Investigation of mechanisms of insulin-regulated lipolysis via ABHD15.

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Acknowledgements

Summary

Adipose tissue (AT) is a multi-functional organ which plays an important role in lipid and glucose homeostasis for whole-body energy metabolism. Dysfunction of energy metabolism in AT leads to insulin resistance and contributes to the development of obesity and type 2 diabetes (T2D) [1]. In mammals, AT is the predominant organ that serves as energy store in form of triglycerides (TGs) in lipid droplets (LD). Upon energy demand, these TGs are rapidly hydrolyzed by lipases (a process known as lipolysis) and the resulting fatty acids (FAs) released from LD are used as energy source in other organs [2]. After food intake, glucose levels increase and activate insulin secretion from pancreatic islets; rise of postprandial insulin inhibits AT lipolysis and promotes glucose uptake for *de novo* lipogenesis. Elevated plasma FA levels, also as a consequence of inappropriately controlled lipolysis, contribute to the development of insulin resistance and T2D [3-8]. Understanding the detailed mechanisms by which insulin suppresses adipocyte lipolysis is critical to develop potential therapeutic strategies to mitigate insulin resistance and T2D.

This work shows that α/β-hydrolase domain-containing (ABHD) 15 is required for the stability of PDE3B in white adipose tissue (WAT). *In vitro* data reveal that ABHD15 associates with and stabilizes phosphodiesterase 3B (PDE3B). Accordingly, PDE3B expression is decreased in WAT of *Abhd15*-ko WAT. However, we also found that ABHD15 per se did not affect lipolysis.

Abbreviations

ABHD15	α/β-hydrolase domain-containing 15
AR	Adrenergic receptor
AT	Adipose tissue
cAMP	Cyclic AMP
DG	Diglyceride
ER	Endoplasmic reticulum
FA	Fatty acid
HFD	High fat diet
IKK	IκB kinase
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrate
LD	Lipid droplets
MCP-1	Monocyte chemoattractant protein 1
MG	Monoglyceride
MMP	Matrix metalloproteinase
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
PDE3B	Phosphodiesterase 3B
PDK-1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB/Akt	Protein kinase B
PM	Plasma membrane
PP2A	Protein phosphatase 2A
T2D	Type 2 diabetes
TG	Trigltcerides
TNFα	Tumor necrosis factor alpha
WAT	White adipose tissue

1. Introduction

1.1 The function of adipose tissues

Obesity has reached epidemic proportions globally, with at least 2.8 million people dying each year as a result of being overweight or obese (http://www.who.int/features/factfiles/obesity/en/). Worldwide obesity has nearly tripled over the past five decades. Obesity, partially due to an overabundance of fat cells, is frequently associated with several complications, the most devastating of which may be type 2 diabetes[9]. Both obesity and type 2 diabetes are associated with the dysfunction of adipose tissue (AT)[10]. Originally, AT has been considered simply as a storage organ for energy in form of triacylglycerols (TAGs), however, since then the interest in AT biology increased substantially. Over the last decades we have gained considerable amount of knowledge about the biology and biochemistry of AT. It is now generally recognized that AT is a significant organ of a complex network that involves the regulation of diverse biological functions. In mammals, there are two distinct types of AT: white adipose tissue (WAT) and brown adipose tissue (BAT)[11]. The adipocytes in WAT and BAT display distinct morphology and functions. WAT serves as a storage depot of lipids whereas BAT burns lipids to generate heat through mitochondrial oxidation. WAT is also a remarkable endocrine organ secreting a number of hormones, known as adipokines, involved in the regulation of diverse metabolic functions and immune responses[12], the endocrine function of BAT has been suggested[13,14], while still unclear.

WAT consists of mature adipocytes and is characterized by unilocular lipid droplets and very few mitochondria, preadipocytes, fibroblasts, small blood vessels, and nerve cells[7]. Mature adipocytes have an important role in buffering nutrient availability and demand by storing excess calories and preventing the toxic accumulation in other

types of cells. Upon energy demand, stored TAGs in mature adipocytes are rapidly hydrolyzed by lipases (a process known as lipolysis). After food digestion, blood glucose levels increase and activate insulin secretion from pancreatic islets; a rise of postprandial insulin inhibits AT lipolysis and promotes glucose uptake for de novo lipogenesis (DNL).

BAT, which is specialized in producing heat, is almost degenerated in adult humans but found at birth[15]. The average diameter of brown adipocytes is smaller than white adipocytes. Brown adipocytes have a number of cytoplasmic lipid droplets of different sizes, a spherical core and numerous mitochondria that release heat by fatty acids (FAs) oxidation. BAT also stores energy as TGs, but more frequently produces heat by oxidizing FAs, rather than supplying energy substrates for other organs. The heat production from BAT is highly dependent on uncoupling protein 1 (UCP1) which is a unique protein located in the inner mitochondrial membrane[16]. It has been known for long time that respiration and mitochondrial ATP synthesis are coupled[17]. It was demonstrated that the mitochondrial electrochemical proton gradient, generated when electrons are passed down the respiratory chain, is the primary source for cellular ATP synthesis[18]. The mitochondrial respiratory chain is made of five complexes. Complexes I, III, and IV pump protons outside the inner membrane and generate a proton gradient which allows the protons to reenter the mitochondria bypassing the ATP synthase[17]. In addition to this coupled proton reentry, an uncoupled proton leak via UCP1 is another mechanism consuming the mitochondrial proton gradient and producing heat which is named non-shivering thermogenesis (NST).

In addition to white and brown adipocytes, another intensively studied cell type are beige adipocytes which are UCP1 positive but predominant in subcutaneous WAT (sWAT)[19]. Similar to brown adipocytes, beige adipocytes also contain multilocular lipid droplets and plentiful mitochondria that express UCP1. Different from the intrinsic

brown adipocytes in BAT, beige adipocyte biogenesis in sWAT is strongly induced in response to some environmental conditions and external cues, including chronic cold stimulation, exercise, long-term treatment of β3-adrenergic receptor (AR) agonists or peroxisome proliferator-activated receptor γ (PPARγ) agonists, and cancer cachexia. This accumulation of beige adipocytes in WAT is often referred to as "browning" or "beiging" of WAT[20]. The idea of activating beige adipocytes and browning of WAT therapeutically has gained a lot of attention, since brown and beige adipocytes play a key role in the regulation of systemic energy homeostasis in mammals[21-23], and the active BAT in adults is barely low and inversely correlated with BMI and age[22,24-30]. For a quite long time, UCP1 has been considered to be the only thermogenic protein that is responsible for NST. However, genetic studies in rodent models suggested an unexpected metabolic phenotype in UCP1-deficient mice. The sWAT of cold-acclimated $Ucp1^{-/-}$ mice exhibits increased oxygen consumption in response to succinate administration[31]. Further, chronic treatment with β3-AR agonists increases respiration of epididymal WAT (eWAT) of $Ucp1^{-/-}$ mice[32]. The creatine-driven substrate cycle[33] has been reported as a novel non-shivering thermogenic mechanism in brown and beige fat further demonstrating that a UCP1-independent thermogenic mechanism exists. A recent study reported sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2b)-mediated calcium cycling regulates beige fat thermogenesis[34], which experimentally confirmed the existence of UCP1-independent thermogenesis in beige fat. Although UCP1 is thermogenic indispensable in BAT, the anti-obesity and anti-diabetic action of beige fat are UCP1-independent.

1.2. Energy metabolism in AT

As the fuel reservoir, AT conserves the heat of the body and controls energy mobilization. As an energy storage organ, AT releases energy and stores excess

energy through lipolysis and lipogenesis, respectively. Systemically, feeding stimulates the lipogenic pathway and storage of TAGs in AT, while fasting induces the activation of the lipolytic pathway and promotes the breakdown of TAGs and the release of FFAs from AT[35].

