Identification and characterization of novel signalling pathways involved in peroxisome proliferation in humans

Submitted by **Afsoon Sadeghi Azadi** to the University of Exeter
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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree
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Abstract

Peroxisomes represent crucial subcellular compartments for human life and health. They are remarkably dynamic organelles which respond to stimulation by adapting their structure, abundance, and metabolic functions according to cellular needs. Peroxisomes can form from pre-existing organelles by membrane growth and division, which results in peroxisome multiplication/proliferation. Growth and division in mammalian cells follows a well-defined multi-step process of morphological alterations including elongation/remodeling of the peroxisomal membrane (by PEX11β), constriction and recruitment of division factors (e.g. Fis1, MFF), and final membrane scission (by the dynamin-related GTPase Drp1) (Chapter 1).

Although our understanding of the mechanisms by which peroxisomes proliferate is increasing, our knowledge on how the division/multiplication process is linked to extracellular signals is limited, in particular in humans. The classical pathway involved in peroxisome proliferation is mediated by a family of ligand-activated transcription factors known as peroxisome proliferator activated receptors (PPARs) (Chapter 1).

This project focused on identifying novel signaling pathways and associated factors involved in peroxisome proliferation in humans. In this study, a cell-based peroxisome proliferation assay using the HepG2 cell model with spherical peroxisomal forms has been developed to investigate different stimuli and their ability to induce peroxisome proliferation (Chapters 2 and 3). In this system, peroxisome elongation has been used as the read-out for peroxisome

proliferation. We also showed that the number of peroxisomes increased after division of elongated peroxisomes indicating peroxisome proliferation.

Different stimuli, such as fatty acids, PPAR agonists and antagonists, have been used in this study. PPAR agonists and antagonists had no stimulatory or inhibitory effect on peroxisome elongation in our assay, suggesting PPAR-independent regulatory processes. However, arachidonic acid and linoleic acid were able to induce peroxisome elongation, whereas palmitic acid and oleic acid were not effective. These findings

indicate that general stimulation of fatty acid β-oxidation is not sufficient to induce peroxisome elongation/proliferation in HepG2 cells. Moreover, mRNA expression levels of peroxismal genes have been monitored during a time course in the HepG2 cell-based assay by qPCR. This analysis shows a regulation of expression of peroxins during peroxisome proliferation in human cells and suggests differences in the regulation pattern of PEX11α and PEX11β.

In Chapter 4, motif binding sites for transcription factors in peroxisomal genes were analyzed. An initial map of candidate regulatory motif sites across the human peroxisomal genes has been developed (Secondment at the University of Sevilla, Spain with Prof. D. Devos). This analysis also revealed the presence of different transcription factor binding sites in the promoter regions of PEX11α and PEX11β, supporting different regulatory mechanisms. Based on the computational analysis, PEX11β contained a putative SMAD2/3 binding site suggesting a novel link between the canonical TGFβ signaling pathway and expression of PEX11β, a key regulator of peroxisome dynamics and proliferation.

Addition of TGFβ to HepG2 cells cultured under serum-free conditions induced elongation/growth of peroxisomes as well as peroxisome proliferation supporting a role for TGFβ signalling in peroxisomal growth and division (Chapter 5). Furthermore, to demonstrate that this induction is through a direct effect of TFGβ on the SMAD binding site found in PEX11β, we performed functional studies using a dual luciferase reporter assay with PEX11β wild type and mutated promoter regions (Secondment at Amsterdam Medical Center, Netherlands with Prof. H. Waterham). Whereas luciferase activity was induced by TGFβ stimulation with the PEX11β wild type promoter, mutation of the SMAD binding site abolished activation. In summary, this study revealed a new signaling pathway involved in peroxisome proliferation in humans and provided a tool to monitor peroxisome morphology and gene expression upon treatment with defined stimuli.

Furthermore, I contributed to a study revealing that ER-peroxisome contacts are important for peroxisome elongation (Chapter 6). Our group identified peroxisomal acyl-CoA binding domain protein 5 (ACBD5), ACBD4 and VABP as a molecular linker between peroxisomes and the ER (Costello et al., 2017). Motif analysis of ACBD4 and ACBD5 promoter regions revealed that unlike PEX11β, these genes do not contain a binding site for SMAD, suggesting they are not co-regulated. Also, ACBD4 and ACBD5 do not share any common transcription factor binding sites suggesting different regulation. An interesting binding motif within the ACBD4 promoter is a glucocorticoid receptor binding site. In our study, we found potential glucocorticoid response elements (GRE) in other peroxisomal genes encoding β-oxidation enzymes. This may suggest an important role for glucocorticoid receptors in activating expression of peroxisomal genes resulting in the stimulation of fatty acid breakdown and energy production.

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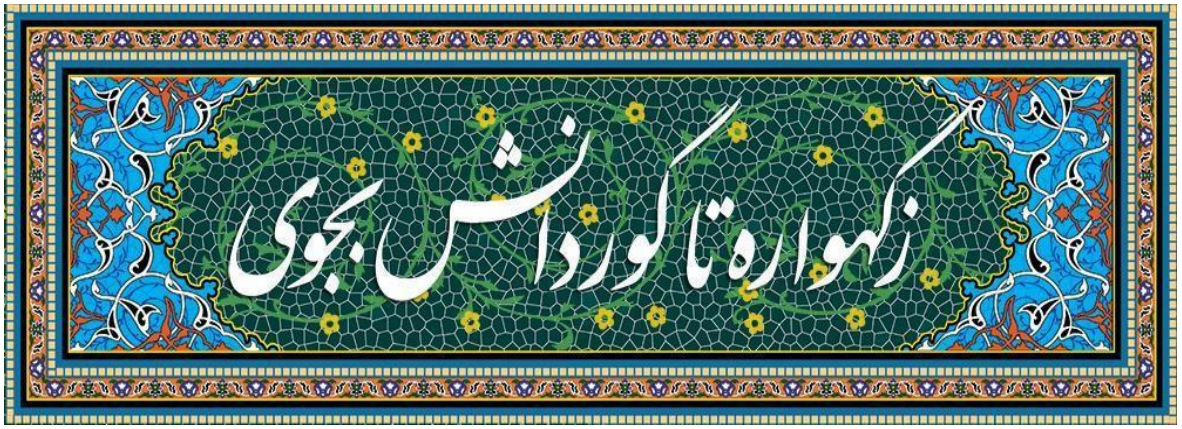

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Author's declaration

This work was performed as part of Marie Curie ITN- PERFUME; in collaboration with partners of the consortium.

Chapter 3

The q-PCR for peroxisomal gene expression in HepG2 cells was supported by Dr. Werner Kovacs, **ETH Zurich Switzerland.**

Chapter 4

The bioinformatics analyses were in part performed during my secondment in Prof. D. Devos laboratory at the **University of Seville (PERFUME member).** The program for searching motif binding sites in promoter regions of peroxisomal genes was generated with support of Ignacio Iberra (ESR4)**.**

Chapter 5

The dual luciferase assay was performed during my secondment at **Amsterdam Medical Center** (with Prof. Hans Waterham) **(PERFUME member).**

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Abbreviations

ACBD5 – acyl-CoA binding domain protein 5
Acyl-CoA – acetyl coenzyme A
ALDP – adrenoleukodystrophy protein
ATAD – ATPase family, AAA domain containing
AFT4 – Activating transcription factor 4
ATM – ataxia telangiectasia mutated protein
ATP – adenosine-5'-triphosphate
BP – Base pair
BSA – bovine serum albumin
DLP1 – dynamin-like protein 1
DLR – Dual luciferase assay
DMEM – Dulbecco's modified eagle medium
DMSO – dimethyl sulfoxide
ECL – enhanced chemiluminescence
EDTA – 2-Amino-2-(hydroxymethyl)-1,3-propanediol
E.coli – *Escherischia coli*
ER – endoplasmic reticulum
FBS – fetal bovine serum
FIS1 – mitochondrial fission 1 protein
GFP – green fluorescence protein
GR- Glucocorticoid receptor
GRE- Glucocorticoid response element
H_2O_2 – hydrogen peroxide
HepG2 – Human hepatoblastoma
HRP – horseradish peroxidase
HSC – heat shock protein
IMF – immunofluorescence
LCFA – long-chain fatty acids
MAPK – Mitogen-Activated Protein Kinase
MAVS – mitochondrial anti-viral signalling protein
MDV – mitochondria-derived vesicle

MFF – mitochondrial fission factor

MITO – mitochondria

mPTS – membrane peroxisome targeting signal

Mut – Mutant

NO• – nitric oxide

NCBI – National Centre for Biotechnology Information

Nfκβ – Nuclear Factor Kappa B Subunit

$O_2^{\bullet -}$ – oxygen superoxide

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffer saline

PCR – polymerase chain reaction

PEX – peroxin

PGC1-α – PPARγ coactivator-1α

PMP – peroxisomal membrane protein

PMV – Position weight matrices

PO – peroxisome

PPAR – peroxisome proliferator-activated receptor

PPRE – peroxisome proliferator response elements PRO – Promoter

PTS – peroxisomal targeting signal

ROS – reactive oxygen species

S. cerevisiae – *Saccharomyces cerevisiae*

SDS – sodium dodecyl sulphate

SMAD – Mothers Against DPP Homolog

TAE – tris-acetate-EDTA

TGFβ – Transforming Growth Factor Beta 1

TNF – Tumor Necrosis Factor

Tris – 2-Amino-2-(hydroxymethyl)-1,3-propanediol

TSC – tuberous sclerosis complex

TSS – Transcription start site

UV - ultraviolet

VAPB – VAMP associated protein B

VLCFA – very-long-chain fatty acids

WT – wild type

Chapter 1 – Introduction

1.1 Peroxisomes

Peroxisomes were first described as spherical microbodies found in mouse kidney by Rhodin in 1954 and later characterized and renamed by Christian de Duve and his group, who identified several enzymes responsible for hydrogen peroxide metabolism (De Duve & Baudhuin, 1966)) Peroxisomes are single membrane-bound organelles lacking DNA and protein synthesis machinery. For this reason, peroxisomal proteins are encoded by nuclear genes and most of them are synthesized on free polyribosomes present in the cytoplasm. Peroxisomes usually have a spherical or rod-like morphology (0.1 to 0.5 μm in diameter), but elongated tubular organelles can be observed (up to 5 μm) (**Figure 1.1**) (Baudhuin *et al.*, 1964; De Duve & Baudhuin, 1966).

Peroxisomes are essential for the breakdown of diverse fatty acids by β-oxidation. Very long chain fatty acids (VLCFA) for example can only be degraded in peroxisomes and not in mitochondria (I. Singh *et al.*, 1984). Defects in genes coding for peroxisomal proteins lead to several peroxisomal disorders with varying degrees of severity.

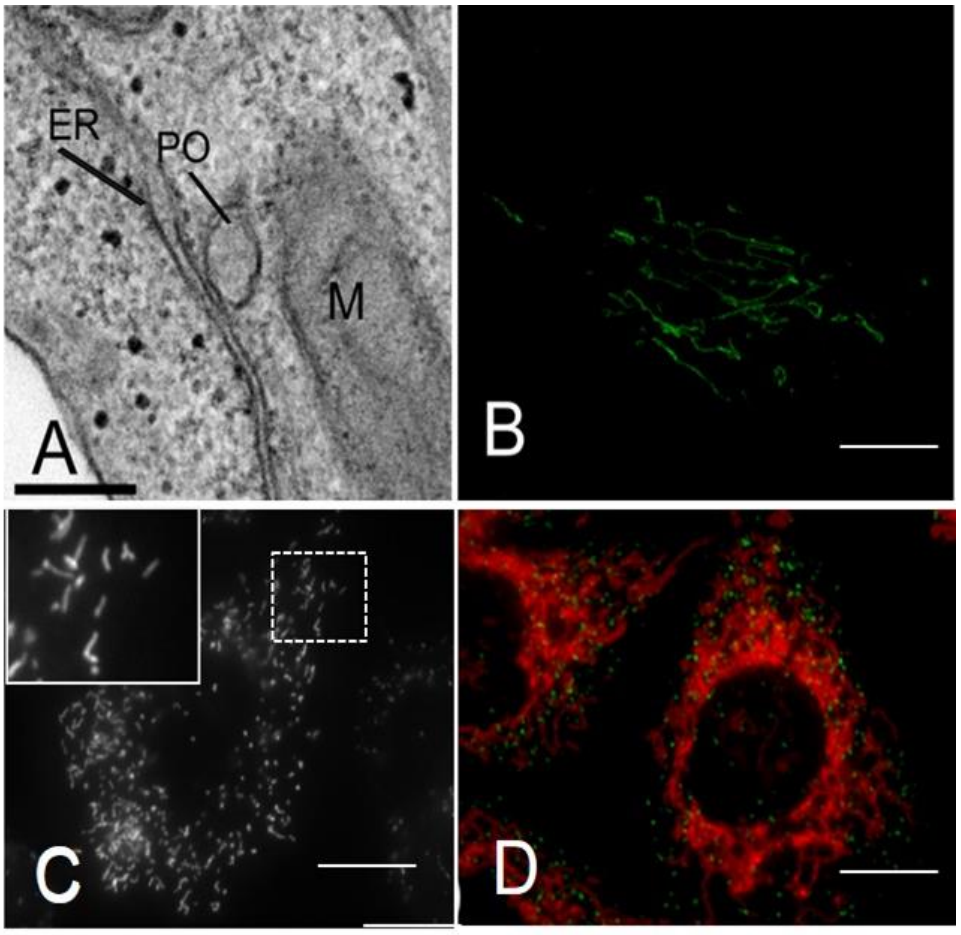

Figure 1.1. Microscopic views of peroxisomes. (A) Electron micrographs of peroxisomes from HepG2 cells. Magnification: x 85,000. (Costello *et al.,* 2017) (B) Highly elongated, tubular peroxisomes in Mff-deficient fibroblasts where peroxisomes are able to elongate but cannot divide. (C) Regular tubular peroxisome morphology in HepG2 cells in normal culture media with serum. (D) HepG2 cells stained with PEX14 (peroxisomal marker, green) and Tom20 (mitochondria, red). Bars= 10 µm (ER = endoplasmic reticulum, PO = peroxisomes, M = mitochondria)

1.2 Peroxisomal functions

Peroxisomes harbour many metabolic functions that vary according to species, cell type, developmental stage and environmental conditions (Circu & Aw, 2010). Peroxisomes in human cells are involved in lipid metabolism pathways which include the synthesis of cholesterol, bile acids and ether lipids such as plasmalogens (Wanders *et al.*, 1987). The synthesis of the latter, which contributes to 80% of the phospholipid content of the white matter in brain and ~ 20% of the total phospholipid mass in humans, is initiated in the peroxisomes and completed in the ER (Biardi & Krisans, 1996; Krisans, 1992). Oxidation of the sterol side chain results in formation of the C_{27}-bile acid intermediates 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA). The C_{24}-bile acids are formed from the C_{27}-bile acid intermediates by peroxisomal β-oxidation of the side chain (Schepers *et al.*, 1988; H. Singh *et al.*, 1994). Peroxisomes also process alpha-oxidation of 3- methyl-branched fatty acids, with phytanic acid (3,7,11,15- tetramethylhexadecanoic acid) as the best known example. Alpha-oxidation is a process in which fatty acids are shortened by one carbon atom (Mannaerts *et al.*, 2000).

Other peroxisomal functions include the oxidation of alcohols, catabolism of purines and polyamines, metabolism of prostaglandins, (Wanders *et al.*, 1987), photorespiration in plants and penicillin synthesis in fungi (Jedlitschky *et al.*, 1991; Tolbert & Essner, 1981). In most organisms, peroxisomes play an essential role in lipid and reactive oxygen species (ROS) metabolism (Ivashchenko *et al.*, 2011). Peroxisomes produce several types of reactive oxygen and nitrogen

species (ROS/RNS) (e.g. H_2O_2, $O_2^{\bullet -}$ and NO•) (Fransen(Fransen *et al.*, 2012), Nordgren (M. Nordgren & Fransen, 2014). For example, H_2O_2 is produced during beta-oxidation through the action of ACOX1, the first enzyme of the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. It donates electrons directly to molecular oxygen, thereby producing hydrogen peroxide (Circu & Aw, 2010).

ROS production can be detrimental for the cells, but ROS also act as intracellular signalling molecules regulating kinase-driven networks leading to cell survival or apoptosis (Circu & Aw, 2010) by acting as messengers in cell signalling and homeostasis.The mechanism of redox signaling involves H_2O_2-mediated oxidation of cysteine residues within proteins (Rhee *et al.*, 2005). Cysteine residues exist as a thiolate anion ($Cys\text{-}S^-$) at physiological pH and are more susceptible to oxidation compared to the protonated cysteine thiol (Cys-SH) (Rhee *et al.*, 2005).

It has also been shown that generating ROS inside peroxisomes disturbs the mitochondrial redox balance, which may lead to mitochondrial fragmentation (Ivashchenko *et al.*, 2011). In order to maintain the cellular redox state, the production of ROS/RNS is balanced by antioxidant enzymes, such as catalase, peroxiredoxin 5 and superoxide dismutase 1 (Fransen *et al.*, 2012). Recently, peroxisomes have been identified as essential signalling platforms, playing key roles in antiviral signalling and ROS-dependent regulation of mTORC1 signalling (Mast, (Mast *et al.*, 2015; Zhang *et al.*, 2013). In one study, Dixit and colleagues showed the dual targeting of the mitochondrial antiviral signalling (MAVS) protein to both peroxisomes and mitochondria, and its ability to induce different signalling cascades at each organelle in response to viral RNA recognition by RIG-I-like receptors (Dixit *et al.*, 2010; Odendall & Kagan, 2013).

In another recent study, the tuberous sclerosis complex (TSC) was reportedly found at the peroxisomal membrane, functioned as a Rheb GTPase-activating protein (GAP), where it upregulates autophagy (and pexophagy) by repressing downstream mTORC1 signalling in response to increases in ROS (Zhang *et al.*, 2013). This ROS-dependent response is still not well understood, and further studies will be needed to better investigate the targeting of these proteins to peroxisomes and their responses to cellular and peroxisomal ROS.

1.3 Peroxisomal diseases and disorders

The peroxisomal disorders include a group of genetic diseases in which there is impairment in one or more peroxisomal functions. The peroxisomal disorders are subdivided into two subgroups comprising: (1) the peroxisome biogenesis disorders (PBDs) including four different autosomal recessive disorders: Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP) type;

(2) The single peroxisomal (enzyme/transporters) protein deficiencies (Hashimoto *et al.*, 1986; Waterham & Wanders, 2012).

PBDs are due to mutation in the PEX genes encoding for peroxin proteins required for peroxisome biogenesis. ZS is an inherited peroxisomal disorder characterised by the absence of functional peroxisomes; hence, peroxisomal substrates (such as VLCFA, THCA and DHCA) accumulate while there is a shortage of essential peroxisomal products (such as plasmalogens which are essential for the formation of the myelin sheath) in every tissue. The consequences are especially destructive in the brain and spinal cord and patients born with Zellweger syndrome will die prematurely (Hashimoto *et al.*, 1986; Wanders *et al.*, 2017).

Mutations in PEX genes can result in the complete absence of functional peroxisomes or the ormation of empty peroxisomal membranes, so called “ghostes”, which are often enlarged and decreased in number. Ebberink *et al*. described the first patient with defective PEX11β resulting in a defect of peroxisome division (Ebberink *et al.*, 2012). The patient showed mild intellectual disability, congenital cataracts, progressive hearing loss and polyneuropathy. These clinical features are also observed in patients with a mild peroxisome biogenesis disorder (Ebberink *et al.*, 2012).

Single-enzyme deficiencies include disorders of peroxisomal beta-oxidation, and X-linked adrenoleukodystrophy (X-ALD). X-ALD is caused by mutations in the ATP binding cassette transporter (ABCD1) gene involved in the uptake of VLCFA in peroxisomes (Biardi & Krisans, 1996). Clinical presentations are diverse, from the cerebral childhood form with difficulty in understanding speech and writing, lethal seizures to symptoms like schizophrenia or other psychiatric disorders in adult cerebral ALD (Waterham & Wanders, 2012). It has been shown that peroxisomes have a role against the development of pulmonary fibrosis (Oruqaj *et al.*, 2015). In idiopathic pulmonary fibrosis, lung injury leads to the production of proinflammatory mediators such as TNF-α, IL-6, and ROS results in the activation of profibrogenic. This damage leads to down-regulation of peroxisomes specifically PEX13, which generates more ROS, thus enabling the persistence of fibrotic phenotype (Oruqaj *et al.*, 2015). The existence of such serious peroxisomal diseases highlights the essential need for correctly functioning peroxisomes in humans. In many cases peroxisome numbers are reduced and stimulation of peroxisome proliferation may help to treat milder cases.

1.4 Biogenesis of peroxisomes

Proteins specifically engaged in peroxisomal biogenesis are referred to as peroxins (encoded by PEX genes). More than 36 PEX genes have been identified in evolutionarily diverse organisms, however, not all organisms possess the full range of PEX genes (32 in yeast, 16 mammalian and 23 plant homologs) (Agrawal *et al.*, 2011; Agrawal & Subramani, 2016; Mano & Nishimura, 2005; Yuan *et al.*, 2016) (**Figure 1.2**). Peroxisome biogenesis is a multistep process and can be considered in three key stages: **1) matrix protein import**, **2) membrane biogenesis**, **3) proliferation of peroxisomes** (Schrader & Fahimi, 2008).

1.4.1 Matrix protein import

Peroxisomal matrix proteins are synthesised in the cytosol and transported to the organelle by the shuttling receptors PEX5 and PEX7 (Braverman *et al.*, 1998; Dodt & Gould, 1996); fully folded, co-factor bound or oligomeric proteins can be transported across the peroxisomal membrane. Matrix proteins have specific peroxisome targeted signal sequences (PTS1 and PTS2), which are recognized by the shuttling receptors PEX5 and PEX7, respectively (Eckert & Erdmann, 2003; Platta & Erdmann, 2007). PEX7 needs to interact with PEX5 for targeting to the peroxisomal membrane. PEX5 and PEX7 receptors form cytosolic complexes with their ligands and efficiently ferry them to the peroxisome membrane. The receptor/cargo complex binds at the peroxisome membrane to the PEX13 and PEX14 docking complex (Schueller *et al.*, 2010). Following cargo release, these receptors are recycled back to the cytosol by an ubiquitin-dependent pathway, which involves PEX2, PEX10 and PEX12, and the AAA ATPases PEX1 and PEX6, in association with PEX26 for receptor export (**Figure 1.2**) (reviewed in (Francisco *et al.*, 2014; Platta & Erdmann, 2007).

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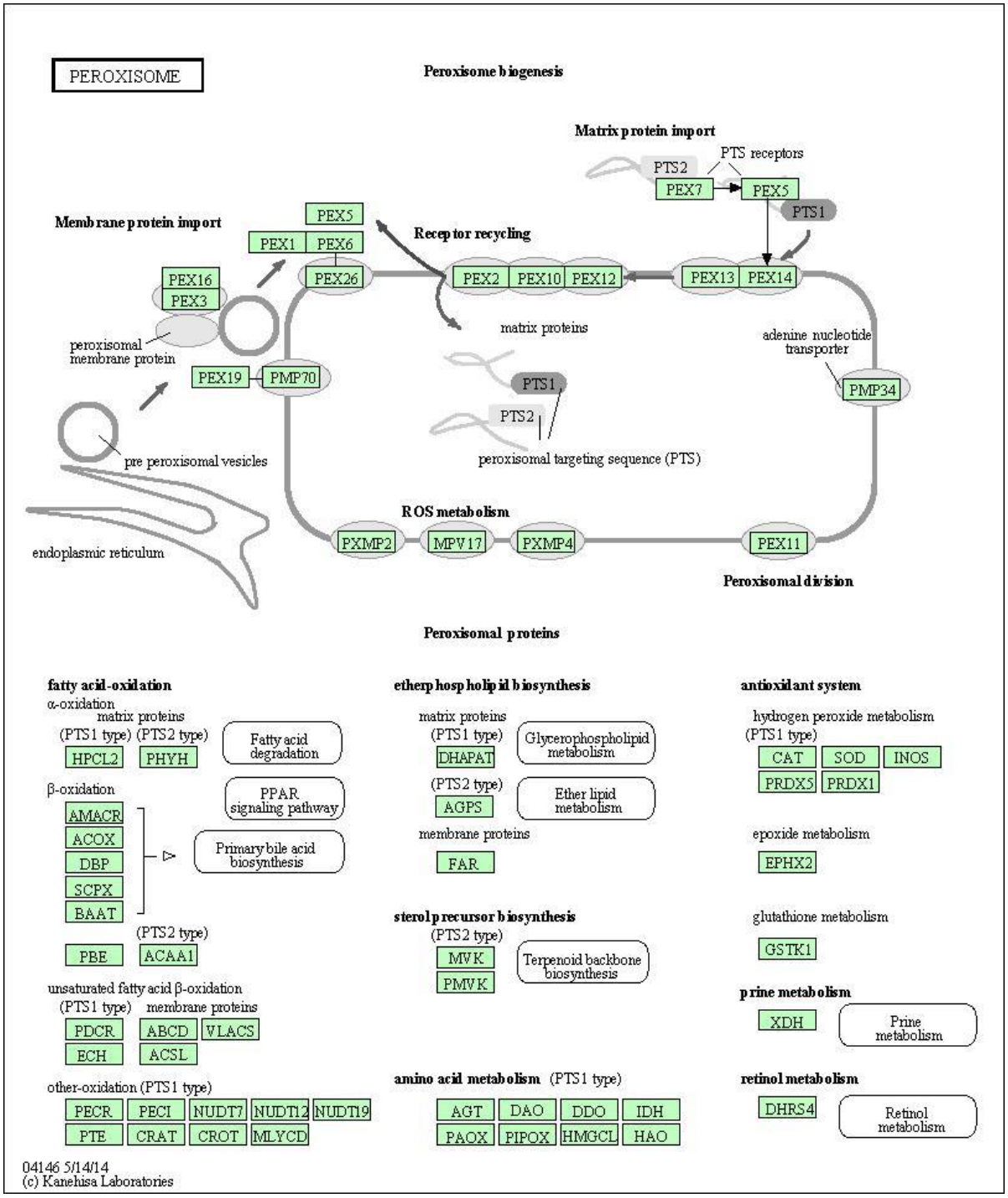


Figure 1.2. Overview of peroxisome genes involved in peroxisomes biogenesis, metabolism and protein import machinery in humans. Proteins specifically engaged in peroxisomal biogenesis are referred to as peroxins. PMPs are recognized by the cytosolic chaperone PEX19, which interacts with the PEX3-PEX16 membrane complex**.** Matrix proteins are being sorted by cytosolic receptors PEX5 and PEX7.Adopted from KEGG Pathway.

1.4.2 Membrane protein import

The import of peroxisomal membrane proteins (PMPs) occurs independently of matrix import via a different import machinery. Most peroxisome membrane proteins (PMPs) are synthesised by free polyribosomes in the cytosol and contain one or more membrane peroxisome targeting signals (mPTS) (Halbach *et al.*, 2006; Jones *et al.*, 2001) . PMPs are recognised and targeted to peroxisomes by the cytosolic chaperone PEX19, which maintains them in a stable and import competent conformation, and keeps them from aggregating (Fang *et al.*, 2004; Shibata *et al.*, 2004). Most PMPs are recognized by PEX19, which then interacts with the PEX3-PEX16 membrane complex (Schmidt *et al.*, 2012). These three peroxins are essential for peroxisome membrane biogenesis in mammals, and loss of either one of them results in the complete loss of peroxisomes (**Figure 1.2**).

The PMP-PEX19 complex docks at the peroxisomal membrane by interacting with PEX3 to insert newly formed proteins (Fang *et al.*, 2004; Fujiki *et al.*, 2006; Giannopoulou *et al.*, 2016). It has been shown in mammalian cells that PEX3 and PEX16 target peroxisomes either directly (Jones *et al.*, 2004; Matsuzaki & Fujiki, 2008), or indirectly via the ER (Kim *et al.*, 2006; Toro *et al.*, 2009). PEX16 promotes peroxisomal growth by enabling the PEX3-dependent integration of peroxisomal membrane proteins (**Figure 1.2**).

1.4.3 Growth and division vs de novo formation

The early steps of peroxisome biogenesis are still controversially discussed (Agrawal & Subramani, 2016; Hettema *et al.*, 2014) confronting a classical view of peroxisome generation by growth and division and the more recent *de novo* formation from the ER. Direct import of matrix and membrane proteins into the peroxisomes led to the classical view that peroxisomes are autonomous organelles and the proposal of the "growth and division" model (Fujiki *et al.*, 2014; Schrader *et al.*, 2016). In this multistep pathway, lipid transfer from the ER leads to peroxisome elongation, constriction and fission (Fujiki *et al.*, 2014). In contrast, the *de novo* synthesis model states that several peroxins, such as PEX3 and PEX16, are inserted in the ER, and segregate to specific ER exit sites and generate pre- peroxisomal vesicles (Agrawal & Subramani, 2016; Dimitrov *et al.*, 2013) that may mature towards complete and functional peroxisomes. These vesicles have been suggested to i) mature into functional peroxisomes, ii) fuse into pre-existing peroxisomes, and iii) fuse with other pre-peroxisomal vesicles to form mature peroxisomes (Agrawal *et al.*, 2011; Kim, 2006; van der Zand *et al.*, 2012).

In summary, a combined model where the contributions of both pathways depend on the cellular state and organism, is the most current view of peroxisome biogenesis. At the moment, growth and division is the major pathway but formation from other organelles is formally possible and may occur under exceptional conditions (**Figure 1.3**) (Agrawal & Subramani, 2016; Hettema *et al.*, 2014; Sugiura *et al.*, 2017).

1.5 Peroxisome proliferation

In mammalian cells, peroxisome proliferation can be regulated by several nutritional and environmental changes, such as increases in free fatty acids and growth factors, hypoxia and cold exposure (Bagattin *et al.*, 2010; Laurenti *et al.*, 2011; Schrader *et al.*, 1998b). These stimuli induce an increase in the number/size of peroxisomes, and alterations in the expression of several peroxisomal proteins, such as ACOX1 or PBFE, in order to increase their metabolic activity (Bagattin *et al.*, 2010; Diano *et al.*, 2011; Gurvitz & Rottensteiner, 2006). The increase in number/size of peroxisomes could also facilitate interactions with other organelles by increasing the available surface area and facilitate metabolism.

The known pathway involved in peroxisome proliferation is mediated by a family of ligand-activated transcription factors known as peroxisome proliferator activated receptors (PPARs) (Rakhshandehroo *et al.*, 2010; Schrader *et al.*, 2012). These transcription factors are typically activated by lipid-ligands and regulate the expression of genes associated with lipid metabolism and adipocyte differentiation (Kliewer *et al.*, 1992; Reddy & Hashimoto, 2001; Varanasi *et al.*, 1996). Additional pathways independent of PPARs have also been described and it is likely that yet unknown mechanisms contribute to the regulation of peroxisome proliferation (Gondcaille *et al.*, 2005; Li & Gould, 2002; Sexton *et al.*, 2010).

1.6 The role of PEX11 in peroxisome proliferation in mammals

In mammalian cells, peroxisome formation by growth and division follows defined steps of morphological alterations: initial membrane elongation of the compartment is carried out by the key peroxisomal membrane protein PEX11pβ, combined with the tail-anchored proteins Mff (mitochondrial fission factor) and Fis1 (fission protein 1) as well as the GTPase DRP1 (dynamin-related protein 1) which mediates final fission into spherical organelles (A. Koch *et al.*, 2005; Motley *et al.*, 2008). DRP1 is recruited to peroxisomes by the tail-anchored (TA) proteins FIS1 and MFF, which facilitate oligomerisation of DLP1 (A. Koch *et al.*, 2005; Lazarow & Fujiki, 1985; Otera *et al.*, 2010).

The three mammalian PEX11 isoforms, PEX11α, PEX11β and PEX11γ, are able to interact with each other and tend to form either homo-oligomers or homo-dimers (**Figure 1.3**) (J. Koch *et al.*, 2010; Tanaka *et al.*, 2003).

PEX11α is mostly expressed in liver, kidney, heart, and testis (Abe & Fujiki, 1998; Li & Gould, 2002). Studies showed that a PEX11α knockout mouse is morphologically indistinguishable from the wild-type mouse, with no obvious effect on peroxisome number or metabolism (Li & Gould, 2002). Moreover, the induction of peroxisome proliferation through PPARα by ciprofibrate does not require PEX11α (Li & Gould, 2002).

However, PEX11β knockout mice showed neonatal defects, neuronal apoptosis and migration defects similar to Zellweger syndrome phenotypes (Li & Gould, 2002). This isoform is expressed in most of the tissues and it is not inducible by

proliferators. Human PEX11β overexpression leads to peroxisome proliferation by tubular peroxisome formation, followed by an increase in peroxisome number through division. PEX11α overexpression has no effect on peroxisome proliferation (Schrader *et al.*, 1998b).

The third isoform, PEX11γ, is constitutively expressed in liver (Kobayashi *et al.*, 2007) and might have a redundant function with PEX11β, although it is with 22% amino acid identity less similar to PEX11β than PEX11α is to PEX11β (40% amino acid identity) (Tanaka *et al.*, 2003).

Studies with synthetic peptide corresponding to the N-terminal amphipathic helix of Pex11β showed that, this part induces the formation of tubule-like structures from artificial membranes in vitro (Opaliński et al., 2011). This membrane-deforming activity has been shown to be dependent on the amphipathic properties of the Pex11pβ α-helical structure by introducing point mutations into its sequence (Yumi Yoshida *et al.*, 2015). In summary, PEX11β is required for constitutive peroxisome biogenesis. PEX11β localizes to the peroxisomal membrane and upon activation assembles in patches at specific sites (Kliewer *et al.*, 1992). The mitochondrial TA protein GDAP1 has also been shown to dually target peroxisomes and mitochondria, and to regulate organelle division (Huber *et al.*, 2013). PEX11β interacts with FIS1 and MFF on the membrane, and activates DRP1 by its GTPase activating protein (GAP) activity (Williams *et al.*, 2015). A summary of peroxisome proliferation is shown in Figure 1.3 (Schrader *et al.*, 2016).

