PEX16: a novel player in adipocyte development and energy metabolism

Master Thesis

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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(date) (signature)

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Abstract

English

Keywords: PEX16, peroxisomes, adipogenesis

Adipose tissue, its formation and function have intensively been studied in the past. It represents a promising target for the treatment of obesity and popular health related problems like type 2 diabetes. New players in adipogenesis and lipid metabolism are often discovered comparing adipose tissues of lean and obese mice using high throughput technologies. *Pex16* is one of these candidate genes, as it was shown to be highly upregulated during the differentiation of brown adipocytes in unpublished microarray data of our group. PEX16 protein is involved in peroxisomal de novo formation, growth and fission [1]. Regulation of peroxisomal growth and fission was shown to occur in concert with mitochondrial reorganization and division under stimulations, such as cold exposure and high fat diets in adipose tissue [2][3], suggesting a role in adipose tissue formation and metabolism. However, PEX16 and peroxisomal function have mainly been studied in the liver. The role of PEX16 in adipose tissue has not been investigated until today.

Pex16-expression profiles of murine tissues revealed that *Pex16* is, besides liver, also highly expressed in brown and white adipose tissue (BAT and WAT) and undergoes upregulation in brown adipose tissue and liver under high-fat dietary stimulation. Additionally, *Pex16* was upregulated during differentiation of several adipose cell lines, such as 3T3-L1, iBACs (immortalized brown adipose cell-line), C3H/10 T1/2, SGBS (Simpson Golabi Behmel Syndrome cells), and hMADS (human multipotent adipose-derived stem cells). The highest expression was observed in brown adipose tissue and in iBACs. Overexpression and silencing experiments were performed in models for white (3T3-L1) and brown (iBACs) adipocytes. Both variations of PEX16-expression provoked reduced lipid accumulations during differentiation. Overexpression of PEX16 in 3T3-L1 cells and iBACs caused a decrease in peroxisomal β-oxidation gene expression, such as *Acox1* and *Ehhadh*, and alterations in the expression of the adipogenesis marker gene *Pparγ*. A reduced cell proliferation was observed for PEX16-overexpressing iBACs. Following hints for direct interactions of *Pex16* and PPARγ, luciferase assays with putative PPARγ-binding sites were performed, uncovering two regions of PPARγ interference. These results indicate a potential role of *Pex16* in (brown) adipogenesis and energy metabolism.

German

Stichwörter: PEX16, Peroxisomen, Adipogenese

Entstehung und Funktion von Fettgewebe wurden aufgrund ihres nachgewiesenen Zusammenhangs mit einer Vielzahl von auf Adipositas zurückgehenden und besonderes herausfordernden Krankheiten des 21. Jahrhunderts, wie zum Beispiel Typ 2 Diabetes, in den letzten Jahren intensiv beforscht. Mittels Hochdurchsatzverfahren konnten bereits einige wichtige Gene ausfindig gemacht werden, die essenziell in der Fettzellentwicklung und im Fettstoffwechsel sind. Im Rahmen eines solchen Microarray-Experiments unserer Arbeitsgruppe wurden wir auf Pex16 aufmerksam, da es in differenzierenden braunen Fettzellen (iBACs) besonders stark exprimiert war. Das Protein PEX16 ist wesentlich an Neubildung, Wachstum und Teilung von Peroxisomen beteiligt [1]. Es wurde gezeigt, dass sich Peroxisomen im Fettgewebe in Anzahl und Aktivität an äußere Gegebenheiten wie Kälteeinwirkung oder sehr fettreiche Ernährung anpassen, wie es auch schon für Mitochondrien bekannt ist, die maßgeblich für die Funktion von braunem Fettgewebe verantwortlich sind [2][3]. Diese Beobachtung lässt vermuten, dass Peroxisomen und daher auch PEX16 eine relevante Rolle im Fettgewebe spielen könnten. Der Einfluss von PEX16 auf Fettzellentwicklung und deren Stoffwechsel ist indes in der Literatur noch nicht beschrieben.

Expressionsprofile von *Pex16* in verschiedenen Maus-Geweben machten deutlich, dass *Pex16* abgesehen von der Leber auch in braunem und weißem Fettgewebe stark exprimiert ist. Die Expression von PEX16 in braunem Fettgewebe und in der Leber von Mäusen, denen eine High-Fat-Diet verabreicht wurde, ist sogar noch höher. Ebenso konnte eine Hochregulierung während der Differenzierung in allen betrachteten Zelllinien (3T3-L1, iBACs (immortalisierte braune Fettzelllinie), C3H/10 T1/2, SGBS (Simpson Golabi Behmel Syndrom Zellen) und hMADS (humane, multipotente Stammzellen, abgeleitet von Adipozyten) festgestellt werden. Die höchste Expression war in braunem Fettgewebe sowie in braunen Fettzellen (iBACs) vorzufinden. Um einen besseren Einblick in die Rolle von PEX16 im Fettgewebe zu erhalten, wurde versucht, PEX16 in weißen (3T3-L1) und braunen (iBACs) Fettzellen überzuexprimieren und zu silencen. Beide Versuchsanordnungen waren durch verringerte Lipideinlagerungen in die Zellen gekennzeichnet. In PEX16- überexprimierenden Zellen konnte ein Trend zu verringerter Expression von peroxisomalen β-Oxidations-Genen (*Acox1* und *Ehhadh*) sowie eine veränderte *Pparγ*-Expression festgestellt werden. In iBACs führte die Überexpression von PEX16 zu einer reduzierten Proliferation der Zellen. Da Hinweise für eine potentielle Interaktion zwischen PPARγ und *Pex16* vorhanden waren, wurde diese im Rahmen von Luciferase Assays untersucht, die schließlich die Existenz von zwei PPARγ- Bindestellen in der genomischen Sequenz von *Pex16* weitgehend bestätigten. Die erhaltenen Ergebnisse deuten an, dass PEX16 eine wesentliche Rolle in der Entwicklung und im Energie-Stoffwechsel von Fettzellen spielen könnte.

Contents

1 Abbreviations

ATP	adenosine-5'-triphosphate
BAT	brown adipose tissue
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma protein 2
BMI	body mass index (kg/m^2)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CDS	coding DNA sequence
ddH_2O	double distilled H_2O
Dex	dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonuceic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ER	endoplasmic reticulum
FA	fatty acid
FBS	fetal bovine serum
FFA	free fatty acid
gDNA	genomic DNA
GM	growth medium
HFD	high fat diet
His-Tag	codon for six histidines in a row
iBAC	immortalized brown adipose cell line
IBMX	3-Isobutyl-1-methylxanthine
Indo	Indomethacin
ko	knockout
mRNA	messenger RNA
MOI	multiplicity of infection
MCS	multiple cloning site
NTC	non targeting control

ob	obese gene
o/e	overexpression
ONC	overnight culture
PBS	phosphate buffered saline
PBST	phosphate buffered saline including Tween20
PCR	polymerase chain reaction
Pex16	Peroxisomal biogenesis factor 16 gene
PEX16	Peroxisomal biogenesis factor 16 protein
PGC-1α	PPARγ-coactivator 1α protein/gene
PIC	protease inhibitor cocktail
pMSCV	murine stem cell virus plasmid
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome prolifer-activated receptor response element
P/S	penicillin/ streptomycin
qRT-PCR	quantitative real-time polymerase chain reaction
RE	restriction enzyme
Rosi	rosiglitazone maleate
RXRα	retinoid X receptor alpha
SGBS	Simpson-Golabi-Behmel syndrome
SD	standard deviation
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TG	triglycerides
TNFα	tumor necrosis factor α
TSS	transcription start site
UCP1	uncoupled protein 1
WAT	white adipose tissue
wt	wild-type

2 Introduction

PEX16, peroxin-16 or peroxisomal biogenesis factor 16 are only a few names of a stunning protein, showing various functions reaching from peroxisomal biogenesis to peroxisomal membrane protein (PMP) import, depending on the expressing organism [1]. Absence or mutations of the protein lead to severe, often lethal neurological and developmental diseases.

In humans, *PEX16* gene is located on chromosome 11 (11p11.2)[1], encoding two protein-isoforms (isoform 1: 336aa, ≈ 38.6 kDa, isoform 2: 346aa, ≈39.3 kDa)[2]. Homologs of the gene can be found in almost all eukaryotes, showing about 15-25% sequence identity [1]. In mice, *Pex16* gene is located on chromosome 2 (2E1;2), encoding two transcript variants[3], whereas only variant 1 encodes the functional PEX16- protein (336aa, ≈38.6 kDa)[4].

The function of PEX16 highly depends on the expressing organism and furthermore on the intracellular localization within these organisms:
PEX16 is commonly targeted to peroxisomes and endoplasmic reticulum [4]. In yeast, PEX16 is a peripheral membrane protein, involved in peroxisomal biogenesis and fission when bound to peroxisomes [1]. This was confirmed by overexpression experiments in yeast, resulting in a decreased number of peroxisomes, which were additionally enlarged in size compared to controls [1][5]. Whereas bound to the endoplasmic reticulum, PEX16 influences cell differentiation and polarity [6][7].

On the contrary, in humans, PEX16 is an integral membrane protein, showing multiple transmembrane domains, with N- and C- termini facing the cytosol [8]. The C-terminal region showed to be essential for the biological function [9], as it seems to play a role in the early phase of peroxisomal de novo synthesis at the endoplasmic reticulum. When integrated into peroxisomes, it influences the attraction of peroxisomal membrane proteins from the cytosol to mature peroxisomes [1]. More precisely, it appears to be a receptor for PEX3 in the endoplasmic reticulum (ER) as well as in mature peroxisomes. Based on this assumption, it enables the PEX3-dependent integration of group I or group II peroxisomal membrane proteins, respectively, either into pre-peroxisomes released from the ER or into mature peroxisomes, promoting growth and fission of these organelles (see figure 2.1). [4][10][11][12].

[1] http://www.ncbi.nlm.nih.gov/gene/9409, July 2014
[2] http://www.uniprot.org/uniprot/Q9Y5Y5, July 2014
[3] http://www.ncbi.nlm.nih.gov/gene/18633, July 2014
[4] http://www.uniprot.org/uniprot/Q91XC9 , July 2014

By taking these assumptions into consideration, PEX16 might be the driving force (or "master"-peroxin) in launching peroxisomal biogenesis de novo [1].

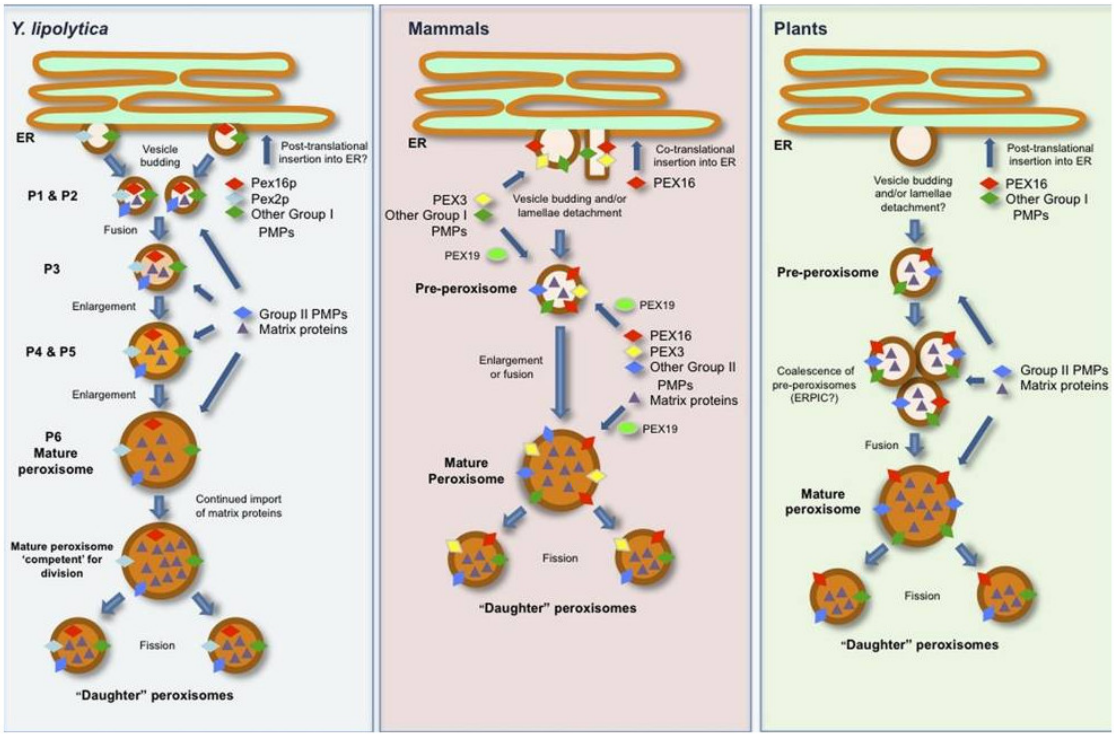

Figure 2.1: Scheme of peroxisomal biogenesis in different organisms: *Y. lipolytica*, *Mammals* and *Plants*. In mammals, PEX16 is co-translationally inserted into the ER by the SEC61-dependent import pathway. At the ER it is integrated into pre-peroxisomes, where it serves as receptor for PEX3 and group 1 PMPs. PEX16 is also directly targeted to pre-peroxisomes, still serving as PEX3 and group 2 PMP receptor and causing the formation of mature peroxisomes, which can now undergo fission for proliferation. [1].

From this point of view, it's no surprise that peroxisomes fail to appear in cells when PEX16 is mutated or absent, which was observed similarly for alterations of PEX3 and PEX19 expression, that are also involved in the early stages of peroxisomal biogenesis [4][8][13][14][15].
Accumulations of toxic substances, like very long-chain fatty acids, phytanic acid and pristanic acid, in the cell caused by a complete or partial loss of peroxisomal functions, culminate in severe developmental and neurological dysfunctions. These are commonly known as peroxisomal biogenesis disorders (PBD) [16][17][18], including fatal Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease [19][20][21].

Peroxisomes are tiny organelles, that are present in almost all eukaryotic cells and show very complex metabolic functions, reaching from α- and β- oxidation of very long fatty acids over the oxidation of ether lipids, bile acids and cholesterol, to the detoxification of H_2O_2 [4][18][22].

In contrast to their complexity of functions and biosynthesis, the peroxisomal final structure is very simple, composing of a nonhomogenous matrix embedded in a single lipid bilayer membrane (see figure 2.2) [22].

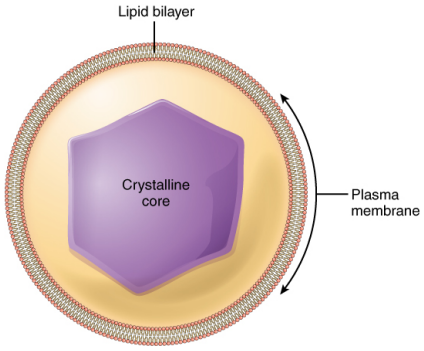

Figure 2.2: Peroxisomal composition: Plasma membrane, nonhomogenous matrix and optional crystaline core. Source: *www.boundless.com/biology/cell-structure/eukaryotic-cells/ peroxisomes*

By defining stationary levels of multiple signaling lipids like phytanic acid, retinoic acid and especially long-chain fatty acids within the cell, activation of RARs (retinoic acid receptors) and PPARs (peroxisome proliferator-activated receptors) is facilitated, resulting in the transcription of genes necessary for embryonic development and differentiation of various tissues, including adipose, brain, skin, and placental tissues in mammals [23][24][25][26].

Although peroxisomes have long been known for their importance in metabolizing lipids, their functions have primarily been investigated in the liver [27]. Based on the observation, that peroxisomes do not participate at the respiratory chain, and FAD-linked oxidases of the peroxisomal β- oxidation are direct donors of electrons to O_2, possible roles of these organelles in thermogenesis in brown adipose tissue (BAT) and in the regulation of body weight have been suggested [3][27][28][29][30].

Three different cell organelles are responsible for the oxidation of fatty acids: 1) mitochondria, 2) peroxisomes, both performing β- oxidation, and 3) the endoplasmic reticulum, performing ω- oxidation of fatty acids [20][31][32][33][34]. Fatty acids exhibit crucial functions in a whole set of processes within the cell, such as storage of energy, cellular membrane synthesis and signal transduction [20]. In mitochondria, β-oxidation of short($<C_8$), medium (C_8-C_{12}) and long (C_{12}-C_{20})-chain fatty acids takes place, generating acetyl-CoA for energy utilization in form of ATP by oxidative phosphorylation or alternatively in activated brown adipose tissue in form of heat [20][34]. Long-chain fatty acids serve as main energy source in conditions of normal feeding and also in fasting state [20].

Peroxisomal β- oxidation, on the contrary, is probably the only possible way of oxidizing very long-chain fatty acids ($>C_{20}$) and trans-unsaturated acids, which can not be metabolized in mitochondria as they do not feature a very long-chain fatty acyl-CoA synthetase [20][33][34][35]. Two ways of peroxisomal β- oxidation are known: The first one, the "traditional" way, was shown to be inducible by PPARα, thyroid hormones, cold exposure and high fat diets. The second way is non-inducible and responsible for oxidation of branched-chain fatty acyl-CoAs. [3][20][33][34].

In peroxisomes, three enzymes are mainly involved in the traditional β-oxidation (see figure 2.3): ACOX1 (straight-chain acyl-CoA oxidase or acyl-Coenzyme A oxidase 1) initiates the peroxisomal β- oxidation by oxidizing very long-chain fatty acyl-CoAs.[20][33][34]. EHHADH (enoyl CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) exhibits two different functions. In the second step of peroxisomal β-oxidation it functions N-terminally as enoyl-CoA hydratase. In the third step, it functions C-terminally as 3-hydroxyacyl-CoA dehydrogenase [5]. ACAA1 (acetyl-CoA acyltransferase 1) serves as catalyst in the reversible thiolytic cleavage of 3-ketoacyl-CoA producing acyl-CoA and acetyl-CoA[6]. Generated acetyl-CoA is utilized in anabolic reactions for cholesterol- and bile acid synthesis [36] or is delivered to mitochondria via a "carnitine shuttle" [30], where it can be used for energy production (see figure 2.3). Energy provided via peroxisomal β-oxidation, which generates H_2O_2, is not preserved in form of ATP, leading to the idea that peroxisomal β- oxidation could eventually be thermogenic, comparable to the energy dissipation in form of heat in mitochondria of brown adipose tissue [20][27].

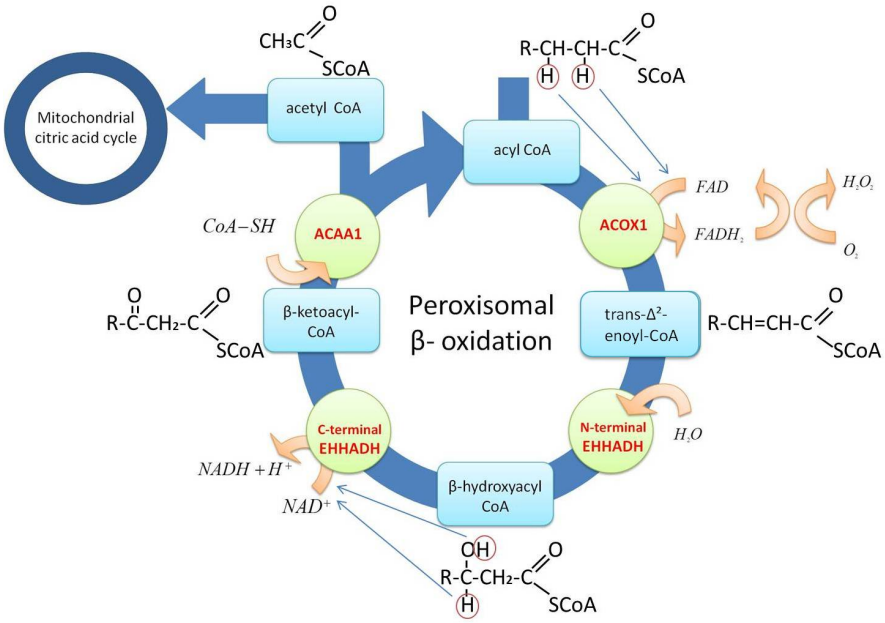

Figure 2.3: Scheme of the peroxisomal β- oxidation. Acyl-CoA is oxidized to acetyl-CoA, which can be delivered to mitochondria where it serves for energy generation. Hydrogen peroxide is generated within the first step of the process.

[5] www.ncbi.nlm.nih.gov/gene/1962, July 2014
[6] www.ncbi.nlm.nih.gov/gene/30, July 2014

It has been shown in the past, that peroxisomes react similarly to mitochondria in response to various stimuli by reorganization and fission [2], suggesting an extensive regulatory linkage between these organelles. Thermogenic, thyroid hormonal stimulation as well as high fat diets and modified expression of PGC-1α, a transcription cofactor in energy metabolism, led to significant morphological and quantitative changes of peroxisomes [2][3]. Elevated numbers of peroxisomes were observed in brown adipose tissue of mice and rats exposed to cold and in fully differentiated murine fat cells overexpressing PGC-1α. This observation was accompanied by increased mRNA-levels of peroxisomal β-oxidation and biogenesis genes (*Acox1*, *Acaa1a*, *Ehhadh*, *Pex11*α, *Pex11*β, *Pex16*, *Pex19*, *PMP70*, *DLP1*) and important mitochondrial genes (*UCP1*, *PGC-1*α).[2][28]. The peroxisomal β-oxidation genes *Acox1*, *Acaa1a* and *Ehhadh* were also found increased in adipose tissues of obesity-resistant black 6 mice under high-fat-diet, suggesting a role of peroxisomal β- oxidation in body-fat control [37]. Peroxisomal β- oxidation activity in brown fat was also studied in perinatal rabbits, with the outcome, that peroxisomal activity was highest prior to birth, suggesting, that peroxisomes might not play a role in thermogenesis itself, but in predetermination of brown fat formation [38]. More recently, aP2-Pex5- knockout in mice was performed. The aP2-Pex5- knockout represents an almost adipose tissue specific knockout of *Pex5*. *Pex5* is a peroxisomal gene involved in peroxisomal matrix protein import. [27]. Experiments in aP2-Pex5- knockout mice revealed increased white fat depots and decreased lipolysis, accompanied by defective shivering thermogenesis, motor activity and elevated insulin resistance. However, thermogenesis in BAT was not affected even under long term cold exposure.[27]. Nevertheless, it is not yet verified, whether the elevated fat mass in aP2-Pex5-knockout mice traces back to the deficiency of peroxisomes [27]. Interestingly, knockout of *Pex7*, an additional gene involved in peroxisomal matrix protein import [7], in mice displayed reduced lipid accumulations in both, white and brown adipose tissue caused by the absence of ether lipids due to a defective peroxisomal β- oxidation. Lipid stores recovered when mice were fed nutrition rich in ether lipid precursors.[27][39]. Amongst others, *Pex5* and *Pex7* together with *Pex16* belong to the group of PEX-genes, which encode proteins responsible for proper peroxisomal assebly [40].

In general, three distinct types of adipose tissues are known: White adipose tissue (WAT), beige or brite ("brown in white") adipose tissue, and brown adipose tissue (BAT) [41]. White adipose tissue represents the biggest part of adipose tissue in humans and is characterized by a single, large lipid droplet, and only few mitochondria. In brown adipose tissue, multiple smaller lipid droplets and a high number of mitochondria can be found [42]. Accumulations of beige adipocytes can be found within white adipose tissue. These adipocytes share several properties with brown adipocytes, although they do not exhibit full "brown capacity". Their occurrence is considered to be inducable by cold exposure or β3-adrenergic stimulation [41].

[7] www.ncbi.nlm.nih.gov/gene/5191

Brown adipose tissue has been of great interest for many research teams in the past few years, because of its ability to increase energy consumption by dissipating energy as heat when activated [43][44]. This heat generating process is mediated by uncoupling protein 1 (UCP1), which is located in mitochondria exclusively in beige and brown adipose tissue. By generating a proton leak across the inner mitochondrial membrane, it "uncouples" the oxidation of fuel from the synthesis of ATP [41], and produces heat. Thereby, the activation of BAT offers a great possibility for the treatment of obesity and its associated disorders like type 2 diabetes, heart disease, hypertension, stroke, and multiple types of cancer, making up a majority of the 21^{st} century's complex diseases responsible for the exploding costs in modern healthcare [45].

Many genes participating in brown adipose tissue formation, mitochondrial development and thermogenesis have already been identified. Now, peroxisomes have been shown to represent a promising new player in the complex network of adipocyte development and metabolism, as they are regulated along with mitochondria. PEX16 is highly upregulated in the brown adipose cell line iBACs during adipogenic differentiation, which was shown by microarray experiments of our group (data not published), and is highly expressed in liver, white and brown adipose tissue in mice [8]. The finding, that it seems to be the initiator of peroxisomal biogenesis and the fact that only little about its role in adipogenesis and lipid metabolism has been described so far, make PEX16 a promising new target to be investigated in the context of adipocyte development and energy metabolism.

