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Angiopoietin-like 8 (Angptl8) controls adipocyte lipolysis and phospholipid composition

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ABSTRACT

Angiopoietin-like 8 (Angptl8) inhibits lipolysis in the circulation together with Angplt3 and controls post-prandial fat storage in white adipose tissue (WAT). It is strongly induced by insulin *in vivo* in WAT and *in vitro* in adipocytes.

In this study we addressed the function of Angptl8 in adipocytes by its stable lentivirus-mediated knock-down in 3T3-L1 cells, followed by analyses of triglyceride (TG) storage, lipid droplet (LD) morphology, the cellular lipidome, lipolysis, and gene expression.

Depletion of Angptl8 did not drastically affect the adipocyte differentiation of 3T3-L1 cells but resulted in a moderate (18–19%) reduction of stored TGs. The lipidome analysis revealed a reduction of alkyl-phosphatidylcholines (PCs) and phosphatidylethanolamine (PE) plasmalogens, as well as saturated PCs and PEs. Importantly, the Angptl8 depleted cells displayed enhanced lipolysis as measured by release of non-esterified fatty acids (NEFAs). Consistently, mRNAs encoding Angptl4 and Leptin, which facilitate lipolysis, as well as Cpt1a, Cpt1b, and Pgc-1α involved in FA oxidation, were elevated. The Angptl8 mRNA itself was suppressed by pharmacologic treatments inducing lipolysis: stimulation with the β-adrenergic agonist isoproterenol or with the adenylate cyclase activator forskolin.

To conclude, knock-down of Angptl8 in adipocytes suggests that the protein acts to inhibit intracellular lipolysis, analogous to its activity in the circulation. Depletion of Angptl8 results in an altered cellular phospholipid composition. The findings identify Angptl8 as a central insulin-regulated controller of adipocyte lipid metabolism.

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1. Introduction

Angiopoietin-like 8 (Angptl8) is a protein predominantly expressed in the liver and adipocytes (Quagliarini et al., 2012). It inhibits lipoprotein lipase (LPL) activity in plasma similar to Angptls 3 and 4 (Dijk and Kersten, 2016; Quagliarini et al., 2012; Santulli, 2014; Wang et al., 2013) and is suggested to act in concert with Angptl3 in the circulation (Quagliarini et al., 2012). Consistently, hepatic overexpression of Angptl8 resulted in elevated plasma triglycerides (TGs) in mice (Dang et al., 2016; Quagliarini et al., 2012; Wang et al., 2013). Angptl8 is markedly induced upon insulin stimulation in both hepatocytes and adipocytes (Nidhina Haridas et al., 2015; Ren et al., 2012). Overexpression of Angptl8 in hepatocytes was reported to enhance insulin signaling *via* the Akt-GSK3β and Akt-FoxO1 pathways (Rong Guo et al., 2016). Characterization of Angptl8 knock-out mice provided evidence for an important role of the protein in the post-prandial distribution of FAs between storage in WAT and tissues which oxidize them (Wang et al., 2013).

Most of the recent studies suggest that plasma ANGPTL8 levels are elevated in type 2 diabetes (T2D), insulin resistance or obesity (Abu-Farha et al., 2016; Chen et al., 2015; Fu et al., 2014). However, ANGPTL8 was found to be the most strongly insulin-induced individual gene in human WAT *in vivo* (Nidhina Haridas et al., 2015; Soronen et al., 2012), suggesting that, apart from its function in the

Abbreviation: Angptl8, angiopoietin-like 8; FA, fatty acid; LD, lipid droplet; LPL, lipoprotein lipase; NEFA, non-esterified FA; PC, phosphatidylcholine; PE, phosphatidylethanolamine; qPCR, quantitative reverse transcriptase PCR; shRNA, short hairpin RNA; TG, triglyceride; WAT, white adipose tissue.

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circulation, it may also execute an unknown intracellular function in adipocytes. Since adipocytes do not express Angptl3, a functional partner of Angptl8 (Conklin et al., 1999; Quagliarini et al., 2012), it is likely that the latter has an independent role in this cell type. Indeed, an RNA interference study in cultured adipocytes suggested that the protein promotes adipocyte differentiation and lipid storage (Ren et al., 2012). Interestingly, exogenous recombinant Angptl8 was shown to downregulate the expression of adipocyte triglyceride lipase (ATGL) in both hepatocytes and adipocytes (Zhang et al., 2016). In contrast, circulating Angptl8 was reported to enhance ATGL expression in the WAT of mice (Dang et al., 2016). To conclude, the function of Angptl8 in adipocyte lipid metabolism is poorly understood and thus warrants detailed study.

In this study, the role of Angptl8 in controlling adipocyte TG content and lipolysis was analyzed by means of its shRNA lentivirus-mediated knock-down in the 3T3-L1 adipocyte model. Lipid droplet morphology was monitored, the lipidome of Angptl8 knock-down and control cells was analyzed, and the expression of relevant genes quantified.

2. Materials and methods

2.1. Cell culture and RNA interference

3T3-L1 fibroblasts were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma-6429, Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS). At 70% confluency cells were infected with lentiviral particles expressing shRNA targeting Angptl8, Clone1: AAGCCCACCAAGAATTTGAGA, Clone2: TATGACAGAGCACTGGAATTC (CSTVRS-Mission™, Sigma-Aldrich) and the transduced cell pools were selected using 3 μg/ml of puromycin. The selected 3T3-L1 cells were further expanded and differentiated. Cells were grown till confluency and differentiated as follows: Cells were treated with 500 μM 3-isobutyl-1-methyl-xanthine-IBMX, 250 nM dexamethasone, 100 nM insulin and 2 μM rosiglitazone in complete medium for the first 48 h, followed by 100 nM insulin and 2 μM rosiglitazone in complete medium for the next 48 h. Fresh complete medium was then changed every 48 h till day 6–8. The experiments were carried out when the cells were completely differentiated as judged from the abundant presence of large cytoplasmic lipid droplets.

