



Wt1 haploinsufficiency induces browning of epididymal fat and alleviates metabolic dysfunction in mice on high-fat diet

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Abstract

Aims/hypothesis Despite a similar fat storing function, visceral (intra-abdominal) white adipose tissue (WAT) is detrimental, whereas subcutaneous WAT is considered to protect against metabolic disease. Recent findings indicate that thermogenic genes, expressed in brown adipose tissue (BAT), can be induced primarily in subcutaneous WAT. Here, we investigate the hypothesis that the Wilms tumour gene product (WT1), which is expressed in intra-abdominal WAT but not in subcutaneous WAT and BAT, suppresses a thermogenic program in white fat cells.

Methods Heterozygous *Wt1* knockout mice and their wild-type littermates were examined in terms of thermogenic and adipocyte-selective gene expression. Glucose tolerance and hepatic lipid accumulation in these mice were assessed under normal chow and high-fat diet conditions. Pre-adipocytes isolated from the stromal vascular fraction of BAT were transduced with *Wt1*-expressing retrovirus, induced to differentiate and analysed for the expression of thermogenic and adipocyte-selective genes.

Results Expression of the thermogenic genes *Cpt1b* and *Tmem26* was enhanced and transcript levels of *Ucp1* were on average more than tenfold higher in epididymal WAT of heterozygous *Wt1* knockout mice compared with wild-type mice. *Wt1* heterozygosity reduced epididymal WAT mass, improved whole-body glucose tolerance and alleviated severe hepatic steatosis upon diet-induced obesity in mice. Retroviral expression of WT1 in brown pre-adipocytes, which lack endogenous WT1, reduced mRNA levels of *Ucp1*, *Ppargc1a*, *Cidea*, *Prdm16* and *Cpt1b* upon in vitro differentiation by 60–90%. WT1 knockdown in epididymal pre-adipocytes significantly lowered *Aldh1a1* and *Zfp423* transcripts, two key suppressors of the thermogenic program. Conversely, *Aldh1a1* and *Zfp423* mRNA levels were increased approximately five- and threefold, respectively, by retroviral expression of WT1 in brown pre-adipocytes.

Conclusion/interpretation WT1 functions as a white adipocyte determination factor in epididymal WAT by suppressing thermogenic genes. Reducing *Wt1* expression in this and other intra-abdominal fat depots may represent a novel treatment strategy in metabolic disease.

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Research in context

What is already known about this subject?

- While subcutaneous white adipose tissue (WAT) is considered to protect against metabolic disease, visceral (intra-abdominal) WAT is deleterious
- Thermogenic genes can be induced, particularly in subcutaneous WAT, a phenomenon referred to as browning
- WT1 is expressed in adipocyte progenitor cells in intra-abdominal WAT, but not in subcutaneous WAT and brown adipose tissue (BAT)

What is the key question?

- Does WT1 function as a white adipocyte determination factor, and if so, what are the consequences of reduced WT1 expression in intra-abdominal WAT for in vivo metabolism?

What are the new findings?

- De novo expression of WT1 in brown pre-adipocytes represses *Ucp1* and other thermogenic genes upon in vitro differentiation
- Heterozygous *Wt1* knockout mice display increased expression levels of thermogenic genes in their epididymal WAT, improved glucose tolerance and reduced diet-related hepatic steatosis
- WT1 is necessary for normal expression of *Aldh1a1* and *Zfp423*, two key suppressors of the thermogenic program in adipocyte progenitor cells

How might this impact on clinical practice in the foreseeable future?

- WT1 in epididymal WAT is identified as a novel candidate target to tackle metabolic disease

Keywords Adipocyte · Browning · Glucose tolerance · Hepatic steatosis · High-fat diet · Obesity · Thermogenesis · Transcription factor · UCP1 · WT1

Abbreviations

BAT	Brown adipose tissue
HFD	High-fat diet
PPAR γ	Peroxisome proliferator-activated receptor γ
PRDM16	PR/SET domain 16
RER	Respiratory exchange ratio
RT-qPCR	Reverse transcription quantitative PCR
SVF	Stromal vascular fraction
UCP1	Uncoupling protein-1
WAT	White adipose tissue
WT1	Wilms tumour gene product

Introduction

Obesity has emerged as a global health problem that increases the prevalence of many life-shortening disorders including type 2 diabetes, cardiovascular disease, chronic kidney disease and cancer [1, 2]. The adverse effects of obesity are dependent not only on the amount of lipids stored in different

fat depots, but also on the site of fat deposition. Lipid accumulation in visceral (intra-abdominal) white adipose tissue (WAT) coincides with increased mortality, even in individuals with normal BMI, whereas subcutaneous obesity is less detrimental [3, 4].

At least two types of brown adipocytes exist besides the energy-storing white fat cells. The classical brown adipose tissue (BAT) fuels energy expenditure by non-shivering thermogenesis [5, 6]. This involves uncoupling protein-1 (UCP1), which dissociates H⁺ fluxes into the mitochondrial matrix from ATP synthesis, thus allowing energy to dissipate as heat [7]. Additionally, a population of brown adipocytes exist in WAT, the so-called beige or brite (brown-in-white) adipocytes, which normally express much lower levels of UCP1 than classical brown fat cells [8–10]. Cold exposure, β -adrenergic agonists and peroxisome proliferator-activated receptor γ (PPAR γ) activators can induce thermogenic genes in beige adipocytes, a phenomenon referred to as browning [8, 9, 11, 12]. Adipocyte-specific deletion of the transcriptional co-regulator PR/SET domain 16 (PRDM16) inhibits browning of subcutaneous WAT [13]. Mice without PRDM16 in their white fat cells develop severe insulin resistance and

subcutaneous macrophage accumulation on a high-fat diet (HFD), resembling the phenotype of visceral obesity [13]. The appearance of visceral fat-like features in *Prdm16*-deficient subcutaneous adipocytes is also indicated by their reduced thermogenic and enforced proinflammatory gene expression pattern [13].

The transcription factor Wilms tumour gene product (WT1) is expressed *de novo* in subcutaneous WAT of mice lacking *Prdm16* [13]. WT1 is normally restricted to intra-abdominal and epicardial white fat, which originates from the mesothelium—an epithelial tissue that lines the major body cavities. WT1 was initially identified as a suppressor of renal childhood cancer (Wilms tumour, nephroblastoma). Subsequent gene knockout studies in mice revealed that WT1 is required for embryonic survival and normal development of the genitourinary system and several other tissues [14]. Ubiquitous deletion of *Wt1* in adult mice reduced the intra-abdominal WAT mass, underlining its critical role in the homeostasis of visceral fat [15].

Little is known about the molecular mechanisms that maintain white adipose identity, particularly in the visceral fat. In general, intra-abdominal WAT of mice is largely resistant to browning, whereas beige adipocytes can be readily induced in subcutaneous WAT [11, 16, 17]. The fact that PRDM16 levels in visceral WAT are rather low is unlikely to account for the resistance to browning because transgenic overexpression of *Prdm16* induced brown adipocyte genes in subcutaneous, but not in visceral fat [18]. Instead, additional factors may exist in visceral WAT that limit its susceptibility to browning stimuli.