A recent study identified a patient with a mutation in PEX11β. Peroxisomes in cells derived from this patient appear enlarged and undivided, supporting a role for PEX11β in peroxisome proliferation and division (Ebberink *et al.*, 2012).

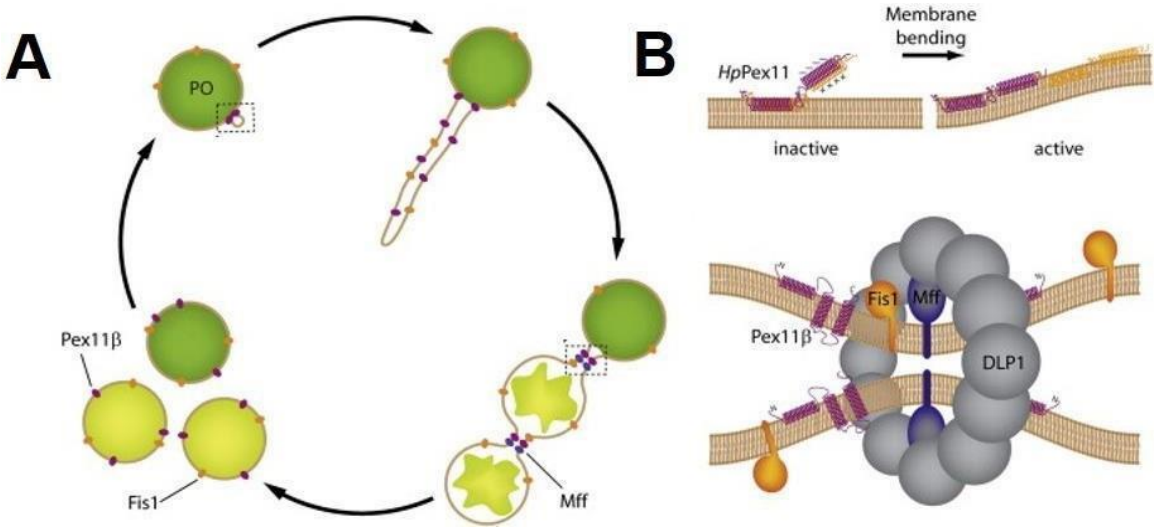


Figure 1.3. Model for peroxisome proliferation. (A) Peroxisomes multiply by elongation (growth), constriction, and final fission (division) contributes to peroxisome proliferation in mammalian cells. (B) PEX11β is involved in the elongation/tubulation of peroxisomes (Schrader *et al.*, 1998a). PEX11β localizes to the peroxisomal membrane and upon activation assembles in patches at specific sites (A. Koch *et al.*, 2005). PEX11 proteins interact with the adaptor proteins Mff and Fis1, which recruit the fission GTPase DRP1 forming a ring-like structure (DLP1) around the organelle (Thoms & Erdmann, 2005). Adapted from Schrader (Schrader *et al.*, 2012).

1.7 Regulation of peroxisome proliferation in different organisms

Peroxisome proliferation is supposed to be controlled by different types of signalling cascades that trigger the transcription of proliferation related genes. These cascades usually start with external stimuli such as fatty acids or fibrates and lead to an increase in peroxisome number (Gurvitz & Rottensteiner, 2006; Issemann & Green, 1990). Due to their critical metabolic functions, regulation of peroxisomal functions and signalling is vital for human health. This has been studied extensively in yeast but knowledge of the pathways in humans is scarce. Therefore, a better understanding of the signals involved in peroxisome proliferation and metabolic activity is necessary, not only to gain a deeper insight

into peroxisome biology but also as a potential starting point for generating drugs which could be used to treat peroxisome related diseases.

In yeast, peroxisomes are crucial for the primary metabolism of several unusual carbon sources and various secondary metabolites such as penicillins, polyketides and terpenes. Pip2 and Oaf1p transcription factors form heterodimers and activate the transcription of peroxisomal genes containing oleate response elements (ORE (Gurvitz & Rottensteiner, 2006). Under glucose depression and oleate induction conditions, Adr1p binds to upstream activating sequence 1 (UAS1) promoter sites in the proximity of OREs. Adr1p binding enhances the affinity of the Pip2/Oaf1p complex to these promoters (**Figure 1.4**) (Rottensteiner (Rottensteiner *et al.*, 1996).

Plant peroxisomes are involved in numerous processes, including primary and secondary metabolism, development, and responses to abiotic and biotic stresses. Like in mammals, PEX11 and DRP1 have a role in peroxisomal elongation and division. The proliferation can be induced with ROS, UV radiation, light, salt stress and clofibrate (Babu *et al.*, 2003; H. Hu *et al.*, 2008; Oikawa *et al.*, 2015). Light induces peroxisome proliferation and up-regulation of the PEX11 gene. Activation of PEX11 requires the far-red light receptor phyA, as well as the bZIP transcription factor HYH, which binds directly to the promoter of PEX11 (J. Hu & Desai, 2008). It has been shown that during photomorphogenesis, both the import of leaf-peroxisome enzymes from the cytosol and the induction of peroxisome proliferation take place to prepare seedlings for photosynthesis and photorespiration (J. Hu & Desai, 2008; Issemann & Green, 1990; Lopez-Huertas *et al.*, 2000).

In mammals, upon binding of ligand, PPARs hetero-dimerise with its binding partner, the 9-cis-retinoic acid receptor, RXRα, and bind to specific cis-acting DNA response elements known as peroxisome proliferator response elements (PPRE). PPREs consist of tandem repeats of the consensus hexameric motif TGACC (T/C) separated by one base pair (DR1). PPREs all have a third half-site with high homology to the TGACCT consensus half-site either two nucleotides 5' or three nucleotides 3' to the PPRE (Issemann & Green, 1990).

The sequence specificity of each PPRE is not strict, as the sequence of the PPRE can deviate from the consensus sequence by as many as 5 nucleotides (Issemann *et al.*, 1993). Despite a study reporting a PPRE shared within the PEX11α and perilipin gene, there is no information so far on transcriptional regulation of PEX genes in humans/mammals (Shimizu *et al.*, 2004).

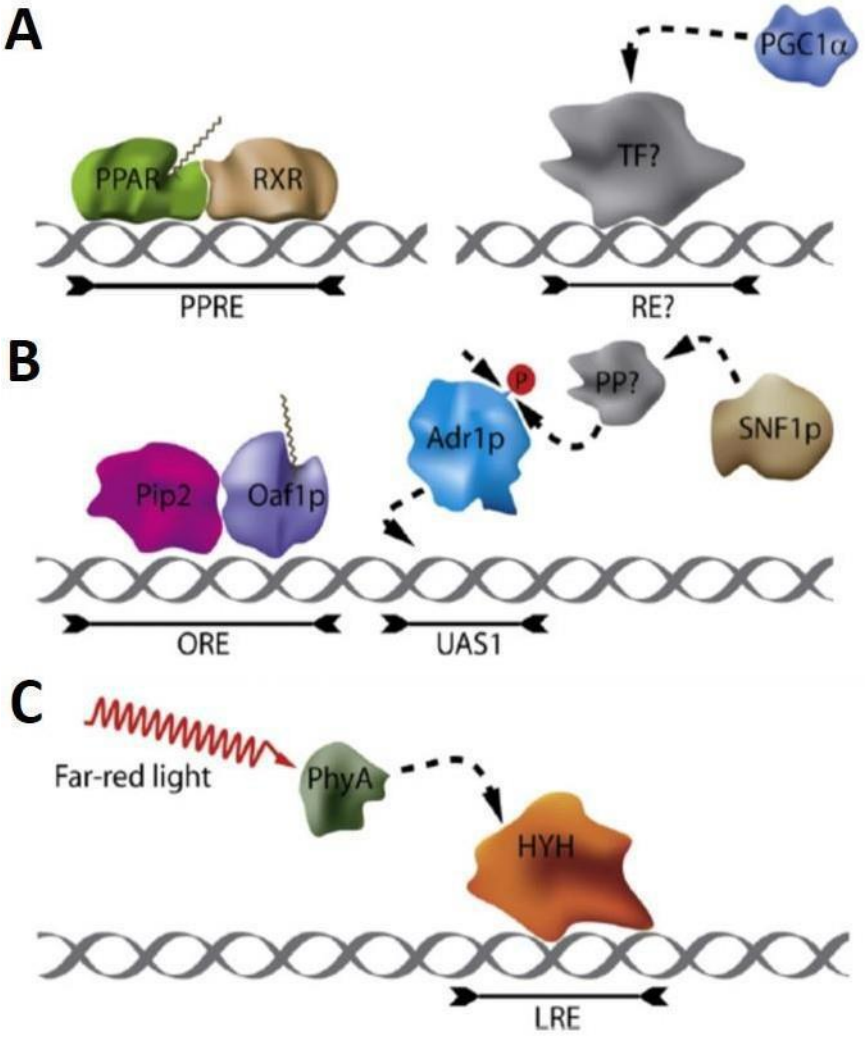


Figure 1.4: Transcriptional regulation of peroxisome proliferation in different organisms (A) In mammals, PPAR and its binding partner RXR form a heterodimer and interact with PPAR response elements which lead to transcription of PPAR target genes. (B) In *S. cerevisae* Oaf1p and Pip2- form a heterodimer, bind to ORE and induce the transcription of peroxisomal genes. (C) In plants, far-red light causes binding of HYH transcription factor to light response elements upon Phytochrom A activation. Figure from (Schrader *et al.*, 2012).

1.7.1 PPAR mechanism of action

In mammals, the major known pathway involved in PO proliferation is the peroxisome proliferator-activated receptor (PPAR) dependent pathway (Kliewer *et al.*, 1992). PPAR represents a family of nuclear receptors composed of three members: PPARα, PPARβ and PPARγ. PPARs respond to a broad class of structurally diverse ligands, including xenobiotic chemicals called peroxisome proliferators and both natural and synthetic fatty acids (**Figure 1.5**) (Rakhshandehroo *et al.*, 2010).

1.7.2 Role of PPAR and differences between activation in rodents and humans

Peroxisome proliferation in mammals in response to specific stimuli was first described in rats treated with the fibrate ethyl-chlorophenoxy-isobutyrate (Hess *et al.*, 1965). Further, PPARα is activated by binding fatty acid ligands, especially polyunsaturated fatty acids with 18-22 carbon atoms and 2-6 double bounds and forms heterodimers with its binding partner, retinoid X receptor alpha (RXRα) (Berger & Moller, 2002; Green, 1995). Endogenous ligands for PPARα include linoleic acid, arachidonic acid and leukotriene B4 (Berger & Moller, 2002; Schrader & Fahimi, 2006). Binding of these fatty acids and synthetic ligands causes a conformational change in PPARα leading to the recruitment of its co-activators (CBP-SRC-HAT complex, ASC complex, and TRAP-Mediator complex) which initiate the transcription of the target genes. So far it has been shown that target genes of PPARα participate in fatty acid

transport, fatty acid oxidation, triglyceride clearance, lipoprotein production, and cholesterol homeostasis (Kersten *et al.*, 2000).

A comparison of response to PPAR agonists in rat and human cells showed that rat hepatocytes responded to PPARα agonist treatment by inducing the mRNA for several peroxisome proliferation-related genes including FACO, HD, THIO, CYP4A, and PMP-70 and had increased fatty acyl-CoA oxidase (Lawrence *et al.*, 2001b). However, there was no change in expression of these genes in human hepatoblastoma (HepG2) cells treated with this agonist (Lawrence *et al.*, 2001a).

Mukherjee *et al*. have observed that WY14,643 (PPARα agonist) was more potent at activating the rat PPARα than the human PPARα (Mukherjee *et al.*, 1997). In addition, they observed that ETYA was more potent on the human PPARα than on the rat PPARα. There are two amino acids that differ in the ligand-binding domain of PPARα between rat and human. These differences in amino acid sequence may explain the differences in the potency of the various ligands for each of the species receptors (Rakhshandehroo *et al.*, 2010).

In humans, PPARα plays a role in the regulation of lipid and glucose homeostasis and inflammatory responses (Rakhshandehroo *et al.*, 2010). ChIP-seq analysis in human hepatoblastoma (HepG2) cells revealed peroxisome proliferator response elements (PPREs) in peroxisomal genes such as ACOX1, ACADs (acyl-CoA dehydrogenases) and G0S2 (G0/G1 Switch Regulatory Protein 2) (van der Meer *et al.*, 2010), suggesting they are regulated by PPARα. Furthermore, studies with the synthetic PPAR activator GW7647, demonstrate that ACAD and ACOX1 are up-regulated upon treatment with this PPARα agonist (P. J. Brown *et al.*, 2001; McMullen *et al.*, 2014). Among other slightly up-regulated genes in primary human hepatocytes treated with GW7647, are metabolism-related genes

such as PLIN1 (Perilipin 1), PDK4 (Pyruvate Dehydrogenase Kinase, Isozyme 4), as well as PEX11α, highlighting the role of this pathway in regulating expression of genes involved in lipid metabolism (McMullen *et al.*, 2014). However, none of these studies found an upregulation of genes involved in peroxisome dynamics/proliferation. The few peroxisomal related genes found to be upregulated by PPARa agonists (ACOX1, ACAD, PEX11a) are related to lipid metabolism and their upregulation does not induce peroxisome proliferation in humans (McMullen *et al.*, 2014).

The mechanism by which PPARα signalling could stimulate an increase in peroxisome numbers in humans remains unclearl. It has been established that one of the major factors involved in proliferation is PEX11β (introduction, 1.1.2) (Li & Gould, 2002) and currently there is no evidence for up-regulation of PEX11β following stimulation with PPARα agonists.

This suggests involvement of PPAR independent pathways in the regulation of peroxisome proliferation.

1.7.3 PPAR independent pathways

Besides the PPAR dependent pathway, a PPAR independent pathway has recently been discovered involving the peroxisome proliferator activated receptor γ coactivator-1α (PGC1-α) in humans and mice. PGC1-α can activate gene expression by binding to other transcription factors such as NRF2 (Kemper *et al.*, 2014; Scarpulla, 2008).

External signals such as growth factors (GF) can induce peroxisome proliferation, suggesting involvement of GF dependent cascades such as mTOR in peroxisome proliferation (Schrader *et al.*, 1996). The mTOR protein forms two complexes, mTORC1 and mTORC2, each of which contains distinct components and signals through a different set of downstream effectors. mTORC1 is also induced by growth factors. TSC inhibits the activity of the small GTPase Rheb to repress mammalian target of rapamycin complex 1 (mTORC1) signalling, a negative regulator of autophagy. Recently, the mTOR pathway has been linked to peroxisomes and the localization of TSC1, TSC2 and Rheb (major components of mTOR signalling pathway) to peroxisomes has been reported. In this study, it has been shown that TSC1 and TSC2 bind PEX19 and PEX5, respectively, and that these mTORC1 regulators localize to the cytoplasmic surface of the peroxisomal membrane (Zhang *et al.*, 2013). So far, it has been shown that peroxisome proliferation in mammals depends on PPARα, but there is more to investigate on other extracellular and intracellular signalling cascades involved in peroxisome proliferation which can be activated by extracellular signals such as ROS and growth factors (**Figure 1.5**).

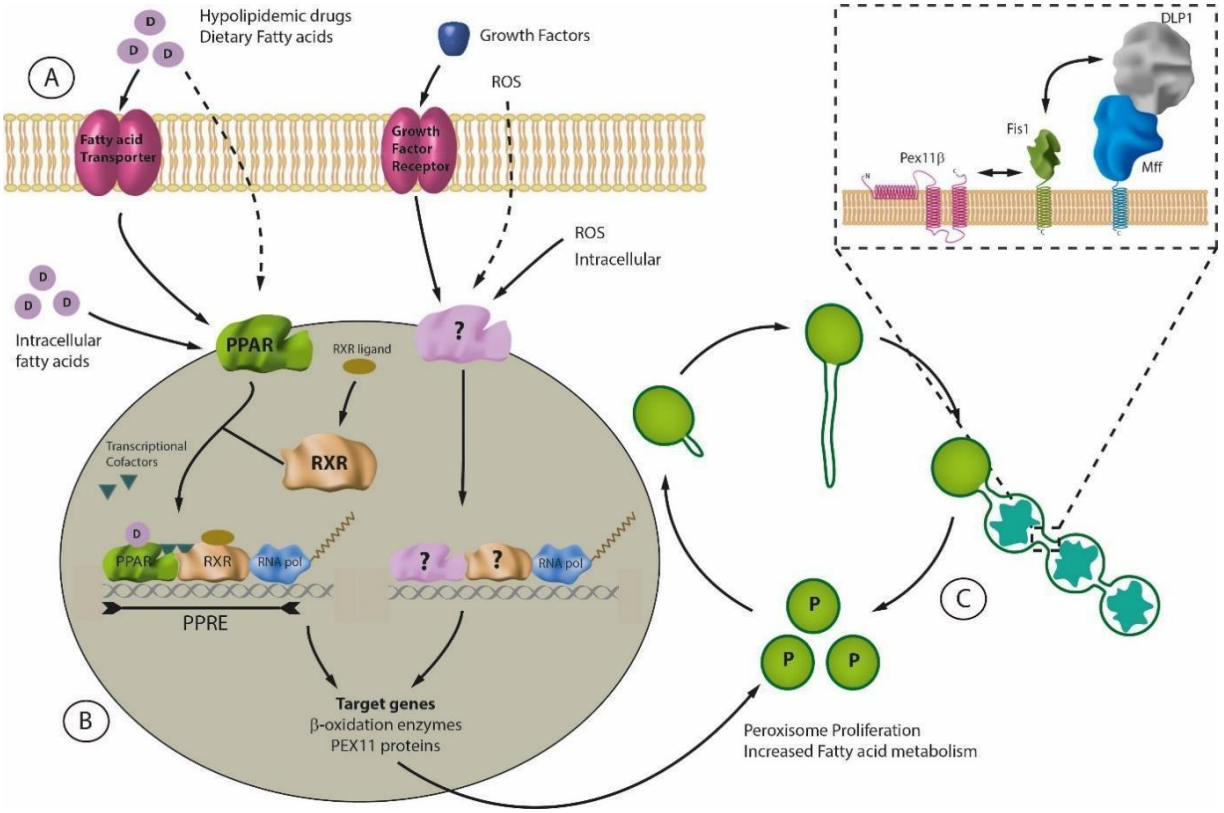


Figure 1.5: Model for peroxisome proliferation. (A) In mammals, peroxisome proliferator-activated receptor alpha (PPARα) is activated by fatty acid-ligands. (B) PPARs and retinoid X receptor (RXR) coordinately bind to the PPAR response element (PPRE) of peroxisomal genes to initiate gene expression resulting in peroxisome proliferation. However, PPARα-independent mechanisms have also been described. (C) The activation of PPARα/RxR usually promotes expression of peroxisomal enzymes (e.g. for peroxisomal fatty acid β-oxidation) and biogenesis factors (e.g. PEX11) (X. Xie *et al.*, 2015). Key division proteins are the Dynamin-like large GTPase DLP1 and its receptors Mff and Fis. Other extracellular and intracellular signalling cascades involved in peroxisome proliferation in mammals are largely unknown.

1.8 Peroxisome degradation

The major process for peroxisome degradation in mammalian cells is Pexophagy, (reviewed in (Honsho *et al.*, 2016; Marcus Nordgren *et al.*, 2013); which allows cells to remove excessive or damaged peroxisomes to maintain organelle homeostasis and can be induced by cellular stresses such as altered redox state and hypoxia (Iwata *et al.*, 2006; Walter *et al.*, 2014; T. Zhang *et al.*, 2015). In general, autophagic processes are regulated by conserved ATG genes, supported by additional organism-specific factors which specifically regulate pexophagy (Meijer *et al.*, 2007; Schroder *et al.*, 2007). Around 30 proteins have been shown to be involved in these pathways (Platta & Erdmann, 2007; Sakai *et al.*, 2006). Two distinct mechanisms have been described: macropexophagy and micropexophagy. The majority of peroxisomes in mammalian cells are degraded by macropexophagy, a process in which a double membrane structure, the autophagosome, grows and engulfs the target peroxisome and delivers it to lysosomes for recycling (Iwata *et al.*, 2006; Mizushima *et al.*, 2011). Organelles tagged for degradation are recognised by specific autophagy adaptors that form a bridge between the organelle and the growing autophagosomal membrane (Behrends & Fulda, 2012).

Few studies reported the ubiquitination of PEX5 as a signal for pexophagy. Nordgren and colleagues showed that an export-incompetent version of EGFP-tagged PEX5 became mono-ubiquitinated at the membrane and triggered pexophagy (M. Nordgren *et al.*, 2015). This study, reported a role for ATM kinase in the phosphorylation of PEX5 in response to intracellular ROS. In another study, a role for the tail-anchored protein ACBD5 in pexophagy in mammalian cells has been suggested (Nazarko *et al.*, 2014).

1.9 Peroxisome motility

In eukaryotic cells the regulation of organelle movement and distribution is an important factor to reach the optimal activity and inheritance during the cell cycle (Jongsma *et al.*, 2015). Whereas in yeast and plant cells peroxisomes move along actin filaments by interacting with myosin motors (Gao *et al.*, 2016; Tower *et al.*, 2011), in mammalian cells, two main populations of peroxisomes can be observed by live-cell imaging: the majority of peroxisomes (85-95%) exhibits slow oscillatory movement, whereas the remaining 5-15% display fast, directional, and microtubule-dependent movement (Costello *et al.*, 2017b; Rapp *et al.*, 1996; Wiemer *et al.*, 1997). This low percentage of directed movement is sufficient to maintain a homogeneous peroxisomal distribution at minimum energy state (Bonekamp *et al.*, 2012).

Cells from patients with peroxisomal disorders possess enlarged and less abundant peroxisomes, which later on cluster and detach from microtubules (Nguyen *et al.*, 2006). Overexpression of PEX11β in these cells promotes peroxisome proliferation and redistribution throughout the cell, suggesting that peroxisome dynamics are essential for their activity (Li & Gould, 2002).

Further studies are needed to elucidate the correlation between peroxisome motility and function.

1.10 Objectives

Peroxisomes represent crucial subcellular compartments for human life and health. They are remarkably dynamic organelles which respond to stimulation by adapting their structure, abundance, and metabolic functions according to cellular needs. An inability to multiply or proliferate peroxisomes has been linked to severe human disorders, which were only recently discovered. Although key players of peroxisomal fission have been identified at the organelle level, it is not clear how they are connected to cellular signalling pathways, in particular in humans. The well-known regulatory pathway regulating peroxisome-related genes such as ACOX1 in humans is the PPAR cascade. However, PPARα-independent mechanisms have also been described. Hence, the establishment and characterization of a cell model which allows reliable monitoring of peroxisome dynamics and proliferation after application of a defined, well-characterized stimulus is desirable.

The aim of this thesis is to address new signalling pathways regulating peroxisome proliferation. In chapter 3, the effect of different stimuli on peroxisome proliferation was analysed, a cell-based peroxisome proliferation assay has been developed to study the effect of this stimuli on peroxisome morphology and peroxisomal gene expression. In chapter 4, transcription factor binding sites of peroxisomal genes have been identified, a bioinformatic approach has been used to identify these regulatory elements and link them to specific signalling networks. In chapter 5, functional studies have been performed to investigate the predicted novel signalling pathway (TGFβ) in peroxsiome proliferation. Molecular cell biology approaches and pharmacological studies have been combined with transcriptome analyses in order to identify novel regulatory networks.

Our results have led to the identification of a novel signalling pathway triggering peroxisome proliferation and may lead to novel leads for therapeutic options to improve peroxisome abundance.

Chapter 2 – Material and Methods

2.1 Buffers and solutions

General buffers and solutions used in performed experiments are listed in table 2.1. All buffers and solutions used were sterilised by autoclaving or filtration prior to use.

Buffers and solutions	**Recipe/Composition**
Blocking solution for IMF	1% (w/v) BSA in PBS
Blocking solution for WB	5% (w/v) low-fat milk powder (Marvel) in TBS-T
Bradford Dye reagent	Biorad) 1.5 dilution in water
Cell Freezing media	MEM/life technologies, 10% DMSO
Collagen-R solution	Type I rat tail collagen; 4 mg/ml in 0.1 % acetic acid, 0.4% (Serva)
DEAE (50mg/ml)	25 mg/ml in deionized H_2O
ECL reagent	ECL prime GE healthcare
FBS 10%	Life technologies, south American origin
Fixative for IMF, pH 7.4	4% (w/v) Paraformaldehyde in PBS
LB medium	2.5% (w/v) LB-Broth Miller
LB Agar	2.5% (w/v) LB-Broth Miller 1% (w/v) Agar
Lysis buffer pH 7.5	25 mM TrisHCl 150 mM NaCl 0,5 mM EDTA Add fresh: 0.5% (w/v) Triton X-100, 1 mM PMSF, 1 mini protease inhibitor cocktail tablet (Roche)

Mowiol mounting media	12 g Mowiol 4-88 (Sigma) 40 ml PBS, stir over night + 20 ml Glycerol, stir over night Centrifuge 1 hour, 15,000 rpm, 4°C Add sodium azide to the supernatant
N1 Supplement	0.5mg/ml recombinant human insulin, 0.5mg/ml human transferrin (partially iron-saturated), 0.5µg/ml sodium selenite, 1.6mg/ml putrescine, and 0.73µg/ml progesterone (Sigma) + 0.25% BSA
Phosphate buffered saline (PBS) pH 7.4	140 mM NaCl 2.5 mM KCl 6.5 mM Na_2HPO_4 1.5 mM K_2HPO_4
Permeabilisation solution	0.2% (v/v) Triton X-100 in PBS
Laemmli-buffer	60 mM Tris, pH 6.8 2% (w/v) SDS 10% (v/v) Glycerol 0.005% (w/v) Bromophenol blue 20 mM DTT 5% (v/v) β-Mercaptoethanol (fresh)
TGS- running buffer	25 mM Tris 190 mM Glycine 0.1% (w/v) SDS
Transfer-buffer	48 mm Tris 39 mM Glycine 0.4% (w/v) SDS 20% (v/v) Methanol
50x TAE –buffer pH 8.0	40 mM Tris 20 mM Acetic acid 1 mM EDTA
TBS-T	50 mM Tris, pH 7.5 150 mM Sodium chloride 0,05% Tween20
Tris buffer, pH 8.8 (separating gel)	2 M Tris in water
Tris buffer, pH 6.8 (stacking gel)	1 M Tris in water

Table 2.1 list of buffers and solutions and their composition/recipe

2.2 Cell culture

2.2.1 Cell passage

HepG2 cells were routinely cultured in Dulbecco's Minimum Essential medium (MEM, Life technologies) low glucose (1.5 g/L) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life technologies) at 37°C with 5% CO_2 and 95% humidity. Cell passage was performed twice a week, after the cells reached 75% confluency. Cells were washed once with 1x PBS and incubated for 3 minutes with 1 ml TrypLE Express at 37°C. Upon resuspension in medium, cells were centrifuged for 3 minutes at 1000 rpm. Cell pellets were resuspended in 10 ml of fresh MEM medium and seeded at 1:10 to 1:3 dilution. Cells were routinely grown on 10ø cm tissue cell culture dishes (Greiner), for immunofluorescence, cells were seeded on round 19ø mm glass coverslips 24 hours prior to fixation. When using HepG2 cells, coverslips were coated with collagen (1/10 in d H_2O, Serva) prior to seeding. To ensure reproducibility between experiments, cell number was determined using a Neubauer improved counting chamber or a TC20™ Automated Cell Counter (Bio-Rad, USA).

Cell line	Organism	Tissue	Cell type	Morphology	Source
COS7	*Cercopithecus aethiops*	Kidney	SV40 transformed	Fibroblast-like	ATCC (CRL-1651)
HepG2	*Homo sapiens*	Liver	carcinoma	Epithelial	ATCC (HB-8065)

Table 2.2 – Cell lines

2.2.2 Cell freezing and thawing

HepG2 cells, early passages, were kept through cryopreservation in liquid nitrogen. Cell pellets were resuspended in freezing medium (MEM supplemented with 10% FBS and 10% DMSO). 1 ml aliquots in cryovials (Greiner) were kept overnight at -80°C, prior to storage in a liquid nitrogen tank (Biorack). For thawing, cells were quickly resuspended with pre-warmed culture medium and seeded in a 10ø cm tissue cell culture dish. The culture medium was change after cell adhesion to remove debris and DMSO.

2.3 Transfection methods

Several transfection methods were used, depending on the cell line and experimental objective. COS7 cells were routinely transfected with (DEAE)-dextran. HepG2 cells were transfected using Lipofectamine® 3000 (Invitrogen). For all transfection methods used, cells were either seeded in dishes or 19ø mm glass coverslips 24 hours prior to transfection.

2.3.1 DEAE

For transfection of 10ø cm dishes, 10 µg of DNA and 18 µl of DEAE-dextran were diluted in 1.5 ml of complete medium and incubated at room temperature for 20 minutes. Cells were washed twice with PBS and incubated with the DNA-DEAE-dextran mixture for 90 minutes, at 37ºC and 5% CO_2. During incubation, the dishes were shaken every 15 minutes. After 90 minutes, the DNA-DEAE-dextran mixture was removed and 10 ml of complete medium supplemented with 10 µl of chloroquine were added to the dishes for 3 hours. Lastly, the cells were washed 3 times with PBS and incubated with fresh medium for 24/48 hours.

2.3.2 Lipofectamine® 3000

Transfection protocols for lipofectamine were optimized for DNA and siRNA following the manufacturer's protocol and scaled up or down as necessary. For DNA transfection in a 6ø cm dish, one tube was prepared with 16.5 µl of lipofectamine diluted in 250 µl of Opti-MEM medium, and another with 11 µg of DNA and 22 µl of P3000 reagent in 250 µl of Opti-MEM medium (Life technologies) and mixed well. The DNA-P3000 mix was added to the diluted lipofectamine and incubated for 5 minutes at room temperature. Lastly, the DNA-lipid mixture was added to the previously seeded cells and incubated at 37 ^{0} C, 5% CO_2 for 24-48 hours.

2.4 Immunofluorescence (IMF)

Cells grown on glass coverslips were processed for IMF 24/48 hours after seeding or transfection, and 72h after silencing. Cells were routinely fixed for 20 minutes with 4% paraformaldehyde (PFA), followed by 3 washes in 1x PBS for 5 minutes each and permeabilized with 0.2% Triton X-100 for 10 minutes. After 3 washes in 1x PBS for 5 minutes, cells were blocked with 1% BSA for 10 minutes. Followed by primary antibody incubation for 1 hour in a humid chamber (Table 2.3). Cells were washed with 1x PBS for 5 minutes. This step was repeated for the secondary antibody, protected from light. Coverslips were washed with dH_2O to remove PBS and mounted using 1:4 Mowiol/ Propyl gallate mounting medium on glass slides (Fisher Scientific). All immunofluorescence steps were performed at room temperature.

Antibodies	Type	Dilution		Source
		IMF	WB	
GAPDH	mc rb	-	1:5000	ProSci, San Diego, USA
PEX14	pc rb	1:1400	1:4000	Kind gift from D.Crane Griffith University, Australia
PMP70	mc ms	1:500	1:5000	Sigma-Aldrich, Schnelldorf, Germany
AlexaFluor 488 IgG	dk anti-rb	1:400	-	Molecular Probes (ThermoFisher Scientific, Waltan, USA)
AlexaFluor 488 IgG	dk anti-ms	1:400	-	Molecular Probes (ThermoFisher Scientific, Waltan, USA)
HRP IgG	gt anti-ms	-	1:5000	Bio-Rad, Munich, Germany
HRP IgG	gt anti-rb	-	1:5000	Bio-Rad, Munich, Germany

Table 2.3 – Primary and secondary antibodies. Abbreviations: IMF, immunofluorescence; WB, western blot; mc, monoclonal; pc, polyclonal; ms, mouse; rb, rabbit; gt, goat; dk, donkey; HRP, horseradish peroxidase.

2.5 Microscopy

2.5.1 Epifluorescence and confocal microscopy

Routine cell imaging was performed using an Olympus IX81 microscope equipped with an UPlanSApo 100x/1.40 oil objective (Olympus Optical, Hamburg, Germany). Digital images were taken with a CoolSNAP HQ2 CCD.

2.5.2 Image processing

Images were adjusted for contrast and brightness using the Olympus Soft Imaging Viewer software (Olympus Soft Imaging Solutions GmbH) and MetaMorph 7 (Molecular Devices, USA).

2.5.3 Live-cell imaging

For live-cell imaging, HepG2 cells labelled with GFP-SKL were plated in collagen coated 3,5ø cm glass bottom dishes (Cellvis, USA and MatTek, USA). Live-cell imaging data was collected using an Olympus IX81 microscope equipped with a Yokogawa CSUX1 spinning disk head, CoolSNAP HQ2 CCD camera, 60x/1.35 oil objective.

A controlled-temperature chamber and objective warmer were set-up on the microscope stage at 37ºC. During image acquisition, cells were kept at 37ºC and in CO_2–independent medium (HEPES buffered) (Life Technologies). For each cell, 250 stacks of 9 planes (0.5µm thickness, 100ms exposure) were taken in a continuous stream. All conditions and laser intensities were kept between experiments. Digital images were taken and processed using VisiView software (Visitron Systems, Germany).

2.6. Protein assays

2.6.1 Cell lysis

Routinely, cells were transfected in 6ø cm dishes (Greiner) and collected after 48/72 hours. For lysis, cells were washed with 1x PBS 2 times, and 0.5 ml of lysis

buffer was added. To remove all cells, a cell scraper (Greiner, 25cm) was used and the cells were collected in a 1.5 ml micro-centrifuge tube. To improve lysis efficiency, cells were incubated at 4^0 C for 30 minutes, using a rotating shaker. Lysate was centrifuged at 15,000 g for 15 minutes at 4ºC to remove debris and the supernatant was kept. Protein concentration was determined by Bradford assay (Bradford 1976).