2.2. Quantitative reverse transcriptase PCR (qPCR)

Total RNA from 3T3-L1 control and Angptl8 knock-down adipocytes cells was isolated using the PureLink® RNA Mini (Ambion/Thermo Fisher, Waltham, MA) or the QIAGEN (Germantown, MD) RNeasy Mini Kit (Cat No./ID: 74104). For Lipolysis related genes both control and silenced cells were treated with isoproterenol (10 μM) for 2 h. cDNA was prepared using the VILO® reverse transcriptase kit (Invitrogen, Carlsbad, CA). Real time quantitative PCR (qPCR) was carried out in Light Cycler 480 II (Roche Applied Science, Penzberg, Germany) using Roche SYBR-Green® master mix and gene-specific primers (Table 1). The data was normalized to the expression of two housekeeping genes, Succinate dehydrogenase complex, subunit A (SDHA) and the acidic ribosomal phosphoprotein 36B4.

2.3. Triglyceride analysis

Total lipids were isolated from 3T3-L1 control and Angptl8 knock-down adipocytes using the Bligh and Dyer method and the extracts, together with TG standards, were separated by automatic thin layer chromatography (Camag, Berlin, Germany) by using hexane/diethyl ether/acetic acid (80:20:1) as the solvent system.

Table 1
The primers employed for qPCR analyses.

Genes	Primer sequence
ANGPTL8	(Forward) F = ccctcaatggcgtgtacaga
	(Reverse) R = ccacctgaatctccgacagg
ANGPTL4	F = caaaacagcaagatccagca
	R = ttggaagagttcctggcagt
ATGL	F = caacgccactcacatctacgg
	R = ggacacctcaataatgttggcac
PNPLA3	F = attcccctcttctctggccta
	R = atgtcatgctcaccgtagaaa
HSL	F = caacggataccgtagtttggtg
	R = cgggatttgtgtagtaggtgtg
PGC1-α	F = tgctagcggttctcacagag
	R = agtgctaagaccgctgcatt
CPT1a	F = tggcatcatcactggtgtgtt
	R = gtctagggtccgattgatctttg
CPT1b	F = tacaacaggtggtttgaca
	R = gtctagggtccgattgatctttg
UCP1	F = aggcttccagtaccattaggt
	R = ctgagtgaggcaaagctgattt
LEPTIN	F = tgctccagcagctgcaaggtgcaag
	R = tcagcattcagggctaacatccaactgtt
36B4	F = catgctcaacatctccccttctcc
	R = gggaaggtgtaatccgtctccacag
PBGD	F = gtgagtgtgttgcacgatcc
	R = tgggtcatcttctggaccat
MOGAT1	F = ctcgtgcaggtgtgcattg
	R = gcgttttgacaagacagattgg
GPAT1	F = acagttggcacaatagacgttt
	R = ccttccatttcagtgttgcaga
DGAT1	F = tccgtccagggtggtagtg
	R = tgaacaaagaatcttgcagacga

The plates were stained with 8% H_3PO_4 (aq) and 3% cupric sulfate (aq) and heated at 180 °C. The TG standards and the corresponding bands in control and knock-down cells were quantified using Image J software, followed by normalization for total cell protein.

2.4. Lipolysis assay

Non-esterified fatty acid (NEFA) release from 3T3-L1 control and Angptl8 silenced adipocytes were measured under basal and isoproterenol (10 μM) treated conditions after a 2-h incubation. Cell supernatants were collected and NEFA release quantified using HR Series NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA) according to the manufacturer's protocol.

2.5. Lipidome analysis

Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode using the analytical setup and strategy described previously (Liebisch et al., 2004). A precursor ion of m/z 184 was used for PC, sphingomyelin (SM) (Liebisch et al., 2004) and lysophosphatidylcholine (LPC) (Liebisch et al., 2002). The following neutral losses were applied: PE 141, phosphatidylserine (PS) 185, phosphatidylglycerol (PG) 189 and phosphatidylinositol (PI) 277 (Brugger et al., 1997; Matyash et al., 2008). PE-based plasmalogens (PE P) were analyzed according to the principles described in (Zemski Berry and Murphy, 2004). Lipid species were annotated according to the recently published proposal for shorthand notation of lipid structures that are derived from mass spectrometry (Liebisch et al., 2013). Glycerophospholipid species annotation was based on the assumption of even numbered carbon chains only.

2.6. Staining of lipid droplets

3T3-L1 control and Angptl8 knock-down adipocytes were washed and fixed with 4% paraformaldehyde. Cells were then

permeabilized with 0.1% Triton x-100 in Phosphate-buffered saline, washed and then stained with 100 nM Bodipy® 493/503 (Molecular Probes/Life Technologies, Eugene, OR) for one hour. Nuclei were stained with DAPI. Cells were imaged using Zeiss Axio Observer Z1 microscope (Carl Zeiss Imaging Solutions GmbH, Oberkochen, Germany) equipped with Colibri laser.

