BBA - Molecular Basis of Disease 1865 (2019) 1567–1578

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Intestinal vitamin D receptor modulates lipid metabolism, adipose tissue inflammation and liver steatosis in obese mice

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ARTICLE INFO

Keywords:
Mouse model
Overweight
Non-alcoholic fatty liver disease
Hepatic

ABSTRACT

Objective: Hypovitaminosis D is common in the obese population and patients suffering from obesity-associated disorders such as type 2 diabetes and fatty liver disease, resulting in suggestions for vitamin D supplementation as a potential therapeutic option. However, the pathomechanistic contribution of the vitamin D-vitamin D receptor (VDR) axis to metabolic disorders is largely unknown.

Methods: We analyzed the pathophysiological role of global and intestinal VDR signaling in diet-induced obesity (DIO) using global *Vdr−/−* mice and mice re-expressing an intestine-specific human *VDR* transgene in the *Vdr* deficient background (*Vdr−/−* hTg).

Results: *Vdr−/−* mice were protected from DIO, hepatosteatosis and metabolic inflammation in adipose tissue and liver. Furthermore, *Vdr−/−* mice displayed a decreased adipose tissue lipoprotein lipase (LPL) activity and a reduced capacity to harvest triglycerides from the circulation. Intriguingly, all these phenotypes were partially reversed in *Vdr−/−* hTg animals. This clearly suggested an intestine-based VDR activity on systemic lipid homeostasis. Scrutinizing this hypothesis, we identified the potent LPL inhibitor angiopoietin-like 4 (*Angptl4*) as a novel transcriptional target of VDR.

Conclusion: Our study suggests a VDR-mediated metabolic cross-talk between gut and adipose tissue, which significantly contributes to systemic lipid homeostasis. These results have important implications for use of the intestinal VDR as a therapeutic target for obesity and associated disorders.

1. Introduction

Observational clinical studies demonstrate that hypovitaminosis D is very common in the obese population and low vitamin D levels are associated with obesity-associated metabolic disorders such as type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) [1–3]. With regard to the strongly increasing prevalence of these diseases, vitamin D supplementation is currently considered as a potential therapeutic option [4–6]. However, there is partially conflicting evidence regarding the therapeutic effects of vitamin D from interventional human studies. While some clinical trials found improvement of metabolic parameters and/or amelioration of NAFLD (e.g. measured by liver fat content or certain serum markers of liver damage) by vitamin D treatment [7–10], other studies could not confirm these positive effects [11–13]. Thus, the precise mechanistic contributions of vitamin D to metabolic disorders including NAFLD and its putative therapeutic relevance in humans have so far remained unclear.

On the biochemical level, activated vitamin D (1,25-dihydroxyvitamin D) binds to the nuclear receptor superfamily member, vitamin D receptor (VDR, NR1I1), which serves as a ligand-activated

Abbreviations: Angptl4, angiopoietin-like 4; CLS, crown-like structures; eWAT, epididymal white adipose tissue; HFD, high-fat diet; LFD, low-fat diet; LPL, lipoprotein lipase; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified fatty acid; PPAR, peroxisome proliferator-activated receptor; PTH, parathyroid hormone; T2DM, type 2 diabetes mellitus; TG, triglyceride; VDR, vitamin D receptor

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https://doi.org/10.1016/j.bbadis.2019.03.007
Received 18 September 2018; Received in revised form 6 March 2019; Accepted 19 March 2019
Available online 21 March 2019
0925-4439/

transcription factor controlling a large number of genes in various tissues [14]. Currently available preclinical studies in rodents are, however, partially controversial with regard to the metabolic effects of vitamin D [15–23], and indicate so far not fully understood cell type- and context-specific effects of VDR signaling [24–28]. Thus, more experimental data on the tissue-specific effects of vitamin D and the underlying modes of action are required to translate the currently available knowledge from observational and interventional clinical studies into future treatment strategies.

In this study, we report a novel role of the intestinal vitamin D-VDR axis as an important metabolic regulator of obesity in mice. Using a villin promoter-driven human *VDR* transgene in a *Vdr* deficient background, we show that gut-specific VDR enhances weight gain, adipose tissue inflammation and the development of hepatic steatosis induced by high-fat diet feeding. With respect to the underlying mechanism, our data indicate that VDR modulates the expression of intestinal factors controlling lipid metabolism in peripheral organs thus providing an unexpected physiological link between VDR signaling in the gut and systemic lipid homeostasis.

2. Materials and methods

2.1. Animals

The generation of mice expressing an intestine-specific, human *VDR* transgene (in the following referred to as hTg) has been reported previously [29]. These animals were crossed with *Vdr*−/− mice (B6.129S4-Vdrtm1Mbd/J, The Jackson Laboratory) which are deficient for the endogenous murine VDR [30] and maintained on a C57BL/6J background.

Experimental transgenic animals were generated by crossing *Vdr*−/− hTg males with *Vdr*+/− females to produce *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg offspring. Tissue specific expression of human VDR was validated by qPCR analyses. Confirming previous findings [29], the transgene was strongly expressed in the small intestine (jejunum) of *Vdr*−/− hTg mice (qPCR C_t-values ~23–24), but was undetectable in liver and adipose tissue of *Vdr*−/− hTg mice as well as in *Vdr*+/− and *Vdr*−/− samples (mean $C_t > 35$ in qPCR). The absence of transcripts encoding the endogenous murine VDR was confirmed in *Vdr*−/− and *Vdr*−/− hTg animals (jejunum). Intestinal protein levels of VDR were assessed by Western blot. Expression of the human transgene in *Vdr*−/− hTg animals resulted in a ~2-fold increase of total VDR protein levels compared to *Vdr*+/− control animals (Fig. S1).

To analyze the pharmacological effects of high-dose vitamin D supplementation in genetically-unaltered animals (Fig. 7A–D), wild-type C57BL/6J mice were obtained from Charles River.

2.2. Diets

Diets were purchased from ssniff Spezialdiäten GmbH (Table S1). Food and water was provided ad libitum. Organs were harvested after overnight fasting and either snap-frozen and stored at −80 °C or fixed with formalin and embedded in paraffin (FFPE). Animal experiments were performed in accordance with the local animal protection laws and were approved by the local authorities (Regierung von Unterfranken, Würzburg, Germany).

All *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg animals received “rescue diet” containing 2% calcium, 1.25% phosphorus and 20% lactose irrespective of their genotype and throughout breeding. This rescue diet (in the following termed “low-fat diet” or “LFD”) has been shown to prevent hypocalcemia and bone abnormalities associated with the loss of VDR [31]. The same amounts of calcium, phosphorus and lactose were also present in the respective high-fat diet (“HFD”) used for the transgenic animals (*Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg). For the induction of obesity, *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg animals (male, aged 8–9 weeks) were fed with this HFD for eight weeks. Age-matched *Vdr*+/− mice receiving LFD served as lean controls.

For the pharmacological studies in C57BL/6J wild-type mice (male, aged 8 weeks; Fig. 7A–D), we used two different high-fat diets with either low (500 IU per kg diet; i.e. “500 IU VD3-HFD”) or high levels of vitamin D (10,000 IU per kg diet; i.e. “10,000 IU VD3-HFD”).

2.3. Human intestinal samples

Human intestinal samples from a previously established cohort [32] were used in this study. The study protocol was approved by the local ethics committee (reference number: EK-1744), and informed consent was obtained from all patients. A total number of 17 patients (9 males, 8 females) were included in the present study. Median patient's age was 62 years, ranging from 41 to 77 years. Median BMI was 26.2 kg/m^2, ranging from 16.2 to 34.4 kg/m^2. All patients were examined at one single institution (University of Zurich, Switzerland, Department of Gastroenterology). Patients underwent gastroduodenoscopy, combined gastroduodenoscopy and ileocolonoscopy, or ileocolonoscopy as part of a routine medical checkup. Exclusion criteria were: age below 18 or above 80 years, severe pathologies of the gastrointestinal tract (including coeliac disease, inflammatory bowel disease), carcinomas, kidney- or hepatic insufficiency, bleeding disorders, infectious diseases, oral anticoagulation, drug- or alcohol abuse and/or mental retardation. Mucosal biopsies were taken from the descending and inferior duodenum, from the terminal ileum, as well as from the ascending colon. Duodenal samples were used from 13 patients for the present analysis, terminal ileum samples from 5 patients, and ascending colon samples from 9 patients. After removal, tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. RNA extraction and qPCR analysis was performed as described in the supplements of this manuscript.