Lipolysis is the catabolic process leading to the breakdown of TAGs stored in adipocytes and the subsequent release of FFAs and glycerol[36-39]. This catabolic pathway is activated by fasting and supplies glycerol for hepatic gluconeogenesis and FFAs for oxidation according to energy needs in other organs[40]. Several hormones have been shown to regulate the lipolytic pathway. During fasting, decreased circulating levels of insulin result in suppression of lipogenesis as well as activation of the lipolytic pathway. Consistently, elevated circulating glucagon during fasting is also responsible for the activation of cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway and lipolysis in adipocytes. Additionally, catecholamines release by the sympathetic nervous system (SNS) is also stimulated by fasting; those catecholamines bind to the β_3-AR and activate PKA and lipolytic pathways[38,41]. Lipolysis proceeds in a precisely regulated manner, with different enzymes acting at each step. TAG is sequentially hydrolyzed into diacylglycerol (DAG), then monoacylglycerol (MAG), with the liberation of one FFA at each step. MAG is hydrolyzed to release the final FFA and glycerol[41]. While adipose triglyceride lipase (ATGL) is mainly responsible for the first step of TAG breakdown, hormone-sensitive lipase (HSL) mainly converts DAGs to MAGs[42,43]. The lipid droplet-associated protein perilipin is phosphorylated by PKA and then recruits activated HSL to lipid droplets for lipolysis[44,45]. Monoglyceride lipase (MGL) hydrolyzes the 1(3) and 2-ester bonds of MAG at equal rates but owns no *in vitro* catalytic activity against TAG, DAG, or cholesteryl esters[46].

Lipogenesis is the process that encompasses *de novo* fatty acid synthesis from acetyl-coenzyme A (acetyl-CoA), eventually also leading to the synthesis of TAGs. After a

meal, increased blood glucose is transported into adipocytes, then oxidized to acetyl-CoA, which is further converted to malonyl-CoA and then to palmitate. Palmitate is modified by elongases and desaturases to produce diverse FAs and derivatives (fig. 4). Glucose provides its own metabolite acetyl-CoA as the substrate for *de novo* synthesis of FAs, induces the expression of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme of lipogenesis and stimulates the release of pancreatic insulin which promotes lipogenesis. As a result, insulin stimulates glucose uptake into adipocytes, activates glycolytic and lipogenic enzymes, and stimulates expression of the lipogenic gene sterol regulatory element-binding protein 1 (SREBP1) that further controls expression of genes required for cholesterol, FA, TAG, and phospholipid synthesis[47,48]. In addition to SREBP1, another transcriptional factor namely carbohydrate response element binding protein (ChREBP) promotes DNL genes expression and has been shown to modulate both lipid and glucose metabolism in AT and substantially impacts whole-body insulin sensitivity[49-51]. Under normal conditions, DNL is relatively low in WAT compared with liver and BAT in rodents and even lower in humans[52,53]. By contrast, BAT can take up a decent amount of glucose during the cold exposure or after fasting[21,54-56]. Thereby, BAT has been considered as an important glucose sink able to defend against the negative effects of obesity on glucose homeostasis.

1.3 Obesity related inflammation and insulin resistance.

Obesity causes a state of chronic, low-grade inflammation in metabolic tissues (such as liver and AT) often associated with macrophage infiltration and the local production of pro-inflammatory cytokines and chemokines that attenuate insulin sensitivity[57-60]. It was first demonstrated that obesity increased the expression and secretion of TNFα from AT both in rodents and humans[61-63], thus TNFα has been considered as a prototypical inflammatory cytokine. Subsequently, the expression of more than a dozen

genes that encode inflammatory proteins in AT, including IL-6, MCP-1, iNOS, MMPs, and lipocalin were found to be correlated with adiposity[64]. Genetic deletion or pharmacological inhibition of those inflammatory pathways can dissociate obesity from insulin resistance, suggesting that local inflammation is a key factor causing insulin resistance. In addition to inflammatory cytokines and intercellular signaling molecules, recent data have implicated intracellular pathways that regulate inflammation in the development of obesity-induced insulin resistance. The nuclear transcription factor NF-κB is activated in metabolic organs and thought to have an important role in the development of insulin resistance. NF-κB is a multi-protein transcription factor whose regulatory targets include secreted pro-inflammatory proteins like TNFα and MCP-1. Many inflammatory stimuli including activation of Toll-like receptors, reactive oxygen species, ultraviolet radiation, and pro-inflammatory cytokines lead to the phosphorylation and subsequent degradation of the inhibitory component of NF-κB[65-68]. Once relieved from its inhibitory component, the NF-κB complex is activated and translocated to the nucleus where it activates NF-κB-dependent transcription in a cell-type specific manner. Activation of NF-κB is triggered by phosphorylation of IκB kinase[69] which includes four members: IKKα, IKKβ, IKKε and TBK1. IKKα and IKKβ act with the scaffolding partner NEMO to activate NF-κB[70]. Obesity increases nuclear translocation of NF-κB in liver and skeletal muscle and its transcriptional target genes. Targeted deletion of IKKβ in hepatocytes reduces the obesity-induced hepatic inflammation and the circulating concentration of inflammatory cytokines IL-1β and IL-6[71,72]. Pharmacologic inhibition of NF-κB through use of high doses salicylates also improves insulin sensitivity in obese rodent models of insulin resistance and in humans[73]. While knockout or pharmacological inhibition of IKKβ defined a role for this kinase in insulin resistance[72,73], the roles of the non-canonical kinases IKKε and TBK1 are less certain. It has been reported that both mRNA and protein expression of TBK1

and IKKε are increased during a high-fat diet (HFD) in AT, and the expression of IKKε is increased in liver[74]. In addition, deletion of the *Ikbke* rendered mice partially resistant to the HFD-dependent development of obesity, insulin resistance, hepatic steatosis, and inflammation[74]. This previous work led to the search for small molecule inhibitors of these kinases as a potential therapeutic option to treat these conditions, e.g. the discovery of amlexanox, which selective inhibits IKKε and TBK1. Amlexanox has been previously developed for the treatment of asthma, allergic rhinitis and aphthous ulcers but has an unclear mechanism of action[75,76]. A recent work published by Saltiel laboratory identified amlexanox as an inhibitor of TBK1 and IKKε improving obesity-related metabolic dysfunction in mice. Administration of amlexanox to obese mice produces reversible weight loss and improved insulin sensitivity, reduced inflammation and attenuated hepatic steatosis[77]. The discovery of new function of amlexanox suggest that TBK1 and IKKε are part of a counter-inflammtory process that sustains energy storage in the context of insulin resistance[78]. A further study revealed that TBK1 suppresses inflammation and energy expenditure in AT via two separate pathways, indicating that TBK1 is positioned at the crossroad between energy sensing and inflammatory signaling pathways[79].

1.4 Insulin-regulated lipolysis

For mammals, AT is the predominant organ to store energy in the form of TGs. When energy is demanded, the stored TGs are hydrolyzed by lipases (a process known as lipolysis), and leading to the release of energy substrates – fatty acids (FAs) and glycerol. After a meal digestion, carbohydrates enter the circulation and blood glucose levels increase thereby activating pancreatic islets´ insulin secretion; a rise of postprandial insulin inhibits energy release and activates energy storage via *de novo* lipogenesis in AT.

Complete lipolysis requires three consecutive steps with different enzymes acting at each step. TGs are sequentially hydrolyzed into diglycerides (DGs) and monoglycerides (MGs), with the liberation of one FA by each step. MG is hydrolyzed to release the final FA and glycerol. Alteration of lipolysis is often accompanied with obesity and metabolic disorders. Elevated circulating FA levels (mainly release from AT) has been considered as a key contributor to insulin resistance in both animals and humans[3,8,80,81]. Recently, it has been reported that the failure of insulin to suppress the supply of FA as substrate for hepatic gluconeogenesis leads to systemic insulin resistance[82]. Whereas many aspects have been elucidated, the detailed molecular mechanisms of how insulin suppresses lipolysis in adipocytes are still elusive. Since it became increasingly evident that elevated FA levels lead to ectopic lipid deposition and insulin resistance, the search for regulators of insulin-mediated inhibition of lipolysis might pave the way for therapeutic applications.