3. Results

3.1. Stable knock-down of Angptl8 in 3T3-L1 adipocytes reduces the cellular TGs

To elucidate the functional role of Angptl8 in adipocyte lipid metabolism, we generated stably transduced 3T3-L1 adipocyte pools by using two independent shRNAs targeting the mRNA, designated Angptl8 KD1 and KD2. The knock-down efficiency was evaluated by qPCR, revealing an approximately similar, 70–75% reduction of the Angptl8 message for KD1 and KD2 (Fig. 1A). Analysis of the TG content of the transduced KD adipocyte pools by thin-layer chromatography (TLC), by normalizing the TGs for total cell protein, revealed a 18–19% reduction in the Angptl8 KD cells as compared to controls transduced with a non-targeting shRNA lentivirus (Fig. 1B). However, no reduction in the mRNAs encoding the TG synthesizing enzymes (Monoacylglycerol O-Acyltransferase 1-MOGAT1, Glycerol-3-phosphate acyltransferase-GPAT1, Diacylglycerol O-Acyltransferase-DGAT1) was observed (data not shown). Upon fluorescence microscopy imaging of the lipid droplets (LD) stained by using the Bodipy 493/503 dye, the accumulation of large LDs in all cell pools, the non-targeting controls, KD1 and KD2, was evident (Fig. 1C, E). While the control and KD2 cells appeared indistinguishable from each other, the distribution of LD and cell size in the KD1 cell pool was more heterogeneous, being characterized by a large number of relatively small LDs in some cells and only few very large ones in others. (Fig. 1D).

3.2. Knock-down of Angptl8 reduces ether phospholipids as well as saturated PCs and PEs

To obtain an overview of the effects of Angptl8 reduction on the cellular lipidome, we subjected the extracted total cell lipids to quantitative direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) analysis. The data obtained for the Angptl8 KD1 and KD2 were individually compared to the non-targeting shRNA controls. While there was quite considerable variation between many individual lipid molecular species between the KD1 and KD2 cell pools, a number of consistent alterations in the lipid classes were observed. The major finding was a reduction of ether phospholipids (alkyl-PCs and PE plasmalogens) in the KD cell pools (Fig. 2A–E). In addition, saturated PCs, lysoPCs and PEs, which however are very low abundancy species, were significantly reduced in both Angptl8 KD pools (Fig. 2F–H).

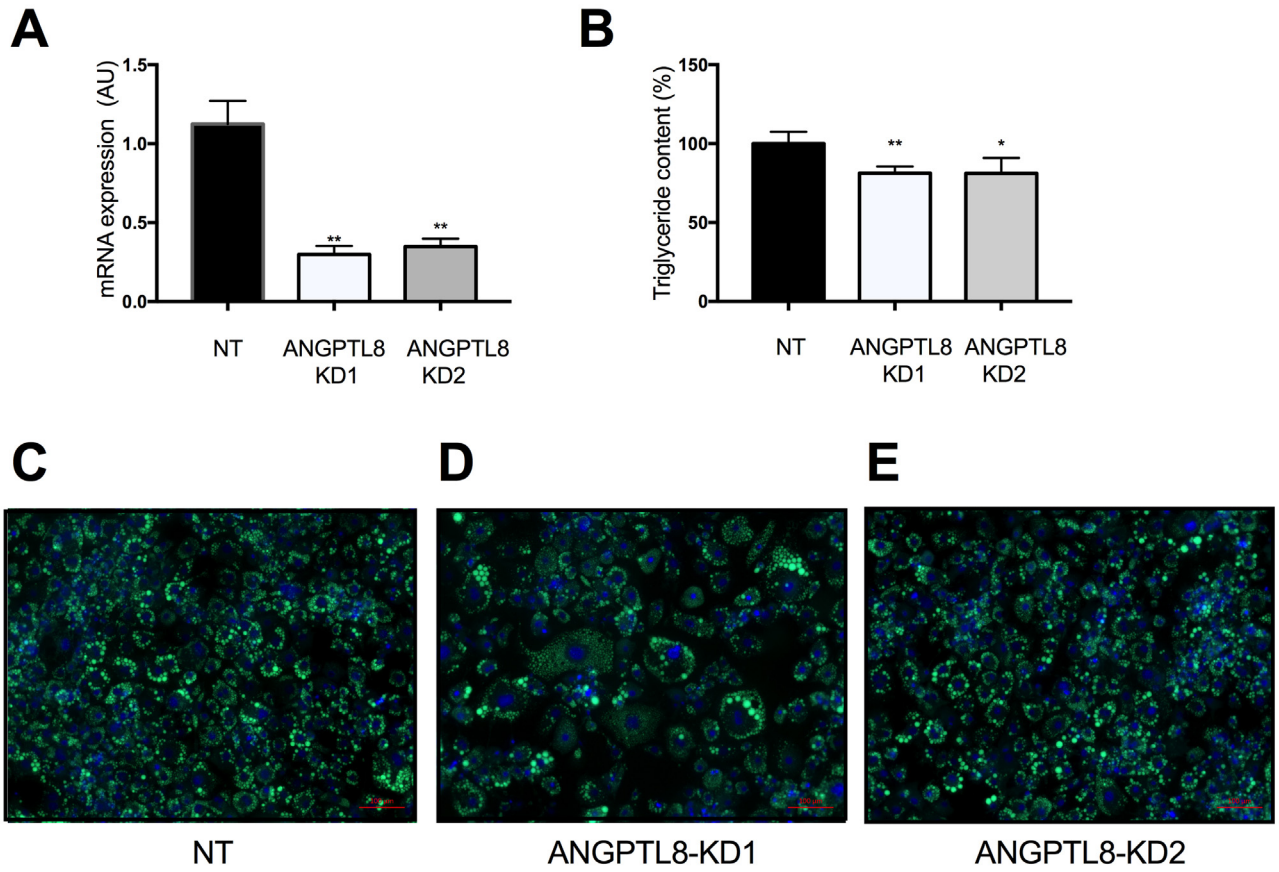

Fig. 1. Knock-down of Angptl8 in 3T3-L1 adipocytes reduces cellular TG storage. **A.** The degree of Angptl8 knock-down in cell pools genarated by using two independent shRNA lentiviruses was evaluated by qPCR **B.** The cellular TG content normalized for total cell protein is depicted (n = 5). In A, the data is for a representative experiment performed in triplicate (mean ± SD), and triplicate PCR amplifications for each specimen; $^{**}p < 0.01$. The data in B represents mean ± SD; N = 8-9 from 2 to 3 independent experiments; $^{**}p < 0.01$. **C–E.** Fluorescence microscopy of lipid droplets in the control (C), KD1 (D) and KD2 (E) cells. LDs were stained with Bodipy 493/503 (green) and nuclei with DAPI (blue), Bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