In this study, we test the hypothesis that WT1, which correlates inversely with PRDM16 levels in differentiating pre-adipocytes [13], determines a white adipose cell fate in epididymal WAT by suppressing thermogenic genes.

Methods

Animal care and protocol All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals and approved by the local authorities (permit no. G003/16 and T0308/12 issued by the Landesamt für Gesundheit und Soziales, [LAGeSo], Berlin, Germany). Pathogen-free C57BL/6 J mice with a heterozygous *Wt1* knockout gene (B6;129S4-*Wt1*^{tm1Jae}/J, The Jackson Laboratory, Bar Harbor, ME, USA) were bred in the local animal house (25°C, 12 h light/dark cycle). Animals were assigned to the specific treatment protocols (see ESM Methods) without randomisation. Blinding of the experiments was not carried out.

Cell culture and retroviral transductions Mycoplasma-negative immortalised pre-adipocytes from murine BAT were

kindly provided by Y-H Tseng (Joslin Diabetes Center, Harvard Medical School Affiliate, Boston, MA, USA) [19]. Routine maintenance, retroviral transduction and differentiation of the cells are described in ESM Methods [20].

Isolation and differentiation of precursor cells from murine WAT and BAT Isolation and maintenance of precursor cells from murine WAT and BAT are reported in ESM Methods [21].

Silencing of *Wt1* in stromal vascular cells isolated from epididymal WAT Established protocols were adapted for silencing of *Wt1* in stromal vascular fraction (SVF) cells isolated from the epididymal WAT [22]. See ESM Methods.

RNA isolation and reverse transcription quantitative PCR Isolation of total RNA and reverse transcription quantitative PCR (RT-qPCR) from mouse liver and fat, SVF cells and immortalised brown preadipocytes were performed as reported previously [22]. See ESM Methods, ESM Fig. 1. The PCR primer sequences are shown in ESM Table 1.

SDS-PAGE Adipose tissues of wild-type and heterozygous *Wt1* knockout mice were lysed in RIPA buffer, and SDS-PAGE was carried-out as described in ESM Methods [23].

Immunohistochemistry Immunostaining of formalin-fixed immortalised brown preadipocytes and paraffin-embedded tissue sections (1.5 µm thick) from epididymal WAT and BAT was performed as described elsewhere [24]. See ESM Methods.

RNAscope RNAscope (Advanced Cell Diagnostics, Newark, CA, USA) was performed on 1.5 µm thick formalin-fixed, paraffin-embedded tissue sections from epididymal WAT of wild-type mice with *Wt1* probe (#432711) using the RNAscope 2.5 HD Detection Reagent – BROWN Kit (Document No. 322310-USM) according to the manufacturer's instructions. Probes for *dapB* (#310043) and *Ppib* (#313911) were used as negative and positive controls, respectively.

Determination of the mean adipocyte area Mean adipocyte area was determined in epididymal WAT of wild-type and heterozygous *Wt1* knockout mice under either chow diet or HFD conditions using Adiposoft within the Fiji software [25]. See ESM Methods.

H&E and Oil Red O staining H&E staining was performed on paraffin-embedded adipose tissue following routine protocols [26]. Oil Red O was used for lipid staining [27].