[8] www.biogps.org/#goto=genereport&id=18633, July 2014

3 Materials & Methods

3.1 Materials

3.1.1 Buffers, Chemicals and Reagents

- ▷ 1 kb DNA Ladder, Fermentas Inc.
- ▷ 5-(3-aminoallyl)-2´-deoxyuridine-5´-triphosphate (AA-dUTP); Sigma-Aldrich Handels GmbH
- ▷ Aqua bidestillata sterilis. "Fresenius"; Fresenius
- ▷ UltraPure^{TM} Agarose; Life Technologies, Invitrogen corp.
- ▷ Benzonase® Nuclease; Merck Chemicals
- ▷ Bovine Serum Albumine (BSA, Lot.Nr. K00110-1227); PAA Laboratories GmbH
- ▷ Chloroform; Sigma-Aldrich Handels GmbH
- ▷ Dimethylsulfoxide (DMSO); Sigma-Aldrich H. GmbH
- ▷ DEPC treated H2O for molecular biology; Karl Roth GmbH + Co. KG
- ▷ Dexamethasone (Dex); Sigma-Aldrich Handels GmbH
- ▷ Diethylether; Karl Roth GmbH + Co. KG
- ▷ Dithiothreitol (DTT) 0.1M; Life Technologies, Invitrogen corp.
- ▷ Dithioerythritol (DTE); VWR International (Merck Chemicals)
- ▷ dNTP Set (100mM); Fermentas
- ▷ ECL prime; Amersham, GE Healthcare
- ▷ Ethanol absolute, for analysis; Lactan chemicals and laboratory devices
- ▷ Ethidiumbromidlösung 1%; Karl Roth GmbH + Co. KG
- ▷ 5x First Strand Buffer (5xFS); Life Technologies, Invitrogen corp.
- ▷ Foetal Bovine Serum (FBS, Lot.Nr. 09SB032); Lonza Inc.
- ▷ Formaldehyde; Sigma-Aldrich Handels GmbH
- ▷ Formamide; Sigma-Aldrich Handels GmbH
- ▷ Glycerol 98%; Lactan chemicals and laboratory devices
- ▷ Geneticindisulfat (G418-Sulfat); Karl Roth GmbH + Co. KG
- ▷ Hepes buffer 1M; Life Technologies, Invitrogen corp.
- ▷ Hexadimethrine (Polybrene 8mg/mL); Sigma-Aldrich Handels GmbH

- ▷ 3-isobutyl-1-methylxanthine (IBMX, 1g); VWR International (Merck Chemicals)
- ▷ Indomethacin crystalline (Indo); Sigma-Aldrich Handels GmbH
- ▷ Insulin (10mg/mL); Sigma-Aldrich Handels GmbH
- ▷ Isopropyl alcohol; VWR International (Fisher Scientific U.K. Ltd.)
- ▷ Isoproterenol; Sigma-Aldrich Handels GmbH
- ▷ K_2HPO_4; Sigma-Aldrich Handels GmbH
- ▷ KH_2PO_4; Sigma-Aldrich Handels GmbH
- ▷ LDS Sample Buffer 4x; Life Technologies, Invitrogen corp.
- ▷ L-Glutamine (L-Glut) 200mM; Life Technologies, Invitrogen corp.
- ▷ Lentiviral MISSION® Transmission Particles NM_145122; Sigma - Aldrich Handels GmbH
- ▷ Mouse anti-β-actin antibody; Sigma-Aldrich H. GmbH
- ▷ Metafectene; Biontex Laboratories GmbH
- ▷ Methanol Normapur for analysis; VWR International (Merck Chemicals)
- ▷ NaCl; Karl Roth GmbH + Co. KG
- ▷ Na_2CO_3; VWR International (Merck Chemicals)
- ▷ NaF; VWR International (Merck Chemicals)
- ▷ NaOAc; Sigma-Aldrich Handels GmbH
- ▷ Natriumorthovanadate; Sigma-Aldrich Handels GmbH
- ▷ n-hexane; Karl Roth GmbH + Co. KG
- ▷ Normocin (Normo); Eubio
- ▷ NuPAGE® Antioxidant; Life Technologies, Invitrgen corp.
- ▷ NuPAGE® 10% Bis-Tris Gel 1.0mm X 10 well; Life Technologies, Invitrogen corp.
- ▷ NuPAGE® 4-12% Bis-Tris Gel 1.0mm X 10 well; Life Technologies, Invitrogen corp.
- ▷ NuPAGE® MOPS SDS Running Buffer 20x; Life Technologies, Invitrogen corp.
- ▷ NuPAGE® MES SDS Running Buffer 20x; Life Technologies, Invitrogen corp.
- ▷ Oil Red O; ICN
- ▷ Oligo-dT Primers; Life Technologies, Invitrogen corp.
- ▷ PBS pH 7.4, Life Technologies, Invitrogen corp.
- ▷ Phosphate buffered saline (PBS, pH 7.4); Life Technologies, Invitrogen corp.
- ▷ Penicillin / Streptomycin (P/S) Sol 10.000U/mL per 10.000µg/mL; Life Technologies, Invitrogen corp.
- ▷ Poly(A)-DNA; Life Technologies, Invitrogen corp.
- ▷ Polyclonal Goat Anti-Rabbit IgG / HRP, DakoCytomation
- ▷ Polyclonal Goat Anti-Mouse IgG / HRP, DakoCytomation

- ▷ Protease Inhibitor Cocktail Tablets (PIC); Roche Austria GmbH
- ▷ Puromycin dihydrochloride CELL CULTURE; Sigma-Aldrich Handels GmbH
- ▷ Rabbit Polyclonal Anti-PEX16 IgG; Acris Antibodies GmbH
- ▷ Random Hexamer Primers 3µg/µL; Life Technologies, Invitrogen corp.
- ▷ RNaseOUT^{TM}, Life Technologies, Invitrogen corp.
- ▷ Roentogen EUKOBROM (b/w paper developer); Tetanal
- ▷ Roentogen Superfix (fixing bath for rapid processing of b/w materials); Tetanal
- ▷ Rosiglitazone Maleate (Rosi), Ebio
- ▷ 20x Saline-Sodium Citrade (SSC); Sigma-Aldrich Handels GmbH
- ▷ Seeblue® Plus2 Prestained Standard; Life Technologies, Invitrogen corp.
- ▷ Skim Milk Powder (NFDM); Fluka analytical AG
- ▷ Sodium Dodecylsulfate (SDS); VWR International (Merck Chemicals)
- ▷ 10% Sodium Dodecyl Sulfate (SDS), Life Technologies, Invitrogen corp.
- ▷ Super Script II Reverse Transcriptase (200U/µL); Life Technologies, Invitrogen corp.
- ▷ Super Signal West Pico Chemoluminescent Substrate; VWR International (Fisher Scientific U.K. Ltd.)
- ▷ SYBR QPCR Supermix W/Rox; Life Technologies, Invitrogen corp.
- ▷ TLC silica gel 60, Merck Chemicals
- ▷ 0.5% Trypsin / EDTA (10x); Life Technologies, Invitrogen corp.
- ▷ Triiodthyronine (T3); Sigma-Aldrich Handels GmbH
- ▷ Tris Glycine Buffer (TGS; 10x), Bio-Rad Laboratories GmbH
- ▷ Tris Ultra Quality, Lactan chemicals and laboratory devices
- ▷ Tween20, VWR International (Merck Chemicals)

TAE-buffer:	242 g Tris Ultra Quality 57,1 g Acetate 16,8 g EDTA 1 L dH_2O	LB-medium:	10 g Peptone 10 g NaCl 5 g Yeast 1 L dH_2O
LB-Medium for agar-plates:	10 g Peptone 10 g NaCl 5 g Yeast 1 L dH_2O 15g Agar	Glycerolstock:	25mL LB-medium 25mL Glycerin (>98%)

3.1.2 Cell Lines

3H/10 T1/2: mouse embryonic fibroblasts; muscle, adipose, bone or cartilage like
3T3-L1: mouse embryonic fibroblasts; adipose like
Cos7: fibroblasts; recovered from green vervet monkey
hMADS: human multipotent adipose-derived stem cells
iBACs: SV40 T-large antigen immortalized brown adipose cell line, kind gift of Patrick Seale, University of Pennsylvania School of Medicine, Philadelphia
Phoenix: human embryonic kidney cell line, retroviral expression system
SGBS: human Simpson-Golabi-Behmel Syndrome cells, adipose like

3.1.3 Culture Media

Standard Growth Medium (**DMEM++++**) for 3T3-L1, Cos7 & Phoenix cells:

DMEM (Dulbeccos Modified Eagle Medium, 4,5g Glucose), Invitrogen	
+ FBS	10%
+ Normocin	1:500
+ L-Glut	2mM
+ P/S	100U/mL / 100µg/mL

Differentiation/Induction Medium (**DM1**) for 3T3-L1 cells:

DMEM++++	
+ Insulin	2µg/mL
+ Dex	1µM
+ IBMX	0,5mM

Differentiation/Induction Medium 2 (**DM2**) for 3T3-L1 cells:

DMEM++++	
+ Insulin	2µg/mL

Differentiation/Induction Medium 3 (**DM3**) for 3T3-L1 cells:

DMEM++++	
+ Insulin	2µg/mL
+ Dex	1µM
+ IBMX	0,5mM
+ Rosi	1µM

iBACs Growth Medium (**iBACs-GM**):

DMEM (Dulbeccos Modified Eagle Medium, 4,5g Glucose), Invitrogen	
+ FBS	10%
+ HEPES	20mM
+ P/S	100U/mL / 100µg/mL

iBACs Maintenance Medium (**iBACs-MM**):
iBACs GM
+ Insulin 20nM (1,161 μL/mL-GM)
+ T3 1nM

iBACs Induction Medium (**iBACs-IM**):
iBACs MM
+ Dex 500nM
+ IBMX 0,5mM
+ Indo 0,125nM

Freeze Medium (**FM**) for 3T3-L1, Cos7 & Phoenix cells:
DMEM++++
+ DMSO 5%

iBACs Freeze Medium (**iBACs-FM**):
iBACs-GM 9%
+DMSO 10%
+FBS 81%

FFA- free Medium:
DMEM (Dulbeccos Modified Eagle Medium, 4,5g Glucose), Invitrogen
+FFA-free BSA 2%

FFA-free Medium + Isoproterenol:
DMEM (Dulbeccos Modified Eagle Medium, 4,5g Glucose), Invitrogen
+FFA-free BSA 2%
+Isoproterenol 10µM

3.1.4 Enzymes

- ▷ DNA Polymerase I, Lg (Klenow) Fragment, New England Biolabs, Inc.
- ▷ FastAP; Fermentas
- ▷ Phusion High-Fidelity DNA Polymerase; Thermo Fisher Scientific, Inc.
- ▷ Q5® High Fidelity DNA Polymerase; New England Biolabs, Inc.
- ▷ T4 DNA Ligase; Fermentas
- ▷ Taq DNA Polymerase (recombinant); Fermentas
- ▷ HindIII-HF®, BamHI-HF®, XhoI, KpnI-HF®; New England Biolabs, Inc.
- ▷ BglII, EcoRI, HpaI, XhoI, NotI; Fermentas

3.1.5 Vectors

- ▷ pMSCVpuro; Clontech Laboratories Inc.
- ▷ pHisMaxC; Invitrogen corp.
- ▷ pMSCVhygro; Clontech Laboratories Inc., kind gift from E.D. Rosen
- ▷ pPPRE X3-TK-luc,(Addgene Plasmid 1015); Addgene, Inc.
- ▷ pGL4.26; Promega, Madison, USA
- ▷ pGL4.75; Promega, Madison, USA

3.1.6 Kits and technical devices

- ▷ 1L 0.22µm cellulose acetate (CA) Filter System; Corning Costar
- ▷ ABI Prism 7000 Sequence Detection System
- ▷ BCA Protein Assay Kit; VWR International (Fisher Scientific U.K. Ltd.)
- ▷ Dual-Luciferase® Reporter Assay System; Promega GmbH
- ▷ FLUOstar Omega microplate reader; BMG LABTECH GmbH
- ▷ Microscope cover glass; Fisher Scientific U.K. Ltd.
- ▷ Mini Trans-Blot® Cell and PowerPac HC Power Supply; Bio-Rad Laboratories, Inc.
- ▷ NanoDrop ND-1000 Spectrophotometer; NanoDrop Technologies,Inc.
- ▷ NEFA-HR R1 Set; Wako Chemicals GmbH
- ▷ NEFA-HR R2 Set; Wako Chemicals GmbH
- ▷ Orion II Microplate Luminometer; Berthold Detection Systems GmbH
- ▷ peqGOLD Total RNA Kit; PEQLAB Biotechnologie GmbH
- ▷ peqGOLD Gel Extraction Kit; PEQLAB Biotechnologie GmbH
- ▷ PureLink® PCR Purification Kit; Life Technologies, Invitrogen corp.
- ▷ PureLink^{TM} Quick Plasmid Miniprep Kit; Life Technologies, Invitrogen corp.
- ▷ QuantiTect Reverse Transcription Kit; QIAGEN, Inc.
- ▷ Sonificator SONOPULS; Bandelin
- ▷ Thermomixer compact, Eppendorf
- ▷ Triglycerides-kit, Thermo Scientific / Fisher Diagnostics
- ▷ UV-Transilluminator Universal Hood; Bio-Rad Laboratories, Inc.
- ▷ Veriti® 96-Well Thermal Cycler; Applied Biosystems®

3.1.7 Primers

Cloning Primers

Targetvector	Primername (F = forward, R = reverse)	Sequence	Restriction site
pMSCVpuro:	*mPex16_BglII_F*	CATCAGAGATCTATGGAGAAGCTACGGCTCC	BglII
	mPex16_EcoRI_R	CGGATCGAATTCTCAGCCCCAACTGTAGAAATAG	EcoRI
pMSCVhygro:	*mPex16_BglII_F*	CATCAGAGATCTATGGAGAAGCTACGGCTCC	BglII
	mPex16_HpaI_R	CGGATCGTTAACTCAGCCCCAACTGTAGAAATAG	HpaI
	mPex16_XhoI_R	CGGATCCTCGAGTCAGCCCCAACTGTAGAAATAG	XhoI
pHisMaxC:	*mPex16_EcoRI_pHM_F*	CATCAGGAATTCATGGAGAAGCTACGGCTCC	EcoRI
	mPex16_NotI_pHM_R	CGGATCGCGGCCGCTCAGCCCCAACTGTAGAAATAG	NotI
pPPRE X3-TK-luc:	*mPex16_Peak1+2_HindIII_F*	GCCAAGCTTCTGTTCAGCCTGCCCGCAAGTTGT	HindIII
	mPex16_Peak1_BamHI_R	AGAGGATCCGACGAGTCACATACTCCTGGTAGCG	BamHI
	mPex16_Peak2_HindIII_F	GCCAAGCTTCTGTCGGCCGGTGGGACTGTC	HindIII
	mPex16_Peak1+2_BamHI_R	AGAGGATCCCAGAGTCCAACTTACCCAGTTCAGAC	BamHI
	mPex16_Peak3_HindIII_F	GCCAAGCTTGAGAGCAAAGATGAGTTGGTGGGAC	HindIII
	mPex16_Peak3short_BamHI_R	AGACTCGAGTGGGCATGCCTCACTCTCTGAGAC	BamHI
	mPex16_Peak3long_XhoI_R	GAGCTCGAGATGGGCATGCCTCACTCTCTGAGAC	XhoI
pGL4.26:	*mPex16_Peak1+2_KpnI_F*	GCCGGTACCCTGTTCAGCCTGCCCGCAAGTTGT	KpnI
	mPex16_Peak1_XhoI_R	AGACTCGAGGACGAGTCACATACTCCTGGTAGCG	XhoI
	mPex16_Peak2_KpnI_F	GCCGGTACCCTGTCGGCCGGTGGGACTGTC	KpnI
	mPex16_Peak1+2_XhoI_R	AGACTCGAGCAGAGTCCAACTTACCCAGTTCAGAC	XhoI
	mPex16_Peak3long_KpnI_F	GCCGGTACCGAGAGCAAAGATGAGTTGGTGGGAC	KpnI
	mPex16_Peak3long_XhoI_R	GAGCTCGAGTGGGCATGCCTCACTCTCTGAGAC	XhoI

qRT-PCR Primers

Target Gene (h=human, m=murine)	Forward Sequence	Reverse Sequence
hβ-Actin	CGCCGCATCCTCCTCTTC	GACACCGGAACCGCTCATT
hPex16	ATGGAGAAGAAGCTGCGGCTCCTG	TCAGCCCCAACTGTAGAAGTA
hPex16_2	GTGCGGGGCTTCAGTTACC	GGTTAGAGGCAGAGTACACCA
hTBP	ACGCCAGCTTCGGAGAGTTC	CAAACCGCTTGGGATTATATTCG
mAcaa1a	GATGACCTCGGAGAATGTGG	GCACAATCTCAGCACGGAAG
mAcox1	CACTTGGGCATGTTCCTGCC	CCTCGAAGATGAGTTCCATGAC
mATGL	GTCCTTCACCATCCGCTTGTT	CTCTTGGCCCTCATCACCAG
mEhhadh	CCAATGCAAAGGCTCGTGTTG	AACAGGAACTCCAACGACCC
mPex16	GGATGGAGAAGCTACGGCTC	ACCAGTTCAGACAGCTCGTG
mPPARγ2	TGCCTATGAGCACTTCACAAGAAAT	CGAAGTTGGTGGGCCAGAA
mTFIIβ	GTCACATGTCCGAATCATCCA	TCAATAACTCGGTCCCCTACAA
mUcp1	CTGAGTGAGGCAAAGCTGATTT	TAGGCTGCCCAATGAACACT

3.1.8 Western Blot reagents and buffers

Blocking solution (Pex16 antibody):	TBST	1x	
	+ Milk	5%	
PBST buffer:	PBS		
	+ Tween	0.05%	
SDS Lysis Buffer:	Aqua bidest. steril.	*Fresenius*	
	+ Glycerol	10%	v/v
	+ β-Glycerophosphate	10mM	
	+ NaF	10mM	
	+ Na orthovanadate	10µM	
	+ SDS	2,5%	v/v
	+ Tris-HCL	50mM	
Tris buffered saline (TBS) 10x, pH 7.5:	MilliQ water		
	+ Tris	10mM	
	+ NaCl	150mM	
TBST buffer:	TBS	1x	
	+ Tween20	0.1%	
Transfer buffer	10x TGS buffer	10%	(100mL)
	Methanol	20%	(200mL)
	MilliQ water	70%	(700mL)

Antibody solutions

PEX16 antibody was diluted 1:500 in TBST buffer containing 1% milk. β-Actin antibody was diluted 1:250000 in PBST buffer. Anti-rabbit antibody was diluted 1:2000 in TBST containing 1% milk for PEX16 antibody and anti-mouse antibody was diluted 1:3000 in PBST containing 1% milk for β-Actin antibody.

3.2 Methods

3.2.1 Cloning

For the purpose of PEX16-overexpression in various cell lines, plasmids including the coding sequence of *Pex16* had to be designed. pMSCVpuro-Pex16 vector constructs could be used for the PEX16 overexpression in 3T3-L1 cells, which received a puromycin resistance for later selection due to the vector-properties. As iBACs show a partial resistance against puromycin, a pMSCVhygro-Pex16 vector construct was designed for the overexpression of PEX16 in this cell line. HisMaxC-Pex16-plasmid was used for transfections of Cos7-cells as a control for PEX16-overexpression on protein level, due to the high expression rate of HisMaxC. The used vectors can be seen in figure 3.1.

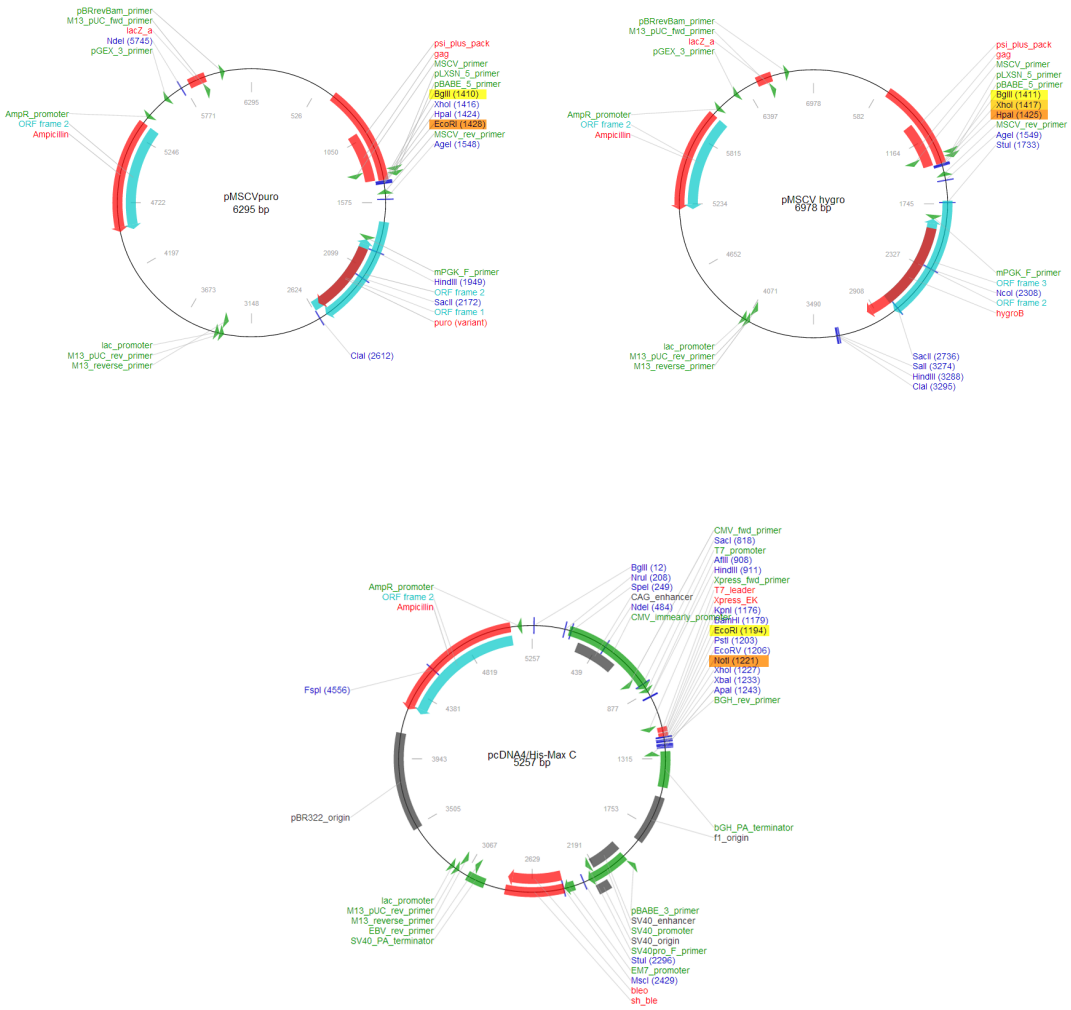

Figure 3.1: Vector maps of cloning plasmids pMSCVpuro, pMSCVhygro and HisMaxC. Yellow and orange labels represent the used restriction sites. Source: www.addgene.org/vector-database

The potential PPARγ- binding sequences of *Pex16* were cloned into pPPRE- X3-TK-luc and pGL4.26 luciferase reporter vectors for subsequent luciferase reporter assays (see figure 3.2). By removing the 3x PPRE-sequence, the empty pTK-luc vector served as control in luciferase reporter assays. These vectors constructs encode the firefly luciferase reporter gene and can be used for the detection of PPARγ binding sites. The pGL4.26-vector also contains a minimal promoter sequence to initiate the transcription of the inserted sequences.

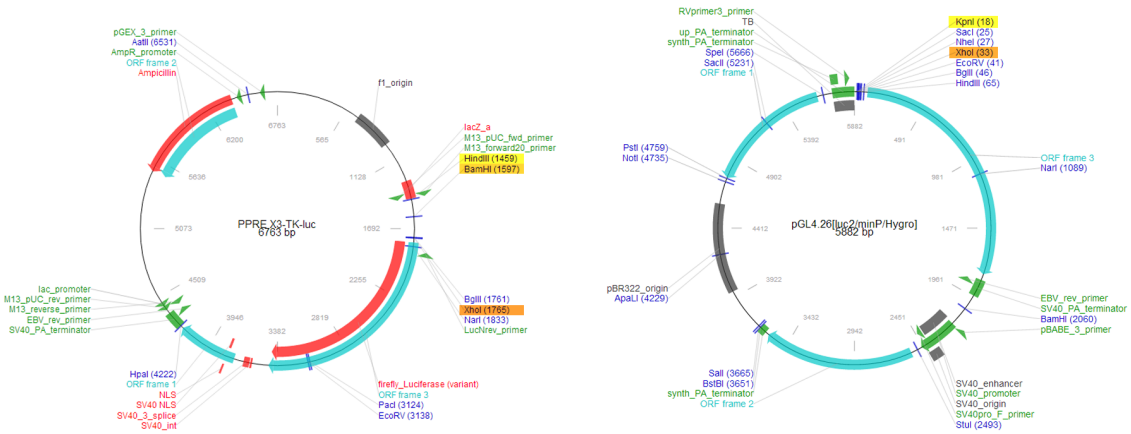

Figure 3.2: Vector maps of cloning plasmids pPPRE X3-TK-luc and pGL4.26. Yellow and orange labels represent the used restriction sites. Source: www.addgene.org/vector-database

All of the vectors harboured ampicillin resistance. This characteristic was used in the transformation of NEB5α Competent E.Coli cells for selection.

Sequence analysis

Genome organization around the *Pex16* transcription start site (TSS) was visualized using the UCSC genome browser (NCBI37/mm9). Custom tracks include ChIP-Seq data (chromatin immunoprecipitation followed by sequencing) for PPARγ in iWAT and eWAT derived adipocytes and 3T3-L1 cells on day 6 of differentiation, see figure 4.17. Sequences of three potential PPARγ targets downstream TSS were generated and used for primer design.

Primerdesign

For cloning *Pex16*-inserts in sense direction into the multiple cloning site (MCS) of pMSCVpuro, pMSCVhygro, pHisMaxC, pPPRE-X3-TK-luc, and pGL4.26-vectors, restriction enzyme cutting sequences corresponding to the cutting sequences provided in the vector-MCS had to be attached to the CDS during PCR, which could be achieved by a well-considered primer design. The used restriction enzymes are listed in section 3.1.7 and labelled yellow and orange in figure 3.1.
For designing the cloning primers, various programs and online-tools were used. [1,2,3]
It was important to avoid self-complementarity and non-specificity of the primers, in order to get high yields of specific PCR products. It was also necessary to make sure that the chosen restriction enzymes do not cut in the *Pex16*-sequence. Ideal PCR primers have a length of 18-22 bp and melting temperatures around 70°C, primer-pairs (forward and reverse primer) should be similar in melting temperature and size.

Based on the UCSC Genome Browser information (see figure 4.17), primers for four luciferase reporter assay constructs were designed, one for each peak in the graph ("Peak1", "Peak2", "Peak3") and one construct covering the first two peaks ("Peak1+2").

PCR

100ng of gDNA (for luciferase assay constructs) or cDNA from murine brown adipose and liver tissue served as PCR templates. 0.3µL of Phusion or Q5® High-Fidelity DNA Polymerase, 5µL of (5x) Phusion HF Buffer or (5x) Q5 Reaction Buffer, 0.5µL of 10mM dNTP-mix and 1µL of 20µM primer-mix were used in the PCR reaction. The implemented PCR temperature profiles are shown in table 3.1, steps 2-4 were repeated 35x. Temperature profile 1 was used for pMSCVpuro, pMSCVhygro and pHisMaxC inserts, temperature profile 2 for pPPRE X3-TK-luc and pGL4.26 inserts.

	Temperature profile 1			Temperature profile 2		
Steps	Temperature in °C	Time		Temperature in °C	Time	
1	98	3 min	1x	98	3 min	1x
2	98	10 s		98	10 s	
3	62	30 s	35x	64	30 s	35x
4	72	90 s		72	40 s	
5	72	10 min	1x	72	10 min	1x
6	4	∞		4	∞	

Table 3.1: Temperature profiles used in PCR reactions.