2.4. Statistical analyses

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc). Unless indicated otherwise, data was analyzed by One-way ANOVA with Holm-Sidak post-hoc test. Differences between groups were considered statistically significant for $P < 0.05$. Sample sizes given in the figure legends are referring to the number of biological replicates.

2.5. Other methods

Details on further methods used in this study can be found in the supplements of this manuscript.

3. Results

3.1. Intestine-specific re-expression of VDR in Vdr−/− mice modulates body weight and HFD-induced obesity

To investigate the role of global and intestinal VDR in the regulation of energy metabolism, male *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg mice received HFD-feeding for eight weeks. *Vdr*+/− mice on LFD served as lean controls. Compared to heterozygous animals and as previously shown [24,25], *Vdr*−/− mice were leaner at baseline and gained less weight during HFD feeding (Fig. 1A, B). This was associated with the absence of increased adipose tissue mass, normal fasting glucose levels and low serum leptin in *Vdr*−/− mice (Fig. 1C–E). Interestingly, these metabolic changes of *Vdr*−/− mice could be partly reversed by the intestine-specific re-expression of a human VDR in the *Vdr*−/− background: Transgenic *Vdr*−/− hTg mice showed more weight gain and more epididymal fat mass as well as increased glucose and leptin levels compared to full *Vdr* knock-out animals (Fig. 1A–E). Quantitative PCR confirmed the restricted expression of human VDR in the intestine and the absence in liver and adipose tissue (see methods). These data

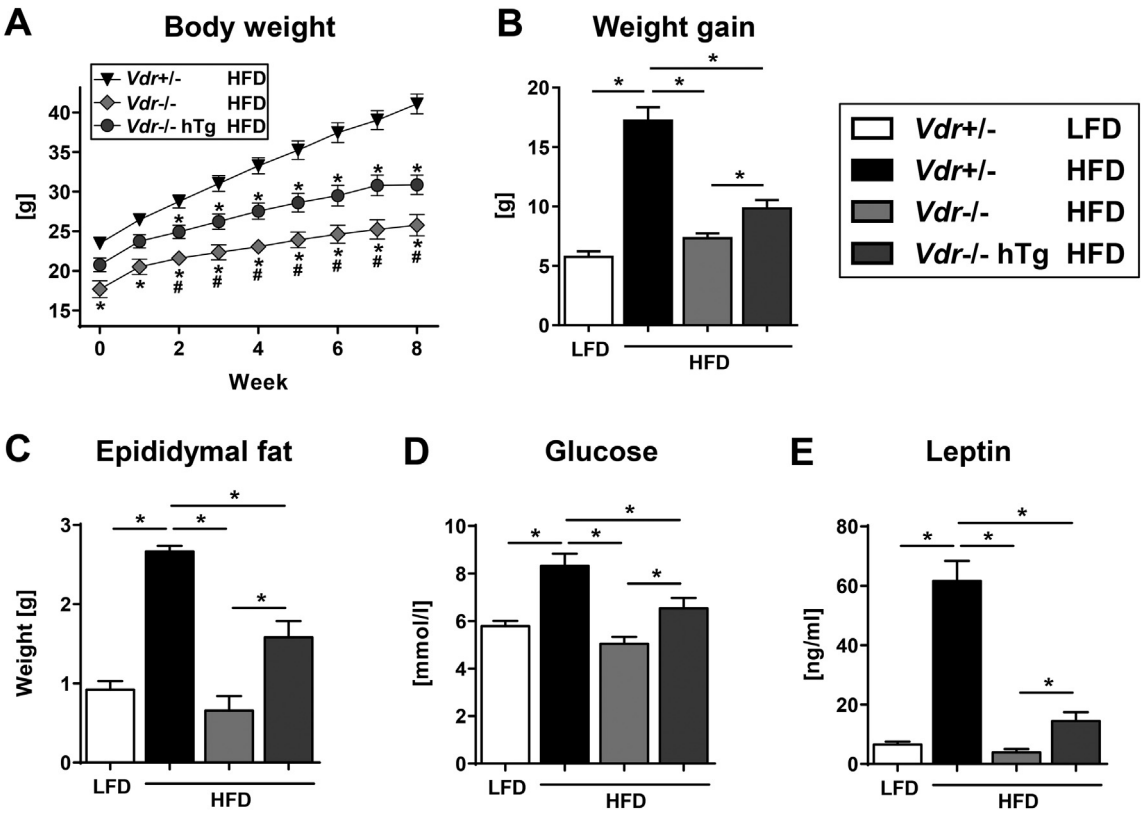

Fig. 1. Intestine-specific expression of VDR in *Vdr−/−* mice modulates body weight and HFD-induced obesity. (A) Body weight, (B) weight gain, (C) epididymal fat mass, (D) fasting serum glucose and (E) serum leptin levels after 8 weeks of HFD feeding. Data represent mean +/− SEM derived from n = 8 for *Vdr+/−* LFD (n = 3 for weight gain), n = 6 for *Vdr+/−* HFD, n = 7 for *Vdr−/−* HFD and n = 9 for *Vdr−/−* hTg HFD. (*) in (A) indicate significant differences from *Vdr+/−* HFD, while (#) indicate significant differences between *Vdr−/−* HFD and *Vdr−/−* hTg HFD ($P < 0.05$, Two-way ANOVA with Holm-Sidak post-hoc test). In panels (B-E), asterisks mark significant differences between the indicated groups ($P < 0.05$, One-way ANOVA with Holm-Sidak post-hoc test).

suggest that intestinal VDR contributes to energy and lipid metabolism and affect metabolic homeostasis in extra-intestinal tissues.

3.2. Intestinal VDR expression promotes adipose tissue expansion and increases HFD-induced adipose tissue inflammation

Further metabolic alterations associated with intestinal VDR status were analyzed in adipose tissue. In line with the reduced weight gain, adipocytes of HFD-fed *Vdr−/−* mice were smaller than those of HFD-fed heterozygous controls. Interestingly, this effect was partly reversed in the *Vdr−/−* hTg group as cells from these animals were significantly larger than *Vdr−/−* adipocytes (Fig. 2A, B). Consistent with these changes on the histological level, the expression of selected metabolic target genes was distinct between the HFD groups. Most importantly, the expression of the triglyceride synthesizing enzyme diglyceride O-acyltransferase 2 (Dgat2) as well as the levels of hypoxia-inducible factor 1-alpha (Hif1a) were inversely regulated in *Vdr−/−* and *Vdr−/−* hTg mice (Fig. 2C). As up-regulation of Hif1a has been found to define hypertrophic adipocytes and expanding adipose tissue in obesity [33,34], these observations are consistent with a significant contribution of intestinal VDR to HFD-induced adipose tissue accumulation.

A further hallmark of obesity is a chronic, low-grade inflammatory state which is characterized by an increased infiltration of macrophages into adipose tissue [35–37]. This is detected histologically as so-called crown-like structures (CLS) surrounding individual adipocytes (Fig. 2A). In line with the general reduction of obesity in *Vdr−/−* mice, significantly less CLS were detected compared to obese heterozygous controls (Fig. 2D). This reduction of adipose tissue inflammation was also reflected by markedly decreased expression of CC-chemokine ligand 2 (Ccl2) and the murine macrophage marker F4/80 in *Vdr−/−* mice. In transgenic *Vdr−/−* hTg mice, however, a clear re-expression of these inflammatory markers became obvious (Fig. 2E). Gene expression analysis of the macrophage antigens Cd11c (associated with a pro-inflammatory M1 phenotype) and Cd206 (associated with an anti-inflammatory M2 phenotype) demonstrated that re-expression of VDR in the intestine results in a strong increase of Cd11c and decrease of Cd206 mRNA in adipose tissue similar to HFD-diet fed *Vdr+/−* mice (Fig. 2F). Therefore, a molecular shift towards a pro-inflammatory state appears to be present in *Vdr−/−* hTg animals despite the lack of a clear-cut histological manifestation after eight weeks of HFD. Together, these data suggest that intestine-specific VDR has a considerable impact on HFD-induced adipose tissue expansion and inflammation.