Food intake attenuates AT lipolysis mainly via the potent anti-lipolytic actions of insulin. Rapid, acute suppression of lipolysis by insulin involves both cyclic AMP (cAMP)-dependent and -independent mechanisms. cAMP-dependent inhibition of lipolysis by insulin requires the activation of phosphodiesterase 3B (PDE3B)[83,84] which is well understood. Insulin binds to the insulin receptor (IR), resulting in phosphorylation of IR substrate (IRS) and the activation of phosphoinositide 3-kinase (PI3K) which generates phosphatidylinositol (3, 4, 5)-trisphosphate (PIP$_3$). PIP$_3$ thereby recruits protein kinase B (PKB/Akt) to the plasma membrane where it is activated by phosphoinositide-dependent kinase 1 (PDK-1). Upon activation, Akt phosphorylates/activates PDE3B which decreases cAMP levels in adipocytes, leading to the inactivation of PKA and reduction of lipolysis via reducing the phosphorylation of HSL and perilipin. The mechanism has been generally accepted that phosphorylation and activation of PDE3B by insulin-activated Akt is the key node of the insulin-suppressed lipolysis

pathway. However, it has been argued that phosphorylation of PDE3B at its Akt or PKA phosphorylation sites is dispensable for the anti-lipolytic action of insulin, whereas those phosphorylation sites are still necessary for the fully activation of PDE3B enzymatic activity by insulin[84]. While the experimental evidence is still missing, one explanation indicated by literature supporting this discrepancy is that both Akt-dependent and -independent activations of PDE3B exist in adipocytes, and depending on the experimental conditions, one of the pathways predominate.

Phosphodiesterases (PDEs) were identified right after the discovery of cAMP[85-87]. PDEs represent a super family of widely expressed hydrolases that regulate the intracellular levels of cyclic nucleotides by hydrolyzing cAMP and cGMP to 5'AMP and 5'GMP, respectively[88]. The PDE3 subfamily displays a unique structure of a 44-amino acid insert in the catalytic domain which is highly conserved in the C-terminal region, and the N-terminal is considered as regulatory regions which contain multiple phosphorylation sites and distinct between isoforms[89-91]. The PDE3 subfamily consists of two isoforms: PDE3A and PDE3B. PDE3A is mostly expressed in heart, smooth muscles, while PDE3B is more abundant in energy metabolism tissues including ATs, liver, pancreatic β-cells and hypothalamus[89,92]. A very important role of PDE3B is its capacity to be rapidly activated in response to insulin and agents which increase cAMP levels. In adipocytes, mechanisms for activation of PDE3B by insulin or cAMP-elevating agents involve formation of distinct signalosomes. Subcellular fractionation studies indicate that in 3T3-L1 adipocytes, approximately 35% of total membrane PDE3 activity was found in the plasma membrane (PM)/caveolae fraction and around 65% in endoplasmic reticulum (ER)/Golgi fraction[93-95]. In adipocytes, the β_3-adrenergic receptor (AR) agonist-CL316,243 (CL) and insulin differentially regulate PDE3B in PM/caveolae and ER/Golgi fractions, separately. CL preferentially activates PDE3B in PM/caveolae fractions, whereas insulin preferentially phosphorylates PDE3B in

ER/Golgi fractions, suggesting different roles of PDE3B at different microdomains in cells[94]. Insulin-induced signalosomes included IRS-1, PI3K p85, HSP90, and Akt; CL-induced signalosomes included β$_3$-AR, HSL and PKA-RII, while protein phosphatase 2A (PP2A), 14-3-3, perilipin and caveolin-1 were common to both signalosomes[94]. Although studies indicate that phosphorylation of PDE3B is associated with its recruitment into signalosomes, it's not certain whether phosphorylation is required for recruitment or whether recruitment of PDE3B into signalosomes is required for effective phosphorylation of PDE3B.

1.5 ABHD15 and its function

Our lab utilized high through techniques to uncover novel players in adipogenesis[96,97]. Based on previous observations, α/β-hydrolase domain containing protein 15 (ABHD15) was found as being strongly increased during adipocyte differentiation[98,99]. ABHD15 belongs to the a/b-hydrolase superfamily which consist of various lipase, esterase, and proteases that share a common structural feature[100]. Typically, ABHD proteins harbour a catalytic triad build up with a nucleophile (Ser, Cys, or Asp), an acid (aspartate or glutamate), and a conserved histidine residue enabling hydrolase activity[100]. However, ABHD15 lacks the nucleophile; therefore a hydrolytic activity is less possible. Moreover, ABHD15 misses the Ser-X4-Asp motif, making a prediction of its enzymatic function difficult. In human tissues, *ABHD15* is widely expressed, with the highest expression in AT and to a lower level in liver[99,100]. In mice, *Abhd15* is widely expressed, with highest expression in AT and to a lower level in liver[99,100]. Those tissues are primary sites of postprandial insulin action, and ABHD15 has been described as a potential novel player in insulin signalling[98,101]. Our previous publication showed ABHD15 is a direct and functional target gene of PPARγ, the master regulator of adipogenesis[99]. In 3T3-L1 adipocytes, ABHD15 has been identified as a

phosphorylation substrate of protein kinase B (PKB)/Akt[101]. ABHD15 also forms a protein complex with cyclic AMP phosphodiesterase 3B (PDE3B) in 3T3-L1 adipocytes, and PDE3B protein expression is related with ABHD15[98]. Although those studies proposed ABHD15 as a substrate of Akt and an interacting protein with PDE3B in adipocytes. The *in vivo* study to confirm those assumptions and the further study on its physiological function are still missing.

2. Material and Methods

Reagents.

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. All cell culture reagents were purchased from Invitrogen. The following primary antibodies were used in this study: Anti-Flag and anti-βACTIN (Sigma), anti-HIS (GE Healthcare), anti-ABHD15 (ProteinTech). Anti-GAPDH, Anti-phospho-PKA substrate, anti-AKT, anti-phospho-AKT (Ser473), anti-HSL, anti-phospho-HSL (Ser660) antibodies were from Cell Signaling Technology. Anti-PDE3B antibody and pAcSG2-mPde3b plasmid were generous gifts from Dr. Vincent Manganiello (NHLBI, NIH) and Dr. Eva Degerman (Lund University). Abhd15-his and LacZ-his plasmids were constructed in our lab. Enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare. EDTA-free protease inhibitor cocktail (PIC) tablets, phosphatase inhibitor cocktail (PhosSTOP®) tablets, Collagenase D and Dispase II were purchased from Roche Diagnostics.

Western blot analysis.

Cell were lysed by scraping with RIPA buffer supplemented with PIC. Frozen tissues were minced and homogenized with an electrical homogenizer in RIPA buffer supplemented with PIC and PhosSTOP®. Cell or tissue lysates were incubated on ice for 30 min, then centrifuged at 16,000 g, 4 °C for 30 min and the clean middle layer were collected and frozen at -20 °C until usage. Protein concentrations were determined with the BCA protein assay kit (Pierce). Protein samples were diluted in sodium dodecyl sulfate (SDS) loading buffer and boiled for 10 min at 80 °C. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Individual proteins were detected with the specific

antibodies and visualized on film using horseradish peroxidase-conjugated secondary antibodies (DAKO) and ECL reagents with the G:Box detection system (Syngene).

Cell culture.

3T3-L1 fibroblasts (American Type Culture Collection) were cultured and differentiated as described previously[97]. Control non-targeting siRNA and siRNA directed against *Abhd15* were purchased from Sigma (MISSION siRNA NM_026185), Control non-targeting siRNA (cat mo. D-001810-10) and siRNA corresponding to murine *Pde3b* mRNA (cat no. L-043781-00) were purchased from (Dharmacon). 450,000 differentiated 3T3-L1 cells (5 days after differentiation start) were electroporated per 100 µL reaction with control siRNA or a mixture of siAbhd15 #1 and #2 (400 nM) using the Neon Transfection System (Invitrogen), at 1400 V, 20 ms, 2 pulse. Cells were harvested for lipolysis and western blotting 72 hours after electroporation. The cDNA of mouse Pde3b-flag was cut from pAcSG2-mPde3b plasmid with XhoI and inserted into a murine stem cell virus vector (pMSCVpuro; BD Biosciences Clontech). The generation of Pde3b-flag recombinant retrovirus was described before[99]. Viral supernatants were supplemented with 8 µg/mL polybrene and added to 3T3L-L1 cells (30 % confluence) for infection for 18-24 hours. Cells were selected with 3 µg/mL puromycin, expanded, and seeded for further experiments. If not otherwise stated, cells were used 7 days after induction of differentiation. Successful overexpression of PDE3B protein was confirmed by Western blot analysis. COS7 and BnICl.2 cells (American Type Culture Collection) were maintained in DMEM (4.5 g/L glucose, glutamine) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C, 5% CO_2. For transfection, 200,000 cells per well were seeded into 12-well plates, the cells were transfected with 1 µg DNA together with 2 µL metafectene overnight (o/n) in complete DMEM medium. 48 hours after transfection,

cells were harvested for co-immunoprecipitation or treated with 5 µg/mL cycloheximide (CHX) for indicated times.

