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Fatty acid transport across cell membranes

Slc43a3 is a regulator of free fatty acid uptake and efflux

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Contents

Abstract

Adipocytes take up long chain fatty acids through diffusion and through a low capacity protein mediated transport system on the plasma membrane, whereas fatty acid efflux is considered to occur by diffusion. Although studies have revealed the importance of fatty acid transport proteins (FATPs), long chain acyl coenzyme A synthetases (ACSLs) and fatty acid translocase (FAT, also known as CD36) in fatty acid uptake, other membrane proteins could be involved, and no membrane proteins have been associated with fatty acid efflux. To identify potential membrane proteins that are involved in regulating fatty acid flux in adipocytes, the expression levels of 55 membrane transporters without known function were screened in subcutaneous adipose samples from obese patients before and after bariatric surgery using branched DNA methodology. Among the 33 solute carrier (SLC) transporter family members screened, the expression of 14 members showed significant changes before and after bariatric surgery. One of them, Slc43a3, increased about 2.5-fold after bariatric surgery. The expression levels of Slc43a3 in various mouse adipose depots and during adipocyte differentiation were explored in C57Bl6J mice and OP9 murine adipocyte cells. The functional significance of Slc43a3 in regulating fatty acid transport was studied using overexpression or silencing of Slc43a3 in differentiated OP9 cells. Slc43a3 is highly expressed in adipose tissue and induced during adipocyte differentiation in OP9 cells. Knockdown of Slc43a3 with siRNA in differentiated OP9 adipocytes reduced both basal and forskolin-stimulated fatty acid efflux. Interestingly, knockdown of Slc43a3 with siRNA in differentiated OP9 adipocytes also increased fatty acid uptake and lipid droplet accumulation. In contrast, overexpression of Slc43a3 decreased fatty acid uptake in differentiated OP9 cells and resulted in decreased lipid droplet accumulation. Therefore, Slc43a3 seems to regulate fatty acid flux in adipocytes, functioning as a positive regulator of fatty acid efflux and as a negative regulator of fatty acid uptake.

1 Introduction

1.1 Functions of fatty acids

In general, fatty acids (FAs) have a negative connotation since they are known to be related not only to cardiovascular disease, but also to influence metabolic diseases such as type 2 diabetes, inflammatory diseases and cancer (1). Nevertheless, FAs play important roles in a variety of cellular functions, such as the production and storage of energy, synthesis of phospholipids, glycolipids, and cellular signaling messengers, modification of proteins for targeting to cellular membranes, and regulation of gene expression (2–4).

FAs are built of hydrocarbon chains, which may differ in length as well as in their degree of unsaturation. They are composed of a methyl group on one and a carboxyl group on the other end. FAs are considered saturated, if there are only single bonds between neigboring carbons in the hydrocarbon chain. Unsaturated FAs have carbon-carbon double bonds and can be further classified in monounsaturated (only one double bond) and polyunsaturated FAs (multiple double bonds) (5).

FAs are stored as triacylglycerols (TAG), a chemical compound derived from glycerol and three fatty acids, in adipocytes during energy abundance and released into the plasma during times of energy need (6). While stored in adipocytes, FAs are an important source of thermal and electrical insulation, as well as protection against mechanical compression. Since “a gram of nearly anhydrous fat stores more than six times as much energy as a gram of hydrated glycogen” it is not surprising that “TAG rather than glycogen were selected in evolution as the major energy reservoir” (2). In order to function as an energy source, FAs are released from TAG and degraded to FAs and glycerol, which are released from the adipose tissue and carried to energy-requiring tissues. To make sure that the FAs reach their assigned tissue, they are bound to one of the following five lipoproteins: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) or HDL high density lipoproteins (HDL). This form of transport also assures that the hydrophobe lipids are not precipitating in the blood. The lipoproteins consist of a core of hydrophobe lipids which is surrounded by a coat of more hydrophile lipids and proteins (the so-called apolipoproteins). The demanding cell must

then “free” the FAs from their transport vehicles. This step is mainly performed by the hormone-sensitive lipase (HSL) which gradually separates the FAs from the glycerol by hydrolysis. For further processing the FAs are being activated and then transported into mitochondria for degredation. In the mitochondria the FAs are broken down into acetyl-CoA, which then will be fed into the citric acid cycle for energy production (2).

Another main task of FAs is the composition of the plasma membrane (PM). The PM surrounds the cytoplasm and therefore seperates the inside of the cell from its environment. It consists of a lipid bilayer, allowing the cell to control the extent of communication and exchange of substances between the intracellular and extracellular space. It is built of various biological molecules, especially lipids and proteins. With an amount of more than 50%, phospholipids (typically composed of two FAs and a hydrophilic head comprised of a phosphate group) are the major component of the PM. Glycolipids (lipids containing sugar) account for 5-10% of lipid in the PM and are vital for intracellular communication.

FAs are both: modulators and messengers in signal transduction. They are particulary involved in the communication at the level of the PM. FAs activate and/or inhibit enzymes and proteins of the cyclic adenosine monophosphate (cAMP) and the protein kinase C signalling pathways as well as those involved in ion fluxes and mobilization (7). As “secondary messengers” lipid molecules (especially in form of arachidonic acids) can bind to intracellular enzymes and function as an arbitrator between the extra- and intracellular space.

The modification of proteins by attachment of FAs occurs on many proteins of various structure and function. The two most common types of fatty acylation of proteins are modifications with myristate (14-carbon saturated FA) and palmitate (16-carbon saturated FA). Whereas the major function of myristate is to help in guiding the modified protein to the PM, palmitate serves as an important driving force for proteins to connect with lipid rafts (specialized membrane microdomains) (8,9).

One of the other key functions of FAs is the regulation of the expression of genes involved in lipid and energy metabolism, cellular proliferation and apoptosis. Polyunsaturated FAs for example reduce the expression of genes

involved in FA and cholesterol synthesis (10).

1.2. Transport of fatty acids across the plasma membrane

Most cells, with the exception of adipocytes, have a very limited capacity for storing FAs, in the form of triglycerides (or for de novo synthesis of FA), therefore circulating plasma FAs are the most important source of FA for most tissues (11). Short and medium chain FAs have high PM permeability and can diffuse freely and easily through the PM of cells. Long chain FAs (LCFAs), however, have much lower water solubility. Hence LCFAs are bound to albumin extracellularly and to fatty-acid-binding-protein (FABP) intracellularly in order to be buffered for their extremely low aqueous concentration (12). The transport of FAs into and out of adipocytes is controlled by a complex series of proteins and enzymes and influenced by a variety of metabolic and hormonal factors (6).

1.2.1 Fatty acid uptake

Different rates of FA uptake are observed among various cell types, with metabolically active cells having increased rates of FA uptake (13). The rates of cellular FA uptake are also regulated acutely and chronically by hormones (such as insulin) and metabolic status (in case of obesity) (14,15).

The uptake of LCFAs involves multiple steps. First, the LCFA is separated from serum albumin, before diffusing through the outer aqueous phase. After an attachment of the LCFA to the outer leaflet of the PM, a translocation or flip-flop from the outer to the inner leaflet occurs. The LCFA then gets released from the inner leaflet into the inner aqueous phase. The predominantly discussed step of this process is the fatty acid translocation and the question if it is diffusion- or protein-mediated (6). In addition to the bidirectional flip-flop model for transporting FAs across the plasma membrane, studies have demonstrated the importance of specific LCFA transport systems in metabolically active tissues, such as intestine, heart, adipose tissue, and the liver (16). This transport appears to involve membrane proteins that can mediate FA uptake via a rapid, saturable, substrate specific and hormonally related mechanism.

In the heart, the use of the main metabolic substrates: glucose and LCFA is not only regulated by substrate availability and competition at the level of mitochondria (Randle-cycle), but also at the site of cellular entry. CD36 is

responsible for the majority of LCFA uptake into the cardiomyocyte. CD36 is an integral membrane protein and belongs to a family of class B scavenger receptors. Moreover, CD36 is thought to bind many ligands like collagen, thrombospondin, sickle cell erythrocytes, oxidized low-density lipoproteins and apoptotic cells (6). Nowadays, CD36 is known to be the main protein involved in FA transport into adipocytes, enterocytes and cardiac/skeletal myocytes (17). In the heart, the translocation of CD36 from intracellular compartments to the sarcolemma (PM of a striated muscle fiber cell), has been found to be responsible for stimulus-induced LCFA uptake. High contractile activity and circulating insulin are the two leading stimuli responsible for an acute increase in cardiac substrate uptake (18). Whereas conditions such as obesity and diabetes are chronically regulating the LCFA uptake (15).

In adipocytes, permeation of LCFAs across the plasma membrane relies on a high affinity, low capacity protein facilitated transport system. Studies have shown the significance of several different proteins, such as FATPs (in adipocytes predominantly FATP1), ACSLs and CD36, in the protein-facilitated process of FA transport.

Fatty acid transport proteins (FATPs, SLC27A1-6) facilitate the transport of LCFAs across the PM and intracellular membranes. They are a group of six related integral membrane proteins that are expressed in all cells with high levels of FA uptake and active lipid metabolism. The mRNA expression and/or protein levels of FATPs are regulated by hormones, inflammatory mediators, such as endotoxin, tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1) and by activators of the peroxisome proliferator activated receptor (PPAR) alpha or gamma (19).

Long chain acyl coenzyme A synthetases (ACSLs) are enzymes of the ligase family that activate the breakdown of complex FAs, by catalyzing the formation of fatty acyl-CoA, so they can enter their designated metabolic pathway. ACSLs consist of five different isoenzymes, each in charge of different tasks regarding their expression in particular tissues. Besides being linked to various diseases (fatty liver disease, atherosclerosis, obesity and diabetes), they also regulate apoptosis and cell proliferation through special mechanisms (20,21).

Even though there are numerous studies and discussions on how FAs traverse the PM to enter the soluble cytoplasm, the exact mechanisms are not yet fully understood (22). Specifically, there is a debate on the rate-limiting step in the overall process of FA uptake and if, and to what extent, one or more membrane-associated proteins could facilitate and/or regulate FA uptake.

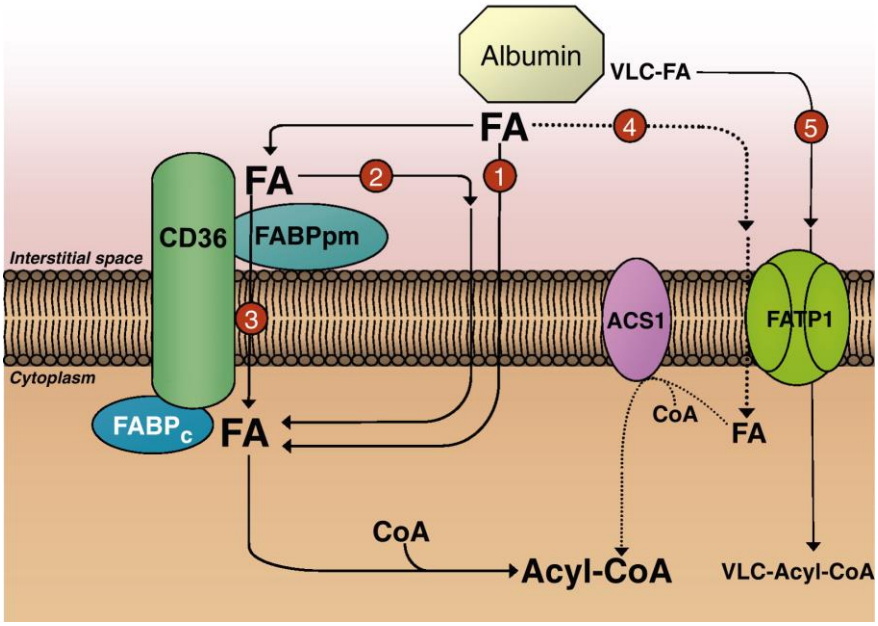


Figure 1. Schematic representation of the current view of FA transport across the cell membrane.

Because the exact mechanism of transmembrane translocation of FAs is still unknown, different models have been suggested. **(1)** In view of their hydrophobic nature, FAs could cross the membrane by simple diffusion. **(2)** Alternatively, CD36 (88 kDa; also referred to as 'fatty acid translocase'), alone or together with the peripheral membrane protein FABPpm (plasma membrane-associated fatty-acid-binding-protein; 43 kDa) accepts FAs at the cell surface to increase their local concentration and thus increase the number of fatty acid diffusion events. **(3)** It is also possible that CD36 itself actively transports fatty acids across the membrane. Once at the inner side of the membrane fatty acids are bound by cytoplasmic FABP (FABPc) before entering metabolic or signalling pathways. **(4)** Additionally, a minority of FAs are thought to be transported by fatty acid transport proteins and rapidly activated by plasma membrane acyl-CoA synthetase (ACS1) to form acyl-CoA esters. **(5)** Very-long-chain FAs (>C22) are preferentially transported by FATPs and by action of their

synthetase activity directly converted into very-long-chain acyl-CoA esters (uptake by vectorial acylation).
Figure 1 (and the associated explanation) are used with permission from Schwenk et al. “Fatty acid transport across the cell membrane: Regulation by fatty acid transporters” (12).

1.2.2 Fatty acid efflux

Although current studies oppose the idea that FA uptake is diffusion-mediated, FA efflux is still thought to occur by diffusion, without any proteins being associated with facilitating the process. In contradistinction to this hypothesis, Henkin et al. reported evidence that suggests the possibility of the involvement of proteins in FA efflux. Henkin et al. developed different assays and a special cell line to quantify FA efflux to generate evidence suggesting that adipocytes express an as yet unidentified FA transporter.
In the search for these FA efflux mediating proteins the major FA uptake proteins (FATP, ACSL, CD36) were examined. Since flip-flop transport of FAs across the PM is bidirectional by nature, this mode could have also been true for protein-mediated transport. However, there is hardly any evidence that FATP, ACSL or CD36 are acting bidirectionally. FATP1 can be ruled out as a FA exporter since previous studies have shown that knockdown of FATP1 results in a decrease of FA uptake and shows no effect on FA efflux (23). In CD36 knockout animals no obvious deficiencies in FA release from adipocytes can be observed (24). Febbraio et al. could even demonstrate that the FA serum levels in CD36 KO animals are actually elevated (25). ACSLs are also not probable candidates for FA exporters since by trapping FAs via activiation to acyl-CoA, they are acting undirectionally (24).
Considering this situation, it is necessary to further explore the mechanisms and proteins involved in FA transport, especially the ones that could mediate FA efflux. The description of such a mechanism will not only help to understand an important pathway but could also have an important impact on the treatment of obesity-related diseases which are characterized by malfunctioning lipid transport (24).