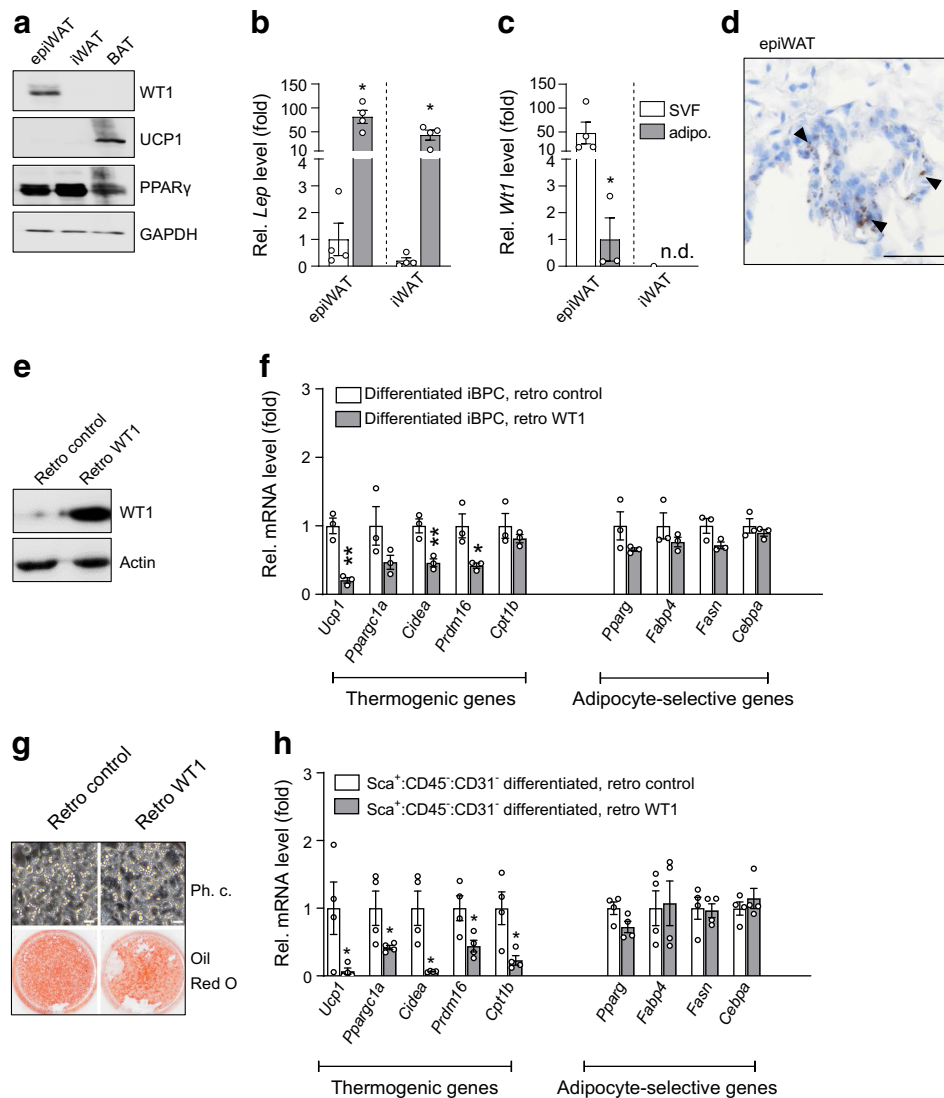


Fig. 1 WT1 suppresses thermogenic genes in differentiating brown precursor cells. **(a)** Representative immunoblot of different fat depots from adult male C57BL/6 J mice: epididymal WAT (epiWAT), inguinal WAT (iWAT), interscapular BAT. **(b, c)** Relative (Rel.) leptin (*Lep*) **(b)** and *Wt1* **(c)** mRNA levels measured by RT-qPCR in the isolated SVF and the adipocyte (adipo.) fraction of epiWAT and iWAT of adult mice. Leptin mRNA in adipocytes is presented as fold difference vs transcript levels in SVF cells of epiWAT **(b)**; *Wt1* transcripts in SVF cells of epiWAT are shown as fold increase vs mRNA levels in adipocytes **(c)**. Bars represent means \pm SEM, $n = 4$. * $p < 0.05$ vs SVF, Student's *t* test; n.d., not detectable. **(d)** *Wt1* mRNA in stromal vascular cells (arrowheads) in murine epiWAT detected by RNAscope. Scale bar, 50 μ m. **(e)** Representative immunoblot of immortalised brown pre-adipocytes (iBPC) with (retro WT1) and without (retro control) retroviral expression of WT1. **(f)** Relative mRNA levels in differentiated immortalised brown

pre-adipocytes with (black bars) and without (white bars) retroviral over-expression of *Wt1*. Bars represent means \pm SEM, $n = 3$. * $p < 0.05$, ** $p < 0.01$ vs retro control, Student's *t* test. **(g)** Phase contrast (Ph. c.) microscopy and Oil Red O lipid staining of immortalised brown pre-adipocytes. After transduction with either *Wt1*-expressing retrovirus or empty vector retrovirus, the cells were induced to differentiate for 5 days. Scale bars, 20 μ m. **(h)** Relative mRNA levels in primary brown adipocytes. Precursor cells were isolated by FACS from the SVF of interscapular BAT of adult mice. *Sca1*⁺:*CD45*⁻:*CD31*⁻ cells were transduced with *Wt1* or empty vector retrovirus, respectively, and grown to confluence for 3 days. Thereafter, the cells were induced to differentiate for 5 days. Transcript levels were measured in differentiated cells by RT-qPCR and normalised to *Actb* mRNA. Bars represent means \pm SEM, $n = 4$. * $p < 0.05$ vs retro control, Student's *t* test

Quantification of fatty acids in tissues and sera Triacylglycerols were extracted from homogenised liver tissue from wild-type and heterozygous *Wt1* knockout mice and measured with the colorimetric Triglycerides FS reagent kit (DyaSys Diagnostic Systems, Waterbury, CT, USA). NEFA were determined in sera using the

NEFA-HR(2) colorimetric assay kit (Fujifilm, Tokyo, Japan).

Measurement of glycogen content in the liver Frozen liver tissue was homogenised with a pestle in liquid nitrogen, dissolved in distilled H₂O and boiled for 10 min. Glycogen

was determined in the supernatant using the Glycogen Colorimetric/Fluorometric Assay kit (BioVision, Milpitas, CA, USA).

Quantification of serum insulin concentration Serum insulin was determined using the colorimetric rat/mouse insulin 96-well ELISA kit according to the supplied protocol (Millipore, Burlington, MA, USA).

Statistics All data obtained are included in the analyses. Values are presented as means \pm SEM. GraphPad PRISM software (Version 9.01) was used for calculation of statistics (GraphPad Software, San Diego, CA, USA). ANOVA with Tukey's post hoc test and two-tailed Student's *t* test were performed as indicated. If not otherwise indicated, unpaired Student's *t* test was used. A *p* value less than 0.05 was considered statistically significant.

Results

WT1 suppresses thermogenic genes in differentiating brown precursor cells WT1 protein was detected in epididymal WAT but neither in inguinal (subcutaneous) WAT nor in interscapular BAT (Fig. 1a) [15, 28]. Within epididymal WAT, *Wt1* was highly expressed in the SVF, but not in the leptin-enriched adipocyte fraction (Fig. 1b–d).

To test the hypothesis that WT1 suppresses the BAT genetic program, an immortalised brown pre-adipocyte cell line was transduced with a *Wt1*-expressing retrovirus and subsequently differentiated (Fig. 1e) [19]. While lipid accumulation looked alike (Fig. 1g), *Wt1*-expressing brown adipocytes had significantly lower mRNA levels of the thermogenic genes *Ucp1*, *Cidea* and *Prdm16* than non-expressing cells (Fig. 1f). Transcript levels of adipocyte-selective genes that play a role in both white and brown adipocyte differentiation, i.e.

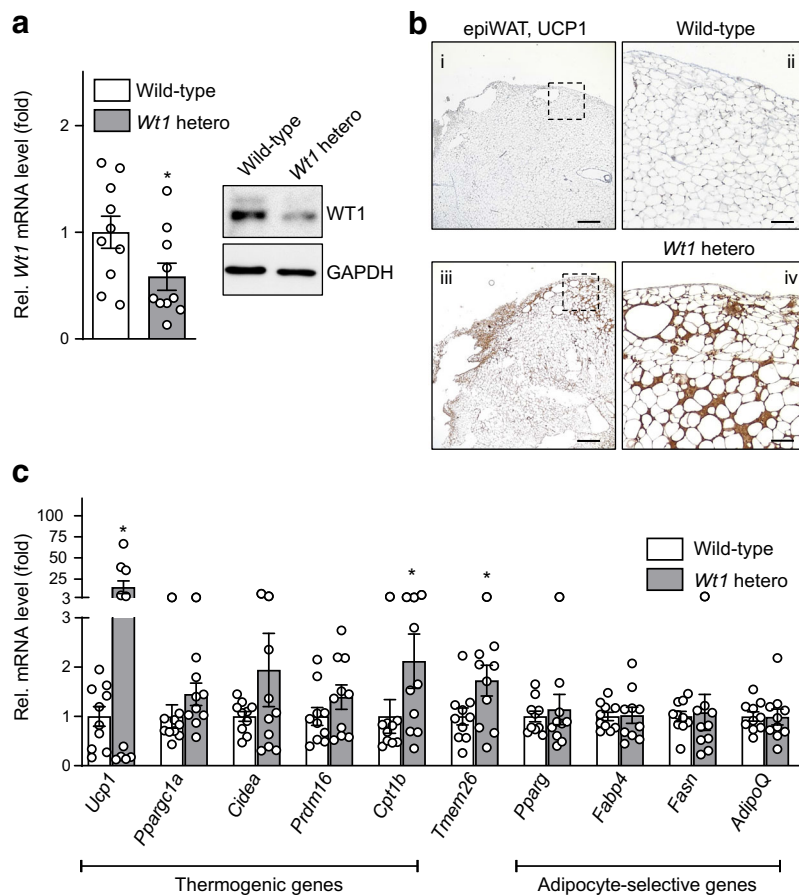


Fig. 2 Heterozygous *Wt1* knockout mice display molecular and morphological signs of browning in their epididymal fat. **(a)** Relative (Rel.) *Wt1* mRNA levels (left) and WT1 protein (right) in epididymal WAT (epiWAT) of wild-type and heterozygous *Wt1* knockout mice. Transcript levels were measured by RT-qPCR. Bars indicate means \pm SEM, *n* = 10. **p* < 0.05 vs wild-type, Student's *t* test. **(b)** Representative UCP1 immunostaining in epiWAT of a wild-type and a heterozygous *Wt1* knockout mouse (*Wt1* hetero). Scale bars, 400 μ m (i,