3.3. Intestinal VDR expression promotes HFD-induced fat accumulation in the liver

Phenotypic changes associated with global and intestinal VDR function were further analyzed in the liver. As expected, HFD-feeding induced a strong hepatic lipid accumulation in heterozygous controls on the histological level (Fig. 3A, B). Accordingly, HFD-fed *Vdr+/−* mice showed a significantly increased liver weight compared to lean heterozygous controls (Fig. 3C) and a marked increase of steatosis area (Fig. 3D). In contrast to this, *Vdr−/−* mice did not show hepatic fat accumulation upon HFD feeding (Fig. 3A–D). This was also reflected by a differential gene expression since *Vdr−/−* animals had significantly decreased mRNA levels of monoacylglycerol O-acyltransferase 1 (Mgat1) compared to obese *Vdr+/−* controls (Fig. 3E). Concomitantly, HFD-fed *Vdr−/−* mice showed a decreased hepatic expression of Ccl2 and tumor necrosis factor-alpha (Tnfa) indicating that global loss of VDR also reduced liver inflammation (Fig. 3F). In line with the partial

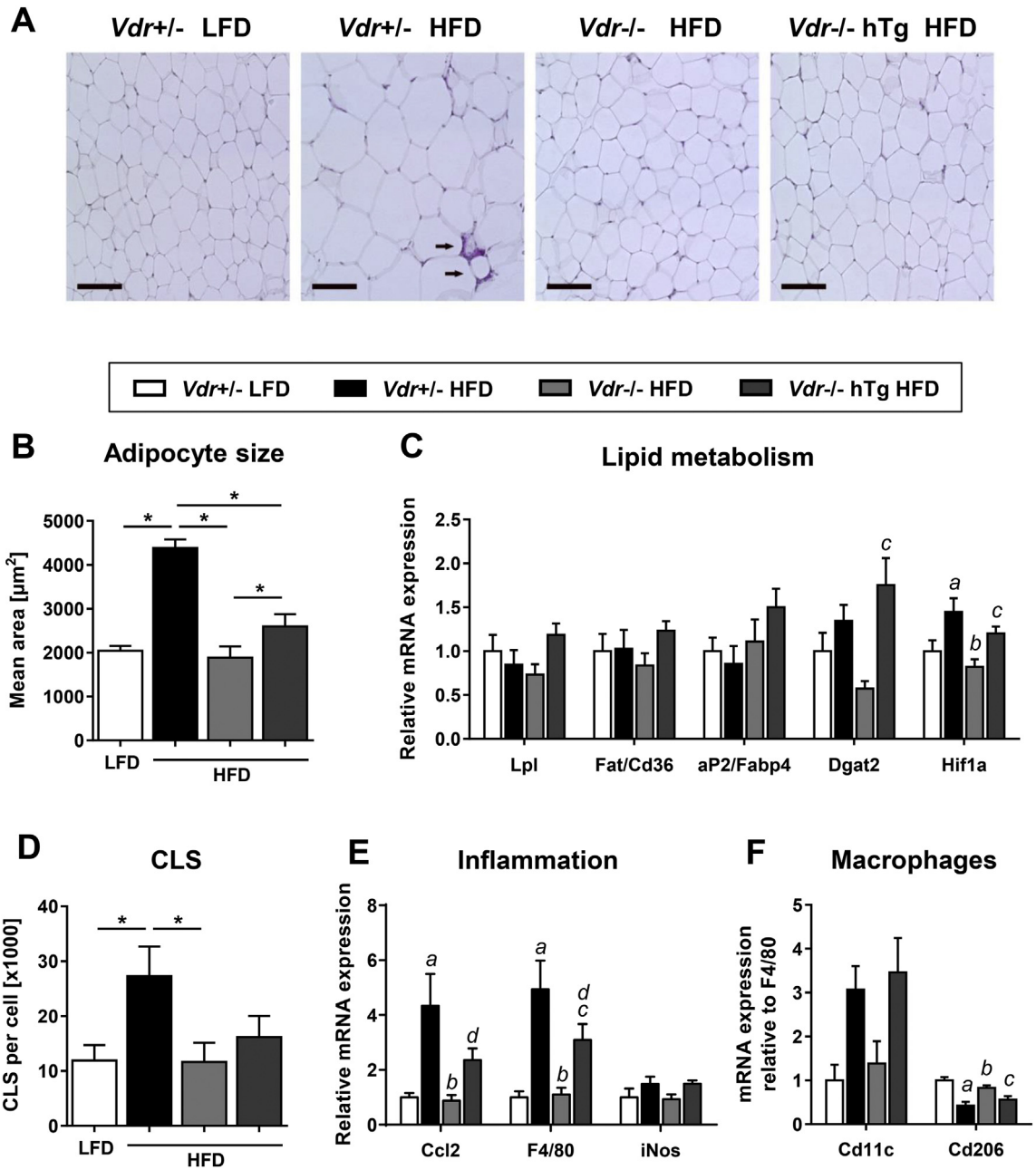

Fig. 2. Intestine-specific expression of human VDR promotes adipose tissue expansion and alters HFD-induced adipose tissue inflammation. (A) Representative sections of H&E-stained adipose tissue. Arrows indicate crown-like structures (CLS). Bar, 100 µm. (B, D) Morphometric analyses of adipocyte size and CLS frequency. (C, E–F) mRNA expression in adipose tissue. Characters indicate significant differences ($P < 0.05$) between: a, *Vdr*+/− LFD vs *Vdr*+/− HFD; b, *Vdr*+/− HFD vs *Vdr*−/− HFD; c, *Vdr*−/− HFD vs *Vdr*−/− hTg HFD; d, *Vdr*+/− HFD vs *Vdr*−/− hTg HFD. Data represent mean +/− SEM derived from n = 8 for *Vdr*+/− LFD, n = 6 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD and n = 9 for *Vdr*−/− hTg HFD.

reversal of the obesity phenotype in *Vdr*−/− hTg, these animals were more susceptible to the development of HFD-induced fatty liver than *Vdr*−/− mice. This was reflected by increased hepatic steatosis (Fig. 3D) and differential expression of metabolic and inflammatory target genes (Fig. 3E, F) in comparison to global *Vdr* knock-outs.

3.4. VDR status impacts the expression of metabolic target genes in the small intestine

The data described above demonstrate that the re-expression of VDR in the intestine of *Vdr*−/− mice modulates body weight and obesity-associated changes in adipose tissue and liver. This finding suggested an intestine-based mechanism by which VDR could regulate lipid metabolism in distal organs. To detect potential changes in lipid metabolism associated with intestinal VDR status, mRNA expression of various factors involved in lipid absorption and processing were determined in jejunal samples (Fig. 4A). In line with a putative impact of VDR on intestinal lipid metabolism, several of the analyzed genes with a known role in fatty acid transport (Fig. 4B), cholesterol transport (Fig. 4C) or chylomicron assembly (Fig. 4D) were differentially expressed. One candidate gene with a markedly distinct regulation between *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg animals was the lipase regulator angiopoietin-like 4 (Angptl4). Angptl4 is a secreted protein and an important metabolic regulator produced in the gut and various other tissues in response to changes of nutritional status [38]. Interestingly, jejunal expression of Angptl4 was increased by > 3-fold in