[1] Serial Cloner 2.5
[2] OligoCalc: http://www.basic.northwestern.edu/biotools/oligocalc.html
[3] NCBI Primer-BLAST: www.ncbi.nlm.nih.gov/tools/primer-blast/

Enzymatic digestion of vectors and inserts

The amplification step was followed by the digestion of PCR products and vectors. Ideal digestion conditions for the particular enzymes (see section 3.1.4) were determined via "Double Digest" online-tools [4,5]. The samples were cut for 1h at 37°C. Vectors were then dephosphorylated for 10min at 37°C using 1µL (= 1U) of FastAP enzyme and subsequently purified via agarose-gel-electrophoresis (1% agarose gel, 105V, 45min). Inserts were purified using the PureLink® PCR Purification Kit.

Generation of blunt ends

In order to produce blunt ends of digested pPPRE X3-TK-luc vector, restriction enzymes were deactivated after 1h of digestion at 70°C for 10min. 0.5µL 20mM dNTP-mix, 1µL Klenow Polymerase (= 5U) and 18.5µL aqua bidest. sterilis "Fresenius" were added to the sample, followed by incubation for 10min at 37°C to fill up the sticky ends. The enzyme was inactivated for 10min at 75°C afterwards. The product was purified via agarose gel electrophoresis.

Ligation

The received, purified products were ligated for 1h at room temperature using 0.5µL (= 2.5U) of T4 DNA Ligase and 1x T4 DNA Ligase Buffer. For blunt end ligations, 2µL PEG4000 were added. Required insert amounts for 40ng of vector DNA were determined using the "Ligation Calculator"-online-tool[6] . The chosen molar vector/insert ratio was 1:3.

Transformation of NEB5α Comp. E-Coli cells

The transformation of NEB5α Comp. E-Coli cells for the propagation of the plasmids was realized according to the "High Efficiency Transformation Protocol (C2987H/C2987I)"[7]. The transformation was followed by an overnight selection on ampicillin selection plates (50µg/mL), spreading 100µL of each dilution per plate.

Colony PCR

In order to test the colonies for successful ligations, a colony PCR was performed and the tested colonies were plated on new selection plates. The colony PCR was performed using 2µL (= 10U) of Taq DNA Polymerase (recombinant), 1.2µL (25mM) $MgCl_2$, 1.6µL (2.5mM) dNTP-mix, 2µL (2µM) primer-mix, 1x Taq Buffer (+KCl - $MgCl_2$) and Aqua bidest. ster. "Fresenius". The temperature profile used for colony PCR reactions is shown in table 3.2.

[4] Double Digest Finder; New England Biolabs,Inc.
[5] Double Digest; Thermo Fisher Scientific, Inc.
[6] Ligation Calculator: www.insilico.uni-duesseldorf.de/Lig_Input.html
[7] "High Efficiency Transformation Protocol (C2987H/C2987I)"; New England Biolabs, Inc.

Temperature °C	Time	
94	2 min	1x
94 62 72	30 s 1 min 4 min	35x
72 4	10 min ∞	1x

Table 3.2: Temperature profile for Colony PCR reactions.

Minipreps and glycerolstocks

The amplified plasmids were isolated and purified, using the PureLink^{TM} Quick Plasmid Miniprep Kit. Therefore, cultures (5mL LB-medium + 50µg/mL ampicillin per colony) were prepared and incubated overnight at 37°C, rotating at 250rpm. For glycerol stocks, 1mL per overnight- culture was centrifuged for 20 min at 4°C and 1500xg. The resulting pellet was then re-suspended in 1mL of glycerol + 1µL of ampicillin and stored at -80°C. The remaining 4mL of the overnight cultures (ONCs) were used for minipreps.

Sequencing

For sequencing, 1.2µg of miniprep-DNA + 6µL 5µM forward or reverse primer, filled up with ddH_2O to 21µL total volume were sent to Microsyth AG for sequencing. Results were viewed with *Sequence Scanner Software v1.0*, Applied Biosystems® and tested via *Align Sequences Nucleotide BLAST*-search[8].

3.2.2 Cell culture

Handling

Storage, freezing and thawing: Cells were held in special cryovials in liquid nitrogen tanks for long time storage. In order to avoid cell death due to crystallization of the media, special DMSO-containing freeze media were used (FM and iBACs-FM).
Before freezing the cells, cultured cells were washed with PBS twice, and trypsin solution (1mL for a 75cm^2 culture flask) was added in order to detach the cells from the bottom of the culture plates. Either DMEM++++ or iBACs-GM was used to stop the trypsin reaction and suspend the cells in medium. The cell-suspensions were centrifuged for 5min at 1200rpm and the resulting pellet was then re-suspended in FM or iBACs-FM. The cell-suspension was aliquoted in special cryovials, each 1mL (or 0.5mL for smaller cell amounts). For gentle freezing, cells were stored in isopropanol chambers at -80°C overnight before transferring them to the liquid nitrogen tanks.

[8] http://blast.ncbi.nlm.nih.gov/Blast

During the thawing process, it was essential to dilute the still partially frozen cells immediately in a ratio of about 1:12 with fresh, preheated media. 24h after thawing, the medium was changed in order to remove the cell-toxic DMSO residues.

Cultivation: 3T3-L1, Cos7, and Phoenix cells were cultivated in DMEM++++ and iBACs in iBACs-GM. Medium change was carried out every 2-3 days as long as splitting was not required.

Splitting: To preserve full differentiation potential, it was necessary to avoid 100% cell confluence, especially for 3T3-L1 and iBACs cell lines, by spitting the cells at 60-80% of confluence. For this purpose, all medium was removed, the cells were washed gently with PBS, and trypsin solution (1mL for a 75cm^2 culture flask) was added to detach the cells from the bottom of the culture plates. Either DMEM++++ or iBACs-GM was used to stop the trypsin reaction and to seed the cells onto appropriate culture plates.

Differentiation

3T3-L1 cells were induced to differentiation 48h after 100% of confluence (day 0), using the standard hormonal cocktail DM1. Medium was changed on day 3 to DM2, and on day 5 to DMEM++++. 3T3-L1 cells were differentiated longest until day 8.

iBACs were induced to differentiation at 100% of confluence or latest 24h after confluence, using iBACs-IM (day 0). The medium was changed to iBACs-MM after 48h and from day 4 every day. iBACs were differentiated until day 7.

Stimulation with rosiglitazone and isoproterenol

The effect of rosiglitazone on 3T3-L1 gene expression has been reviewed during differentiation via qRT-PCR. The stimulation was done using 1µM Rosiglitazone in DM1 permanently applied to the cells.

The influence of isoproterenol on FFA- release and TG- content during differentiation was measured using free fatty acid and triglyceride-assays. Therefore, the medium had to be changed 1h before stimulation to iBACs-MM. The stimulation was done by applying 10µM isoproterenol (final concentration) in FFA-free Medium for 4h and/or 12h to the cells.

Transfection

METAFECTENE® PRO-transfection was used to transiently integrate plasmid DNA into Cos7 and Phoenix cells. The poly-cationic molecular characteristic of Metafectene leads to the formation of DNA/lipid-complexes, which can easily enter the cells. Furthermore, the transfection reagent leads to the destabilisation of the DNA-coating lipid membrane by repulsive electrostatic forces and makes the DNA available in the cell. Phoenix cells were transfected at 30-50%, Cos7 cells at 60-80% of confluence. One hour before transfection, a medium change was performed (DMEM++++). For 24-well plates, 400ng DNA and 1µL metafectene (1µg : 2.5µL) per well, each in 50µL PBS, were combined and pipetted up and down twice. The DNA/metafectene-mix was incubated for 20min at room temperature and then pipetted dropwise into the cell medium. Empty vectors were transfected as negative controls. After 24h, a medium change to DMEM++++ was performed and 48-72h after the transfection, the maximal expression results were obtained.
Via transfection of Phoenix cells with pMSCVpuro and pMSCV-Pex16, amphotrophic lentiviral particles were produced in the supernatant, which were collected and used for transduction of 3T3-L1 cells and iBACs. The lentiviral particles were stored at -80°C in order to preserve the transduction capacity. The transfection of Cos7 cells with pHixMaxC and pHixMaxC-Pex16 was used to control protein production via Western Blot.

Transduction

Lentiviral transduction is a common method to stably integrate foreign DNA into the genomic DNA of 3T3-L1 cells and iBACs.

Overexpression(o/e) and silencing (si) of PEX16 in 3T3-L1 cells: 3T3-L1 cells were seeded in 6-well plates and transduced with lentiviral particles at about 30% of confluence. The addition of polybrene (8µg/mL final conc.) to the medium can increase the retroviral transduction efficiency by neutralizing the charge interactions between the pseudoviral capsid and the sialic acid of the cell membrane due to its poly-cationic characteristic. In order to achieve a stable overexpression of PEX16 in 3T3-L1 cells, 1mL of lentiviral supernatant obtained from transfected phoenix cells and 1mL of DMEM++++ containing 8µg/mL polybrene per well of a 6-well, were applied to the cells for 16-24h. Testing for successful overexpression of *Pex16* was done via qRT-PCR.
For stable silencing of PEX16, 3T3-L1 cells were infected with approximately 7.5 MOI (multiplicity of infection) of shRNA lentiviral particles (NM_145122) and non-targeting control (NTC) per cell (=90.000 MOI per well). The lentiviral particles were pipetted directly into 1mL DMEM++++ plus 8µg/mL polybrene per well of a 6-well and incubated for 16-24h. After the transduction, medium was changed and a selection was performed with 3µg/mL (final conc.) puromycin for at least 5-6 days for overexpression. Selection during silencing was performed with 1.5mg/mL geneticin (G418) for 72h. Cells were split at 60-70% of confluence in order to preserve their capacity to differentiate. Testing for successful silencing of *Pex16* was done via qRT-PCR.

Overexpression (o/e) and silencing(si) of PEX16 in iBACs: For overexpression of PEX16 in iBACs, cells were seeded into 6-well plates and transduced at about 30% of confluence with pMSCVpuro (=controls) and pMSCV-Pex16 lentiviral particles. In order to achieve a stable overexpression of PEX16 in iBACs, 1mL of the lentiviral supernatant from transfected phoenix cells was added to 1ml of iBACs-GM containing 8µg/mL polybrene and applied to the cells for 16-24h, followed by a medium change to iBACs-GM. After another 24h, cells were detached from the bottom, counted and two dilution steps were carried out: 1) 100 cells/10mL iBACs-GM, 2) 10 or 20 cells/mL iBACs-GM ($\approx$ 1 or 2 cells/100µL). Then 100µL of cell suspension were seeded per well in a 96-well plate. Cells were grown with iBACs-GM and split at 60-70% of confluence. Testing for successful overexpression of *Pex16* was done via qRT-PCR.
For stable silencing of Pex16, iBACs were infected with approximately 7.5 MOI per cell (=90.000 MOI per well) of shRNA lentiviral particles and NTC for 16-24h followed by a medium change. As about one third of iBACs show puromycin resistance, selection was done using 1.5mg/mL geneticin (G418) for 7 days. Testing for successful silencing of *Pex16* was done via qRT-PCR.

Proliferation assay

For testing the proliferation of transduced iBACs, 20.000 and 40.000 cells of each, iBACs-puro and iBACs-Pex16, were seeded into 6-well plates and grown with iBACs-GM. As soon as cells reached 100% of confluence, they were induced to differentiate with iBACs-IM and differentiated as described above. Starting 24h after cell-seeding, cells of one well per sample were counted every 48h.

3.2.3 RNA-Isolation

Before RNA was harvested using 400µL of RNA Lysis Buffer T (in a 12-well or 6-well plate), cells were washed with PBS once. For the isolation of RNA, the "peqGOLD Total RNA Kit" was used. In contrast to the kit-instructions, RNA was eluted with 30µL DEPC treated H_2O and incubated for 3 minutes at room temperature before centrifugation at 5000xg for 1min. RNA concentrations were measured using NanoDrop spectrophotometer. The samples were stored at -20°C.

3.2.4 cDNA synthesis

Reverse transcription was performed using the QuantiTect Reverse Transcription Kit. 400ng of RNA were transcribed into cDNA per sample following the kit-instructions. After the transcription, samples were diluted to a final concentration of 1ng/µL with DEPC treated H_2O and stored at -20°C.

3.2.5 qRT-PCR

Via PCR (polymerase chain reaction), DNA sequences up to 3000bp can be amplified by the use of typical polymerases. Special polymerases allow the amplifications of even longer sequences. Based on fluorescence technology, a quantification of the amplified sequences is possible (qRT-PCR). SYBR-green fluorescent absorbs blue light (maximum wavelength = 498nm) and emits green light (maximum wavelength = 522nm) when bound to double stranded DNA. Measurable fluorescence signal increases proportionally to the amount of double stranded DNA generated during PCR. Correct quantification can only be done during the exponential phase of the dsDNA-formation. Housekeeping genes used in qRT-PCR reactions as reference were *TFII*β for murine samples, β-*actin* and *TBP* for human samples. All qRT-PCR primer sequences can be found in section 3.1.7. Per reaction, 4.5µL of 1ng/µL cDNA, 4.5µL of 800nM primer-mix and 9µL of SYBR green were used.
The temperature program for qRT-PCR reactions is shown in table 3.3.

Temperature °C	Time	
50	2 min	1x
95	10 min	
95	15 s	40x
60	1 min	

Table 3.3: Temperature profile for qRT-PCR reactions.

3.2.6 Oil Red O staining

For staining of cells with Oil Red O, cells had to be washed with PBS twice and fixed with 10% formaldehyde (≈ 1mL in a 6-well) for 30min. For the Oil Red O stock, Oil Red O powder (0.25g) was diluted in 50mL isopropyl alcohol. The Oil Red O dilution was filtered through filter paper. The staining was performed for one hour using 1mL Oil Red O stock, diluted 3:2 with ddH_2O, per well in a 6-well. The dyed cells were stored covered with 1mL ddH_2O at 4°C.

3.2.7 Protein quantification

Protein quantification was done using the *BCA Protein Assay Kit*, Pierce. The sample-quantities were measured with FLUOstar Omega microplate reader; BMG LABTECH GmbH at a wavelength of 562nm.

3.2.8 Triglyceride quantification in cells

Triglyceride (TG) quantification was done using the *Triglycerides*-kit from Thermo Scientific / Fisher Diagnostics. Cells were harvested with 150µL of PBS per well in a 6-well after washing the cells twice with PBS. Cell- samples had to be treated with ultrasound for 10s twice, cooling in ice, in order to open the cells and release the triglycerides. 10µL per sample were used for the measurement. The TG content was measured with the FLUOstar Omega microplate reader; BMG LABTECH GmbH at a wavelength of 500nm.

3.2.9 Free fatty acid quantification in the supernatant of cells

Free fatty acid (FFA) quantification was done using *NEFA-HR R1 Set + NEFA-HR R2 Set*, Wako Chemicals GmbH. To measure FFA in the supernatant of cells, cells were washed with PBS twice and incubated at 37°C for either 4h or 12h with FFA-free Medium. Afterwards, the supernatant was harvested and centrifuged for 15min at 4°C at 12,000xg. The supernatant was transferred to new Eppendorf tubes. 20µL per sample were mixed with 100µL of Reagent 1 (0.06g/mL buffer 1) and incubated for 10min at 37°C. Afterwards, 150µL of Reagent 2 (0,01g/mL buffer 2) were added and again incubated at 37°C for 10min. The FFA-content was measured with the FLUOstar Omega microplate reader; BMG LABTECH GmbH at a wavelength of 546nm.

3.2.10 Western Blot analysis

After washing cells 3x with PBS, protein samples were harvested with 100µL SDS-Lysis Buffer + 1x Protease Inhibitor Cocktail (PIC), in order to improve protein stability. The samples had to be stored on ice for further processing. Protein samples were inactivated at 95°C for 5min and placed back on ice afterwards. For an easier handling, protein samples were digested using 3-5µL benzonase per 100µL sample. After the digest, samples were centrifuged for 3min at 13.000xg.

Gel electrophoresis

Proteins can be separated by gel electrophoresis based on differences in charge and size. Therefore, 50-70µg of protein was used for Western Blot experiments. Gels were inserted in the Bio-Rad Western Blot chamber, which was filled with 700mL of diluted (1x) NuPAGE® MOPS buffer. Additionally, 500µL antioxidant were pipetted directly into the buffer. Protein samples, 8µL (4x) LDS and 1µL DTE were filled up to 35µL with ddH_2O, mixed gently and incubated at 70°C for 10 min, with the purpose of protein denaturation. The protein standard consisted of 10µL of Seeblue® Plus2, 20µL sterile H_2O and 5µL of 4x LDS. Before sample and standard were applied to the gel, the gel slots had to be rinsed with a pipette tip in order to remove antioxidant-residues from the slots. The gel was run at 175V for ≈1h.

Transfer

Proteins need to be blotted onto a nitrocellulose membrane to get accessible to the detection with antibodies. The negative net charge of proteins in the gel enables the transfer from the gel to the membrane within an electric field.
The membrane had to be incubated in ddH_2O for ≈5min before the transfer. Three filter papers, nitrocellulose membrane, gel and two sponges were applied to the transfer buffer and fixed in the right order in a gel holder cassette (as to see in figure 3.3).

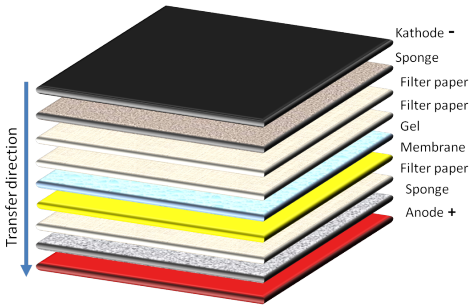

Figure 3.3: Arrangement of sponges, filters, gel and membrane for the blotting procedure.

Gel holder cassettes and cooling unit were placed in the transfer tank, which was then filled with transfer buffer. The transfer was performed within 1.5h at an electric current of 500mA and at 4°C.

Blocking: To avoid the binding of antibodies to the nitrocellulose membrane instead of the antigen, the membrane had to be blocked for 1h with an antibody-dependent blocking solution (see 3.1.8).

Incubation with antibodies

The membrane was incubated with two different types of antibodies. Incubation with a primary antibody specific to the target protein was done over night, rotating at 4°C. Thereafter, the membranes were washed three times with TBST (PEX16) or PBST (β-ACTIN) for 10min on a shaking plate. Incubation with a secondary antibody, α-rabbit for PEX16 and α-mouse for β-ACTIN, was done for 2h, rotating the blots at room temperature. The secondary antibody binds to the primary antibody and can be used for chemiluminescent detection of the target protein due to an attached enzymatic reporter molecule. The last step was once more a three times washing procedure using the appropriate buffer.

Developing the blot

Western Blots were developed using *Amersham ECL prime* or *Super Signal West Pico*- Western Blot detection reagents. For both types of detection reagents, 750-850µL of each of the chemiluminescent substrates were mixed and applied to the membranes for 5min. Chemiluminescence signals at the location of the target protein hit the photo paper, which could be developed with Roentogen EUKOBROM and Roentogen Superfix. The exposure time at the developing chamber was dependent on the signal intensity. The photo paper was washed with ddH_2O.

3.2.11 Luciferase reporter assays

Four regions downstream the *Pex16* - TSS (transcription start site) (Peak1: 1052-1379bp, Peak2: 1349-1676bp, Peak1+2: 1052-1676bp, Peak3: 2160-2554bp) were cloned into luciferase reporter vectors (pGL4.26 and pPPRE X3-TK-luc). The 3x PPRE- sequence of the pPPRE X3-TK-luc vector was removed in order to get an empty control vector for the assays, see section 3.2.1.

Cos7 cells were transfected according to section 3.2.2 in 24-well plates. 200ng of the cloned plasmids or empty luciferase reporter vectors were co-transfected with 100ng/well pCMX_PPARγ2 and 100ng/well pCMX_RxRα expression vectors. In controls, 200ng of empty pCMX vector were co-transfected instead of pCMX_PPARγ2 and pCMX_RxRα. Co-transfection of renilla luciferase reporter vector pGL4.75 in a ratio of 1:50 (=2ng/well) to the luciferase reporter vectors in all experiments served as control for varying efficiencies in transfections.
Luciferase reporter assays were performed 48h after transfection using the *Dual-Luciferase*® *Reporter Assay System*, Promega. Before starting, buffers and substrates had to be prepared. Passive lysis buffer (5x) was diluted with aqua bidest. sterilis "Fresenius" in a ratio of 1:5. LARII reagent was diluted with Luciferase Assay buffer, remaining substrate was stored in 2mL-aliquots at -80°C. Stop&Glo substrate (50x) was diluted with Stop&Glo Buffer in a 1:50 ratio. Both substrates had to be protected from light.
Cells were washed with PBS twice and lysed with 100µL (1x) passive lysis buffer per well on a shaking-plate for 20min at room temperature. 10µL per sample were used for measurement in a 96-well assay-plate.
Luminescence measurement was done using "Berthold Orion II" luminometer. Relative luciferase activities were calculated by relating renilla-normalized values of PPARγ2/RxRα - cotransfected cells to measured values for pCMX-transfected cells for each construct individually.

3.2.12 Statistics

Mean value and standard deviation (SD) for qRT-PCR results were calculated from 2-3 biological replicates. For representative experiments, SD was calculated from technical replicates. Significance levels were set to $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ only for n=3.

4 Results

In humans, *Pex16* gene (peroxisomal biogenesis factor 16, [Homo sapiens], gene ID: 9409)[1] is located on chromosome 11 (11p11.2). Two human protein encoding transcript variants are known (isoform 1: 336 aa, ≈ 38.6 kDa, isoform 2: 346 aa, ≈39.3 kDa)[2]. All herein shown experiments were carried out with murine models, and for that reason, further mentioned "*Pex16* gene" refers to "*Pex16* peroxisomal biogenesis factor [Mus musculus]" (gene ID:18633), located on mouse chromosome 2 (2 E1;2)[3]. According to NCBI[3], only transcript variant 1 of 2 existing variants in mus musculus encodes the functional PEX16-protein (336 aa, ≈38.6 kDa[4]).

4.1 Pex16 expression in murine tissue

Various murine tissues were screened for *Pex16* mRNA-expression via qRT-PCR. Expression was highest in white adipose tissue (WAT) (ct-levels: 25-26) and in brown adipose tissue (BAT) (ct-levels: 24-25), and lower in cardiac muscle (CM) (ct-levels: 27-28), skeletal muscle (SM) (ct-levels: 27-28) and liver (ct-levels: ≈26) in fed samples (see figure 4.1A). A trend for elevated *Pex16* mRNA-expression could be determined in brown adipose tissue and liver in high-fat-diet fed mice compared to chow diet fed mice (see figure 4.1B). No difference in WAT and BAT between ob/ob and wildtype (wt) mice could be observed. Additionally, the nutrition status did not change the expression pattern (see figure 4.1C).

[1] http://www.ncbi.nlm.nih.gov/gene/9409
[2] http://www.uniprot.org/uniprot/Q9Y5Y5
[3] http://www.ncbi.nlm.nih.gov/gene/18633
[4] http://www.uniprot.org/uniprot/Q91XC9

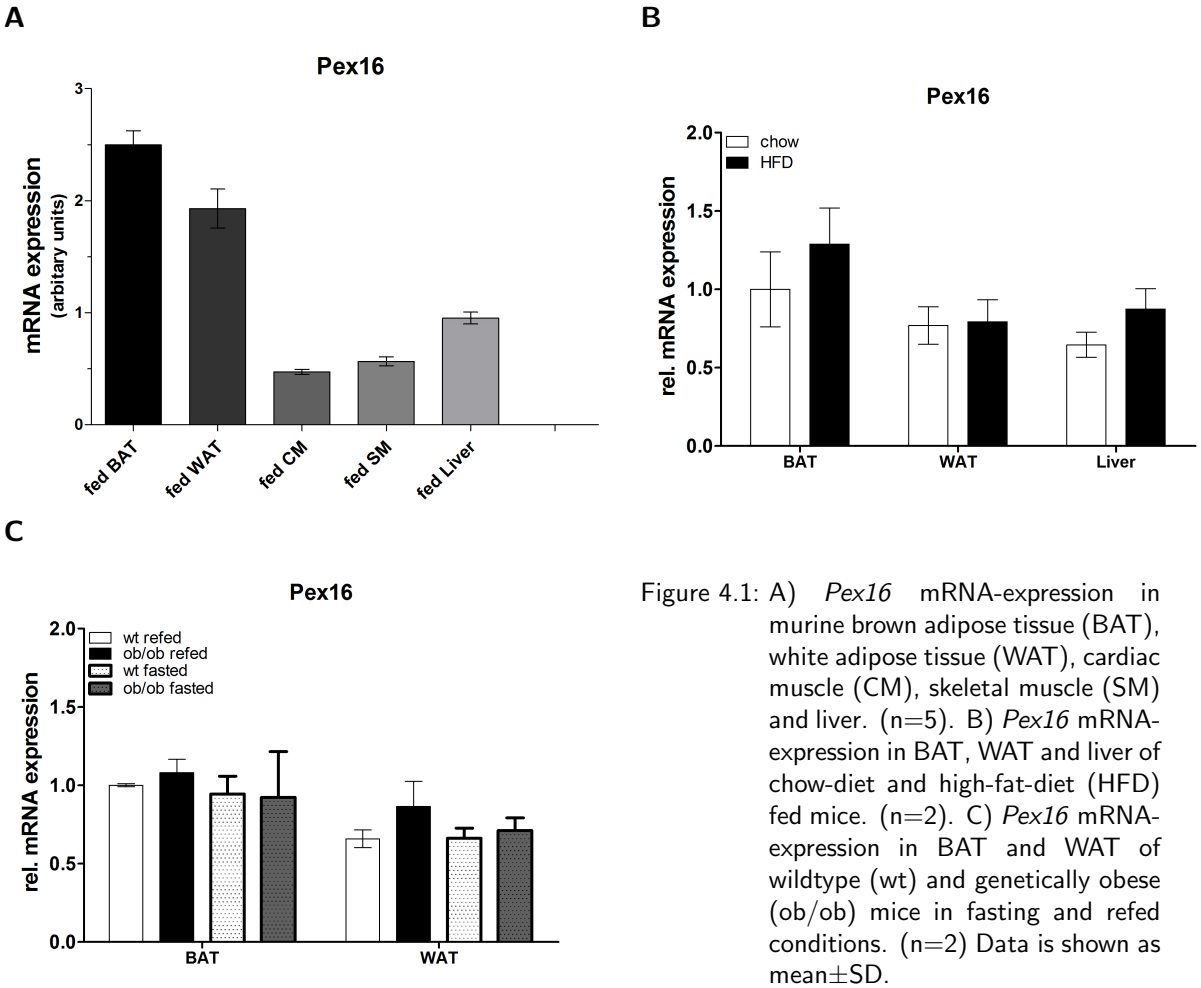

Figure 4.1: A) *Pex16* mRNA-expression in murine brown adipose tissue (BAT), white adipose tissue (WAT), cardiac muscle (CM), skeletal muscle (SM) and liver. (n=5). B) *Pex16* mRNA-expression in BAT, WAT and liver of chow-diet and high-fat-diet (HFD) fed mice. (n=2). C) *Pex16* mRNA-expression in BAT and WAT of wildtype (wt) and genetically obese (ob/ob) mice in fasting and refed conditions. (n=2) Data is shown as mean±SD.