2.6.2 SDS-PAGE Electrophoresis

Standard 1D-SDS **P**oly**A**crylamide **G**el-**E**lectrophoresis (SDS-PAGE) was performed with 10-12.5% separating and 4% stacking gels (Table 4). A pre-stained molecular weight marker (Precision Plus, Biorad) was used to determine the protein size. The sample running front was visualized by bromophenol blue added to the Laemmli-buffer. Gels were conducted in chambers (Biorad, Minicell) containing TGS-buffer for 30 minutes at 80 V until the proteins were focused and entered the separating gel, followed by an increase to 130 V for approximately 90 minutes.

2.6.3 Immunoblotting

Protein transfer to nitrocellulose membrane (GE-Healthcare,) was performed by semi-dry western blotting for 60 minutes at 14 V (Transblot, BioRad). After protein transfer, membrane was blocked with 5% low fat powdered milk in TBS-T for 1 hour at room temperature. Membrane was incubated with the primary antibody diluted in TBS-T, overnight at 4°C on a rotation shaker (VWR). Membrane was washed three times for 10 minutes with TBS-T. Secondary antibody incubation was performed for 60 minutes at room temperature, after which membrane was washed three times for 10 minutes with TBS-T.

	Separating Gel		Stacking Gel
	10%	12.5%	4%
30% Polyacrylamide	3.33 ml	4.17 ml	0.83 ml
2 M Tris pH 8.8	1.86 ml	1.86 ml	-
1 M Tris pH 6.8	-	-	0.63 ml
20% SDS (0.1%)	50 µl	50 µl	25 µl
dH_2O	4.73 ml	3.89 ml	3.43 ml
10% APS	30 µl	30 µl	40 µl
TEMED	5 µl	5 µl	5 µl
Total volume	10 ml	10 ml	5 ml

Table 2.4 – Recipe for acrylamide gels

2.6.4 ECL detection

For protein detection, membrane was incubated for 2 minutes with 1:1 ECL prime and ECL (GE-Healthcare) and exposed to photographic film (GE-Healthcare) for 1 to 10 minutes developed protected from light.

2.7 Molecular biology techniques

2.7.1 Gene synthesis

Gene synthesis of PEX11β promoter regions (1302bp for the wild type and 1296bp for the mutant) of human PEX11β was performed by Eurofins, sequences are shown in **Table 2.5**.

PEX11β Wt (1302 bp)
GAG CTC CCC CAA GCG CAC CCT TTA ACT CAT GCT ATG GAC CCA AAT CCC TGT CCT GCC CGG TCC TGA GTC CTA GGT CAC TCA CCT GGG GGA AGG CAA GCA GGA AAC TAA GTC CCC AGG CTG CCC CCA GAA GTT TCC TTA CAC CTG AAC GGG ATC CAA GCG GGT TGA GTA CTG CTG CCT GGC GGT CCA ATC CAA TGA CCA CAG GCA GGA AAG CTG CAG AAT ACG TGG CCA TTA GTT TCA GGA ACA TCA GTG TCC GAC ATG CGA TGT CCA CAG CCA GCC ATT GAA CAG TGA TAT TCC AGG TGG CAT CTA GGG GCA TAA CCA CAA AAG TGA CTA GTA AGT CGG CGG CTG CTA AAT GGA TGA AGA GTC TCC TGA CCG GAG AGG GGC GGA GCT GGC TGG GTT CCC GCC GTG TCA CTG ACC ACA GGA CTG CCA GGT TCC CTC CAG CCG AAG AAA CAA ACA GCA CAA TGG TCA CTC CCA CTC GGA CCT TGG CTG CTG CCG AGA AGG TGG GCA GCT CTG AGC CCT CCA CCT CCA CTC CTG ATC CAG CCC AGA CCT CCT CCC CCG CTG CTG ACC CCA AGG GGT GCC GTT GCC TGC AGA CAT GGT GGC CTG GAG AGA AAC TGA GGA GGA TGA AGT GTG GGT ATG AAG CGG TTA GCT TTG GCC ACT GAT TCT TCG CCC TCC TTT TCA GGA TCT GGA GCT GAT TAT TGC GAA TTT CGT TCA CTA AAG CCT CTG CTT CTG GAG ACT CTC CAT CTG TTC TGG AGG AGG TCA CTT GTT CAA CTG TCA AGG TTC TGC AGG GAA CGA AGA TCT GGT ACC AAT CAC GCT CCT TTT TCC TTG GAC AGA CCA GCC TGG GCT TCA GGC CTC TAC TGA CCT AGA CCT CTT TAG CCC CTA CAG CCC TGG ATT CAG TCG GGG CCT GCG TGC ATG TGT ATT TGT GAG GTT CTG CCA GGC GCA CAC CTA TGC CGG CGG CCT CCA AGA CGT GGA GAT CAC TGC GGC GCT TTC TCG CCC CGC CCC GGC CCC TGC AGG CCC CGC CCT TGG TCA TGA ATA TTT AAA GAA GAA GGT GCC GCT GGA GGC GTG CTA GGG AGT AGG GGT CGT CTG ATA AGG GGA AGC TGT GAC GCA GAC ACG CAC AGT AAT ACA CAG ATG GAG GCT CAA AAG ACA CGA GTT TCG CGT CCT GAA ATT CCG CTT CCA GGG CCA AGC TTT CTT TTC TGA TAC TGT TTG TCC CTC GCG AGG CAC CGT TGG GTC GCG CAG TAG GCG TGA CTA GGG GCG GGA AGT GGG GCG GGA GCA GGG CCG CGG AGC CTG GGC TGC GGC TGT CAT CTC GAG
PEX11β Mut (1296bp)
CGG TCC TGA GTC CTA GGT CAC TCA CCT GGG GGA AGG CAA GCA GGA AAC TAA GTC CCC AGG CTG CCC CCA GAA GTT TCC TTA CAC CTG AAC GGG ATC CAA GCG GGT TGA GTA CTG CTG CCT GGC GGT CCA ATC CAA TGA CCA CAG GCA GGA AAG CTG CAG AAT ACG TGG CCA TTA GTT TCA GGA ACA TCA GTG TCC GAC ATG CGA TGT CCA CAG CCA GCC ATT GAA CAG TGA TAT TCC AGG TGG CAT CTA GGG GCA TAA CCA CAA AAG TGA CTA GTA AGT CGG CGG CTG CTA AAT GGA TGA AGA TGA CCG GAG AGG GGC GGA GCT GGC TGG GTT CCC GCC GTG TCA CTG ACC ACA GGA CTG CCA GGT TCC CTC CAG CCG AAG AAA CAA ACA GCA CAA TGG TCA CTC CCA CTC GGA CCT TGG CTG CTG CCG AGA AGG TGG GCA GCT CTG AGC CCT CCA CCT CCA CTC CTG ATC CAG CCC AGA CCT CCT CCC CCG CTG CTG ACC CCA AGG GGT GCC GTT GCC TGC AGA CAT GGT GGC CTG GAG AGA AAC TGA GGA GGA TGA AGT GTG GGT ATG AAG CGG TTA GCT TTG GCC ACT GAT TCT TCG CCC TCC TTT TCA GGA TCT GGA GCT GAT TAT TGC GAA TTT CGT TCA CTA AAG CCT CTG CTT CTG GAG ACT CTC CAT CTG TTC TGG AGG AGG TCA CTT GTT CAA CTG TCA AGG TTC TGC AGG GAA CGA AGA TCT GGT ACC AAT CAC GCT CCT TTT TCC TTG GAC AGA CCA GCC TGG GCT TCA GGC CTC TAC TGA CCT AGA CCT CTT TAG CCC CTA CAG CCC TGG ATT CAG TCG GGG CCT GCG TGC ATG TGT ATT TGT GAG GTT CTG CCA GGC GCA CAC CTA TGC CGG CGG CCT CCA AGA CGT GGA GAT CAC TGC GGC GCT TTC TCG CCC CGC CCC GGC CCC TGC AGG CCC CGC CCT TGG TCA TGA ATA TTT AAA GAA GAA GGT GCC GCT GGA GGC GTG CTA GGG AGT AGG GGT CGT CTG ATA AGG GGA AGC TGT GAC GCA GAC ACG CAC AGT AAT ACA CAG ATG GAG GCT CAA AAG ACA CGA GTT TCG CGT CCT GAA ATT CCG CTT CCA GGG CCA AGC TTT CTT TTC TGA TAC TGT TTG TCC CTC GCG AGG CAC CGT TGG GTC GCG CAG TAG GCG TGA CTA GGG GCG GGA AGT GGG GCG GGA GCA GGG CCG CGG AGC CTG GGC TGC GGC TGT CAT CTC GAG

Table 2.5 PEX11β Wt /Mut Sequences, in yellow is binding site for SMAD transcription factor.

2.7.2 Digestion

DNA and vectors were digested with restriction enzymes following the manufacturer's suggested buffers at 37°C, for 4h or overnight. Enzyme inactivation was performed at 65°C for 20 minutes. Upon digestion, samples were run in an agarose gel to remove unwanted DNA fragments. Vector DNA was dephosphorylated with antarctic phosphatase for 40 minutes at 37°C, followed by enzyme inactivation for 20 minutes at 65°C. DNA concentration was determined using the Qubit2.0 fluorometer (Invitrogen, USA). To calculate the exact amount of DNA to be used, the following formula was used:

$$insert\ (ng) = \frac{vector\ (ng) \times insert\ (kb)}{vector\ (kb)} \times ratio$$

The complementary sticky DNA ends created by digestion of vector and insert hybridize with each other and the DNA backbone is ligated by the DNA ligase by using T4 ligase (NEB) at 4 °C overnight or at room temperature for 2 hours.

Digestion reactions were run in agarose gels prior to purification. DNA electrophoresis was routinely performed in an agarose gel (1% agarose in 1x TAE buffer) stained with ethidium bromide (0.5 µg/ml). Separation was performed at 75 V for 45 to 60 minutes in TAE 1x buffer. DNA samples were mixed with 6x purple loading dye buffer and loaded to individual wells. A DNA ladder was also loaded to one well to compare band sizes. Digital images were taken using the BioDoc-It Imaging System (UVP, USA). Specific DNA bands were excised with a scalpel under UV light and purified using the NucleoSpin Extract II kit (Macherey-Nagel, Germany), following the manufacturer's protocol

2.7.3 Transformation

Plasmid amplification was performed in competent DH5α *Escherichia coli* (*E.coli*) following a standard heat shock protocol. For each plasmid, 50 µl of competent *E.coli* bacteria were mixed with 2 µl of ligation mixture and incubated for 30 minutes on ice, followed by a 90 seconds heat shock at 42°C, and a short incubation on ice. The DNA plasmid binds to the membrane of the competent bacteria and the uptake is induced by a heat shock. Cells were incubated with 950 µl of LB medium at 37°C for 1 hour with low agitation (300rpm). Cells were centrifuged for 2 minutes at 5000 rpm and the pellets were resuspended in 100 µl of LB medium. Cells were spread using glass beads on LB agar plates containing Kanamycin (30 µg/ml) or Ampicillin (100 µg/ml). Plates were incubated overnight at 37°C.

2.7.4 Plasmid isolation

Colonies were tested by DNA digestion following plasmid DNA isolation using the NucleoSpin Plasmid Miniprep kit (Macherey-Nagel, Düren, Germany). A single positive clone for each plasmid was selected and sequenced (**Table 2.6**). For positive clones, DNA yield was increased by performing midi preparations using the NucleoBond Xtra Midi kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

Table 2.6 – Primers for cloning and sequencing

Name	Sequence (5' to 3')
PGL3-forward (RVprimer3)	5' CTAGCAAAATAGGCTGTCCC 3'
PGL3- reverse (GL2)	5' CTTTATGTTTTTGGCGTCTTCC 3'
PEX11β- forward	5' CTAAATGGATGAAGACTCCTGACCGGAGAG 3'
PEX11β- reverse	5' CTCTCCGGTCAGGAGTCTTCATCCATTTAG 3'

Chapter 3 – Results

Generation and application of a cell-based peroxisome proliferation assay

Summary:

Peroxisomes respond to external signals and changes in the cellular environment with alterations in their morphology, number and enzyme composition. How the regulation of these processes is integrated into the cell's response to different stimuli, the signaling pathways and factors involved, remains unclear, in particular in human cells. Here, a cell-based peroxisome proliferation assay using the human hepatoblastoma cell line HepG2 has been developed to investigate different stimuli and their ability to induce peroxisome proliferation. This assay allows culture of HepG2 cells under serum-free conditions and can be applied for different approaches, including studying the expression of peroxisomal genes, the correlation between gene expression and peroxisome morphology and quantification of peroxisome number. As shown before (Schrader *et al.*, 1998a), addition of serum/growth factors stimulated the membrane elongation of peroxisomes, which is a pre-requisite of peroxisomal division, multiplication/ proliferation, and was used as a morphological read-out via immunofluorescence. We demonstrated that growth of HepG2 cells was not affected under serum-free culture condition. Furthermore, autophagic processes were not induced. We also showed that the number of peroxisomes increased after division of elongated peroxisomes which is indicative for peroxisome proliferation.

Different stimuli, such as fatty acids, PPAR agonists and antagonists, have been used in this study. PPAR agonists and antagonists had no stimulatory or inhibitory effect on peroxisome elongation in our assay, suggesting PPAR-independent regulatory processes. However, arachidonic acid and linoleic acid were able to induce peroxisome elongation, whereas palmitic acid and oleic acid were not

effective. These findings point to a stimulatory mechanism, which is different from mere stimulation of peroxisomal fatty acid β-oxidation.

Analysis of peroxisome morphology and peroxisome gene expression during a time course showed an increase in PEX11β expression which reaches a maximum at 24 hours before declining. This correlates very well with the induction of peroxisome elongation and subsequent division. No such increase was observed for PEX11α and PEX11y, suggesting different roles of PEX11 isoforms. Besides PEX11β there are several other factors whose activity could be modified to control peroxisome proliferation. Deficiencies in peroxisome proliferation have been associated with a variety of disease states, including liver diseases and neurological dysfunction, as well as cellular ageing (Cimini *et al.*, 2009; Titorenko & Terlecky, 2011). A clear understanding of the mechanisms and signaling pathways that control peroxisome plasticity could allow modulation of peroxisome abundance to improve cellular function in health and disease.

3.1 Introduction

3.1.1 Peroxisome plasticity and proliferation

Peroxisomes are responsive organelles which show a remarkable plasticity with respect to morphology, number, protein composition and metabolic function. All those parameters can vary depending upon organism, cell type, developmental stage, and environmental conditions (Islinger *et al.*, 2010). Peroxisomes in almost all organisms have the ability to proliferate and multiply or be degraded in response to nutritional and extracellular environmental stimuli (Schrader *et al.*, 2012).

Several morphologically distinct types of peroxisomes have been described in mammalian cells either by immunofluorescence or by electron microscopic studies (Schrader *et al.*, 1996; Yamamoto & Fahimi, 1987). Peroxisomes can appear as spherical organelles, but are also observed to form elongated, tubular structures (**Figure 3.1**). Over the years it became evident, that elongation (tubulation) is the first step in a sequence of morphological changes which occur during peroxisome proliferation/multiplication. This step contributes to the expansion and growth of the peroxisomal membrane, which then constricts and divides into new, spherical peroxisomes, which import newly synthesized matrix proteins (see **Introduction 1.6; Figure 1.3**) (reviewed in (Schrader *et al.*, 2016)). PEX11 proteins are key factors in the elongation and division process. A striking increase in elongated peroxisomal forms on expression of PEX11 has been observed in all organisms studied including yeast, trypanosomes and mammalian cells (Erdmann & Blobel, 1995; Schrader *et al.*, 1996). It is supposed that human PEX11β, which is very well studied, remodels and deforms the peroxisomal membrane by oligomerisation and

interaction with membrane lipids via its amphipathic helixes at the N-terminus of the protein (Delille *et al.*, 2010; Opaliński *et al.*, 2011; Y. Yoshida *et al.*, 2015). It then recruits and interacts with components of the division machinery such as the adaptor proteins Fis1 and Mff, which are involved in the recruitment of the fission GTPase Drp1 (reviewed in (Schrader *et al.*, 2016)). Recently, it was found that PEX11 also acts as a GTPase activating protein (GAP) towards Drp1 and stimulates Drp1 GTPase activity and membrane scission (Williams *et al.*, 2015). Mutations in Drp1 and Mff have been linked to new disorders resulting in highly elongated peroxisomes which are unable to divide (Shamseldin *et al.*, 2012; Wanders & Waterham, 2006; Waterham *et al.*, 2007). Patients with a loss of PEX11β function have also been identified (Ebberink *et al.*, 2012; Taylor *et al.*, 2017). They present with short stature, eye problems, progressive hearing loss and neurological defects. Similar to Drp1 or Mff deficiency, the metabolic functions of peroxisomes are either not or only slightly affected in patients with defects in peroxisome dynamics. This suggests that the symptoms relate to altered peroxisome plasticity, highlighting the importance of proper regulation of peroxisome abundance for cell function. In line with this, altered peroxisome abundance in PEX11β-deficient epidermal cells resulted in abnormal mitosis and organelle inheritance, thus affecting cell fate decisions (Asare *et al.*, 2017).

Elongated peroxisomes can be observed during rapid cellular growth, for example, after hepatectomy (Yamamoto & Fahimi, 1987) or in mammalian cells under conditions of low cell density (Schrader *et al.*, 1998b). Stimulation of cultured cells with defined growth factors, fatty acids or free radicals also promotes peroxisome elongation (Schrader *et al.*, 1996; Schrader *et al.*, 1999) as well as depolymerisation of microtubules (Passmore *et al.*, 2017; Schrader *et al.*, 1996), suggesting the involvement of intracellular signalling in peroxisome elongation. Motor-driven pulling forces, e.g. mediated by the Kinesin-adaptor

Miro1 along microtubules, can also contribute to peroxisomal membrane expansion (Castro *et al.*, 2018).

Despite their importance to cell physiology, the mechanisms that mediate and regulate peroxisome membrane dynamics and abundance in humans are poorly understood. Despite progress in understanding of peroxisomal growth and division, the relation of these mechanisms with cellular signalling cascades and the transcriptomics behind this action is still not clear (see **Introduction 1.7.3; Fig. 1.5**).

3.1.2 Peroxisome proliferator activated receptors (PPARs)

The known pathway involved in peroxisome proliferation is mediated by a family of ligand-activated transcription factors known as peroxisome proliferator activated receptors (PPARs) (Rakhshandehroo *et al.*, 2010; Schrader *et al.*, 2012; Y. X. Wang, 2010).

PPARs are ligand-activated nuclear receptors which function in the regulation of genes involved in glucose and lipid homeostasis. PPARs represent a family of nuclear receptors composed of three members: PPARα, PPARβ, PPARγ which have tissue -specific expression, and vary in the responses they mediate through binding to their interaction partners. The PPAR:RXR heterodimer binds peroxisome proliferator receptor elements (PPREs) in and around target genes (Berger & Moller, 2002). These transcription factors are typically activated by lipid- ligands and regulate the expression of genes associated with lipid metabolism and adipocyte differentiation (Kliewer *et al.*, 1992; Reddy & Hashimoto, 2001).

Additional pathways independent of PPARs have also been described and it is likely that yet unknown mechanisms contribute to the regulation of peroxisome proliferation (Gondcaille *et al.*, 2005; Li & Gould, 2002; Sexton *et al.*, 2010).

3.1.3 PPAR independent mechanisms

Even though there is evidence that peroxisome proliferation is regulated by PPARs it is likely that other regulatory mechanisms also play a role. Interestingly, peroxisome numbers have been shown to increase in response to external stimuli in a PPAR-independent manner. A set of chemical compounds (e.g. BM 15766 and 4-phenylbutyrate) was shown to induce peroxisome proliferation in mammalian (Ribeiro *et al.*, 2012; Schrader *et al.*, 2012). BM 15766, an inhibitor of cholesterol biosynthesis at the 7-dehydrocholesterol-Δ7-reductase step, was shown to induce peroxisome proliferation without significantly elevating fatty acid β-oxidation enzyme levels, thus implying a PPARα-independent signal transduction (Baumgart *et al.*, 1990).

Phenylbutyrate was also shown to induce peroxisome proliferation in mice in the absence of the nuclear receptor PPARα although a potential role for PPARγ has not been ruled out (Baumgart *et al.*, 1990; Gondcaille *et al.*, 2005; Song *et al.*, 2013). In a high content screen probing more than 15,000 drugs, 10 new compounds were reported to induce peroxisome proliferation in HepG2 cells, which are supposed to be refractory to PPARα-mediated peroxisome proliferation (Sexton *et al.*, 2010). Thus, there is increasing evidence for the existence of numerous PPARα independent pathways controlling peroxisome abundance, which could be exploited for therapeutic use in humans. For most of the chemicals

described so far in the literature, the mechanism of action remains unknown (Ahmadian *et al.*, 2013) However, unlike PPARα-ligands, which induce peroxisome proliferation in the absence of PEX11α, 4- phenylbutyrate does require PEX11α to induce peroxisome proliferation indicating the existence of alternative pathways involving different molecular players in order to control peroxisome abundance (Li & Gould, 2002). Besides the peroxisome proliferators reported up to now, other factors such as growth factors, polyunsaturated fatty acids (e.g. arachidonic acid) and ROS have been shown to induce peroxisomal elongation (a pre-requisite for proliferation) in human cells (Diano *et al.*, 2011; Schrader *et al.*, 1998a).

Another PPAR independent pathway has recently been discovered involving PPARγ coactivator-1α (PGC1-α) in humans and mice (Bagattin *et al.*, 2010). Although, as the name suggests, the obvious receptor for PGC1-α would indeed be PPARγ, this was excluded in this case. PGC1-α can activate gene expression by binding to other transcription factors such as NRF2 (Schrader *et al.*, 2012; Vega *et al.*, 2000).

External signals such as growth factors can also induce peroxisome proliferation, suggesting the involvement of growth factor-dependent cascades such as mTOR (Schrader & Fahimi, 2006). Indeed, the mTOR pathway has recently been linked with peroxisomes (Zhang *et al.*, 2013; J. Zhang *et al.*, 2015). The mTOR protein forms two complexes, mTORC1 and mTORC2, each of which contains distinct components and signals through a different set of downstream effectors. A key function of mTORC1 is to coordinate nutrient availability with autophagy. When growth factors are present, the action of mTORC1 ensures that autophagy is inhibited, whilst under starvation conditions, or in response to ROS, mTORC1

inhibition causes upregulation of autophagy (Sengupta *et al.*, 2010). The tuberous sclerosis complex (TSC) is a signalling node which represses mTOR1 signalling by inhibiting the activity of the small GTPase Rheb, leading to activation of autophagy (Watanabe *et al.*, 2011; Zhang *et al.*, 2013). Recently, the peroxisomal localization of TSC1, TSC2 was reported (J. Zhang *et al.*, 2015). The authors also suggest that the peroxisomal TSC functions as a signalling node which is able to repress mTORC1 and to induce autophagy when peroxisomal ROS production exceeds tolerable levels (Zhang *et al.*, 2013).

3.1.4 Human hepatoblastoma cells (HepG2) as a model to study peroxisomes

In the past decades, huge efforts have been made to establish cell lines that express differentiated hepatic functions. Among them, a number of human hepatoma cell lines, including HepG2, Hep3B, HuH7, and HepaRG, are commonly used for drug metabolism and hepatotoxicity studies (Costantini *et al.*, 2013). Although the metabolic functions of hepatoma cells are more limited than those of primary hepatocytes, they offer advantages for in vitro studies, such as high availability, easy handling, and stable phenotype.

The hepatoblastoma cell line HepG2 is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male who had a well-differentiated hepatocellular carcinoma (Aden *et al.*, 1979; Donato *et al.*, 2015). HepG2 is the most widely used human hepatoma cell line in pharmaco-toxicological research (Natarajan & Darroudi, 1991). These cells are non-tumorigenic and highly proliferative and have been grown successfully in large-scale culture systems. HepG2 cells secret many plasma proteins, such as

transferrin, fibrinogen, plasminogen and albumin (Knowles *et al.*, 1980). They are adherent, epithelial-like cells growing as monolayers and in small aggregates. HepG2 cells can also form bile canaliculi-like structures between adjacent cells (Sormunen *et al.*, 1993). Bile canaliculus formation in cultured HepG2 cells (Bader *et al.*, 1992; Natarajan & Darroudi, 1991) indicating the potential for differentiation (Stier *et al.*, 1998).

Due to their important role in lipid metabolism, peroxisomes are very prominent in hepatocytes in the liver. The peroxisomal compartment in HepG2 cells has been well characterised (Schrader *et al.*, 1996), and the cell line has been used in several studies on peroxisome dynamics (A. Koch *et al.*, 2003; Schrader *et al.*, 2000). HepG2 cells contain an elaborate peroxisomal compartment with high plasticity, showing distinct peroxisome morphologies (Grabenbauer *et al.*, 2000; Schrader *et al.*, 1996). Furthermore, peroxisome elongation can be stimulated in HepG2 cells by low cell density or microtubule depolymerisation (Schrader *et al.*, 1996). Furthermore, addition of growth factors, fatty acids or UV irradiation has been shown to promote peroxisomal tubule formation in HepG2 cells (Schrader *et al.*, 1998b; Schrader *et al.*, 1999). In addition, peroxisomal enzyme activities are comparable in HepG2 and human liver (Wanders *et al.*, 1991), and peroxisomes can be isolated from HepG2 cells using density gradient centrifugation (Manner & Islinger, 2017). Hence, HepG2 cells represent a suitable model system for the investigation of peroxisome proliferation and the identification of additional stimuli which alter peroxisome morphology and numbers in human cells.

3.2 Specific Methods

3.2.1 Cell culture and cell-based peroxisome proliferation assay

HepG2 cells (ATCC HB8065) were maintained under standard conditions in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO_2-humidified incubator. To test certain stimuli and their effect on peroxisome dynamics and proliferation, a serum-free cell culture assay was developed, which is based on a previous system using HepG2 cells (Schrader *et al.*, 1998b). Culture conditions were tested and optimised (e.g. cell batches, cell culture media, time points, cell density). Cells were incubated in serum-free MEM supplemented with N1 (Sigma) containing 0.5 mg/ml recombinant human insulin, 0.5 mg/ml human transferrin (partially iron-saturated), 0.5 µg/ml sodium selenite, 1.6 mg/ml putrescine, and 0.73 µg/ml progesterone.

For morphology analysis, cells were plated at a defined cell density (2×10^5 cells/ml) in 6 cm ø dishes with collagen-coated glass coverslips in standard or serum-free media. After 6 hours, compounds/inhibitors were added at the indicated concentrations. Stimulation with 20% FBS served as a positive control for peroxisome elongation/proliferation. HepG2 cells were incubated with 20-80 nM of rapamycin for 2 hours upon serum stimulation. PPAR agonists and antagonists were also tested in this system. Summary of compounds and the concentrations used for this experiment are shown in **Table 3.1.** Fatty acid stimulation was performed by addition of 25-50 µM of arachidonic acid (C20:4(ω-6), linoleic acid (C18:2 ω-6), oleic acid (C18:1 ω-9) and palmitic acid (C16:1 ω-7) to cells incubated for 6 hours in serum-free media. Fatty acids (Sigma) were dissolved in in isopropyl alcohol at a stock concentration of 40 - 80 mM and stored

at -20. Prior to the experiment, the stocks were diluted in MEM serum free media. Cells were incubated for 6, 24 and 48 hours and processed for immunofluorescence or immunoblotting (coverslips were omitted). For immunofluorescence, peroxisomes were labelled with a combination of anti-PEX14 and Alexa-488 antibodies, and at least 100 cells per preparation were quantified. YFP-LC3 was transfected into HepG2 cells using Lipofectamine (see general methods). All experiments were performed in triplicates. Table

Type of Compounds	Component	Concentration
m TOR inhibitior	Rapamycin	10 nM,15 nM, 20 nM, 40 nM,80 nM
PPARγ agonist	Trosiglitazone	100 nM, 80 nM,40 nM
PPARγ agonist	Rosiglitazone	100 nM, 80 nM,40 nM
PPARα agonist	Wy-14,643	100 nM,80 nM,40 nM

Table 3.1 List of compounds used in cell-based assay

3.2.2 Cell density and growth curve

HepG2 cells were cultured under serum-free conditions supplemented with N1 or under standard conditions in MEM/FBS. For analysis of cell growth, cell numbers were determined every 24 hours for a period of 4 days after seeding. In order to determine the cell density over time, the absorbance of a cell suspension at OD_{600} was measured using a spectrophotometer. The cell number was also determined using a Neubauer chamber. Cell growth experiments were performed in triplicates.

3.2.3 RNA extraction

Total RNA was extracted from HepG2 cells cultured under standard conditions using the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). Samples were collected at 6, 12, 24, 48 and 72 hours after cultivation in quadruples. The isolation steps were followed according to the manufacturer's protocol. DNA was removed by on-column digestion with rDNase. The concentration of the extracted RNA was determined with a Nanodrop-1000 spectrophotometer (NanoDrop Technologies, Inc).

3.2.4 Quantitative real-time PCR

First-strand cDNA was synthesized from 2 µg of RNA with random hexamer primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and diluted 1:10 in sterilized miliQ water. The real-time PCR reaction was set up in a final volume of 20 µl using 2x light cycler 480 SYBR Green I MasterMix (Roche Diagnosis, Mannheim, Germany). PCR reactions were performed in duplicate using a Roche LightCycler 480. Thermal cycling was carried out with a 5 minutes denaturation step at 95°C, followed by 45 three-step cycles: 10 sec at 95°C, 10 sec at 60°C, and 10 sec at 72°C. The relative mRNA amount was calculated using the comparative threshold cycle (C_T) method. 18S rRNA was used as the invariant control. The quantitative real-time RT-PCR was performed in cooperation with Dr. W. Kovacs (ETH Zurich, Switzerland). Primers used are listed in Supplementary **Figure S 3.1.**

3.3. Results

3.3.1 Implementation of a cell-based peroxisome proliferation assay

In order to develop an inducible system for the investigation of peroxisome dynamics, proliferation and signalling in human cells, HepG2 cells were used and culture conditions tested and optimised (e.g. cell batches, media composition, cell density, time points) to reproduce earlier, published results (Schrader *et al.*, 1996; Schrader *et al.*, 1998b). When seeded under standard culture conditions in MEM/10% FBS at low cell density, peroxisomes in HepG2 cells undergo a series of morphological alterations, which have been linked to peroxisome proliferation/multiplication by growth and division (Schrader *et al.*, 1996; Schrader *et al.*, 2016; Schrader *et al.*, 1998b). We seeded a selected stock of HepG2 cells at a density of 2×10^5 cells/ml on collagen-coated coverslips. Alterations in peroxisome morphology were then monitored and quantified over time (4-72 hours) by immunofluorescence microscopy using antibodies against the peroxisomal membrane marker PEX14 (Grant *et al.*, 2013; Nguyen *et al.*, 2006) (**Figure 3.1 A, B**). As described before, HepG2 cells exhibiting tubular, elongated peroxisomes increased in a time-dependent manner after seeding reaching a maximum after 24 hours (**Figure 3.1 A, B**). This is due to a stimulation of peroxisomal membrane growth. Then cells with elongated peroxisomes declined, giving rise to cells with spherical peroxisomes. This decline is due to division of elongated peroxisomes into spherical organelles and contributes to peroxisome multiplication/proliferation. These findings show that peroxisomes in HepG2 cells respond to growth-stimulating culture conditions with peroxisomal growth and division.

As shown, peroxisomes change their abundance and morphology in response to external stimuli. Formation of tubular peroxisome can be an indicator of inducing peroxisome proliferation by adding specific stimuli to the cells. The aim was now to develop an inducible cell culture assay to investigate different stimuli (or inhibitors) and their effect on peroxisome dynamics and proliferation in order to identify signalling pathways involved in these processes. We previously showed that HepG2 cells can also be cultured under serum-free conditions, where a change in peroxisome morphology from spherical shapes to tubular forms can be achieved upon serum stimulation (Schrader *et al.*, 1998b). Culturing HepG2 cells under serum-free (but supplemented) culture conditions has the advantage that peroxisomes remain spherical under those conditions making it possible to readily monitor peroxisome elongation when a specific stimulus is added (Schrader *et al.*, 1998b).

This cell-based peroxisome proliferation assay can be used for stimulus or inhibitor screening (see chapters 3 and 5) in serum-free conditions, but also for the monitoring and analysis of peroxisome dynamics by fluorescence microscopy and live cell imaging. In addition, it opens possibilities for RNA profiling (e.g. monitoring the RNA level of peroxisomal genes during the time course and correlation of expression to peroxisome morphology; see 3.3x), metabolomics and proteomics under standard and serum-free culture conditions to further study peroxisome dynamics and proliferation (see **Figure 3.1 C**).