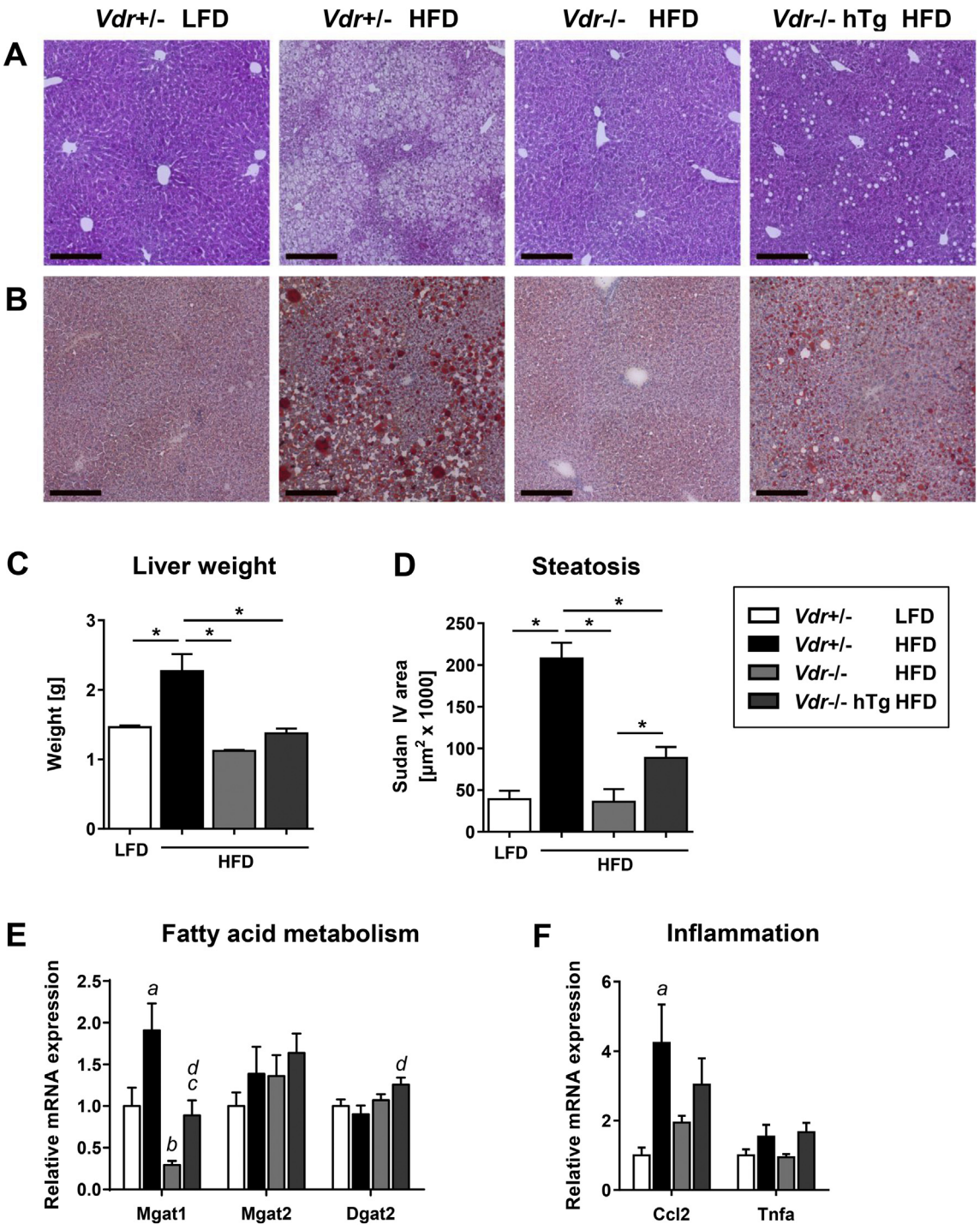

Fig. 3. Intestinal VDR promotes HFD-induced fatty liver. (A, B) Representative liver sections stained with H&E (A) or Sudan IV (B). Bars, 200 µm. (C) Liver weight and (D) morphometric quantification of steatosis area in Sudan IV-stained liver sections. (E, F) Liver mRNA expression. Characters indicate significant differences ($P < 0.05$) between: a, *Vdr*+/− LFD vs *Vdr*+/− HFD; b, *Vdr*+/− HFD vs *Vdr*−/− HFD; c, *Vdr*−/− HFD vs *Vdr*−/− hTg HFD; d, *Vdr*+/− HFD vs *Vdr*−/− hTg HFD. Data represent mean +/− SEM derived from n = 8 for *Vdr*+/− LFD, n = 6 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD (n = 5 for data in D) and n = 9 for *Vdr*−/− hTg HFD (n = 8 for data in D).

HFD-fed *Vdr*−/− animals compared to obese, heterozygous controls and this effect was partially reversed in *Vdr*−/− hTg. Angptl4 levels showed similar changes in the ileum (Fig. 4E).

3.5. Analysis of fecal lipid content in *Vdr*+/−, *Vdr*−/− and *Vdr*−/− *hTg mice*

Endogenous Angptl4 can act as a negative regulator of intestinal lipid digestion by inhibiting luminal lipase activity [39]. Based on this, we analyzed whether increased intestinal Angptl4 and concomitant changes in fecal lipid excretion may contribute to the lean phenotype of *Vdr*−/− mice. To test this, total lipid weight was determined from crude fecal extracts of LFD-fed *Vdr*−/− mice and heterozygous controls. Interestingly, fecal lipid content was in fact increased in *Vdr*−/− mice (Fig. 5A). In contrast, fecal output (Fig. 5B) and food intake (Fig. 5C) were not significantly different, indicating that the lean

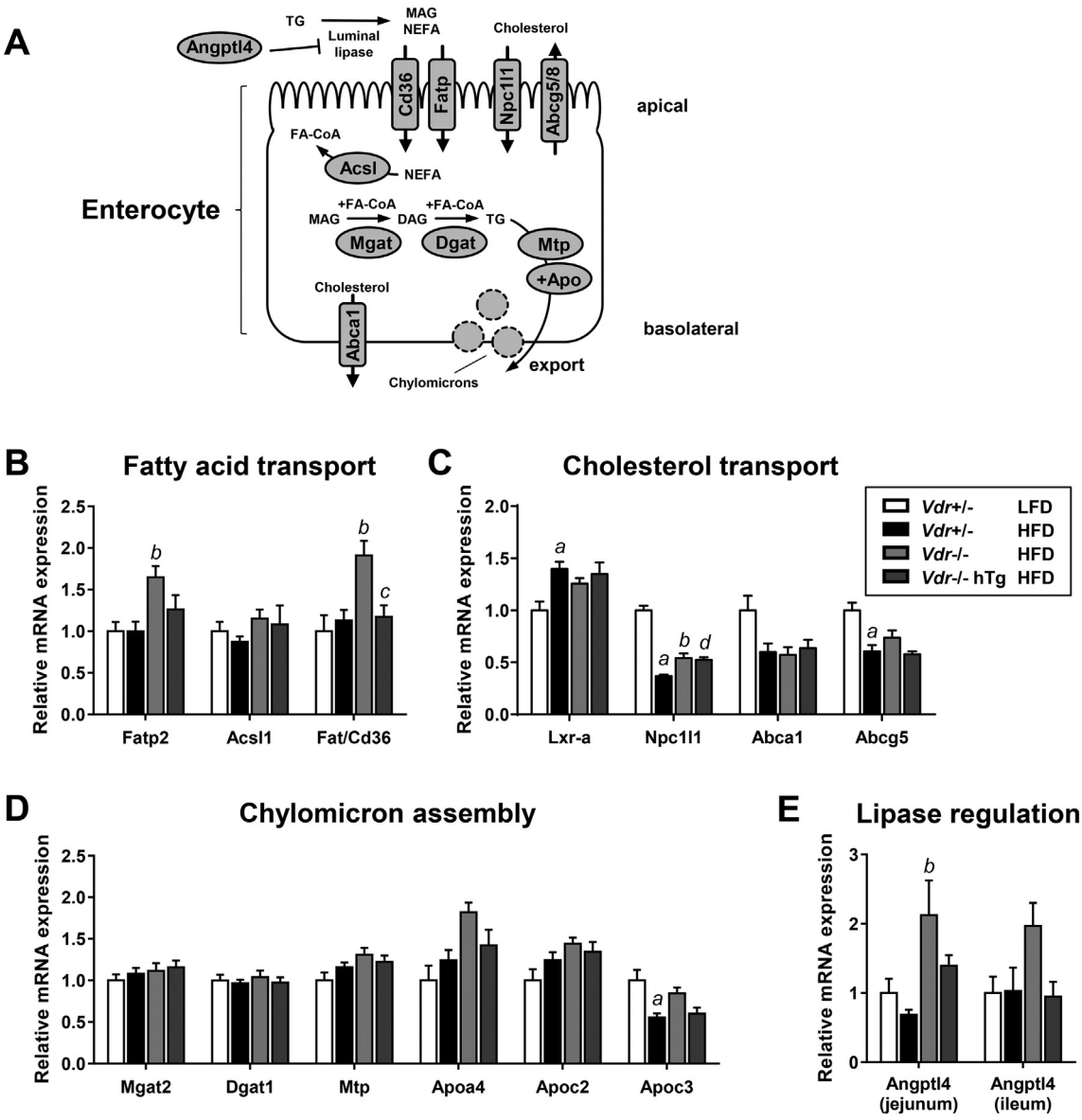

Fig. 4. VDR status impacts on the expression of metabolic target genes in the small intestine. (A) Overview of key factors implicated in intestinal lipid metabolism. (B-E) Jejunal mRNA expression was analyzed by qPCR. Expression of *Angptl4* was also measured in the ileum. Characters indicate $P < 0.05$ between: a, *Vdr*+/− LFD vs *Vdr*+/− HFD; b, *Vdr*+/− HFD vs *Vdr*−/− HFD; c, *Vdr*−/− HFD vs *Vdr*−/− hTg HFD; d, *Vdr*+/− HFD vs *Vdr*−/− hTg HFD. Data on jejunal gene expression represent mean +/− SEM derived from n = 8 for *Vdr*+/− LFD, n = 6 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD and n = 9 for *Vdr*−/− hTg HFD. Data on ileal *Angptl4* are derived from n = 4 for *Vdr*+/− LFD, n = 4 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD and n = 5 for *Vdr*−/− hTg HFD.

phenotype of *Vdr*−/− animals is not due to diarrhea-like symptoms or inadequate caloric intake.