4.2 PEX16-expression undergoes upregulation during adipogenic differentiation of murine and human cell models

In addition to murine tissues, also human and murine cell models for adipogenesis were screened for *Pex16* mRNA-expression during their differentiation process. The qRT-PCR results showed an up-regulation of *Pex16* during early stages of adipogenesis in the murine cell models 3T3-L1, iBACs and C3H/10 T1/2 (see figure 4.2A-C). Stimulation of *Pparγ*, an important marker gene of adipogenesis, by rosiglitazone in 3T3-L1 cells lead to an increased *Pex16* mRNA-expression on day 3 and day 5 of differentiation (see figure 4.2A). In iBACs, β3-adrenergic stimulation by isoproterenol led to an elevated mRNA-expression of *Pex16* on day 5 of differentiation (see figure 4.2B). *PEX16* expression in human SGBS cells and hMADS was lower (ct-levels: 26-29), when compared to murine adipocytes (ct-levels: 24-26), but increased with progress of differentiation (see figure 4.2D,E).

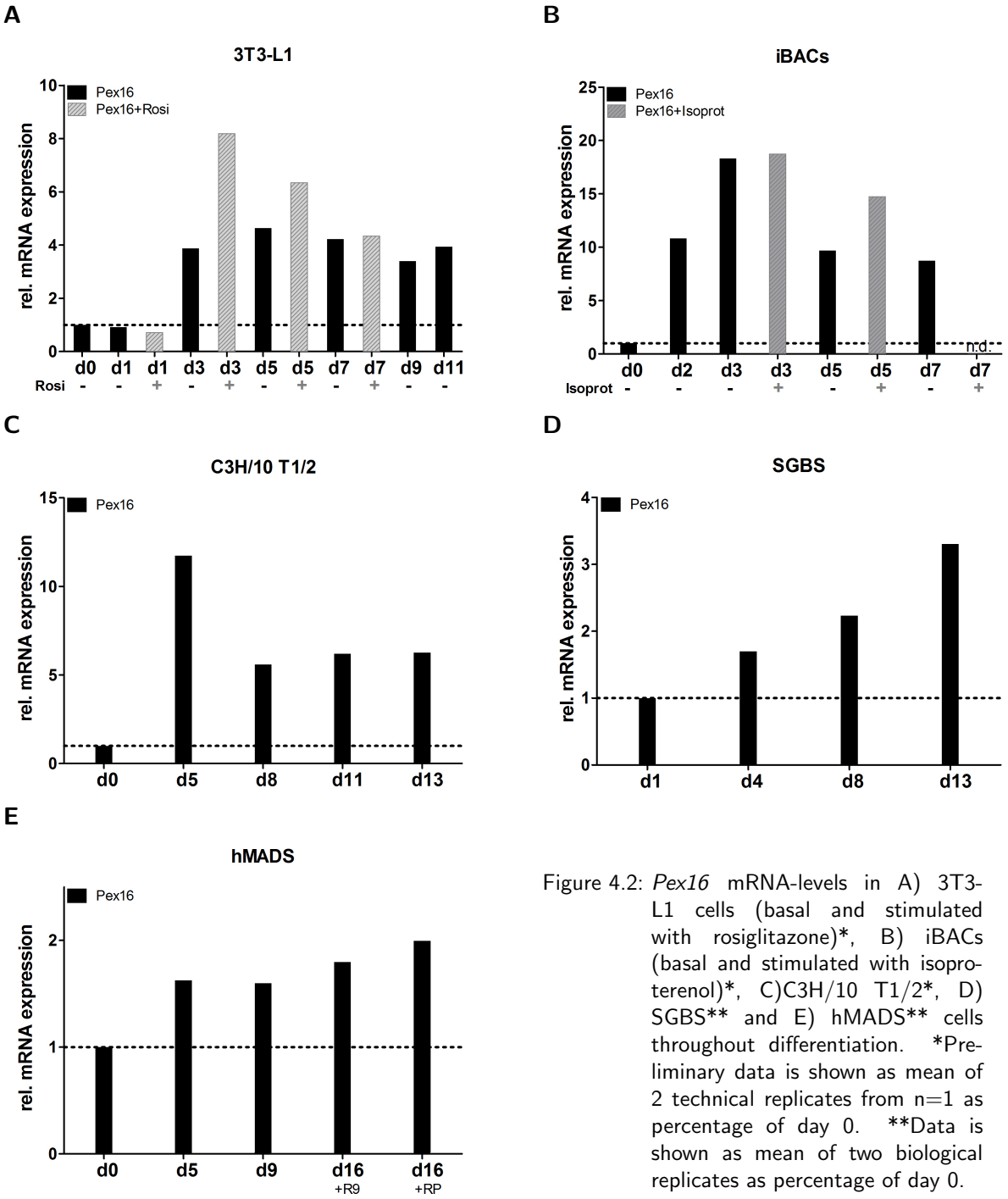

Figure 4.2: *Pex16* mRNA-levels in A) 3T3-L1 cells (basal and stimulated with rosiglitazone)*, B) iBACs (basal and stimulated with isoproterenol)*, C)C3H/10 T1/2*, D) SGBS** and E) hMADS** cells throughout differentiation. *Preliminary data is shown as mean of 2 technical replicates from n=1 as percentage of day 0. **Data is shown as mean of two biological replicates as percentage of day 0.

4.3 Overexpressing PEX16 in white and brown adipocyte models

As an up-regulation of PEX16 during adipogenic differentiation of cellular models for adipogenesis as well as a high expression of PEX16 in murine brown and white adipose tissue was found, PEX16 was further investigated in order to gain insight into the function of PEX16 in adipocyte development and energy metabolism.
The first step was to test the effects of overexpression. PEX16 was overexpressed in 3T3-L1 cells (white adipose cells), and iBACs (brown adipose cells). To accomplish the overexpression of PEX16 in these cell lines, several preparative steps had to be done: The *Pex16*-CDS had to be cloned into transporter vectors pMSCVpuro and pMSCVhygro (see section 3.2.1). By transfection of Phoenix cells with the generated pMSCV-Pex16 plasmids, amphotrophic lentiviral particles were produced, which could then be used for transduction of 3T3-L1 cells and iBACs according to section 3.2.2. Cells transduced with pMSCVpuro plasmid served as control.

4.3.1 Overexpression of PEX16 in 3T3-L1-cells

Cloning of Pex16-CDS into pMSCVpuro and pHisMaxC-vector

Cloning of *Pex16*-CDS into pMSCVpuro and pHisMaxC was done during a practical prior to the master thesis. Positive clones were confirmed via colony PCR and sequencing. Sequencing results can be found in the appendix.

Phenotypical changes due to overexpression of PEX16 in white adipocytes

In order to overexpress PEX16 in 3T3-L1 cell line, two distinct lines of amphotrophic lentiviral particles were tested. As transduction with line 1 exhibited the highest *Pex16*-expression in 3T3-L1 cells, this line was used for all further experiments (see figure 4.3A).
First, three biological replicates of Pex16-o/e cells and controls from line 1 were differentiated ("time-series 1") and mRNA-levels of *Pex16* ($\pm$ rosiglitazone-stimulation) were measured. The overexpression was confirmed over the whole differentiation process (see figure 4.3B). PEX16-o/e cells showed no further increase in *Pex16* mRNA-levels upon rosiglitazone-stimulation when compared to controls (figure 4.3B). Furthermore, reduced lipid accumulations were found for PEX16-o/e cells on day 7 of differentiation in pictures of Oil-red-O stained cells when compared to controls (figure 4.3C).
Again, 3 biological replicates of PEX16-o/e cells and controls from line 1 were differentiated ("time-series 2") to investigate *Pex16* mRNA-expression, protein expression, free fatty acid (FFA) release and triglyceride (TG) content. The overexpression was confirmed throughout differentiation on mRNA-level (see figure 4.3D). Moreover, the overexpression was shown on protein level within Western Blots on day 0, day 3 and day 8 of differentiation as well (see figure 4.3E). Pictures of Oil-red-O (ORO) stained cells on day 8 of differentiation showed again a reduction in lipid accumulation in PEX16-o/e cells compared to controls (see figure 4.3F).

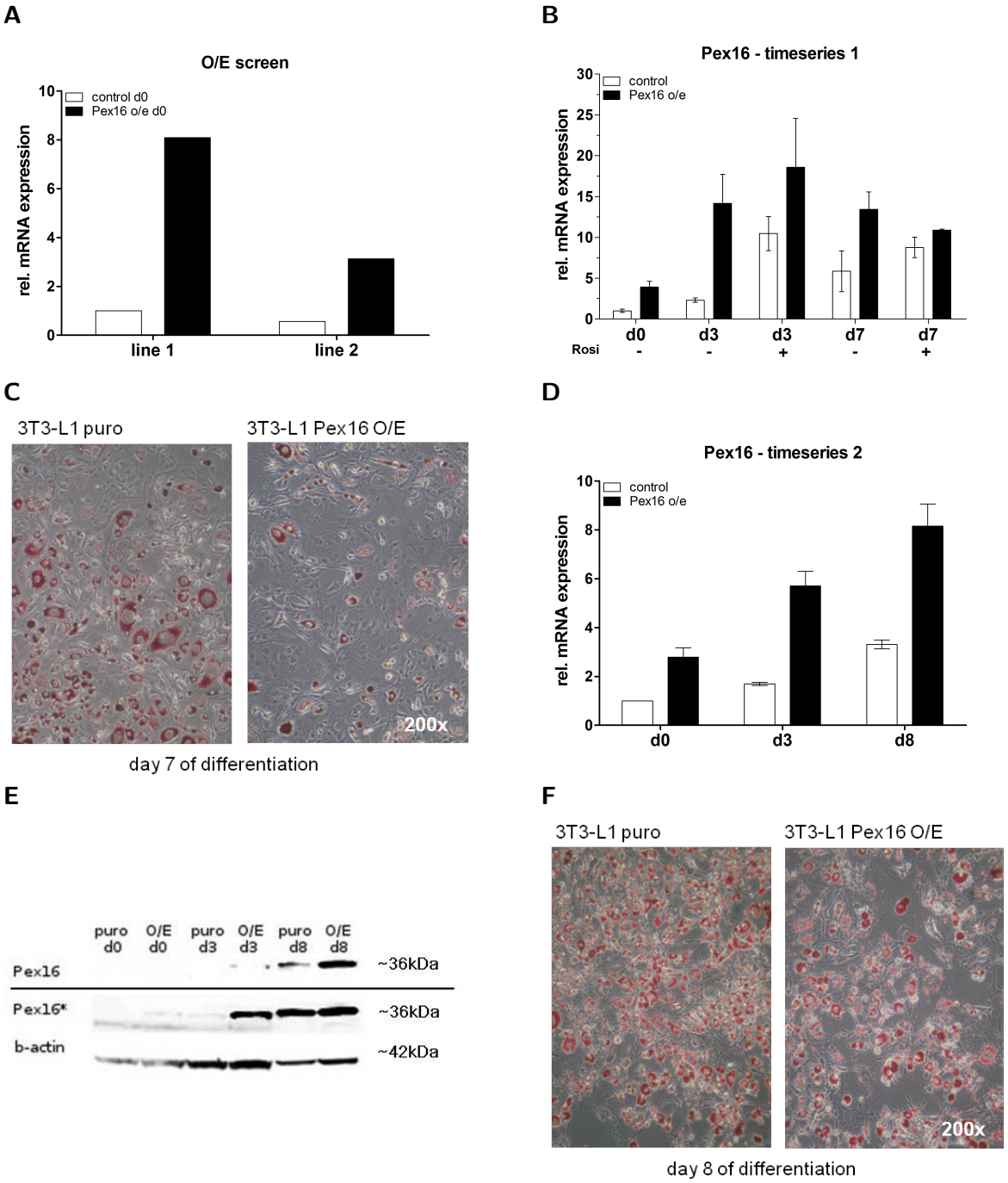

Figure 4.3: A) mRNA-level-screen for *Pex16*-overexpression on day 0 of differentiation. 3T3-L1 cells were transduced with lentiviral particles. Preliminary data is shown as mean of two technical replicates as percentage of pMSCV-puro control on day 0 of differentiation. B) *Pex16* mRNA-levels during differentiation of PEX16-o/e line 1-3T3-L1 cells and controls with and without rosiglitazone (=Rosi) stimulation, referred to as "timeseries 1". C) Oil-red-O stainings from PEX16-o/e 3T3-L1 cells and controls of timeseries 1 on day 7 of differentiation. D) *Pex16* mRNA-levels during the second differentiation of PEX16-o/e line 1-3T3-L1 cells and controls, referred to as "timeseries 2". E) Western Blot of timeseries 2-protein samples (puro= control, O/E= PEX16-o/e, "Pex16": developed with Super Signal, "Pex16*": developed with ECL Prime). F) Oil-red-O stainings from PEX16-o/e 3T3-L1 cells and controls of timeseries 2 on day 8 of differentiation. qRT-PCR-data is shown as mean±SD as percentage of pMSCVpuro-control (d0) which was set to 1. (n=3)

As ORO staining showed reduced lipid accumulations in PEX16-o/e cells, triglyceride (TG) content and free fatty acid (FFA) release were determined as well. Results of the measurement show hardly any differences in TG content as well as in FFA release between PEX16-o/e cells and controls under basal conditions. However, on day 8 of differentiation a small but significant decrease in FFA release was observed (see figure 4.4A,B). After β3-adrenergic stimulation with isoproterenol a trend to a reduction in TG content and to an increase in FFA release in PEX16-o/e cells could be seen on day 8 of differentiation (see figure 4.4A,B).

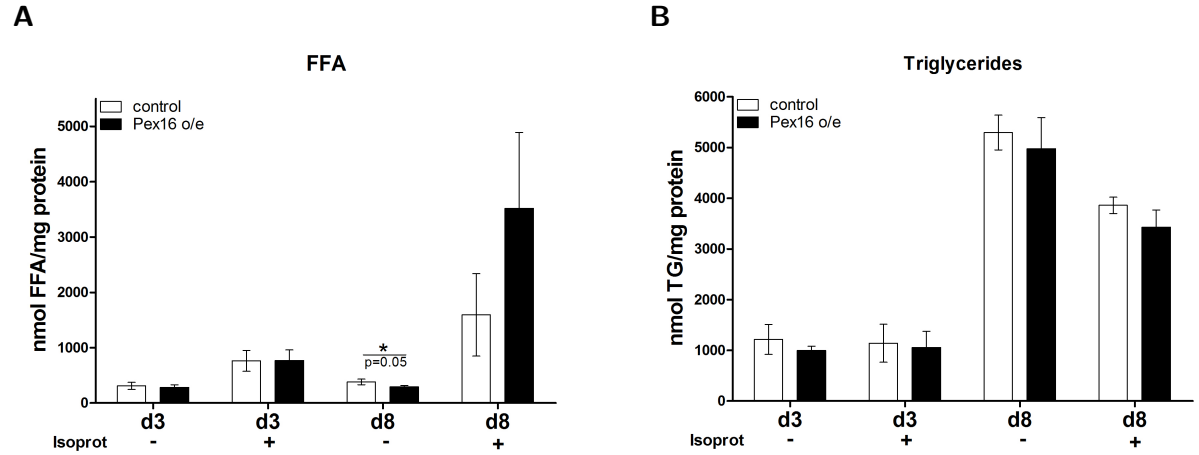

Figure 4.4: A) Free fatty acid and B) triglyceride measurement on day 3 and day 8 of differentiation of Pex16-o/e 3T3-L1 cells and controls (timeseries 2) with and without isoproterenol (=Isoprot) stimulation. Free fatty acid contents are presented in nmol FFA/mg protein, triglyceride contents in nmol TG/mg protein. (n=3) *p<0.05

In addition, marker gene expression for adipogenesis (*Pparγ2*) and lipolysis (*ATGL*) as well as peroxisomal β-oxidation gene expression (*Acox1*, *Ehhadh*, *Acaa1a*) were tested via qRT-PCR. Corresponding to the noticed reduction in lipid accumulation of PEX16-o/e cells, *Pparγ2* mRNA-levels showed a trend to reduction as shown in figure 4.5A, hinting at a reduced differentiation of PEX16-o/e cells. mRNA-expression of lipolysis gene *ATGL* was significantly reduced in PEX16-o/e cells compared to controls on day 0 and day 3 of differentiation (see figure 4.5B). Peroxisomal β-oxidation gene *Acox1* mRNA-levels were decreased in PEX16-o/e cells throughout differentiation (figure 4.5C). mRNA-levels of another peroxisomal β-oxidation gene *Ehhadh* were also decreased in PEX16-o/e cells (figure 4.5D), but the expression of *Ehhadh* in 3T3-L1 cells was very low, as only one of three biological replicates showed detectable mRNA-levels (ct-values: 25-31). The third peroxisomal β-oxidation gene *Acaa1a* showed a trend to elevated mRNA-levels in PEX16-o/e cells on day 3 of differentiation (figure 4.5E).

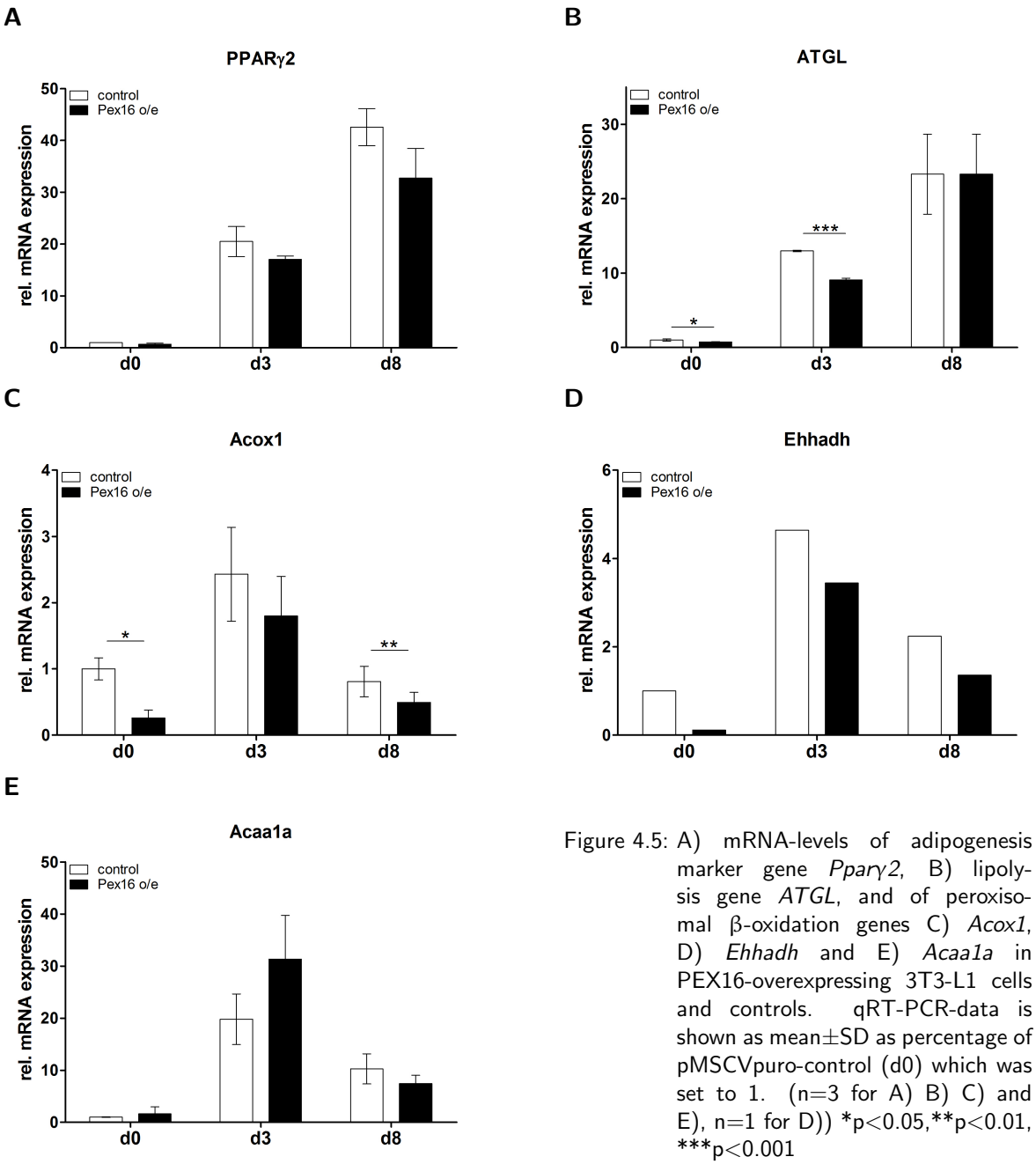

Figure 4.5: A) mRNA-levels of adipogenesis marker gene *Pparγ2*, B) lipolysis gene *ATGL*, and of peroxisomal β-oxidation genes C) *Acox1*, D) *Ehhadh* and E) *Acaa1a* in PEX16-overexpressing 3T3-L1 cells and controls. qRT-PCR-data is shown as mean±SD as percentage of pMSCVpuro-control (d0) which was set to 1. (n=3 for A) B) C) and E), n=1 for D)) *$p<0.05$,**$p<0.01$, ***$p<0.001$

Western Blots for PEX16 did not show the expected size of 38.6 kDa. Instead a band at ≈36 kDa was detected (see figure 4.6A). The His-tagged control showed the right size of ≈39kDa (figure 4.6A). We used *SignalP 4.1*[5], an online tool for protein cleavage-site predictions, to find reasons for the reduced protein size. However, no potential cleavage sites were detected (see figure 4.6B).

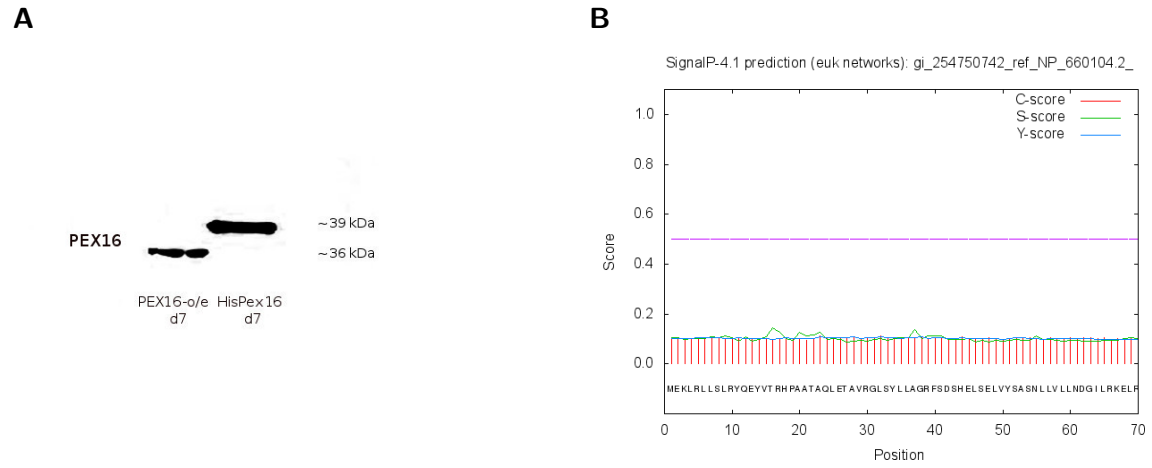

Figure 4.6: A) Western Blot of PEX16 overexpressing 3T3-L1 cells (PEX16 o/e) and Cos7 cells (HisPex16) on day 7 of differentiation using α-PEX16-antibody. The pMSCV-Pex16 transduced 3T3-L1 cells express a ≈36 kDa- protein, HisPex16 transfected Cos7 cells express a protein at the size of ≈39 kDa. B) Cleavage site prediction for PEX16-protein generated via *SignalP4.1* prediction tool: no cleavage sites could be detected within the aminoacid sequence. C-score: raw cleavage site score, S-score: signal peptide score, Y-score: combined cleavage site score.

4.3.2 Overexpression of PEX16 in iBACs

For further elucidation of PEX16 function in adipocyte development and metabolism, overexpression of PEX16 in the immortalized brown adipose cell line "iBACs" was performed.

Cloning of Pex16-CDS into pMSCVhygro-vector

Cloning of *Pex16*-CDS into pMSCVhygro at BglII/HpaI or BglII/XhoI- restriction sites was not successful. No positive NEB5α Comp.E-Coli colonies could be found within several repetitions of the experiment.

Phenotypical changes due to overexpression of PEX16 in brown adipocytes via pMSCVpuro

iBACs exhibit a partial resistance against puromycin, as they were selected with puromycin throughout the immortalization process and developed a resistance [46]. In addition, integration of *Pex16*-CDS into pMSCVhygro for selection with hygromycin could not be accomplished. Thus, another option for stably overexpressing PEX16 in iBACs had to be found: A dilution experiment. iBACs were transduced with pMSCVpuro and pMSCV-Pex16. Transduced cells were diluted to a final concentration of approx. 1 cell per 100µL. Single cells were then grown in 96-wells without puromycin

[5] http://www.cbs.dtu.dk/services/SignalP/

selection and subsequently screened for PEX16 overexpression on mRNA-level at 100% of confluence. Three promising PEX16-overexpressing cell lines (H2, E12, F5) resulted from two iterations of this experiment, as shown in figure 4.7.