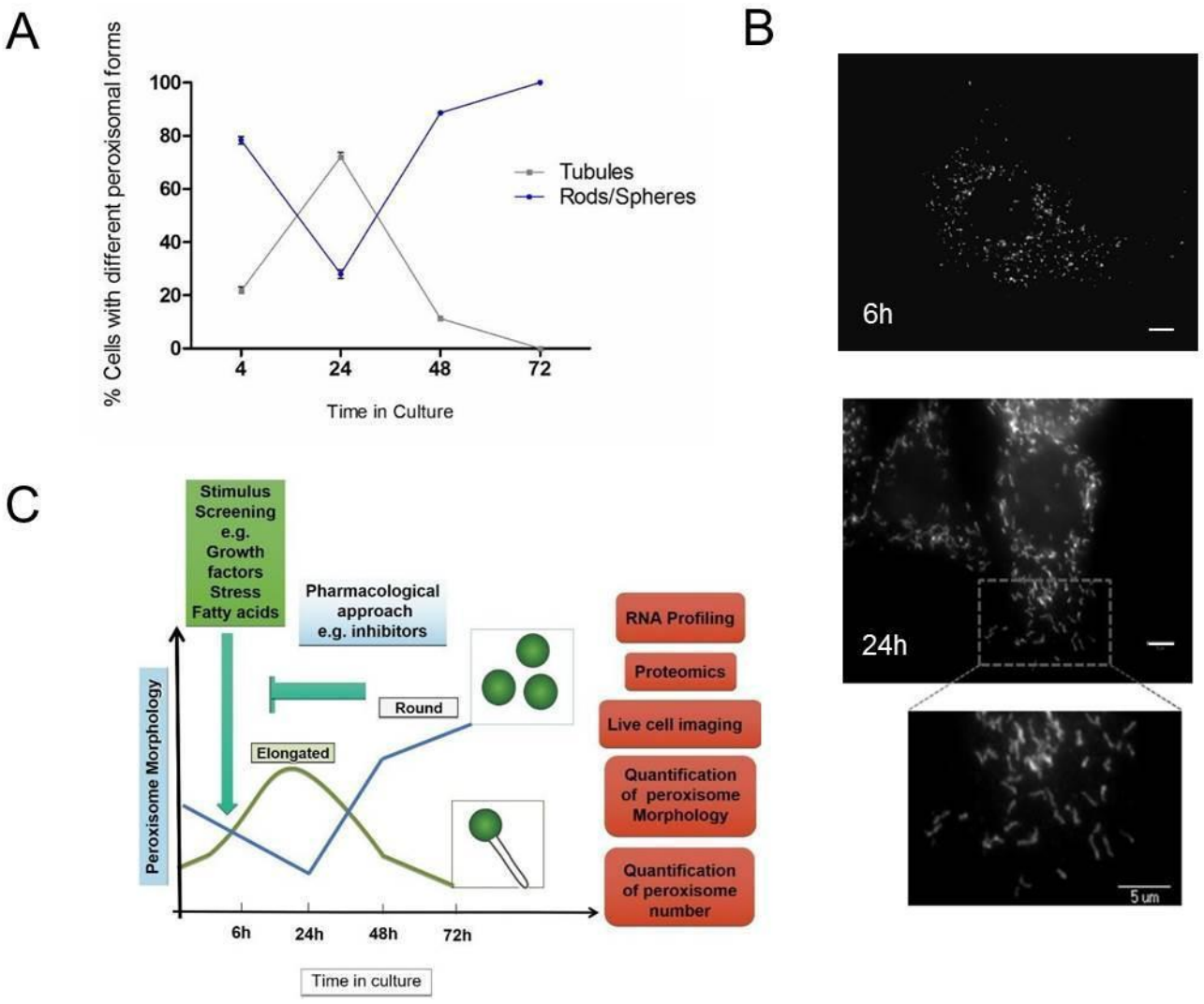


Figure 3.1. Overview of the cell-based peroxisome proliferation assay. (A) Quantification of peroxisome morphology over time in HepG2 cells grown under standard culture conditions and processed for immunofluorescence. **(B) Immunofluorescence of HepG2 cells showing tubular peroxisomes.** Cells were cultured for 6 and 24 hours under standard conditions, processed for immunofluorescence and stained with anti-PEX14 antibodies. Bars, 5 µm. **(C) Cell-based peroxisome proliferation assay and its applications under standard and serum-free culture conditions** (for example, stimulus screening, pharmacological approaches, RNA profiling, live cell imaging, protein expression, quantification of peroxisome dynamics and number).

To establish this assay in our laboratory in Exeter with our current HepG2 cells required optimization, including the routine cell culture conditions, serum-free

conditions, time-points, and cell density. After testing different types of media, the ATCC recommended medium (MEM) was selected based on the healthy cell morphology and the highest rate of peroxisomal membrane tubulation/proliferation. To select optimal conditions for the serum-free culture, we switched to a commercially available N1 supplement (Sigma). For optimization, 0.25% BSA was added to the N1 solution (N1 final composition: 0.5 mg/ml recombinant human insulin, 0.5 mg/ml human transferrin (partially iron-saturated), 0.5 µg/ml sodium selenite, 1.6 mg/ml putrescine, 0.73 µg/ml progesterone, 0.25% BSA). Addition of BSA to serum-free MEM/N1 medium had a beneficial effect on the induction of elongated peroxisomes by stimulation with serum (**Figure 3.2**).

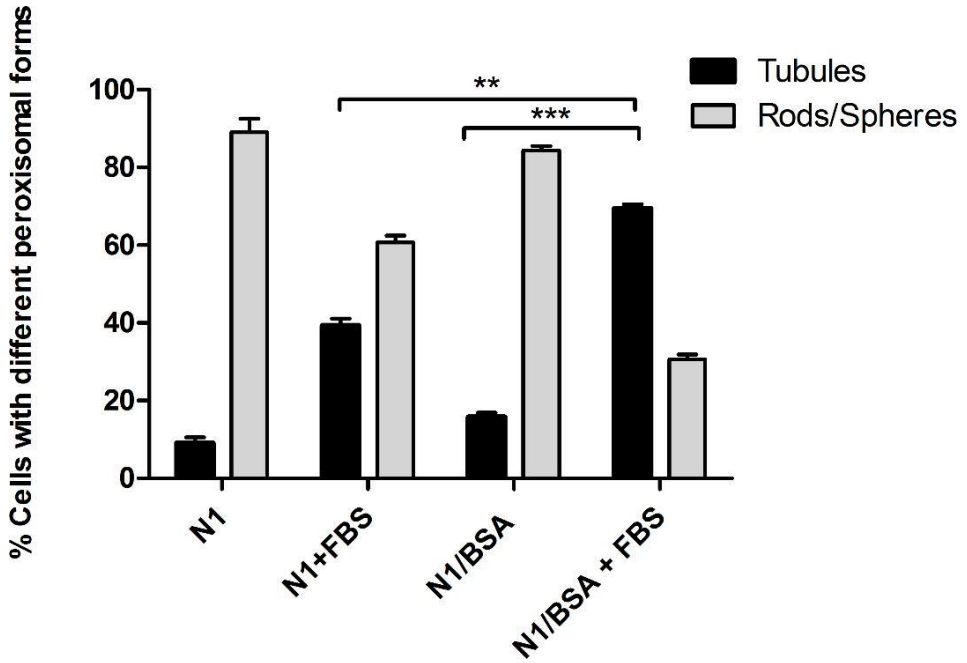


Figure 3.2. Morphological changes of peroxisomes with time in culture in HepG2 cells cultured in MEM/N1 with and without BSA. HepG2 cells were seeded in different media, processed for immunofluorescence after 24 hours using anti-Pex14 antibodies, and peroxisome morphology quantified. Addition of BSA to serum-free MEM/N1 medium

is beneficial for the induction of elongated peroxisomes by the addition of serum. Data represent mean ± SEM of 3 independent experiments (n=3 cells). *** p<0.001, ** 0.001 to 0.01, two-tailed unpaired t-test

For analysis of peroxisome morphology and number, 2 × 10^5 cells/ml were plated in 6 cm dishes with collagen-coated glass coverslips in standard or serum-free media. The cells were incubated for 6 hours in serum-free medium supplemented with N1 and either stimulated by the addition of 10% FBS or left untreated. After 24 hours, the cells were processed for immunofluorescence, and peroxisomes were labelled with a combination of anti-PEX14 (pcR) and Alexa-488 (DaR). Peroxisome morphology was analysed under the fluorescence microscope and at least 100 cells/coverslip were quantified. Peroxisomes were either scored as “Rods /Spheres” or “Tubules” based on visual analysis (**Figure 3.3**). The data indicates that in HepG2 cells grown under serum-free conditions only around 12% of cells contain tubular peroxisomes after 24 hours. However, stimulation with 10% FCS increases the number of cells with tubular peroxisomes to 70-80% (**Figures 3.2 and 3.3B**). Furthermore, peroxisome numbers increased almost 3-fold after 48 hours when compared to 6 hours. This indicates that elongated peroxisomes, which form through serum-stimulation with a maximum after 24 hours, divide by fission and contribute to peroxisome proliferation (**Figure 3.3C**). These observations indicate that peroxisomal growth and division is reduced under serum-free culture conditions, and that the peroxisomal compartment is responsive to serum stimulation.

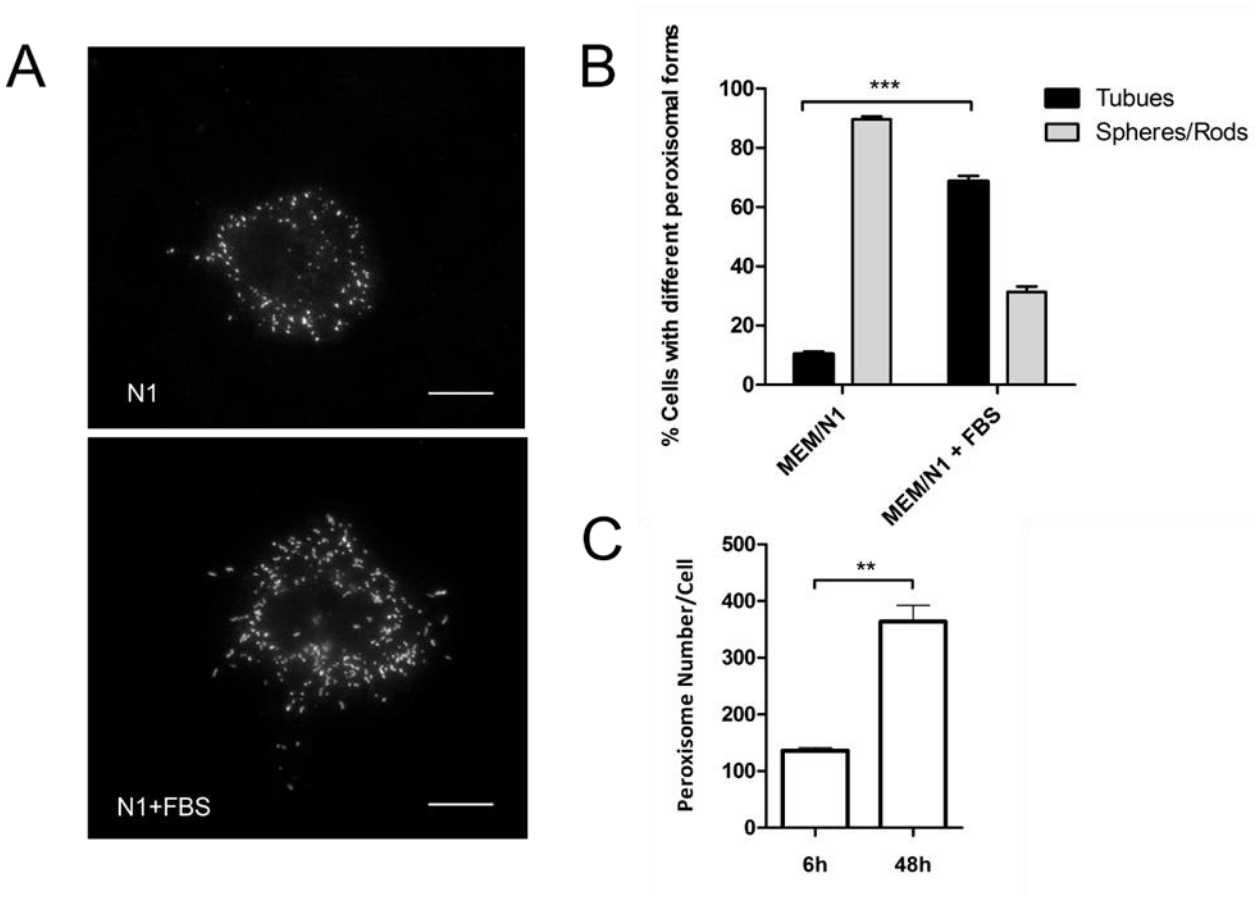


Figure 3.3. Peroxisome morphology analysis of HepG2 cells in serum-free N1 medium upon serum stimulation. (**A**) Representative images of cells cultured in N1 and cells stimulated with FBS (N1+FBS) after 24 hours. HepG2 cells cultured in MEM media with and without FBS stimulation were processed for immunofluorescence after 24 h and stained with anti-PEX14 (pcR) and Alexa-488 (DaR) antibodies. Bars, 20 µM (**B**) Quantitative analysis of peroxisome morphology.) **(C)** Quantitative analysis of peroxisome number. Data represent mean ± SEM of 3 independent experiments (n=3 cells in each condition). *** $p<0.001$ two-tailed unpaired t-test.

3.3.2 HepG2 cells cultured in MEM/N1 medium grow similar to cells cultured in MEM/FBS

Cells in conditions of complete nutrient deprivation cease to grow exponentially.

In our system, the cells are grown under serum-free condition with N1

supplement. To test if cellular growth is comparable to standard growth conditions or disturbed, we determined and compared cellular growth over time. In our assay, two methods (counting number of cells in a Neubauer chamber and OD measurement) has been used. Cells cultured in N1 medium (serum-free medium (MEM) + N1 supplement) display slightly lower growth rates when compared to standard conditions. However, the cells are actively proliferating and an exponential increase in cell density arises, indicating that the growth conditions are suitable for the study of peroxisome dynamics. It should, however, be noted that the cells appear smaller and are not as extensively spread as HepG2 cells grown under standard conditions (**Figure 3.4**).

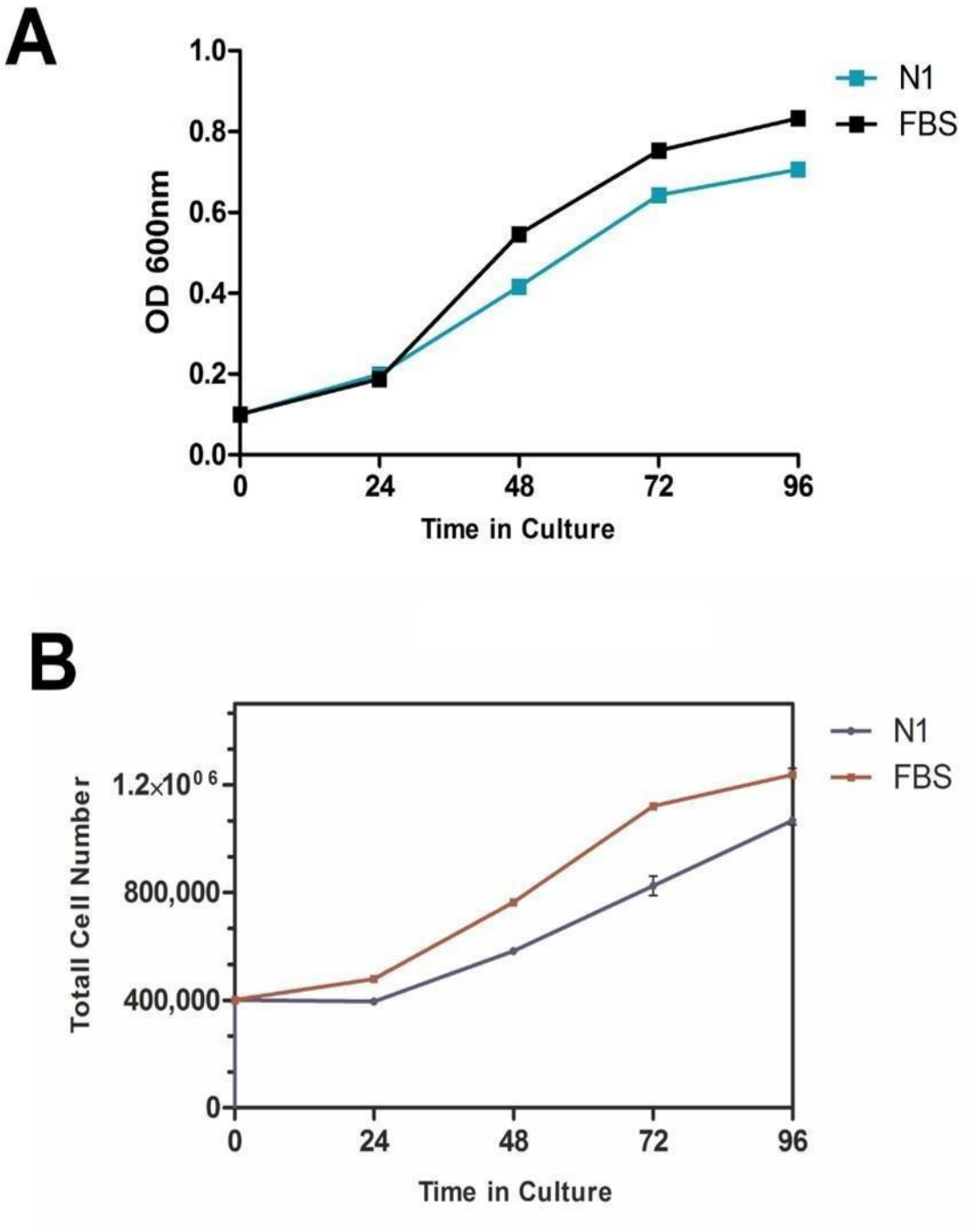


Figure 3.4. HepG2 cell density and cellular growth curve. (**A**) Cellular growth curve based on measuring absorbance at OD_{600} at the indicated time points. (**B**) Cellular growth curve based on counting cell number at the indicated time points (Neubauer chamber). An exponential increase in cell density is observed in both N1 and standard (FBS) conditions. All measurements were from experiments performed in triplicate. Data are expressed as mean ± SD of cell densities determined in three independent experiments.

3.3.3 Culture of HepG2 cells in MEM/N1 does not induce autophagy

In order to exclude increased autophagic processes in HepG2 cells cultured under serum-deprived MEM/N1 conditions, which would have an impact on peroxisome number and proliferation and reduce the effectiveness of the cell-based proliferation assay, the autophagic marker LC3 was used. Autophagy can be measured by changes in LC3 localization: tracking the level of conversion of LC3-I to LC3-II provides an indicator of autophagic activity (Tanida *et al.*, 2005). The levels of LC3-II correlate with autophagosome formation, due to its association with the autophagosome membrane. These can be detected as punctate forms in cells displaying autophagy (Tanida *et al.*, 2004).

HepG2 cells were transfected with an YFP-LC3 plasmid using lipofectamine 3000, and were examined using fluorescence microscopy (**Figure 3.5 A**). Transfected HepG2 cells with LC3 puncta (as determined by visualisation of LC3-YFP) were quantified in MEM/N1 and MEM/N1+FBS as well as after treatment with rapamycin. Rapamycin, an inducer of autophagy, was used as a positive control. Addition of DMSO (solvent) did not alter LC3 puncta when compared to conditions without DMSO (**Figure 3.5 B**). LC3 puncta were clearly observed when rapamycin was added (**Figure 3.5 A, c-d**). In contrast, only 11% of cells in MEM/N1 showed LC3 puncta which was not significantly different from controls with FBS (**Figure 3.5 B**). This clearly shows that growth in MEM/N1 does not induce autophagy.

Growth factors induce the activation of p70 S6 kinase and the subsequent phosphorylation of the S6 ribosomal protein (An *et al.*, 2003). Phosphorylation of S6 correlates with an increase in translation of mRNA transcripts required for cell cycle progression and synthesis of ribosomal proteins as well as elongation

factors necessary for translation. Inhibition of mTORC1 by rapamycin can cause a decrease in PS6 level (An *et al.*, 2003). To determine the level of phosphorylated S6, lysates from HepG2 cells cultured in MEM/N1, MEM/N1 + FBS or 20 nM rapamycin were analysed by immunoblotting (**Figure 3.5 C**). As described in the literature, the level of PS6 using the phospho-specific PS6 antibody was decreased by addition of rapamycin (blocking mTOR) compared to the control containing FBS. Cells growing in MEM/N1 exhibit the same level of phosphorylated S6 ribosomal protein compared to controls containing serum, indicating that the translation of proteins involved in cell cycle progression and ribosome formation is active under our experimental conditions.

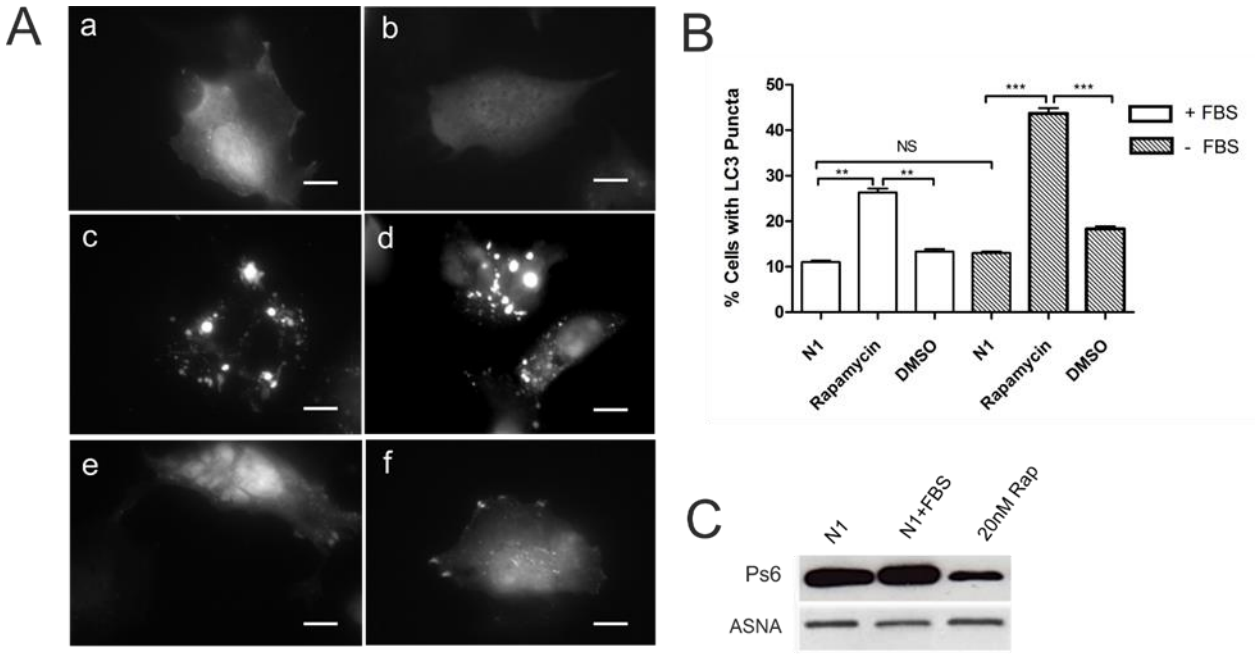


Figure 3.5. Autophagy is not induced in MEM/N1 condition (A) HepG2 cells transfected with YFP-LC3 (lipofectamine transfection) and controls were incubated for 24 hours in (a) MEM/N1 supplemented with FBS, (b) plain MEM/N1 with no FBS ,(c) FBS+rapamycin, (d) MEM/N1 containing rapamycin , (e) FBS+DMSO (solvent), or (f) plain MEM/N1 containing DMSO. Cells were fixed and prepared for fluorescence

microscopy after 24 hours of treatment. Bar= 5µm **(B)** Quantitative analysis of cells with LC3 puntca under different conditions. Data represent mean ± SEM of 3 independent experiments (n=300 cells in each condition). *** p<0.001 two-tailed unpaired t-test. **(C)** Immunoblot of lysates from HepG2 cells cultured as indicated. 40 ug of protein was loaded on 10% SDS-PAGE and immunoblots were incubated with anti-Phospho-S6 Ribosomal Protein (PS6) and anti-ASNA (loading control), an ATPase required for the post-translational delivery of tail-anchored (TA) proteins to the endoplasmic reticulum.

3.3.4 Investigation of different stimuli and their ability to induce peroxisome proliferation in mammalian cells

Following optimization of the cell-based peroxisome proliferation assay, we now have an inducible system in hand to test different stimuli and their effect on peroxisome dynamics and proliferation. A set of stimulating compounds such as fatty acids, PPAR agonists and antagonists have been used in this study. Applications of this assay including the testing of inhibitors and stimuli on peroxisome proliferation and alterations in peroxisomal gene expression are illustrated in **Figure 3.1**.

3.3.4.1 Distinct fatty acids are potent inducers of peroxisome elongation/proliferation in HepG2 cells

It is known that fatty acids regulate cellular function by modulating the rates of transcription of various target genes including the peroxisomal acyl-CoA oxidase (ACOX1) through activation of the PPAR-RXR complex (Rakhshandehroo *et al.*, 2010). Several studies have reported the transactivation of PPAR family members through the administration of several fatty acids, in different organisms and cell models (Benjamin *et al.*, 2013). However, despite some knowledge

on the regulation of peroxisomal enzymes, the effect of different fatty acids on peroxisome dynamics and proliferation in humans remains scarce. Making use of our cell-based assay, we analysed the effect of saturated and unsaturated fatty acids on peroxisome dynamics in HepG2 cells. The fatty acids used were: arachidonic acid (AA) (C20:4(ω-6), linoleic acid (LA) (C18:2 ω-6), palmitic acid (PA) (C16:1 ω-7) and oleic acid (OA) (C18:1 ω-9).

Arachidonic acid (AA), a 20-C polyunsaturated fatty acid of the 0-6 class, serves as a precursor for the synthesis of a number of biologically active lipid mediators (Irvine, 1982). Depending on chain length and degree of unsaturation, fatty acid β-oxidation occurs in either the mitochondria or peroxisomes (Schulz *et al*., 1991).

Linoleic acid (LA**)** is a polyunsaturated fatty acid used in the biosynthesis of arachidonic acid (AA) and thus some prostaglandins. It has been shown that treatment of HepG2 cells with linoleic acid leads to an increase in PPARγ, PPARβ and ACOX1 (Lu *et al.*, 2015).

We used AA as a positive control since it has been reported to induce peroxisome elongation/proliferation (Schrader *et al.*, 1998b) and indeed our results show that AA causes a prominent increase in the percentage (45-70%) of cells with tubular peroxisomes (**Figure 3.6**). In fact, when adding 50 µM AA to the medium, the percentage of cells with tubular peroxisomes is of the same magnitude as when serum is added to the N1 medium. The addition of LA to the medium had a less pronounced effect on peroxisome elongation with about 44% of the cells exhibiting that phenotype. On the other hand, OA and PA were not able to induce peroxisome elongation at the concentrations applied (**Figure 3.6**). However, as observed for AA and LA, the number and size of lipid droplets was increased in

the cells (not shown), indicating that the different fatty acids are properly taken up and stored by the cells. Whereas PA is a target for mitochondrial β-oxidation, OA is supposed to be mainly degraded by peroxisomal fatty acid β-oxidation. Even though OA is likely able to trans-activate PPAR family members and to undergo peroxisomal β-oxidation in HepG2 cells, it seems that the addition of general fatty acid substrates for peroxisomal β-oxidation is not sufficient to induce peroxisome elongation. The effect appears to be more specific for LA and AA, which link to the synthesis of cellular lipid mediators.

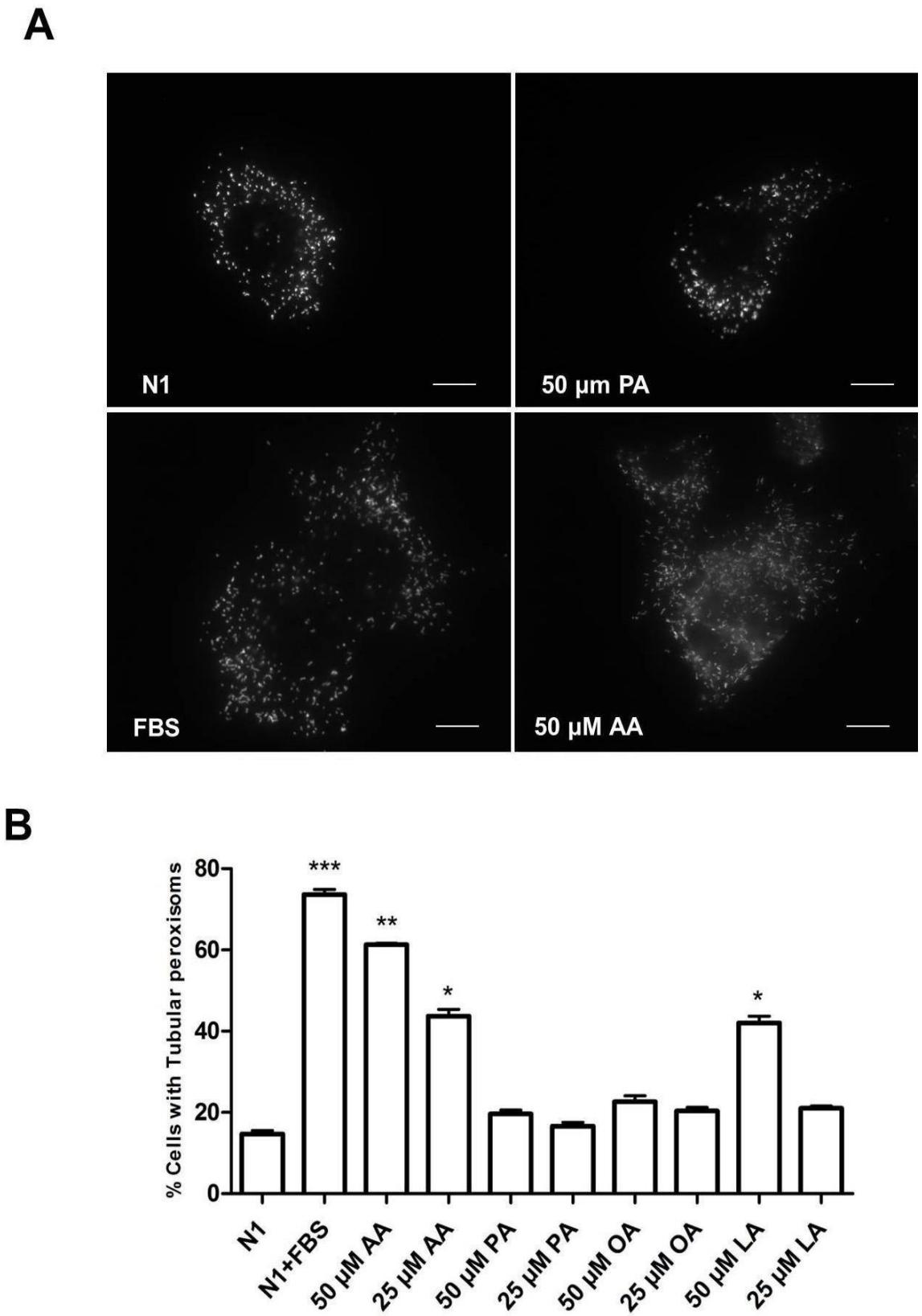


Figure 3.6. Arachidonic acid induces the formation of tubular peroxisome in our system, whereas palmitic acid and oleic acid have no effect (A) Representative images of immunofluorescence preparations with PEX14-Alexa488 antibodies of HepG2

cells cultured in MEM/N1 (negative control), MEM/N1 supplemented with FBS (positive control) or MEM/N1 supplemented with fatty acids. Bars, 20 µM **(B)** Quantitative analysis of cells with tubular peroxisomes upon fatty acid stimulation in HepG2 cells seeded in MEM/N1 (N1). Data represent mean± SEM of 3 independent experiments (n=3).

3.3.4.2 PPARα Agonists do not induce peroxisome elongation in HepG2 cells cultured in N1 medium

Previous studies have shown that fibrates such as bezafibrate, fenofibrate, Wy-14,643 and clofibrate are PPARα agonists and significantly increase peroxisome number and the levels of fatty acid β-oxidation enzymes (L. Guo *et al.*, 2006; Lazarow & De Duve, 1976; Schrader *et al.*, 2012). The effect of these compounds on peroxisome proliferation in mammals is restricted to the superfamily of *Muroidea*, whereas the effect in humans is very mild (Islinger *et al.*, 2010; Lawrence *et al.*, 2001a). Testing physiological concentration (10µM, 15µM and 20 µM) of this agonist in our HepG2 assay did not induce peroxisome tabulation.

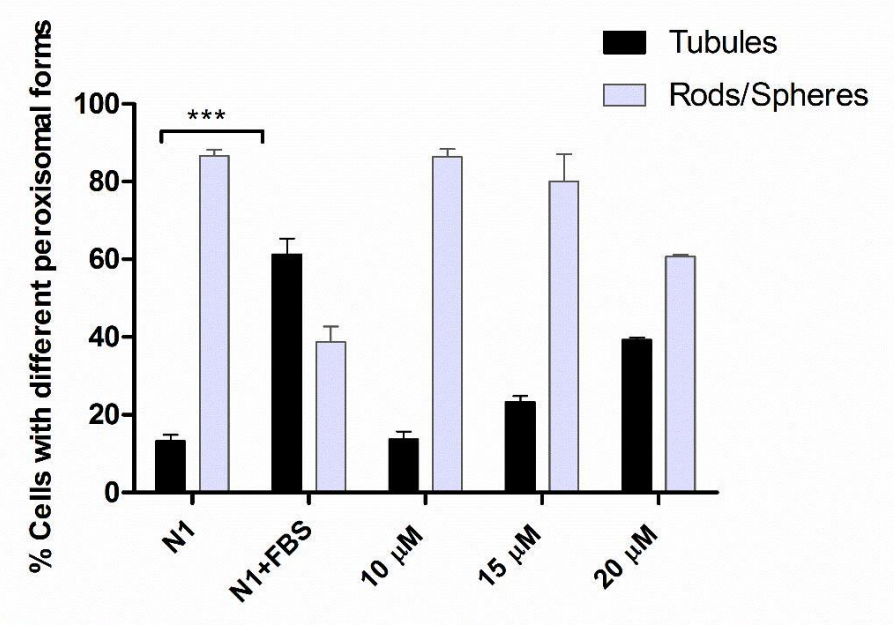


Figure 3.7 The PPARα agonist Wy-14,643 **does not induce the formation of tubular peroxisome in our cell model.** Quantitative analysis of peroxisome morphology in HepG2 cells seeded in N1, stimulated with FBS, or 10-15 and 20 µM of Wy-14,643. Data represent mean ± SEM of 3 independent experiments) *** $p<0.001$; two-tailed unpaired t-test (n=3).