To address the relevance of these findings for the HFD condition and define the individual lipid species present in feces, the amounts of non-esterified fatty acids (NEFA), triglycerides (TG) and total cholesterol were measured in colonic contents of HFD-fed *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg animals. In line with previous observations [40], NEFA were the most abundant lipids while only minor amounts of TG and cholesterol were detectable (Fig. 5D). Consistent with our observations in LFD-fed animals, HFD-fed *Vdr*−/− mice excreted slightly more NEFA than heterozygous controls on the same diet although this effect was no longer significant. Importantly, fecal NEFA excretion was, however, not normalized in *Vdr*−/− hTg animals. With regard to fecal TG and cholesterol levels, no considerable increase could be detected in HFD-fed *Vdr*−/− mice compared to HFD-fed heterozygous controls. Overall, these findings suggest that although a slight increase in fecal fat loss (primarily NEFA) may occur in *Vdr*−/− mice, this phenotype is probably not mediated by a direct effect of VDR in enterocytes as it is not reversed in *Vdr*−/− hTg animals. Moreover, the observed changes may be too small to fully explain the marked phenotypic differences in body weight and epididymal fat mass observed between the different genotypes. In line with this notion, a previous study did not find significant differences between wild-type and *Vdr*−/− animals with regard to intestinal lipid absorption (i.e. measured as the increase of blood triglycerides after an oral lipid gavage in the presence of a lipoprotein lipase inhibitor) [24]. Together with our data, this indicates that global loss of VDR is not associated with a general defect of intestinal lipid absorption.

3.6. Adipose tissue lipoprotein lipase activity and serum triglycerides are modulated by global and intestinal VDR status

Besides its local effect on luminal lipase activity in the gut, Angptl4 also inhibits lipoprotein lipase (LPL) activity in peripheral tissues

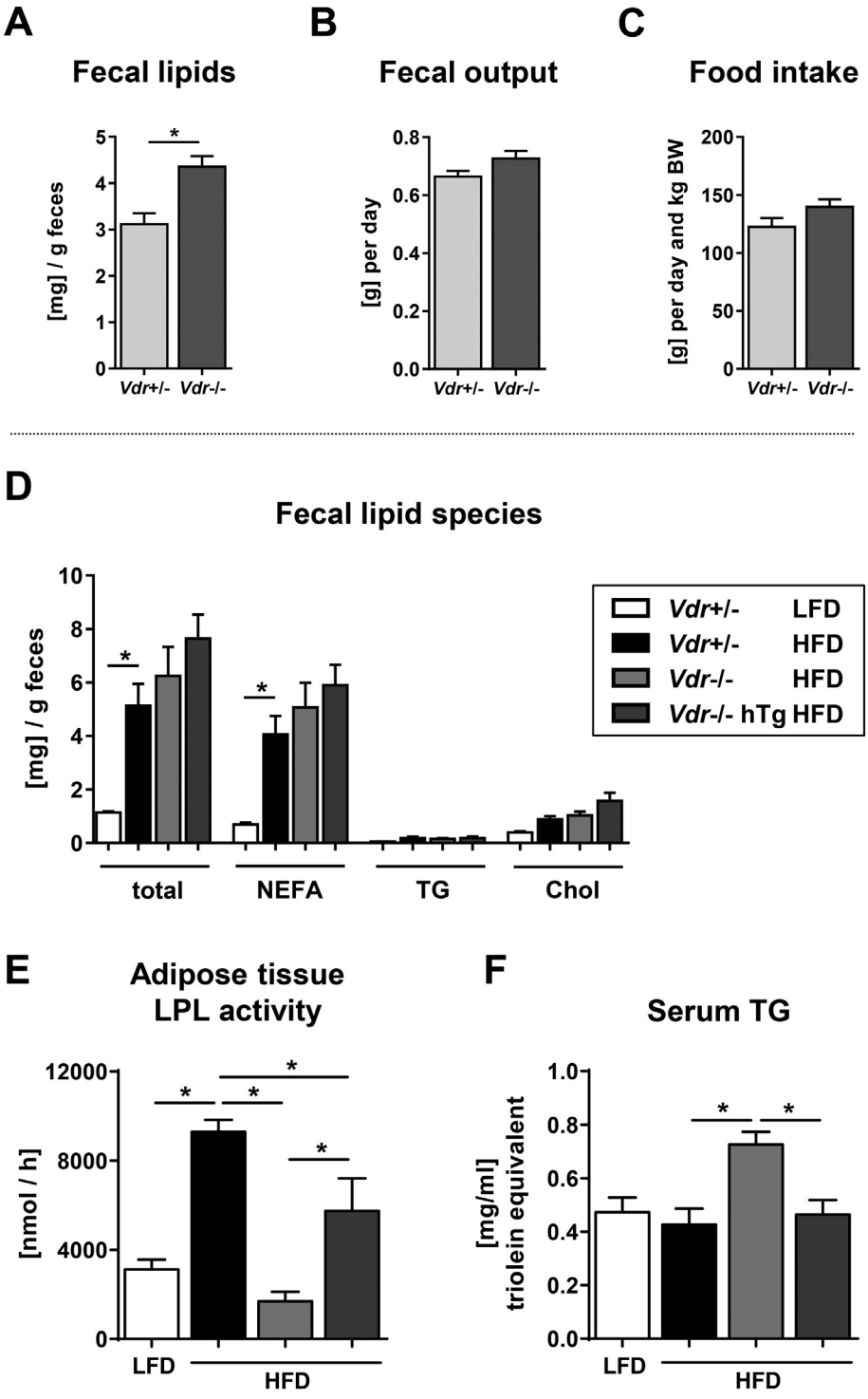

Fig. 5. Effect of intestinal VDR on fecal lipid excretion, adipose tissue LPL activity and serum TG levels. (A–C) The total amount of fecal lipids (A), fecal output per day (B) and food intake (C) were determined in *Vdr*+/− and *Vdr*−/− mice on LFD. Data in (A, B) represent the mean +/− SEM of n = 5 (*Vdr*+/−) and n = 6 (*Vdr*−/−). Data in (C) represent the mean +/− SEM of n = 4 (*Vdr*+/−) and n = 6 (*Vdr*−/−). Statistical significance in (A-C) was tested by Mann-Whitney test. (D) Amounts of non-esterified fatty acids (NEFA), triglycerides (TG) and cholesterol (Chol) in feces of LFD- and HFD-fed mice were measured individually by specific colorimetric assays. "Total" represents the sum of NEFA, TG and cholesterol values. Data in (D) represent mean +/− SEM derived from n = 4 for *Vdr*+/− LFD, n = 6 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD and n = 5 for *Vdr*−/− hTg HFD. (E) Lipoprotein lipase (LPL) activity of epididymal fat pads. (F) Fasting serum triglyceride (TG) levels. Data in (E) and (F) represent mean +/− SEM of n = 8 for *Vdr*+/− LFD, n = 6 for *Vdr*+/− HFD, n = 7 for *Vdr*−/− HFD and n = 9 for *Vdr*−/− hTg HFD.

thereby contributing to the regulation of post-prandial plasma TG clearance [38]. To test whether a related mechanism may contribute to the metabolic changes associated with the loss of VDR, LPL enzyme activity was measured in extracts of epididymal white adipose tissue (eWAT) samples. In heterozygous controls, HFD-feeding increased the activity of LPL compared to LFD-fed animals (Fig. 5E) which is likely reflecting a physiological adaption to the elevated lipid intake and the concomitant ability to extract more lipids from the circulation [41]. In line with this, serum TG levels remained unaltered in *Vdr*+/− animals irrespective of the consumed diet (Fig. 5F). Interestingly, global absence of VDR resulted in a markedly decreased LPL activity in *Vdr*−/− mice (Fig. 5E). As a consequence, serum TG levels were significantly increased despite the absence of obesity in these animals (Fig. 5F). In line with a potential role of intestinal VDR in the control of systemic lipid homeostasis, adipose tissue LPL activity was significantly increased in *Vdr*−/− hTg animals compared to the full knock-outs and, consistently, serum TG levels were normalized (Fig. 5E, F).