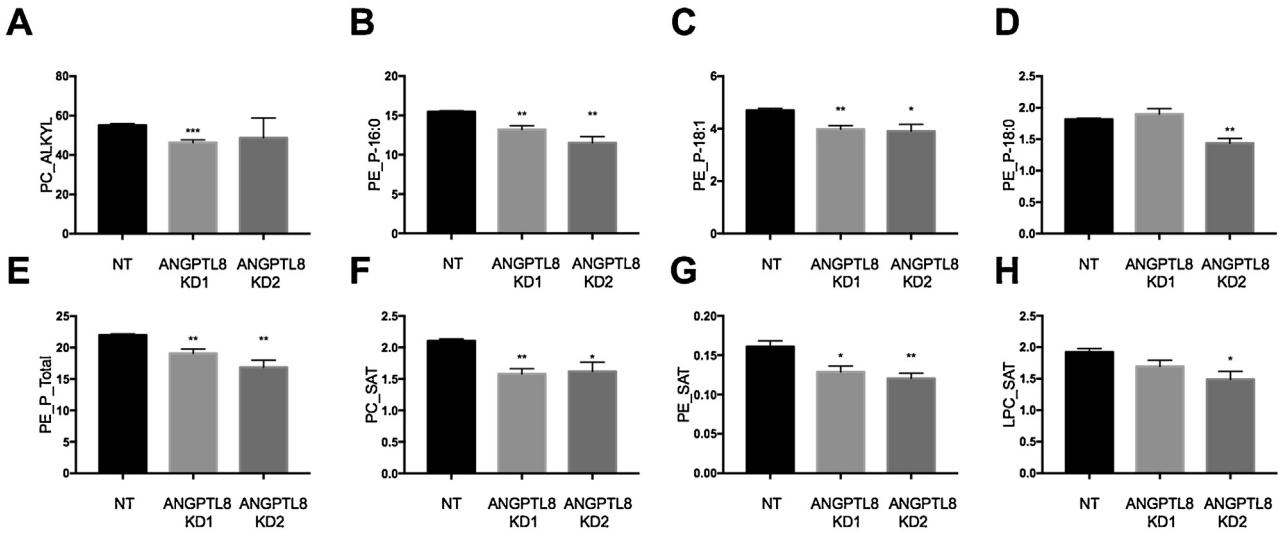

Fig. 2. The lipid classes significantly affected by Angptl8 knock-down. Total lipids of Angptl8 KD and control 3T3-L1 adipocytes were subjected to quantitative mass spectrometric lipidome analysis. The phospholipid classes consistently affected in both KD1 and KD2 cell pools are depicted. **A.** Alkyl-PCs with an ether-bonded FA. Annotation is based on the assumption of even numbered carbon chains only. **B.** PE-plasmalogens (PE_P-16:0; vinyl-ether-16:0). **C.** PE_P-18:1. **D.** PE_P-18:0. **E.** Total PE_Ps. **F.** Saturated PCs. **G.** Saturated PEs. **H.** Saturated lyso PCs. The data represent a mean ± SD, N = 6; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. Angptl8 knock-down enhances lipolysis in 3T3-L1 adipocytes

Since we saw in the Angptl8 KD adipocytes a reduction of stored TGs and because Angptl8 together with Angptl3 inhibits LPL activity in the circulation (Quagliarini et al., 2012; Wang et al., 2013), we decided to measure the intracellular lipolysis in the Angptl8 silenced and control adipocytes in the presence or absence of β-adrenergic (isoproterenol) stimulation. Measurement of NEFA released into the culture medium revealed a significant enhancement of lipolysis in both KD1 and KD2 cell pools as compared to the non-targeting control, the volume of the effect being more pronounced for KD1. This enhancement of lipolysis was evident under both the unstimulated and the isoproterenol-stimulated conditions (Fig. 3A). The Angptl8 mRNA in naive 3T3-L1 adipocytes was significantly suppressed by both isoproterenol and forskolin, which stimulates adenylate cyclase resulting in enhanced protein kinase A activity and lipolysis (Fig. 3B). Together, these data suggest that the endogenous Angptl8 inhibits adipocyte lipolysis, and pharmacologic stimulation of lipolysis down-regulates the expression of Angptl8.

3.4. Effects of Angptl8 knock-down on the expression of genes controlling lipolysis and lipid oxidation

Prompted by the observed enhancement of lipolysis in the Angptl8 KD adipocytes, we analyzed mRNA expression of key genes involved in lipolysis. After 2-h isoproterenol treatment (induction of lipolysis) the mRNAs encoding Angptl4, Pnpla3, Pnpla2/adipose triglyceride lipase (ATGL), and hormone-sensitive lipase (HSL) were quantified. In the both Angtpl8 KD cell pools, the Angptl4 message was significantly increased as compared to the non-targeting control, while the Pnpla2/ATGL and HSL mRNAs