1.3 Changes in fatty acid metabolism after bariatric surgery

Bariatric Surgery is the most effective treatment of obesity and obesity-related disorders. Obese patients who have been unable to lose weight by non-surgical means profit from this intervention, because it reduces the risk for cardiovascular disease and others. The ultimate benefit of weight reduction comes with the reduction of the co-morbidities, quality of life and all-cause mortality (26). These co-morbidities include, among many others, hypertension, type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), inflammation and obstructive sleep apnea. While pharmaceutical treatments and lifestyle changes show only minor effects, bariatric surgery significantly reduces body weight and insulin sensitivity. Whereas in former days these effects were only attributed to malabsorption and restriction, it is now presumed that modifications in the anatomy of the gastrointestinal (GI) tract have an important effect on GI physiology, including changes in GI hormone secretion, which are necessary for regulating the feeding and energy metabolism. After bariatric surgery an increase of FFA outflow in the circulation can be observed. Due to a major deficiency of calories the body switches to fatty acid oxidation. The reesterfication of FFAs is reported to increase as much as sixfold during fasting (27). That’s why it’s not surprising that various studies demonstrate, that the FFA levels are elevated in the first few months after bariatric surgery (28,29). These changes in lipid homeostasis make pre- vs. post-bariatric surgery metabolism a great model for studying alterations in lipid flux.

2 Aim of this study and research strategy

2.1 Aim of this study

Studies have demonstrated the importance of several different proteins, such as FATPs, ACSLs and CD36, in the protein-faciliated process of FA transport. Nevertheless, the precise mechanisms how FAs cross the PM to enter the soluble cytoplasm are not yet fully understood. Especially, the impact of one or more membrane-associated proteins on the rate-limiting step in the global process of FA uptake is still being discussed. In contradistinction, FA efflux from adipocytes is attributed to diffusion, which does not involve any proteins facilitating the process, though Henkin et al. previously reported evidence to suggest the opposite; the possibility of a protein-mediated transport across the PM.

The aim of this study is to find possible candidates for these mechanisms. It will be investigated if particular proteins, which are involved in FA efflux, exist and if special membrane-associated proteins can be shown to regulate the rate of FA uptake.

2.2 Research strategy/approach

To find potential candidates for these mechanisms an in-silico study of all transporter genes fulfilling the following criteria: unknown function, plasma membrane location and significant expression in adipose tissue, will be performed. One of the transporter gene families that comes into focus is the family of the ATP-binding cassette (ABC) transporters, of which almost half are thought to facilitate the ATP-dependent translocation of lipids or lipid-related compounds (30–32). Another family of potentially relevant membrane transport proteins is the family of the Solute Carriers (SLC), with over 400 members, most of which are located in the cell membrane (33–36).

The mRNA expression levels of gene candidates from the in-silico study in patients before and after bariatric surgery will be examined. Bariatric surgery will therefore be used as model for a physiological change in FA flux. If the mRNA expression level of the selected gene candidates increases by more than 1.5-fold pre- vs. post-op, they will be further analyzed in epicutaneous, subcutaneous and brown adipose tissue as well as in liver tissue. Genes that

express highly in these tissues will be studied in OP9 murine adipocyte cells and primary preadipocytes throughout differentiation. The ones that show significant changes during adipocyte differentiation in OP9 cells and primary preadipocytes will be further investigated. It will be analyzed if the knockdown/overexpression of one of the identified genes from the in-silico study will have a significant effect on FA uptake and/or FA efflux. Furthermore, a possible interaction between newly identified transporter proteins and already existing ones, such as CD36, will be explored.

3 Material and Methods

3.1 Chemicals and reagents

Reagents were obtained from the following sources: cAMP Complete ELISA kit from Enzo Life Sciences, Farmingdale, NY, USA; Non-esterified fatty acid HR Series NEFA-HR(2) from Wako Diagnostics, Wako Life Sciences, Inc., Mountain View, CA, USA; QBT Fatty Acid Uptake Assay Kit from Molecular Devices Corporation, Sunnyvale, CA, USA; All other chemicals were from Sigma-Aldrich, St. Louis, MO.

3.2 Animals

Wildtype C57Bl6/J and CD36 knockout (KO) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used for the collection of liver and fat tissues. Mice were housed in the animal facility at the VA Palo Alto Health Care System on a 12/12 h light/dark cycle. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee of the VA Palo Alto Health Care System.

3.3 Patient sample collection and RNA preparation

Patients undergoing an elective VSG (vertical sleeve gastrectomy) procedure were recruited and informed consent was obtained from each study participant. Inclusion criteria included men and women older than 21 years of age with a body mass index (BMI) ≥35.0 kg/m2, and meeting criteria by American Diabetes Association standards for pre-diabetes or type 2 diabetes mellitus (T2DM). For laparoscopic VSG surgery, a 4.8mm stapler load was used to divide the greater curvature of the stomach 5cm from the pylorus and remaining 3cm from the angularis incisura. 3.5mm stapler loads were fired thereafter progressing up to the angle of His to complete the VSG. Details of the surgical procedures have been described by Jahansouz et al. (37). Demographic data on sex, age, and T2DM were collected for obese patients at the time of surgery and seven days following bariatric surgery. Weight and height were measured immediately prior to surgery (pre-op) and during the postoperative visit. BMI was calculated as weight (kg) divided by height (m2). Additional details as to the molecular changes occurring following bariatric surgery have been published by

Jahansouz et al. (38). The University of Minnesota and St. Cloud Hospital Institutional Review Boards approved all investigations and informed consent was obtained from each participant. Abdominal subcutaneous adipose tissue biopsies from patients were processed for tissue analysis. Approximately 2-3g of fat was obtained from each subject and immediately frozen with liquid nitrogen and stored at -80°C until analyzed. For RNA preparation, approximately 0.3g of adipose tissue was extracted using TRIzol© (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

QuantiGene2.0 RNA assays for RNA level analysis: RNA levels were assayed using QuantiGene2.0 RNA assays from Affymetrix, which are hybridization-based assays that utilize a branched DNA technology for signal amplification for the direct quantitation of gene expression transcripts. The QuantiGene Plex assay combines branched DNA (bDNA) signal amplification with Luminex multi-analyte profiling bead (xMAP®) technology to enable the capture and quantitation of multiple RNA targets simultaneously. First, samples are lysed to release the target RNA. Second, the lysates are incubated overnight with an oligonucleotide probe set and color-coded bead mix. During this incubation, the probes cooperatively hybridize to both the target RNA and the capture probes conjugated to the microspheres, capturing the target RNA onto designated beads. Third, signal amplification is achieved through sequential hybridizations of the bDNA pre-amplifier, amplifier, and biotinylated label probe molecules to the target. Finally, addition of streptavidin-phycoerythrin generates a fluorescent signal that is proportional to the amount of target RNA captured. Fluorescence intensity is measured using a Luminex instrument. This assay system provides a robust and accurate measurement utilizing xMAP Luminex beads for multiplexing RNA targets. We selected 22 members of the ABC transporter family and 33 members from the SLC family with unknown functions together with control genes (Supplemental Table S1) for analysis simultaneously on the Luminex® platform. 250ng total RNA for each sample was incubated overnight at 54°C with unique fluorescent beads which have target-specific capture extenders and label extenders. The beads are coated with capture probes specific to the capture extenders, thus cooperatively hybridizing each target gene to a unique bead. After overnight incubation, the bDNA signal amplification portion was initiated by first washing the beads, followed by a

1-hour incubation with the preamplifier DNA mix at 50°C. The beads were next washed, followed by a 1-hour incubation with the amplifier DNA solution mix at 50°C. Biotinylated DNA label probe was added to the wells and incubated at 50°C for 1 hour (h). Finally, the beads were washed, followed by incubation at room temperature for 30 minutes (min) with streptavidin phycoerythrin. The beads were washed and read on the Luminex® instrument.

3.4 Cell Cultures

3.4.1 OP9 cells

OP9 cells, a stromal cell line from mouse bone marrow (39) (obtained from ATCC), were grown to confluence in Dulbecco's modified Eagle's medium, containing 20% fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin. Before RNA isolation, OP9 cells were treated with 0.5mM isobutyl-methylxanthine and 1µM dexamethasone on day 0 and with 1µg/ml insulin on day 2. Cells were harvested on day 0, day 3 (72 hours) and day 9 (216 hours) for further experiments.

3.4.2 Primary preadipocytes

Primary preadipocytes were isolated by collection of white adipose tissue from wildtype C57Bl6/J mice. Fat pads were briefly washed with phosphate-buffered saline (PBS) and minced quickly with small scissors into small pieces for ca. 5min. Collagenase buffer (10ml) was added to the minced fat pads from 2 mice, which were then incubated at 37°C for 20min in small plastic cornical flasks. Fat cell wash buffer (5ml) was added to the flasks. The digested mix was passed through a nylon mesh and the tubes were centrifuged gently at 1500 rounds per minute (rpm) for 3-4 min. The fat cells were floating on top, so the solution could easily be sucked out with long needles on top of 50ml syringes. The cells were washed with fat cell wash buffer 2-3 times, with gentle centrifugation in between to pack the cells, before being cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin. Before RNA isolation, primary preadipocytes were differentiated as described above and harvested on day 0, day 7 and day 14.

Buffers for Collagenase

A) Hepes Stock Solution	
1 – HEPES (Sigma H-3375)	24.0g
2 – NaCl	7.0g
3 – KCl	0.385g
4 – CaCl2	0.147g
5 – Glucose	0.9008g
6 – Water	500ml
B) Albumin Solution: 3g/100ml	
C) Equal parts of A (Hepes Stock) and B (Albumin Solution) were mixed and adjusted to a pH of 7.4.	
D) 100ml of Collagenase was weighed and then mixed with 100 ml of the A and B buffer mix, before being filtered with a sterile 0.45µm and then 0.22 µm syringe filter.	

3.5 Transfections

The pEZ-M51-Slc43a3-His plasmid was purchased from GeneCopoeia (Rockville, MD) for overexpression and the Slc43a3 Silencer Pre-designed siRNAs were purchased from Ambion Life Technologies, Inc. (Austin, TX, USA) for knockdown of Slc43a3. Scrambled siRNA was purchased from Santa Cruz Biotechnology and used as a negative control. OP9 cells were used for transfection with Slc43a3 siRNA and scrambled siRNA. Cells were plated in 96-well culture plates and transfection of siRNA or plasmid was performed with PolyJetTM reagent (SignaGen Laboratories, Gaithersburg, MD, USA) following a reverse transfection protocol. Cells were cultured at 37°C, 5% CO2 for 5h, then the medium was replaced with fresh medium containing 0.5mM isobutyl-methylxanthine and 1µM dexamethasone and subsequently cultured according to the differentiation protocol. After 120h (5 days), the cells were used for FA efflux- and uptake assays, as well as cAMP assays.

3.5.1 Construction of Slc43a3 clone

The plasmid pEZ-M51-Slc43a3-His was transformed into DH5alpha competent cells (engineered E. coli cells which maximize transformation efficiency).
The DH5alpha cells were taken from the -80°C freezer and thawed on ice. The cells were gently mixed using the tip of a pipette and 35µl of cells were aliquoted into a 1.5ml tube. 1.5µl (= ca. 300ng) of Slc43a3 plasmid DNA was added to the cells and mixed gently. For the pUC19 (a commonly used cloning vector) control, 2.5µl (250pg) of DNA was added and mixed gently. The tubes were incubated on ice for 30min. The cells were shock heated for 20 seconds (s) in a 42°C water bath and afterwards placed on ice for 2min. A volume of 500µl of pre-warmed lysogeny broth (LB) was added followed by an incubation for 1h at 225rpm. 200µl of the culture were spread on pre-warmed selective LB-Ampicillin (LB+Amp) plates and incubated overnight at 37°C. A single colony was picked after 12-16h and grown in 200ml of LB+Amp media for another 12-16h.

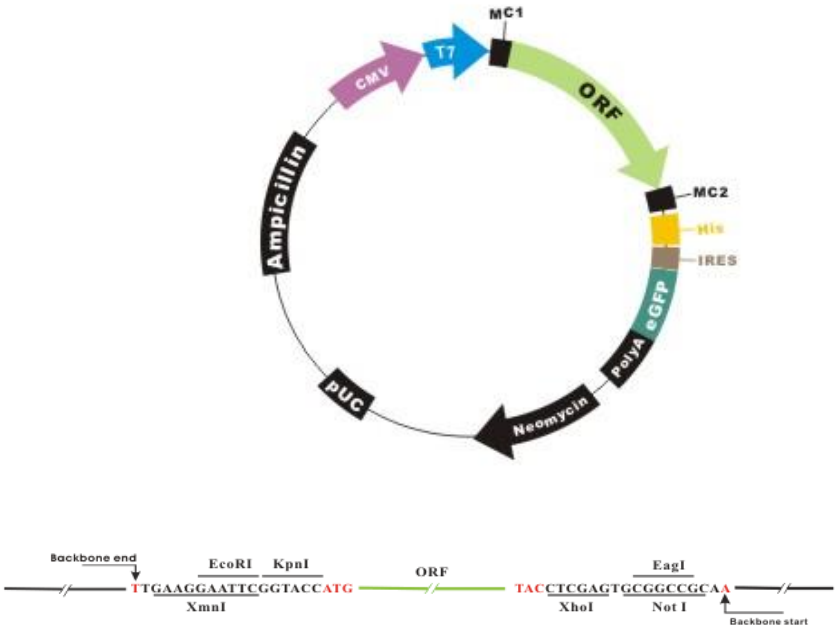


Figure 2. Slc43a3 plasmid DNA from GeneCopoeia.
(A) Vector information. **(B)** Restriction enzyme information.