3.7. Angiopoietin-like 4 is transcriptionally regulated by vitamin D/VDR

The data described above provide a potential mechanistic explanation for the differences in body weight between HFD-fed *Vdr*−/−

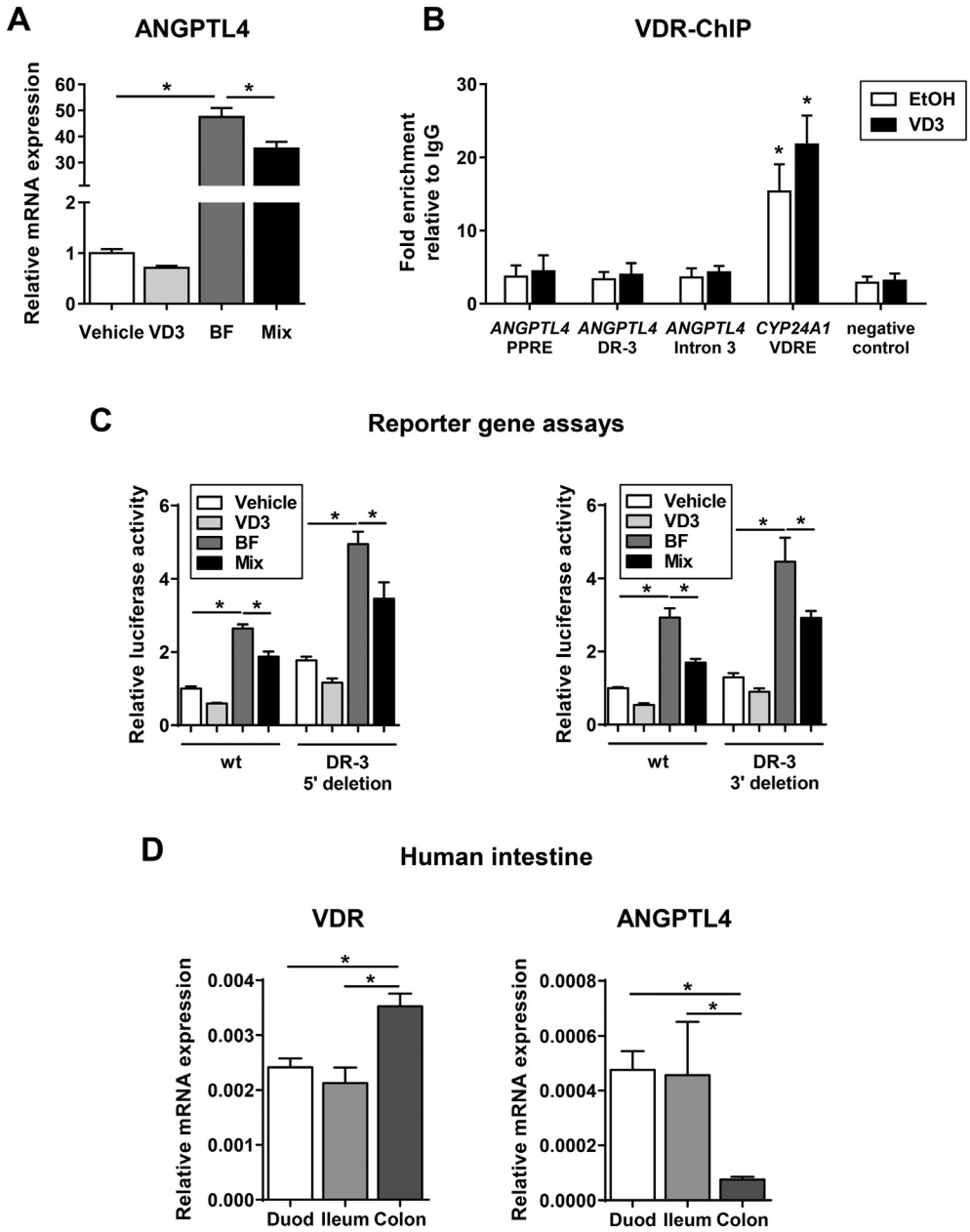

Fig. 6. Vitamin D/VDR-dependent regulation of Angptl4 expression. (A) mRNA expression of *ANGPTL4* in human HT-29 cells after 24 h treatment with 100 nM 1α,25-dihydroxyvitamin D3 (VD3) and/or 60 μM bezafibrate (BF) (mean +/− SEM, n = 6 per condition). (B) Chromatin immunoprecipitation (ChIP) using anti-VDR antibodies. HT-29 cells were pre-treated with either 100 nM 1α,25-dihydroxyvitamin D3 (VD3) or vehicle (EtOH). Binding of VDR to *ANGPTL4* was quantified by qPCR and expressed as fold enrichment over normal rabbit IgG. *CYP24A1* and a gene desert on chromosome 12 served as a positive control and negative control, respectively. Asterisk indicate significant difference ($P < 0.05$) from negative control (n = 3). (C) Reporter gene assay in HT-29 cells transfected with luciferase-based constructs containing intron 3 of *Angptl4* and mutant versions harboring deletions of either half-site (5′ or 3′) of the putative DR-3 response element. Cells were treated with 1α,25-dihydroxyvitamin D3 (VD3, 100 nM) and/or bezafibrate (BF, 60 μM) (mean +/− SEM, n = 9 per condition). (D) Expression of *VDR* and *ANGPTL4* along the longitudinal axis of the human intestine (n = 13 for duodenum, n = 5 for ileum, n = 9 for colon).

and *Vdr*−/− hTg mice. In this scenario, signaling via vitamin D/VDR modulates the expression of the LPL regulator Angptl4 and can thus affect lipid homeostasis in extra-intestinal tissues. Since VDR is a ligand-activated transcription factor, we speculated that the alterations of Angptl4 expression may be driven by a direct transcriptional impact of vitamin D/VDR on the *Angptl4* gene. To test this, cell culture experiments were performed in human intestinal HT-29 cells. In line with our hypothesis, treatment of cells with the VDR agonist 1α,25-dihydroxyvitamin D3 led to a ~30% reduction of ANGPTL4 mRNA expression. Furthermore, *ANGPTL4* has been previously defined as a bona fide peroxisome proliferator-activated receptor (PPAR) target gene [42] and, interestingly, co-treatment with 1α,25-dihydroxyvitamin D3 significantly reduced the induction of ANGPTL4 mediated by the pan-PPAR agonist bezafibrate (Fig. 6A).

To study this regulation of Angptl4 by vitamin D in more detail we searched for potential VDR binding sites within the mouse *Angptl4* gene (http://jaspar.binf.ku.dk/). This revealed a predicted VDR response element (5`-GGG GCA CAG AGT CAG-3`; DR-3) located in intron 3 and 428 bp upstream of a functional PPAR response element (PPRE) which was previously shown to control *Angptl4* [42]. A putative VDR response element was also detected in the human *ANGPTL4* gene at a similar position. Direct binding of VDR to the chromatin of intron 3 of *ANGPTL4* was tested by chromatin immunoprecipitation (ChIP) in HT-29 cells treated with 1α,25-dihydroxyvitamin D3. As expected, VDR was strongly enriched at a genomic region corresponding to a bona fide VDR response element (VDRE) in the *CYP24A1* gene [43,44]. However, VDR was not significantly enriched in any of the tested regions of the *ANGPTL4* gene (Fig. 6B).

The responsiveness of intron 3 of *Angptl4* to vitamin D and the requirement of the predicted VDRE was further tested by reporter gene assay. As a positive control, cells transfected with a respective luciferase-based reporter construct were treated with the pan-PPAR agonist bezafibrate. This led to a ~3-fold increase in luciferase activity indicating increased PPAR-mediated transcription. In contrast, treatment with 1α,25-dihydroxyvitamin D3 had the opposite effect and resulted in a reduction of the luciferase activity. Moreover, 1α,25-dihydroxyvitamin D3 co-treatment significantly blunted the bezafibrate/PPAR-mediated induction of the reporter construct. However, deletion of

either of the two half-sites of the putative VDRE did not abrogate these inhibitory effects of vitamin D (Fig. 6C). Together with the ChIP data, this indicates that direct DNA binding of VDR is not required for its transcriptional regulation of the angiopoietin-like 4 gene. This notion is in line with an earlier mechanistic report describing a VDR-mediated repression of PPAR activity that was independent of VDR binding to DNA and also independent of direct protein-protein interactions between both transcription factors [45]. Irrespective of this, we analyzed expression of VDR and ANGPTL4 in intestinal samples of human patients (Fig. 6D). Intriguingly, this revealed that VDR and ANGPTL4 are inversely expressed along the longitudinal axis of the human intestine (duodenum, ileum and colon). Together with our in vitro analyses, this corroborates that angiopoietin-like 4 is transcriptionally regulated by VDR and indicates that this mechanism is also physiologically relevant in the human gut.