The PPARγ ligand troglitazone, one of the thiazolidinediones which are used in the treatment of type II diabetes, is a potent and selective activator of PPARγ (Blaschke *et al.*, 2006). The role of troglitazone in the growth of cancer cells has been elucidated in some studies (Shimada *et al.*, 2002). It has been shown that troglitazone has negative effects on the proliferation of malignant tumor cells, including colon cancer cells (Lucarelli *et al.*, 2002). The effect of this agonist on peroxisome proliferation has not been tested. An effect on fatty acid cycling in HepG2 cells has, however, been described (W. N. Lee *et al.*, 1998). Here, different concentrations of PPARγ agonist have been tested to induce peroxisome elongation/proliferation. Our results showed that the maximal dose of PPARγ

agonist (15 µM) only induced the number of cells with tubular peroxisomes to 30% compared to the control group in N1, which is 20% (**Figure 3.8**).

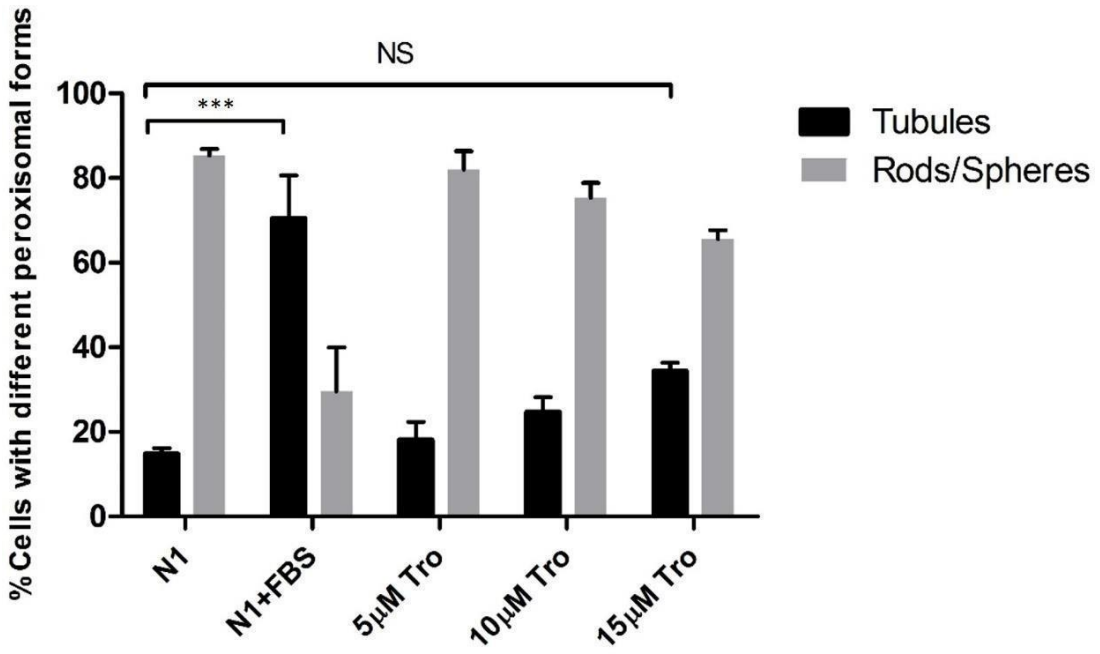


Figure 3.8 The PPARγ agonist Troglitazone does not induce the formation of tubular peroxisome in our cell model. Quantitative analysis of peroxisome morphology in HepG2 cells seeded in N1, stimulated with FBS, or 5-15 µM of Troglitazone. Data represent mean ± SEM of 3 independent experiments) *** $p<0.001$; NS $p \geq 0.05$; two-tailed unpaired t-test (n=3).

PPARγ antagonists such as GW9962 inhibit the PPARγ binding to its binding elements (X. Liu *et al.*, 2016). In this study, we used 20 - 80 µM of GW9962 to monitor the peroxisome morphology in our assay, when the agonist was applied in combination with or without FBS. The cells were cultured for 4 hours, pre-incubated for additional 2 hours with the PPARγ antagonist, and 6 hours after cultivation serum stimulation was performed (**Figure 3.9**). Based on our immunofluorescence and quantification data, GW9962did not repress

peroxisome elongation/proliferation induced by serum. This implies that serum contains factors which can induce peroxisome proliferation independently of PPARγ.

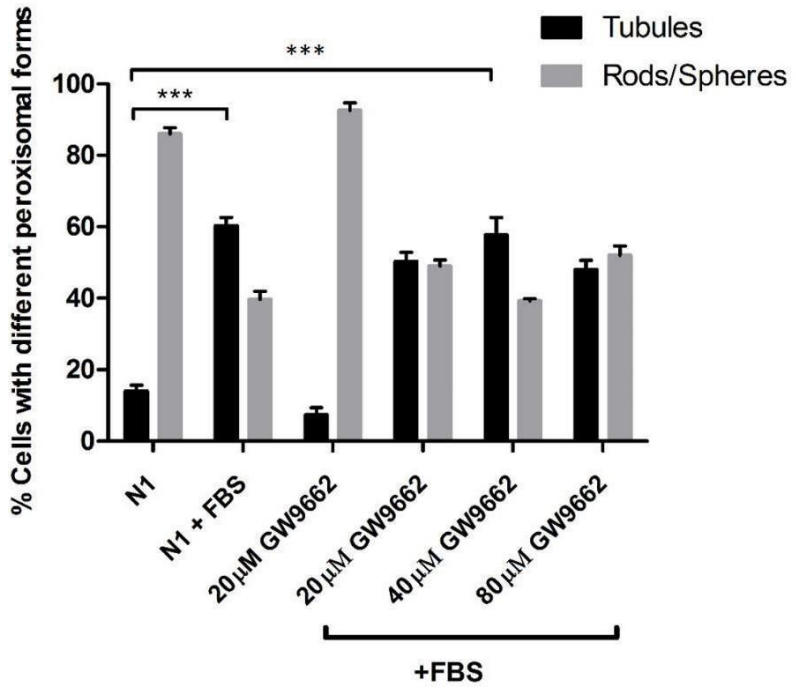


Figure 3.9. The PPARγ antagonist GW9962 does not repress serum-induced peroxisome elongation/proliferation. Quantitative analysis of peroxisome morphology in HepG2 cells seeded in N1, stimulated with FBS, and pre-treated with 20 μM, 40 μM and 80 μM of GW9962. Data represent mean ± SEM of 3 independent experiments) *** $p<0.001$; ** $p<0.01$; two-tailed unpaired t-test (n=3).

3.3.4.3 mTOR signalling plays a role in the initiation of peroxisome elongation and proliferation

Our previous studies revealed a role for serum/growth factors in the elongation and proliferation of peroxisomes in mammalian cells (Schrader *et al.*, 1998b) and experiments above). We also exploited our cell-based assay for live cell analyses of peroxisomal alterations under N1 and serum-stimulated conditions. Imaging of peroxisomes in MEM/N1 revealed overwhelmingly spherical peroxisome with little elongation or protrusion-forming activity (not shown). Interestingly, addition of serum promoted peroxisome elongation and protrusion-formation as well as overall motility of peroxisomes (data under analysis). Overall, these findings clearly demonstrate a role for serum factors in peroxisome dynamics and proliferation and suggest the involvement of growth factor-dependent pathways like the mTOR pathway. In order to investigate the involvement of the mTOR pathway in peroxisome elongation/proliferation in our assay, cells were treated with the mTOR inhibitor rapamycin (Rap). HepG2 cells were cultured for 4 hours, pre-incubated for additional 2 hours with rapamycin (20 – 80 nM), and 6 hours after cultivation serum stimulation was performed. Quantification of immunofluorescence preparations (**Figure 3.10**) revealed that rapamycin treatment diminished peroxisome elongation in a dose-dependent manner (**Figure 3.10 A-B**). These data complement our previous observations and support our hypothesis that the mTOR pathway plays a role in the initiation of peroxisome proliferation during cellular growth. Higher concentration of rapamycin (2.5 µM and 5µM) have been used in our laboratory to study pexophagy in human fibroblasts. Even with these concentrations no effect on

peroxisome number was detected after 24 hours (Josiah Passmore, unpublished data)

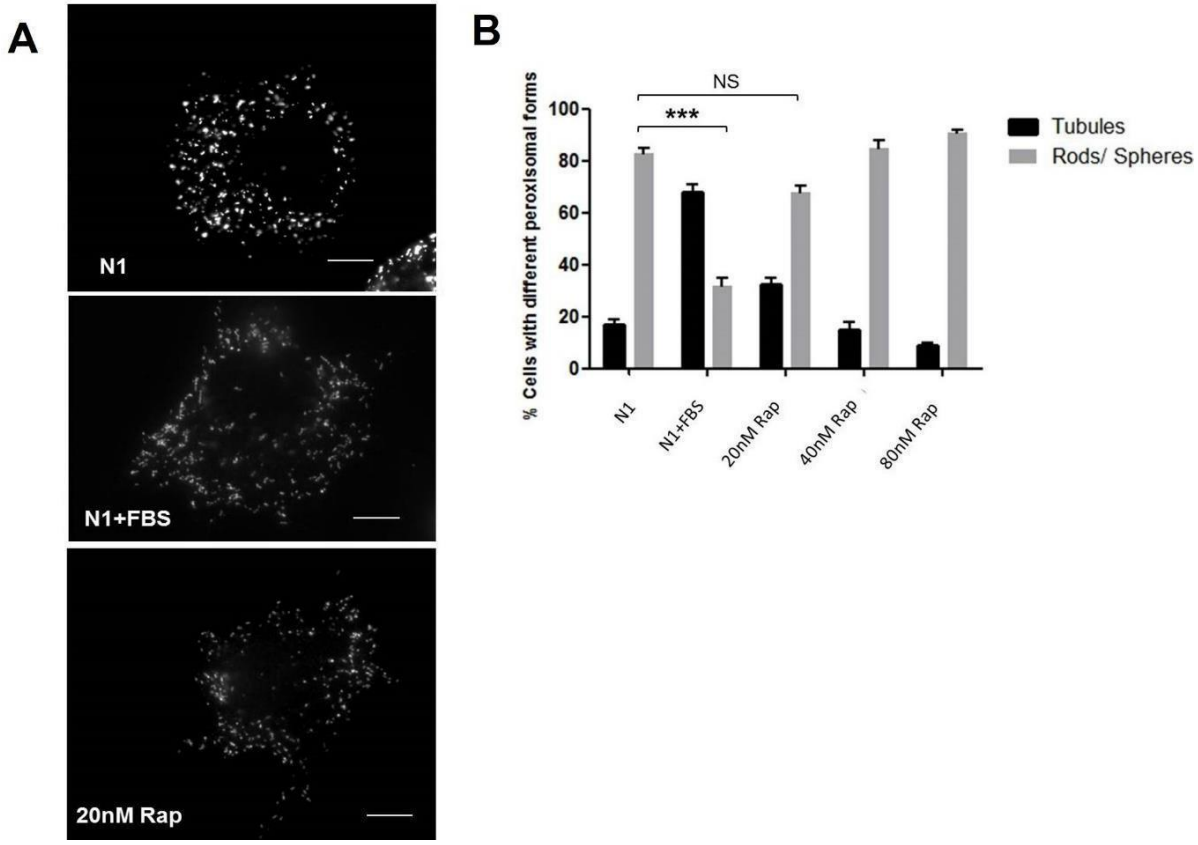


Figure 3.10. The mTOR inhibitor rapamycin inhibits peroxisome elongation. **(A)** Representative images of HepG2 cells cultured in MEM/N1 (N1), MEM/N1+FBS and 20 nM rapamycin+FBS. Cells were processed for immunofluorescence after 24 hours and labelled with anti-PEX14/Alexa-488 antibodies. Bars, 20 µM. **(B)** Quantitative analysis of peroxisome morphology after treatment of HepG2 cells with rapamycin (20 nM—80 nM) and subsequent serum-stimulation. Data represent mean ± SEM of 3 independent experiments (n=3). *** $p<0.001$, ^{NS} $p \geq 0.05$ two-tailed unpaired t-test.

3.4 Correlation of peroxisome morphology and peroxisomal gene expression

Little is known about the regulation of peroxins and peroxisomal membrane proteins and the relation of gene expression with peroxisome proliferation, in particular in humans. The only peroxin whose expression has so far been linked to peroxisome proliferation in humans is PEX11 (Schrader *et al.*, 2016). As indicated before (see **Figure 3.1 C**), our cell-based assay provides an opportunity to monitor peroxisome morphology as well as peroxisomal gene expression by RNA analysis.

In order to investigate and correlate alterations in the gene expression (and potential signaling factors) with peroxisome proliferation events, we exploited our established assay which shows proliferation-associated alterations of peroxisome morphology in standard medium over a time course in culture (**Figure 3.1 A**).

As shown before (**Figure 3.1 A)**, the number of elongated peroxisome reaches a maximum after 24 hours before declining giving rise to spherical peroxisome produced by fission. At each of the time points indicated in **Figure 3.11** (6-72 hours) cell samples were taken, RNA was extracted and then qPCR was performed to determine changes in mRNA levels over the time course. The experiment was performed in 4 replicates for each time point with the same cell number at the start point. For each tested gene the mean Ct value was determined for each time point. The change in expression of a subset of peroxisomal genes was normalized to18S rRNA. $\Delta\Delta$Ct was calculated for each time point compared to 6 hours (eg, $\Delta\Delta Ct=\Delta Ct12-\Delta Ct6$). The fold change for each time point was calculated using $2^{-\Delta\Delta Ct}$ (**Figure 3.11C**). Furthermore,

morphological changes of peroxisomes over time in the same cohort of cell samples (seeded on coverslips) was assessed by immunofluorescence. Samples were stained with anti-PEX14 antibodies, and peroxisome morphology was quantified (**Figure 3.11 A**).

Interestingly, an increase in the expression level of PEX11β was observed with a maximum at 24 hours and subsequent decline in expression. These alterations correlate nicely with the morphological changes of peroxisomes, supporting the view that PEX11β expression promotes peroxisome elongation and subsequent division (**Figure 3.11 A-B**). Furthermore, we monitored the PEX11β protein level in this assay by immunoblotting, as it is illustrated in **Figure 3.11 c**. We also observed an increase in PEX11β protein up to 24-48 hours before protein levels declined. In contrast to PEX11β, the mRNA levels of PEX11α and PEX11γ were not observed to increase and to correlate with peroxisome elongation, but rather declined (**Figure 3.11**). The PEX11 peroxisomal membrane proteins are supposed to promote peroxisome proliferation and division in multiple eukaryotes. As indicated in the introduction (**Section 1.6**) in humans/mammals, the three PEX11 isoforms PEX11α, β, γ are the only peroxins identified which are thought to be involved in peroxisome proliferation. This makes PEX11 an ideal candidate for our proliferation studies. As part of our effort to understand the molecular and physiological functions of PEX11β in peroxisome proliferation, we already revealed that PEX11β expression promotes peroxisome elongation prior to fission, whereas PEX11α expression has no or little effect (our unpublished observations). We therefore suggest that PEX11α is not directly involved in peroxisome multiplication/proliferation, and may fulfill other functions, e.g. in peroxisomal metabolism.

Interestingly, expression of the early peroxins PEX19 and PEX3 was also observed to correlate with peroxisome elongation/proliferation, whereas PEX16 remained unchanged and declined at later time points. As these early peroxins are required for the insertion of peroxisomal membrane proteins including PEX11β, an increased expression of those early peroxins would be meaningful.

Other peroxins such as PEX12, PEX10 and PEX14, which are involved in peroxisomal matrix import, showed a minor increase after 24 hours followed by a decline in expression (PEX10, PEX14) or remained unchanged (PEX12).

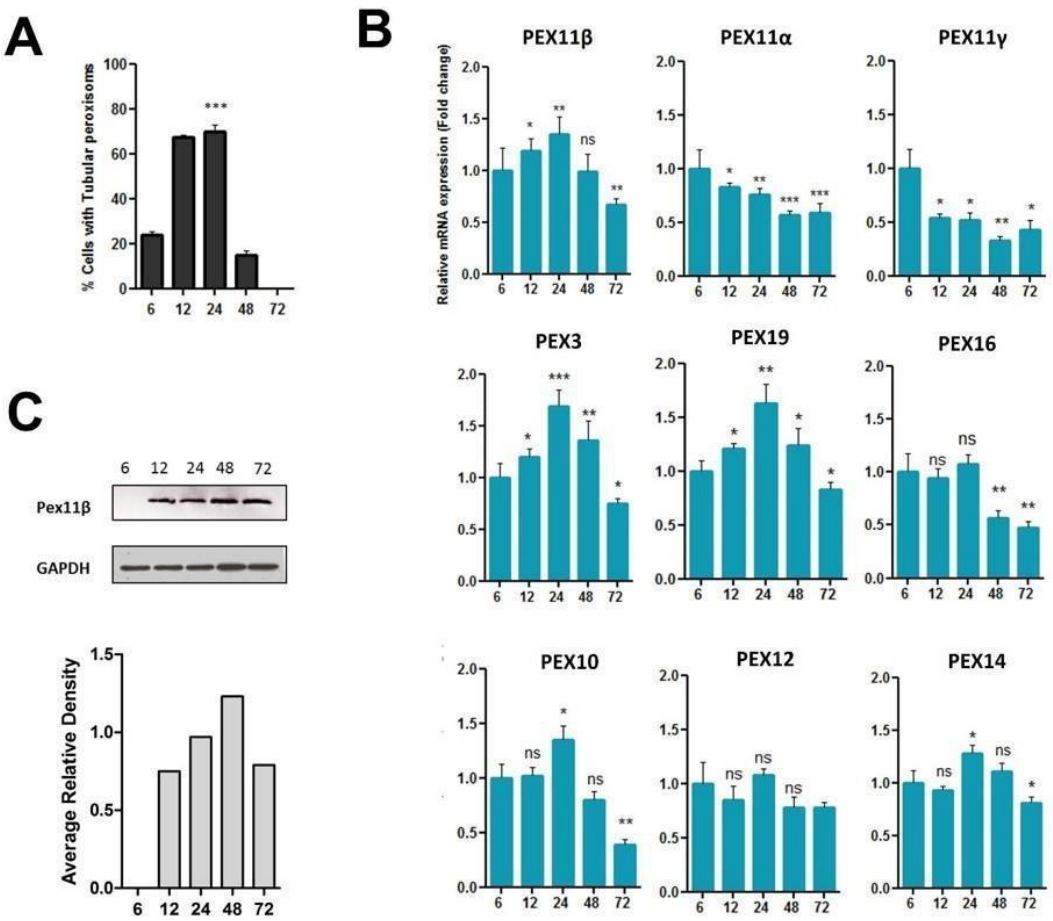


Figure 3.11. Analysis of peroxisome morphology and peroxisomal gene expression during a time course. (A) Quantitative analysis of tubular peroxisomes in HepG2 cells monitored over time in standard culture medium and processed for immunofluorescence. Data represent mean ± SEM of 3 independent experiments (n=300 cells in each condition). **(B)** qPCR, relative mRNA expression levels of a set of peroxisomal genes normalized to 18S rRNA expression in a time course experiment. Note that PEX11β expression follows the tubular peroxisome morphology, emphasizing its role in peroxisome elongation and division. In contrast, PEX11α expression does not correlate with changes in peroxisome morphology. **(C)** Immunoblot of HepG2 lysates (6 – 72 hours) using anti-PEX11β antibodies. GAPDH was used as a loading control. Density plot of protein bands normalized to GAPDH. Data represent mean ± SEM of 3 independent experiments) *** $p<0.001$; ** $p<0.01$; two-tailed unpaired t-test.

The expression profile of the transcription factors PPARα, PPARγ1 and PGC-1α which are involved in the upregulation of peroxisomal matrix protein expression (in particular β-oxidation enzymes) did not correlate with peroxisomal morphology alterations, and expression of those transcription factors rather declined over time or remained unchanged (**Figure 3.12 A).** These findings may indicate that the observed morphological alterations are independent of those transcription factors. It has however been reported that PPARα expression is upregulated in HepG2 cells at prolonged incubation times in culture (Baumgart *et al.*, 1990).

Interestingly, the expression of the peroxisomal ABC transporter ABCD3 (PMP70) follows the morphological changes of peroxisomes, whereas ABCD1, the ABC transporter for VLCFA remains unchanged.

Expression of ACOX1b, a key enzyme in the first step of peroxisomal fatty acid β-oxidation, remained unchanged, whereas MFP1, which catalyzed the 2nd and 3rd step, was slightly upregulated after 24 hours.

It should be noted that besides PEX11α regulation by PPARα, there is currently no information available on transcriptional regulation of peroxins in humans/mammals.

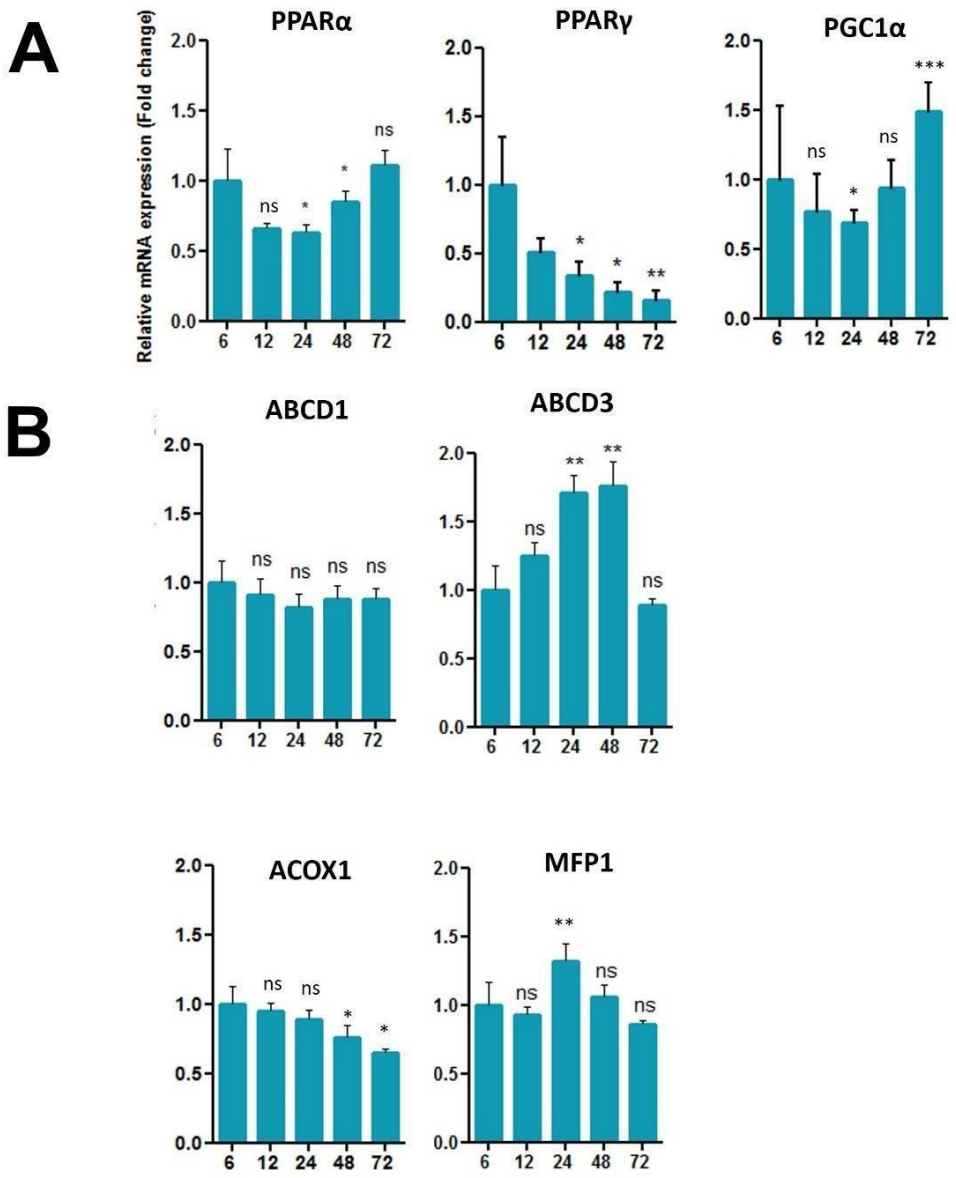


Figure 3.12. Analysis of peroxisome morphology and peroxisomal gene expression during a time course. (A) qPCR, relative mRNA expression levels of a set PPARα, PPARγ and PGC1α genes normalized to 18S rRNA expression in a time course experiment. **(B)** qPCR, relative mRNA expression levels of a set of ABCD1 and ABCD2 genes, and ACOX1 MFP1, normalized to 18S rRNA expression in a time course experiment. Data represent mean ± SEM of 3 independent experiments) *** p<0.001; ** p<0.01; two-tailed unpaired t-test.

3.5 Discussion

Peroxisome can respond to external stimuli such as fatty acids and fibrates with an increase in their number or size ((Kaur & Hu, 2009; Platta & Erdmann, 2007). Due to their critical metabolic functions, regulation of peroxisomal functions and number is vital for human health. Therefore, a better understanding of the signals involved in peroxisome proliferation and metabolic activity is necessary. It is still not clear how these organelles are connected to cellular signaling pathways, particularly in humans.

In this study, HepG2 cells were used as a model system to assess the ability of different stimuli to induce peroxisome proliferation in a cell-based approach. As previously shown, the well-differentiated human hepatoblastoma cell line HepG2 has a very plastic peroxisome compartment, displaying small spherical (0.1-0.3 µm) and rod-shaped (0.3 µm) peroxisomes as well as elongated-tubular ones (up to 5 µm and more) (Schrader et al., 1996). In this study, HepG2 cells grown under standard cell culture conditions form tubular peroxisomes (around 70% of cells) 24 hours after cultivation when seeded at a defined density. Elongation of peroxisomes is a pre-requisite of peroxisome proliferation and is followed by constriction and division of tubular peroxisomal membranes resulting in new spherical peroxisomes (Schrader *et al*., 2016) (**Figure 3.1**). Formation of tubular peroxisomes can be almost completely inhibited by culturing HepG2 cells under serum-free conditions (MEM) supplemented with N1, with HepG2 cells still demonstrating cellular growth (**Figure 3.4**) and normal levels of autophagy (**Figure 3.5**). We have established a cell-based assay using the HepG2 cell model with spherical peroxisomal forms to analyze peroxisome proliferation upon stimulation. In this system, peroxisome elongation has been used as the read-out

for peroxisome proliferation, as peroxisomes first form tubular structures and then divide (**Figure 3.3 C)**. Cells seeded in MEM/N1 showed similar growth to cells cultured under standard conditions (MEM/FBS) (**Figure 3.4**). Also, based on our results with LC3 transfection, this condition does not induce autophagy (**Figure 3.5 A-C**). Thus, the assay is a valuable tool to monitor the effect of different stimuli on peroxisome proliferation.

Quantification of different peroxisome morphologies in MEM/N1 and serum-stimulated conditions showed induction of tubular peroxisomes upon stimulation with serum which contains growth factors. One of the main growth factor-dependent pathways is mammalian target of rapamycin (mTOR) (Guri & Hall, 2016). In order to investigate the involvement of the mTOR pathway in peroxisome elongation/proliferation, rapamycin was used to inhibit this pathway through mTORC1 inhibition. Upon serum stimulation, rapamycin-treated cells showed a reduced number of tubular peroxisomes compared to non-treated cells, suggesting that the mTOR pathway may be involved in the regulation of peroxisome proliferation and tubule formation we observe under conditions of cellular growth (**Figure 3.13**). Rapamycin is functional in HepG2 cells, as Phospho-S6 Ribosomal Protein was reduced in immunoblots compared to controls when rapamycin was added (**Figure 3.5 C**).

In skeletal muscle tissues and cells, the mTOR inhibitor rapamycin decreases the gene expression level of mitochondrial transcriptional regulators, PPARγ coactivator-1 (PGC1-α) (Cunningham *et al.*, 2007). This study indicates that mTORC1 controls the transcriptional activity of PPARγ coactivator-1 (PGC1a), by altering its physical interaction with another transcription factor, yin-yang 1

(YY1) initiating expression of genes involved in mitochondrial biogenesis (Cunningham *et al.*, 2007).

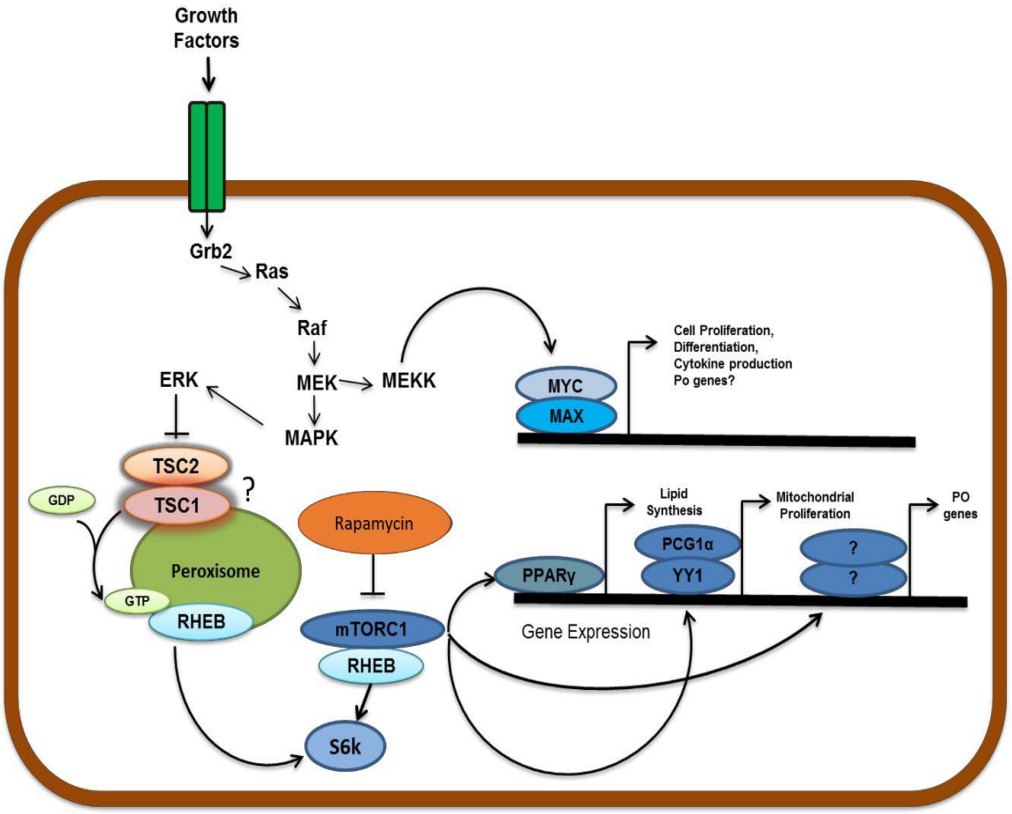


Figure 3.13. Schematic diagram of pathways being investigated and how they are related. Growth factor stimulation can activate different cascades in the cell. The Ras-Raf-MEK - cascade is one of the probable pathways which cause MAX-MYC complex activation. Activation of ERK1/2 by growth factors depends on the MAPK c-Raf. This can inhibit TSC1/TSC2. Rapamycin can block the mTOR pathway through mTORC1 inhibition.

Various types of stimuli including fatty acids, PPAR agonists and antagonists were used in our system to analyze the effect of these stimuli on peroxisome elongation/proliferation. Peroxisomal fatty acid β-oxidation involves the degradation of long chain and very long chain fatty acids (Baumgart *et al.*, 1990). It has been shown that fatty acids regulate cellular function by modulating the rates of transcription of various target genes including acyl-CoA oxidase (ACOX1)

through activating the PPAR:RXR complex. As discussed in the introduction (**See Introduction 1.7.2**), PPARs are the known regulators of lipid homeostasis and peroxisome proliferation.

The effect of several fatty acids on peroxisome proliferation has been tested in our cell-based assay, including arachidonic acid (AA), linoleic acid (LA), palmitic acid (PA) and oleic acid (OA). Based on our results, AA and LA, a precursor of AA, caused an increase in tubular peroxisomes (from 20% to 70% of total cells), comparable to the increase with serum, whereas palmitic acid or oleic acid had no effect (**Figure 3.6**). The addition of LA to the medium had a less pronounced effect on peroxisome elongation with about 44% of the cells exhibiting that phenotype

PA is a saturated fatty acid found in olive oil and palm oil and it is not a primary substrate for peroxisomal β-oxidation and is the target for mitochondrial β-oxidation, thus likely does not induce peroxisome proliferation. Although, OA is also substrates for peroxisomal β-oxidation but treatment with OA in HepG2 cell-based assay did not induce peroxisome elongation (**Figure 3.6**). This suggests that a general stimulation of peroxisomal β-oxidation by fatty acids is not the main trigger of peroxisome elongation/proliferation in HepG2 cells. The AA effect may thus not be related to peroxisomal β-oxidation. AA is involved in several pathways as a signaling molecule – e.g. in inflammation initiating gene expression of the target genes or activating the PPAR pathway (Varga *et al.*, 2011). In previous studies, it has been shown that AA can act as a second messenger in T-cells through NFκB activation. Activation of the oxidative cascade of PUFAs has been associated with cancers, including B-CLL (Solomon *et al.*, 2016). B-CLL tumor lymphocytes have been shown to produce leukotriene B4 (LTB4). LTB4 binds

PPARα, which in turn regulates the oxidative degradation of PUFAs and their derivatives, including LTB4. Moreover, PPARγ, another member of the PPAR family, contributes to the regulation of inflammation (Stienstra *et al.*, 2007).