For the isolation of plasmid DNA from E. coli the QIAprep Spin Midiprep Kit was used. The overnight bacterial culture was harvested by centrifugation (6000 x g, 15min, 4°C). The bacterial pellet was resuspended in 4ml of Buffer P1. 4ml of

Buffer P2 was added and mixed thoroughly by vigorously inverting the mix for 6 times. The mixture was incubated at room temperature for 5min before 4ml of prechilled Buffer P3 was added and mixed by vigorously inverting the tubes 6 times. The mix was incubated for 15min on ice, followed by centrifugation (20000 x g, 30min, 4°C). The supernatant was re-centrifuged at 20000 x g for15 min at 4°C. The QIAGEN-tip 100 was equilibrated by applying 4ml of QBT Buffer and allowing the column to empty by gravity flow. The supernatant was added to the QIAGEN-tip, which was then washed with 2 times 10ml of QC Buffer. The DNA was eluted with 5ml of QF Buffer into a clean 15ml vessel. By adding 3.5ml of isopropanolol to the eluted DNA, the DNA was precipitated. After a centrifugation at 15000 x g for 30min at 4°C the supernatant was carefully decanted. The DNA pellet was washed with 2ml of 70% ethanol and centrifuged at 15000 x g for 10min. The supernatant was discarded. The pellet was air-dryed for 5-10min and the DNA was redissolved in TE buffer (pH 8.0).

3.5.2 Reverse transfection protocol

siRNA (20pmol) or plasmid (0.2µg) were diluted in 5µl serum free DMEM high-glucose medium and gently pipetted up and down. Three times as much Polyjet was diluted in 5µl serum free DMEM high-glucose medium and carefully pipetted up and down. The PolyJet was added all at once to the siRNA/plasmid dilution and pipetted up and down 3-4 times. It is critical to not mix the solutions in reverse order. The mixture was incubated for 10-15min at room temperature. 10µl of the PolyJet-siRNA/clone-mixture was added to each well of a 96-well culture plate. 2 x 10^4 cells/well in 20% FBS were added to each well. After 5h the medium was replaced with the day 0 differentiation media, as described above.

3.6 RNA isolation and quantitative real time PCR analysis

For RNA isolation OP9 cells and primary preadipocytes were cultured and then collected in 1ml TRIzol reagent (if cultured in a 6-well plate) or using the Direct-zol RNA isolation kit from Zymo Research, Irvine, CA, USA (if cultured in a 96-well plate).

3.6.1 TRIzol – RNA isolation

The media of confluent OP9 cell or primary preadipocytes 6-well culture plates was sucked off using a 1000µl-pipette. The cells were washed 2 times with PBS, which was removed afterwards. To each well 1ml of TRIzol reagent was added, the cells were scraped from the well, the lysate was pipetted up and down several times to homogenize and was then placed into a 1.5ml cornical plastic flask. The lysate was centrifuged for 5min at 12000 x g at 4°C and the clear supernatant was transferred to a new tube, followed by a 5-min-incubation at room temperature to permit complete dissociation of the nucleoprotein complex. Per 1ml of TRIzol reagent used for lysis, 0.2 ml of chloroform was added, and the mix was incubated for 2-3min at room temperature. Then the sample was centrifuged for 15min at 12000 x g at 4°C. The mixture seperated at this step into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase. The aqueous phase, containing the RNA, was transferred to a new tube by angling the tube at 45°C and pipetting the solution out. Per 1ml of TRIzol reagent used for lysis, 0.5ml of isopropranolol was added to the aqueous phase and was incubated for 10min at room temperature. After centrifuging the mixture for 10min at 12000 x g at 4°C the RNA precipitate formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded with a micropipettor. The pellet was resuspended in 1ml of 75% ethanol per 1ml of TRIzol Reagent used for lysis. The sample was briefly vortexed and then centrifuged for 5min at 7500 x g at 4°C. The last step was repeated one more time. After centrifugation the supernatant was discarded with a micropipettor and the RNA pellet was air dryed for 5-10min. After drying, the RNA pellet was resuspended in 30µl RNAse free water.

3.6.2 Direct-Zol – RNA isolation

The cells were lysed in 100µl of TRI Reagent and mixed thoroughly. An equal volume of ethanol (95-100%) was added to the sample lysed in TRI Reagent and mixed completely. The mixture was transferred into a Zymo-Spin IIC Column in a collection tube and centrifuged at 12000 x g for 30s. The column was moved to a new collection tube and the flow-through was discarded. RNA Wash Buffer (400µl) was added to the column and centrifuged at 12000 x g for 30s. In an RNase-free tube a mixture of 5µl DNase I and 75µl DNA Digestion Buffer was prepared, which was then added directly to the column matrix. The column was incubated at room temperature for 15min. Direct-zol RNA PreWash (400µl) was added to the column and centrifuged at 12000 x g for 30s. The flow-through was discarded and the previous step was repeated one more time. RNA Wash Buffer (700µl) was added to the column and centrifuged at 12000 x g for 2 min to ensure complete removal of the Wash Buffer. The column was carefully transferred to a RNase-free tube. To elute the RNA, 50µl of DNase/RNase-free water was directely added to the column matrix and centrifuged at 12000 x g for 30s.

3.6.3 Measurement of RNA concentration

For measuring the RNA concentration, the NanoDrop 1000 Spectrophotometer by Thermo Scientific was used. A volume of 1µl of the sample was pipetted onto the end of a sampling arm. This sampling arm contains a fibre optic cable. The sampling arm was closed and therefore the second fibre optic cable came into contact with the sample causing the liquid to bridge between both fibre optic ends. By using a pulsed xenon flash lapm a s a light source a spectral measurement was made. The NanoDrop 1000 Spectrophotometer is linked to a computer with a special software, which saves the measured RNA concentration directly in a file.

3.6.4 First-Strand cDNA synthesis using SuperScript II Reverse Transcriptase

A 20-µl reaction volume was used for 1ng to 5µg of total RNA

1) The following components were added to a nuclease-free microcentrifuge tube	
50 to 250ng random primers	1µl
1ng to 5µg total RNA	x µl (calculated based on the NanoDrop RNA concentration of sample)
1µl dNTP mix (10 mM each)	1µl
Sterile, distilled water	up to 12µl
2) The mixture was heated to 65°C for 5min and quickly chilled on ice before the contents of the tube were collected by brief centrifugation and the following components were added	
5X First-Strand Buffer	4µl
0.1M DTT	2µl
RNaseOUT (40 units/µl)	1µl
3) The contents of the tube were mixed gently and the tube was incubated at 25°C for 2min	
4) 1µl (200 units) of SuperScript II RT was added and mixed by pipetting gently up and down and then incubated at 25°C for 10min	
5) The mixture was incubated at 42°C for 50min	
6) The reaction was inactivated by heating the mix at 70°C for 15min	

3.6.5 Real-time PCR

After converting RNA into cDNA a real-time PCR was performed using an ABI Prism 8500 System using SYBR green maxter mix reagent. The relative mass of specific RNA was calculated by the comparative cycle of threshold detection method according to the manufacturer's instruction. Genes examined included: Plin1, Hsl, Fabp4, Slc22a15, Slc43a3, Slc16a6, Slc45a3, Slc25a33, Slc35c2, Abcd4.

Supplemental Table S2 shows the primer sets used for each gene.

3.7 Fatty acid uptake assay

OP9 cells were seeded into a black-wall/clear-bottom 96-well plate (Costar), transfected with Slc43a3 Pre-designed Silencer Select siRNA or scrambled siRNA following a reverse transfection protocol and then cultured for 120h (5 days) according to the differentiation protocol (described above). On day 5, a FA uptake assay was performed according to the manufacturers protocol. Briefly, the medium was removed from the wells and replaced with 90µl/well serum free medium and 10µl/well HBSS (1x) containing 0.2% fatty acid free bovine serumalbumin (BSA). The assay plate was then incubated for 1h at 37°C, 5% CO2. Before the test compound was added, a time zero read was performed. 100µl of the test compound, diluted in HBSS (1x) and 0.2% BSA was added to each well and immediately read in a fluorescence microplate reader for a kinetic reading at 485mm (every 30s for 30min) using a bottom-read mode.

3.8 Quencher-based free fatty acid efflux assay

The efflux assay was conducted as previously described (24) with minor modifications. OP9 cells (2×10^4) were seeded into a black-wall/clear-bottom 96-well plate (Costar), transfected with Slc43a3 Pre-designed Silencer Select siRNA or scrambled siRNA following a reverse transfection protocol and then cultured for 120h (5 days) according to the differentiation protocol for OP9 cells. The cells were then loaded with 2µM of the fluorescent lipid BODIPY C1-C12 bound to 1%BSA in HBSS (1x) for 16h. After washing the assay plates with PBS, 100µl of fresh buffer containing HBSS (1x) with 1%BSA buffer and BackDrop GREEN Background Supressor Ready Probe Reagent 2 drops/ml (Molecular Probes by Life Technologies) were added and the wells were once read for an initial time zero reading at 24°C. Half of the wells of each group were then stimulated with 20µM forskolin and 1mM isobutyl-methylxanthine. The assay plates were read for 30min in a fluorescent plate reader utilizing a bottom-read setting (kinetic, 485mm).

3.9 Cyclic adenosine monophosphate assay

A cyclic adenosine monophosphate (cAMP) complete enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure forskolin-induced cAMP formation intra- and extracellularly. Differentiated OP9 cells were incubated for 60min with or without lipolytic stimuli (20µM forskolin, 1mM isobutyl-methylxanthine) bound to 1%BSA in HBSS (1x) at 37°C and 5% CO2. The incubation media was collected, and the cells were extracted using 0.1M HCl to avoid degradation of cAMP, and cAMP levels were measured in whole cell lysates as well as the media by using the complete cAMP immunoassay kit (Enzo Life Sciences) according to the manufacturer's instructions. The level of cAMP production was normalized to the amount of total protein.

3.10 Western blot

Cells were lysed with 100-150µl of Ripa Buffer (with proteinkinase inhibitor) per 1 well of 6 well culture plate and sonicated with 10 bursts per tube (on ice). The tubes were spun for 10min at 1000 x g at 4°C. The supernatant was pipetted to a new Eppendorf tube and the protein concentration was determined using a protein concentration assay kit.

BSA was used for the standard curve (blank, 0.2µg, 0.4µg, 0.6µg, 1µg, 1.5µg, 2µg, 3µg – added up with 150µl of autoclaved, distilled water). 1 well was only pipetted with Ripa Buffer. 1µl of sample was added up to 150µl of autoclaved, distilled water and incubated for 2h and then read at 652nm. After plotting the standard curve, the protein concentration of each sample was calculated.

15-20µg of protein was used for the western blot. 1.5-2 times as much 4X loading buffer was added to each sample and heated at 100°C for 10min. The tubes were quickly spun at 12000 x g for 30s. The gel was placed in the voltage equipment and loaded with the proteins and the marker. It was run at 125-200 Volt (V) for ca. 1h. The gel was then washed in 1X transfer buffer with 20% methanol. Membrane, polster and gel were prepared in a clip-bag and placed in the voltage machine and ran for 45min – 1h in 4°C with 80V. The membrane was peeled from the clip-bag, placed in a little black box and blocked with blocking gel (no antibody was added at this point) for 30min on a plate shaker. Afterwards the old blocking gel was discarded and a new one was added as well as the antibody, mostly in a 1:1000 dilution (housekeeping antibody: beta-

actin). The box with the membrane was placed on a plate shaker at 4°C overnight. The next day the membrane was washed 3 times for 10min with phosphate-buffered saline with Tween 20 (PBST) and then incubated for 1h at room temperature with the secondary antibody (diluted 1:15000). The membrane was washed again 2 times for 10min with (PBST) and then one time with PBS before being scanned.

3.11 Statistics

Data are expressed as means ± SEM. Statistical analyses were performed by one-way ANOVA using Prism 6.02 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). To evaluate the differences in Table 1, a t-test was performed. Differences between groups were considered statistically significant when $P<0.05$.

4 Results

4.1 mRNA expression levels of membrane transport proteins in patients before and after bariatric surgery

Patients who undergo bariatric sugery show major changes in fat metabolism. Since caloric intake is significantly reduced, FA efflux is expected to be substantially increased. In search of genes that are potentially involved in FA transport, particularly any potentially involved in FA efflux, the mRNA expression levels of 22 membrane transport proteins from the ABC transporter family and 33 from the SLC family (see list in Supplemental Table S1) were examined in the adipose tissue of patients post bariatric surgery at this critical time. At the time of the study these membrane transport proteins did not have a known ligand. Table 1 shows the expression levels, determined using branched DNA analysis, of genes that displayed statistically significant differences before (pre-op) and one-week after bariatric surgery (post-op). Of the genes examined, eight genes from the SLC family showed significant increases after bariatric surgery and six showed significant decreases. The functions of these SLC family members have not yet been determined.