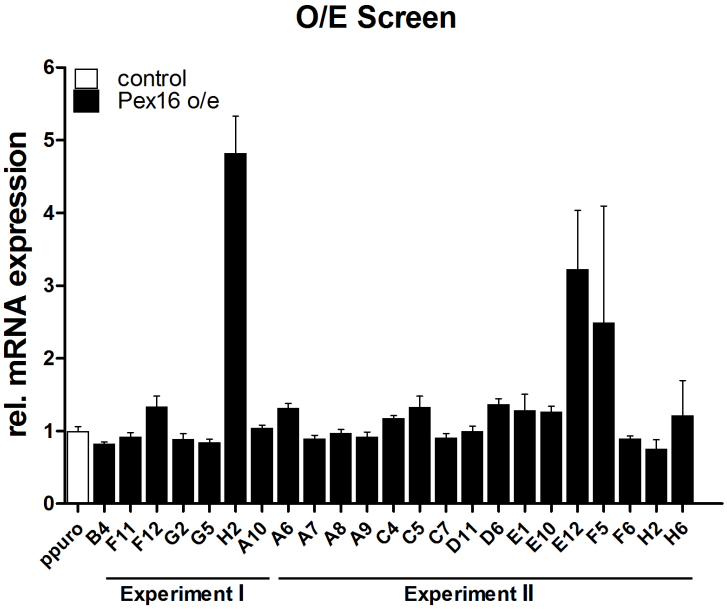

Figure 4.7: qRT-PCR-screen for PEX16 overexpression in iBACs compared to controls after two repetitions of a dilution experiment ("Experiment I", "Experiment II"). iBACs were transduced with lentiviral particles (pMSCVpuro and pMSCV-Pex16) and diluted to 1 cell/100µL. Single cells were grown without antibiotic selection and tested for overexpression at 100% of confluence via qRT-PCR. mRNA-levels of PEX16-o/e iBACs are shown as percentage of pMSCVpuro-control, which was set to 1. Preliminary data is shown as mean±SD of two technical replicates from n=1.

A 1:1:1 mix of these three cell lines was grown to confluence and differentiated until day 7. qRT-PCR analysis of *Pex16* mRNA-levels throughout this differentiation process show a loss of the overexpression on day 3, which was partially regained on day 7 of differentiation (see figure 4.8A). Oil-red-O stainings of the cells show again a reduction in lipid accumulation of PEX16-o/e cells comparable to the one seen in 3T3-L1 cells overexpressing PEX16 (see figure 4.8E,F). However, and that was true for puro and PEX16-o/e cells, many cells died during day 3 and day 7 of differentiation, as visually observed. The surviving cells displayed a high content of lipid accumulations (see figure 4.8E,F). Only on day 3 of differentiation, Western Blot showed a signal around the expected protein size of ≈36kDa, but the signal for PEX16-o/e samples was even reduced when compared to controls (see figure 4.8B). The reduced protein amount seen in the β-actin control on day 7, probably due to a biased protein-amount determination caused by the high lipid-content of the samples, could be responsible for not detecting PEX16-protein on day 7 (see figure 4.8B). Differences between PEX16-o/e cells and pMSCVpuro controls concerning free fatty acid-release were hardly existent (see figure 4.8C). However, there was a trend to increased fatty acid release and reduced triglyceride content nearly throughout all timepoints measured (see figure 4.8C,D). On day 3 of differentiation, TG content was even significantly reduced after 12h incubation with FFA-free Medium in PEX16-o/e cells when compared to controls (see figure 4.8D).

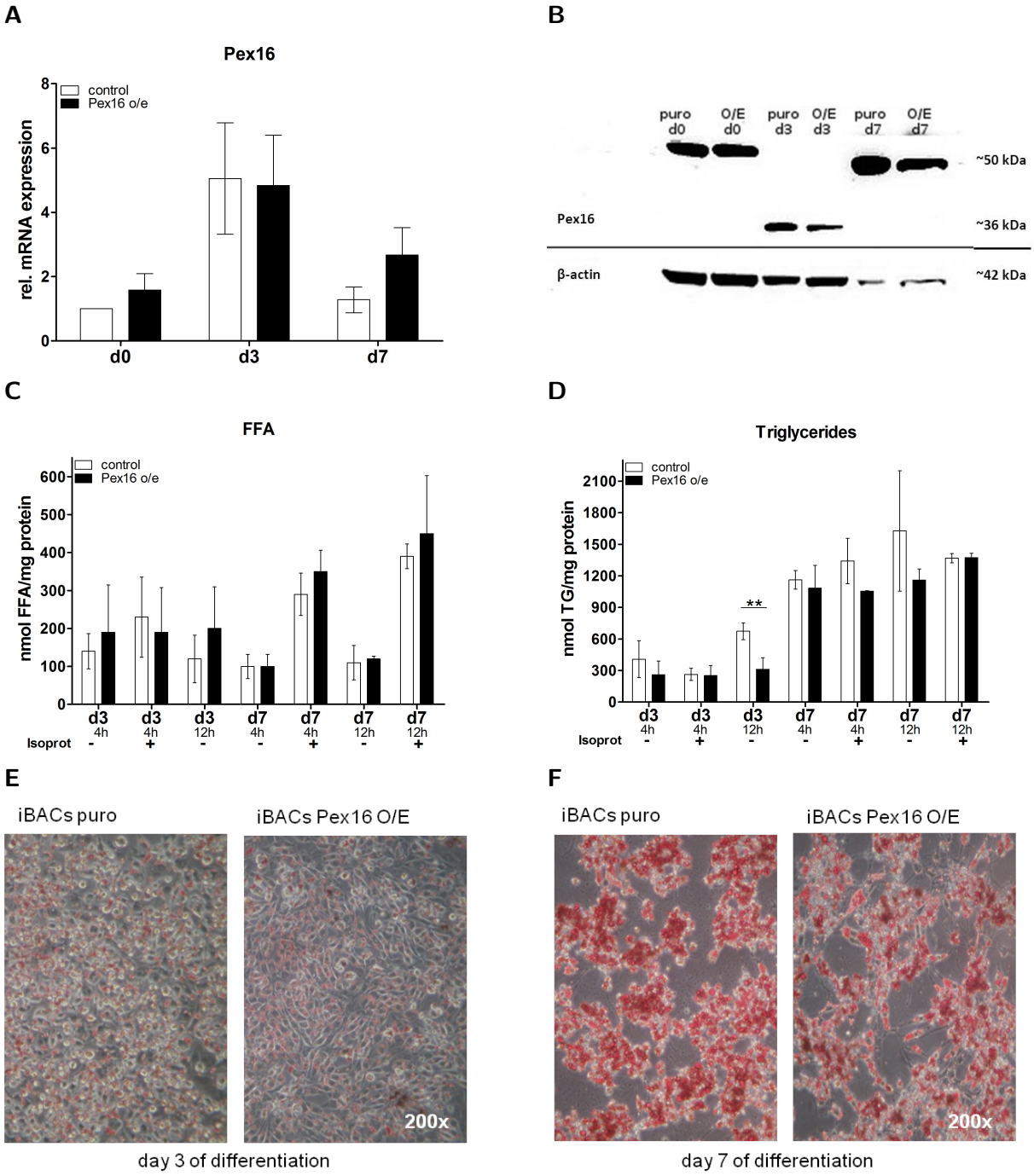

Figure 4.8: Combination of the three highest PEX16-o/e iBACs lines H2, E12 and F5 compared to pMSCVpuro-controls: A) mRNA-levels of *Pex16* in PEX16-overexpressing cells compared to controls throughout differentiation. B) Western Blot analysis of protein samples from PEX16-o/e cells (=O/E) and controls (=puro) on d0, d3 and d7 of differentiation, developed with ECL Prime. C) Free fatty acid (FFA) and D) triglyceride-measurement of PEX16-o/e cells compared to controls after 4h or 12h incubation with FFA-free Medium with and without stimulation with isoproterenol (=Isoprot) on day 3 and day 7 of differentiation. Oil-Red-O staining of PEX16-o/e cells and controls E) on day 3 and F) on day 7 of differentiation. qRT-PCR-data is shown as mean±SD as percentage of pMSCVpuro-control (d0) which was set to 1. Free fatty acids are presented in nmol FFA/mg protein, triglycerides are presented in nmol TG/mg protein.(n=3) **$p<0.01$

Additionally, mRNA-expression of adipocyte marker gene *Pparγ2*, brown adipocyte marker gene *UCP1* and peroxisomal β-oxidation genes *Acox1*, *Ehhadh* and *Acaa1a* was investigated via qRT-PCR. Interestingly, *Pparγ2* expression was elevated in PEX16-o/e cells throughout the differentiation (see figure 4.9A), contradicting the impression gained in Oil-red-O-pictures, that PEX16-o/e cells showed less differentiation and would therefore express less *Pparγ* (figure 4.8E,F). A trend of elevated *UCP1*-expression could be observed on day 7 of differentiation (figure 4.9B). Differences of *Acox1*, *Ehhadh* and *Acaa1a* expression between PEX16-o/e cells and controls were not significant due to very high standard deviations (figure 4.9C,D,E). However, a trend to reduced *Acox1* and *Ehhadh* mRNA-levels and to elevated *Acaa1a* mRNA-levels in PEX16-o/e iBACs was detected nearly throughout all measured timepoints when compared to controls (figure 4.9C,D,E).

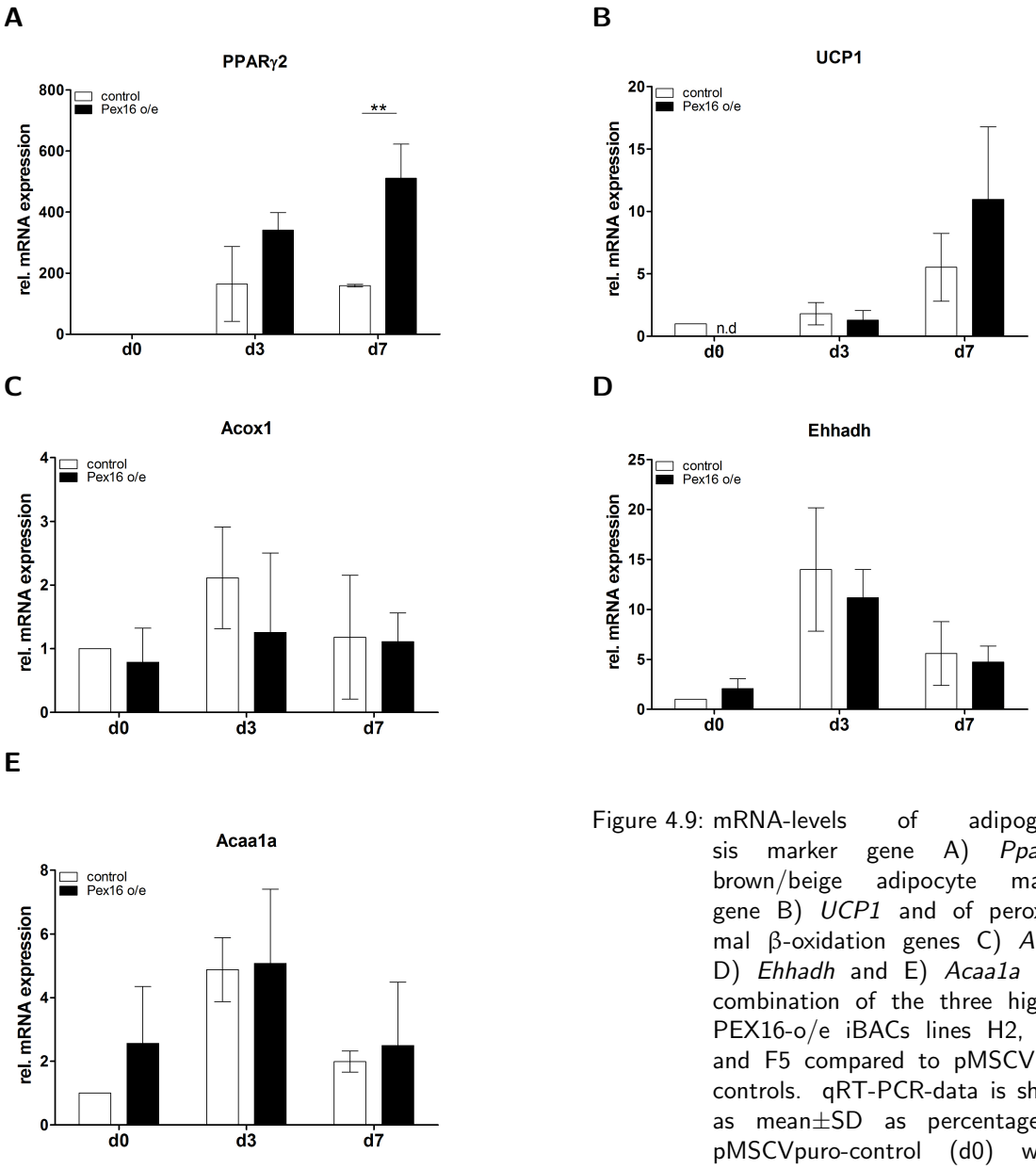

Figure 4.9: mRNA-levels of adipogenesis marker gene A) *Pparγ2*, brown/beige adipocyte marker gene B) *UCP1* and of peroxisomal β-oxidation genes C) *Acox1* D) *Ehhadh* and E) *Acaa1a* in a combination of the three highest PEX16-o/e iBACs lines H2, E12 and F5 compared to pMSCVpuro controls. qRT-PCR-data is shown as mean±SD as percentage of pMSCVpuro-control (d0) which was set to 1. (n=3)

As the overexpression of PEX16 on day 0 was already less than 2-fold in the combined PEX16-o/e iBACs, in persuasive experiments only the highest PEX16-o/e iBACs-line H2 was used. During differentiation of the H2-cell line a clear overexpression of *Pex16* mRNA could be seen (figure 4.10A). Again, PEX16-o/e cells seemed to accumulate less lipids than controls (figure 4.10B). Furthermore, mRNA expression of *Pparγ2* was measured to see, whether the changes in cell morphology can be related to an altered adipogenesis, but again elevated *Pparγ2* mRNA-levels were found in PEX16-o/e iBACs when compared to controls (figure 4.10C). From visual observation, we concluded that cells died throughout differentiation, which gave reason to perform a proliferation assay. The assay confirmed the observation of decreasing cell amounts for PEX16-o/e iBACs starting about 72h after induction of differentiation (figure 4.10D).

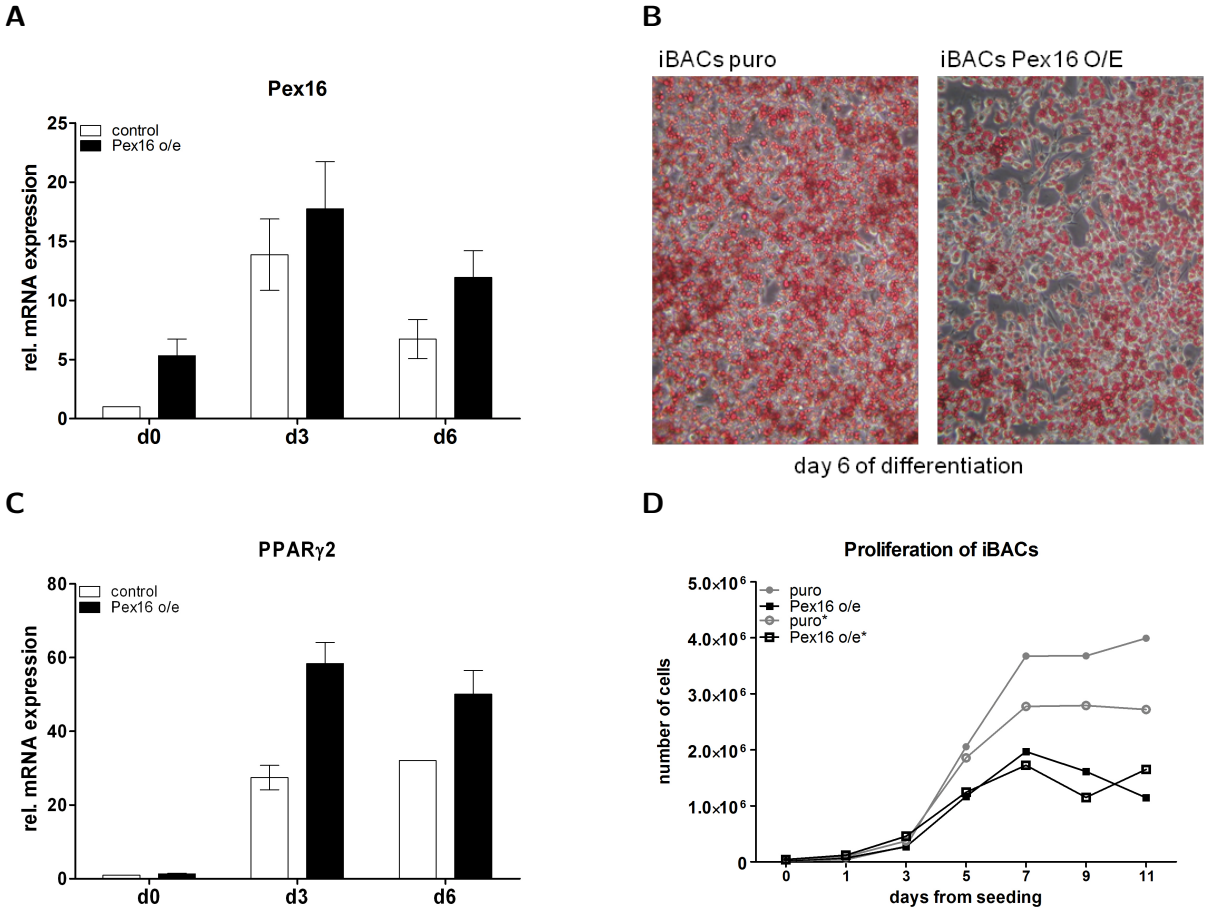

Figure 4.10: A) mRNA levels of *Pex16* in PEX16-o/e iBACs cell line H2 compared to pMSCVpuro controls during differentiation. B) Oil-red-O pictures of control cells and PEX16-o/e cells on day 6 of differentiation. C) mRNA levels of *Pparγ2* in PEX16-o/e iBACs cell line H2 compared to controls during differentiation. D) Proliferation testing by counting the cell amount of iBACs during growth and differentiation ("puro"/"Pex16 o/e": 20.000 cells seeded, induction on day 5, "puro*"/"Pex16 o/e*": 40.000 cells seeded, induction on day 4). Preliminary qRT-PCR-data is shown as mean±SD of two technical replicates as percentage of pMSCVpuro-control (d0), which was set to 1.

Furthermore, mRNA-expression of brown adipocyte marker gene *UCP1* and peroxisomal β-oxidation genes *Acox1*, *Ehhadh* and *Acaa1a* in the PEX16-o/e iBACs line H2 and controls was measured via qRT-PCR. Intriguingly, *UCP1* mRNA- levels were clearly decreased in PEX16-o/e cells on day 3 and on day 6 of differentiation when compared to controls (ct-levels: PEX16-o/e: d3: ≈34.7, d6: ≈29.6, control: d3: ≈29, d6: ≈26.7), as to see in figure 4.11A. *Acox1* mRNA-expression showed again a trend to reduction in PEX16-o/e iBACs throughout all measured timepoints (figure 4.11B). Furthermore, a trend to increased *Acaa1a* mRNA-expression on day 0 and day 3 of differentiation was observed again, *Ehhadh* was not changed in PEX16-o/e cells compared to controls (figure 4.11C,D).

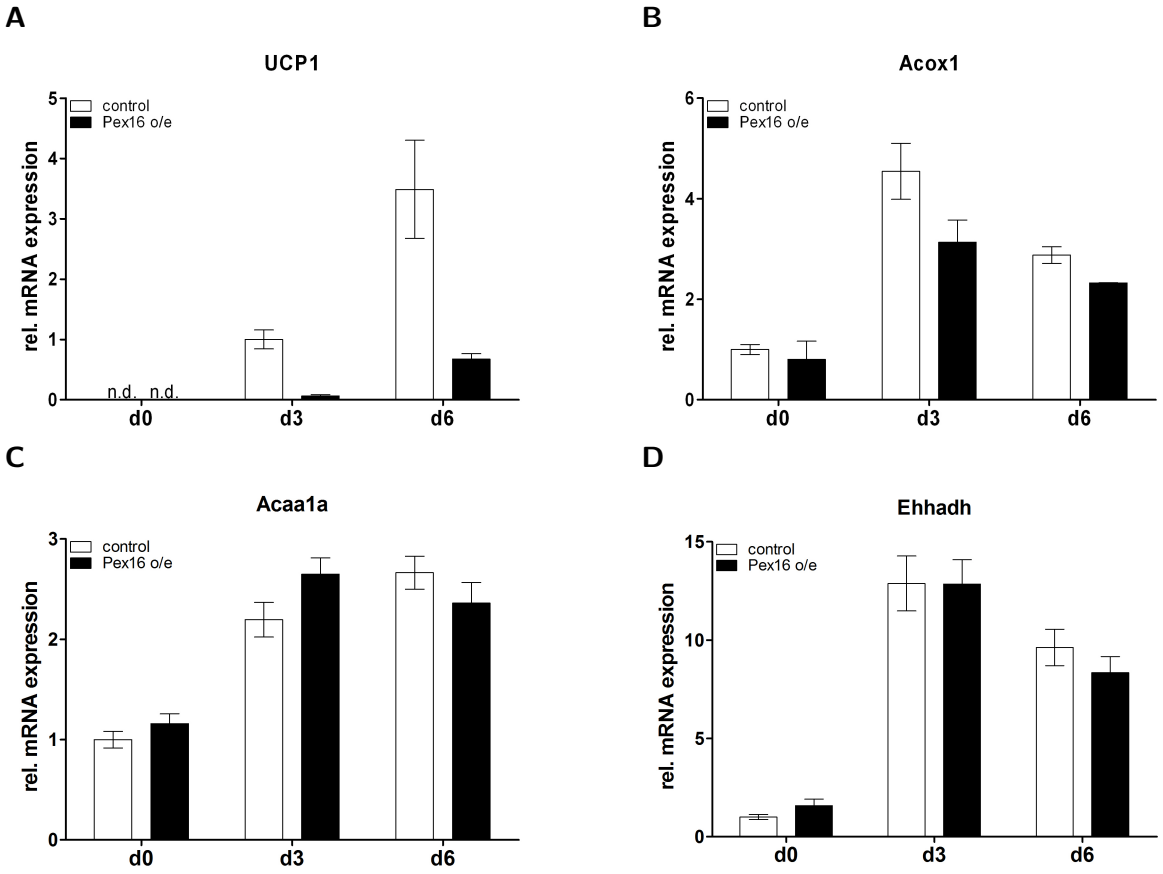

Figure 4.11: A) mRNA levels of *UCP1* in PEX16-o/e iBACs cell line H2 compared to pMSCVpuro controls. Preliminary qRT-PCR-data is shown as mean±SD of two technical replicates as percentage of pMSCVpuro-control (d3), which was set to 1. B) mRNA levels of *Acox1* C) *Acaa1a* and D) *Ehhadh* in PEX16-o/e iBACs cell line H2 compared to pMSCVpuro controls. Preliminary qRT-PCR-data is shown as mean±SD of two technical replicates as percentage of pMSCVpuro-control(d0), which was set to 1.

Based on the observation, that PEX16-overexpression gets almost lost during the differentiation process, cells were differentiated until day 4 and RNA samples were harvested every day. The qRT-PCR results show a loss of overexpression already 24h after induction and a regeneration on day 4 of differentiation (figure 4.12A). *Pparγ2* expression of PEX16-o/e cells was clearly decreased on day 0 and day 1 of differentiation (ct-levels for PEX16-o/e cells: d0: ≈38, d1: ≈33, ct-levels for controls: d0: ≈33, d1: ≈31) (figure 4.12B). However, starting on day 2 of differentiation, *Pparγ2*-expression of overexpressing cells was boosted to higher levels than in controls (figure 4.12B).

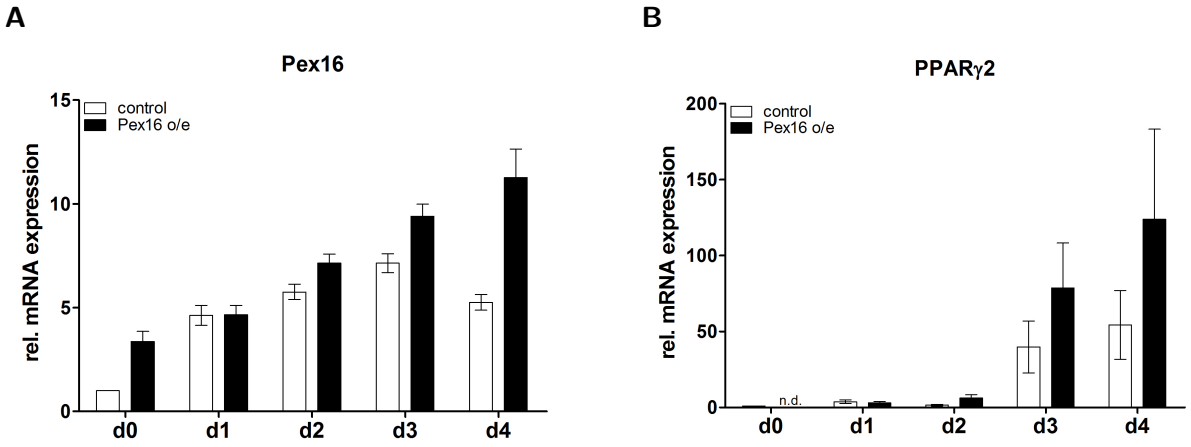

Figure 4.12: mRNA-levels of A)*Pex16* and B)*Pparγ2* during differentiation of the PEX16-o/e iBACs line H2 compared to pMSCVpuro-controls. The graphic represents mean±SD of 2 technical replicates as percentage of controls on day 0, which were set to 1.

In order to find an explanation for the cell death of PEX16 overexpressing iBACs after induction, Western Blots with day 0-protein samples for pro- and anti-apoptotic markers BAX and BCL-2 were performed by Madeleine Göritzer, Medical University of Graz. PEX16-o/e cells of passage 19 showed an approx. 50%-reduced expression of the pro-apoptotic protein BAX when compared to controls (figure 4.13A). With higher passages, the BAX-expression in PEX16-o/e cells increased, resulting in an even higher BAX expression in PEX16-o/e cells of passage 21 than in controls (figure 4.13A). Anti-apoptotic BCL-2 expression in PEX16-o/e cells of passage 19 was higher than in controls, and decreased with elevating passage (figure 4.13B). PEX16-o/e cells of passage 21 showed BCL-2 expression levels that were decreased to approximately 70% of the levels observed in controls (figure 4.13B).