iii) and 100 μ m (ii, iv). Panels ii and iv are high power magnifications of the boxed areas in panels i and iii, respectively. **(c)** Relative transcript levels of thermogenic genes, the beige adipocyte gene *Tmem26* and adipocyte-selective genes in epiWAT of wild-type and heterozygous *Wt1* knockout mice. Transcripts were measured by RT-qPCR and normalised to *Actb* mRNA. Data are shown as fold difference between mRNA levels in wild-type and heterozygous *Wt1* knockout mice. Bars indicate means \pm SEM, *n* = 10. **p* < 0.05 vs wild-type, Student's *t* test

Pparg, *Fabp4*, *Fasn* and *Cebpa*, were also slightly reduced in *Wtl* expressing brown adipocytes, but this effect did not reach statistical significance (Fig. 1f).

To validate these findings in primary cultures, we isolated $\text{Sca1}^+:\text{CD45}^-:\text{CD31}^-$ cells by FACS from the SVF of interscapular BAT of adult mice. Purified cells were transduced with *Wtl*-expressing retrovirus or empty vector and differentiated for 5 days. *Wtl*-expressing brown adipocytes had significantly lower transcript levels of *Ucp1*, *Pparg1a*, *Cidea*, *Prdm16* and *Cpt1b* compared with *Wtl*-negative cells, but no significant differences in adipocyte-selective transcripts were detected (Fig. 1h). The fraction of Ki-67 immunopositive cells was not significantly different between *Wtl*-expressing and non-expressing brown pre-adipocytes as well as between SVF cells isolated from epididymal WAT of wild-type and heterozygous *Wtl* knockout mice (ESM Fig. 2).

Heterozygous *Wtl* knockout mice display signs of browning in their epididymal WAT

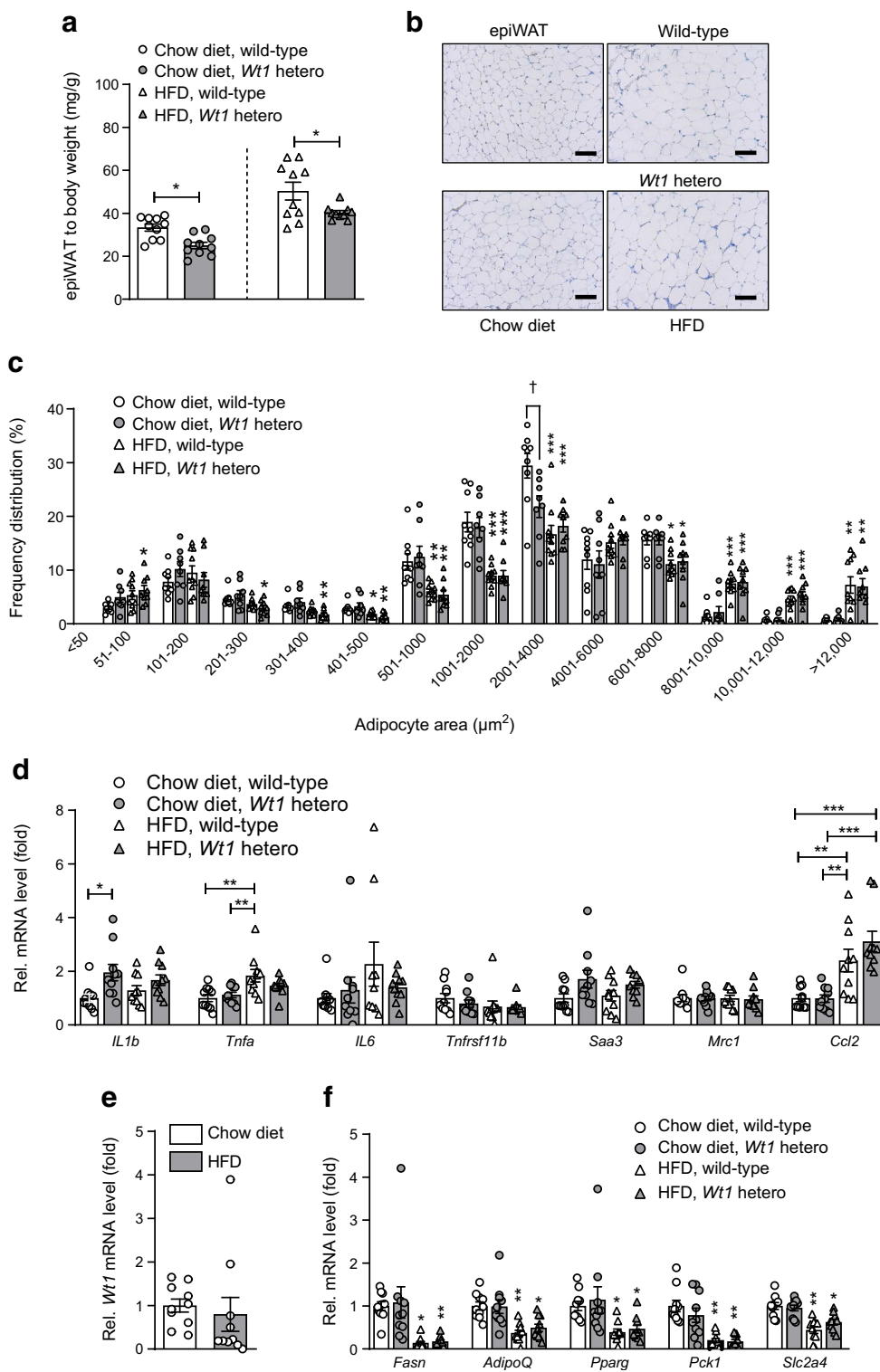
Heterozygous *Wtl* knockout mice and their wild-type littermates were analysed to explore whether WT1 affects thermogenic gene expression in adipose tissues in vivo. *Wtl* mRNA and protein were lower in epididymal WAT of heterozygous *Wtl* knockout compared with wild-type mice (Fig. 2a). *Wtl* heterozygotes had significantly higher transcript levels of the thermogenic genes *Ucp1* and *Cpt1b*, and the beige adipocyte marker gene *Tmem26* in their epididymal WAT, whereas mRNA levels of adipocyte-selective genes (*Pparg*, *Fabp4*, *Fasn*, *AdipoQ*) were similar (Fig. 2c). Transcript levels of the tested genes were not significantly different in inguinal WAT and interscapular BAT of wild-type and *Wtl* mutant mice (ESM Fig. 3), i.e. in fat depots that do not express *Wtl* (Fig. 1a) [13, 15, 29]. β 3-Adrenergic stimulation with CL316,243 (1 $\mu\text{g/g}$ body weight) significantly increased *Ucp1*, *Cidea*, *Cpt1b* and *Adrb3* transcripts in epididymal WAT to a level that was not significantly different ($p > 0.05$, *t* test) between wild-type and heterozygous *Wtl* knockout mice (ESM Fig. 4). In accordance with the increased transcript levels (Fig. 2c), UCP1 immunoreactivity was clearly visible in epididymal WAT of heterozygous *Wtl* knockout mice, but not of wild-type mice (Fig. 2b). Interscapular BAT was used as a positive control for UCP1 immunostaining (ESM Fig. 5a). To corroborate the in vivo findings in cell culture, SVF cells were freshly isolated from epididymal WAT of wild-type and heterozygous *Wtl* knockout mice, expanded and induced to differentiate into adipocytes. All cultures looked similar in terms of cellular lipid accumulation. Notably, adipogenic differentiation was associated with an increase in *Ucp1* mRNA levels that was statistically significant in cells obtained from heterozygous *Wtl* knockout mice (ESM Fig. 5b).