	Pre-op ave. (/TBP)	Post-op ave. (/TBP)	Fold Post/Pre	T test
ABC transporter family				
ABCC10	0.157	0.246	1.567	0.002
ABCD4	0.789	1.181	1.497	0.009
ABCB10	0.646	0.942	1.459	0.016
ABCA6	4.636	2.273	0.490	0.017
ABCA5	0.460	0.196	0.426	4.78E-04
ABCA10	1.680	0.586	0.349	1.51E-04
ABCA9	3.122	1.073	0.344	0.002
ABCA8	3.878	1.229	0.317	0.002
ABCC6	1.242	0.208	0.167	9.49E-05
ABCD2	3.385	0.564	0.167	7.99E-06

	Pre-op ave. (/TBP)	Post-op ave. (/TBP)	Fold Post/Pre	T test
SLC transporter family				
SLC22A15	0.005	0.062	11.808	6.96E-06
SLC16A6	0.060	0.379	6.271	0.014
SLC45A3	0.066	0.244	3.680	0.006
SLC35F2	0.065	0.226	3.460	0.044
SLC43A3	1.151	2.791	2.424	0.012
SLC16A5	0.136	0.280	2.058	0.001
SLC35C2	0.922	1.734	1.880	0.001
SLC35E3	0.372	0.531	1.428	0.054
SLC25A46	1.740	1.197	0.688	4.48E-05
SLC25A30	0.863	0.562	0.652	1.22E-04
SLC25A36	0.975	0.531	0.545	0.001
SLC25A42	0.441	0.191	0.432	0.001
SLC25A33	1.780	0.627	0.352	2.19E-04
SLC6A16	0.073	0.011	0.153	0.001
P450 superfamily				
TBXAS1	0.942	2.882	3.059	0.001
Leukotriene metabolism				
LTA4H	2.191	3.617	1.651	0.028
LTB4R	0.258	0.422	1.636	0.026
CYSLTR1	0.873	0.614	0.703	0.040
Others				
SIRT3	0.770	0.619	0.804	0.012
GSTA4	0.895	0.493	0.550	0.001
PDE3B	3.764	0.810	0.215	0.005

Table 1 mRNA expression levels of membrane transport proteins in patients before and after bariatric surgery showing statistically significant changes. Genes that will be further examined are printed in bold.

Genes that showed the most significant changes were Slc22a15, Slc43a3, Slc16a6, Slc45a3, Slc25a33 and Slc35c2 and Abcd4. In further experiments it will be studied how these genes expess in varios tissues involved in the energy metabolism (liver and adipose tissues) and how their expression levels change throughout differentiation.

4.2 mRNA expression of membrane transporters in mouse tissues, OP9 cells and primary preadipocytes

4.2.1 mRNA expression of membrane transporters in mouse tissues

Liver and fat tissue are the main participants in energy metabolism. Hence, it was assumed that genes, which are involved in FA transport, must express highly in liver and adipose tissue. Therefore, mRNA expression levels of the six plasma membrane proteins of the SLC family that showed substantial basal expression levels and more than a 1.5-fold increase (see Table 1) after bariatric surgery were further analyzed in murine liver and various fat tissues (epicutaneous, subcutaneous and brown adipose tissue). For this analysis, four months old male wild type C57Bl6 mice were sacrificed and their liver and fat tissues were collected for analyis of mRNA expression by RT-qPCR. From all analyzed genes Slc43a3 showed the most significant expression in epicutaneous, subcutaneous and brown adipose tissue (Figure 3). Its expression pattern is comparable to genes that are involved in control of lipid homeostasis in adipose tissue, ie. Plin1, Hsl and Fabp4.

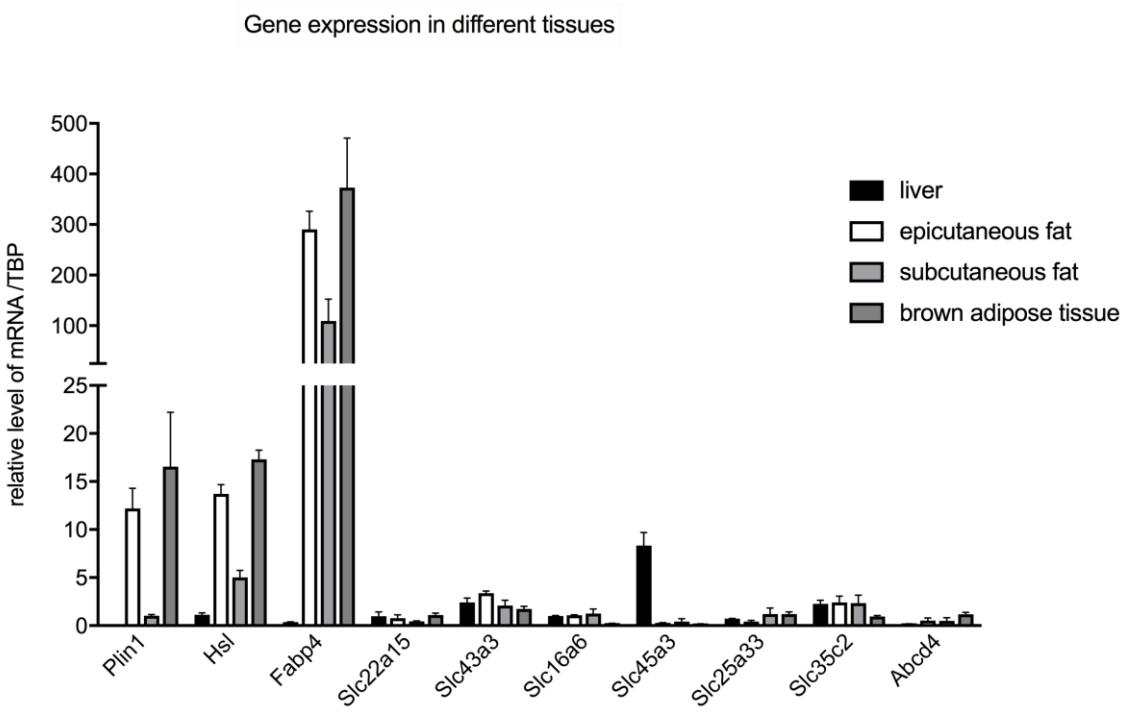
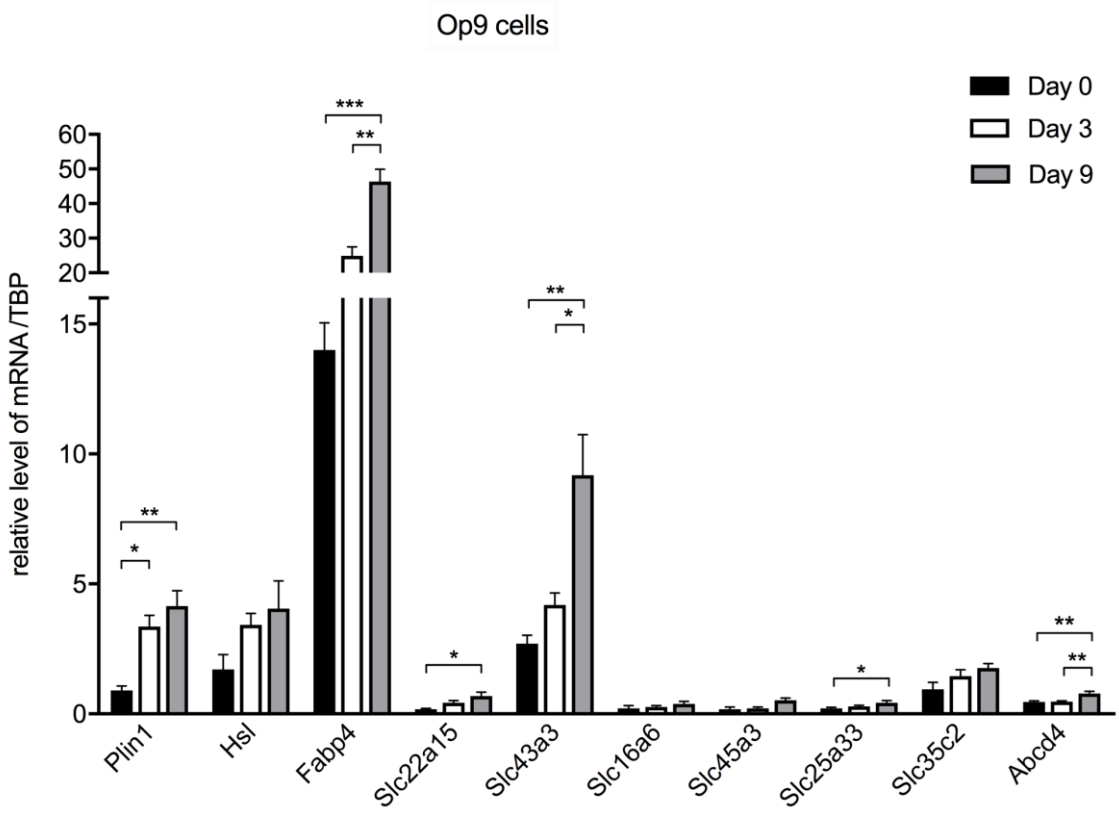


Figure 3. Levels of membrane transporter mRNA in wildtype mouse liver, epicutaneous fat, subcutaneous fat and brown adipose tissue, each with n=4. Plin1, HSL and Fabp4 are known genes involved in fat metabolism. Slc22a15, Slc43a3, Slc16a6, Slc45a3, Slc45a3, Slc22a33, Slc35c2 and Abcd4 are genes that showed a significant change in mRNA expression levels before and after bariatric surgery. The function of these genes has not been determined yet.

4.2.2 mRNA expression of membrane transporters in OP9 cells and primary preadipocytes

During differentiation from preadipocytes to adipocytes mRNA expression levels of genes that are involved in energy metabolism are expected to increase. This effect can be seen in Figure 4A and 4B: apart from Plin1, the lipid-droplet associated protein, also Fabp4, the common indicator for lipid accumulation/adipocyte maturity, increases significantly throughout differentiation. Though not only the familiar genes (Plin1, Hsl and Fabp4) but also the so far unknown genes like Slc22a15, Slc43a3, Slc16a6, Slc25a33, Slc35c2 and Abcd4 showed significant changes. Out of all the genes that are named above, Slc43a3 showed the most significant changes in mRNA expression levels. The differentiation of OP9 cells from preadipocytes to adipocytes showed that expression of Slc43a3 increased significantly between day 0 and day 9 ($P<0.01$) and between day 3 and day 9 ($P<0.05$) (Figure 4A). The gene expression was also studied in primary preadipocytes, which were isolated from wildtype mice, and differentiated for 14 days. As shown in Figure 4B, again Slc43a3 displayed the most significant changes in expresssion throughout differentiation: between day 0 and day 3 ($P<0.05$), between day 3 and day 9 ($P<0.001$) and between day 0 and day 9 ($P<0.001$). These results imply that Slc43a3 plays a significant role in lipid metabolism.

4 A


Op9 cells
Day 0
Day 3
Day 9
relative level of mRNA/TBP
60
50
40
30
20
15
10
5
0
***
**
*
**
*
*
**
*
**
Plin1
Hsl
Fabp4
Slc22a15
Slc43a3
Slc16a6
Slc45a3
Slc25a33
Slc35c2
Abcd4

4 B

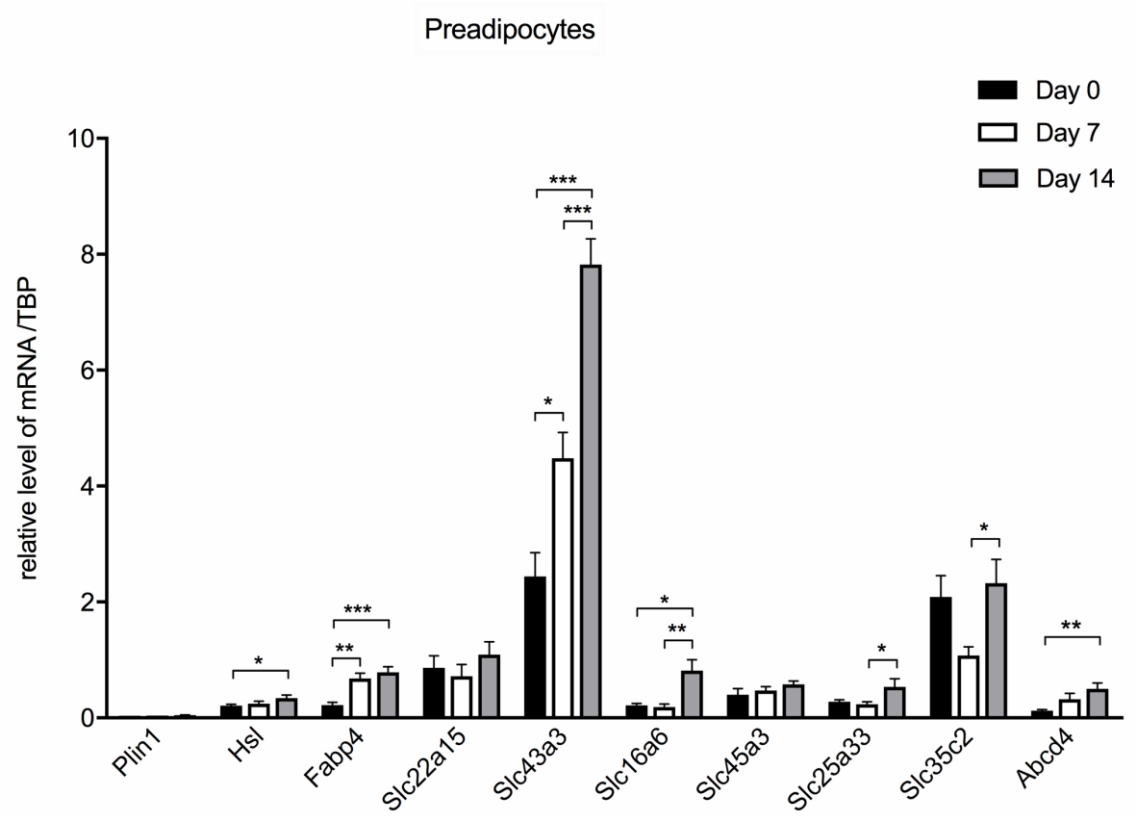

Preadipocytes
Day 0
Day 7
Day 14
relative level of mRNA/TBP
10
8
6
4
2
0
***
***
*
*
***
**
*
*
**
*
*
**
Plin1
Hsl
Fabp4
Slc22a15
Slc43a3
Slc16a6
Slc45a3
Slc25a33
Slc35c2
Abcd4