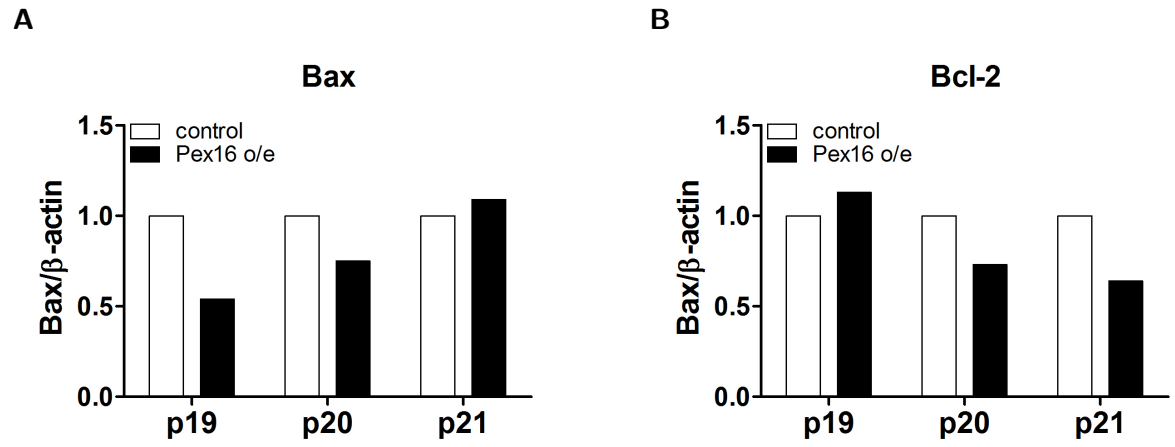

Figure 4.13: Results obtained from densiometric determination of BAX-and BCL-2 expression on day 0 of differentiation via Western Blots, normalized to β-actin. Controls were set to 1. The graphic shows 3 biological replicates.

4.4 Silencing of PEX16 in models for white and brown adipocytes

As PEX16 overexpression in 3T3-L1 cells and iBACs already showed slight alterations in growth, morphology and lipid metabolism, we were also interested in the effects of silencing PEX16 in these cell lines. Therefore, lentiviral transduction particles, encoding *Pex16*-targeting small hairpin RNA (shRNA), were used for generation of 3T3-L1 and iBACs cell lines with stably silenced PEX16 expression. Cells transduced with non targeting control (NTC)-lentiviral particles served as control.

4.4.1 Silencing of PEX16 in 3T3-L1 adipocytes

Silencing of PEX16 in 3T3-L1 cells was performed with 7.5 MOI (multiplicity of infection) per cell using 5 different shRNA lentiviral constructs. Subsequent selection with G418 had to be abandoned 72h after selection-start due to a massive loss of cells. Surviving cells were grown to 100% of confluence, and differentiated until day 7. qRT-PCR analysis was performed with day 0 and day 7 samples. On day 0 and on day 7 of differentiation a clear reduction of *Pex16* mRNA expression up to more than 50% was observed for all 5 lentiviral particles (figure 4.14A,B).

PEX16 silenced 3T3-L1 cells differentiated worse than control cells, as to see in ORO-pictures in figure 4.15. Thus, PEX16 seems to play a critical role in white adipocyte differentiation. On protein level silencing has not been verified yet.

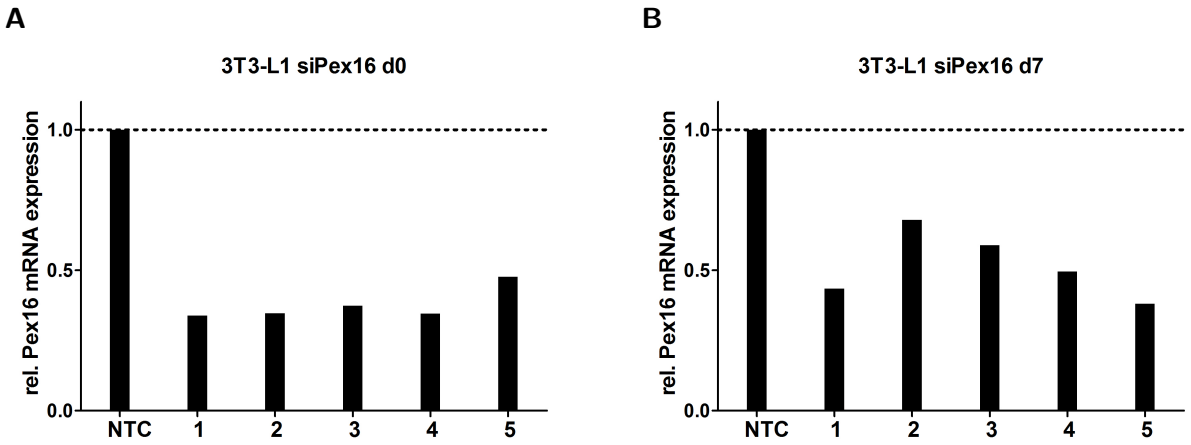

Figure 4.14: mRNA-levels of *Pex16* in 3T3-L1 cells on A) day 0 and B) day 7 of differentiation, silenced with 5 different lentiviral lines or transduced with non targeting control determined by qRT-PCR. Data is shown as mean of two technical replicates as percentage of not targeting control (NTC), which was set to 1.

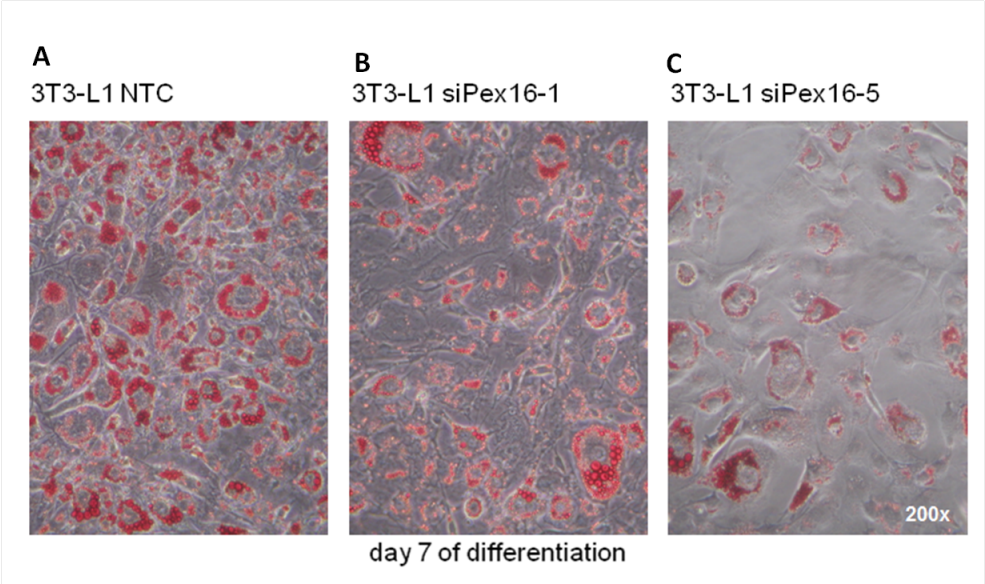

Figure 4.15: Effect of PEX16-silencing in 3T3-L1 cells on lipid accumulation shown in Oil-Red-O pictures of cells on day 7 of differentiation. A) Non targeting control (NTC) B) silencing construct 1 C) silencing construct 5. 200x magnification.

4.4.2 Silencing of PEX16 in iBACs

As PEX16-expression is even higher in BAT than in WAT (figure 4.1A), silencing effects in iBACs were expected to be more significant than in 3T3-L1 cells. Silencing of PEX16 in iBACs was tried with the same *Pex16*-targeting shRNA-expressing lentiviral particles used for 3T3-L1 cells. Due to the partial resistance of iBACs to puromycin, selection was done using 1.5mg/mL G418, but cells did not respond to the selection. Nevertheless, a screen for transduction efficiency was made on day 7 of differentiation via qRT-PCR, which confirmed that selection was inefficient. No reduction in *Pex16* mRNA-expression could be observed (figure 4.16).

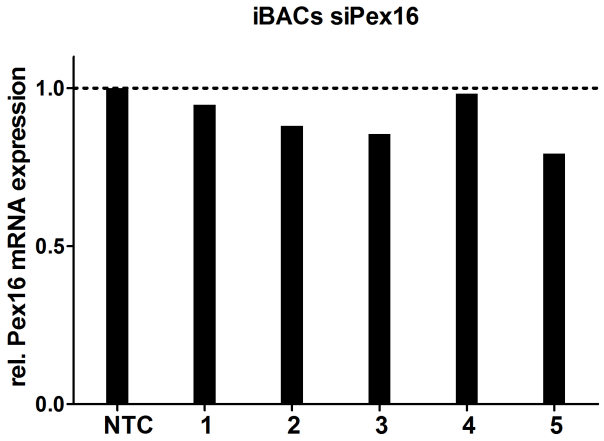

Figure 4.16: mRNA-levels of *Pex16* in iBACs on day 7 of differentiation, transduced with 5 different PEX16-targeting lentiviral lines and non targeting control. Data is shown as mean of two technical replicates as percentage of not targeting control (NTC), which was set to 1.

4.5 Pex16 - a possible PPARγ target

As elevated levels of *Pex16* mRNA-expression during adipogenesis and an increase of *Pex16* mRNA-expression under PPARγ-stimulation with rosiglitazone could be observed by qRT-PCR, a linkage between PPARγ and *Pex16* seemed likely. For that purpose, the region around TSS of *Pex16* gene was observed via UCSC genome browser and three estimated PPARγ binding targets downstream the TSS were discovered, referred to as "Peak1" (1052-1379bp downstream TSS) and "Peak2" (1349-1676bp downstream TSS), both located in intron 1, and "Peak3" (2160-2554bp downstream TSS), located in intron 2/exon 3 -transition (see figure 4.17).

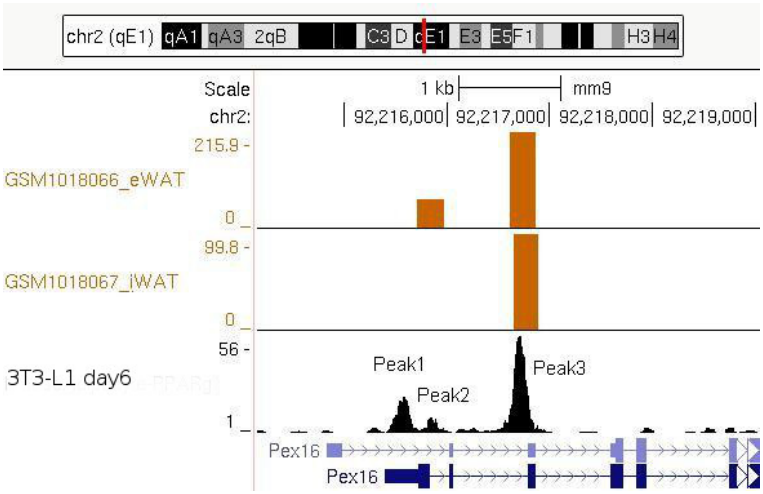

Figure 4.17: UCSC genome browser information on *Pex16* showing the region around *Pex16*-transcription start site. Three potential PPARγ-targets, labelled as "Peak1", "Peak2" and "Peak3" were detected.

The genomic regions a) 1052-1379bp (Peak1), b) 1349-1676bp (Peak2), c) the combined version 1052-1676bp (Peak1+2), and d) 2160-2554bp (Peak3) downstream the TSS, according to figure 4.18 were cloned into luciferase reporter vector pTK-luc.

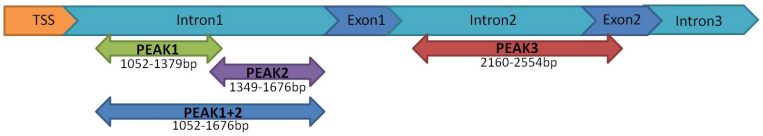

Figure 4.18: Location of the potential PPARγ-target regions "Peak1", "Peak2", "Peak1+2", and "Peak3" downstream the transcription start site.

Luciferase assays with these constructs were performed, but only very low read-outs could be achieved in these experiments due to a missing minimal promotor in the vector system. This realization gave reason to re-clone the sequences into another vector system (pGL4.26), providing a minimal promotor in its sequence, and to repeat the luciferase assays.

4.5.1 Luciferase assays using pTK-luc - luciferase reporter vector

Cloning of putative PPARγ - target sequences into pPPRE-X3-TK-luc- vector

The genomic regions were successfully amplified via PCR using primers with restriction-site extensions (see section 3.1.7) for HindIII (fw) and BamHI (rv) restriction enzymes (see figure 4.19A). Enzymatic digestion, ligation and transformation of NEB5α Comp. E.Coli-cells were carried out as described in section 3.2.1. In order to have an empty control vector of pTK-luc for subsequent luciferase assays, the triple PPRE-region in the pPPRE-X3-TK-luc plasmid was removed and the empty plasmid was then ligated via blunt end ligation. Ampicillin-selection on agar-selection-plates was performed over night at 37°C and resulted in growth of several potentially plasmid-containing colonies. Three colonies per plasmid were used for miniprep-production. To evaluate whether the generated plasmids contain the desired DNA-sequences, control cuts with HindIII and BamHI were performed (see figure 4.19B). Positive results were obtained for Peak1 (2 colonies), Peak2 (1 colony) and Peak1+2 (3 colonies). Peak3 was ≈150bp shorter than expected. This observation was confirmed by the sequencing results, which can be found in the appendix. An overlooked restriction site of BamHI within the Peak3-sequence was found to be responsible for the sequence-abbreviation. Cloning of full Peak3-sequence was finally successful at HindIII (fw) and XhoI (rv)-restriction sites (data not shown).

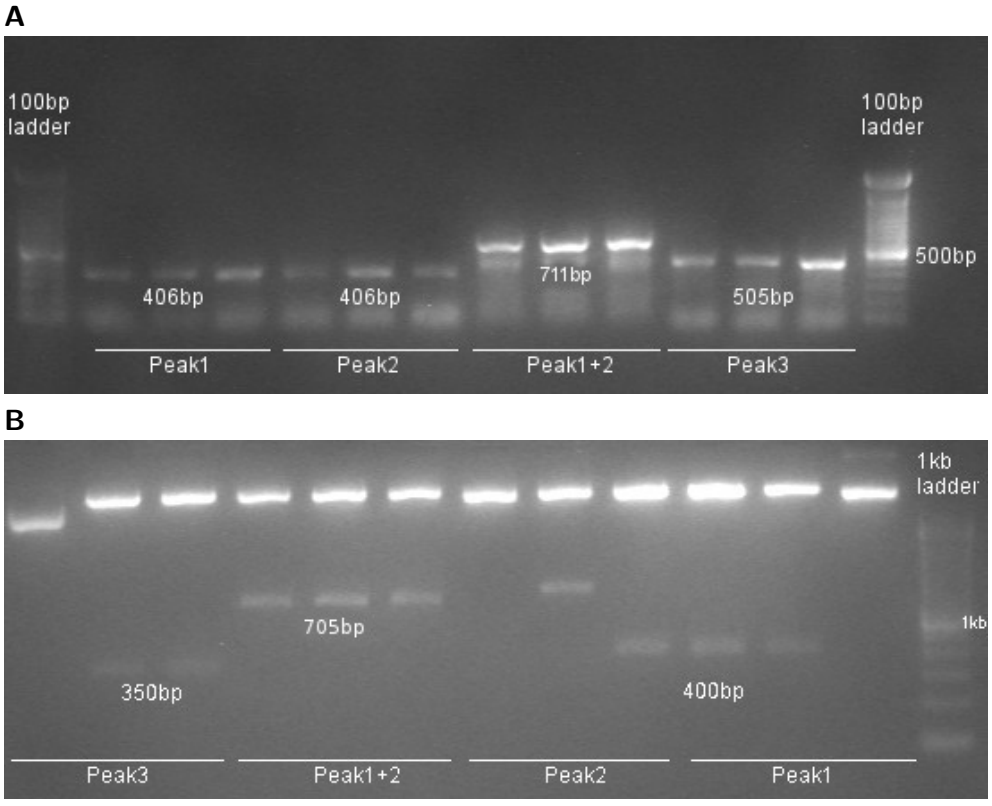

Figure 4.19: Electrophoresis gel: A) Genomic regions of *Pex16* used for testing of PPARγ-interactions amplified via PCR. B) Control digest of minipreps containing the putative PPARγ target regions using HindIII and BamHI restriction enzymes. Peak3 sequence is ≈150bp shorter than expected.

Luciferase-assays using pTK-luc- luciferase reporter vector suggest a potential interaction of PPARγ and Pex16

The luciferase activities of pTK-luc-Peak3l construct of PPARγ/RxRα- cotransfected cells with and without rosiglitazone-stimulation were significantly elevated compared to the pCMX-cotransfected samples (see figure 4.20). Stimulation with rosiglitazone resulted in a significant increase in luciferase activity at Peak3s- and Peak3l- constructs compared to the unstimulated construct of the PPARγ/RxRα cotransfected samples (see figure 4.20). In addition, luciferase activity of rosiglitazone-stimulated pTK-luc-Peak3s construct of PPARγ/RxRα- cotransfected cells was significantly increased compared to the pCMX-cotransfected samples and a trend for elevated luciferase activities under basal conditions was observed as well (figure 4.20). Peak1+2 of PPARγ/RxRα- cotransfected cells showed a trend to increased luciferase activity with and without rosiglitazone-stimulation. Peak1- and Peak2-constructs did not react to PPARγ- stimulation. These results suggest, that *Pex16* might be a target of PPARγ.

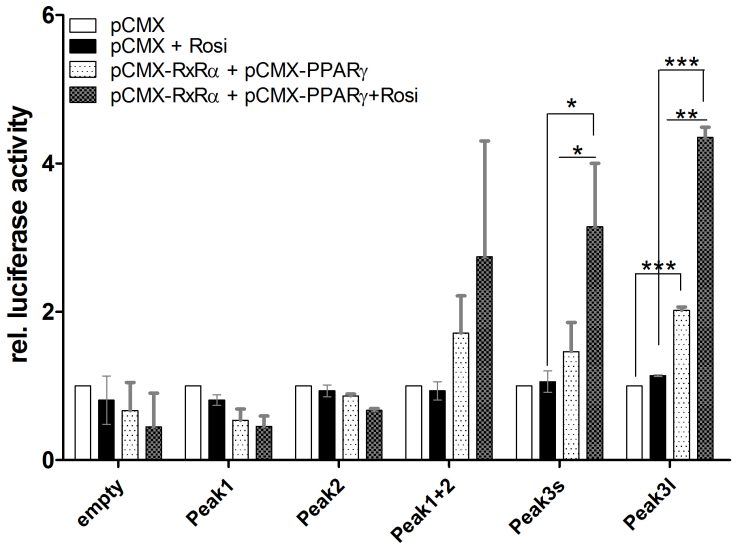

Figure 4.20: Luciferase assay results for PPARγ binding. Five potential target regions downstream transcription start site were cloned into luciferase reporter vector pTK-luc (Peak1, Peak2, Peak1+2, Peak3s (truncated sequence) and Peak3l (full sequence)). pCMX-vectors expressing PPARγ and RxRα, or the empty pCMX-vecor respectively, were co-transfected into Cos7 cells. Preliminary data show two (Peak1, Peak2, Peak3l) or three (empty pGL4.26 and Peak3s) biological replicates. Luciferase activity is shown as percentage of pCMX-luciferase activity, which was set to 1, for each construct individually. *$p<0.05$, **$p<0.01$, ***$p<0.001$

As the number of counts in these experiments was very low, the results could not be seen as reliable and had to be repeated, using an appropriate luciferase reporter vector, which harbours a minimal promoter.

4.5.2 Luciferase assays using pGL4.26 - luciferase reporter vector

Cloning of PPARγ target sequences into pGL4.26-vector

To solve the problem of low read-outs obtained from previous luciferase assays, the sequences were re-cloned at KpnI (fw) and XhoI (rv) restriction sites into pGL4.26 vector, containing a minimal promoter. The four generated constructs are represented schematically in figure 4.21. The figures were generated via *Serial Cloner*-program, which was used for imaginary test-runs of the cloning procedure including PCR, enzymatic digestion of vectors and inserts, and ligation, in order to avoid mistakes as in previous cloning attempts of the Peak3-sequence into pTK-luc vector.

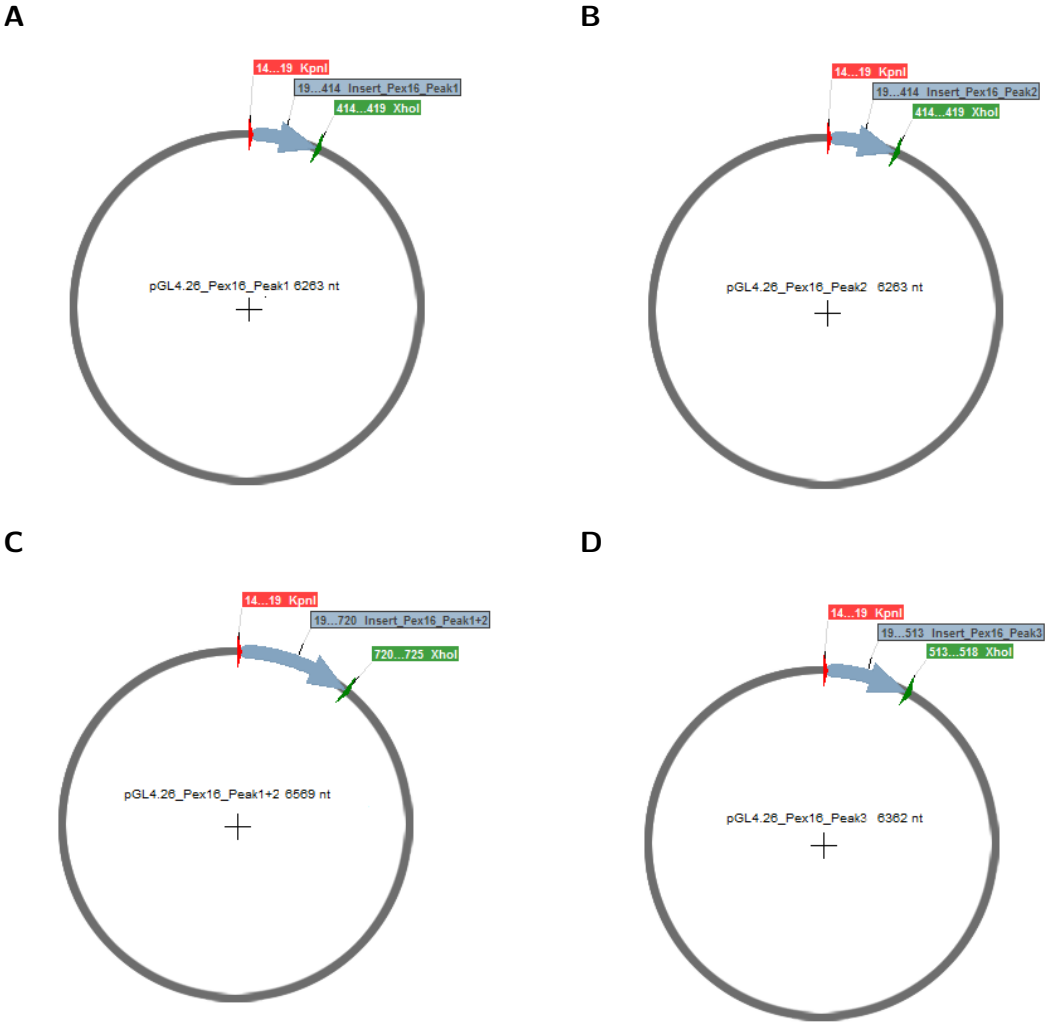

Figure 4.21: Scheme of plasmids generated by cloning *Pex16*-regions A) Peak1, B) Peak2, C) Peak1+2, and D) Peak3 into pGL4.26 vector at KpnI and XhoI restriction sites.

Pex16-inserts were amplified via PCR. Minipreps of pTK-luc constructs served as template, and primers with restriction-site extensions for KpnI and XhoI restriction enzymes (see section 3.1.7) were used for the amplification (see figure 4.22).

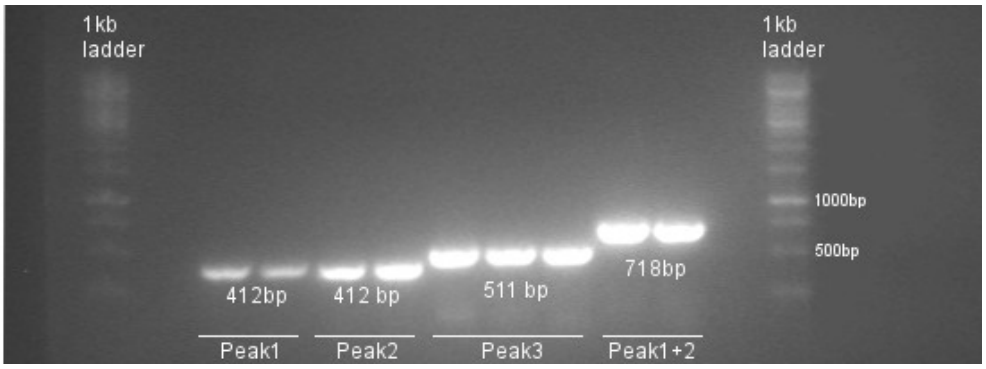

Figure 4.22: Electrophoresis gel: Inserts for pGL4.26-vector amplified via PCR.

Transformation of NEB5α Comp. E.Coli-cells with the generated plasmids and subsequent colony PCR of 6 colonies per construct revealed a well-functioning cloning of *Pex16*-inserts into pGL4.26-vector (see figure 4.23).

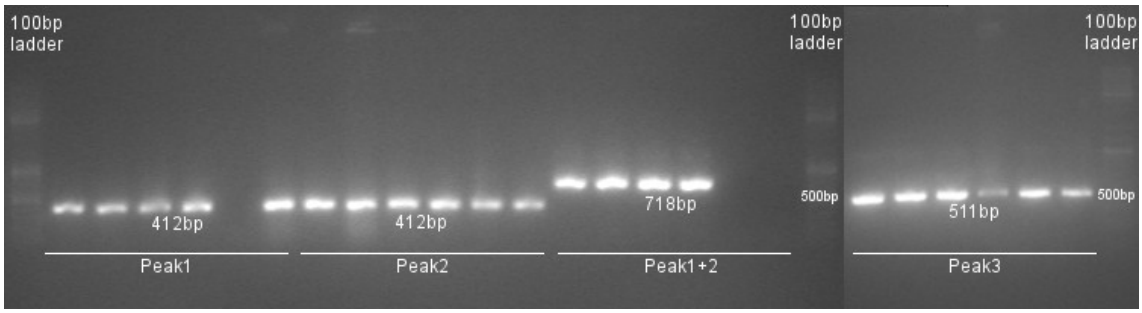

Figure 4.23: Electrophoresis gel: Colony PCR results for pGL4.26-Pex16 (Peak1, Peak2, Peak1+2, and Peak3) constructs.