Heterozygous *Wtl* knockout mice receiving HFD have a lower epididymal WAT to body weight ratio

To test whether wild-

Fig. 3 Heterozygous *Wtl* knockout mice fed with HFD have lower epididymal WAT to body weight ratios. Wild-type and heterozygous *Wtl* knockout mice ($n = 40$ total) were kept on either chow diet (10% of kJ from fat) or HFD (60% of kJ from fat) for 11 weeks. **(a)** Epididymal WAT (epiWAT) weight to body weight ratios. Bars indicate means \pm SEM, $n = 10$, each. $*p < 0.05$ between wild-type and *Wtl* mutant animals (Student's *t* test). Note that, for better data visualisation, statistical significance between mice receiving chow diet and HFD is not indicated. **(b)** Representative H&E staining of epiWAT of a wild-type and heterozygous *Wtl* knockout mouse fed with either chow or HFD. Scale bars, 100 μm . **(c)** Frequency distribution of adipocyte areas in epididymal WAT of wild-type and heterozygous *Wtl* knockout mice receiving either chow diet or HFD. Measurements were performed with tissue sections from $n = 40$ animals analysing more than 2500 cells per group. Statistical differences between mice of identical genotype receiving HFD vs chow diet are indicated by asterisks ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ANOVA with Tukey post hoc test). $\dagger p < 0.05$, statistical differences between wild-type and heterozygous *Wtl* knockout mice (ANOVA with Tukey post hoc test). Note that the adipocyte areas were not significantly different between wild-type and heterozygous *Wtl* knockout mice except for the size range 2001–4000 μm^2 . In this particular range, *Wtl* knockout mice fed with chow diet had significantly smaller adipocytes in their epididymal WAT than wild-type mice. **(d)** Relative mRNA levels of inflammation-related genes in epididymal WAT of normal and *Wtl* mutant mice. Bars represent means \pm SEM, $n = 10$ in each group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ as shown, ANOVA with Tukey post hoc test. **(e)** Relative *Wtl* mRNA levels in epididymal WAT of wild-type mice receiving either chow diet or HFD. Transcripts were measured by RT-qPCR and normalised to *Actb* mRNA. Bars indicate means \pm SEM, $n = 10$. **(f)** Relative transcript levels of genes involved in fatty acid and glucose homeostasis in epididymal WAT of wild-type and heterozygous *Wtl* knockout mice receiving either normal diet or HFD. Bars show means \pm SEM, $n = 10$ in each group. $*p < 0.05$ and $**p < 0.01$ between mice of the same genotype receiving HFD vs chow diet, ANOVA with Tukey post hoc test

type and heterozygous *Wtl* knockout mice respond differently to metabolic stress conditions, they received either HFD or chow diet for 11 weeks. Minor differences in daily food intake, body weight, relative lean body mass, serum concentrations of insulin and NEFA and locomotor activity were observed between wild-type and heterozygous *Wtl* knockout mice (ESM Fig. 6). Mice with a single intact *Wtl* allele had a significantly lower epididymal WAT to body weight ratio (Fig. 3a). As expected, mean adipocyte areas were larger in epididymal WAT of mice receiving HFD vs chow diet (Fig. 3b, c). However, significant differences in adipocyte areas were generally not observed between wild-type and heterozygous *Wtl* knockout mice except for the range from 2001 to 4000 μm^2 . In this size range, the adipocytes of chow-fed heterozygous *Wtl* knockout mice were significantly smaller than those of wild-type mice (Fig. 3c). *I11b* transcripts were approximately twofold higher in epididymal WAT of heterozygous *Wtl* knockout mice than wild-type mice on chow diet (Fig. 3d). *Tnfa* mRNA levels in epididymal WAT increased upon HFD feeding, and this effect was significant ($p < 0.01$) in wild-type but not in *Wtl* mutant mice (Fig. 3d). *Wtl* transcripts were slightly ($p > 0.05$) higher with a broader

