozen at -80°C . Cryosections of $10\mu\text{m}$ were then processed for haematoxylin-eosin staining and Oil Red O staining. Oil Red O staining is described in the supplementary information online.

Luciferase promoter assay. Plasmids and procedures of the assay are described in the supplementary information online. We thank

J. Auwerx, ETH Lausanne, for sharing PPAR γ reporter plasmids with our laboratory.

Data analysis and statistics. Statistical calculations and data handling were performed using Graph Pad Prism 5 software (Graph Pad Prism software). For comparison of two data sets, Student's *t*-test was used. A *P*-value below 0.05 was accepted to deny the null hypothesis.

Fig 4 | Forced HR expression promotes adipogenesis in NIH3T3 and C2C12 cells. (A) Relative expression of *Hr* in NIH3T3 and 3T3-L1 cells using quantitative reverse transcription–polymerase chain reaction (RT–PCR). (B,C) Analysis of expression of hHR–GFP and GFP alone in NIH3T3 cells (B) and C2C12 cells (C) 24 h after transfection and in non-transfected cells (control), using flow cytometry (upper panels) and fluorescence microscopy (lower panels). Scale bars, 50 μ m. Histograms are representative of three flow cytometry analyses. (D,E) Relative expression of adipogenic genes in NIH3T3 cells (D) and C2C12 cells (E) ectopically expressing hHR–GFP or GFP alone prior differentiation, using quantitative RT–PCR. (F) Visualization of lipid incorporation by Oil Red O stains (upper panels) or phase-contrast microscopy (lower panels) in NIH3T3 cells 8 days after differentiation induction. Scale bars, 100 μ m. (G) Relative expression of adipogenic genes in NIH3T3 cells on day 8 of differentiation using quantitative RT–PCR. (H) Visualization of lipid incorporation by Oil Red O stains (upper panels) or phase-contrast microscopy (lower panels) in C2C12 cells 8 days after differentiation induction. Scale bars, 100 μ m. (I) Relative expression of adipogenic genes in C2C12 cells on day 8 of differentiation using quantitative RT–PCR. (J) Relative expression of myogenic genes in C2C12 cells on day 6 of myogenic differentiation using quantitative RT–PCR. Error bars indicate standard deviations. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 3$, Student's t -test). GFP, green fluorescent protein; HR, hairless; PPAR γ 1, peroxisome proliferator-activated receptor- γ 1.

Fig 5 | Hairless promotes *PPAR γ 1* expression and drives adipogenesis independent of its JmJC domain. (A) Schematic representation of structural and functional domains of human HR (hHR). Repression domains (RD1, RD2, RD3); thyroid hormone receptor (TR)-interacting domains (TR-ID1, TR-ID2), RAR-related orphan receptor alpha (ROR α)-interacting domains (ROR α -ID1, ROR-ID2) cysteine-rich domain (Cys) and JmJC domain. (B) Visualization of lipid incorporation by phase-contrast microscopy in NIH3T3 cells ectopically expressing GFP alone, human full-length HR or a mutant form of HR lacking the JmJC domain (hHR Δ JmJC) after 8 days of differentiation. Scale bars, 50 μ m. (C) Visualization of lipid incorporation by phase-contrast microscopy in HR-silenced (shHr1 and shHr2) and non-silenced (ns) 3T3-L1 cells 8 days after differentiation induction in the absence (upper panels) and presence (lower panels) of rosiglitazone. Scale bars, 100 μ m. (D) Luciferase reporter assay in NIH3T3 cells coexpressing hHR–GFP or GFP alone and either an empty luciferase reporter or luciferase under the control of the *PPAR γ 1* or *PPAR γ 2* promoter. One day after transfection, luciferase activity was measured and plotted relative to the activity of the empty reporter. Error bars indicate standard deviations. *** $P < 0.001$ ($n = 3$, Student's t -test). GFP, green fluorescent protein; HR, hairless; JmJC, jumonji; NS, nonsignificant; PPAR γ 1, peroxisome proliferator-activated receptor- γ 1; sh, short hairpin; VDR, vitamin D receptor.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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