Control digest with KpnI and XhoI restriction enzymes (see figure 4.24) and sequencing of the minipreps confirmed the integration of all of the four potential PPARγ target sequences into pGL4.26 vector. Sequencing results can be found in the appendix.

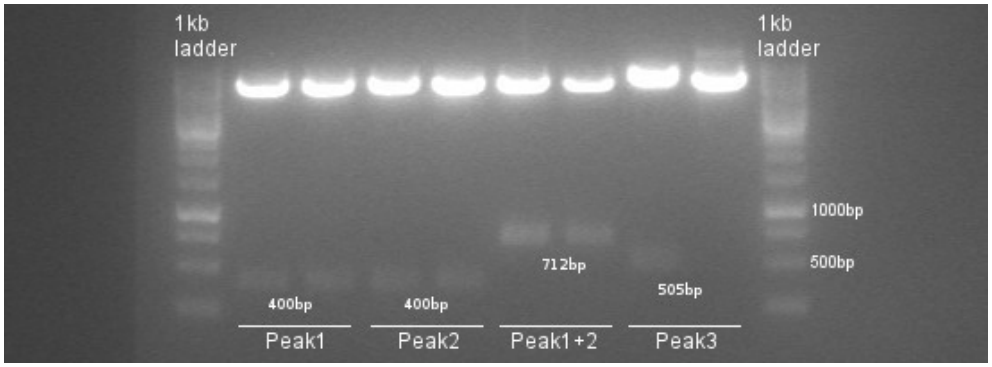

Figure 4.24: Electrophoresis gel: Control digest of the generated pGL4.26- constructs with KpnI and XhoI restriction enzymes confirms the integration of all putative PPARγ target regions (Peak1, Peak2, Peak3, and Peak1+2).

Luciferase-assay using pGL4.26- luciferase reporter vector confirms the suggested binding of Pex16 by PPARγ

Corresponding to the results from previous luciferase assays using pTK-luc-vector, the luciferase activity of pGL4.26 - Peak1+2- and Peak3- constructs of PPARγ/RxRα- cotransfected cells was elevated compared to the pCMX-cotransfected samples (figure 4.25). A clear increase under stimulation with rosiglitazone was detected for Peak3-samples cotransfected with PPARγ/RxRα compared to the unstimulated samples. In addition, a tendency to increase was observed for Peak1+2-constructs when stimulated with rosiglitazone (figure 4.25).

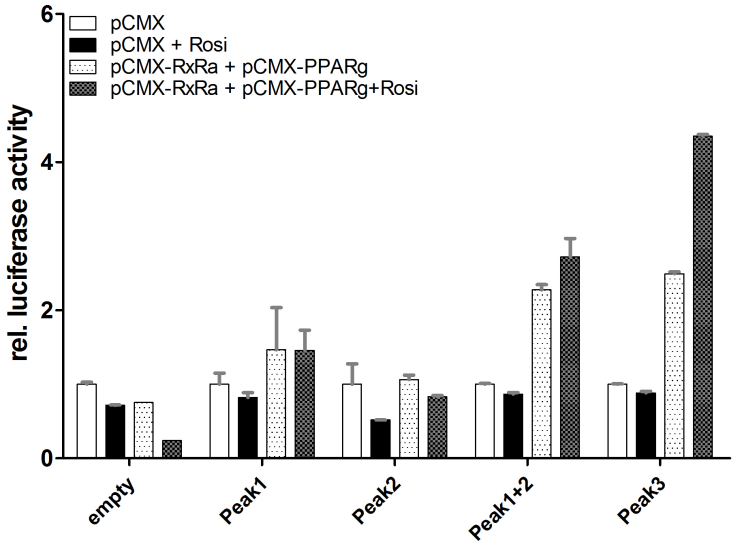

Figure 4.25: Luciferase assay results for PPARγ binding. Four potential target regions downstream transcription start site were cloned into luciferase reporter vector pGL4.26 (Peak1, Peak2, Peak1+2 and Peak3 (full sequence)). pCMX-vectors expressing PPARγ and RxRα, or the empty pCMX-vecor respectively, were co-transfected into Cos7 cells. Preliminary data show the mean$\pm$SD of two technical replicates. Luciferase activity is shown as percentage of pCMX-luciferase activity, which was set to 1, for each construct individually.

5 Discussion

PEX16 is known to be essential for peroxisomal biogenesis, growth and fission. Although function and composition of peroxisomes as well as several peroxisomal proteins have been studied in the liver extensively in the past, not much is known about the role of peroxisomes, and especially PEX16, in adipose tissue. As PEX16 was highly upregulated in differentiating iBACs as observed via microarray experiments performed in our group and hints for an influence of peroxisomes in adipocyte development and energy metabolism were found in literature, *Pex16* seemed to represent an interesting new candidate gene to investigate.

5.1 Pex16 is highly expressed in murine adipose tissue and elevated during adipogenesis

The first step of investigation was to screen various tissues for *Pex16* mRNA-expression.
High levels of *Pex16*-mRNA could be detected in white and brown adipose tissue, while expression in liver, skeletal muscle and cardiac muscle tissue was rather low. Additionally, *Pex16*-expression was significantly higher in BAT than in WAT. Further, mice on high-fat diet for 16-20 weeks showed trends of elevated *Pex16* mRNA-expression in brown adipose tissue as well as in the liver (see figure 4.1). Thus, a role of PEX16, and consequentially peroxisomes, in adipocyte, and especially brown adipocyte energy metabolism was assumed. Reports of elevated numbers of peroxisomes and increased *Pex16* mRNA-expression in brown adipose tissue in literature, as consequence of various stimuli, including high-fat diets and cold exposure [2][3], substantiate the suspicion, that PEX16 might contribute to adipocyte lipid metabolism and eventually also to thermogenesis. In BAT and WAT, *Pex16*-expression was the same in fasting (overnight) or refed (1h free access to food after overnight fasting) conditions of wildtype mice. Also genetic obesity, like in ob/ob-mice, had no impact on BAT and WAT expression of *Pex16*- mRNA. Eventually a longer fasting for 24h would lead to perceivable changes of *Pex16* mRNA-expression.

Further, we were interested in *Pex16* mRNA-expression in various adipose cell lines during differentiation and eventual expression-changes under stimulation of PPARγ by rosiglitazone or by β3-adrenergic stimulation with isoproterenol. In murine 3T3-L1 cells, iBACs and C3H/10 T1/2, an increase in *Pex16*-expression on the first few days of differentiation could be observed. Stimulation of PPARγ in 3T3-L1 cells with rosiglitazone and β3-adrenergic stimulation of iBACs with isoproterenol lead to an further increase in *Pex16*-expression. PPARγ represents the "master-regulator"

of adipogenesis. A linkage of *Pex16* mRNA-expression and PPARγ-stimulation gives evidence to a contribution of PEX16 to adipogenic differentiation. The increase of Pex16 expression under β3-adrenergic stimulation suggests a role of PEX16 also in brown fat metabolism and eventually even in thermogenesis. In human Simpson-Golabi-Behmel-syndrome cells (SGBS) and human multipotent adiopse-derived stem cells (hMADS), *Pex16*-expression was lower compared to murine cell lines, although mRNA-levels were also increasing with progressive differentiation. This result is congruent with the finding on *www.biogps.org*, that *Pex16* seems to be hardly expressed in human tissues[1].

5.2 PEX16 overexpression influences adipogenesis

Although expression in human cell lines was comparatively low, we wanted to further investigate the role of PEX16 in murine adipose cell lines, as the results for *Pex16*-expression in murine tissues and cell lines seemed very promising. Therefore, we overexpressed PEX16 in models for brown and white adipocytes, namely iBACs and 3T3-L1 cells.

As pMSCV-plasmids including the *Pex16* coding sequence for the overexpression in 3T3-L1 cell line were already cloned within a practical prior to the master thesis, overexpression of PEX16 was first accomplished in 3T3-L1 cells. Cells stably overexpressing PEX16 were differentiated twice (three passages each). The first trial of differentiation was characterized by low lipid accumulations in control and PEX16-o/e cells. However, PEX16-overexpressing cells accumulated even less lipid droplets. Application of an unnoticed wrong differentiation cocktail or a too early induction could be possible reasons for the weak differentiation. Nevertheless, overexpression of PEX16 could still be observed and stimulation with rosiglitazone led to an even higher *Pex16* mRNA-expression in PEX16-overexpressing cells compared to controls on day three of differentiation. The second trial showed enhanced differentiation, but as in the first trial, PEX16-overexpressing cells seemed to exhibit reduced lipid accumulation. This observation was also accompanied by reduced *Pparγ2* mRNA-expression, suggesting a role of PEX16 in adipogenic differentiation. Here it has to be mentioned that also the reduced differentiation of PEX16-o/e cells might be responsible for the reduced *Pparγ* expression. When stimulated with isoproterenol PEX16- overexpressing cells showed a trend to reduced triglyceride content and increased free fatty acid release on day 8 of differentiation, indicating a possible contribution of PEX16 to an elevated lipid turnover in response to β3- adrenergic stimulation. Quite the contrary was witnessed for basal conditions, as on day 8 free fatty acid release was significantly decreased in overexpressing cells. This observation was supported by a tendency to reduced mRNA-expression of *ATGL*, the main enzyme for adipogenic lipolysis, and a reduction in peroxisomal β-oxidation genes (*Acox1*, *Acaa1a*, *Ehhadh*) in PEX16-o/e cells. We expected, that overexpressing PEX16 would result in a higher peroxisomal activity and subsequently a higher peroxisomal β- oxidation within cells, but this was at least not the case under basal conditions. In future, isoproterenol stimulated mRNAs have to be measured. Taken together, overexpression of PEX16

[1] www.biogps.org/#goto=genereport&id=9409

seems to interfere with peroxisomal function. In which way this happens remains to be investigated. Experiments in yeast revealed an increase of peroxisomes in size, and a reduction of peroxisomal number caused by the overexpression of PEX16 [5]. Eventually, electron microscopy- experiments could illuminate the effect of PEX16-overexpression on peroxisomal structure, size and morphology.

PEX16- overexpression in iBACs, a brown adipocyte model, seemed to be more difficult than PEX16-o/e overexpression in 3T3-L1 cells. iBACs already possess a resistance against puromycin [46], making a selection of transduced cells using puromycin impossible. Therefore, *Pex16*-CDS had to be cloned into the vector system pMSCVhygro, providing a hygromycin-resistance-gene as selectable marker. Multiple trials of cloning the sequence into pMSCVhygro vector at various restriction sites failed, presumably due to a malfunctioning ligase. Thus, an alternative way for overexpressing PEX16 in iBACs had to be found. For this purpose, a dilution experiment with cells transduced with lentiviral particles for pMSCVpuro and pMSCV-Pex16 plasmids was performed. The subsequent screening for PEX16-overexpressing cell lines via qRT-PCR revealed three candidate cell lines, exhibiting an up to 5-fold overexpression of PEX16. Since we did not want to base further conclusions only on results received from one single overexpressing cell line, differentiation of the cells was performed with a combination of all three overexpressing cell lines. Interestingly, the previously explored 3- to 5-fold overexpression of *Pex16* on mRNA-level almost vanished and accordingly differences in FFA- release and TG- content, as seen in 3T3-L1 cells, could not be observed. A slight trend to augmented "browning" of the overexpressing cells was detected on day 7 of differentiation by elevated *UCP1* mRNA-levels. The expression of β- oxidation genes was not changed in PEX16-o/e iBACs.

Although Oil-red-O pictures suggested a worse differentiation of PEX16 overexpressing iBACs, *Pparγ2* mRNA-expression was elevated although the opposite would have been expected. The experiments were repeated with the H2-overexpressing iBAC line. Overexpression was maintained throughout differentiation, although the overexpression on day 3 was not significant. Again, reduced lipid accumulation was found in PEX16 overexpressing iBACs, although *Pparγ2*- expression was still increased in these cells. Interestingly, *UCP1*-expression in PEX16-o/e cells was not comparable to the previous results observed in the combined PEX16-overexpressing iBACs, as mRNA levels of *UCP1* were hardly detectable. Peroxisomal β-oxidation gene expression was again hardly altered in PEX16-o/e iBACs compared to controls. RNA samples from day 0 to day 4 were harvested in order to investigate, at which point of differentiation the overexpression disappears. The clear 3 to 4-fold overexpression of PEX16 on day 0 disappeared on day 1, and was slowly regained in the next few days. I assume, that this observation might be caused by a reduced differentiation or proliferation of overexpressing iBACs. *Pparγ2*- levels in PEX16-o/e cells were lower than in control cells on day 0 and day 1. On day 2, the overexpressing cells executed a delayed boost of *Pparγ2*- expression, which was then also preserved on day 3 and 4. This delayed *Pparγ* expression could eventually explain the delayed differentiation and the accompanied loss of PEX16 overexpression on day 2 and day 3 of differentiation. The boost in *Pparγ2*-expression might justify the regain of PEX16 overexpression on day 4.

A proliferation assay, which should be repeated in the future for more reliable results, revealed that growth of PEX16-overexpressing iBACs was actually reduced compared to controls, or that some of the cells died in the first few days of growth. Additionally, a massive cell death starting three days after induction could be observed visually for PEX16-o/e overexpressing cells and controls. Microscopy pictures from ORO-stainings show that there were holes in the cell monolayer of PEX16-o/e cells and controls.

In order to find a reason for the enormous loss of cells, we asked Madeleine Göritzer from the Medical University of Graz to screen our day 0 protein samples for the apoptose markers BAX (pro-apoptotic) and BCL-2 (anti-apoptotic). Intriguingly, hardly a difference or even reduced pro-apoptotic protein-levels for overexpressing cells could be detected. The pro-apoptotic BAX protein levels increased with every passage of the cells. I assume, that another screen for day 3 and/or day 7 protein samples for apoptotic marker genes would be necessary to detect alterations, as the massive cell death started three days after induction.
The facts, that many control cells died as well and that detached cells were still connected to the bottom by structures, reminding of extracellular matrix, indicate another reason for the cell death. Possible explanations could be the exorbitant lipid accumulations in the cells, causing cells to detach from the bottom, or the way the dilution experiment was performed, as cells were exposed to trypsin repeatedly during this process, or, not to forget, an unidentified infection. However, typical infections like mycosis or bacterial infections could not be observed by microscopy, and a mycoplasma-test was negative.

As the overexpression of PEX16 in iBACs was very conflicting, the repetition of these experiments with an appropriate vector-system would be reasonable. By considering that the accomplished overexpression in 3T3-L1 cells was also not very high, a repetition of this experiments with higher PEX16-overexpressing cells would eventually lead to more significant results.

5.2.1 Western Blot analysis reveils a reduced protein size of PEX16

Western Blot analysis was performed for both overexpression experiments in 3T3-L1 cells as well as in iBACs. The observed protein bands were found 2-3kDA below the expected protein size of 38.6kDA. Neither cleavage site analysis using *SignalP4.1* prediction tool could explain the reduction in size, nor could the sequencing results of the pMSCV-Pex16 plasmids. If there was an undetected cleavage of PEX16, it could only be C-terminally, as the N-terminally His-tagged control could be detected and had the right size using anti-His-antibody (data not shown) and anti-PEX16-antibody. According to Honsho et al. (1998), the C-terminal region of PEX16 is responsible for the biological function of PEX16. The loss of a C-terminal region would subsequently lead to a loss of PEX16 protein function. There was apparently no cleavage in the His-tagged PEX16-proteins. Thus, a cleavage in the untagged samples seems unlikely. Until now we have no explanation for the reduced protein size. Mass spectrometry could eventually clarify whether the detected protein is PEX16 or not.

In iBACs, PEX16 overexpression could not be verified on protein level. For day 0 and day 7, signals at the size of ≈50kDA were observed, indicating a possible dimerization of PEX16, which has not been described so far in literature. However, on day 3 a band at the right size was detected, but this band suggested reduced PEX16 concentration in PEX16-o/e cells.

Anyway, reasons for the reduced PEX16-protein size should be further investigated, as drawing conclusions regarding PEX16-overexpression based on an eventually malfunctioning protein would quite simply be wrong.

5.2.2 FFA and Triglyceride measurement

Free fatty acid release and triglyceride content were determined using intracellular protein-amounts for normalization. As protein amounts were consistently reduced in PEX16-overexpressing cells, variations of FFA-release and TG- content could also arise from this normalization method. It would be necessary to consider additional normalization parameters, such as the cell number, for the determination of more reliable results.

5.3 Stable silencing could be accomplished for 3T3-L1 cells, iBACs were not selectable

As the overexpression of PEX16 in 3T3-L1 cells and iBACs already interfered with adipocyte differentiation, we were also interested in silencing effects of PEX16. Mutations and absence of PEX16 were shown to result in the complete loss of peroxisomal structures [4][8][13][14][15].

Five different lentiviral strains were tested for their silencing efficiencies in 3T3-L1 cells and iBACs. Silencing of PEX16 in 3T3-L1 was successful for all five lentiviral particles compared to the non targeting control, although antibiotic selection had to be abandoned 72h after selection start, due to the appearance of an enormous cell death. It remains to be investigated, whether the cell death under selection pressure traces back to bad transduction efficiencies, or if this occurrence represents the first effect of complete silencing of PEX16. Non targeting controls remained in large parts alive during selection. Silencing efficiencies up to $>50\%$ could be observed for all lentiviral strains on day 0, and for strains 1 and 5 also on day 7 of differentiation. Additionally, reduced lipid accumulations of silenced cells could be observed using Oil-red-O-stainings. In future experiments, silencing should be confirmed on protein level. Additionally, the expression of adipogenic and peroxisomal marker genes and FFA- release and TG- content in basal and stimulated conditions should be in be investigated in these cells. Transduced iBACs were not selectable with geneticin (G418), possibly caused by a developed resistance against the antibiotic, or due to the use of defective G418 antibiotic. The unselected cells were differentiated until day 7. Silencing of PEX16 could not be detected. Selection should be repeated with new antibiotic in the future.

5.4 Luciferase assays reveal Pex16 as a PPARγ target gene

As multiple hints for possible interactions of PPARγ and *Pex16* were found in literature and previous experiments, we wanted to investigate these interactions using luciferase assays.

Cloning of putative PPARγ target sequences (Peak1, Peak2, Peak1+2, and Peak3) into luciferase reporter vector pTK-luc and the blunt end ligation for the generation of an empty vector turned out to be very time consuming, as in initial trials the same malfunctioning ligase as for cloning *Pex16*-CDS into pMSCVhygro was used. Additionally, an overlooked restriction site for BamHI in the Peak3-sequence lead to another necessary cloning repetition of the complete Peak3-sequence into pTK-luc vector.

However, luciferase assay results received for experiments using the generated pTK-luc plasmids indicate a PPARγ interaction at Peak1+2 and Peak3 sequence, as significantly increased firefly luciferase activities could be observed compared to controls. The luciferase activities for cells transfected with Peak1 and Peak2 sequences were hardly altered when PPARγ/RxRα were present, suggesting that the putative PPARγ- target sequence of Peak1+2 might be located between Peak1 and Peak2 region. In these experiments, only very low read outs (several hundred counts) for firefly luciferase activity were generated. A non-existent minimal promoter in the pTK-luc sequence might be the cause of these low read outs. The fact, that still differences in luciferase activity could be observed, can be explained by the existance of a thymidine kinase promoter lying several hundred basepairs upstream the target region in the vector [47], which could be sufficient for generation of at least some transcripts of these regions.

In order to get more reliable results, the sequences were re-cloned into pGL4.26 vector, providing a minimal promoter in its sequence. The performed luciferase assay confirmed the results obtained for pTK-luc constructs, finally showing reliable counts for firefly luciferase activity. The regions of Peak1+2 and Peak3 seem to represent direct targets of PPARγ. By knowing this, a linkage of PEX16 to adipogenesis seems very likely. This results needs to be verified within prospective repetitions of the experiment in the future.

5.5 Model for PEX16 contribution to lipid metabolism in adipose tissue

The idea of how PEX16 might contribute to (brown) adipocyte metabolism, is drawn schematically in figure 5.1. PEX16 is integrated into pre-peroxisomes at the endoplasmic reticulum. It contributes to peroxisomal formation and growth by serving as receptor for PEX3, which subsequently attracts peroxisomal membrane proteins from the cytosol to the peroxisomal membrane [1][4][10][11][12]. Peroxisomes perform β-oxidation of very long chain fatty acids, in an acetyl-CoA providing and potentially thermogenic way [3]. Acetyl-CoA is than transported into mitochondria, where it is processed in order to generate ATP or heat (only in brown adipocytes). The expression of PEX16 can be regulated by PPARγ.

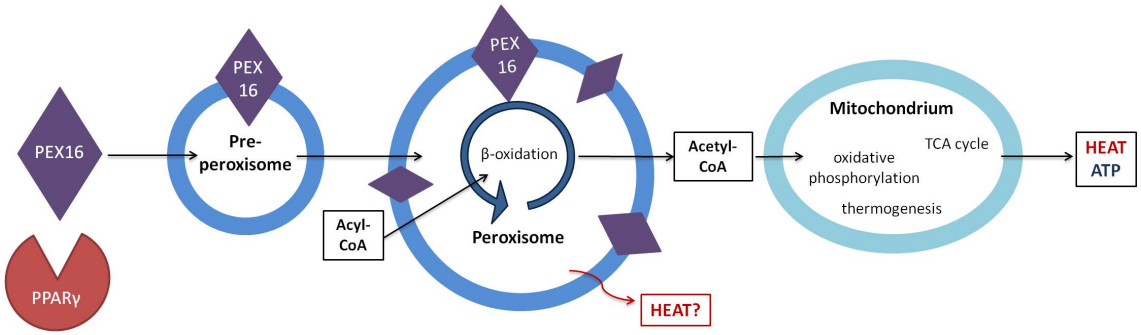

Figure 5.1: Scheme of the potential influence of PEX16 to peroxisomal development and lipid metabolism in (brown) adipose tissue.

5.6 Concluding remarks

Many fascinating facts about PEX16 and its possible contribution to adipogenesis and energy metabolism have been collected throughout the research for this thesis. *Pex16* seems to be a direct target of PPARγ, which makes it a promising new player in adipogenesis. Overexpression and silencing of PEX16 in models for white and brown adipocytes also gave hints for this assumption. Nevertheless, for unraveling questions, like the impact of PEX16 in priming adipocyte development and if so, how it might contribute to adipocyte metabolism, more research needs to be done in the future.

List of Figures

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6 Appendix

Sequencing results (Query = Pex16-sequence, Subject= Sequencing results)

pMSCVpuro_Pex16_fw

Alignments

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Sbjct  479   AATGACGGGATCCTGAGGAAGGAGCTTCGAAAAAAGTTGCCTGTGTCGCTGTCCCAGCAG  538

Query  241   AAGTTGCTGACATGGCTGAGTGTACTGGAGTGTGTGGAGGTGTTCATGGAGATGGGGGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  539   AAGTTGCTGACATGGCTGAGTGTACTGGAGTGTGTGGAGGTGTTCATGGAGATGGGGGCT  598

Query  301   GCCAAGGTGTGGGGCGAAGTAGGCCGTTGGCTCGTCATTGCTCTCATCCAGTTGGCCAAG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599   GCCAAGGTGTGGGGCGAAGTAGGCCGTTGGCTCGTCATTGCTCTCATCCAGTTGGCCAAG  658

Query  361   GCCGTCCTGCGCATGCTCCTGTTGATCTGGTTCAAGGCTGGCATTCAGACATCACCCCCC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659   GCCGTCCTGCGCATGCTCCTGTTGATCTGGTTCAAGGCTGGCATTCAGACATCACCCCCC  718

Query  421   ATTGTTCCACTGGACAGGGAAACTCAGGCACAGCCCTTGGATGGTGACCACAATCCGGGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  719   ATTGTTCCACTGGACAGGGAAACTCAGGCACAGCCCTTGGATGGTGACCACAATCCGGGC  778

Query  481   AGCCAGGAGCCATCATATGTGGGGAAACGGTCACACAGAGTGGTGCGAACCCTCCAGAAC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  779   AGCCAGGAGCCATCATATGTGGGGAAACGGTCACACAGAGTGGTGCGAACCCTCCAGAAC  838

Query  541   AGCCCATCCCTGCACTCACGGTACTGGGGAGCCCCTCAGCAGCGGGAAATTCGGCAGAAA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  839   AGCCCATCCCTGCACTCACGGTACTGGGGAGCCCCTCAGCAGCGGGAAATTCGGCAGAAA  898

Query  601   CAGCAGCAGGAGGAACTGAGCACACCCCCAACACCTCTGGGGTTGCAGGAGACCATTGCA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  899   CAGCAGCAGGAGGAACTGAGCACACCCCCAACACCTCTGGGGTTGCAGGAGACCATTGCA  958

Query  661   GAGTCTTTGTACATCGCCCGGCCCCTGCTGCACTTGCTCAGCCTGGGCTTATGGGGTCAG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  959   GAGTCTTTGTACATCGCCCGGCCCCTGCTGCACTTGCTCAGCCTGGGCTTATGGGGTCAG  1018

Query  721   CGATCGTGGACACCCTGGCTACTGTCTGGTGTGGTAGATATGACTAGCCTGAGTCTCCTG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019  CGATCGTGGACACCCTGGCTACTGTCTGGTGTGGTAGATATGACTAGCCTGAGTCTCCTG  1078

Query  781   AGTGACAGGAAGAACTTGACCCGGAGGGAGCGGCTAGAACTGA  823
             ||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1079  AGTGACAGGAAGAACTTGACCCGGANGGAGCGGCTAGAACTGA  1121
```

HisMaxC_Pex16_rv

Alignments

[2_puro_Pex16_1_r_pMSCV_rv.fasta.txt - 1131 bp] 2_puro_Pex16_1_r_pMSCV_rv - Selection From [1] To [113
Sequence ID: lcl|39299 Length: 1131 Number of Matches: 1
Range 1: 209 to 1130

Score	Expect	Identities	Gaps	Strand	Frame
1668 bits(903)	0.0()	914/922(99%)	0/922(0%)	Plus/Minus	

Features:

```
Query  90    GGGCCTCAGTTACCTGCTGGCAGGTCGCTTCTCCGATTCACACGAGCTGTCTGAACTGGT  149
             ||||| |||||||||||||||||||| | |||||||||| ||||||||||||||||||
Sbjct  1130  GGGCTTCAGTTACCTGCTGGCAGGTCGNNTTTCCGATTCANMCGAGCTGTCTGAACTGGT  1071