Primary cell culture.

The isolation of adipocytes and stromal vascular cells (SVCs) was described previously[102] with the following modifications. 1g sWAT from 8-10 weeks old female mice was dissected, washed, minced, and digested in 1 mL PBS containing 0.125 U/mL Collagenase D, 2.4 U/mL Dispase II, 10 mM $CaCl_2$ (added just prior to digestion of the tissue) at 37 °C with constant agitation at 180 rpm for 25-30 min. To stop digestion, complete DMEM/F12 media containing Glutamax (LifeTechnology), 10 % FBS, 1% P/S was added to the digestion mixture then filtered through a 100-µm cell strainer to remove undigested tissue. The flow-through was centrifuge for 10min at 200 g, the floating adipocyte layer was collected for protein isolation, while the left medium and cells was re-suspended and filtered through a 70-µm cell strainer. After centrifugation for 10 min at 700 g, the cell pellet containing the SVCs was resuspended in complete DMEM/F12 and seeded on a 10-cm cell culture dish. At a confluency of ~80%, cells were propagated and seeded for further experiment. To re-express Abhd15 in KO-SVCs, 50,000 cells in 12-well plates were subjected to either pMSCV-puro or pMSCV-Abhd15 retrovirus medium (prepared as described above). 48 hours after reaching confluency, adipocyte differentiation was induced by using complete DMEM/F12 media supplemented with 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), 5 µg/mL insulin, and 1 µM rosiglitazone. Three days after induction, medium was changed to complete DMEM/F12 supplemented with 5 µg/mL insulin for two days, afterwards cells were maintained in complete DMEM/F12 medium. On day 7, fully differentiated cells were harvested for Western Blotting.

Animal Study

C57BL/6J mice were housed in groups of 4 in filter-top cages in a pathogen-free barrier facility. The animals were maintained in a 14 hours light/ 10 hours dark cycle, light on at 7:00 a.m., and had *ad libitum* access to food and water, except when food was restricted during fasting. The o/n fasting is around 12 to 14 hours during the dark cycle. At the age of 8-10 weeks, they were either fed a chow diet (calories 11 kJ% from fat, 53 kJ% from carbohydrates, and 36 kJ% from protein, #V1126, Ssniff Spezialdiäten, Germany) or put, at the age of 8-10 weeks on HGD (calories 7 kJ% from fat, 72 kJ% from carbohydrates, and 21 kJ% from protein, #E15629-34, Ssniff Spezialdiaten, Germany) or on HFD (Sniff, Germany, #E15744-34, 45 kJ% calories from fat, 35 kJ% from carbohydrates, and 20 kJ% from protein) until experiments were done. Tissues were collected after 12 weeks on the according diet, or mice were maintained on diets until experiments were finished.

Membrane isolation.

Cells from 10-cm dishes were washed with ice-cold PBS, then scraped in 1 mL hypotonic lysis medium (HLM) containing 50 mM HEPES, 50 mM sucrose, 1 mM EDTA, 100 mM NaCl and 1 x PIC and were lysed using a Dounce homogenizer (~50 strokes). Around 50 mg frozen tissue were minced in 1 mL HLM, and thoroughly dounced. Lysates were centrifuged at 5000 g, 4 °C for 10 min. The supernatant was centrifuged at 100,000 g, 4 °C for 30 min. The resulting supernatant represented the cytosolic fraction; membrane pellets were resuspended in RIPA buffer with 1 x PIC for Western blotting.

Co-immunoprecipitation.

Pde3b-Flag overexpressing cells in 35-mm dishes were washed twice with ice-cold PBS and then lysed in 1mL pulldown buffer (50 mM Tris-HCl pH 7.4-7.5; 300 mM NaCl;

1% Triton X-100; 1x PIC and PhosSTOP$^@$). Lysates were cleared from cell debris via centrifugation and protein content was measured by BCA as described above. 1 mg protein lysate was used for pulldown with Anti-FLAG M2 affinity gel (Sigma Aldrich) according to manufacturer's guidelines. After o/n incubation, beads were washed thoroughly and affinity-bound proteins were eluted by boiling the samples with 2x SDS lysis buffer (100 mM Tris/HCl pH 6.8, 10% glycerol, 2.5% SDS, 1 x PIC). Pulldown-products were directly subjected to Western blot analysis.

Free fatty acids and glycerol release

3T3-L1 pre-adipocytes were seeded and differentiated in 6-well plates. Fully differentiated 3T3-L1 adipocytes were wash twice with DPBS. 2% FFA-free BSA in DMEM medium (BSA medium) was pre-warmed in CO_2 incubator. Cell were incubated in 2 mL BSA medium in the presence or absence of 100 nM insulin for 120 min at 37 °C, 5% CO_2 and 95% humidified atmosphere. Thereafter, the medium was removed and used to measure NEFA (WAKO) and free glycerol (Sigma-Aldrich) release. For protein determination, cells were washed with PBS twice and 120 µL lysis buffer were added. Cell lysates were further used for western blotting.

RNA isolation and quantitative-RT-PCR

Total RNA from cells was isolated with TRIzol® reagent (Invitrogen) according to the manufacturer's protocols. cDNA was generated using the cDNA Reverse Transcription Kit (Thermo Fisher Scientific). mRNA expression was assessed using real-time PCR using the StepOne Plus Detector system and SYBR Green PCR master mix (Invitrogen). Gene expression was normalized to *βactin*. Relative mRNA expression levels were calculated using averaged 2^{-ddCt} values for each biological replicate[103]. Primers used for qRT-PCR:

Abhd15 (Forward-TATGAACGTGGGTTCTTGCT, Reverse-

TTGGTGTGACAGAACAGGGT);

Pde3b (Forward-CGTCTGTCATATGTAGCA, Reverse-

GTGTCATCTGTTCTGTTTG);

Acsl1 (Forward-TCCTACAAAGAGGTGGCAGAACT, Reverse-

GGCTTGAACCCCTTCTGGAT);

Dgat1 (Forward-GACGGCTACTGGGATCTGA, Reverse-

TCACCACACACCAATTCAGG);

Acc1 (Forward-TGACAGACTGATCGCAGAGAAAG, Reverse-

TGGAGAGCCCCACACACA);

Fasn (Forward- GCTGTAGCACACATCCTAGGCA, Reverse-

TCGTGTTCTCGTTCCAGGATC).

Statistical analysis

If not otherwise stated results are mean values ± SEM of at least three independent experiments or results show one representative experiment out of at least three. Statistical significance was determined using the unpaired 2-tailed student's t-test or the two-way ANOVA test. For statistical analysis GraphPad prism software was used. * ($p < 0.05$), $$, ** ($p < 0.01$), *** ($p < 0.001$).