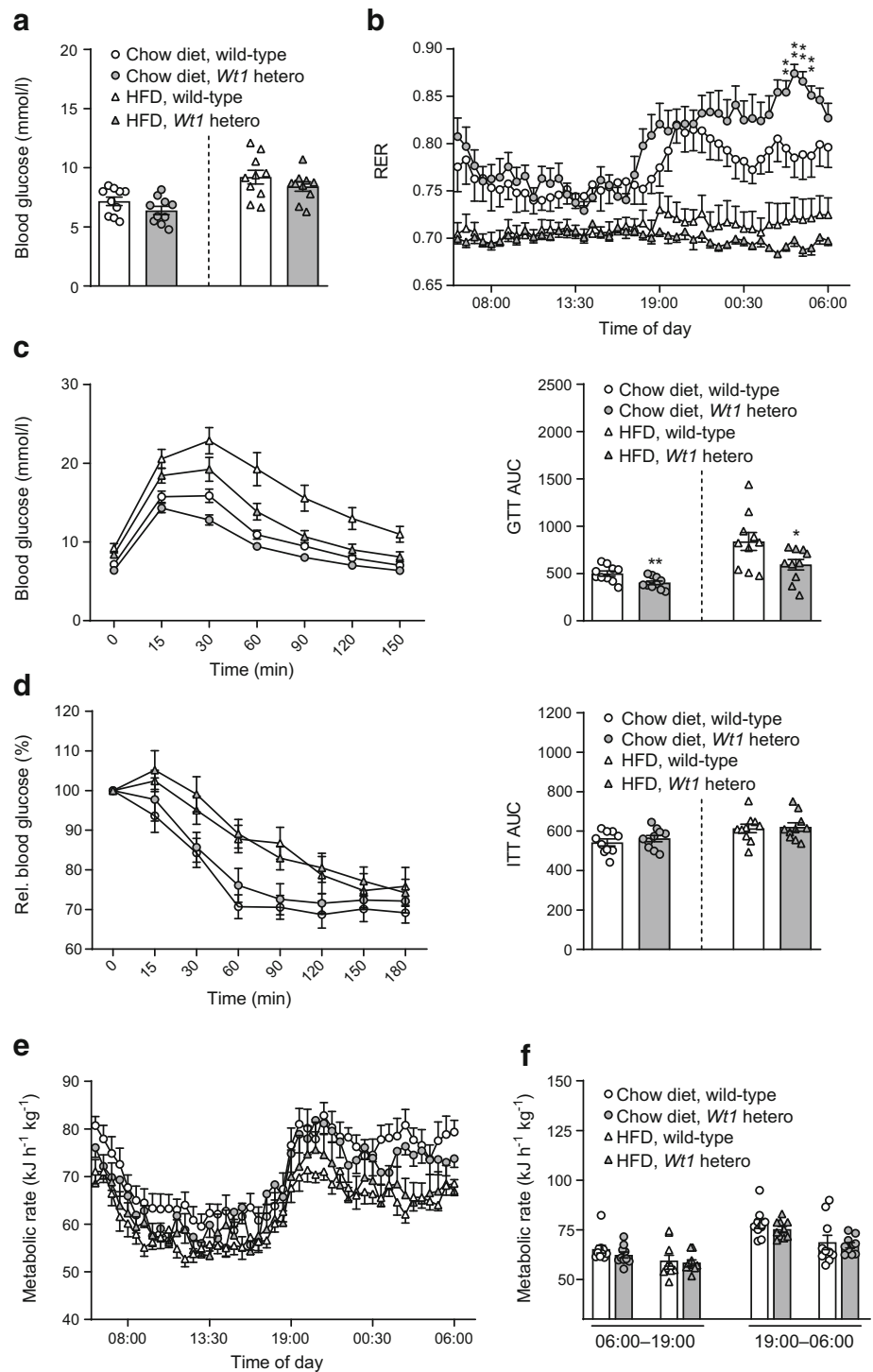


value distribution in HFD-fed animals (Fig. 3e). Transcript levels of genes involved in fatty acid and glucose metabolism, i.e. *Fasn*, *AdipoQ*, *Pparg*, *Pck1* and *Slc2a4*, were significantly lower in epididymal WAT of HFD- vs chow-fed mice (Fig. 3f). Heterozygous *Wt1* knockout mice on HFD had higher *Ucp1* mRNA levels

in their epididymal WAT than wild-type mice (ESM Fig. 7).

Heterozygous *Wt1* knockout mice show improved whole-body glucose tolerance and reduced hepatic steatosis We reasoned that enhanced *Ucp1* expression in epididymal

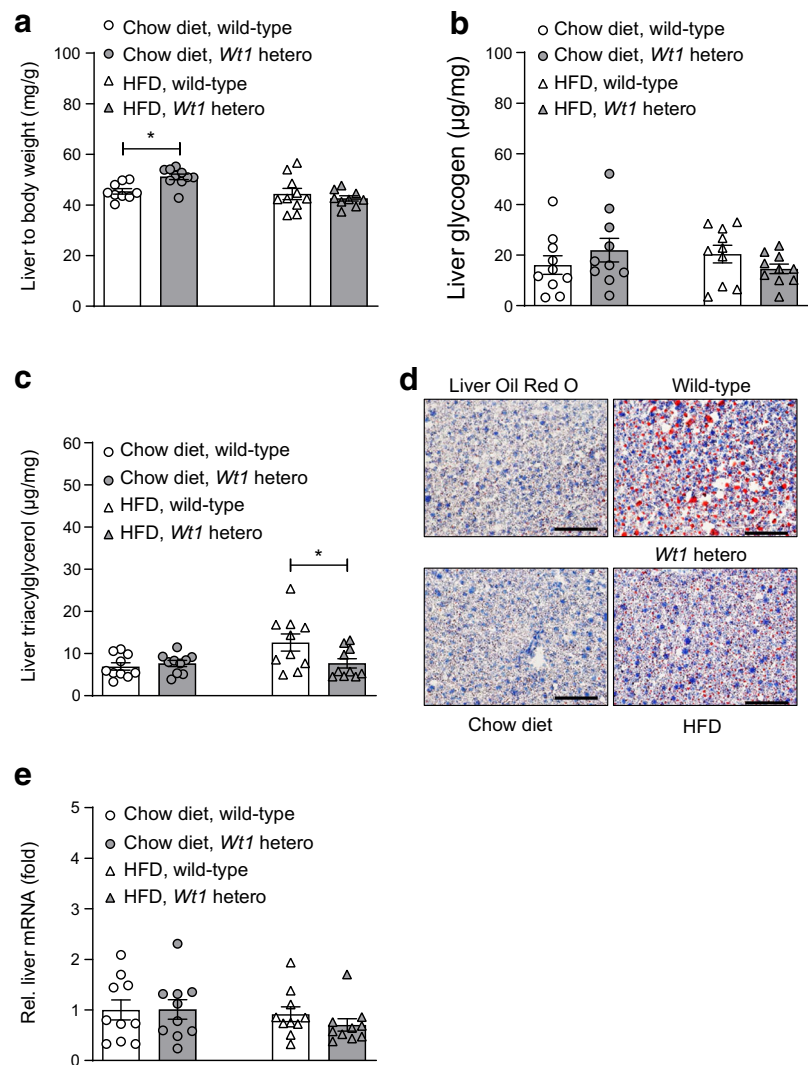
Fig. 4 Heterozygous *Wt1* knockout mice show improved whole-body glucose tolerance. Fasting blood glucose levels (a) and RER (b) in wild-type and *Wt1* mutant mice kept on either chow diet or HFD for 11 weeks. Asterisks indicate statistical differences between wild-type and heterozygous *Wt1* knockout mice fed with chow diet. $**p < 0.01$, ANOVA with Tukey post hoc test, $n = 10$. Glucose (c) and insulin (d) tolerance test. Values are means \pm SEM, $*p < 0.05$, $**p < 0.01$ vs wild-type, Student's *t* test, $n = 10$ in each group. Diurnal (e) and phasic (f) metabolic rates of wild-type and *Wt1* mutant mice on HFD and chow diet



WAT might protect heterozygous *Wt1* knockout mice against HFD-induced metabolic dysfunction. Animals receiving HFD had indeed higher blood glucose concentrations than mice on chow diet, but no significant differences were observed between wild-type and heterozygous *Wt1* knockout mice (Fig. 4a). The respiratory exchange ratio (RER, $\dot{V}CO_2/\dot{V}O_2$)

was elevated between 03:00 h and 05:30 h in chow-fed *Wt1* heterozygotes suggesting that carbohydrates were their predominant fuel source during the final stages of the dark phase (Fig. 4b). *Wt1* mutant mice showed a significantly improved whole-body glucose tolerance (Fig. 4c). Serum insulin concentrations (ESM Fig. 6e) and insulin sensitivity