Using the cell-based assay, the expression of several peroxisomal genes was analyzed in HepG2 cells cultured under standard culture conditions during the time course 6-72 hours after cultivation. Cells from the same set of experiments were also fixed for peroxisome morphology analysis. According to the q-PCR results, PEX11β expression followed the changes in peroxisome morphology, whereas PEX11α expression did not. This indicates that PEX11α may not directly be involved in peroxisome elongation/proliferation and may have other functions in metabolism, e.g. a role in fatty acid b-oxidation or as a pore forming protein. It also indicates that different regulatory pathways and different transcription factors may control the expression of these genes.

Although our understanding of the mechanisms by which peroxisomes proliferate is increasing, our knowledge on how the division process is linked to extracellular signals is limited. In mammals, a key player in peroxisome proliferation is PEX11β (Itoyama *et al.*, 2012; Schrader *et al.*, 1998b; Y. Yoshida *et al.*, 2015). When PEX11β is over-expressed, it causes first elongation and then subsequent division of peroxisomes (Delille *et al.*, 2010). This makes PEX11β an ideal target for signaling pathways which lead to an increase in peroxisome number. So far, there are no studies focusing on transcriptomics of genes inducing peroxisome proliferation. The function of PEX11α is less clear (Delille *et al.*, 2010) but may be linked to more specialized functions such as in lipid homeostasis or membrane contact sites (Dulermo *et al.*, 2015; Mattiazzi Usaj *et al.*, 2015). Further clarification of the role of the different PEX11 isoforms is

required to understand how transcriptional signal cascades produced by external stimuli correlate with an increase in peroxisomal number. Despite an in-depth, mechanistic understanding of factors involved in peroxisome-related signaling, such as the PPARs, there is still some uncertainty as how this pathway leads to an increase in peroxisome number.

So far, we established a system which allows us to assess changes in peroxisome proliferation/morphology and to correlate this with the expression profile of peroxisomal genes involved in the regulation of peroxisome proliferation. Future studies are needed, using large scale expression profiling in this system which may then allow the identification of novel components regulating peroxisome morphology and dynamics. Several key players involved in peroxisome dynamics and proliferation have been identified in previous studies; however, regulatory mechanisms need further investigation. In this study, a possible link between novel regulatory pathways such as NF-κB / mTOR and peroxisome proliferation has been suggested based on testing different stimuli inducing peroxisome proliferation. There are questions to be answered about how external stimulation affects the expression of the three mammalian PEX11 isoforms, e.g are they all regulated in a different manner? (see chapter 4). This work may also have the outcome of revealing novel diagnostics and/or treatment targets for peroxisomal disorders. Further investigation is required to validate the involvement of novel PPAR-independent pathways in peroxisomal gene expression by computational analysis, e.g. of promoter regions and transcription factor binding sites (see chapter 4).

Chapter 4 – Results

Identifying regulatory elements involved in peroxisomal gene expression in humans

Summary

Although our understanding of peroxisome proliferation mechanisms is increasing, our knowledge on how this process is regulated and linked to signaling pathways is limited.

The known pathway inducing peroxisome proliferation is through peroxisome proliferator-activated receptor (PPARS), however the exact mechanism by which this pathway regulates expression of peroxisomal genes is not yet known, in particular in humans. So far, we established a cell-based assay in which we monitor peroxisome proliferation upon extracellular stimulation (Chapter 3). This assay gives us the opportunity to study gene expression and its correlation with peroxisome morphology. Based on these results, expression of genes such as PEX11β, PEX19 and PEX3 correlates with the formation of tubular peroxisomes. In this chapter we have employed bioinformatics approaches, including screening of promoter regions of 100 promising human candidate peroxisomal genes for regulatory elements. A Python-based program has been used to identify regulatory elements in the promoter regions of candidate peroxisomal genes. In this initial analysis more than 7000 transcription factor binding sites were identified in our gene cohort. Furthermore, the chromosome structure for each site identified was analyzed for its histone marks. This approach eliminated sites which were located on transcriptionally inactive regions. An initial map of candidate regulatory motif sites showed a difference between binding sites in PEX11α and PEX11β promoters suggesting that these genes are differentially regulated. One of the common transcription factors shared by PEX11β and PEX11γ was SMAD2/3. Moreover, differences in transcription factor binding sites

between metabolic and biogenetic peroxisomal genes suggest differential regulation of these genes.

In order to link these motif sites to certain signaling pathways, the Top 10 shared motifs among our gene cohort were run through the Enrichr database. A prominent SMAD2/3 binding site in PEX11β was suggestive for the involvement of the TGFβ signalling pathway in the expression of this important gene and in peroxisome proliferation and membrane dynamics.

4.2 Method pipeline and data filtering

4.2.1 Peroxisome proliferator response element (PPRE) search

The known pathway involved in peroxisome proliferation in mammals is through PPAR. Most of these studies used rodent models and studied the response to peroxisome proliferators (Kadowaki, 2001; McMullen *et al.*, 2014). Despite the evidence linking PPARα signalling with a variety of other signalling pathways there are currently few reports on how this links to peroxisome homeostasis in humans (Kadowaki, 2001; McMullen *et al.*, 2014). Treatment with PPAR agonists and microarray studies on human hepatocytes showed PPAR binding sites in the promoter regions of ACOX1 and PEX11α (Rakhshandehroo *et al.*, 2010). There is currently a gap in our understanding of the involvement of this regulatory pathway in controlling the transcription of peroxisomal genes for peroxisome proliferation under different conditions and precisely how this up-regulation of peroxisome genes is linked to peroxisome function (Kadowaki, 2001; McMullen *et al.*, 2014). Here, we investigated the promoters of 100 peroxisome genes (**Table 4.1**) searching for PPAR binding sites, starting by extracting -10kb from the TSS (transcription start site) and scanning the promoter area.

Ensembl Gene ID	Associated Gene Name	Chromosome Name	Gene Start (bp)	Gene End (bp)	Strand
ENSG00000101986	ABCD1	X	153724868	1.54E+08	1
ENSG00000173208	ABCD2	12	39550033	39619751	-1
ENSG00000117528	ABCD3	1	94418455	94518666	1
ENSG00000119688	ABCD4	14	74285423	74303056	-1
ENSG00000060971	ACAA1	3	38103129	38137242	-1
ENSG00000167315	ACAA2	18	49782167	49813960	-1
ENSG00000240303	ACAD11	3	132558138	1.33E+08	-1
ENSG00000181513	ACBD4	17	45132600	45144181	1
ENSG00000107897	ACBD5	10	27195214	27242130	-1
ENSG00000184227	ACOT1	14	73537114	73543794	1

ENSG00000119673	ACOT2	14	73567620	73575658	1
ENSG00000177465	ACOT4	14	73591706	73596496	1
ENSG00000101473	ACOT8	20	45841721	45857406	-1
ENSG00000161533	ACOX1	17	75941507	75979363	-1
ENSG00000168306	ACOX2	3	58505136	58537319	-1
ENSG00000087008	ACOX3	4	8366282	8440723	-1
ENSG00000176715	ACSF3	16	89088375	89155846	1
ENSG00000151726	ACSL1	4	184755595	1.85E+08	-1
ENSG00000123983	ACSL3	2	222860934	2.23E+08	1
ENSG00000068366	ACSL4	X	109624244	1.10E+08	-1
ENSG00000197142	ACSL5	10	112374018	1.12E+08	1
ENSG00000164398	ACSL6	5	131949973	1.32E+08	-1
ENSG00000281938	ACSL6	5	131807143	1.32E+08	-1
ENSG00000018510	AGPS	2	177392644	1.78E+08	1
ENSG00000072210	ALDH3A2	17	19648136	19677598	1
ENSG00000242110	AMACR	5	33986178	34008108	-1
ENSG00000276559	BAAT	CHR_HSCHR9_1_CTG5	101371093	1.01E+08	-1
ENSG00000136881	BAAT	9	101360417	1.01E+08	-1
ENSG00000121691	CAT	11	34438925	34472062	1
ENSG00000095321	CRAT	9	129094810	1.29E+08	-1
ENSG00000005469	CROT	7	87345681	87399795	1
ENSG00000110887	DAO	12	108858932	1.09E+08	1
ENSG00000203797	DDO	6	110391771	1.10E+08	-1
ENSG00000274296	DECR2	CHR_HSCHR16_CTG2	401826	412487	1
ENSG00000242612	DECR2	16	401826	412487	1
ENSG00000087470	DNM1L	12	32679200	32745650	1
ENSG00000113790	EHHADH	3	185190624	1.85E+08	-1
ENSG00000120915	EPHX2	8	27490779	27545564	1
ENSG00000197601	FAR1	11	13668670	13732346	1
ENSG00000064763	FAR2	12	29149103	29340980	1
ENSG00000214253	FIS1	7	101239458	1.01E+08	-1
ENSG00000160097	FNDC5	1	32862268	32872482	-1
ENSG00000116906	GNPAT	1	231241207	2.31E+08	1
ENSG00000197448	GSTK1	7	143244093	1.43E+08	1
ENSG00000131373	HACL1	3	15560704	15601852	-1
ENSG00000101323	HAO1	20	7882981	7940474	-1
ENSG00000116882	HAO2	1	119368779	1.19E+08	1
ENSG00000117305	HMGCL	1	23801885	23838620	-1
ENSG00000133835	HSD17B4	5	119452443	1.20E+08	1
ENSG00000119912	IDE	10	92451684	92574076	-1
ENSG00000138413	IDH1	2	208236227	2.08E+08	-1

ENSG00000067064	IDI1	10	1039908	1049170	-1
ENSG00000148377	IDI2	10	1018907	1025859	-1
ENSG00000102910	LONP2	16	48244296	48363122	1
ENSG00000103150	MLYCD	16	83899126	83927026	1
ENSG00000115204	MPV17	2	27309492	27325680	-1
ENSG00000110921	MVK	12	109573255	1.10E+08	1
ENSG00000007171	NOS2	17	27756766	27800499	-1
ENSG00000112874	NUDT12	5	103548855	1.04E+08	-1
ENSG00000148832	PAOX	10	133379234	1.33E+08	1
ENSG00000115425	PECR	2	215996329	2.16E+08	-1
ENSG00000127980	PEX1	7	92487020	92528531	-1
ENSG00000157911	PEX10	1	2403964	2413797	-1
ENSG00000166821	PEX11A	15	89677764	89690783	-1
ENSG00000131779	PEX11B	1	145911350	1.46E+08	-1
ENSG00000104883	PEX11G	19	7476875	7497449	-1
ENSG00000108733	PEX12	17	35574795	35578863	-1
ENSG00000162928	PEX13	2	61017225	61051990	1
ENSG00000142655	PEX14	1	10472288	10630758	1
ENSG00000121680	PEX16	11	45909669	45918812	-1
ENSG00000162735	PEX19	1	160276812	1.60E+08	-1
ENSG00000164751	PEX2	8	76980258	77001044	-1
ENSG00000215193	PEX26	22	18077920	18131138	1
ENSG00000034693	PEX3	6	143450807	1.43E+08	1
ENSG00000139197	PEX5	12	7188685	7218574	1
ENSG00000114757	PEX5L	3	179794958	1.80E+08	-1
ENSG00000124587	PEX6	6	42963870	42979220	-1
ENSG00000112357	PEX7	6	136822564	1.37E+08	1
ENSG00000107537	PHYH	10	13277796	13302412	-1
ENSG00000179761	PIPOX	17	28950513	29057220	1
ENSG00000163344	PMVK	1	154924734	1.55E+08	-1
ENSG00000115138	POMC	2	25160853	25168903	-1
ENSG00000186951	PPARA	22	46150521	46243756	1
ENSG00000132170	PPARG	3	12287368	12434356	1
ENSG00000117450	PRDX1	1	45511036	45523047	-1
ENSG00000126432	PRDX5	11	64318088	64321811	1
ENSG00000176894	PXMP2	12	132687606	1.33E+08	1
ENSG00000101417	PXMP4	20	33702754	33720319	-1
ENSG00000155366	RHOC	1	112701106	1.13E+08	-1
ENSG00000116171	SCP2	1	52927229	53051703	1
ENSG00000172250	SERHL	22	42500579	42512560	1
ENSG00000197375	SLC22A5	5	132369752	1.32E+08	1

ENSG00000100372	SLC25A17	22	40769630	40819399	-1
ENSG00000140284	SLC27A2	15	50182196	50236395	1
ENSG00000142168	SOD1	21	31659622	31668931	1
ENSG00000112096	SOD2	6	159669057	1.60E+08	-1
ENSG00000166575	TMEM135	11	87037844	87323758	1
ENSG00000108395	TRIM37	17	58982638	59106921	-1
ENSG00000158125	XDH	2	31334321	31414715	-1
ENSG00000180011	ZADH2	18	75195108	75209348	-1

Table 4.1 List of 100 peroxisome genes

To study the core promoters and proximal promoters of candidate genes in more detail, new databases (such as DECODE) (https://www.decode.com) were added to the approach. Ultimately, a program for checking PPREs in our gene cohort was generated. This program was used to search for and to assess the probability of different forms of PPREs by running through the sequences base pair by base pair. Histograms of potential PPREs that have been used for this study are shown in **Figure 4.1**. The results include the position of probable binding sites, the PPRE pattern and the percentage of binding efficiency (calculated based on the number of base pairs in the gene sequence matching the PPRE consensus sequence) (**Table 4.2**).

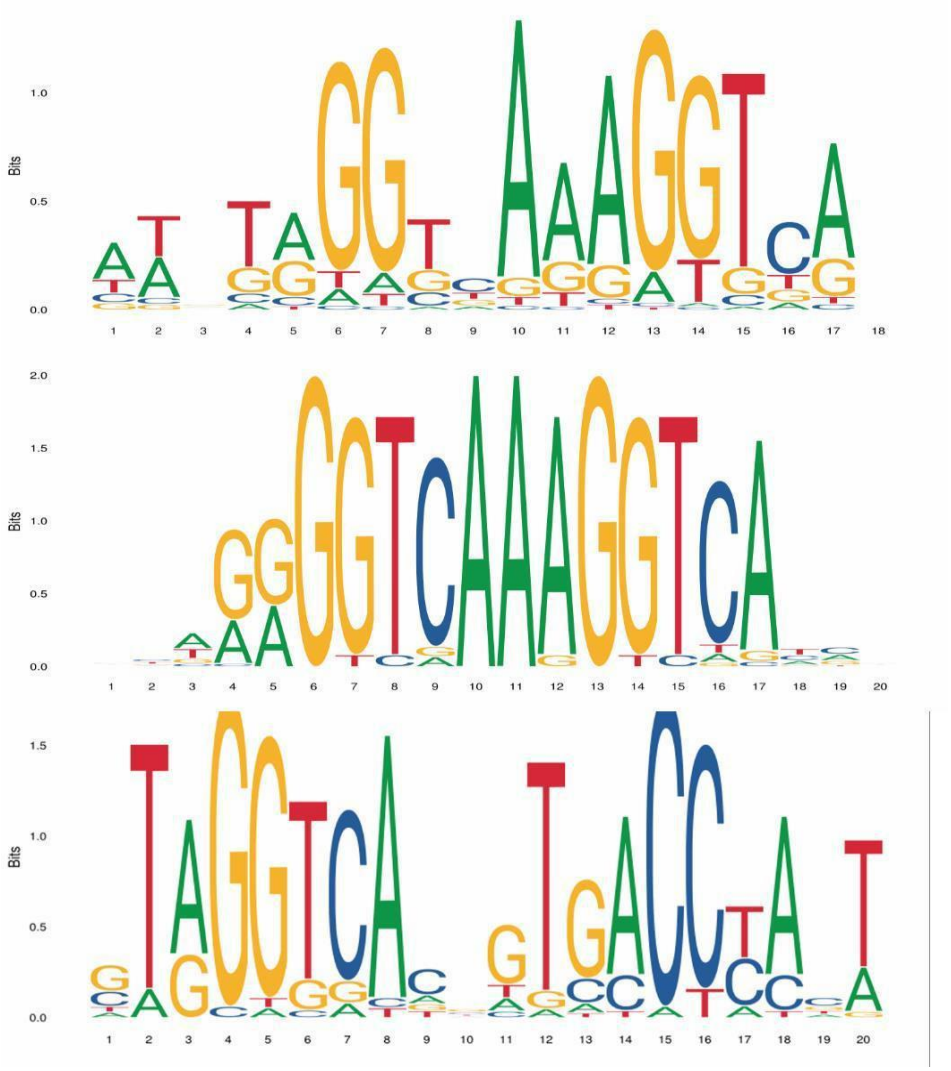


Figure 4.1. Pictograms of PPAR motif binding sites (JASPAR CORE), position weight matrices of PPARα, PPARβ and PPARγ.

In total, 34 PPAR motif sites were found in 29 peroxisomal genes with a binding efficiency higher than 70%, with some genes (PEX1, PEX11γ, ACAA1) containing more than one PPRE. An overview of the genes carrying at least one potential PPRE is shown in Table 4.2. The PPRE search results reveal the presence of PPARγ binding sites in promoter regions of genes involved in peroxisome biogenesis such as PEX11β, PEX16, PEX19, or PEX14 and PPARα binding sites mostly in the promotor regions of peroxisomal genes involved in metabolic functions of peroxisomes such as ACOX1, ACAD, CAT and PGC-1α.

Gene Name	PPRE Name	Binding efficiency	PPRE pattern
PEX1	PPAR γ	89	AGGTCANNAGGTCG
PEX1	PPAR β	72	AGGTGANNAGGTCA
PEX2	PPAR γ	75	AGGTCAAGGGTCA
PEX3	PPAR γ	91	AGGCCANNAGGTCA
PEX5	PPAR γ	85	AGGTCCNNAGTTCA
PEX6	PPAR γ	81	AGGTCANNAGTTCA
PEX10	PPAR γ	89	AGGTCANNAGGTCG
PEX11 α	PPAR α	100	AGGCCANNAGGTCA
PEX11β	PPAR γ	81	AGGTCANNAGTTCA
PEX11 γ	PPAR α	72	AGGTCANNAGGTCA
PEX11 γ	PPAR γ	81	AGGTCANNAGTTCA
PEX14	PPAR γ	100	AGGCCANNAGGTCA
PEX16	PPAR γ	91	AGGCCANNAGGTCA
PEX19	PPAR γ	89	AGGTCANNAGGTCG
SLC27A2	PPAR γ	81	AGGTCANNAGTTCA
ACSL5	PPAR γ	81	AGGTCANNAGTTCA
ACOX1	PPAR α	100	AGGCCANNAGGTCA
ACOX2	PPAR γ	81	AGGTCANNAGTTCA
EHHADH	PPAR γ	81	AGGTCANNAGTTCA
ACAA1	PPAR α	79	TGGTCANNAGGTCA
ACAA1	PPAR γ	91	AGGCCANNAGGTCA
ACOT1	PPAR α	96	GGGGCANNAGGGCA
SLC25A17	PPAR α	100	AGGCCANNAGGTCA
ACBD4	PPAR γ	81	AGGTCANNAGTTCA
BAAT	PPAR α	72	AGGTCANNAGGTCA
GSTK1	PPAR α	75	AGGTGANNAGGTCA
ABCD2	PPAR γ	81	AGGTCANNAGTTCA
PPAR α	PPAR γ	93	GGGGCANNAGGGCA
ABCD1	PPAR α	96	GGGGCANNAGGGCA
CAT	PPAR α	83	AGCTCANNAGGTCA
ACAD	PPAR α	83	AGCTCANNAGGTCA
PGC1	PPAR α	100	AGGCCANNAGGTCA

Table 4.2. Summary of PPREs found in human peroxisomal genes. Binding sites for PPARγ appear in the promoter regions of 10 peroxins, while binding sites for PPARα were found mostly in promoter regions of genes encoding enzymes involved in metabolic pathways.

4.2.2 Identification of novel transcriptional regulatory elements

In order to search for additional potential non-PPAR regulatory elements in peroxisomal genes in humans, we used a Python based program (Gimmemotif). (http://gimmemotifs.readthedocs.io/en/master/index.html). The available motif sequences were extracted from major transcription factor binding sites in PWS (position weight matrices) format JASPARE Core (http://jaspar.genereg.net/). In total 700 transcription factor binding sites were represented in the form of PWS matrices. For each candidate gene, we analysed -10kb upstream of the start codon. The sequences of candidate genes, including 100 peroxisomal r genes (PEX genes, genes encoding metabolic enzymes etc), and 100 randomly selected control genes were downloaded from Biomart (https://www.ensembl.org/biomart). The full list of the selected peroxisomal genes is shown in **Table 4.1**, and control genes in **Table 4.3**.

Gene stable ID	Gene name	Gene stable ID	Gene name	Gene stable ID	Gene name
ENSG00000120437	ACAT2	ENSG00000282513	FMN1	ENSG00000100162	CENPM
ENSG00000153107	ANAPC1	ENSG00000053254	FOXN3	ENSG00000172757	CFL1
ENSG00000149311	ATM	ENSG00000116717	GADD45A	ENSG00000165410	CFL2
ENSG00000175054	ATR	ENSG00000111640	GAPDH	ENSG00000149554	CHEK1
ENSG00000089685	BIRC5	ENSG00000105968	H2AFV	ENSG00000183765	CHEK2
ENSG00000012048	BRCA1	ENSG00000188486	H2AFX	ENSG00000122966	CIT
ENSG00000139618	BRCA2	ENSG00000164032	H2AFZ	ENSG00000136108	CKAP2
ENSG00000169679	BUB1	ENSG00000164104	HMGB2	ENSG00000173207	CKS1B
ENSG00000156970	BUB1B	ENSG00000198830	HMGN2	ENSG00000123975	CKS2
ENSG00000154473	BUB3	ENSG00000072571	HMMR	ENSG00000124207	CSE1L
ENSG00000198668	CALM1	ENSG00000165704	HPRT1	ENSG00000006634	DBF4
ENSG00000110104	CCDC86	ENSG00000144381	HSPD1	ENSG00000155368	DBI
ENSG00000145386	CCNA2	ENSG00000136273	HUS1	ENSG00000168393	DTYMK
ENSG00000134057	CCNB1	ENSG00000125968	ID1	ENSG00000147155	EBP
ENSG00000110092	CCND1	ENSG00000149503	INCENP	ENSG00000114346	ECT2
ENSG00000082258	CCNT2	ENSG00000091409	ITGA6	ENSG00000074800	ENO1
ENSG00000010278	CD9	ENSG00000138160	KIF11	ENSG00000174371	EXO1
ENSG00000136807	CDK9	ENSG00000079616	KIF22	ENSG00000106462	EZH2
ENSG00000100526	CDKN3	ENSG00000090889	KIF4A	ENSG00000105202	FBL

ENSG00000138778	CENPE	ENSG00000237649	KIFC1	ENSG00000280548	FBL
ENSG00000117724	CENPF	ENSG00000233450	KIFC1	ENSG00000112029	FBXO5
ENSG00000153044	CENPH	ENSG00000204197	KIFC1	ENSG00000160752	FDPS
ENSG00000123219	CENPK	ENSG00000056678	KIFC1	ENSG00000196924	FLNA
ENSG00000198554	WDHD1	ENSG00000130640	TUBGCP2	ENSG00000136068	FLNB
ENSG00000122952	ZWINT	ENSG00000175063	UBE2C	ENSG00000248905	FMN1
ENSG00000111057	KRT18	ENSG00000073111	MCM2	ENSG00000037042	TUBG2
ENSG00000106683	LIMK1	ENSG00000135679	MDM2	ENSG00000197275	RAD54B
ENSG00000131899	LLGL1	ENSG00000148773	MKI67	ENSG00000068028	RASSF1
ENSG00000284137	LLGL1	ENSG00000100714	MTHFD1	ENSG00000067560	RHOA
ENSG00000073350	LLGL2	ENSG00000100345	MYH9	ENSG00000175793	SFN
ENSG00000164109	MAD2L1	ENSG00000106631	MYL7	ENSG00000005022	SLC25A5
ENSG00000112062	MAPK14	ENSG00000109805	NCAPG	ENSG00000250254	PTTG2
ENSG00000101367	MAPRE1	ENSG00000106268	NUDT1	ENSG00000166974	MAPRE2
ENSG00000173598	NUDT4				

Table 4.3 List of 100 control genes

Having extracted the promoter sequences for our 100 peroxisomal and 100 control genes, we then analysed the promoter sequences using Gimmemotif.

Gimmemotif contains commands to collect sequences of known motifs and predict and score for motif enrichment on sequences. The commands were adopted for the purpose of this research.

The candidate gene sequences were run through the program which displayed a potential match to each motif. There are several parameters to be considered including the best match of each motif in each sequence, and stringency thresholds that were set to maximum to only include the highest probable motifs. The threshold for calling a match was fixed at 89%. As controls we used 100 random gene sequences (**Table 4.3**) and in addition, each candidate gene sequence was randomized (scrambled 3 times in succession) and run through the same set up as an additional control. To select for transcription factor binding motifs which were enriched in the peroxisomal genes compared to controls, and

to test if the numbers of each motif match were significant, a ratio was obtained from the number of sites compared to the control group. These values were normalized and the obtained hypergeometric p-value ("log10 (p-value)" >1.3 = significant) was used to remove the false positives. This analysis showed the set of transcription factor binding sites enriched/over-represented in peroxisomal genes compared to the control groups. The results were collected as BED or FASTA. The motif search process is summarized in **Figure 4.2**.

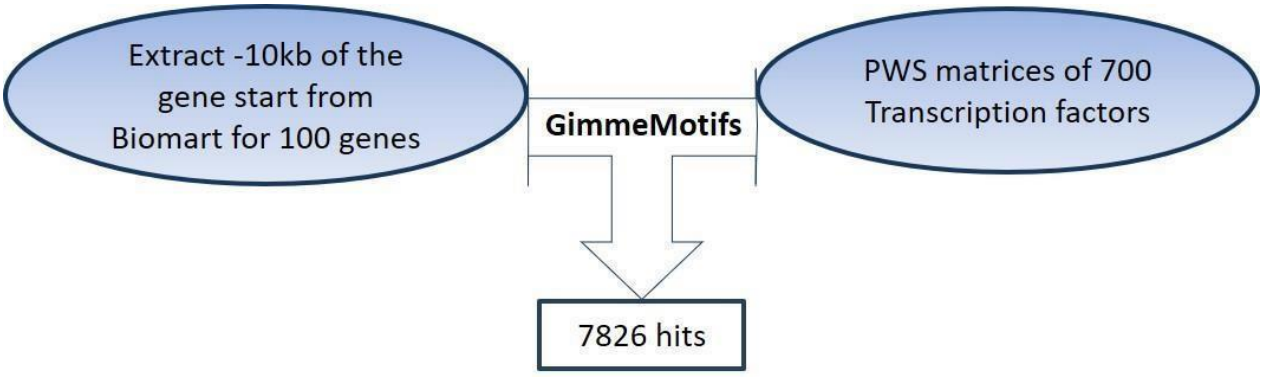


Figure 4.2. Schematic view of the bioinformatics approach to scan TF motif sites in the promoter region of peroxisomal genes. -10kb of the peroxisomal genes were extracted from Biomart, along with JASPAR matrices for transcription factor binding sites and run through the GimmeMotif program resulting in 7826 hits.

4.2.3 General summary of motif search Data filtering

In total, 7826 sites belonging to 105 transcription factors were found to be enriched in our gene cohort. Transcription factors such as VDR, TEAD3 or TBX20 did not have a binding consensus in the promoter regions of these 100 genes.

In order to eliminate non-active motif binding sites based on chromosome structure, we used the USCS transcription factor binding tracks to choose transcriptionally active sites in each gene. This database shows probable accessible binding sites of the specified transcription factors in the given cell

types as determined by chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq).

Transcriptionally active and silent chromatin is characterized by posttranslational modifications on histones. Active genes typically carry high levels of lysine acetylation on the histone3 and histone4 tails (H3K27ac), trimethylation of lysine 4 histone3 subunit (H3K4me3), trimethylation of H3 lysine 79, ubiquitylation of H2B, and trimethylation of histone 3 lysine 36 (H3K36me3). These histone marks may regulate transcription by creating an open chromatin structure and recruit effectors that mediate a transcriptionally competent state (Bannister & Kouzarides, 2011; T. Zhang *et al.*, 2015).

The presence of H3K4me3, H3K36me3, H3K27ac (histone marks) and absence of H3K27m3 near the potential motif was checked with the USCS genome browser as indicators of transcriptionally active sites in HepG2 cells. Our cohort of 100 peroxisomal genes was analyzed by USCS TF binding tracks. Each track shows a graph of enrichment for motif binding (signal), along with sites that have the greatest evidence of transcription factor binding (Peaks). These tracks are color coded by a binding strength score on a scale 0 to 1000, with higher score indicative of stronger binding. The peaks were grouped based on the peak height plotted to the same scale. From 0 to 200 a low peak was considered, , 200 to 500 as medium score, and > 500 was considered as the high binding score. Based on this analysis, we selected for a high binding score meaning that among the potential motif sites, 3271 sites were excluded out of 7826. In total 105 transcription factor binding sites among 700 transcription factors were found in our cohort. In the control group only 1102 binding sites for these 105 transcription factors were identified (instead of 4555 within the peroxisomal group), indicating

these motifs are enriched in peroxisomal genes and may play a role in regulating peroxisomal gene expression.

Whilst many transcription factors have multiple binding sites, none bind to the promoter regions of all peroxisomal genes, and others have multiple sites in one promoter, e.g. FOXA1 has 117 binding sites in the promoter regions of 42 peroxisomal genes (Table 4.3). However, this was not equally distributed, i.e. some genes had 10 FOXA1 binding sites and some had one.

On the other hand, none of the peroxisomal genes showed active motif sites for GLI2 (belongs to the C2H2-type zinc finger protein subclass of the Gli family) but it was present in the control set.

Motif Name	Number of sites	Motif Name	Number of sites	Motif Name	Number of sites
FOXA1	117	TBP	68	NFE2L1::MafG	39
SP1	116	GABPA	68	Sox3	39
Gata1	114	JUND	68	Hltf	39
MYC	110	Myc	65	Sox5	39
NFKB1	90	JunD	64	CEBP/A	39
Myb	78	CREB1	61	ELK1	39
JUN::FOS	78	TFAP2A	58	Mafb	39
RUNX1	78	ELK4	56	FOXP2	39
ZEB1	78	STAT1	56	NFATC2	39
SPI1	78	Tcfcp2l1	52	ETS1	39
Nkx2-5	78	HNF4A	50	Ddit3::CEBPA	39
CEBPA	78	Sox2	45	SOX10	39
EBF1	78	E2F1	43	MZF1_5-13	39
Foxa2	77	Gfi1b	39	En1	39
Klf4	77	FOXL1	39	Bhlhe40	39
Zfx	76	RORA_1	39	Sox17	39
MEF2A	76	FOXI1	39	MEF2C	39
GATA2	74	Prrx2	39	HLF	39
MAX	74	ZNF354C	39	MyOg	39
USF1	74	KLF5	39	FOXC1	39
Esrrb	72	BRCA1	39	TCF12	39
GATA3	71	CDX2	39	ATF4	39
YY1	71	HIF1A::ARNT	39	REL	39
NR3C1	69	Erg	39	Nobox	39
Myc-MAX	25	HINFP	17	RREB1	11
NR2C2	23	STAT2::STAT1	16	SRF	10
NR5a2	22	znf143	16	POU5f1::Sox2	8

NRF1	22	RXRA	15	HSF1	7
Nr1h3::Rxra	20	TP63	15	MAFF	7
Tal1::Gata1	19	ZNF263	14	Bach1::Mafk	7
NFYB	19	Mecom	13	RORA_2	6
Bcl6	18	PLAG1	13	ESR1	6
PRDM1	18	TP53	12	SMAD2/3	5
TAL1::GATA1	18	RXR::RAR_DR5	12	Rfx1	5
ESR2	18	Pax5	12	Cc NFE2::MAF	5

Table 4.4 Summary of motif binding sites and number of their repeats in our peroxisomal gene cohort. 4555 sites in total were found in transcriptionally active parts of our 100 candidate genes.

Interestingly, NR3C1, encoding the glucocorticoid receptor, had 69 motif sites in our peroxisomal gene cohort. Upon activation of NR3C1, the expression of enzymes involved in fatty acid metabolism is increased (**Table 4. 3)** (LeBleu *et al.*, 2014).

4.2.5 The expression of PEX11 isoforms is differentially regulated

The PEX11 family of proteins were shown to directly participate in peroxisome proliferation in yeasts, plants and mammals (Abe & Fujiki, 1998; Erdmann & Blobel, 1995; Lingard & Trelease, 2006; Marshall *et al.*, 1995; Orth *et al.*, 2007). In mammalian cells, three PEX11-related genes have been identified, PEX11*α,* PEX11*β* and Pex11*γ*. Based on our qPCR results studying the expression of PEX11 isoforms in HepG2 cells during a time course, these genes are not following the same pattern (**Chapter 3, section 3.3.5**). PEX11β expression follows the tubular morphology pattern of peroxisomes in our assay, with a higher expression after 24 hours, during the time course experiment

in HepG2 cells, whilst PEX11*α* and PEX11*γ* do not. This different expression pattern suggests differential regulation; we therefore searched for differences in motif sites in these genes. Interestingly, as shown in **Figure 4.3**, PEX11β and PEX11α have no common motifs for transcription factor binding, however, ATF4 and SMAD2/3 sites were found both in PEX11β and PEX11γ, whilst each gene also had other unique motifs. Overall, this suggests differential regulation of the three PEX11 genes but also implies potential co-regulation of PEX11β and PEX11γ by the ATF4/SMAD2/3 pathways.