Figure 4. mRNA expression of membrane transporters in OP9 cells and primary preadipocytes.
(A) Levels of membrane transporter mRNA in OP9 cells. OP9 cells were treated with 0.5mM isobutyl-methylxanthine, 1µM dexamethasone and 1µg/ml insulin for differentiation for 5 days before mRNA analysis. Results are representative of 3 independent experiments, each with n=6. **(B)** Levels of membrane transporter mRNA in differentiated primary mouse preadipocytes. Results are representative of 3 independent experiments, each with n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

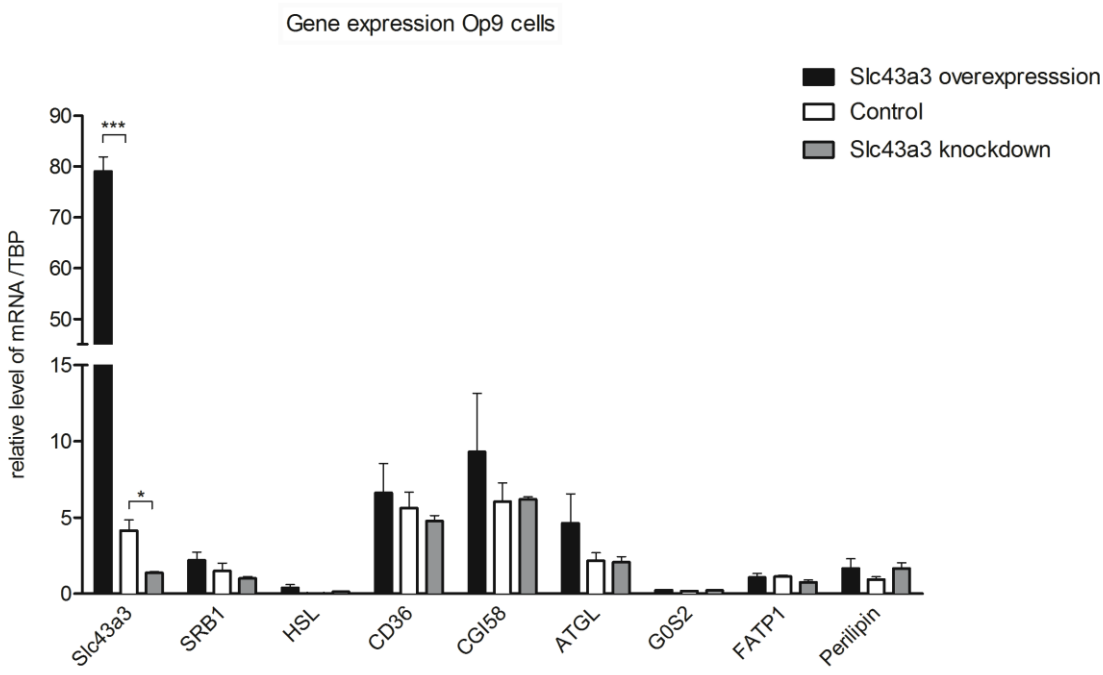
4.3 Knockdown and overexpression of Slc43a3

To characterize the function of Slc43a3, the levels of Slc43a3 in OP9 cells were manipulated by knockdown using siRNA or overexpression (pEZ-M51-Slc43a3) and the cells were examined after 5 days of differentiation.

4.3.1 Confirmation of the expression levels of Slc43a3

To further study the effect of Slc43a3 on fat metabolism, Slc43a3 was inhibited/overexpressed in OP9 cells. The expression levels of Slc43a3 in transfected and control (scrambled siRNA; pcDNA6) cells were detected by RT-qPCR (Figure 5A) and western blot (Figure 5B). When Slc43a3 siRNA was transfected into the cells, Slc43a3 mRNA expression significantly decreased ($P<0.05$), whereas transfection of cells with pEZ-M51-Slc43a3 significantly increased Slc43a3 expression compared to control ($P<0.001$) (Figure 5A). In this experiment it was also investigated if knockdown/overexpression of Slc43a3 has a significant effect on the mRNA expression levels of popular genes involved in lipid transport, including SR-B1, CD36, HSL, ATGL, CGI58, G0S2, FATP1 and Plin1, but no link could be found. This data suggests that Slc43a3 is independantly affecting fat metabolism.

5 A



5 B

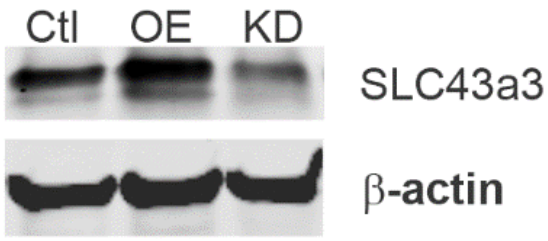


Figure 5. Confirmation of the expression levels of Slc43a3.
(A) Levels of Slc43a3 mRNA and other genes involved in adipose metabolism in OP9 cells after transfection with scrambled siRNA (control), Slc43a3 siRNA, pcDNA6 (control) or pEZ-M51-Slc43a3-His clone. Results are representative of 3 independent experiments, each with n=4. **(B)** Western blot of Slc43a3 in OP9 cells transfected with scrambled siRNA (control), Slc43a3 siRNA, pcDNA6 (control) or pEZ-M51-Slc43a3-His clone.

4.3.2 Difference in lipid droplet accumulation

During the differentiation process, the synthesis of TAG increases and the differentiated cells obtain the behaviour of adipose cells. TAG, especially, gather in lipid droplets embedded in the cytoplasm. Lipid droplets are cellular organelles that manage the storage and hydrolysis of neutral lipids, including TAG and cholesterol esters (40). Figure 6 shows the difference in lipid droplet accumulation using fluorescent microscopy. There was significantly more accumulation of lipid droplets in OP9 cells at day 5 after differentiation with knockdown of Slc43a3, whereas overexpression of Slc43a3 resulted in a significant decrease in lipid accumulation (Figure 6).

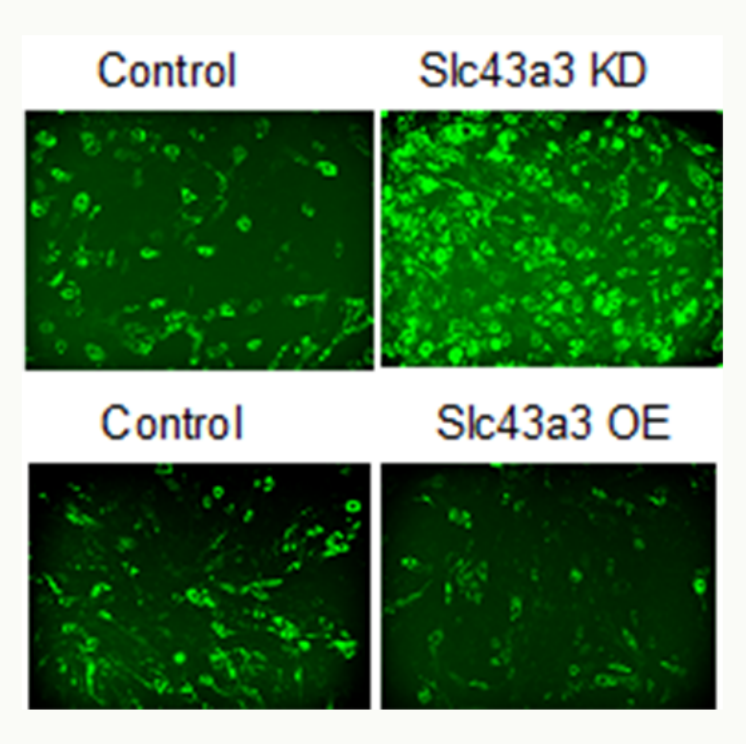


Figure 6. Lipid accumulation in differentiated OP9 cells transfected with scrambled siRNA (control), Slc43a3 siRNA, pcDNA6 (control) or pEZ-M51-Slc43a3-His clone. OP9 cells were transfected with the scrambled siRNA (control), Slc43a3 siRNA, pcDNA6 (control) or pEZ-M51-Slc43a3-His clone and differentiated for 5 days. 1 nM Bodipy 493/503 were added to the media before fluorescent microscopy. Results are representative of 3 independent experiments.

4.3.3 Effects of Slc43a3 knockdown and overexpression on free fatty acid uptake

The aim of this study was to find genes that are involved in FA transport. Figure 7 shows the result of direct measurements of FA uptake with knockdown and overexpression of Slc43a3. When cells were transfected with Slc43a3-siRNA a significantly increased rate of FA uptake (P<0.001) was observed, whereas overexpression of Slc43a3 significantly decreased the rate of FA uptake (P<0.001). As a result, it can be concluded that Slc43a3 is a negative regulator of FA uptake.

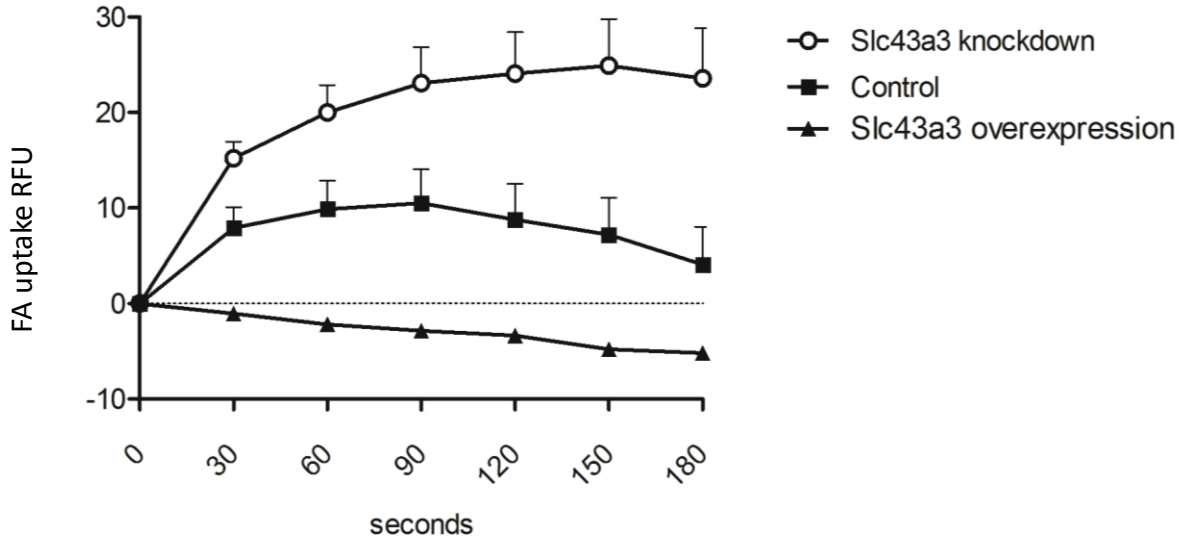


Figure 7. Effects of knockdown and overexpression of Slc43a3 in OP9 cells on free FA uptake. Results are representative of 2-3 independent experiments. Slc43a3 knockdown: n=22; Slc43a3 overexpression: n=12; Control combined: n=36. * p<0.05, ** p<0.01, *** p<0.001. RFU = relative fluorescence units.

4.3.4 Effects of Slc43a3 knockdown and overexpression on free fatty acid efflux

The impact of Slc43a3 on FA efflux was also examined. Interestingly, knockdown of Slc43a3 significantly ($P<0.05$) decreased the rates of FA efflux compared to control cells under basal conditions and substantially ($P<0.01$) blunted FA efflux when cells were treated with forskolin to stimulate lipolysis and FA efflux (Figure 8). This data suggests that Slc43a3 is a positive regulator of FA efflux. Although a final conclusion can't be drawn yet, since until today it wasn't possible to study the effect of Slc43a3 overexpression on FA efflux. These experiments should be conducted in the future to better comprehend the possible influence of Slc43a3 overexpression on FA efflux.