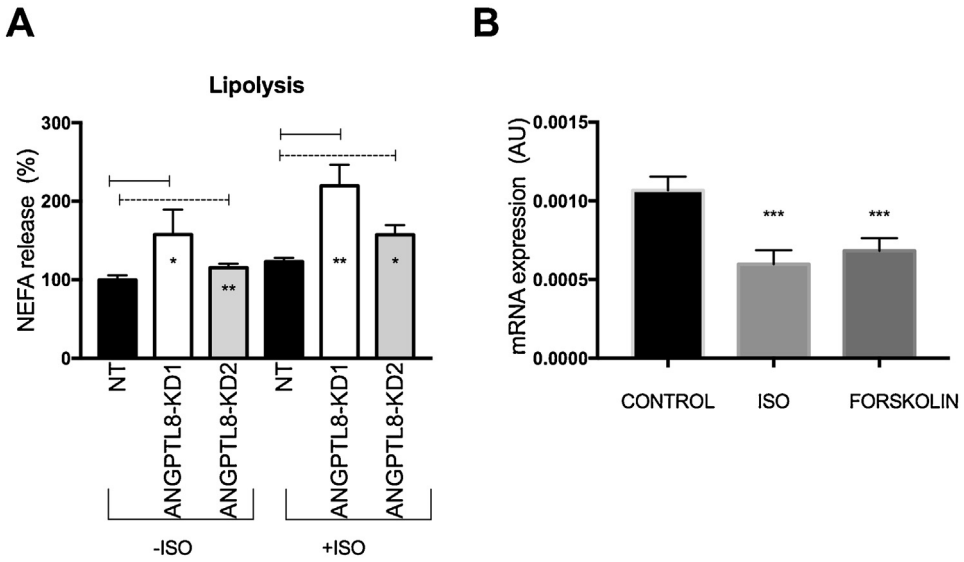

Fig. 3. Depletion of Angptl8 enhances adipocyte lipolysis. **A.** Lipolysis in mature 3T3-L1 adipocytes was induced by a 2-h incubation in the absence (-ISO) or presence (+ISO) of the β-adrenergic agonist isoproterenol, and NEFA released into growth medium was quantified. The data represent a mean ± SD; N = 4, $^{*}p < 0.05$, $^{**}p < 0.01$. **B.** Naive 3T3-L1 adipocytes were incubated for 24 h with 10 μM isoproterenol or 0.2 μM forskolin, followed by qPCR quantification of the Angptl8 mRNA. The data represent a mean ± SD, N = 6, $^{***}p < 0.001$.

remained unchanged. Moreover, there was a marked increase of the Pnpla3 message in KD1 which was, however, not observed in the KD2 cells (Fig. 4A).

We next analyzed genes associated with FA oxidation, PGC-1α, Cpt1a, Cpt1b, and Ucp1. The Cpt1a and Cpt1b mRNAs were significantly increased in both Angptl8 KD cell pools, while PGC-1α was elevated in KD1 and showed a similar trend in KD2 (Fig. 4B). We also analyzed the expression of the adipokine Leptin, which is known to play a role in adipocyte lipolysis in addition to its role as a satiety signal (Flak and Myers, 2016; Kim et al., 2008). The Leptin mRNA was remarkably induced in the Angptl8 KD adipocytes (Fig. 4B).

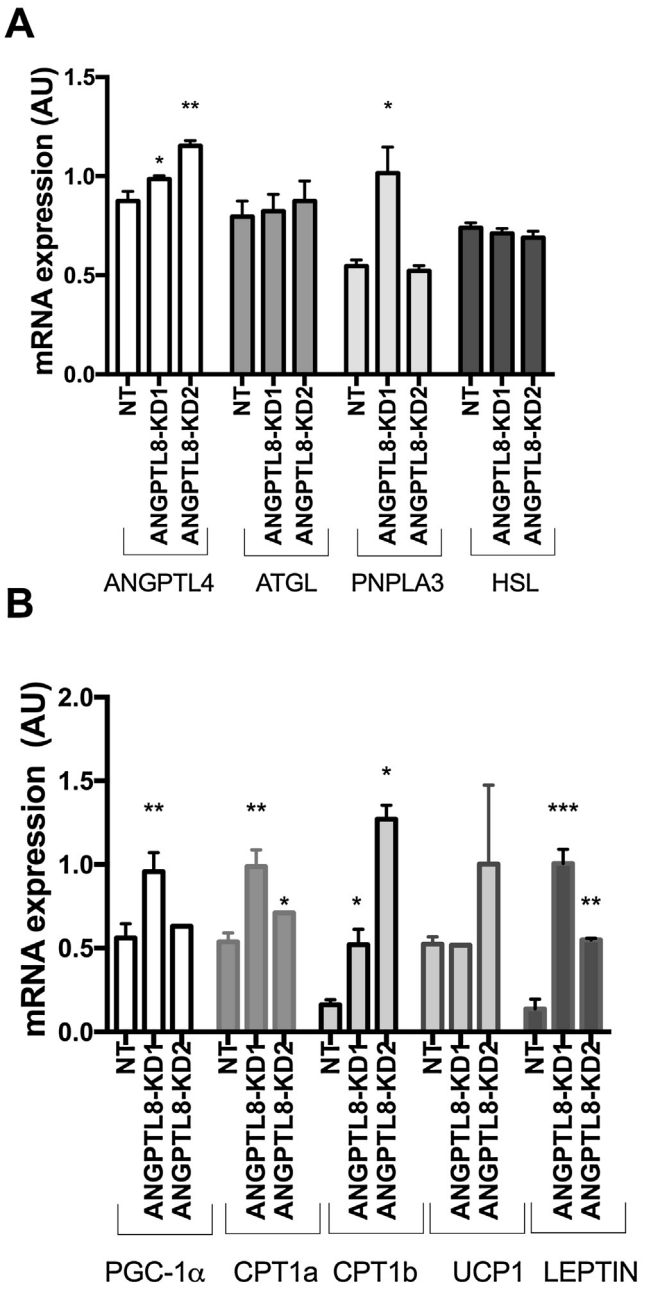

Fig. 4. Gene expression changes observed in the Angptl8 KD cell pools as compared to non-targeting (NT) controls: qPCR analyses. **A.** Quantities of mRNAs involved in lipolysis, in 3T3-L1 adipocytes after 2-h treatment with 10 μM isoproterenol (+ISO). **B.** Quantities of mRNAs involved in FA oxidation and Leptin (identified on the right) in 3T3-L1 adipocytes under the normal growth conditions. The data represent a mean ± SD, N = 8–9, from 2 to 3 independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The cell pools are identified at the bottom; the mRNA quantities are expressed in arbitrary units (AU).