3. Results

3.1 The expression pattern of Abhd15 protein *in vivo* and *in vitro*.

In line with previously reported *Abhd15* mRNA expression[99,100], ABHD15 protein was mainly expressed in 3T3-L1 adipocytes and adipose depots, followed by a weak expression in liver (Figure 1A). ABHD15 was not detected in skeletal (SM) and cardiac muscle (CM) (Figure 1A). In WAT, ABHD15 was only expressed in mature adipocytes and primary adipocytes differentiated from stromal vascular cells (SVCs), but not in undifferentiated SVCs, suggesting that ABHD15 is not expressed in macrophages (Figure 1B). In mouse WAT, ABHD15 expression was reduced after o/n fasting, while it is significantly increased upon 1 and 2 hours of refeeding (Figure 1C-D). In comparison to chow diet (CD)-fed C57BL/6J mice, 12 weeks of either high fat (HFD, 45% fat) or high glucose diet feeding (HGD, 50% glucose) massively induced ABHD15 protein expression in WAT (Figure 1E-F). The abundant expression of ABHD15 in adipose tissue and its robust increase upon HGD/HFD feeding indicating that this protein may play an important role in AT function and diet related diseases. Previously, we showed that *Abhd15* mRNA expression is upregulated during 3T3-L1 adipocyte differentiation and regulated upon obesity, aging, and varying fatty acid concentrations in mice and murine cells[99]. Here, we confirm that ABHD15 protein expression is detectable 2 days after adipogenic induction, and maximal expression is reached in fully differentiated 3T3-L1 adipocytes (Figure 1G-H). Like in differentiating 3T3-L1 cell line, ABHD15 expression was also increasing during the beige adipocytes differentiation of SVCs (Figure 1I). Thereby, in this study we applied animal models and cell line models to comprehensively investigate the contribution of ABHD15 to AT function.
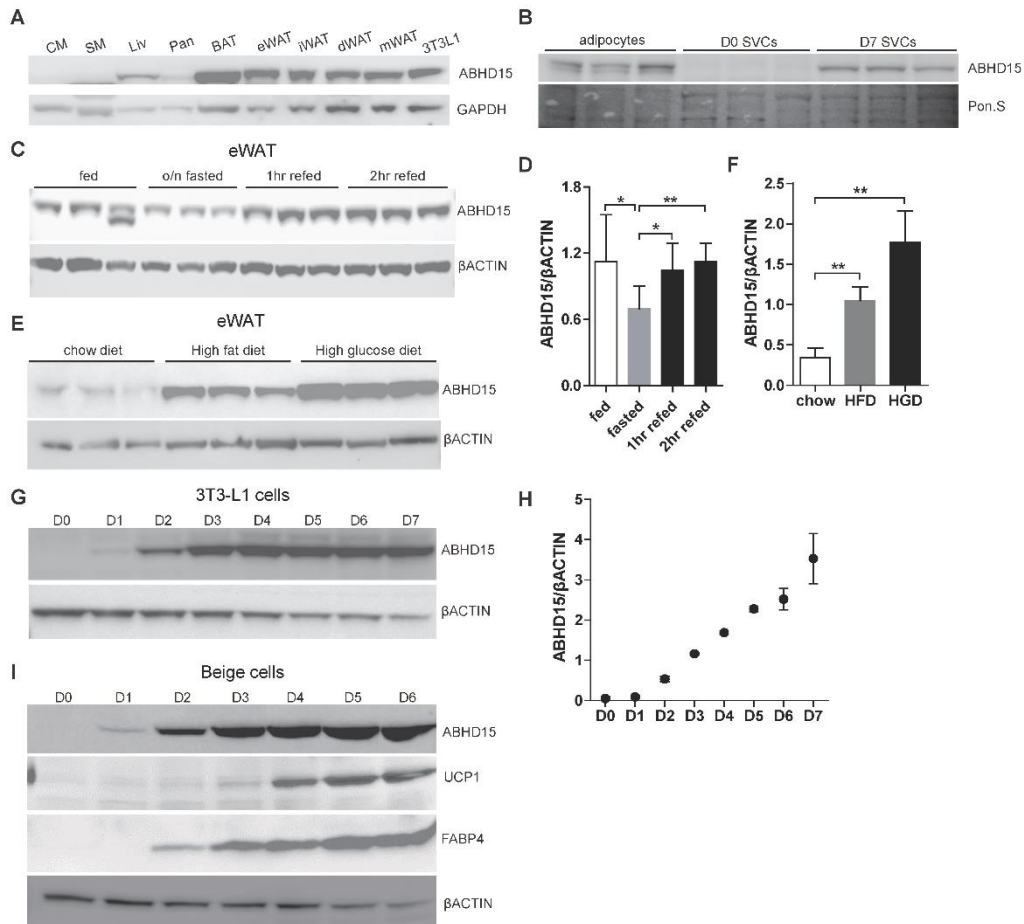
Figure 1. The expression profile of ABHD15 in vivo and in vitro. (A) ABHD15 expression in cardiac muscle (CM), skeletal muscle (SM), liver (Liv), pancreas (Pan), brown AT (BAT), eWAT, inguinal WAT (iWAT), dorsal WAT (dWAT), and mesenteric WAT (mWAT) of 18 weeks old CD fed C57BL6/J mice and in 3T3-L1 adipocytes. (B) ABHD15 expression in mature adipocytes and SVCs isolated from sWAT of 10 weeks old female C57BL6/J mice. SVCs were differentiated into adipocytes and harvested on day 7 (n = 3). (C&D) ABHD15 expression in eWAT of 18 weeks old CD fed C57BL6/J mice harvested at fed ab libitum, o/n fasted, 1 hour refed and 2 hours refed states. (E&F) ABHD15 expression in eWAT of 20 weeks old C57BL6/J mice fed with CD, HGD, HFD for 12 weeks. (G&H) ABHD15 expression in murine 3T3-L1 cells during differentiation from day 0 to 7 (D0-D7). One representative replicate is shown. (I) ABHD15 expression in SVCs during the differentiation into beige cells. *<0.05, **<0.01.

3.2 The impact of ABHD15 on lipid metabolism *in vitro*.

To apply a different but complementary approach to study lipolysis in 3T3-L1 adipocytes, we generated the *Abhd15* (or puro as control) stable overexpressing 3T3-L1 fibroblast cell line. The adipogenic markers, lipogenic genes expression and lipid accumulation in ABHD15 overexpressed cells didn't differ from control cells during differentiation (Figure 2A-E). Overexpression of ABHD15 *per se* had no influence on basal or stimulated (with insulin or isoproterenol) FA and glycerol release (Figure 2F-G), when under the co-treatment of insulin and 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor, Abhd15 overexpressing adipocytes had higher glycerol release than control cells (Figure 2G).
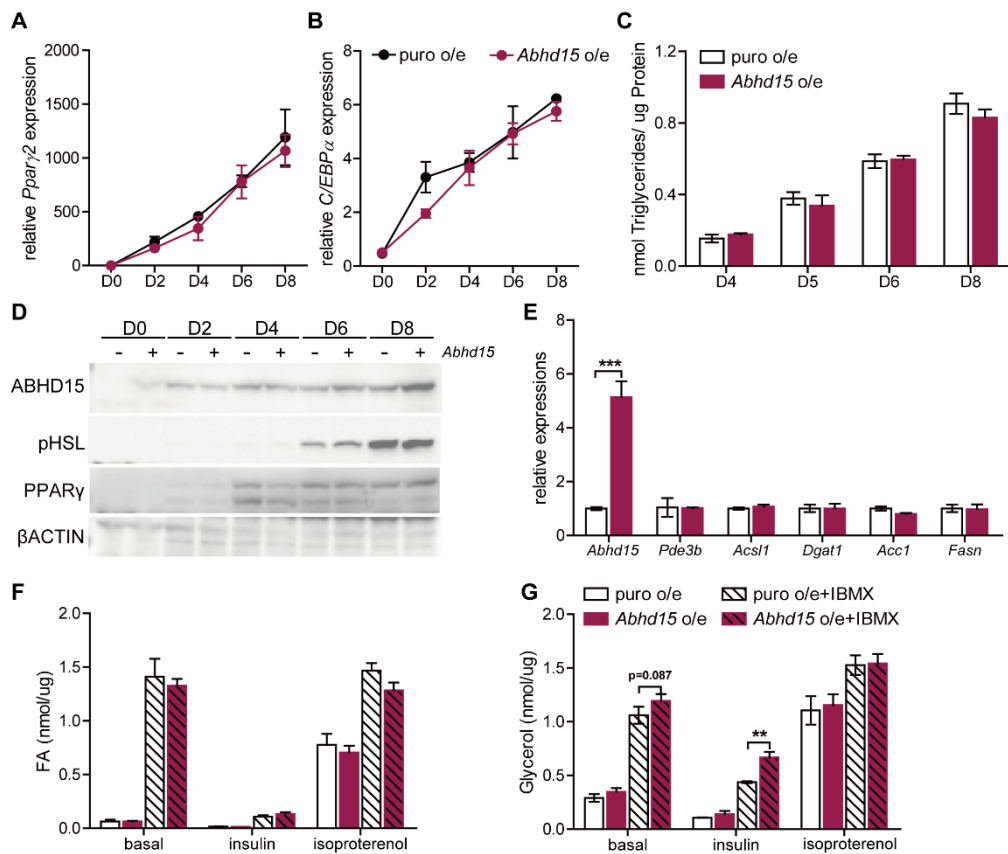
Figure 2. The overexpression of Abhd15 in 3T3-L1 adipocytes. (A-B) Relative Pparγ2 and C/EBPα expression during the differentiation of cells (day 0-8) (n = 3). (C) Intracellular TG content during the differentiation of cells (day 4-8) (n = 3). (D) PPARγ and phosphorylated HSL expression during the differentiation. (E) Relative genes expression in fully differentiated cells (day 8). (F-G) FA and glycerol release in control and Abhd15 o/e cells under basal, insulin and isoproterenol stimulation with or without 50 µM IBMX treatment (n = 3). **<0.01, ***<0.001.