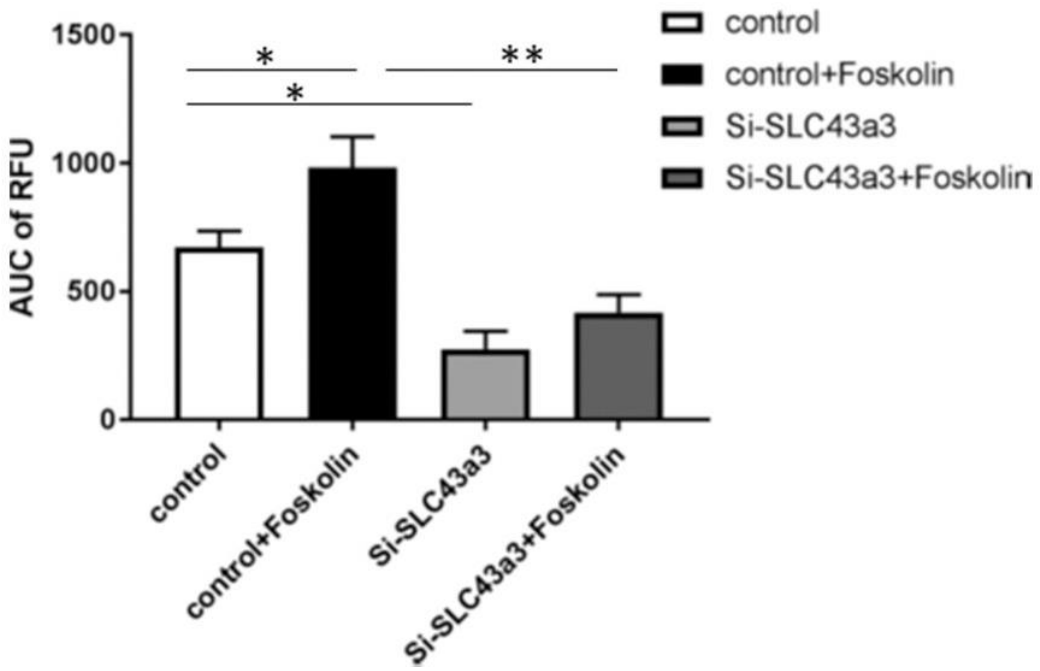
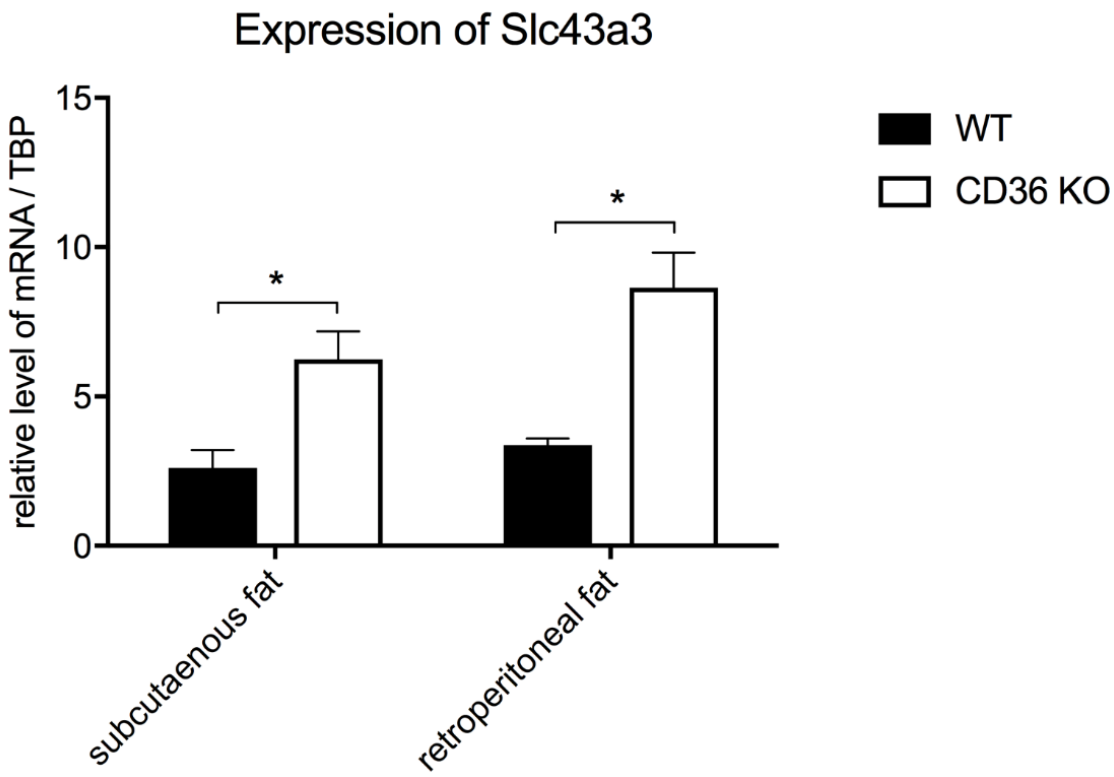


Figure 8. Effects of knockdown of Slc43a3 in OP9 cells on FA efflux. Results are representative of 3-4 independent experiments with replication of 9 in each treatment. * $p<0.05$, ** $p<0.01$. AUC = area under the curve.

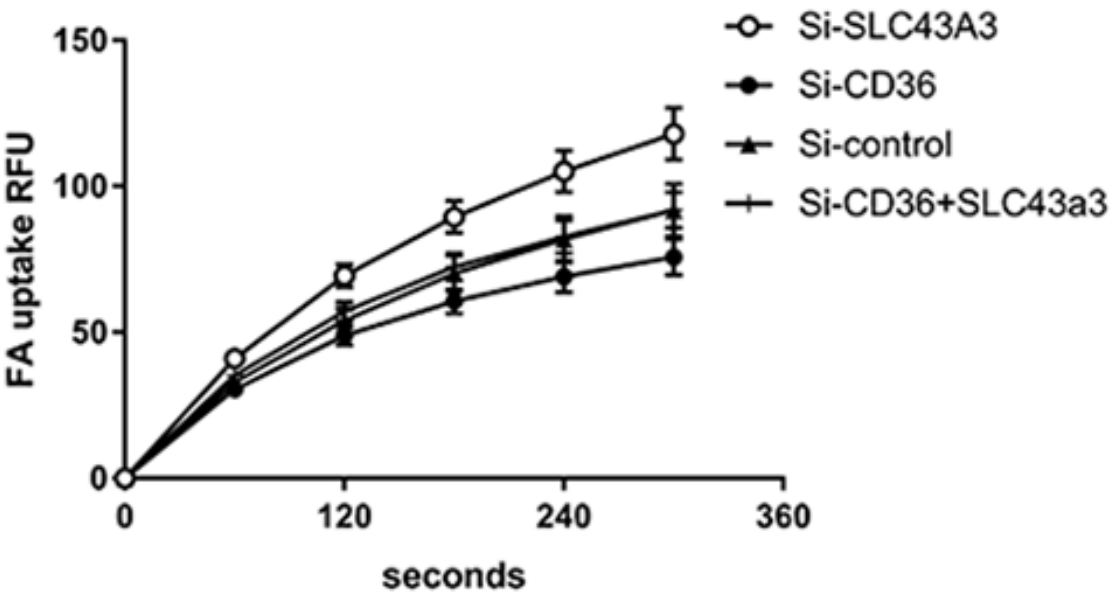
4.4 Expression of Slc43a3 in CD36 knockout mice

CD36 is known to be important in FA transport and regulation, such that there is decreased FA uptake in adipose tissue of CD36 KO mice. Therefore, the expression levels of Slc43a3 were examined in adipose tissues of CD36 KO mice. As shown in Figure 9A, there is a significant upregulation of expression of Slc43a3 ($P<0.05$) compared to control (wildtype mice) in both, the subcutaneous and retroperitoneal adipose tissue of CD36 KO mice. To further explore this possible relationship, the expression of both, Slc43a3 and CD36, were manipulated in OP9 cells. OP9 cells were transfected with Slc43a3 siRNA, CD36 siRNA, both Slc43a3 and CD36 siRNA at the same time or with control scrambled siRNA. Data presented in Figure 9B shows that knockdown of CD36 decreases the rate of FA uptake as compared to control, consistent with prior observations (41). Compatible with prior data shown in Figure 7, knockdown of Slc43a3 again increased the rate of FA uptake compared to control. Figure 9C shows the accumulative effect on FA uptake of CD36, Slc43a3 and CD36+Slc43a3 knockdown compared to control. Interestingly, when both CD36 and Slc43a3 were knocked down, the rate of FA uptake was similar to control, suggesting that the effects of Slc43a3 are mediated independently from CD36.

9 A


Expression of Slc43a3
relative level of mRNA / TBP
15
10
5
0
*
*
WT
CD36 KO
subcutaenous fat
retroperitoneal fat

9 B


FA uptake RFU
150
100
50
0
0
120
240
360
seconds
Si-SLC43A3
Si-CD36
Si-control
Si-CD36+SLC43a3

9 C

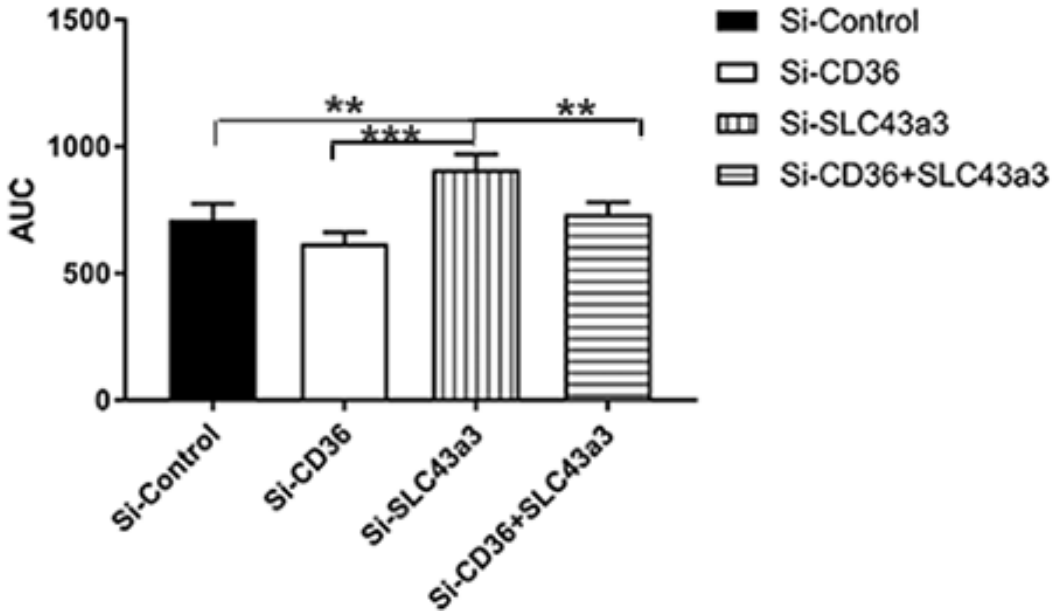


Figure 9. Exploration of the relation between Slc43a3 and CD36.
(A) Levels of Slc43a3 RNA in adipose tissue of wildtype and CD36 KO mice. Total RNA was prepared from subcutaneous and retroperitoneal adipose tissues from wild type and CD36 knockout mice. Levels of Slc43a3 were assayed using Taqman RT-PCR as described in the methods. Results are a summary of three independent assays with n=3-4 in each group. **(B)** Effects of Slc43a3, CD36 and double knockdown in OP9 cells on free FA uptake compared to control (scrambled siRNA). **(C)** AUC of FA uptake for the first 6 minutes. Results are representative of 3 independent experiments, each with n=8. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.5 Effects of Slc43a3 on cAMP levels

In exploring potential mechanisms through which Slc43a3 might influence cellular homeostasis, the changes in cAMP levels with either knockdown or with overexpression of Slc43a3 were examined, since cAMP is known to be a component in lipogenesis and lipolysis. Interestingly, the knockdown of Slc43a3 caused a reduction in cAMP extracellularly ($P<0.001$), as well as intracellularly ($P<0.05$) following treatment with forskolin and isobutyl-methylxanthine. Surprisingly, overexpression of Slc43a3 also caused a small reduction in extracellular cAMP following treatment with forskolin and isobutyl-methylxanthine (17.45±0.04 pmol/ml control versus 15.14±0.62 pmol/ml overexpression, $P<0.05$).

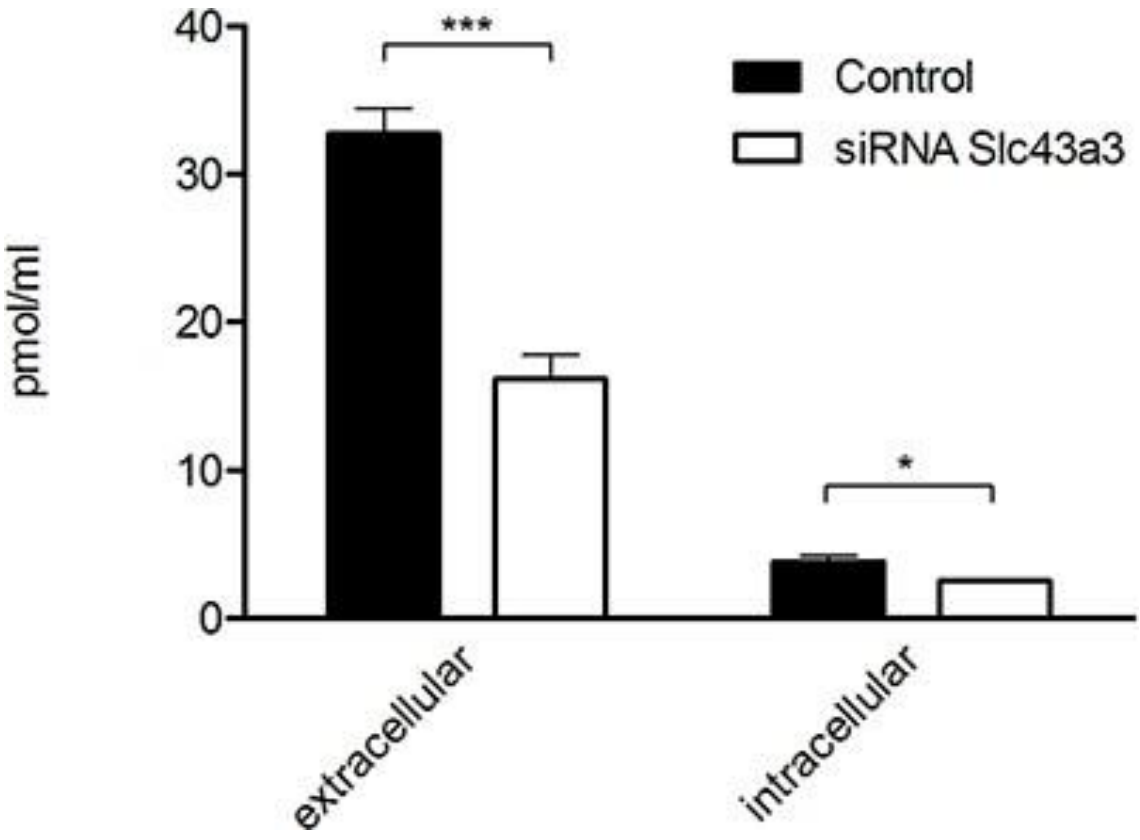


Figure 10. Effects of Slc43a3 on cAMP levels. AMP was measured by ELISA. Changes of extra- and intracellular cAMP levels after transfection with Slc43a3 siRNA and pEZ-M51-Slc43a3-His clone in OP9 cells and treatment with forskolin and isobutyl-methylxanthine. Results are representative of 2 independent experiments, each with n=4.

5 Discussion

5.1 Slc43a3 was identified as a major player in lipid metabolism

As a cell with endocrine function, adipocytes actively take up and release FAs under different physiological conditions, contribute to the regulation of lipid homeostasis, and, in turn, participate in whole body metabolism. Dysregulation of FA metabolism is a major factor contributing to the development of disorders such as diabetes, cardiovascular disease and non-alcoholic fatty liver disease. In that regard it seemed necessary to further explore possible components of the regulation of lipid homeostasis, considering that their modification could have a major impact on the treatment of obesity-related disorders.