4. Discussion

In the present study, we employed the 3T3-L1 adipocyte model to investigate the functional effects of Angptl8 knock-down *via* stable lentivirus-mediated shRNA expression. Depletion of Angptl8 did not drastically affect the adipocytic differentiation of the cells but resulted in a moderate (18–19%) reduction of stored TGs. Lipidome analysis revealed a reduction of ether phospholipids. Importantly, the Angptl8 depleted cells displayed enhanced lipolysis as measured by the release of NEFAs, and treatments with the lipolysis-inducing agents isoproterenol or forskolin suppressed the Angptl8 mRNA. Increased expression of mRNAs encoding Cpt1a, Cpt1b, and PGC-1α involved in FA oxidation, as well as Leptin, was observed in the Angptl8 KD adipocytes.

We observed in the Angptl8 KD adipocytes a moderate but consistent reduction of stored TGs. Several studies have documented an induction of the Angptl8 message during adipocyte differentiation (Ebert et al., 2014; Ren et al., 2012) and by their treatment with insulin, which favours the storage of FAs as TGs (Ebert et al., 2014; Nidhina Haridas et al., 2015; Ren et al., 2012). In a previous study, Ren et al. (2012) carried out transient siRNA-mediated knock-down of Angptl8 in the 3T3-L1 adipocyte model and observed a defect in their adipocyte differentiation. We observed in the stable knock-down cell pools no drastic defect in adipogenesis; However, the moderate reduction of stored TGs we detected could reflect a mild defect in the terminal stages of adipocytic differentiation.

The present lipidome analyses revealed a significant reduction of alkyl-PCs and PE plasmalogens with an ether-linked FA chain, usually in the *sn-1* position. Synthesis of ether phospholipids begins by peroxisomal enzyme machineries (Braverman and Moser, 2012). We therefore considered it possible that Angptl8 depletion could result in a peroxisomal dysfunction. Synthesis of alkyl-glycerol ether lipids or exogenous treatment with these lipids promote adipocytic differentiation (Homan et al., 2011). Consistently, Pex7 KO mice, which lack plasmalogens, have reduced body fat and display an abnormal morphology of both white and brown fat, characterized by small LD (Brites et al., 2011). Besides the defect in ether phospholipids, we observed a reduction of saturated PCs and PEs, which, however, are very minor species. Since no significant change was observed in the total saturation degree of phospholipids (data not shown), the reason for this observation remains unknown. Nevertheless, we find it possible that the observed depletion of ether phospholipids, which usually have a polyunsaturated FA chain in the *sn-2* position, might decrease the fluidity of cellular membranes (Pietiläinen et al., 2011; Saitoh et al., 2009), and the reduction of saturated PCs and PEs could represent a compensatory response aimed at resisting the fluidity change.

A major functional effect of Angptl8 depletion was an enhancement of lipolysis within adipocytes, suggesting that the endogenous Angptl8 acts to inhibit lipolysis. This would be fully consistent with its robust induction by insulin in WAT (Nidhina Haridas et al., 2015; Soronen et al., 2012). Under circumstances favouring FA deposition as TGs in WAT, it is plausible that hydrolysis of the stored TGs should simultaneously be suppressed. It thus seems that, analogous to the activity of Angptl8 as an inhibitor of lipolysis in the circulation (Quagliarini et al., 2012; Wang et al., 2013), it could execute a similar function intracellularly. We failed to detect changes in the mRNA expression of central mediators of lipolysis, ATGL and HSL, while a small increase of the Angptl4 message was evident; Angptl4 acts as stimulator of adipocyte lipolysis (Gray et al., 2012), so its induction could

contribute to the observed increase of lipolysis. In KD1 cells, which displayed a more potent enhancement of lipolysis than KD2, also a significant increase of the Pnpla3 mRNA was observed; Of note, Pnpla3 is known to enhance lipolysis and TG remodeling in hepatocytes (He et al., 2010; Ruhanen et al., 2014). Its elevated expression could thus facilitate the potent increase of lipolysis in KD1. At present we do not know the reason for this discrepancy between KD1 and KD2. However, we find it possible that an shRNA off-target effect in KD2 could alleviate the lipolysis phenotype. Plasmalogens act as a source of prostaglandin E2 (PGE2), which inhibits lipolysis in adipocytes (Chatzipanteli et al., 1992; Fain et al., 2000). Reduction of PGE2 increases the cellular cAMP, resulting in enhanced lipolysis (Jaworski et al., 2009). Consistently, patients with the Zellweger syndrome, a genetic peroxisome defect, display a deficiency in plasmalogens; Their fibroblasts contain an increased number of β-adrenergic receptors and are therefore more responsive to isoproterenol-induced cAMP responses (Tiffany et al., 1990).

Interestingly, we observed a robust induction of the Leptin mRNA in the Angptl KD cells under the basal culture conditions, but the mRNA was hardly detectable under the lipolytic conditions employed. This finding is controversial since PGE2 is known to enhance leptin secretion by adipocytes (Fain et al., 2000; Harris, 2014). Thus, the observed induction of Leptin mRNA could represent a compensatory response to reduced synthesis of PGE2, or depletion of Angptl8 might affect Leptin expression through another, thus far unknown mechanism. In addition to the above lipolysis phenotype, we also found a number of genes associated with FA oxidation significantly elevated in the Angptl8 KD cells. This is consistent with the observed increase of lipolysis releasing free FAs, which can be used as an energy source *via* oxidation, and on the other hand, need to be degraded to reduce FFA-mediated toxicity to cells (Saha et al., 2004).