Fig. 5 Heterozygous *Wt1* knockout mice on HFD show reduced hepatic steatosis. Liver to body weight ratio (a), hepatic glycogen (b) and triacylglycerol content per milligram liver tissue (c) of wild-type and heterozygous *Wt1* knockout mice fed with either chow diet or HFD. * $p < 0.05$, ANOVA with Tukey post hoc test. Note that for better data visualisation, statistical significance between mice receiving chow diet and HFD is not indicated. (d) Representative Oil Red O lipid staining of liver sections from wild-type and heterozygous *Wt1* knockout mice receiving either chow diet or HFD. Scale bars, 100 μm . (e) *Wt1* transcript levels measured by RT-qPCR and normalised to *Actb* mRNA in the livers of wild-type mice receiving either chow diet or HFD. Bars represent means \pm SEM, $n = 10$. $p > 0.05$, ANOVA with Tukey post hoc test



(Fig. 4d) were not significantly different between normal and *Wt1* mutant mice. Metabolic rates followed a circadian rhythm with higher values during the dark period (Fig. 4e, f).

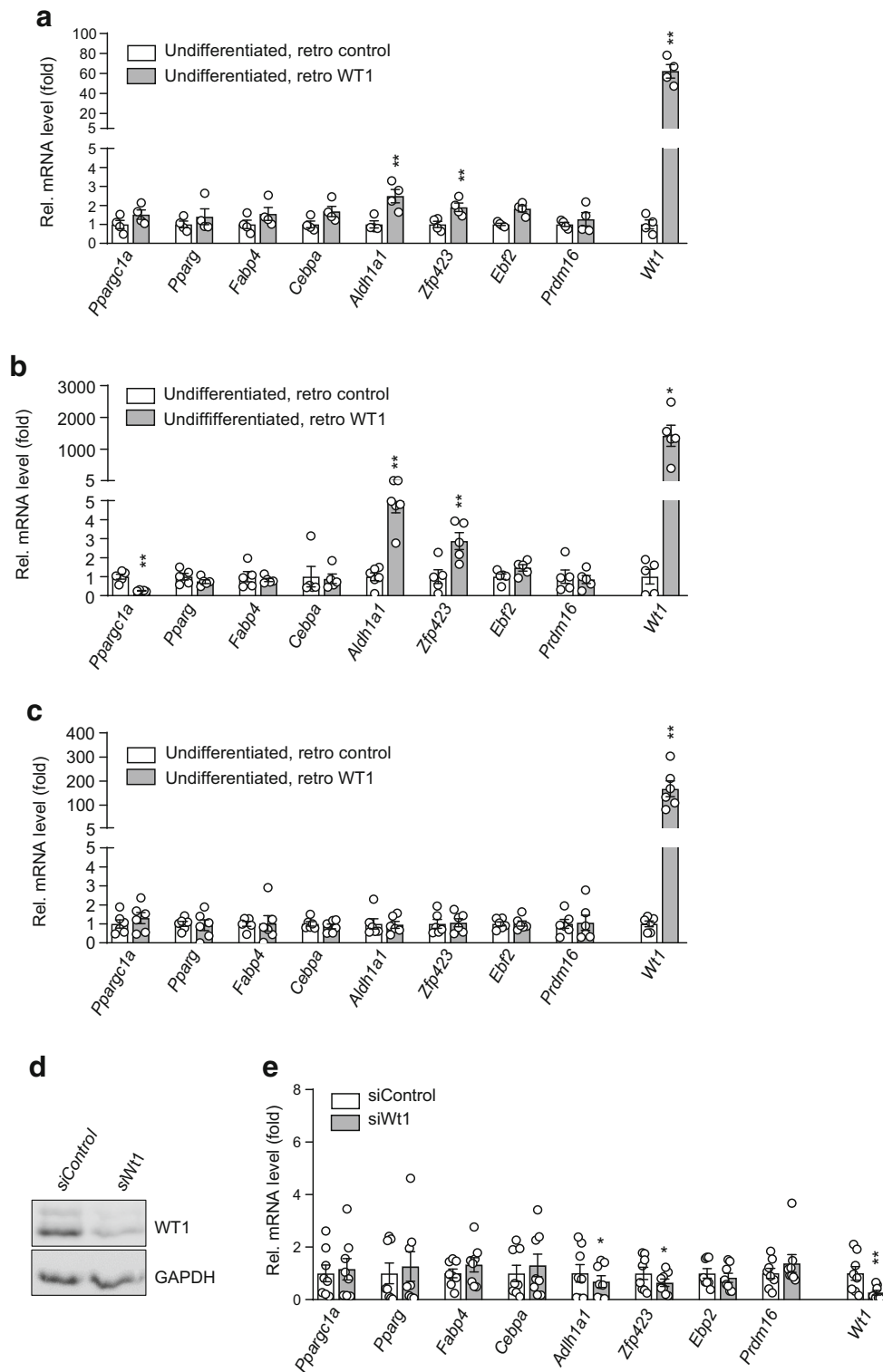
Heterozygous *Wt1* knockout mice on chow diet, but not on HFD, had significantly higher liver to body weight ratios than their wild-type littermates (Fig. 5a). The amount of glycogen stored per gram liver tissue was not significantly different between the groups (Fig. 5b). Hepatic triacylglycerol content was similar in wild-type and *Wt1* mutant mice on chow diet (Fig. 5c). HFD feeding increased triacylglycerol levels nearly twofold in the livers of wild-type but not of *Wt1* mutant mice (Fig. 5c). Accordingly, wild-type mice showed severe hepatic steatosis with clearly milder changes in the *Wt1* heterozygotes (Fig. 5d). HFD feeding did not significantly alter *Wt1* mRNA levels in the liver of wild-type and *Wt1* mutant mice (Fig. 5e).

WT1 increases *Aldh1a1* and *Zfp423* expression in adipogenic precursor cells Finally, we sought to identify potential downstream pathways, along which WT1 may repress a brown

adipocyte differentiation program. Notably, transcript levels of *Aldh1a1* and *Zfp423*, two key determinants of white adipose identity [30–32], were significantly increased upon retroviral expression of *Wt1* in undifferentiated SVF cells from interscapular BAT and in immortalised brown pre-adipocytes (Fig. 6a, b). *Wt1* expressing pre-adipocytes also had reduced mRNA levels of *Ppargc1a* (Fig. 6b), which interacts with nuclear receptor PPAR γ in the regulation of BAT mitochondrial biogenesis [33]. Retroviral delivery of WT1 did not affect mRNA levels in SVF cells isolated from inguinal WAT (Fig. 6c). Silencing of *Wt1* significantly reduced *Aldh1a1* and *Zfp423* transcripts in SVF cells from epididymal WAT (Fig. 6d, e).