Bariatric surgery has been shown to be the most effective treatment of obesity and obesity related type 2 diabetes (45–47). One of the interesting observations is that one-week post-surgery, most patients have improved insulin sensitivity without substantial weight loss (48). Studies have displayed that in response to the major changes of bariatric surgery, several main regulators of adipose tissue metabolism, such as PPARγ, PPARδ and UCP2, have altered expression during the first week post-surgery, attenuating lipid storage and promoting fatty oxidation (38). Indeed, FA efflux would be expected to be markedly increased at this time since caloric intake is substantially reduced. This is why this timepoint of dramatic metabolic changes post-bariatric surgery was choosen to search for potential regulators of FA transport in adipocytes. It was observed that there are many alterations in adipocyte plasma membrane transporters, which currently have unknown functions, during this first week of metabolic transition.

The biggest change in mRNA expression levels before and after bariatric surgery could be observed in genes from the transporter families of the ATP-binding cassette (ABC) transporters and the Solute Carriers (SLC). We could show that from the 22 membrane transport proteins of the ABC transporter family and 33 of the SLC family, 10 and respectively 14 changed significantly, which suggests that they are involved in lipid metabolism. For the advanced search of possible transport proteins, the fold post-op vs. pre-op was calculated and if it was greater than 1.5, the mRNA expression levels of these genes were further analyzed throughout differentiation in murine OP9 cells and primary preadipocytes. On the basis of its significant changes in mRNA expression, the

gene Slc43a3 came into focus and became the core of this study. The hypothesis, that a gene which is highly expressed in adipose tissue and increases its expression throughout differentiation has to be somehow involved in fat metabolism, holds true. The results of the FA uptake as well as the FA efflux assays demonstrated that Slc43a3 significantly influences FA transport. When cells were transfected with Slc43a3-siRNA the uptake rates of FA increased significantly, whereas when Slc43a3 was overexpressed the uptake rates decreased significantly. Furthermore, a knockdown of Slc43a3 resulted in significantly decreased FA efflux rates. To better understand the mode of action of Slc43a3, different approaches were chosen. For now, no final conclusion can be drawn but it seems that Slc43a3 is acting seperatly from popular genes involved in fat metabolism, especially CD36. In addition, it was observed that Slc43a3 affects cAMP production, but it appears that FA flux is independent of this effect.

5.2 Slc43a3 shows significant expression in adipose tissue and throughout adipocyte differentiation

The SLC family is one of the two transporter super families and consists of more than 400 members. SLC transporters are predominantly facilitative or secondary-active. For the movement of substrates across membranes SLCs need an electrochemical gradient. In order for SLCs to transport substrates against a concentration gradient, they need ion gradients that are generated by ATP-dependent pumps (34).

In this study eight genes from the SLC family were identified to increase significantly (SLC22A15, SLC16A6, SLC45A3, SLC35F2, SLC43A3, SLC16A5, SLC35C2, SLC35E3) after bariatric surgery and six to significantly decrease (SLC25A46, SLC25A30, SLC25A36, SLC25A42, SLC25A33, SLC6A16). The functions of these SLC family members have not yet been determined.

One of the SLC transporter genes that demonstrated increased expression after bariatric surgery (2.5 fold) was Slc43a3. The SLC43 family of transporters is composed of only three members: two amino acid system L transporters (LAT3 and LAT4) and the orphan transporter EEG1 (embryonic epithelia gene 1) (49–51). Human LAT3 and LAT4 share ca. 57% amino acid sequence identity, whereas human EEG1 is a distant member of the family with only ca. 27%

amino acid sequence identity with the other two members. Mouse Eeg1 was identified as a gene expressed in a cellular model of renal tubulogenesis (51) and the human gene has been assigned as SLC43A3 (52). However, its specific function in adipose tissue and its involvement in lipid metabolism had not yet been investigated prior to the current studies. Slc43a3 was shown to be expressed during embryogenesis in liver and lung and has recently been reported to be involved in the cellular uptake of extracellular purine nucleobases (53).

The analysis of mRNA expression in normal adult control mice revealed that the expression of Slc43a3 in adipose tissues is similar to that in the liver. Slc43a3 showed in epicutaneous, subcutaneous and brown adipose tissue, compared to all other examined genes, the most significant mRNA expression levels. These expression levels can be compared with genes that are involved in control of lipid homeostasis in adipose tissue, such as Plin1, Hsl and Fabp4. The in vitro study in a murine OP9 adipocyte cell line and in primary mouse preadipocytes indicated that Slc43a3 expression is induced during the differentiation of adipocytes. The differentiation of the OP9 cells and primary mouse preadipocytes with 0.5mM isobutyl-methylxanthine and 1µM dexamethasone on day 0 and with 1 µg/ml insulin on day 2, resulted in increasing levels of Slc43a3 expression. Other, yet unidentified transporter genes, showed not as significant changes at this point.

The continuous and significant increase of Slc43a3 throughout differentiation suggests that Slc43a3 is playing a major role in lipid metabolism.

5.3 Slc43a3 is a regulator of free fatty acid flux

The experiments of this project were designed in order to understand the molecular mechanisms and regulations of FA flux better. Therefore, it was necessary to construct assays that allowed the observation of FA transport, such as FA uptake and FA efflux assays (25). Among the construction of FA transport assays, Slc43a3 needed to be overexpressed/knocked down with an appropriate clone/siRNA.

Interestingly overexpression of Slc43a3 in OP9 cells resulted in significantly lower ($P<0.001$) rates of FA uptake and lipid accumulation in the cells, whereas siRNA mediated knockdown of Slc43a3 resulted in significantly higher

(P<0.001) rates of FA uptake and increased lipid accumulation in cells. Surprisingly, knockdown of Slc43a3 significantly decreased the rates of FA efflux compared to control cells under basal conditions (P<0.05) and substantially blunted FA efflux when cells were treated with forskolin to stimulate lipolysis and FA efflux (P<0.01). Thus, Slc43a3 appears to influence fatty acid flux in adipocytes, functioning as a positive regulator of fatty acid efflux and as a negative regulator of fatty acid uptake.

5.4 Possible mechanisms of Slc43a3

This study could state that Slc43a3 has a significant impact on free fatty acid efflux. To explore potential mechanisms whereby Slc43a3 might be altering rates of FA flux a cAMP assay was performed. cAMP and cAMP-dependant protein kinase A (PKA) signaling pathways are known to be involved in lipogenesis and the lipolysis process (54). The performed cAMP assay displayed that both knockdown, as well as overexpression of Slc43a3, reduced forskolin-stimulated cAMP generation. Although an explanation for how both knockdown and overexpression of SLC43a3 reduce forskolin-stimulated cAMP remains obscure, the observations suggest that any Slc43a3 mediated changes in FA flux are independent of its effects on cAMP production.

In the search for other possible ways how Slc43a3 could be acting, the effect of Slc43a3 knockdown and overexpression was studied compared to expression levels of genes that are known to be involved in cellular FA and lipid metabolism. But neither the knockdown nor the overexpression of Slc43a3 had any significant effect on the mRNA expression of genes, such as SR-B1, CD36, HSL, ATGL, CGI58, G0S2, FATP1 and Plin1.

To make sure that the observed effects were not caused by an unknown relationship between Slc43a3 and CD36, the rate of FA uptake was measured in si-Slc43a3, si-CD36 and si-CD36+Slc43a3 transfected OP9 cells. Knockdown of Slc43a3, according to prior experiments from this study, increased the FA uptake. As expected, knockdown of CD36, decreased FA uptake, since CD36 is known as the predominant transporter of FAs into adipocytes. However, when both genes were knocked out (si-CD36+Slc43a3) the rate of the FA uptake was similar to control. This suggests that actions of Slc43a3 on FA uptake are independent of CD36 expression. The mechanism(s)

through which Slc43a3 mediates these effects on FA flux remains to be elucidated and is the focus of ongoing studies.
Nonetheless, it would appear that Slc43a3 is not directly involved in FA flux, but somehow either influences the function of other proteins involved in facilitating FA flux or affects the diffusion or flip-flop of FAs across the PM. In regard to the latter possibility, we speculate that Slc43a3 might be involved in maintaining the electroneutrality of the cell during FA flux, functioning either as a symporter or antiporter of protons, which would be required to offset the intracellular changes associated with the flux of FA anions.

5.5 Limitations of this study

As with many studies, the experiments performed in this study have limitations. In this study, it was assumed that after bariatric surgery FA storage is attenuated and therefore FA mobilization from adipose tissue is favoured as caloric intake is reduced. In regard to the identification of proteins involved in FA transport, it would be interesting to assess whether the expression of Slc43a3 is differentially modulated in post prandial and fasting stage and whether this modulation is impacted by an obesogenic diet. Moreover, changes in Slc43a3 are described during adipocyte differentiation but no experiments were performed demonstrating if knockdown or overexpression might influence adipocyte differentiation itself. It would be helpful to examine the effect of each treatment on various adipocyte differentiation markers. Furthermore, the FA uptake assay used in this study only investigated the uptake of medium chain fatty acids (BODIPY C1-C12). In additional studies it would be exciting to study if manipulating Slc43a3 expression has a similar impact on the uptake and efflux of long chain fatty acids (i.e. BODIPY FL-C16). Also, it was shown that cAMP levels changed when Slc43a3 was manipulated. The modulation of FA efflux might be related to changes in lipolysis activity, which would make the measurements of glycerol release in parallel to FA in the context of Slc43a3 overexpression and knockdown attractive.

5.6 Summary

This project was designed to find membrane-associated proteins that are not yet known to be involved in fatty acid transport. In an in-silico study genes encoding for proteins with unknown function, a plasma membrane location and a significant expression in adipose tissue, were searched for. These genes were studied in patients before and after bariatric surgery. In summary, more than 20 proteins (predominantly proteins from the ABC and SLC transporter families), without known function in lipid metabolism, could be identified to show significant change ($P<0.05$) after bariatric surgery. This alteration in expression levels indicates a possible involvement of these proteins in lipid homeostasis.
The center of this study became the protein Slc43a3, coding for a membrane transporter of the SLC family. It was found that Slc43a3 is expressed highly in various adipose tissues, in the same extent as popular proteins involved in lipid metabolism, such as Plin1, Hsl and Fabp4. Furthermore, it was observed that Slc43a3 undergoes a significant upregulation throughout differentiation, which was studied in the murine OP9 cell line as well as in primary preadipocytes.
However, the most exciting finding of this study is the substantial effect Slc43a3 has on FA transport. Knockdown of Slc43a3 in OP9 cells lead to a significant increase ($P<0.001$) in FA uptake rates and lipid accumulation, while overexpression resulted in significantly decreased ($P<0.001$) rates of FA uptake and lipid accumulation. Slc43a3 also seems to have an effect on FA efflux since knockdown of Slc43a3 significantly lowered the rates of FA efflux compared to control cells under basal conditions ($P<0.05$) and substantially blunted FA efflux when cells were treated with forskolin to stimulate lipolysis and FA efflux ($P<0.01$).
In the search for possible mechanisms for these actions, different approaches were pursued. First, it was investigated if knockdown/overexpression of Slc43a3 has an effect on cAMP levels. Since, both reduce forskolin-stimulated cAMP levels significantly, it remains unclear how Slc43a3 is related to cAMP. However, the results suggest that any Slc43a3 mediated changes in FA flux are independent of its effects on cAMP production.
Another hypothesis was that Slc43a3 does not influence FA transport itself, but rather influences one of the main proteins involved in lipid metabolism, such as SR-B1, CD36, HSL, ATGL, CGI58, G0S2, FATP1 and Plin1. Nevertheless,

neither knockdown nor overexpression presented any significant effect. Lastly, a potential relationship between Slc43a3 and CD36 was investigated. A FA uptake was performed in si-Slc43a3, si-CD36 and si-Slc43a3+CD36 transfected OP9 cells. The outcome of these experiments suggests that the actions of Slc43a3 on FA uptake are independent of CD36 expression. Whatever its mechanism of action, it can be concluded that Slc43a3 regulates fatty acid flux in adipocytes, functioning as a positive regulator of FA efflux and as a negative regulator of FA uptake. Moreover, the demonstration that manipulating a plasma membrane protein can dramatically affect the ability of a cell to export FA provides a new direction for gaining a deeper understanding of the processes mediating FA flux in mammalian cells.

6 Supplemental data

Supplemental Table S1. List of ABC transporters, SLC transporters and other genes screened.