To conclude, knock-down of Angptl8 in adipocytes suggests that the protein acts to inhibit intracellular lipolysis. Modulation of the cellular ether phospholipid content may be one of the underlying mechanisms. The findings identify Angptl8 as a central insulin-regulated controller of adipocyte lipid storage and metabolism.

Conflict of interest

The authors have no potential conflicts of interest to disclose concerning this study.

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References

Abu-Farha, M., Al-Khairi, I., Cherian, P., Chandy, B., Sriraman, D., Alhubail, A., Al-Refaei, F., AlTerki, A., Abubaker, J., 2016. Increased ANGPTL3, 4 and ANGPTL8/betatrophin expression levels in obesity and T2D. Lipids Health Dis. 15, 181.

Braverman, N.E., Moser, A.B., 2012. Functions of plasmalogen lipids in health and disease. Biochim. Biophys. Acta 1822, 1442–1452.

Brites, P., Ferreira, A.S., da Silva, T.F., Sousa, V.F., Malheiro, A.R., Duran, M., Waterham, H.R., Baes, M., Wanders, R.J., 2011. Alkyl-glycerol rescues plasmalogen levels and pathology of ether-phospholipid deficient mice. PLoS One 6, e28539.

Brugger, B., Erben, G., Sandhoff, R., Wieland, F.T., Lehmann, W.D., 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. 94, 2339–2344.

Chatzipanteli, K., Rudolph, S., Axelrod, L., 1992. Coordinate control of lipolysis by prostaglandin E2 and prostacyclin in rat adipose tissue. Diabetes 41, 927–935.

Chen, X., Lu, P., He, W., Zhang, J., Liu, L., Yang, Y., Liu, Z., Xie, J., Shao, S., Du, T., Su, X., Zhou, X., Hu, S., Yuan, G., Zhang, M., Zhang, H., Liu, L., Wang, D., Yu, X., 2015. Circulating betatrophin levels are increased in patients with type 2 diabetes and associated with insulin resistance. J. Clin. Endocrinol. Metab. 100, E96–100.

Conklin, D., Gilbertson, D., Taft, D.W., Maurer, M.F., Whitmore, T.E., Smith, D.L., Walker, K.M., Chen, L.H., Wattler, S., Nehls, M., Lewis, K.B., 1999. Identification of a mammalian angiopoietin-related protein expressed specifically in liver. Genomics 62, 477–482.

Dang, F., Wu, R., Wang, P., Wu, Y., Azam, M.S., Xu, Q., Chen, Y., Liu, Y., 2016. Fasting and feeding signals control the oscillatory expression of angptl8 to modulate lipid metabolism. Sci. Rep. 6, 36926.

Dijk, W., Kersten, S., 2016. Regulation of lipid metabolism by angiopoietin-like proteins. Curr. Opin. Lipidol. 27, 249–256.

Ebert, T., Kralisch, S., Hoffmann, A., Bachmann, A., Lossner, U., Kratzsch, J., Bluher, M., Stumvoll, M., Tonjes, A., Fasshauer, M., 2014. Circulating angiopoietin-like protein 8 is independently associated with fasting plasma glucose and type 2 diabetes mellitus. J. Clin. Endocrinol. Metab. 99, E2510–2517.

Fain, J.N., Leffler, C.W., Bahouth, S.W., 2000. Eicosanoids as endogenous regulators of leptin release and lipolysis by mouse adipose tissue in primary culture. J. Lipid Res. 41, 1689–1694.

Flak, J.N., Myers Jr., M.G., 2016. Minireview: CNS mechanisms of leptin action. Mol. Endocrinol. 30, 3–12.

Fu, Z., Berhane, F., Fite, A., Seyoum, B., Abou-Samra, A.B., Zhang, R., 2014. Elevated circulating lipasin/betatrophin in human type 2 diabetes and obesity. Sci. Rep. 4, 5013.

Gray, N.E., Lam, L.N., Yang, K., Zhou, A.Y., Koliwad, S., Wang, J.C., 2012. Angiopoietin-like 4 (Angptl4) protein is a physiological mediator of intracellular lipolysis in murine adipocytes. J. Biol. Chem. 287, 8444–8456.

Harris, R.B., 2014. Direct and indirect effects of leptin on adipocyte metabolism. Biochim. Biophys. Acta 1842, 414–423.

He, S., McPhaul, C., Li, J.Z., Garuti, R., Kinch, L., Grishin, N.V., Cohen, J.C., Hobbs, H.H., 2010. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J. Biol. Chem. 285, 6706–6715.

Homan, E.A., Kim, Y.G., Cardia, J.P., Saghatelian, A., 2011. Monoalkylglycerol ether lipids promote adipogenesis. J. Am. Chem. Soc. 133, 5178–5181.

Jaworski, K., Ahmadian, M., Duncan, R.E., Sarkadi-Nagy, E., Varady, K.A., Hellerstein, M.K., Lee, H.Y., Samuel, V.T., Shulman, G.I., Kim, K.H., de Val, S., Kang, C., Sul, H.S., 2009. AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. Nat. Med. 15, 159–168.

Kim, W.K., Lee, C.Y., Kang, M.S., Kim, M.H., Ryu, Y.H., Bae, K.H., Shin, S.J., Lee, S.C., Ko, Y., 2008. Effects of leptin on lipid metabolism and gene expression of differentiation-associated growth factors and transcription factors during differentiation and maturation of 3T3-L1 preadipocytes. Endocr. J. 55, 827–837.