3.4 The regulation and association between ABHD15 and PDE3B *in vitro*.

In accordance with mRNA expression, loss of ABHD15 markedly reduced PDE3B protein expression in eWAT (Figure 3A-B) and fully differentiated SVCs of *Abhd15*-ko mice when compared to controls (Figure 3C-D). Partial loss of ABHD15 in heterozygous *Abhd15*-ko mice (Figure 3A-B) and in differentiated 3T3-L1 cells that were transiently *Abhd15*-silenced was not sufficient to reduce PDE3B levels (Figure 3E-G). Notably, re-expression of ABHD15 in Abhd15-ko SVCs in vitro was able to rescue PDE3B expression by around 50% (Figure 3H-I). Chavez and co-workers[98] suggested that uncomplexed PDE3B undergoes degradation faster than PDE3B complexed with ABHD15. To directly test the stabilizing effect of ABHD15, we analyzed PDE3B protein stability by treating PDE3B overexpressing COS7 cells with CHX which resulted in PDE3B degradation within less than 2 hours (Figure 3J, left blot). Remarkably, co-expression of ABHD15 prevented its degradation and stabilized PDE3B protein throughout the treatment (Figure 3J, right blot).

Although interaction of ABHD15 with PDE3B was proposed[98], here we confirmed the interaction again with a specific antibody for ABHD15, and we also confirmed this interaction in COS7 cells and Bnlcl2 hepatocytes (Figure 3K-L). Fractionation experiments revealed that ABHD15, like PDE3B, is a membrane-associated protein (Figure 3M), making an interaction of these proteins even more likely. Interestingly, silencing of PDE3B in fully differentiated 3T3-L1 cells also reduced the expression of ABHD15 (Figure 4C) indicating that ABHD15 and PDE3B regulate each other's protein expression and/or stability presumably via complex formation.
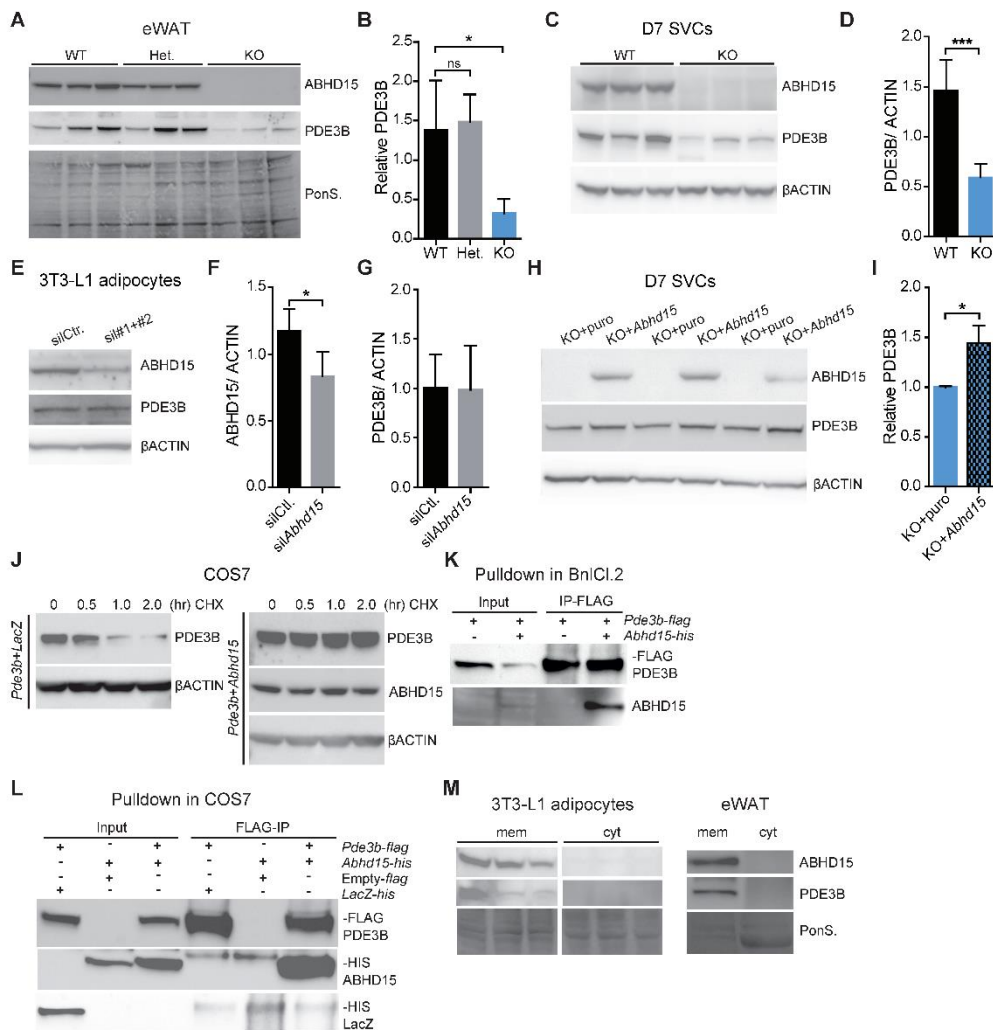
Figure 3. ABHD15 associated with PDE3B and regulates its stability and expression. (A&B) ABHD15 and PDE3B expression in the membrane fraction of eWAT from WT, heterozygous (Het), and homozygous (KO) *Abhd15*-ko mice. (C&D) ABHD15 and PDE3B expression in fully differentiated (day 7) SVCs. (E-G) ABHD15 and PDE3B expression in control and *Abhd15* silenced 3T3-L1 adipocytes. One representative WB is shown (n = 6). (H&I) ABHD15 and PDE3B expression in puro and *Abhd15* re-expressed fully differentiated *Abhd15*-ko SVCs. (J) ABHD15 and PDE3B expression in COS-7 cells co-transfected with *Pde3b* and *LacZ* or *Pde3b* and *Abhd15*, treated with or without 5 µg/mL CHX for 0-2 hours. (K&L) Pull down of ABHD15 and PDE3B in BnlCl2 and COS-7 cells. (M) ABHD15 and PDE3B expression in the membrane faction of 3T3-L1 adipocytes and eWAT. *<0.051, ***<0.001.