3.8. Vitamin D treatment modulates HFD-induced obesity in wild-type mice

The data reported above provide comprehensive evidence that intestinal re-expression of VDR modulates the metabolic effects of HFD feeding in *Vdr*−/− mice and suggest that VDR-mediated transcriptional regulation of *Angptl4* contributes to this phenomenon. To address the relevance of these findings in a clinically more relevant experimental set-up, we analyzed the pharmacological effects of oral high-dose vitamin D treatment on HFD-induced obesity in genetically unaltered animals. To this end, C57BL/6J wild-type mice were fed a HFD containing relatively low levels of vitamin D3 (500IU/kg diet) for six weeks (500 IU VD3-HFD). Then, one group of mice was switched to a HFD that contained 10,000 IU vitamin D3 per kg diet (10,000 IU VD3-HFD) but had an otherwise identical nutrient composition. The control group continued to receive 500 IU VD3-HFD. The experiment was terminated after eight weeks. Interestingly, HFD-induced weight gain was promoted by high vitamin D intake in this set-up (Fig. 7A). This was associated with a significant increase in epididymal fat mass, increased serum 25-OH vitamin D levels and a clear trend towards lowered *Angptl4* mRNA levels in the small intestine (ileum) of high vitamin D-fed animals (Fig. 7B–D). In addition to this, short-term treatment with the physiologically active VDR agonist 1,25-dihydroxyvitamin D for 16 h resulted in a (statistically non-significant) ~70% reduction of duodenal *Angptl4* mRNA expression (Fig. 7E). These data confirm the findings made in the transgenic animals and strongly argue for a relevant physiological role of the intestinal VDR to control *Angptl4* expression and systemic lipid homeostasis.

4. Discussion

This study addresses the importance of the vitamin D-VDR axis as metabolic regulator in obesity. Our data demonstrate that complete loss of VDR expression in *Vdr*−/− mice is associated with a lean phenotype which is in line with similar observations that have been reported previously [24,25]. Extending these findings, we observed that this is accompanied by reduced hepatic fat accumulation and reduced inflammation in adipose tissue and liver of *Vdr*−/− mice. Whether the reduced inflammation in adipose tissue and liver is mainly a result of reduced adiposity of *Vdr*−/− mice or is based on specific functions of VDR in these tissues will require further analysis in future studies. As a further major finding of the current study, the intestine-specific re-expression of VDR could partly reverse the lean phenotype of *Vdr*−/− mice. This effect occurred in conjunction with increased expression of pro-inflammatory genes in adipose tissue and with increased hepatic steatosis. These findings therefore strongly argue for an important and so far unexpected role of intestinal VDR signaling in the control of lipid metabolism in extra-intestinal tissues (Fig. 7F). The physiological relevance of this signaling axis is further supported by the finding that high-dose vitamin D treatment in C57BL/6J wild-type mice reproduced the general metabolic effects of intestinal VDR over-expression.

With regard to the underlying mechanism, our data indicate that VDR-dependent modulation of Angptl4 expression is an important contributing factor. The ability of Angptl4 to control lipid metabolism by inhibiting lipase activities in multiple organs is well documented [38]. In line with this model, up-regulation of Angptl4 in the intestines of *Vdr*−/− mice was associated with a decrease of adipose tissue LPL activity resulting in a reduced ability to extract lipids from the blood and, concomitantly, less adipose tissue mass and smaller adipocytes. There is an ongoing debate on whether gut-derived Angptl4 serves as a classical endocrine factor or rather acts in a locally restricted manner [38,39,46,47]. However, direct evidence from mice with liver-specific overexpression demonstrates that liver-derived Angptl4 can in fact act as a bona fide endocrine hormone regulating LPL activity on a systemic level [48]. Noteworthy, in our scenario increased expression of Angptl4 was also evident in the liver of *Vdr*−/− mice and may therefore additionally contribute to the inhibition of peripheral LPL in these animals (1.01 ± 0.08 in *Vdr*+/− vs. 1.55 ± 0.18 in *Vdr*−/−, $P < 0.05$), particularly if the rather short time period of HFD treatment is taken into consideration.

Our data identify angiopoietin-like 4 as a VDR regulated gene and suggest that the in vivo changes of intestinal Angptl4 expression in *Vdr*−/− mice are due to the lack of gene repression in the absence of VDR. Since this repression also occurred in cultured cells and in reporter gene assays, cell-autonomous effects on the transcriptional level are likely to operate. However, ChIP analyses of the corresponding promoter regions and the use of respective deletion mutants did not confirm the presence of a bona fide VDRE in the angiopoietin-like 4 gene. Although the exact molecular mechanism underlying the VDR-mediated repression of angiopoietin-like 4 requires further analysis, our data obtained from intestinal samples of patients suggest that a similar regulation may also occur in humans.

In addition to these cell-autonomous effects, the gut microbiota may be an important determinant controlling Angptl4 in a VDR-dependent manner. It is known that certain bacterial strains and specific metabolites such as short chain fatty acids can modulate Angptl4 expression [46,49–53]. Recent data also suggest a key role for VDR in the control of the gut microbiota in experimental inflammatory bowel disease [54,55]. Although beyond the scope of the current study, it is therefore tempting to speculate that VDR-dependent changes of the gut microbiota may also be relevant in the context of obesity and that these changes could be linked to differential regulation of Angptl4.

Beyond Angptl4, inhibition of adipose tissue LPL in *Vdr*−/− mice may also be promoted by other factors. As such, jejunal expression of apolipoprotein C3 (Apoc3) was increased in *Vdr*−/− mice compared to *Vdr*+/− animals and this effect was reversed in *Vdr*−/− hTg (Fig. 4D, $P < 0.1$ each). Like Angptl4, Apoc3 is a potent inhibitor of LPL [56]. In addition to decreased lipid uptake by inhibition of LPL, we investigated whether increased adipose tissue lipolysis may contribute to the lean phenotype of *Vdr*−/− mice (Fig. S2). In line with earlier findings [57], *Vdr*−/− mice showed increased levels of parathyroid hormone – a known inducer of adipose tissue lipolysis [58,59]. However, we did neither observe an increase in fasting serum NEFAs in *Vdr*−/− mice nor an adipose tissue-specific up-regulation of the PTH-inducible genes Ucp1 or Dio2 [60]. These findings suggest that PTH and adipose tissue lipolysis are probably not the major physiological factors driving the phenotype of *Vdr*−/− mice.

The independence of our findings from PTH (and calcium) are further supported by data on ten months-old *Vdr*+/−, *Vdr*−/− and *Vdr*−/− hTg mice fed a LFD rather than a HFD (Table S2). In this set-up, *Vdr*−/− animals received a low-fat rescue diet (containing high levels of calcium) resulting in completely normal serum calcium and PTH. Despite this normalization, *Vdr*−/− mice were much leaner and had significantly less fat mass than *Vdr*+/− mice. In line with our findings under HFD, this lean phenotype was partially reversed by intestinal re-expression of VDR in *Vdr*−/− hTg mice which had body weights that were intermediate between *Vdr*+/− and *Vdr*−/−.