Liebisch, G., Drobnik, W., Lieser, B., Schmitz, G., 2002. High-throughput quantification of lysophosphatidylcholine by electrospray ionization tandem mass spectrometry. Clin. Chem. 48, 2217–2224.

Liebisch, G., Lieser, B., Rathenberg, J., Drobnik, W., Schmitz, G., 2004. High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. Biochim. Biophys. Acta 1686, 108–117.

Liebisch, G., Vizcaino, J.A., Kofeler, H., Trotzmuller, M., Griffiths, W.J., Schmitz, G., Spener, F., Wakelam, M.J., 2013. Shorthand notation for lipid structures derived from mass spectrometry. J. Lipid Res. 54, 1523–1530.

Matyash, V., Liebisch, G., Kurzchalia, T.V., Shevchenko, A., Schwudke, D., 2008. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J. Lipid Res. 49, 1137–1146.

Nidhina Haridas, P.A., Soronen, J., Sädevirta, S., Mysore, R., Quagliarini, F., Pasternack, A., Metso, J., Perttilä, J., Leivonen, M., Smas, C.M., Fischer-Posovszky, P., Wabitsch, M., Ehnholm, C., Ritvos, O., Jauhiainen, M., Olkkonen, V.M., Yki-Järvinen, H., 2015. Regulation of angiopoietin-Like proteins (ANGPTLs) 3 and 8 by insulin. J. Clin. Endocrinol. Metab. 100, E1299–1307.

Pietiläinen, K.H., Rog, T., Seppänen-Laakso, T., Virtue, S., Gopalacharyulu, P., Tang, J., Rodriguez-Cuenca, S., Maciejewski, A., Naukkarinen, J., Ruskeepää, A.L., Niemelä, P.S., Yetukuri, L., Tan, C.Y., Velagapudi, V., Castillo, S., Nygren, H., Hyotylainen, T., Rissanen, A., Kaprio, J., Yki-Järvinen, H., Vattulainen, I., Vidal-Puig, A., Oresic, M., 2011. Association of lipidome remodeling in the adipocyte membrane with acquired obesity in humans. PLoS Biol. 9, e1000623.

Quagliarini, F., Wang, Y., Kozlitina, J., Grishin, N.V., Hyde, R., Boerwinkle, E., Valenzuela, D.M., Murphy, A.J., Cohen, J.C., Hobbs, H.H., 2012. Atypical angiopoietin-like protein that regulates ANGPTL3. Proc. Natl. Acad. Sci. U. S. A. 109, 19751–19756.

Ren, G., Kim, J.Y., Smas, C.M., 2012. Identification of RIFL, a novel adipocyte-enriched insulin target gene with a role in lipid metabolism. Am. J. Physiol. Endocrinol. Metab. 303, E334–351.

Rong Guo, X., Li Wang, X., Chen, Y., Hong Yuan, Y., Mei Chen, Y., Ding, Y., Fang, J., Jiao Bian, L., Sheng Li, D., 2016. ANGPTL8/betatrophin alleviates insulin resistance via the Akt-GSK3beta or Akt-FoxO1 pathway in HepG2 cells. Exp. Cell Res. 345, 158–167.

Ruhanen, H., Perttilä, J., Hölttä-Vuori, M., Zhou, Y., Yki-Järvinen, H., Ikonen, E., Käkelä, R., Olkkonen, V.M., 2014. PNPLA3 mediates hepatocyte triacylglycerol remodeling. J. Lipid Res. 55, 739–746.

Saha, P.K., Kojima, H., Martinez-Botas, J., Sunehag, A.L., Chan, L., 2004. Metabolic adaptations in the absence of perilipin: increased beta-oxidation and decreased hepatic glucose production associated with peripheral insulin resistance but normal glucose tolerance in perilipin-null mice. J. Biol. Chem. 279, 35150–35158.

Saitoh, M., Itoh, M., Takashima, S., Mizuguchi, M., Iwamori, M., 2009. Phosphatidyl ethanolamine with increased polyunsaturated fatty acids in compensation for plasmalogen defect in the Zellweger syndrome brain. Neurosci. Lett. 449, 164–167.

Santulli, G., 2014. Angiopoietin-like proteins: a comprehensive look. Front. Endocrinol. (Lausanne) 5, 4.

Soronen, J., Laurila, P.P., Naukkarinen, J., Surakka, I., Ripatti, S., Jauhiainen, M., Olkkonen, V.M., Yki-Järvinen, H., 2012. Adipose tissue gene expression analysis reveals changes in inflammatory, mitochondrial respiratory and lipid metabolic pathways in obese insulin-resistant subjects. BMC Med. Genomics 5, 9.

Tiffany, C.W., Hoefler, S., Moser, H.W., Burch, R.M., 1990. Arachidonic acid metabolism in fibroblasts from patients with peroxisomal diseases: response to interleukin 1. Biochim. Biophys. Acta 1096, 41–46.

Wang, Y., Quagliarini, F., Gusarova, V., Gromada, J., Valenzuela, D.M., Cohen, J.C., Hobbs, H.H., 2013. Mice lacking ANGPTL8 (Betatrophin) manifest disrupted triglyceride metabolism without impaired glucose homeostasis. Proc. Natl. Acad. Sci. U. S. A. 110, 16109–16114.

Zemski Berry, K.A., Murphy, R.C., 2004. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. J. Am. Soc. Mass Spectrom. 15, 1499–1508.

Zhang, Y., Li, S., Donelan, W., Xie, C., Wang, H., Wu, Q., Purich, D.L., Reeves, W.H., Tang, D., Yang, L.J., 2016. Angiopoietin-like protein 8 (betatrophin) is a stress-response protein that down-regulates expression of adipocyte triglyceride lipase. Biochim. Biophys. Acta 1861, 130–137.