3.4.2 The presence of PDE3B for the ABHD15 effect on lipolysis.

To further investigate how the interaction between ABHD15 and PDE3B affect the lipolysis, we silenced *Pde3b* transiently in the *Abhd15* overexpressing 3T3-L1 adipocytes. Both PDE3B mRNA and protein were strongly reduced after 72 hours of *Pde3b*-siRNA (si*Pde3b*) electroporation (Figure 4A-E). Intriguingly, the knocked down PDE3B protein also reduced ABHD15 protein levels in both puro and *Abhd15* overexpressing adipocytes (Figure 4C-E). Despite the successful deletion of PDE3B in ABHD15 overexpressed adipocytes, there is no defect on FA and free glycerol release under basal and insulin conditions (Figure 4F-G). Comparing to the basal state, insulin treatment strongly reduced the phosphorylation of HSL and PKA substrate (Figure 4G). Silencing of Pde3b slightly increased the phosphorylation of HSL and PKA substrate in the puro overexpressing cells when treated with insulin, however, this mild effect is not seen in the ABHD15 overexpressing cells (Figure 4G). Those weak changes might due to the complete effect of PDE3B is highly dependent on the lipolytic state which means that the insulin inhibits lipolysis via PDE3B only at stimulated condition (e.g. isoproterenol, forskolin or CL-314,263), not at basal or suppressed condition (e.g. insulin).
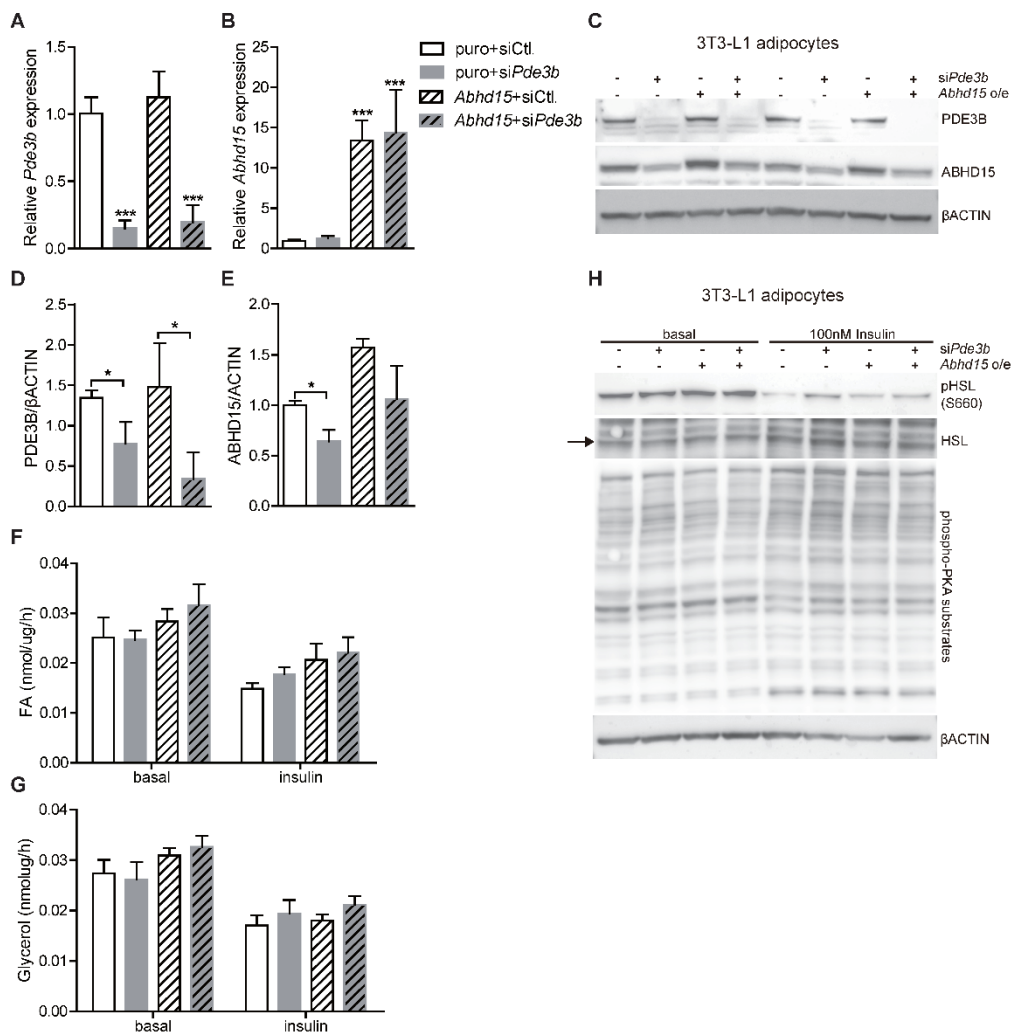
Figure 4. The impact of ABHD15 on lipolysis in the absence of PDE3B. (A&B) Relative *Abhd15* and *Pde3b* expression in adipocytes 48 hours after electroporation (n = 3). (C-E) ABHD15 and PDE3B expression in adipocytes 72 hours after electroporation (n = 4). (F-G) FA and glycerol release from cells 72 hours after electroporation; cells were incubated with 2% BSA medium for 3 hours (basal) or pre-treated with 100 nM insulin in 2% BSA medium for 1 hour and incubated with insulin for 2 hours (n = 3). (H) 72 hours after electroporation cells were serum starved for 6 hours, treated with or without 100nM insulin for 15 min and then harvested for western blotting. Arrow indicate the specific band of HSL. *<0.05, ***<0.001.

3.6 The impact of ABHD15 on PDE3B activity.

In addition to the evidence of reduced PDE3B protein and mRNA levels in *Abhd15* knockout cells, we also explored the impact of ABHD15 on PDE3B activity. Since both ABHD15 and PDE3B are membrane enriched proteins, we isolated the membrane fraction of Abhd15 and control plasmid (HMC) overexpressed COS7 cells and re-suspended the membrane lysates in PDE3B activity assay buffer. Immunoblotting confirmed the enrichment of ABHD15 protein in membrane lysates (Figure 5A). The PDE3B activity assay showed that IBMX, a selective PDE3 inhibitor, significantly reduced PDE3B enzyme activity. However, compared to the control lysates, ABHD15 lysates did not change PDE3B enzyme activity (Figure 5B). We further analyzed PDE3B activity with eWAT tissues from *Abhd15-ko* and WT mice injected with saline or insulin for 1 hour. However, we did not observe differences between WT and *Abhd15*-ko mice (Figure 5C). Even though the impact of ABHD15 on PDE3B activity remains elusive, *Abhd15*-ko lysates showed trend of reduced PDE3B activity. Further sensitive activity assay needs to be applied.
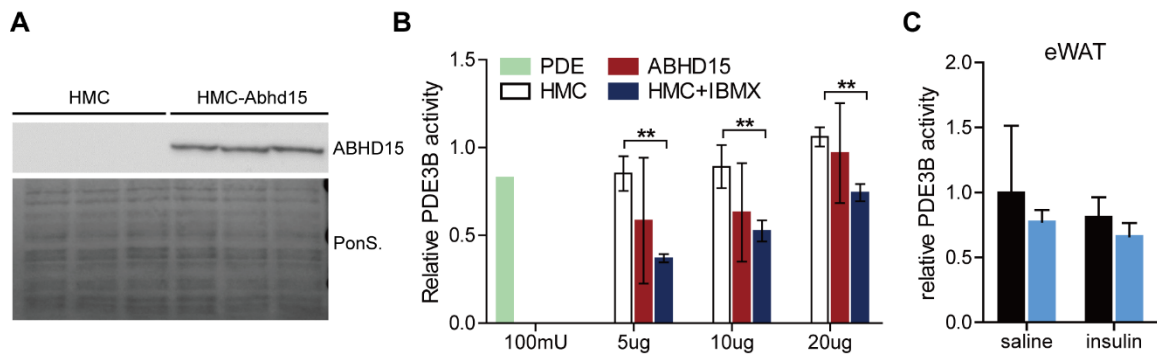
Figure 5. PDE3B activity assay. (A) ABHD15 expression in the membrane lysates used for PDE3B activity assay. (B) Relative PDE3B activity with different amount of lysate from HMC or HMC-Abhd15 transfected COS7 cell, IBMX was used as a negative control. (C) PDE3B activity in the membrane fractions of eWAT from Abhd15-ko and WT mice injected with saline or insulin.

4. Discussion

Dynamic metabolic transition from the fasting to the feeding state and vice versa is of great importance for maintaining energy homeostasis *in vivo*. The major part of my thesis is to investigate the physiological function of ABHD15 in WAT based on global and conditional knockout animal models. ABHD15 belongs to the a/b-hydrolase superfamily which consist of various lipase, esterase, and proteases that share a common structural feature[100]. Typically, ABHD proteins harbour a catalytic triad build up with a nucleophile (Ser, Cys, or Asp), an acid (aspartate or glutamate), and a conserved histidine residue enabling hydrolase activity. However, ABHD15 lacks the nucleophile; therefore a hydrolytic activity is less possible. Moreover, ABHD15 misses the Ser-X4-Asp motif, making a prediction of its enzymatic function difficult. Although there are several studies proposed ABHD15 as a substrate of Akt and an interacting protein with PDE3B in adipocytes. The *in vivo* study to confirm those assumptions and the further study on its physiological function are still missing.

In the global and adipose tissue specific knockout models, we reported ABHD15 as a crucial player in regulating insulin-mediated suppression of lipolysis and the development of insulin resistance. ABHD15 is highly expressed in ATs, which are the main organs where lipolysis occur. We have previously shown that *Abhd15* mRNA expression in WAT is decreased in fasted mice and in genetically obese mice with T2D[99]. In this thesis, we further confirmed that ABHD15 protein is reduced by fasting and induced by refeeding in the WAT of lean and healthy mice. Moreover, *ABHD15* expression is diminished in the WAT of patients with obesity and diabetes when compare to the patients with obesity and normal glucose tolerance. In addition, we showed that *ABHD15* mRNA expression negatively correlated with markers of insulin resistance in humans.