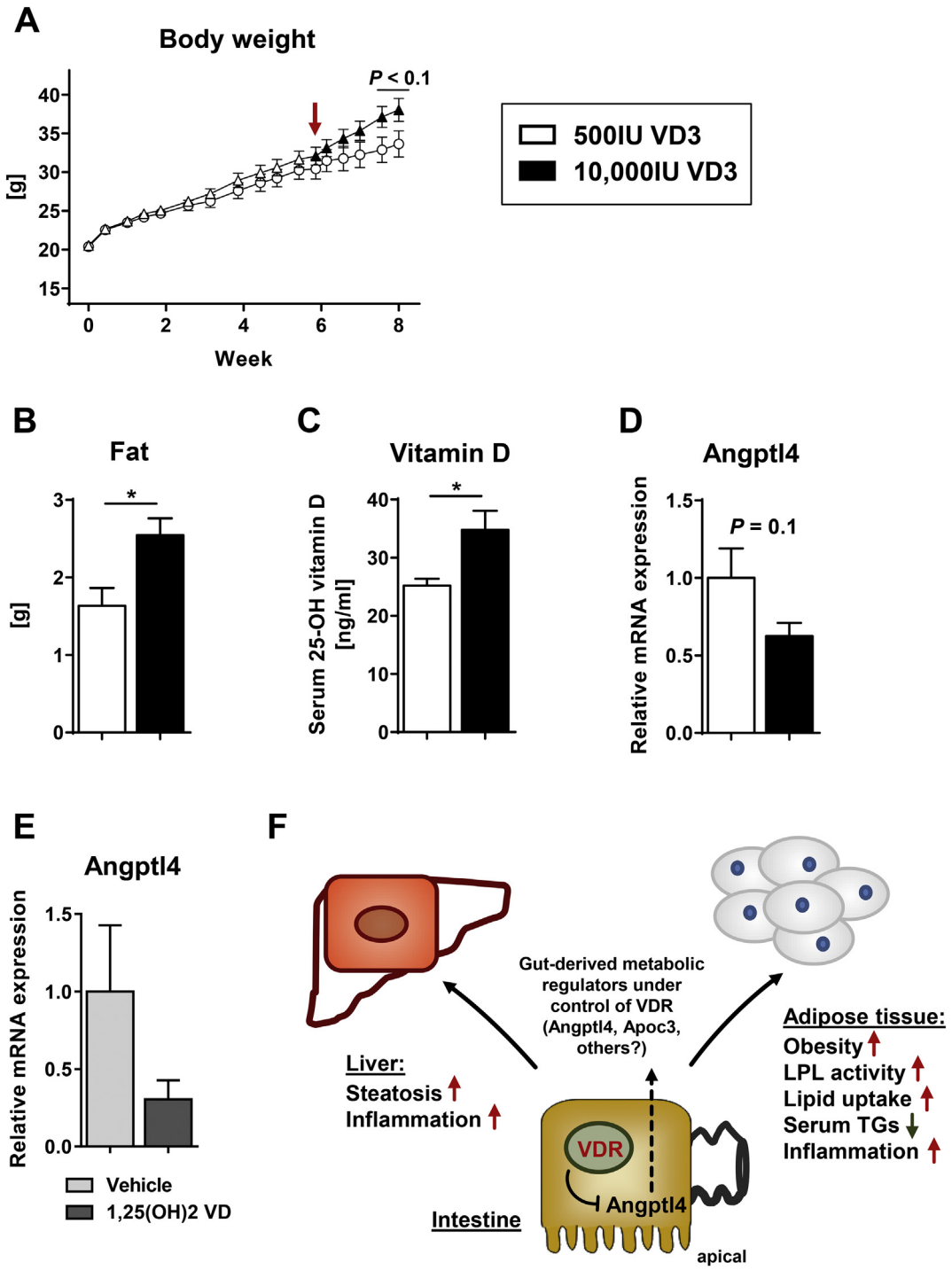

Fig. 7. High-dose vitamin D treatment promotes HFD-induced obesity in wild-type mice. Changes in body weight (A), epididymal fat mass (B), serum vitamin D levels (C) and intestinal (ileum) Angptl4 mRNA expression (D) after 8 weeks of HFD-feeding. Arrow in (A) indicates the beginning of high-dose vitamin D treatment in the 10,000 IU per kg diet group (n = 6 per group; asterisk indicate $P < 0.05$; unpaired *t*-test, two-tailed). (E) Male mice were injected with either vehicle (n = 5) or 1,25-dihydroxyvitamin D (1,25(OH)2 VD; 100 ng/100 g body weight; n = 4). Mice treated with vehicle or 1,25(OH)2 VD were sacrificed 6 h and 16 h post-injection, respectively, and duodenal *Angptl4* mRNA levels were measured by qPCR. (F) Model of the metabolic effects of intestinal VDR activation: This study suggests that intestinal VDR modulates the expression of Angptl4 (and potentially other gut-derived factors) thus contributing to the control of lipid metabolism in extra-intestinal tissues. *Angptl4* is repressed via a VDR-dependent transcriptional mechanism.

Previous mouse studies have demonstrated important functions of vitamin D and VDR in maintaining the intestinal barrier and protecting against intestinal inflammation. In the context of NAFLD, this has for example been suggested by a report from Su et al. showing that nutritional vitamin D deficiency increases gut permeability and plasma endotoxin levels in a HFD mouse model [61]. Mechanistically, this function of vitamin D could partially rely on modulation of certain tight junction proteins as suggested by another report demonstrating direct transcriptional regulation of claudin-2 by VDR [62]. In addition to this, intestinal VDR has been shown to protect against mucosal inflammation in experimental colitis [63,64] and contribute to systemic bile acid homeostasis by regulation of the intestinal hormone fibroblast growth

factor 15 (FGF15) [65]. Whether and how these intestinal functions of VDR may affect the metabolic phenotype of our mouse model beyond the regulation of Angptl4, remains to be studied in the future.

Observational clinical studies suggest an association of vitamin D deficiency with obesity and NAFLD in humans resulting in suggestions for vitamin D supplementation as a therapeutic option [1–6]. Moreover, certain (although not all) previous interventional trials testing the efficacy of vitamin D supplementation in NAFLD patients described positive effects on disease measures such as liver fat content and/or improved serum markers of liver damage [7–10]. This is in apparent contrast to the findings reported in the present study which rather suggests that a complete loss of VDR signaling protects against fat accumulation in adipose tissue and liver. In this context, it is however important to note that – besides gene regulation through VDR – vitamin D exerts non-genomic biological effects through cell surface receptors [66]. Vice versa, VDR can – in addition to vitamin D – also be activated by the secondary bile acid lithocholic acid [67] and has moreover been shown to regulate gene expression in a ligand-independent manner as well [68]. Thus, from a biochemical point of view vitamin D deficiency and inhibition of VDR activity are overlapping but partially distinct phenomena that may not necessarily result in the same physiological outputs.

With regard to the clinical implications of this aspect, an important recent study including data on NAFLD patients reported that hepatic expression of VDR was in fact up-regulated in fatty livers compared to non-steatotic controls [69]. Noteworthy, this up-regulation of VDR was more pronounced in patients with early NAFLD (i.e. steatosis) compared to patients with more progressed disease (i.e. non-alcoholic steatohepatitis, NASH), indicating a putative pro-steatogenic activity of VDR – especially in early NAFLD. The functional relevance of this finding was further supported by experiments in hepatoma cells and mice indicating a pro-steatogenic activity of VDR directly in hepatocytes [69]. These data were recently further validated in a follow-up study, in which the authors could also demonstrate that hepatic angiopoietin-like 8 (ANGPTL8) expression was elevated in NAFLD patients, and that its mRNA levels correlate with VDR mRNA as well as with the grade of liver steatosis [70]. Further mechanistic experiments from the same study suggested that ANGPTL8 is a novel target gene of VDR that promotes triglyceride accumulation in hepatocytes. On the other hand, an earlier paper by Barchetta *et al.* reported that VDR protein expression in the liver was rather decreased in a cohort of NASH patients and that VDR abundance was inversely associated with disease progression (assessed on the histopathological level as the NAFLD activity score, NAS) [71]. Together, these data indicate that the pathophysiological role of VDR in NAFLD may considerably differ between early and more progressed disease stages. Extending these findings, the results reported in our manuscript suggest that activation of VDR in the intestine can suppress Angptl4 as a further important metabolic regulator thereby exerting pro-adipogenic/steatogenic effects in distal tissues such as adipose tissue and liver. Together with the previous studies mentioned above, these findings warrant further preclinical studies testing the metabolic effects of (tissue-specific) pharmacological VDR agonist/antagonists in obesity and NAFLD to further evaluate the suitability of this signaling axis as a future therapeutic target in different disease stages.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.03.007.

Author contributions

DJ, JS, HMH and AG designed the study, planned the experiments and analyzed the data. JCF provided the transgenic mouse model, made important contributions to study design and data interpretation, provided the data shown in Fig. 7E and conducted the studies reported as Supplemental Table S2. DJ, DD, AKS, LG, JS, DK, CM and RNV performed experiments and analyzed the obtained data. DJ, HMH and AG drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

Disclosures

DJ received travel grants from Alexion and Falk. HMH consulted for GlaxoSmithKline and received travel grants from Falk. AG advises for AbbVie, Alexion, BMS, Gilead, Intercept, Novartis, and Sequana, is on the speakers' bureau for AbbVie, Alexion, BMS, Falk, Gilead, Intercept, Novartis and Sequana, received research grants from Intercept and Novartis, and received material resources from Burgerstein Vitamine (study medication for SASL34). The other authors declare no conflict of interests.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

The authors thank Dr. Rinke Stienstra, Dr. Sander Kersten and Dr. Rudolf Zechner for stimulating discussion. This work was supported by grants of the Schweizerischer Nationalfonds SNF (310030_135548) and the Else Kröner-Fresenius-Stiftung EKFS (2014_A67) to AG. DJ was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg. JCF was supported by a grant of the National Institute of Diabetes and Digestive and Kidney Diseases (award #DK54111). A part of this study has been published as conference abstracts at the International Liver Congress (EASL) and the Annual Meeting of the German Association for the Study of the Liver (GASL).

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