Discussion

Visceral adipose tissue of mice is normally less susceptible to browning than subcutaneous WAT [34, 35]. Our current data



suggest that WT1, which is expressed in epididymal but not in subcutaneous WAT (Fig. 1a, [28]), is a direct or indirect repressor of WAT browning. Inhibition of WAT browning by WT1 in vivo is indicated by the upregulation of thermogenic genes in epididymal WAT of heterozygous *Wt1* knock-out mice (Fig. 2). Remarkably, β 3-adrenergic stimulation

with CL316,243 eliminates differences in thermogenic gene expression between wild-type and *Wt1* mutant mice (ESM Fig. 4). This suggests that the repressor activity of WT1 on WAT browning can be antagonised by the effect of a potent β 3-adrenergic stimulus. Hence, WT1 does not limit the overall browning capacity of epididymal WAT, but may rather

◀ **Fig. 6** *Wt1* increases *Aldh1a1* and *Zfp423* expression in adipogenic precursor cells. **(a)** Relative mRNA levels of genes involved in adipose cell fate determination in SVF cells isolated from interscapular BAT of adult mice. Cells were transduced with either *Wt1*-expressing retrovirus (retro WT1) or empty vector control (retro control). **(b)** Transcript levels in immortalised brown pre-adipocytes with and without retroviral expression of WT1. **(c)** Relative mRNA levels in *Wt1*-expressing and non-expressing SVF cells isolated from inguinal WAT of adult mice. WT1 immunoblot **(d)** and relative transcript levels **(e)** of undifferentiated SVF cells prepared from epididymal WAT of adult mice. Primary cells at approximately 50% confluence were incubated with either non-targeting control siRNA or *Wt1* siRNA for 48 h. Transcript levels were measured by RT-qPCR and normalised to *Actb*. In each figure part, mRNA levels are shown as fold difference between cells transfected with *Wt1* siRNA (siWt1) and non-targeting siRNA (siControl). Bars represent means \pm SEM, $n = 4$ **(a)**, $n = 5$ **(b)**, $n = 6$ **(c)** and $n = 8$ **(e)**. * $p < 0.05$, ** $p < 0.01$, Student's paired t test. Note that all data shown were obtained with undifferentiated cells

contribute to the maintenance of WAT identity under unchallenging conditions, i.e. in the absence of a strong browning inducer.

Silencing of endogenous *Wt1* in epididymal SVF cells reduces *Aldh1a1* and *Zfp423* transcripts, whereas retroviral expression of *Wt1* increases these mRNAs in brown precursor cells (Fig. 6). These findings suggest that WT1 stimulates *Aldh1a1* and *Zfp423* expression in epididymal pre-adipocytes either directly or through indirect mechanisms. Deletion of either *Aldh1a1* or *Zfp423* has been reported to induce thermogenic genes in visceral fat depots [30, 32, 36]. Retinal dehydrogenase 1 (ALDH1A1) is the rate-limiting enzyme in the conversion of retinaldehyde (Rald) to retinoic acid. *Aldh1a1* deficiency increases Rald concentrations, particularly in tissues with high endogenous *Aldh1a1* expression, such as WAT [37, 38]. By activating retinoic acid receptor (RAR)-dependent mechanisms, Rald functions as a positive transcriptional regulator of the classical BAT program in white fat cells [30]. Zinc finger protein 423 (*Zfp423*) functions as a transcription factor that maintains white adipocyte identity by suppressing thermogenic genes [31, 32]. It acts, at least partially, by inhibiting the activity of *Ebf2* and thereby represses *Prdm16* [32]. Remarkably, *Zfp423* was among the top 1000 genes (p value 4.7×10^{-23}) bound by WT1 in a genome-wide ChIP-sequencing analysis of mouse embryonic kidneys [39].

Importantly, retroviral delivery of WT1 in brown precursor cells does not inhibit adipogenic differentiation in general nor does WT1 significantly downregulate adipocyte-selective genes that are involved in white and brown adipocyte differentiation (Fig. 1). Instead, ectopic WT1 predominantly represses thermogenic genes in differentiating brown precursor cells (Fig. 1e, g). One can therefore assume that the brown adipose signature in epididymal WAT of heterozygous *Wt1* knockout mice is genuine and not merely due to subtle developmental defects. Furthermore, these findings qualify WT1 as a repressor of brown adipocyte identity.

What are possible functional consequences arising from a BAT-like gene expression pattern in the epididymal fat of heterozygous *Wt1* knockout mice? Strikingly, mice with a heterozygous *Wt1* gene show improved whole-body glucose tolerance and alleviated diet-induced fatty liver disease (Figs 4, 5). The former observation is in keeping with the higher RER of heterozygous *Wt1* mutant mice indicating that carbohydrates are their predominant fuel source on HFD, at least during the last phase of diurnal physical activity (Fig. 4b). Activation of classical BAT and browning of WAT are associated with a favourable metabolic status including improved insulin sensitivity and reduced fatty liver disease [5, 40–42]. Accordingly, inhibition of beige adipocyte function by conditional deletion of *Prdm16* caused severe insulin resistance and hepatic steatosis in mice receiving HFD [13]. Thus, we assume that thermogenic genes expressed in epididymal WAT of heterozygous *Wt1* knockout mice might contribute to their improved glucose tolerance and largely preserved liver morphology upon HFD feeding. However, a causative relationship between epididymal WAT browning and metabolic function in *Wt1* mutant mice cannot be derived from our data. Our findings also do not allow us to exclude that preserved metabolic function is secondary to *Wt1* heterozygosity in the liver or other tissues. More specific approaches using conditional *Wt1* knockout mice are necessary to resolve this important issue.

HFD feeding does not significantly increase *Wt1* mRNA levels in murine epididymal WAT (Fig. 3e). This observation is in agreement with transcriptome analyses in humans reporting similar *Wt1* mRNA levels in the visceral fat of lean and obese individuals [43]. Surprisingly, body weight gain and metabolic rates are not significantly different between wild-type and *Wt1* mutant mice (Fig. 4e, f, ESM Fig. 6b, c). This might seem puzzling at first sight as browning of subcutaneous WAT is normally associated with increased energy expenditure and reduced obesity in mice [44–46]. However, classical inducers of WAT browning, such as exposure to low temperature, PPAR γ agonists or β -adrenergic stimulation usually increase thermogenic genes in subcutaneous WAT to much higher levels than those measured in epididymal WAT of heterozygous *Wt1* knockout mice [8, 11, 13]. One can therefore speculate whether the induction of thermogenic genes in epididymal WAT of *Wt1* mutant mice is not strong enough for eliciting a significant increase in whole-body energy expenditure in the conditions tested.

In summary, our findings demonstrate that *Wt1* haploinsufficiency activates a brown adipocyte genetic program in epididymal WAT of mice. We suggest that browning of epididymal WAT depots contributes to reduced diet-related hepatic steatosis and increased whole-body glucose tolerance in mice with a single intact *Wt1* allele. Thus, our results identify WT1 as a repressor of brown adipocyte identity and a potential therapeutic target in metabolic disorders.

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