In all conditions associated with reduced ABHD15 expression, plasma FA levels are elevated. Plasma FA concentrations are tightly controlled by lipolysis, a process turned on by fasting to provide FAs as energy source for peripheral tissues, while turned off upon (re)feeding by insulin signaling. Elevated circulating FA levels contribute to insulin resistance in both animals and humans[3,4,8,80,81]. Recently, it has been reported that the failure of insulin to decrease the supply of FAs as substrate for liver gluconeogenesis leads to systemic insulin resistance[82]. Although many aspects have been clarified, the detailed molecular mechanisms how insulin suppresses lipolysis in adipocytes is still elusive. Since it became increasingly evident that elevated plasma FA levels lead to ectopic lipid deposition and insulin resistance, the search for regulators of insulin-mediated inhibition of lipolysis might pave the way for therapeutic applications. We therefore used constitutive and AT-specific Abhd15-ko mice to test our hypothesis that ABHD15 is an important player in insulin-mediated suppression of lipolysis and the development of insulin resistance.

Loss of ABHD15 resulted in a failure of insulin to decrease plasma FA levels at both experimental (insulin-injected) and physiological (oral glucose intake) conditions independent of diet and age. However, plasma glycerol concentrations were unchanged in these mice. The underlying reason(s) for this discrepancy between reduced FA but normal glycerol levels might due to the increased uptake by liver. We hypothesize that elevated FA concentrations upon *Abhd15* deletion are at least partially due to the simultaneous reduction of PDE3B in WAT, as the unsuppressed lipolysis phenotype of our Abhd15 knockout mouse models was also described in Pde3b-null mice [83]. Consistently, it has been previously suggested that ABHD15 binds to PDE3B and regulates its expression in 3T3-L1 adipocytes[98]. However, this former study used an antibody generated with a peptide directed against 15 AA at the very C-terminus of ABHD15 that also shows homology with ABHD1 and ABHD3. We used an

antibody directed against the whole protein sequence and confirmed in various cell lines that ABHD15 co-immunoprecipitates with PDE3B. Within this complex, ABHD15 likely regulates PDE3B expression and/or protein stability as its expression is also impaired in WAT of Abhd15-ko mice. A regulation of PDE3B by ABHD15 is also supported by the fact that both proteins are expressed as membrane proteins. Importantly, we could rescue PDE3B expression by re-expressing ABHD15 in differentiating Abhd15-ko SVCs. Supporting our hypothesis, PDE3B protein stability is massively increased when ABHD15 is co-expressed. Vice versa, also PDE3B seems to influence ABHD15 stability, since ABHD15 protein expression is decreased in Pde3b silenced 3T3-L1 cells.

Phosphorylation by PKB/Akt or PKA was shown to increase the activity of PDE3B[104,105]. However, disrupted phosphorylation of PDE3B on both PKB/Akt and PKA phosphorylation sites did not interfere with insulin-mediated suppression of lipolysis[84]. Therefore, we did not further investigate PDE3B phosphorylation in our mouse models, but suggest that ABHD15 plays a crucial role in regulating the amount of PDE3B protein and downstream lipolysis. In adipocytes, activated/phosphorylated PDE3B has also been shown to form large macro-molecular complexes, so-called "signalosomes", with IRS, PI3K, PKB/Akt, caveolin-1, and protein phosphatase 2 (PP2A)[93,95,104,106]. It is, however, unlikely that the reduced amount of PDE3B in Abhd15-ko mice regarding signalosome formation affect the insulin signaling pathway as Pde3b-ko adipocytes neither show reduced Akt phosphorylation nor reduced glucose uptake[83,84]. Nevertheless, reduced PDE3B protein might impact the formation of the signalosome and its localization to the lipid droplet and therefore impact insulin-suppressed lipolysis. Decreased but also increased adipocyte lipolysis have been shown to improve insulin resistance[107-112]. In humans with obesity, however, persistently elevated circulating FA concentrations can account for a large part of insulin resistance[5,10,113]. Unrestrained

lipolysis in humans with mutations in perilipin 1 leads to severe insulin resistance[3,4] underlining a role of increased plasma FA concentrations in the pathogenesis of this disease. Pharmacological inhibition of ATGL or HSL also improves insulin sensitivity in genetically or diet-induced obesity[5,111]. Accordingly, Abhd15-ko mice might develop insulin resistance due to unsuppressed adipocyte FA release.

ABHD15 has been firstly identified as a novel Akt substrate and named as pp47[101]. By that time, the function of this protein was unknown. Later, pp47 has been designated as AS47, Chavez *et al* found that AS47 associates with PDE3B and regulates its protein level in adipocytes[98]. Since we published *Abhd15* as a direct target of PPARγ, this 47 kDa Akt substrate has been named ABHD15[99]. Knockdown of ABHD15 in 3T3-L1 fibroblasts reduced PDE3B protein level, while the authors did not describe whether the cells differentiated well[98]. However, our data showed that silencing of *Abhd15* with shRNA in lentivirus causes strongly defects in 3T3-L1 cells differentiation[99], which might be the cause for reduced PDE3B. When ABHD15 was transiently knocked down in 3T3-L1 adipocytes, there are no effects on lipid accumulation and expression of adipocytes markers[99]. More importantly, neither partial loss nor transient knockdown of ABHD15 in adipocytes was sufficient to reduce PDE3B level. In adipocytes, activated/phosphorylated PDE3B has also been shown to form large macro-molecular complexes, so-called "signalosomes", with IRS, PI3K, PKB/Akt, caveolin-1, and protein phosphatase 2 (PP2A)[93,95,104,106]. It is, however, unlikely that the reduced amount of PDE3B in Abhd15-ko mice regarding signalosome formation affect the insulin signaling pathway as Pde3b-ko adipocytes neither show reduced Akt phosphorylation nor reduced glucose uptake[83,84]. Nevertheless, reduced PDE3B protein might impact the formation of the signalosome and its localization to the lipid droplet and therefore impact insulin-suppressed lipolysis.

Decreased but also increased adipocyte lipolysis have been shown to improve insulin resistance[107-112]. In humans with obesity, however, persistently elevated circulating FA concentrations can account for a large part of insulin resistance[5,10,113]. Unrestrained lipolysis in humans with mutations in perilipin 1 leads to severe insulin resistance[3,4] underlining a role of increased plasma FA concentrations in the pathogenesis of this disease. Pharmacological inhibition of ATGL or HSL also improves insulin sensitivity in genetically or diet-induced obesity[5,111]. Accordingly, Abhd15-ko mice might develop insulin resistance due to unsuppressed adipocyte FA release.

Glucose uptake and DNL in WAT are largely dependent on the insulin/Akt/Glut4 pathway[114]. Interestingly, in WAT of Abhd15-ko mice, phosphorylation of Akt is decreased upon insulin injection and thus might be responsible for the reduced glucose uptake and incorporation into de novo synthesized lipids. In adipocytes, glucose uptake controls DNL, which plays a crucial role in whole body insulin sensitivity[49,115]. Improved insulin sensitivity in HFD-fed heterozygous Hsl-ko mice was associated with increased DNL[5]. Thus, it is also conceivable that the reduced DNL observed in our mouse models contributes to the development of insulin resistance. Furthermore, reduction of adiponectin levels, an early indicator for insulin resistance[116,117], might add to the observed phenotype.

It has been suggested that PDE3B is involved in the secretion of adiponectin in adipocytes by an unknown mechanism[118]. PDE3B is part of the signalosome that also contains PP2A. Very recently, Hatting et al showed that PP2A inhibition increases CREB phosphorylation[119]. pCREB activates ATF3 and this transcription factor inhibits the expression of adiponectin and Glut4, thereby leading to insulin resistance[120]. PDE3B is reduced in adipocytes from patients with diabetes[121] and Pde3b-ko mice develop insulin resistance[83]. These mice, however, have elevated adiponectin levels and the insulin resistance was accounted to their hepatic and pancreatic phenotype[83].

In contrary, our data reveal that the insulin resistance in Abhd15-ko mice is mainly due to the AT phenotype. Although Abhd15-ko mice only show ~50% reduction in PDE3B expression, their WAT phenotype on the molecular level is much stronger than the one observed in Pde3b-ko mice. Underlining these observations, the AdipoQ-Abhd15-ko mouse phenocopies the global Abhd15-ko mouse despite undiminished PDE3B expression in any other tissue than ATs. Thus, we hypothesize that ABHD15 signaling has a mechanism apart from the regulation of PDE3B that warrants future studies.

Together, those data demonstrate that ABHD15 is indispensable for insulin-mediated suppression of lipolysis and its ablation leads to insulin resistance in mice. These findings together with the reduced *ABHD15* expression in humans with obesity and diabetes identify ABHD15 as a potential therapeutic target for treating age- and obesity-associated insulin resistance.

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