	Accession #	Gene Symbol	Species	Locus ID
1	NM_018672	ABCA5	HUMAN	23461
2	NM_080284	ABCA6	HUMAN	23460
3	NM_019112	ABCA7	HUMAN	10347
4	NM_007168	ABCA8	HUMAN	10351
5	NM_080283	ABCA9	HUMAN	10350
6	NM_080282	ABCA10	HUMAN	10349
7	NM_000194	HPRT1	HUMAN	3251
8	NM_015657	ABCA12	HUMAN	26154
9	NM_152701	ABCA13	HUMAN	154664
10	NM_022717	SNRNP35	HUMAN	11066
11	NM_007188	ABCB8	HUMAN	11194
12	NM_019624	ABCB9	HUMAN	23457
13	NM_012089	ABCB10	HUMAN	23456
14	NM_001171	ABCC6	HUMAN	368
15	NM_033450	ABCC10	HUMAN	89845
16	NM_032583	ABCC11	HUMAN	85320
17	NM_033226	ABCC12	HUMAN	94160
18	NM_005164	ABCD2	HUMAN	225
19	NM_002858	ABCD3	HUMAN	5825
20	NM_005050	ABCD4	HUMAN	5826
21	NM_001090	ABCF1	HUMAN	23
22	NM_007189	ABCF2	HUMAN	10061
23	NM_018358	ABCF3	HUMAN	55324
24	NM_022169	ABCG4	HUMAN	64137
25	NM_001442	FABP4	HUMAN	2167
26	NM_001444	FABP5	HUMAN	2171
27	NM_003355	UCP2	HUMAN	7351
28	NM_000922	PDE3B	HUMAN	5140
29	NM_014037	SLC6A16	HUMAN	28968
30	NM_032178	FLJ13291	HUMAN	84138
31	NM_004695	SLC16A5	HUMAN	9121
32	NM_004694	SLC16A6	HUMAN	9120
33	NM_005495	SLC17A4	HUMAN	10050
34	NM_030958	SLCO5A1	HUMAN	81796
35	NM_080866	SLC22A9	HUMAN	114571
36	NM_004803	SLC22A14	HUMAN	9389
37	NM_018420	SLC22A15	HUMAN	55356

	Accession #	Gene Symbol	Species	Locus ID
38	NM_015482	SLC22A23	HUMAN	63027
39	NM_001010875	SLC25A30	HUMAN	253512
40	NM_032315	SLC25A33	HUMAN	84275
41	NM_207348	SLC25A34	HUMAN	284723
42	NM_201520	SLC25A35	HUMAN	399512
43	NM_018155	SLC25A36	HUMAN	55186
44	NM_178526	SLC25A42	HUMAN	284439
45	NM_145305	SLC25A43	HUMAN	203427
46	NM_014655	SLC25A44	HUMAN	9673
47	NM_182556	SLC25A45	HUMAN	283130
48	NM_138773	SLC25A46	HUMAN	91137
49	NM_015945	SLC35C2	HUMAN	51006
50	NM_001008783	SLC35D3	HUMAN	340146
51	NM_024881	SLC35E1	HUMAN	79939
52	NM_018656	SLC35E3	HUMAN	55508
53	NM_001001479	SLC35E4	HUMAN	339665
54	NM_001029858	SLC35F1	HUMAN	222553
55	NM_017515	SLC35F2	HUMAN	54733
56	NM_173508	SLC35F3	HUMAN	148641
57	NM_001080455	SLC35F4	HUMAN	341880
58	NM_025181	SLC35F5	HUMAN	80255
59	NM_014096	SLC43A3	HUMAN	29015
60	NM_033102	SLC45A3	HUMAN	85414
61	NM_001080431	SLC45A4	HUMAN	57210
62	NM_181785	SLC46A3	HUMAN	283537
63	NM_022551	RPS18	HUMAN	6222
64	NM_000942	PPIB	HUMAN	5479
65	NM_003194	TBP	HUMAN	6908
66	NM_000181	GUSB	HUMAN	2990
67	NM_000698	ALOX5	HUMAN	240
68	NM_001629	ALOX5AP	HUMAN	241
69	NM_000895	LTA4H	HUMAN	4048
70	NM_145867	LTC4S	HUMAN	4056
71	NM_000963	PTGS2	HUMAN	5743
72	NM_001061	TBXAS1	HUMAN	6916
73	NM_181657	LTB4R	HUMAN	1241
74	NM_019839	LTB4R2	HUMAN	56413
75	NM_006639	CYSLTR1	HUMAN	10800
76	NM_020377	CYSLTR2	HUMAN	57105
77	NM_001060	TBXA2R	HUMAN	6915
78	NM_006793	PRDX3	HUMAN	10935
79	NM_001512	GSTA4	HUMAN	2941
80	NM_012239	SIRT3	HUMAN	23410

Supplemental Table S2. PCR primers.

Gene	Mouse Accession	Primer	Primer-Sequence
ABCD4	NM_008992	Forward primer	GTTCCTGCCTCAGAAGCCAT
		Reverse primer	CAATCCTCTCATCGTCCGCA
ATGL	AK 031609	Forward primer	AACACCAGCATCCAGTTCAA
		Reverse primer	GGTTCAGTAGGCCATTCCTC
CD36	NM_001159555.1	Forward primer	CTTCACCAGAAATAGACCCTTGT
		Reverse primer	CAGATGCTAATTTGTGGTTGGTTG
CGI58	NM_026179	Forward primer	TCCTACCTGGTGTCCCACAT
		Reverse primer	GGATATGCGCACAGGCTCTT
FABP4	NM_024406.3	Forward primer	GCGTGGAATTCGATGAAATCA
		Reverse primer	CCCGCCATCTAGGGTTATGA
FATP1	XM_006509678.3	Forward primer	TTGCCCACAGCGTTTTCC
		Reverse primer	CACTAGCACGTCACCTGAGAGGTA
G0S2	NM_008059.3	Forward primer	GCGGATCCCGGACAAAAGAT
		Reverse primer	ACTGGCTTTGGCGTTCTTCT
HSL	NM_001039507	Forward primer	TCATGGCTCAACTCCTTCCT
		Reverse primer	CTGCCTCAGACACACTCCTG
SLC16A6	NM_134038	Forward primer	GTTTCCTCCCAACTGTGACCA
		Reverse primer	TAGCTGGTGCGAAAGCAAAC
SLC22A1	NM_001039371	Forward primer	GGGGCAGCATTTATGCCAAC
		Reverse primer	AGAAACGCTGCTAGTGTCCG
SLC25A3	NM_027460	Forward primer	AGACTAGCTCTTCGGACGGT
		Reverse primer	CGTCACAGACGTTGGTCTCA
SLC35C	NM_001252575	Forward primer	GATTCTGGCCTTCGGTTTGG
		Reverse primer	GATGCCAGCGATGGAGAGTG
SLC43A3	NM_021398	Forward primer	CACGTGGTGTGGCTGTCTAT
		Reverse primer	GTCCCCACCGGATAACTTGG
Plin1	NM_175640.2	Forward primer	CATCTCTACCCGCCTTCGAA
		Reverse primer	TGCTTGCAATGGGCACACT
SR-B1	XM_017320764.1	Forward primer	TCCCTTCGTGCATTTTCTCA
		Reverse primer	GTTCATCCCAACAAACAGGC

A1 Zusammenfassung

Adipozyten sind Zellen mit endokriner Funktion, die aktiv freie Fettsäuren aufnehmen und unter unterschiedlichen physiologischen Konditionen freigeben. Sie sind an der Regulation des Fettstoffwechsels beteiligt und somit Teil des Energiestoffwechsels. Eine Fehlregulation dieser Lipidhomöostase ist ein wesentlicher Faktor für das Entstehen von Erkrankungen wie Diabetes, kardiovaskulärer Herzkrankheit (KHK) und nicht-alkoholischer Fettleber (NAFLD, „nonalcoholic fatty liver disease“).
Freie Fettsäuren werden entweder in Form von Triglyceriden in Adipozyten gespeichert oder bewegen sich frei im Plasma. Die meisten Zellen, außer Adipozyten, haben keine ausreichende Kapazität um freie Fettsäuren zu speichern und sind deshalb auf die frei-zirkulierenden Fettsäuren angewiesen. Der Transport von freien Fettsäuren über die Plasmamembran wird von einer umfangreichen Menge an Proteinen und Enzymen kontrolliert und unterliegt verschschiedensten metabolischen und hormonellen Faktoren. Die Aufnahme von freien Fettsäuren scheint nicht durch Diffusion, sondern durch spezielle Transporter, welche an der Plasmamembran lokalisiert sind, zu erfolgen. Verschiedene Studien zur Aufnahme von freien Fettsäuren zeigten, dass Transportproteine wie Fettsäuretransporter (FATP), Thrombozytenglykoprotein 4 (FAT/CD36) und Fettsäure-CoA-Liagesen (ACSL), eine wesentliche Rolle in diesem Transportprozess übernehmen.
Obwohl heutige Arbeiten die diffusions-vermittelte Aufnahme freier Fettsäuren widerlegen, ist man immernoch der Annahme, dass die Freisetzung von freien Fettsäuren nach diesem Mechanismus erfolgt. Henkin et al. präsentierten jedoch Daten, die eine mögliche Beteiligung von speziellen Transportproteinen an dieser Freisetzung, suggerieren. Obwohl das Thema „Transport von freien Fettsäuren“ immer wieder erforscht und diskutiert wird, sind die exakten Mechanismen dieses Vorgangs noch nicht vollständig geklärt. Sind spezielle Membranporteine an der Abgabe freier Fettsäuren aus dem Adipozten beteiligt? Werden bereits erforschte Membrantransporter, wie FATP, CD36 oder ACSL, von bislang unbekannten Proteinen beeinflusst?
Der Versuch diese Fragen zu beantworten, begann mit einer in-silico Suche nach Proteinen mit folgenden Merkmalen: bislang keine gesicherte Funktion,

auf der Plasmamembran lokalisiert, ausreichende Expression im adipösen Gewebe.
Die Veränderung der Expression dieser Proteine wurde dann in Patienten vor- und nach bariatrischer Chirurgie untersucht. Mehr als 20 dieser Proteine zeigten eine signifikante Veränderung ihrer Expression (prä-operativ vs. post-operativ). Dieser Umstand macht es denkbar, dass die untersuchten Proteine eine Rolle im Fettstoffwechsel spielen könnten.

Zum Fokus dieser Studie wurde das Protein Slc43a3. Es zeigte eine hohe Expression in verschiedenen adipösen Geweben, welche vergleichbar mit bereits bekannten Fettstoffwechsel-Proteinen, wie Plin1, Hsl und Fabp4 ist. Außerdem zeigte sich eine vermehrte Expression von Slc43a3 im Verlauf der Zelldifferenzierung von Präadipozyten zu Adipozyten in in-vitro Experimenten mit Zellen der murinen OP9-Zelllinie und Präadipozyten, die aus Mäusen gewonnen wurden.
Besonders beeindruckend war jedoch der Effekt von Slc43a3 auf die Fettsäureaufnahme und Fettsäurefreisetzung. Zellen, in denen künstlich mit si-Slc43a3/ Slc4a3-clone das Protein Slc43a3 herunterreguliert bzw. überexprimiert wurde, zeigten signifikante Unterschiede in der Aufnahme und bei der Abgabe von freien Fettsäuren. Bei einer Überexprimierung von Slc43a3 nahm die Aufnahme von freien Fettsäuren und die Anhäufung von Lipidtropfen signifikant ab ($P<0.001$). Wohingegen bei einer Herunterregulierung von Slc43a3 die Aufnahme von freien Fettsäuren und die Anhäufung von Lipidtropfen signifikant zunahm ($P<0.001$). Auch die Freisetzung von freien Fettsäuren scheint durch eine Herunterregulierung von Slc43a3 beeinflusst zu werden, denn dessen Rate nimmt zum einen im Vergleich zur Kontrolle unter normalen Konditionen ($P<0.05$), als auch in Forskolin-stimulierten Zellen, signifikant ab ($P<0.01$).
Auf der Suche nach Mechanismen für diese Beobachtungen, wurden verschiedene experimentelle Ansätze verfolgt. Zunächst wurde untersucht ob die Herunter-/ Hochregulierung von Slc43a3 die Spiegel von cyclischem Adenosinmonophosphat (cAMP) beinflusst. Tatsächlich zeigten sich signifikante Unterschiede zwischen transfizierten Zellen im Vergleich zur Kontrolle. Da jedoch sowohl die Herunterregulierung als auch die Überexpression von Slc43a3 die Forskolin-stimulierten cAMP Spiegel senkten, scheint der Effekt

von Slc43a3 unabhängig von der Produkution von cAMP zu sein. Desweiteren wurde untersucht ob Slc43a3 eventuell zusammen mit anderen Fettstoffwechsel-Proteinen agiert oder diese beeinflusst. Aber sowohl eine Herunterregulierung als auch eine Überexpression zeigten keine signifikaten Unterschiede in der Expression von Proteinen wie SR-B1, CD36, HSL, ATGL, CGI58, G0S2, FATP1 oder Plin1.

Zuletzt wurde eine mögliche Beziehung von Slc43a3 zu CD36 geprüft. Ein Experiment zur Aufnahme freier Fettsäuren wurde in si-Slc43a3, si-CD36 und si-Slc43a3+CD36 transfizierten Zellen durchgeführt. Das Resultat dieses Experiments macht es jedoch wahrscheinlich, dass Slc43a3 den Fettstoffwechsel unabhängig von CD36 beeinflusst.

Unabhängig vom genauen Aktionsmechanismus lässt sich zusammenfassen, dass Slc43a3 den Transport freier Fettsäuren in Adipozyten (mit-) reguliert. Slc43a3 funktioniert als positiver Regulator bei der Freisetzung von freien Fettsäuren und als negativer Regulator bei der Aufnahme von freien Fettsäuren. Der genaue molekulare Mechanismus bleibt dabei unklar und ist Gegenstand weiterer Forschungsarbeiten.

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A3 List of abbreviations

ABC	ATP-binding cassette
ACSL	long chain fatty acyl coenzyme A synthetase
Amp	ampicillin
AUC	area under the curve
bDNA	branched DNA
BMI	body mass index
BSA	bovine serumalbumin
cAMP	cyclic adenosine monophosphate
EEG1	embryonic epithelia gene 1
FA(s)	fatty acid(s)
FAT	fatty acid translocase (CD36)
FATP	fatty acid transport protein
FABP	fatty acid binding protein
FFA(s)	free fatty acid(s)
GI	gastrointestinal (tract)
h	hour(s)
HDL	high-density lipoprotein
HSL	hormone-sensitive lipase
IDL	intermediate-density lipoprotein
IL	interleukin
KO	knockout
LAT	amino acid system L transporter
LB	lysogeny broth
LCFA(s)	long chain fatty acid(s)
LDL	low-density lipoprotein
min	minute(s)
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PLIN1	lipid droplet-associated protein (Perilipin-1)
PM	plasma membrane
PPAR	peroxisome proliferator activated receptor

RFU	relative fluorescence units
rpm	rounds per minute
s	second(s)
SLC	solute carrier
TAG	triacylglycerol
TNF	tumor necrosis factor
VLDL	very-low-density lipoprotein
VSG	vertical sleeve gastrectomy

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