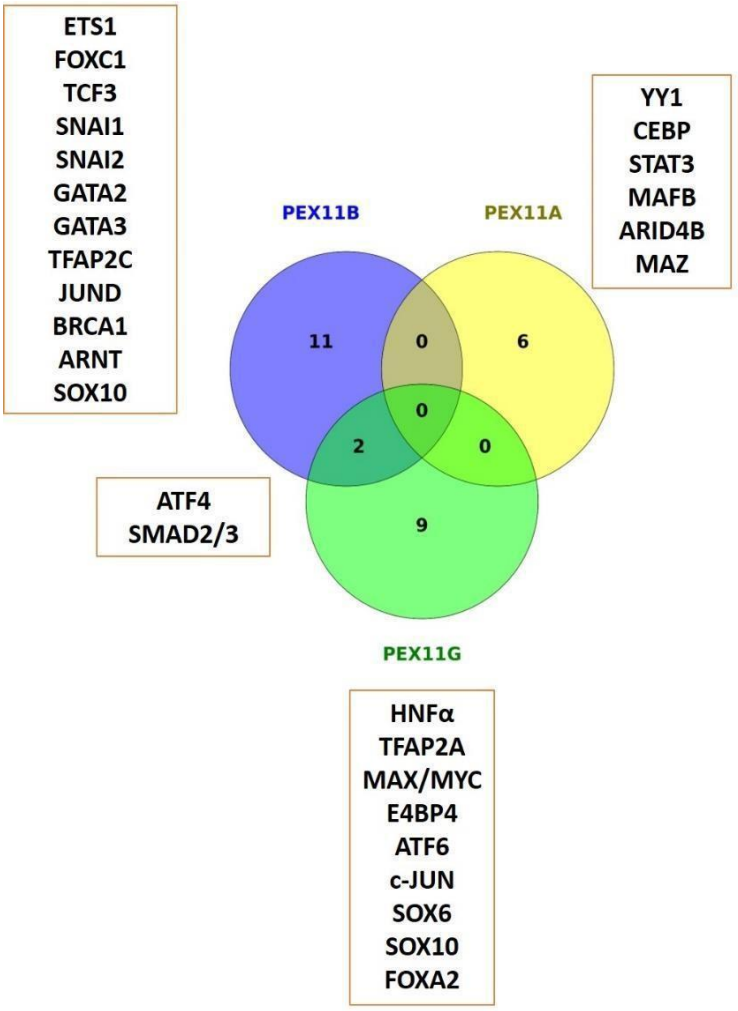


Figure 4.3. Venn diagram of common transcription factor binding sites within the promoter regions of human Pex11 isoforms. Potential binding sites for SMAD2/3 (activated by the TGFβ pathway) in Pex11γ and Pex11β were predicted which are located in a transcriptionally active area according to histone marks.

4.2.3 Network analysis to compare regulation of peroxisomal genes with different functions

Gene expression is the result of an organized network of interacting elements in signaling pathways and gene regulation networks in the cell (Ling et al., 2013). External signals activate signal transduction pathways to initiate transcription factor driven gene expression in gene regulatory networks (Schlitt & Brazma, 2007; D. Xie *et al.*, 2011). Transcription factors are often pleiotropic and involved in gene expression profiles of multiple genes and therefore multiple biological processes and phenotypes (Franco-Zorrilla *et al.*, 2014; Sureshbabu *et al.*, 2016). A list of metabolic genes is shown in Supplementary **Table S4.1**.

In order to find regulatory pathways involved in expression of our candidate genes, the candidate genes were separated into two broad groups based on their known function, namely genes involved in metabolic functions of peroxisomes or peroxins, which were analyzed for common transcription factor binding sites. The top 10 common transcription factors for each group were then run through an open source network analysis, Enricher (http://amp.pharm.mssm.edu/Enrichr/). The program uses the identity of the associated transcription factors to suggest links to specific regulatory pathways using data collated on each factor (e.g., KEGG pathways) (Kuleshov *et al.*, 2016). **Table 4.5** shows the most common transcription factors regulating metabolic genes and peroxins.

Metabolic Genes	Peroxins
NR1H3	ATF1
NR1I2	MAFB
FOXO3A	FOXA2
ELK4	HNF4A
ESR1	SOX10
ELK1	NFAT2
VDR	IRF2
TFAP2D	ATF4
FOXF1	GFI1
MYOD1	SPIB

Table 4.5. Top 10 motifs found in peroxisomal genes encoding metabolic proteins and peroxins.

As discussed in chapter 3, we established a cell-based assay using HepG2 cells to monitor peroxisome morphology and peroxisomal gene expression over a time course under standard or serum-free conditions (**Chapter 3, Section 3.3.5**). Based on the qPCR analysis, peroxins which are required early in peroxisome formation/proliferation for membrane biogenesis and insertion of membrane proteins such as**,** PEX3, PEX19 or PEX11β are following the tubular morphology pattern during a time course of 72 hours upon culture of HepG2 cells. We aimed at clarifying whether certain gene clusters following the tubular peroxisome pattern during the 6-72 hours are enriched with particular functional categories.

In order to further study their regulatory elements, the set of genes used for performing qPCR studies were analyzed for common transcription factor binding sites. As illustrated in **Figure 4.4**, PEX11β and PEX10 have common transcription factor binding sites, including those for ATF4 and SNAI2.

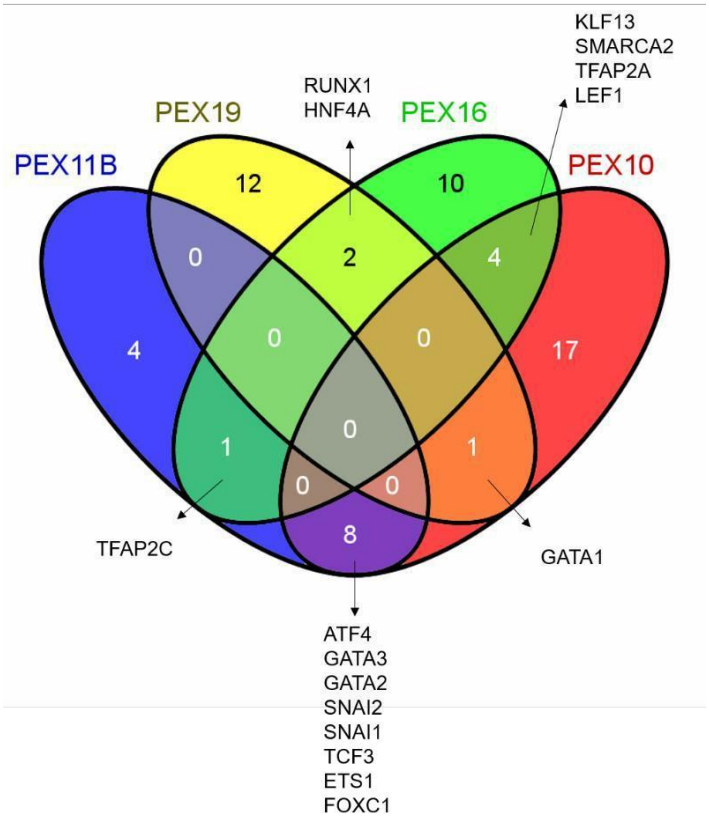


Figure 4.4. Venn diagram of common transcription factor binding sites within the promoter regions of PEX11β, PEX19, PEX16 and Pex10.

The network analysis was performed by using a web-based tool, Enrichr, to link these factors to certain regulatory pathways (Kuleshov *et al.*, 2016). Enrichr uses all available databases for signaling networks and protein/protein interaction, e.g. wikiPathways (Pico *et al.*, 2008), KEGG (Ogata *et al.*, 1998), BioCarta, Reactome (Joshi-Tope *et al.*, 2005) and kinase enrichment analysis (KEA) (Lachmann & Ma'ayan, 2009) to link certain transcription factors to the pathways they are involved in (http://amp.pharm.mssm.edu/Enrichr) and to predict relationships between biological and pharmacological processes. The results showed that the transcription factors involved in the regulation of PEX11β, PEX16, PEX10 and PEX19 are linked to two main pathways, including SMAD2/3 and C-MYC

signaling pathways. However, ABCD1, PEX11α, PPARα and PEX11γ are regulated by the HIF-1α pathway (**Figure 4.5**).

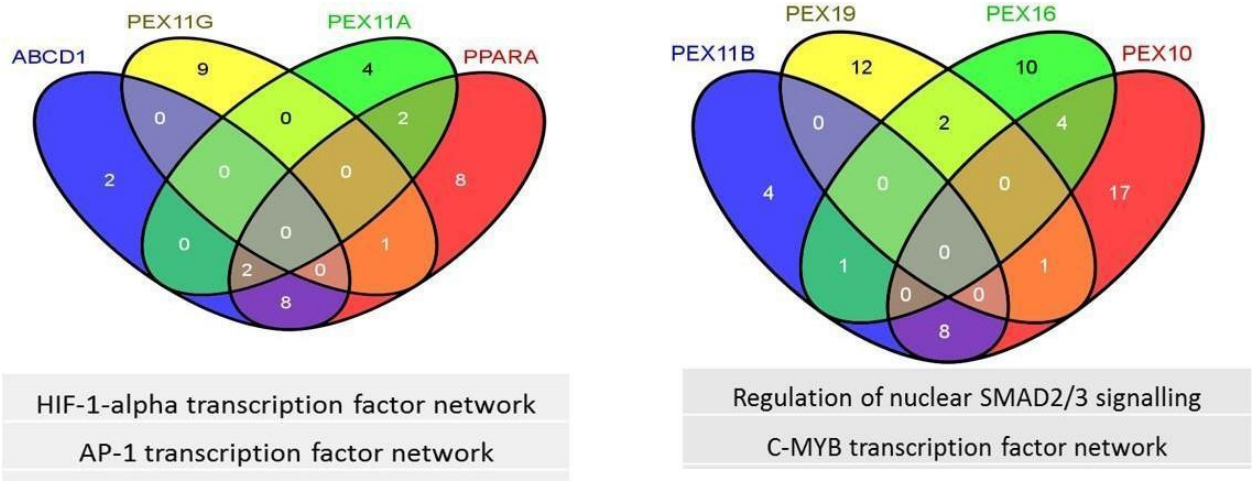


Figure 4.5 Venn diagrams of potential regulatory networks involved in the regulation of ABCD1, PEX11γ, PEX11α and PPARα (gene expression does not correlate with peroxisome elongation/proliferation in HepG2 cells) and PEX11B, PEX19, PEX16, PEX10 (gene expression correlates with peroxisome elongation/proliferation in HepG2 cells).

4.3 Discussion

As highlighted in the introduction, PPARs are the known regulators of lipid homeostasis and peroxisome proliferation. Studies with PPAR antagonists indicate that unlike many other nuclear receptors, PPARα governs the expression of a large set of genes, many of which are involved in fatty acid metabolism in human and rodent liver (Berger & Moller, 2002; Desvergne & Wahli, 1999; van der Meer *et al.*, 2010). However, PPARα agonists have only mild effects on the expression of genes associated with lipid metabolism in HepG2 cells (Vanden Heuvel et al., 2003). Here, we focused not only on PPARα binding sites but also on PPARβ and γ.

Based on our results, 34 PPAR motif sites were found in 29 peroxisomal genes with > 70% binding efficiency, whilst PEX1, PEX11γ and ACAA1 are containing more than one PPRE. It has been shown that fatty acids can regulate cellular function by modulating the rates of transcription of various target genes including acyl-CoA oxidase (ACOX1) through activating PPAR-RXR complex (McMullen *et al.*, 2014). Here we used ACOX1 as a control, and as expected, a PPARα site was found within the promoter of ACOX1 (McMullen *et al.*, 2014). Potential PPARγ sites were identified in the promoter regions of peroxins such as PEX11β, PEX16, PEX19, and PEX14. However, PPARα binding sites were mainly found in promoter regions of genes involved in metabolic functions of peroxisomes. Interestingly, PEX11α, which is likely not directly involved in peroxisome proliferation, contains a potential PPARα binding site, which may further point to a metabolic role of PEX11α. This suggests different regulatory mechanisms for peroxisomal biogenetic and metabolic genes.

In the present study, we further searched for other regulatory elements present in the promoter regions of peroxisomal genes. As a result, common binding sites for transcription factors shared by peroxisomal genes were identified. This data can be used to identify the regulatory elements of peroxisomal genes in different cell lines considering their active transcription sites. Promoter sequences of 100 peroxisomal genes were used to investigate the potential target sites for transcription factors. This study resulted in a large amount of data and here we focus on some interesting individual examples. Our findings showed the difference in regulation of peroxisomal genes involved in metabolic or biogenetic functions of peroxisomes. The metabolic genes mostly are connected to pathways such as HIF-1α, which is a hypoxia related pathway. However, transcription factors which can be activated through cross talk with the SMAD2/3 pathway are main regulators for peroxins.

Another interesting motif found in our cohort is the Glucocorticoid Response Element (GRE). Our results show that PPARα and γ have an active GRE. Glucocorticoids (GR) are a class of steroid hormones that are secreted by cortical cells in adrenal glands and modify the immune responses to diverse stimuli (Scheschowitsch *et al.*, 2017). In our cohort, a binding element for GR was found in 27 genes, including ACOX2, ACAD11, EHHADH and ACBD4. Interestingly, there are studies on direct involvement of the mitochondrially localized glucocorticoid receptor in the regulation of mitochondrial transcription and OXPHOS enzyme biosynthesis by binding to mtDNA and activation of mitochondrial gene expression. Also, it has been shown that GR can indirectly control expression of mitochondrial genes by induction of genes encoding mitochondrial transcription factors (Beato *et al.*, 1996; Psarra & Sekeris, 2008). In addition, the mitochondrial localization of several other transcription

factors which are involved in activating immune responses including NF-κB, p53, AP-1, STAT-3 have been reported (Psarra & Sekeris, 2009). Localization of GR to mitochondria and existence of GR binding sites in peroxisomal genes suggest the involvement of this pathway in the expression of genes contributing to energy production and immune responses (Psarra & Sekeris, 2009).

As a key regulator of peroxisome dynamics, the PEX11 promoters were analyzed for transcription factor binding sites. Comparison between PEX11α, PEX11β and PEX11γ revealed common binding sites for SMAD2/3 and also ATF4, which both can be activated by TGFβ signalling. Activation transcription factor 4 (ATF4) can also bind to cAMP response element (CRE), which is involved in many pathways including activation of autophagy processess upon ER stress (B'chir *et al.*, 2013). Other genes containing SMAD2/3 binding sites are FIS1, PEX13 and PEX14, further indicating the potential involvement of this pathway in the regulation of peroxisome dynamics. Further investigation including functional promoter studies using PEX11β vectors are required to validate the direct effect of TGFβ signaling via SMAD2/3 on peroxisome dynamics and proliferation (see Chapter 5).

Chapter 5 – Results

The canonical TGFβ pathway has a role in peroxisome proliferation in hepatocytes

Summary

Our bioinformatic analysis of peroxisomal genes for transcription factor binding sites indicated a potential SMAD2/3 binding site in the promoter region of PEX11β. As SMAD2/3 can be activated through TFGβ signalling, we investigated whether expression of PEX11β is linked to the canonical, SMAD-dependent TGFβ signalling pathway. PEX11β is a key protein in the regulation of peroxisome dynamics and proliferation, and regulation of PEX11β expression by TGFβ would link this signalling pathway to peroxisome proliferation. We first used our developed cell-based peroxisome proliferation assay to investigate if TGFβ had an effect on peroxisome morphology and number. Interestingly, addition of TGFβ to HepG2 cells cultured under serum-free conditions induced elongation/growth of peroxisomes as well as peroxisome proliferation supporting a role for TGFβ signalling in peroxisomal growth and division. We also predicted that the putative SMAD2/3 binding site in the PEX11β promoter is located in a transcriptionally active region with strong binding efficiency for SMAD2/3. To demonstrate that the SMAD binding site is indeed functional and can induce PEX11β expression, we applied a dual luciferase reporter assay. When we expressed a luciferase reporter under control of the PEX11β promoter, luciferase activity was induced by TGFβ stimulation. However, a PEX11β promoter mutant with altered SMAD binding site was unable to induce luciferase activity after TGFβ stimulation. These findings demonstrate that the SMAD binding site within the PEX11β promoter is functional, and that TGFβ signalling via the canonical SMAD-dependent pathway can activate PEX11β expression, leading to peroxisome growth, division and proliferation in HepG2 cells.

5.1 Introduction

5.1.1 Transforming growth factor β- an overview

The transforming growth factor β (TGFβ) superfamily is composed of a large group of structurally related polypeptide growth factors, including the TGFβ/activin family, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and the glial cell line-derived neurotrophic factor (GDNF) family (Massague, 2012).

The TGFβ family consist of multifunctional proteins that regulate a diverse range of processes during development and tissue homeostasis, such as cell proliferation, apoptosis, autophagy, inflammation, angiogenesis, and epithelial-to-mesenchymal transition (Horbelt *et al.*, 2012; Kitisin *et al.*, 2007; Massague, 2012; Nagaraj & Datta, 2010). There are three known isoforms of TGFβ (TGFβ1, TGFβ2 and TGFβ3) expressed in mammalian tissues; they contain highly conserved regions but are different in several amino acid regions. All of these isoforms function through the same receptor signalling pathways (Horbelt *et al.*, 2012; Kitisin *et al.*, 2007; Massague, 2012; Nagaraj & Datta, 2010).

TGFβ isoforms bind to receptors at the cell surface, and recruit two type I receptors and two type II receptors forming a tetrameric complex (Massague, 1998). Activated TGFβ superfamily receptors induce a phosphorylation cascade, from receptor phosphorylation to subsequent phosphorylation and activation of downstream signal transducer R-Smads transcription factors (receptor-activated Smads) (Derynck & Akhurst, 2007; Miyazawa & Miyazono, 2017) (**Figure 5.1**). Phosphorylated R-Smads form a hetero-oligomeric (often trimeric) complex with Smad4. This R-Smad-Smad4 complex is imported into the nucleus where it

regulates the expression of target genes by direct binding to the target gene promoter and/or through interaction with transcriptional cofactors in a cell-type-specific manner (**Figure 5.1**) (Hariharan & Pillai, 2008; Kamato *et al.*, 2013).

5.1.2 TGFβ mechanism of action

TGFβ is unique amongst the family of growth factors in that they are synthesized as inactive precursors, cleaved into mature ligands and the latency-associated peptides (LAP), which are non-covalently linked to the mature ligands, preventing binding to their respective receptors The three mammalian TGFβ isoforms are normally secreted as part of this inactive complex that consists of an N-terminal latency-associated peptide (LAP) and a C-terminal mature TGFβ monomer (Heldin & Moustakas, 2016; Kamato *et al.*, 2013). Covalent linkage of LAP to one of three latent TGFβ binding proteins (LTBPs) creates a large latent complex that may interact with the extracellular matrix (Hariharan & Pillai, 2008; Kamato *et al.*, 2013). Activated TGFβ signals through a hetero-tetrameric receptor complex composed of two type I and two type II transmembrane receptor subunits with serine/threonine kinase domains (Kamato *et al.*, 2013).

The TGFβ family of ligands have different affinities for different type I and II receptor combinations. TGFβ protein binds to the constitutively active TGFβ type II receptor. In most cell types, following ligand binding, the type II receptor (TGFβ RII) phosphorylates the type I (TGFβ RI), leading to recruitment and phosphorylation of Smad2 and Smad3 at their C-termini, dissociation from the receptor to form a hetero-trimeric complex with other SMAD proteins (**Figure 5.1**) (Hariharan & Pillai, 2008; Kamato *et al.*, 2013).

Activated Smad proteins associate with Smad4 and translocate to the nucleus, where they recruit additional transcriptional regulators, including DNA-binding transcription factors, co-activators, co-repressors, and chromatin remodelling factors, that control the expression of numerous target genes with Smad sequence-specific DNA binding sites (**Figure 5.1**) (Kamato *et al.*, 2013). Differential expression of these factors may be responsible for some of the cell type-specific responses to TGFβ (Heldin *et al.*, 1997; Kamato *et al.*, 2013).

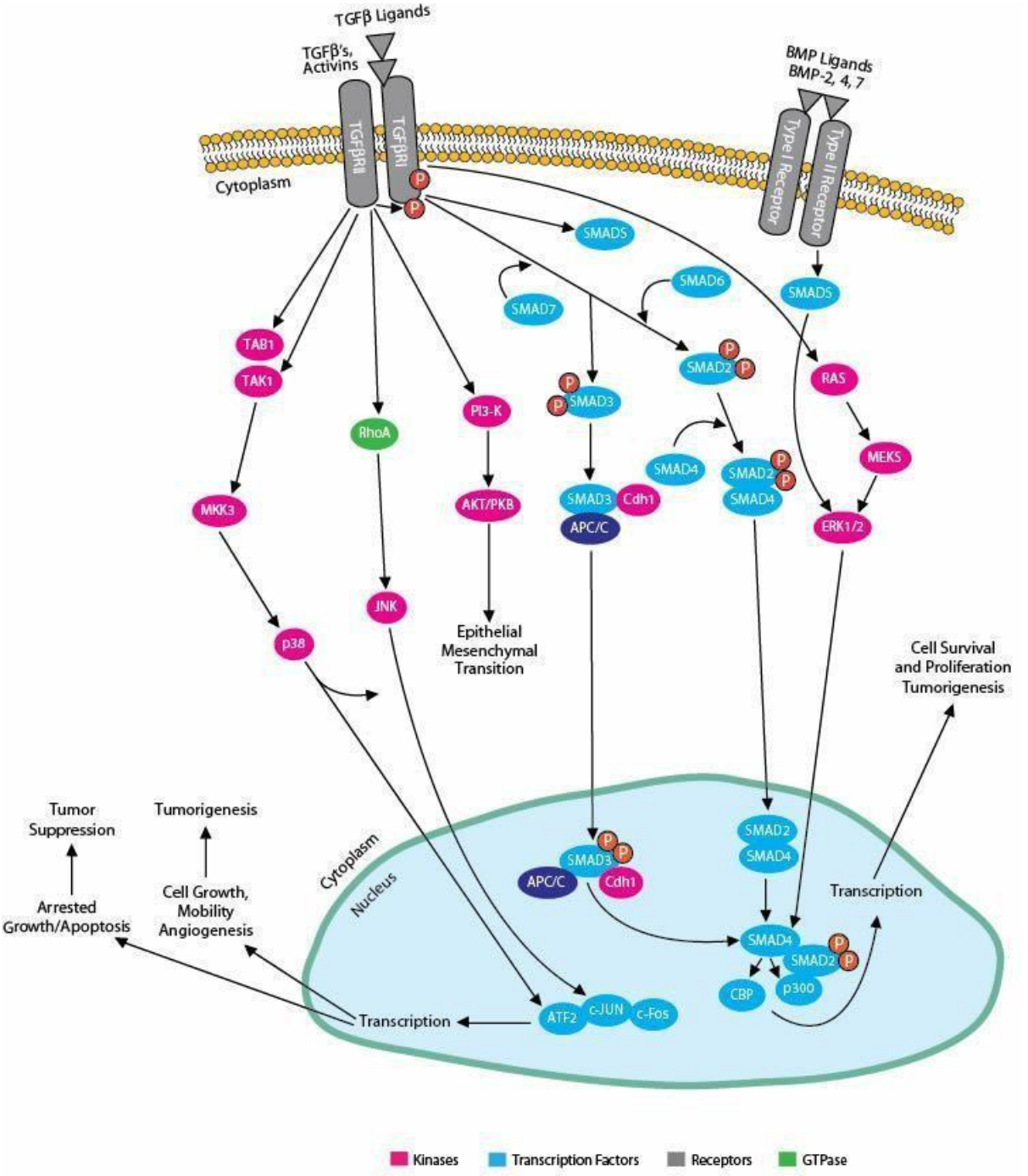


Figure 5.1. TGFβ signalling and cross talk with other signalling pathways. The TGFβ pathway is involved in the regulation of cellular proliferation, differentiation, embryogenesis, apoptosis, inflammation, immunity and cancer pathways. TGFβ binds its receptor type II at the cell surface which then phosphorylates the TGFβ receptor Type I. The activated receptor can phosphorylate various SMAD factors. These molecules then transfer to the nucleus and act as transcription factors, recruiting their co-factors CBP and P300. TGFβ can also activate the AKT/PKB and JNK cascade.

5.1.3 The SMAD protein family

Eight Smad proteins are encoded in the human and mouse genomes, four in *Drosophila*, and three in *C. elegans* (Massague, 1998). The Mad protein in *D. melanogaster*, which was the first identified member of this family (Raftery & Sutherland, 1999) is the ortholog of mammalian Smad1/5, whereas dSmad2, Medea, and Dad are the orthologs of Smad2/3, Smad4, and Smad6/7, respectively. The Smad family in *C. elegans* includes Sma1, Sma2, and Sma3 (J. Wang *et al.*, 2005).

Smad4 serves as a co-factor for the SMAD2/3 complex (**Figure 5.1**). The name “Smad” was coined with the identification of human Smad1 because of its sequence similarity to the Sma and Mad proteins (F. Liu *et al.*, 1997).

5.1.4 TGFβ crosstalk with other signalling pathways

Accessory proteins such as soluble or membrane-bound regulators or co-receptors can also affect TGFβ signalling (J. D. Brown *et al.*, 1999; Kamato *et al.*, 2013). In addition, TGFβ can activate a number of Smad-independent (non-canonical) signalling pathways, including Ras/MAPK, PI 3-K/Akt, p38, JNK, and RhoA/ROCK in a cell type-specific and context-dependent manner (**Figure 5.1**). Activation of these pathways may also contribute to the cellular responses induced by TGFβ (**Figure 5.1**) (Borggrefe *et al.*, 2016; Ji *et al.*, 2014; Mu *et al.*, 2012).

So far, several transcription factors, such as AP1, ETS, basic helix-loop-helix proteins, C/EBPβ, FOXH1 and FOXO have been identified and validated as

important co-factors of TGF-β/BMP signaling pathways (Massague, 2012; Miettinen *et al.*, 1994; Seoane *et al.*, 2001) .

TGFβ also induces activation of Ras, RhoB and RhoA, as well as of TAK1 and protein phosphatase 2A, which leads to the activation of several MAP kinase pathways and the downregulation of S6 kinase activity (**Figure 5.1**).

There is evidence for a crucial role of TGFβ as cytostatic and apoptotic in hepatocytes, which is critical for the control of liver mass (Karkampouna *et al.*, 2012). Liver is a unique organ with high regenerative capacity which is important for homeostasis and tissue repair. As mentioned, this pathway has activator and repressor effects on other pathways such as cell growth. Hence, a balanced TGFβ signaling in terms of both dosage and spatiotemporal activity is crucial to control hepatic gene expression. TGFβ acts in the process of differentiation of hepatoblasts to either hepatocytes or cholangiocytes and biliary morphogenesis in the developing liver parenchyma during liver regeneration after partial hepatectomy, or cholangiocytes in the developing liver parenchyma (**Figure 5.2**) (Karkampouna *et al.*, 2012). This pathway is required at different stages of the process, to allow hepatocyte proliferation at the inductive phase followed by an efficient termination of the regenerative response afterwards.

In cancer, TGFβ acts as tumour suppressor to induce growth arrest, senescence, and apoptosis at the early stages of tumorigenesis, but acts as a tumour promoter to induce epithelial-mesenchymal transition (EMT) and to promote angiogenesis in addition to loss of growth inhibitory effects at the advanced stages of cancer (Katsuno *et al.*, 2013). TGFβ signalling is considered to be an attractive molecular target for cancer therapy (Fransvea *et al.*, 2008).

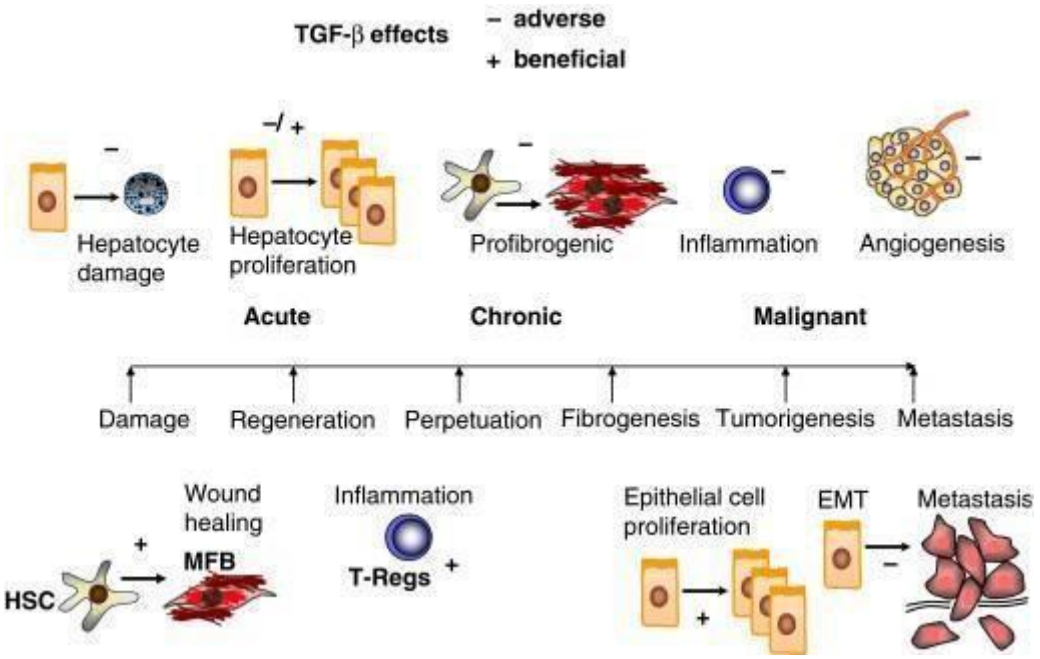


Figure 5.2. TGFβ signalling during the progression of chronic liver diseases. Upon liver damage, TGFβ ligands in the liver induce the downstream signaling. Depending on the disease stage, TGF-β might have a good (+) or bad (−) outcome in the organ. TGFβ enhances damage to epithelial cells by inducing apoptosis and oxidative stress, triggers myofibroblast (*MFB*) activation and a wound-healing response, controls or inhibits liver regeneration, activates regulatory T cells (T_{Reg}) and Th17 differentiation to calm down inflammatory responses, inhibits the proliferation of premalignant cells, inhibits tumour-directed inflammatory responses, facilitates tumour angiogenesis and induces epithelial-mesenchymal transition (*EMT*) of tumour cells (Karkampouna *et al.*, 2012).

5.1.5 TGFβ target genes

In most cell types, TGFβ induces phosphorylation of Smad2 and Smad3 (activin/TGFβ-specific R-Smads, or AR-Smads) and BMPs induce phosphorylation of Smad1, Smad5 and Smad8 (BMP-specific R-Smads, or BR-Smads). Activated R-Smads form hetero-oligomeric complexes with common-partner (co)-Smad (Smad4). The complexes translocate into the nucleus where they regulate the expression of target genes, such as the genes for *Serpine1* (plasminogen activator inhibitor-1), inhibitory (I)-Smads (Smad6 and Smad7) and *Id1* (inhibitor of differentiation-1 or inhibitor of DNA binding-1). It has been shown that elevated TGFβ signalling is associated with systemic insulin resistance and hepatic steatosis by regulating expression of its target genes, such as PGC-1α and PPAR-γ (Sohn *et al.*, 2012; Tan *et al.*, 2012; Yadav *et al.*, 2011). In another study, TGFβ had an inhibitory effect on mRNA production of apolipoprotein B (apoB) as well apolipoprotein M (apoM) in less extent in HepG2 cells (Xu *et al.*, 2004).

5.2 Specific Methods

5.2.1 Cell culture, TGFβ and inhibitor treatment

HepG2 cells were maintained under standard culture conditions as described (**see General Methods section 2.2**). For stimulation experiments, HepG2 cells were cultured in serum-free MEM supplemented with N1 (Sigma) containing 10% BSA. For morphological analysis of peroxisomes, HepG2 cells were seeded at a defined cell density (2×10^5 cells/dish) in 6 cm ø culture dishes (Greiner) on collagen (Serva)-coated glass coverslips in standard or serum-free media. After 6 hours, compounds/inhibitors were added at the indicated concentrations, cells were incubated for 24-48 hours and processed for immunofluorescence or immunoblotting (coverslips were omitted). Stimulation with 10% FBS served as a positive control for peroxisome elongation/proliferation. TGFβ stimulation was performed by addition of 1-2 ng of TGFβ recombinant protein (R&D systems). For inhibitor studies, cells were treated with 20-80 nM of TGFβ inhibitors LY2109761 (Cell Signalling) and SIS3 (Cell Signalling) for 2 hours upon TGFβ stimulation.

5.2.2 Cloning of PEX11β promoter region into the pGL3-basic luciferase vector

For cloning of PEX11β promoter regions, the candidate promoter region (1302bp for the wild type and 1296bp for the mutant) of human PEX11β was synthesized (Eurofins) and then cloned into the pGL3-basic vector (#E1751, Promega) between XhoI and SacI sites. This resulted in the generation of pGL3-Pex11β^{proWT} and pGL3-Pex11β^{proMut}. pGL3-promoter (#E1761, Promega) and

pGL3-control vector (#E1741) were used as controls. pRL-TK vector (#E2231, Promega) was used as internal control vector to normalize luciferase activities. All constructs produced were confirmed by sequencing (Eurofins). Vector maps are shown in supplementary Figure S.5.1.

5.2.3 Lipofectamine Transfection and TGFβ treatment

For transfection of HepG2 cells for the dual luciferase assay, 2×10^5 cells/well were seeded the day before transfection in 6-well plates (Corning, #3548) in 1.5 ml complete growth medium (MEM containing serum and antibiotics) and incubated at 37°C and 5% CO_2. On the day of transfection, pGL3-CMV/ pGL3-Pex11β^{proWT} /pGL3-Pex11β^{prMut} / pGL3-SMAD (100 ng) and pRL-TK vector DNA (4 ng) (ratio 25:1) was diluted in Opti-MEM (Life Technologies) in a 1.5 ml microcentrifuge tube, and 5 µl of P3000 was added to a final volume of 100 µl. In a second microcentrifuge tube, 2.5 µl Lipofectamine 3000 were added to 100 µl Opti-MEM. The DNA mix was carefully added to the Lipofectamine mix, gently pipetted up and down 3-4 times and incubated for 5 minutes at room temperature to allow complex formation. Meanwhile, the growth medium was gently aspirated from the plate, the cells were washed once with 200 µl PBS, and 100 µl fresh complete growth medium was added. Then, 200 µl of the DNA/Lipofectamine mix was added dropwise to the wells, and the plate gently swirled to ensure uniform distribution of the transfection complex. The cells were incubated in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 48 hours to allow expression of the transfected DNA constructs (**Figure 5.4**).