Query  150   GTACTCTGCCTCGAACCTGCTGGTGCTGCTCAATGACGGGATCCTGAGGAAGGAGCTTCG  209
              || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1070  KTATTCTGCCTCGAACCTGCTGGTGCTGCTCAATGACGGGATCCTGAGGAAGGAGCTTCG  1011

Query  210   AAAAAAGTTGCCTGTGTCGCTGTCCCAGCAGAAGTTGCTGACATGGCTGAGTGTACTGGA  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1010  AAAAAAGTTGCCTGTGTCGCTGTCCCAGCAGAAGTTGCTGACATGGCTGAGTGTACTGGA  951

Query  270   GTGTGTGGAGGTGTTCATGGAGATGGGGGCTGCCAAGGTGTGGGGCGAAGTAGGCCGTTG  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  950   GTGTGTGGAGGTGTTCATGGAGATGGGGGCTGCCAAGGTGTGGGGCGAAGTAGGCCGTTG  891

Query  330   GCTCGTCATTGCTCTCATCCAGTTGGCCAAGGCCGTCCTGCGCATGCTCCTGTTGATCTG  389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  890   GCTCGTCATTGCTCTCATCCAGTTGGCCAAGGCCGTCCTGCGCATGCTCCTGTTGATCTG  831

Query  390   GTTCAAGGCTGGCATTCAGACATCACCCCCCATTGTTCCACTGGACAGGGAAACTCAGGC  449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  830   GTTCAAGGCTGGCATTCAGACATCACCCCCCATTGTTCCACTGGACAGGGAAACTCAGGC  771

Query  450   ACAGCCCTTGGATGGTGACCACAATCCGGGCAGCCAGGAGCCATCATATGTGGGGAAACG  509
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  770   ACAGCCCTTGGATGGTGACCACAATCCGGGCAGCCAGGAGCCATCATATGTGGGGAAACG  711

Query  510   GTCACACAGAGTGGTGCGAACCCTCCAGAACAGCCCATCCCTGCACTCACGGTACTGGGG  569
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  710   GTCACACAGAGTGGTGCGAACCCTCCAGAACAGCCCATCCCTGCACTCACGGTACTGGGG  651

Query  570   AGCCCCTCAGCAGCGGGAAATTCGGCAGAAACAGCAGCAGGAGGAACTGAGCACACCCCC  629
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  650   AGCCCCTCAGCAGCGGGAAATTCGGCAGAAACAGCAGCAGGAGGAACTGAGCACACCCCC  591

Query  630   AACACCTCTGGGGTTGCAGGAGACCATTGCAGAGTCTTTGTACATCGCCCGGCCCCTGCT  689
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  590   AACACCTCTGGGGTTGCAGGAGACCATTGCAGAGTCTTTGTACATCGCCCGGCCCCTGCT  531

Query  690   GCACTTGCTCAGCCTGGGCTTATGGGGTCAGCGATCGTGGACACCCTGGCTACTGTCTGG  749
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  530   GCACTTGCTCAGCCTGGGCTTATGGGGTCAGCGATCGTGGACACCCTGGCTACTGTCTGG  471

Query  750   TGTGGTAGATATGACTAGCCTGAGTCTCCTGAGTGACAGGAAGAACTTGACCCGGAGGGA  809
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  470   TGTGGTAGATATGACTAGCCTGAGTCTCCTGAGTGACAGGAAGAACTTGACCCGGAGGGA  411

Query  810   GCGGCTAGAACTGAGGCGTCGCACAATCCTGCTGCTATACTACCTGCTACGCTCACCGTT  869
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410   GCGGCTAGAACTGAGGCGTCGCACAATCCTGCTGCTATACTACCTGCTACGCTCACCGTT  351

Query  870   CTATGACCGCTTCTCTGAAGCCAAGATCCTCTTCCTGCTCCAGCTGCTTACAGACCACAT  929
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350   CTATGACCGCTTCTCTGAAGCCAAGATCCTCTTCCTGCTCCAGCTGCTTACAGACCACAT  291

Query  930   CCCTGGCGTGGGTCTGGTGGCAAGGCCGCTCATGGACTACTTGCCCTCCTGGCAGAAAAT  989
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290   CCCTGGCGTGGGTCTGGTGGCAAGGCCGCTCATGGACTACTTGCCCTCCTGGCAGAAAAT  231

Query  990   CTATTTCTACAGTTGGGGCTGA  1011
             |||||||||||||||||||||
Sbjct  230   CTATTTCTACAGTTGGGGCTGA  209
```

pTK-Luc_Pex16_Peak1_fw, pTK-Luc_Pex16_Peak2_fw

Alignments

[4_pTK_x3_Peak1_M13.fasta - 1110 bp] 4_pTK_x3_Peak1_M13 - Selection From [1] To [1110]
Sequence ID: lcl|236315 Length: 1110 Number of Matches: 1
Range 1: 1 to 372

Score	Expect	Identities	Gaps	Strand	Frame
678 bits(367)	0.0()	372/374(99%)	2/374(0%)	Plus/Plus	

Features:

```
Query  1033  TTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGCTGCAGATCGGGTGCCAGAAG  1092
             ||||  ||||||||||||||  ||||||||||||||||||||||||||||||||||||
Sbjct  1     TTGT-ACTACGCAGGCGCC-AGACAGCCAACCAAGGAGGCTGCAGATCGGGTGCCAGAAG  58

Query  1093  CTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGGCTGGAAGGAGGGAGGGTGGG  1152
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  59    CTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGGCTGGAAGGAGGGAGGGTGGG  118

Query  1153  AGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGAAGGGGCGTTCAGGGGCTCCG  1212
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119   AGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGAAGGGGCGTTCAGGGGCTCCG  178

Query  1213  CGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCGGGAGGCGGGCTTAGGGCCGG  1272
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179   CGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCGGGAGGCGGGCTTAGGGCCGG  238

Query  1273  AAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCAGCCTTGCTGTCGGCCGGTGG  1332
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239   AAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCAGCCTTGCTGTCGGCCGGTGG  298

Query  1333  GACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGCCTCCGCTACCAGGA  1392
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299   GACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGCCTCCGCTACCAGGA  358

Query  1393  GTATGTGACTCGTC  1406
              ||||||||||||||
Sbjct  359   GTATGTGACTCGTC  372
```

Alignments

[3_pTK_x3_Peak2_M13.fasta - 1110 bp] 3_pTK_x3_Peak2_M13 - Selection From [1] To [1110]
Sequence ID: lcl|18831 Length: 1110 Number of Matches: 1
Range 1: 1 to 360

Score	Expect	Identities	Gaps	Strand	Frame
658 bits(356)	0.0()	359/360(99%)	1/360(0%)	Plus/Plus	

Features:

```
Query  1356  TGGAGAA-GCTACGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCC  1414
             |||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TGGAGAANGCTACGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCC  60

Query  1415  GCCACGGCCCAGTTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACA  1474
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GCCACGGCCCAGTTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACA  120

Query  1475  CTGACCTTTGACCCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGC  1534
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTGACCTTTGACCCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGC  180

Query  1535  TGACCCAGGGTAGTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAA  1594
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TGACCCAGGGTAGTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAA  240

Query  1595  ACTAGAGAACCCAAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCT  1654
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   ACTAGAGAACCCAAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCT  300

Query  1655  TCCCTAGGTCGCTTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTGGG  1714
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   TCCCTAGGTCGCTTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTGGG  360
```

pTK-Luc_Pex16_Peak1+2_fw

Alignments

[2_pTK_x3_Peak1_2_M13.fasta - 1130 bp] 2_pTK_x3_Peak1_2_M13 - Selection From [1] To [1130]
Sequence ID: lcl|9665 Length: 1130 Number of Matches: 1
Range 1: 6 to 691

Score	Expect	Identities	Gaps	Strand	Frame
1247 bits(675)	0.0()	685/689(99%)	3/689(0%)	Plus/Plus	

Features:

```
Query  1026  CCGCAAGTTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGCTGCAGATCGGGTG  1085
             ||||  ||||||  ||||||||||||||  ||||||||||||||||||||||||||||||
Sbjct  6     CCGC-AGTTGT-ACTACGCAGGCGCC-AGACAGCCAACCAAGGAGGCTGCAGATCGGGTG  62

Query  1086  CCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGGCTGGAAGGAGGGA  1145
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63    CCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGGCTGGAAGGAGGGA  122

Query  1146  GGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGAAGGGGCGTTCAGG  1205
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123   GGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGAAGGGGCGTTCAGG  182

Query  1206  GGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCGGGAGGCGGGCTTA  1265
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183   GGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCGGGAGGCGGGCTTA  242

Query  1266  GGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCAGCCTTGCTGTCGG  1325
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243   GGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCAGCCTTGCTGTCGG  302

Query  1326  CCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGCCTCCGCT  1385
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303   CCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGCCTCCGCT  362

Query  1386  ACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAGACGGCTGTGCGGG  1445
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363   ACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAGACGGCTGTGCGGG  422

Query  1446  GCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTTCTTGGGGACTCTG  1505
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423   GCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTTCTTGGGGACTCTG  482

Query  1506  ACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTCGCGGTGCTTCAGT  1565
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483   ACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTCGCGGTGCTTCAGT  542

Query  1566  TCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCCGCAGGGTAAAACA  1625
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  543   TCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCCGCAGGGTAAAACA  602

Query  1626  GACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCTTCTCCGATTCACACGAGC  1685
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603   GACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCTTCTCCGATTCACACGAGC  662

Query  1686  TGTCTGAACTGGGTAAGTTGGACTCTGGG  1714
              |||||||||||||||||||| |||||||
Sbjct  663   TGTCTGAACTGGGTAAGTTGGGCTCTGGG  691
```

pTK-Luc_Pex16_Peak3s_fw, pTK-Luc_Pex16_Peak3l_fw

Alignments

[1_pTK_x3_Peak3_M13.fasta - 1207 bp] 1_pTK_x3_Peak3_M13 - Selection From [1] To [1207]
Sequence ID: lcl|73877 Length: 1207 Number of Matches: 1
Range 1: 5 to 321

Score	Expect	Identities	Gaps	Strand	Frame
580 bits(314)	1e-168()	317/318(99%)	1/318(0%)	Plus/Plus	

Features:

```
Query  2145  TTGGCTGTTTGGAACATGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGCA  2204
             ||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5     TTGGCTGTTTGG-ACATGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGCA  63

Query  2205  GAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCAT  2264
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCAT  123

Query  2265  GGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTGT  2324
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTGT  183

Query  2325  GGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAGG  2384
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   GGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAGG  243

Query  2385  GCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTGC  2444
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   GCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTGC  303

Query  2445  TGCTCAATGACGGGATCC  2462
              ||||||||||||||||||
Sbjct  304   TGCTCAATGACGGGATCC  321
```

Alignments

[3_pTK_Luc_peak3_M13.fasta - 1122 bp] 3_pTK_Luc_peak3_M13 - Selection From [1] To [1122]
Sequence ID: lcl|50251 Length: 1122 Number of Matches: 1
Range 1: 5 to 465

Score	Expect	Identities	Gaps	Strand	Frame
846 bits(458)	0.0()	461/462(99%)	1/462(0%)	Plus/Plus	

Features:

```
Query  2144  TTTGGCTGTTTGGAACATGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGC  2203
             ||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5     TTTGGCTGTTTGG-ACATGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGC  63

Query  2204  AGAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCA  2263
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    AGAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCA  123

Query  2264  TGGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTG  2323
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   TGGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTG  183

Query  2324  TGGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAG  2383
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   TGGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAG  243

Query  2384  GGCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTG  2443
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   GGCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTG  303

Query  2444  CTGCTCAATGACGGGATCCTGAGGAAGGAGCTTCGAAAAAAGTTGCCTGTGGTGAGAACT  2503
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   CTGCTCAATGACGGGATCCTGAGGAAGGAGCTTCGAAAAAAGTTGCCTGTGGTGAGAACT  363

Query  2504  TTGCCAGGAGCTGGGGGTGGGAAGCTCAGGGACTTGGGAGGAAAACAGTAATGACCAGCA  2563
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   TTGCCAGGAGCTGGGGGTGGGAAGCTCAGGGACTTGGGAGGAAAACAGTAATGACCAGCA  423

Query  2564  CGTGTGGTACATGTCGTTGTCTCAGAGAGTGAGGCATGCCCA  2605
              |||||||||||||||||||||||||||||||||||||||||
Sbjct  424   CGTGTGGTACATGTCGTTGTCTCAGAGAGTGAGGCATGCCCA  465
```

pGl4.26_Pex16_Peak1_fw, pGl4.26_Pex16_Peak1_rv

Alignments

[1_Pex16_Peak1_fw_Pex16_Peak1_2_fw.fasta - 1137 bp] 1_Pex16_Peak1_fw_Pex16_Peak1_2_fw - Selection From [1] To [1137]
Sequence ID: lcl|33325 Length: 1137 Number of Matches: 1
Range 1: 2 to 349

Score	Expect	Identities	Gaps	Strand	Frame
634 bits(343)	0.0()	348/350(99%)	2/350(0%)	Plus/Plus	

Features:

```
Query  1057  AGCCAACCAAGGAGGCTGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGA  1116
             ||||  ||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2     AGCC-ACC-AGGAGGCTGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGA  59

Query  1117  GAGAAGGAGCCAATGGCTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGC  1176
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60    GAGAAGGAGCCAATGGCTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGC  119

Query  1177  GCTAGAGATGGGGTGAAGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGC  1236
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   GCTAGAGATGGGGTGAAGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGC  179

Query  1237  GGAAGTTGAGCAACCGGGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACG  1296
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   GGAAGTTGAGCAACCGGGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACG  239

Query  1297  CAGGGCGCCGCTGCCAGCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGAT  1356
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240   CAGGGCGCCGCTGCCAGCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGAT  299

Query  1357  GGAGAAGCTACGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTC  1406
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300   GGAGAAGCTACGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTC  349
```

Alignments

[2_Pex16_Peak1_rv_Pex16_Peak1_rv.fasta - 1164 bp] 2_Pex16_Peak1_rv_Pex16_Peak1_rv - Selection From [1] To [1164]
Sequence ID: lcl|109391 Length: 1164 Number of Matches: 1
Range 1: 3 to 359

Score	Expect	Identities	Gaps	Strand	Frame
656 bits(355)	0.0()	356/357(99%)	0/357(0%)	Plus/Minus	

Features:

```
Query  1013  CTGTTCAGCCTGCCCGCAAGTTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGC  1072
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359   CTGTTCAGCCTGCCCGCAAGTTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGC  300

Query  1073  TGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGG  1132
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299   TGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGG  240

Query  1133  CTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGA  1192
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239   CTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGA  180

Query  1193  AGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCG  1252
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179   AGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCG  120

Query  1253  GGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCA  1312
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119   GGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCA  60

Query  1313  GCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGG  1369
              ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  59    GCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGANGGAGAAGCTACGG  3
```

pGl4.26_Pex16_Peak2_fw, pGl4.26_Pex16_Peak2_rv

Alignments

[3_Pex16_Peak2_fw_Pex16_Peak2_fw.fasta - 1146 bp] 3_Pex16_Peak2_fw_Pex16_Peak2_fw - Selection From [1] To [1146]
Sequence ID: lcl|7189 Length: 1146 Number of Matches: 1
Range 1: 5 to 356

Score	Expect	Identities	Gaps	Strand	Frame
643 bits(348)	0.0()	351/352(99%)	1/352(0%)	Plus/Plus	

Features:

```
Query  1362  AGCTACGGCTCCTGAG-CCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACG  1420
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  5     AGCTACGGCTCCTGAGCCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACG  64

Query  1421  GCCCAGTTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACC  1480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65    GCCCAGTTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACC  124

Query  1481  TTTGACCCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCC  1540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125   TTTGACCCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCC  184

Query  1541  AGGGTAGTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGA  1600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185   AGGGTAGTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGA  244

Query  1601  GAACCCAAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTA  1660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245   GAACCCAAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTA  304

Query  1661  GGTCGCTTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTG  1712
              ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305   GGTCGCTTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTG  356
```

Alignments

[4_Pex16_Peak2_rv_Pex16_Peak1_2_rv.fasta - 1158 bp] 4_Pex16_Peak2_rv_Pex16_Peak1_2_rv - Selection From [1] To [1158]
Sequence ID: lcl|6973 Length: 1158 Number of Matches: 1
Range 1: 1 to 355

Score	Expect	Identities	Gaps	Strand	Frame
647 bits(350)	0.0()	354/356(99%)	1/356(0%)	Plus/Minus	

Features:

```
Query  1319  CTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGC  1378
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355   CTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTCCTGAGC  296

Query  1379  CTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAGACGGCT  1438
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295   CTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAGACGGCT  236

Query  1439  GTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTTCTTGGG  1498
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235   GTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTTCTTGGG  176

Query  1499  GACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTCGCGGTG  1558
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  175   GACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTCGCGGTG  116

Query  1559  CTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCCGCAGGG  1618
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  115   CTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCCGCAGGG  56

Query  1619  TAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCTTCTCCGA  1674
              |||||||||||||||||||||||||||||||||||||||||||||||||  |||||
Sbjct  55    TAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCTN-TCCGA  1
```

pGl4.26_Pex16_Peak1+2_fw, pGl4.26_Pex16_Peak1+2_rv

Alignments

[5_Pex16_Peak12_f_Pex16_Peak1_2_fw.fasta - 1147 bp] 5_Pex16_Peak12_f_Pex16_Peak1_2_fw - Selection From [1] To [1147]
Sequence ID: lcl|6007 Length: 1147 Number of Matches: 1
Range 1: 4 to 649

Score	Expect	Identities	Gaps	Strand	Frame
1194 bits(646)	0.0()	646/646(100%)	0/646(0%)	Plus/Plus	

Features:

```
Query  1067  GGAGGCTGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGC  1126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4     GGAGGCTGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGC  63

Query  1127  CAATGGCTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATG  1186
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    CAATGGCTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATG  123

Query  1187  GGGTGAAGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAG  1246
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   GGGTGAAGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAG  183

Query  1247  CAACCGGGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCG  1306
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   CAACCGGGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCG  243

Query  1307  CTGCCAGCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTA  1366
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   CTGCCAGCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTA  303

Query  1367  CGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAG  1426
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   CGGCTCCTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAG  363

Query  1427  TTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGAC  1486
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   TTGGAGACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGAC  423

Query  1487  CCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTA  1546
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   CCATTTCTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTA  483

Query  1547  GTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCC  1606
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   GTGGTCGCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCC  543

Query  1607  AAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGC  1666
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   AAAGCCGCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGC  603

Query  1667  TTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTG  1712
              |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  604   TTCTCCGATTCACACGAGCTGTCTGAACTGGGTAAGTTGGACTCTG  649
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Alignments

[6_Pex16_Peak12_r_Pex16_Peak1_2_rv.fasta - 1158 bp] 6_Pex16_Peak12_r_Pex16_Peak1_2_rv - Selection From [1] To [1158]
Sequence ID: lcl|21799 Length: 1158 Number of Matches: 1
Range 1: 6 to 660

Score	Expect	Identities	Gaps	Strand	Frame
1210 bits(655)	0.0()	655/655(100%)	0/655(0%)	Plus/Minus	

Features:

```
Query  1013  CTGTTCAGCCTGCCCGCAAGTTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGC  1072
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   CTGTTCAGCCTGCCCGCAAGTTGTAACTACGCAGGCGCCAAGACAGCCAACCAAGGAGGC  601

Query  1073  TGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGG  1132
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600   TGCAGATCGGGTGCCAGAAGCTCTAGAGAGCTTCAGGGCGGAGAGAGAAGGAGCCAATGG  541

Query  1133  CTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGA  1192
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540   CTGGAAGGAGGGAGGGTGGGAGCTCCCGGTCTCGGGGAGGAGGCGCTAGAGATGGGGTGA  481

Query  1193  AGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCG  1252
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480   AGGGGCGTTCAGGGGCTCCGCGGGTCTAAGTCCTCCTGGGCTGCGGAAGTTGAGCAACCG  421

Query  1253  GGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCA  1312
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420   GGAGGCGGGCTTAGGGCCGGAAGCAGGAAGGAGGGCTCAGGACGCAGGGCGCCGCTGCCA  361

Query  1313  GCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTC  1372
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   GCCTTGCTGTCGGCCGGTGGGACTGTCAGTGGCCAGAGCAGGATGGAGAAGCTACGGCTC  301

Query  1373  CTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAG  1432
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300   CTGAGCCTCCGCTACCAGGAGTATGTGACTCGTCATCCAGCCGCCACGGCCCAGTTGGAG  241

Query  1433  ACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTT  1492
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240   ACGGCTGTGCGGGGCCTCAGTTACCTGCTGGCAGGTGCCACACTGACCTTTGACCCATTT  181

Query  1493  CTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTC  1552
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   CTTGGGGACTCTGACCCAGGAACCCTATGGCTCTTTGCCTGCTGACCCAGGGTAGTGGTC  121

Query  1553  GCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCC  1612
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   GCGGTGCTTCAGTTCTGTCTGGGTTCCTGTGGCGTAGAGGAAACTAGAGAACCCAAAGCC  61

Query  1613  GCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCT  1667
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60    GCAGGGTAAAACAGACGCTCACCAACGCCTTCTATACCTTCTTCCCTAGGTCGCT  6
```

pGl4.26_Pex16_Peak3_fw, pGl4.26_Pex16_Peak3_rv

Alignments

[7_Pex16_Peak3_fw_Pex16_Peak3_fw.fasta - 1199 bp] 7_Pex16_Peak3_fw_Pex16_Peak3_fw - Selection From [1] To [1199]
Sequence ID: lcl|8669 Length: 1199 Number of Matches: 1
Range 1: 1 to 461

Score	Expect	Identities	Gaps	Strand	Frame
843 bits(456)	0.0()	460/462(99%)	1/462(0%)	Plus/Plus	

Features:

```
Query  2145  TTGGCTGTTTGGAACATGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGCA  2204
             ||||||||||| || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TTGGCTGTTTGG-ACNTGACAGGCAGTTTTGAGCAGCTCAGTAACCAGTGCTGTCCAGCA  59

Query  2205  GAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCAT  2264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60    GAGCAAGGGCTGGCTTGTCACTCCAATCAGCTCATTTCTTACTGGCTCAGATGTCATCAT  119

Query  2265  GGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTGT  2324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120   GGAGAGAATGGGGCTCTGAGGTAGTGGGCGGAGAGGCTTGTTGGTTTCCACACAGTCTGT  179

Query  2325  GGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAGG  2384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180   GGGTAGCTGTGATCGGTAGGGCTCTGGGGTAAGGGTGAGAGGGGTGAAGGCCACAGCAGG  239

Query  2385  GCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTGC  2444
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240   GCCGGCCTCTCAAGACCACAATCTGCTTCTCAGTGTACTCTGCCTCGAACCTGCTGGTGC  299

Query  2445  TGCTCAATGACGGGATCCTGAGGAAGGAGCTTCGAAAAAAGTTGCCTGTGGTGAGAACTT  2504
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300   TGCTCAATGACGGGATCCTGAGGAAGGAGCTTCGAAAAAAGTTGCCTGTGGTGAGAACTT  359

Query  2505  TGCCAGGAGCTGGGGGTGGGAAGCTCAGGGACTTGGGAGGAAAACAGTAATGACCAGCAC  2564
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   TGCCAGGAGCTGGGGGTGGGAAGCTCAGGGACTTGGGAGGAAAACAGTAATGACCAGCAC  419

Query  2565  GTGTGGTACATGTCGTTGTCTCAGAGAGTGAGGCATGCCCAC  2606
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  420   GTGTGGTACATGTCGTTGTCTCAGAGAGTGAGGCATGCCCAC  461
```

Alignments

[8_Pex16_Peak3_rv_Pex16_Peak3_rv.fasta - 1130 bp] 8_Pex16_Peak3_rv_Pex16_Peak3_rv - Selection From [1] To [1130]
Sequence ID: lcl|181849 Length: 1130 Number of Matches: 1
Range 1: 8 to 460

Score	Expect	Identities	Gaps	Strand	Frame
833 bits(451)	0.0()	452/453(99%)	0/453(0%)	Plus/Minus	

Features:

```
Query  2113  GAGAGCAAAGATGAGTTGGTGGGACCACACATTTGGCTGTTTGGAACATGACAGGCAGTT  2172
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460   GAGAGCAAAGATGAGTTGGTGGGACCACACATTTGGCTGTTTGGAACATGACAGGCAGTT  401

Query  2173  TTGAGCAGCTCAGTAACCAGTGCTGTCCAGCAGAGCAAGGGCTGGCTTGTCACTCCAATC  2232
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400   TTGAGCAGCTCAGTAACCAGTGCTGTCCAGCAGAGCAAGGGCTGGCTTGTCACTCCAATC  341

Query  2233  AGCTCATTTCTTACTGGCTCAGATGTCATCATGGAGAGAATGGGGCTCTGAGGTAGTGGG  2292
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340   AGCTCATTTCTTACTGGCTCAGATGTCATCATGGAGAGAATGGGGCTCTGAGGTAGTGGG  281

Query  2293  CGGAGAGGCTTGTTGGTTTCCACACAGTCTGTGGGTAGCTGTGATCGGTAGGGCTCTGGG  2352
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280   CGGAGAGGCTTGTTGGTTTCCACACAGTCTGTGGGTAGCTGTGATCGGTAGGGCTCTGGG  221

Query  2353  GTAAGGGTGAGAGGGGTGAAGGCCACAGCAGGGCCGGCCTCTCAAGACCACAATCTGCTT  2412
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220   GTAAGGGTGAGAGGGGTGAAGGCCACAGCAGGGCCGGCCTCTCAAGACCACAATCTGCTT  161

Query  2413  CTCAGTGTACTCTGCCTCGAACCTGCTGGTGCTGCTCAATGACGGGATCCTGAGGAAGGA  2472
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160   CTCAGTGTACTCTGCCTCGAACCTGCTGGTGCTGCTCAATGACGGGATCCTGAGGAAGGA  101

Query  2473  GCTTCGAAAAAAGTTGCCTGTGGTGAGAACTTTGCCAGGAGCTGGGGGTGGGAAGCTCAG  2532
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100   GCTTCGAAAAAAGTTGCCTGTGGTGAGAACTTTGCCAGGAGCTGGGGGTGGGAAGCTCAG  41

Query  2533  GGACTTGGGAGGAAAACAGTAATGACCAGCACG  2565
             ||||||||||||||||||||||||||||||||
Sbjct  40    GGACTTNGGAGGAAAACAGTAATGACCAGCACG  8
```

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