After 48 hours upon transfection, growth media was removed and cells were washed with 500 µl of 1×PBS. Cell were were transferred to 96 well luminometer plates (Greiner Bio-One Inc, #655083) in 5 columns. Cells were seeded in MEM/N1 media in 96 cell plates. After 6 hours incubation in N1, cells were treated with TGFβ, FBS or left un treated and were incubated for 24 hours prior to performing the dual luciferase assay.

5.2.5 Dual Luciferase Reporter Assay (DLR)

A dual luciferase assay (Promega, #E1910) was used to measure the luciferase and Renilla activity within the transfected cells. Luciferase assays were performed with a MicroLumat Plus LB 96V luminometer (Berthold Technologies). Cells were incubated in 100 µl of 1× Passive Cell Lysis Buffer for 20 minutes at room temperature for cell lysis. First, 50 µl of Luciferase Reagent II was injected to the lysate and luciferase activity was measured for 10 seconds. Then, 50 µl of Stop and Glo® reagent was added to the lysate to stop the luciferase activity and catalyse the Renilla reaction, incubated for 1.6 seconds followed by measurement of Renilla activity for 10 seconds. Each reaction was measured 3 times. For analysis, luciferase activities were normalized to Renilla luminescence in each well (**Figure 5.4**).

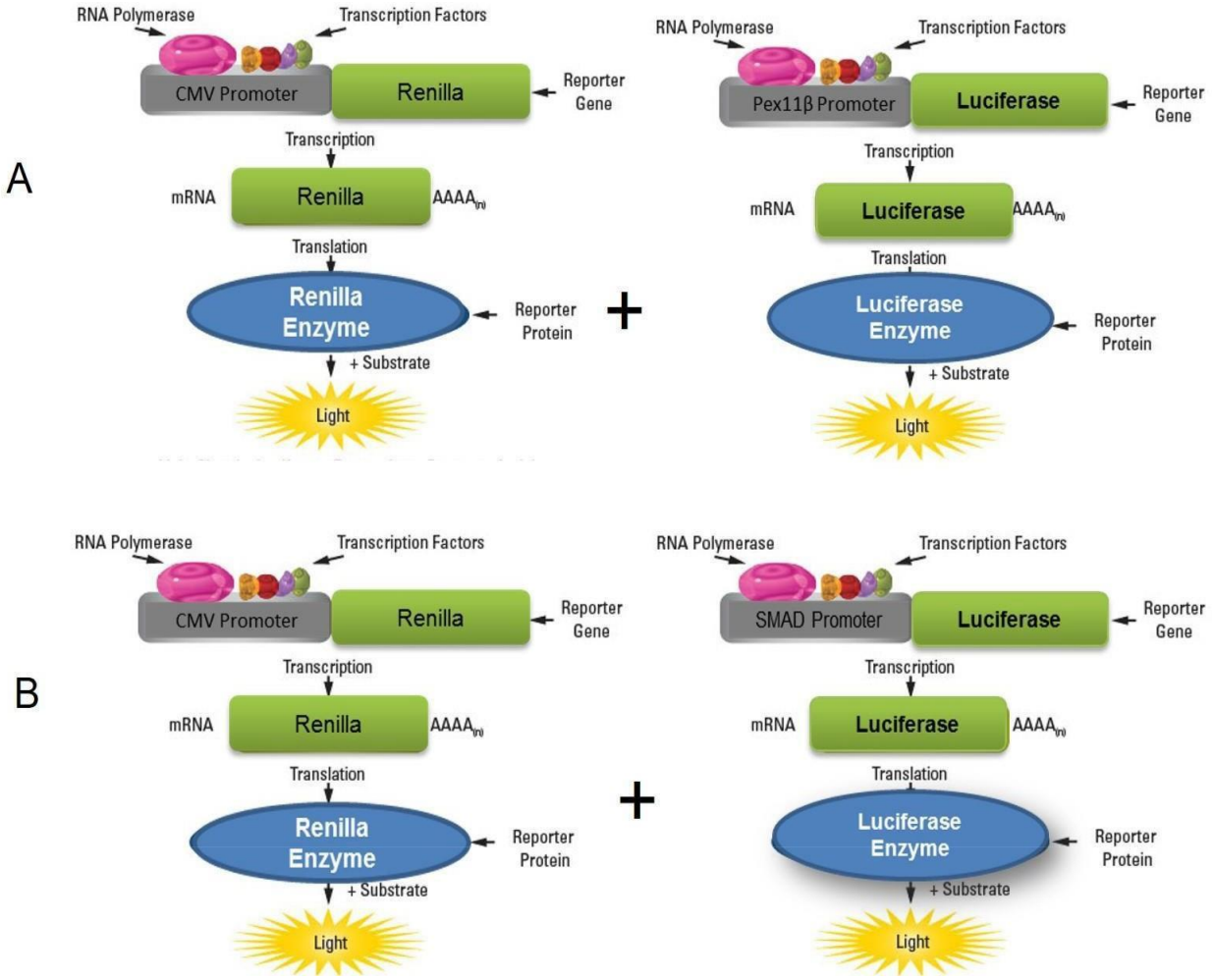


Figure 5.4. Experimental design for lipofectamine transfection with luciferase constructs. (A) HepG2 cells were transfected with PEX11βWT or PEX11βMut luciferase and Renilla control. **(B)** HepG2 cells were transfected with SMAD luciferase and Renilla control.

5.3 Results

5.3.1 *Hs*PEX11β possesses a SMAD binding site

Our analysis of peroxisomal genes for transcription factor binding sites revealed the presence of putative SMAD2/3 binding sites in the promoter regions of PEX11β and PEX11γ, but not PEX11α. The SMAD binding motif consensus sequence was previously defined as two inverted repeats of GTCT (Jonk *et al.*, 1998), and it was suggested that a single GNCN would be sufficient for SMAD DNA-binding (Sandelin *et al.*, 2004). A more complete model of the SMAD binding site including the probability of each base pair and its role in the binding efficiency has been proposed as position-specific scoring matric on the JASPAR CORE data base, which has been used in this study (**Figure 5.5**) (Sandelin *et al.*, 2004; Stormo, 2013) . In our bioinformatic approach, we searched for SMAD2/3 binding sites for in promoter of 100 peroxisomal genes (**Chapter 4. Section 4.2.3**). A potential binding site for SMAD2/3 binding site within the PEX11β promoter was found, this factor also showed a potential binding motif in PEX11γ promoter. For comparison, the known SMAD regulated genes PGC-1α and PPARγ obtained as control in our system. This indicates that PEX11β possesses a strong putative SMAD2/3 binding site.

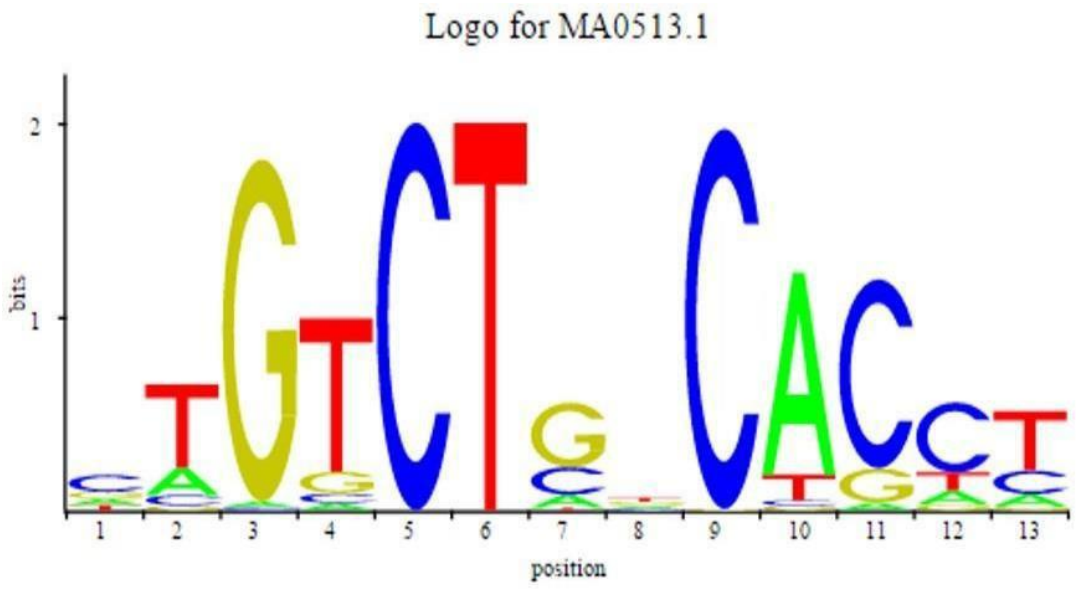


Figure 5.5: Pictogram of SMAD binding motif: Graphical representations of the SMAD matrix model, based on information content. The information content of a matrix column ranges from 0 (no base preference) and 2 (only 1 base used). This barplot shows the total information content in each position, where the bar is replaced by stacked letters (A, C, G, T), which are sized and sorted relative to their occurrence Taken from (Stormo, 2013).

To confirm that the SMAD binding sites in the PEX11β and PEX11γ promoters were located in transcriptionally active regions, the ENCODE Histone mark database was consulted indicating that these sites were located in chromatin-free, transcription-active regions and thus represent feasible locations for transcription factor binding in many cell lines including HepG2. (**Chapter 4, Section 4.2.3**)

A comparison of the promoter regions of all three human PEX genes revealed that PEX11β and PEX11γ shared two putative transcription factor binding sites for SMAD and ATF4 (Activating Transcription Factor 4), which belongs to the

large ATF/CREB family of transcription factors. In contrast, no transcription factor binding sites were shared between PEX11β and PEX11α or PEX11α and PEX11γ (**Figure 5.5**). These findings indicate that PEX11β and PEX11α are differently regulated and further support our notion that both proteins fulfil different functions at peroxisomes.

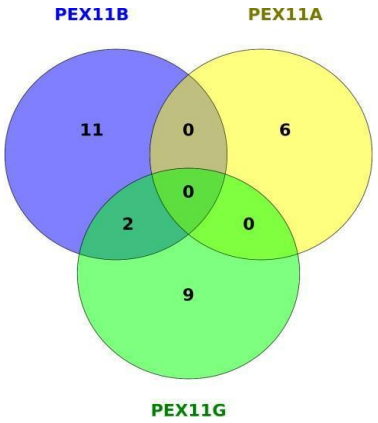


Figure 5.6 Venn diagram of shared transcription factor binding sites of *Hs*PEX11 genes alpha, beta and gamma. Potential transcription factor binding site for SMAD2/3 (activated by TGFβ pathway) in PEX11β and PEX11γ were predicted which are located in the transcriptionally active areas according to histone marks.

SMAD2/3 can be activated through the TFGβ pathway (Dennler *et al.*, 1998). The prediction, with high probability, of SMAD binding sites in the promoter regions of Pex11β and Pex11γ suggests that expression of these two genes might be linked to TGFβ signalling. PEX11β is a key protein in the regulation of peroxisome dynamics and proliferation; it is involved in all steps of the growth and division process including remodelling and elongation of the peroxisomal membrane, recruitment of the division machinery, and activation of the fission GTPase Drp1

as a GAP **(Introduction section 1.6**). We therefore focused our studies on the possible link between TGFβ signalling and peroxisome proliferation.

5.3.2 TGFβ induces peroxisome elongation and proliferation in HepG2 cells

To study the effect of TGFβ on peroxisomes, we used our developed cell-based peroxisome proliferation assay (see chapter 3). HepG2 cells cultured under serum-free conditions (N1) were mock treated or treated with recombinant TGFβ and processed for immunofluorescence 24 hours after treatment using anti-PEX14 as a peroxisomal marker (**Figure 5.7 A**). Recombinant TGFβ has been shown to activate TGFβ receptors on the cell surface and to signal through the SMAD2/3 complex (Dituri *et al.*, 2013). Whereas control cells (N1) showed overwhelmingly spherical peroxisomes (12% ± 0.88), addition of TGFβ resulted in a pronounced elongation of peroxisomes (75% ± 0.66), which is a pre-requisite of peroxisomal growth and division (**Figure 5.7 A-B**). As a positive control for peroxisomal growth/elongation, the cells were treated with 20% FBS (57%±1.6). Interestingly, treatment with TGFβ was as efficent or more efficient than with 20% FBS (**Figure 5.7 A-B**).

To validate that the TGFβ effect was specific to the SMAD-mediated TGFβ pathway, we used specific chemical inhibitors which inhibit different steps of the TGFβ signalling pathway. The inhibitor SIS3 selectively inhibits TGFβ signalling by suppressing SMAD3 phosphorylation (IC_{50} = 3 μM) without affecting the MAPK/p38, ERK, or PI3-kinase signalling pathways (Dituri *et al.*, 2013). We also examined the effect of TGFβ receptor type I/II inhibitor LY2109761, which acts

upstream of SIS3. LY2109761 has a selective dual inhibitor effect on both TGFβ receptors (type I/II) resulting in reduced phosphorylation of SMAD2 (Dituri *et al.*, 2013). HepG2 cells were pre-treated with TGFβ inhibitors for 2 hours before adding recombinant TGFβ, and processed for immunofluorescence as descried above. Both inhibitors reduced peroxisome elongation significantly when compared to TGFβ-treated controls (**Figure 5.7 B**) (LY210976 - 33% ± 1.2; SIS3
- 18% ± 1.3) indicating a specific TGFβ-mediated response.

We also quantified the number of peroxisomes in controls and TGFβ-treated cells after 6 hours (addition of TGFβ or FBS) and 48 hours using a MATLAB script. Whereas in unstimulated control cells no increase in peroxisome numbers was observed (6 hours - 131 ± 3.8, 48 hours - 239 ± 5.1), TGFβ-treatment resulted in a 3-fold increase in peroxisome numbers (6 hours - 131 ± 3.8, 48 hours - 522 ± 21.32) (**Figure 5.7 C-D**), which is indicative of peroxisome proliferation. Interestingly, peroxisome numbers were less prominently induced by treatment with FBS when compared to TGFβ (6 hours - 131 ± 3.8, 48 hours – 393 ± 17.94) (**Figure 5.7 D**). Overall, these findings support a role for TGFβ signalling in peroxisome proliferation, potentially via SMAD2/3 binding to the PEX11β promoter.

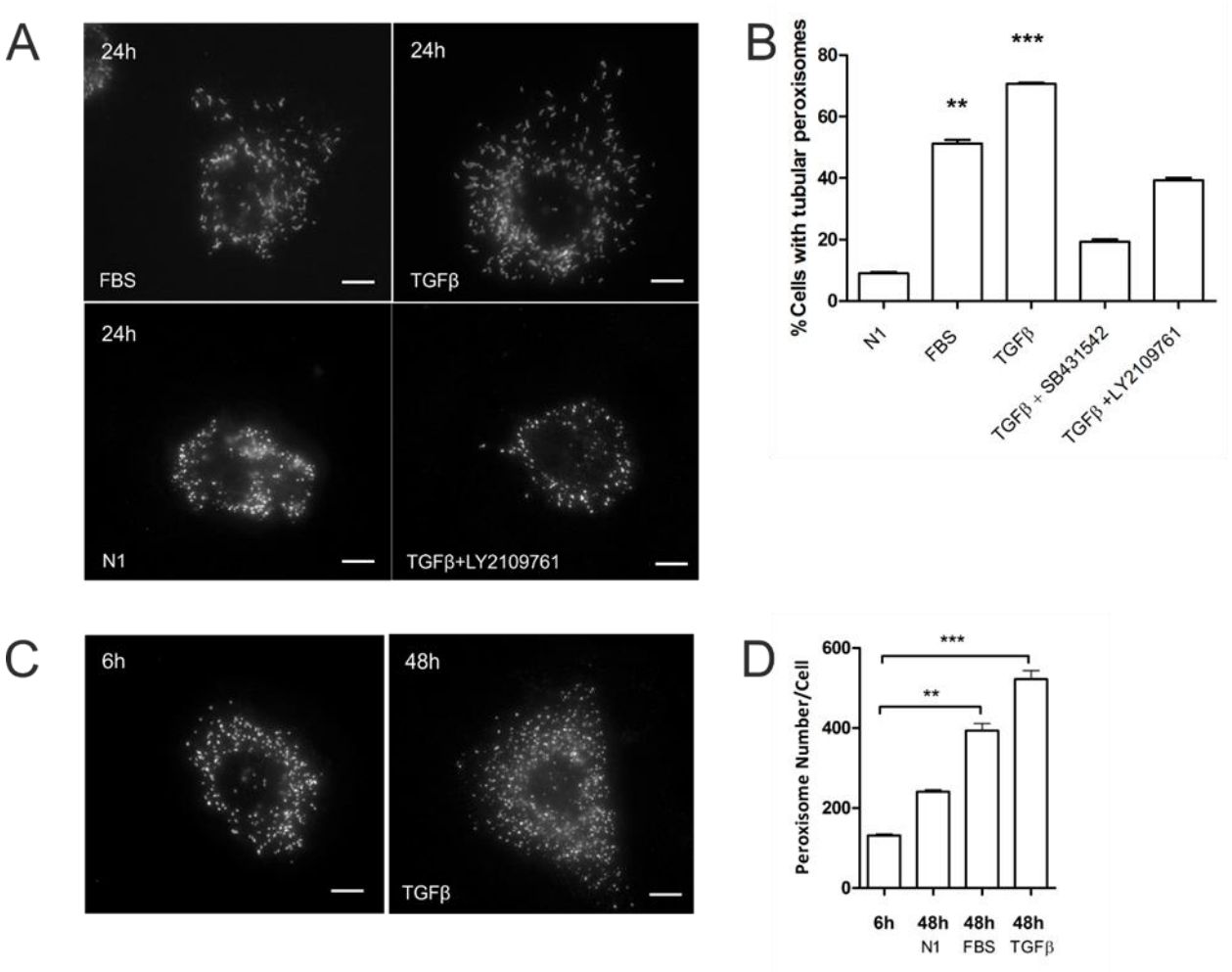


Figure 5.7. TGFβ increases peroxisome elongation and number in HepG2 cells. (**A**) HepG2 cells were cultured in serum-free αMEM-N1 medium, treated with 10% FBS, or TGFβ (2 ng) (with or without inhibitors LY2109761 or SB431542) or mock treated and processed for immunofluorescence after 24 hours using anti-PEX14 (pcR) and Alexa-488 (DaR) antibodies. **(B)** Quantitative analysis of peroxisome morphology (elongation) for the conditions described. Data represent mean ± SEM of 3 independent experiments (n=300 cells for each condition). *** p<0.001; ** p<0.01; two-tailed unpaired t-test. (**C**) HepG2 cells were cultured in αMEM-N1medium for 6h prior to the addition of TGFβ (2 ng) or 10% FBS, incubated for 48 hours and processed for immunofluorescence after 6 and 48 hours as described in (A). **(D)** Quantitative analysis of peroxisome number/cell after 6 and 48 hours. Data represent mean ± SEM of 3 independent experiments (n=10 cells for each condition).*** p<0.001; ** p<0.01; two-tailed unpaired t-test. Bars, 5 µm.

5.3.2 TGFβ induces PEX11β expression through its SMAD binding motif

The effect of TGFβ on peroxisome morphology and number is indicative for an involvement of TGFβ signalling in peroxisome proliferation via expression of PEX11β. To demonstrate that the SMAD binding motif within the PEX11β promoter is functional and can activate PEX11β expression after TGFβ stimulation, we applied a dual luciferase reporter assay (**Figure 5.8**). This assay relies on the co-expression of two different reporter genes, Renilla and firefly luciferase, to evaluate regulated gene expression. The plasmid encoding Renilla luciferase (pRL-TK) is under control of a constitutive CMV promoter and served as internal control. The PEX11β promoter region was cloned into a pGL3 vector encoding firefly luciferase (pGL3-Pex11β^{proWT}). In addition, a construct encoding a mutated promoter was generated by site directed mutagenesis inducing a two base pair deletion in the putative PEX11β SMAD binding site (pGL3-Pex11β^{proMut}) (**Figure 5.8**).

HepG2 cells were co-transfected with pRL-TK and pGL3-Pex11β^{proWT}, pGL3-Pex11β^{proMut}, or Basic-pGL3 and treated with recombinant TGFβ. As a positive control, cells were co-transfected with pRL-TK and pGL3 encoding the SMAD promoter (pGL3-SMAD). It has been shown that TGFβ has a direct effect on SMAD protein expression. After 24 hours, the luciferase activity, which reflects promoter activity, was measured in cell lysates after addition of luciferin. The dissimilarity in the substrates for the two luciferases makes it possible to selectively distinguish between the luminescent reactions for each enzyme. The luminescence of the firefly luciferase can be measured by addition of the luciferin reagent, and this reaction is subsequently quenched while simultaneously activating the luminescence of the Renilla luciferase. Thus, one can sequentially

measure the luminescence of both reporters in the same well. The data from firefly luciferase measurement (FLuc) was then normalised to the Renilla luciferase signal (RLuc) and the results expressed as the mean ratio of firefly to Renilla luciferase activity (FLuc/RLuc) (**Figure 5.8**).

We found that TGFβ augmented the luciferase activity in cells transfected with wild type PEX11β promoter (pGL3-Pex11β^{proWT}) (20.75 ± 0.32), which indicates that TGFβ increases the level of PEX11β expression (**Figure 5.8 B**). However, treatment with TGFβ failed to induce luciferase activity in the PEX11β promoter mutant (pGL3-Pex11β^{proMut}) (**Figure 5.8 B**) (1.1 ± 0.27) . Negative controls transfected with basic luciferase vector (pGL3) showed no inducible luciferase activity after TGFβ (0.98 ± 0.07) , whereas TGFβ induced luciferase activity in cells transfected with the SMAD promoter construct (pGL3-SMAD) (12.95 ± 0.12) (**Figure 5.8 B**).

When the mean FLuc/RLuc ratios in the absence of TGFβ stimulation were compared, only very low basal activity was detected for the empty luciferase vector (pGL3) (- control), pGL3-SMAD (+ control) and pGL3-Pex11β^{proMut} (**Figure 5.8 C**). Expression of pGL3-Pex11β^{proWT} resulted in some luciferase activity (1.4 RLU ± 0.03), suggesting that other factors may be able to trigger signalling via the SMAD binding site within the Pex11β promoter in the absence of TGFβ (**Figure 5.8 C**). However, treatment with TGFβ resulted in a prominent increase in luciferase activity (24.51RLU ± 0.32). For pGL3-SMAD, an increase from 0.2 ± 0.01 (control) to 1.6 RLU ± 0.01 (+ TGFβ) was detected, whereas the FLuc/RLuc ratios remained unchanged for pGL3 (- control) (0.28 RLU ± 0.07 to 0.30 ± 0.07) and pGL3-Pex11β^{proMut} (0.21 RLU ± 0.02 to 0.24 RLU ± 0.02) (**Figure 5.8 C**). These findings indicate that the SMAD binding site within the Pex11β promoter

is functional, and that TGFβ signalling via the canonical SMAD-dependent pathway can activate PEX11β expression, leading to peroxisome growth, division and proliferation in HepG2 cells.

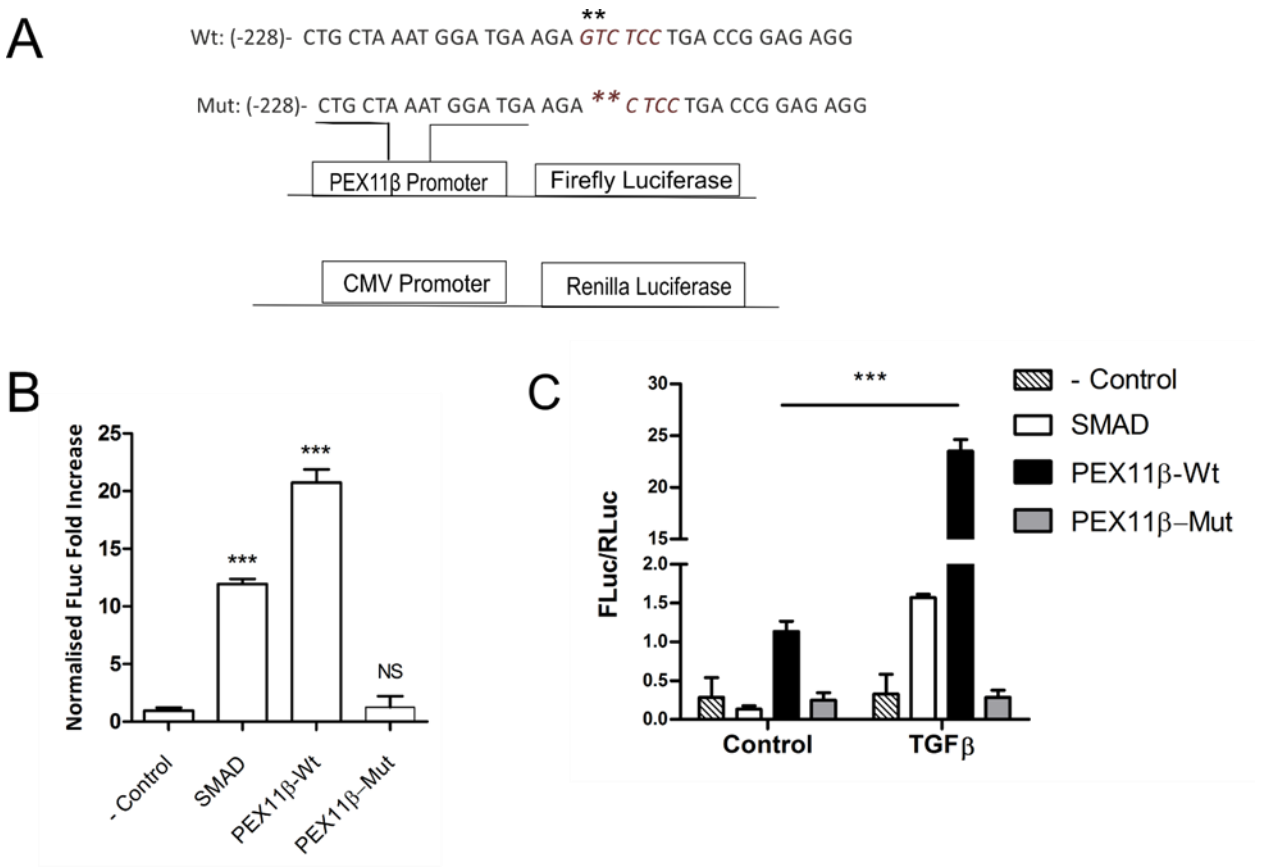


Figure 5.8. TGFβ activates PEX11β expression by direct binding of SMAD transcription factor to the PEX11β promoter. Luciferase reporter assays in HepG2 cells transfected with 100 ng/well of SMAD or PEX11β (Wt/Mut) Firefly-Luc reporter and 4 ng/well of *Renilla*-Luc reporter. As controls, empty Firefly Luc vector (- Control) was used. Cells were treated with TGFβ (2 ng/ml) or left untreated. Data are represented as mean ±SEM of the ratio firefly/*Renilla* luciferase, relative to control conditions. Each experiment was performed in triplicates (n=3). (**A**) Reporter vectors include the wild-type or mutant PEX11β promoter downstream of the firefly luciferase. The potential SMAD binding motif is in red and the mutated base pairs are marked as **. pR-TLK construct with CMV promoter downstream of *Renilla* luciferase was used as an internal control in all the experiments. (**B**) Firefly luciferase activity (FLuc) was first normalized to *Renilla*

luciferase activity (RLuc). Fold increase was then calculated by normalizing the values in the presence of TGFβ. **(C)** The mean ratio of Firefly luciferase and *Renilla* luciferase activity in cells transfected with pGL3-Pex11β^{proWT} in untreated and TGFβ-treated cells was approximately 1.4 and 25 RLU (relative light unit), respectively. The mean ratio of FLuc/RLuc for cells transfected with the SMAD promoter construct pGL3-SMAD (positive control, inducible promoter by TGFβ) in untreated and TGFβ-treated cells was approximately 0.2 RLU and 1.6 RLU.

5.4 Discussion

The canonical TGFβ pathway is involved in peroxisome proliferation in HepG2 cells

In this chapter, we provided convincing evidence for a role of the canonical, SMAD-dependent TGFβ signalling pathway in the control of peroxisome proliferation in HepG2 cells via PEX11β. We showed that i) addition of TGFβ induces peroxisome elongation/growth and proliferation in HepG2 cells in our cell-based proliferation assay; ii) the stimulatory effect of TGFβ on peroxisomes could be inhibited by specific kinase inhibitors which interfere with TGFβ signalling; iii) the promoter region of PEX11β contains a SMAD2/3 binding site in a transcriptionally active region with a predicted strong binding efficiency; iv) the SMAD2/3 binding site is functional and required to induce luciferase activity of a reporter after TGFβ stimulation. These findings indicate that TGFβ signalling through the canonical SMAD-dependent pathway can activate PEX11β expression, leading to peroxisome growth, division and proliferation in HepG2 cells. This study therefore links a new signalling pathway to peroxisome proliferation in human cells (**Figure 5.9**).

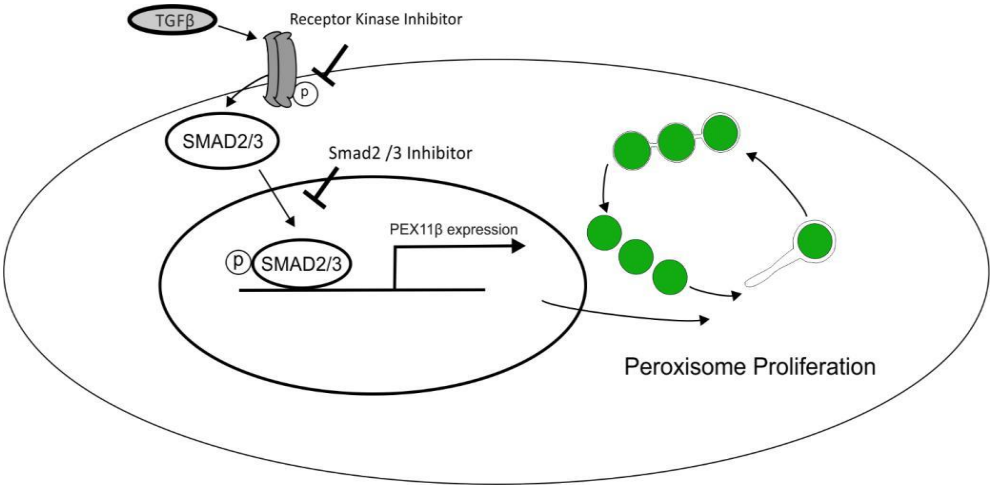


Figure 5.9. Schematic diagram of TGFβ signaling and peroxisome proliferation in HepG2 cells. TGFβ binds a complex of transmembrane receptor serine/threonine kinases (types I and II) at the cell surface and induces phosphorylation of the receptor kinases. The activated receptors phosphorylate SMAD2/3 (R-SMAD) at C-terminal serines. Activated Smad complexes translocate into the nucleus, where they regulate transcription of target genes/PEX11β, through physical interaction and functional cooperation with DNA-binding transcription factors. Activation of R-Smads by type I receptor kinases can be inhibited by receptor kinase inhibitors or SMAD2/3 inhibitor.

The TGFβ superfamily of signalling proteins regulates a variety of biological processes including cell growth, differentiation, apoptosis, migration, extracellular matrix production, angiogenesis, immunity, and development (Massague, 1998; Zhu *et al.*, 2004). The cellular response to many TGFβ superfamily members can be variable, and promote as well as antagonise responses such as proliferation, apoptosis, and differentiation depending on the cell type and stimulation context (Massague, 1998; Massague *et al.*, 2000). A well known function of TGFβ is the induction of growth arrest and apoptosis in epithelial cells, which suppress carcinogenesis. However, TGFβ can also induce epithelial-mesenchymal transition (EMT) and mediate fibroblast

activation, thus promoting carcinogenesis and fibrotic diseases (Siegel & Massague, 2003).

TGFβ is also a key regulator of liver physiology and pathology. It contributes to hepatocyte proliferation and differentiation, but also to all stages of disease progression, from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma (Fabregat *et al.*, 2016).

HepG2 cells have been shown to express TGFβ and to respond to TGFβ treatment or silencing of TGFβ with alterations in cell growth, apoptosis and cell cycle (X. H. Wang *et al.*, 2017). In our experimental set up, HepG2 cells were treated with physiological concentrations of TGFβ for short periods of time under serum-free conditions. HepG2 cells responded with a pronounced elongation/growth and subsequent division and proliferation of peroxisomes. This response was often more pronounce than stimulation with FBS indicating that TGFβ initiates a strong, peroxisome-specific response. This response is likely mediated by PEX11β expression via its SMAD transcription factor binding site. PEX11β is a key protein in the regulation of peroxisome dynamics and proliferation, and its expression induces peroxisome elongation, division and proliferation (Schrader *et al.*, 1998b). Those processes have been linked to cellular growth and are observed under standard growth conditions in HepG2 and other cell lines, in particular when the cells are seeded at low cell densities (Schrader *et al.*, 1996). It is suggested that peroxisomes, which are involved in fatty acid β-oxidation and cellular energy control, multiply under those growth-stimulating conditions to support cell proliferation and/or differentiation. We therefore assume that TGFβ signalling in our experimental system contributes to the proliferation and/or differentiation of HepG2 cells rather than to growth arrest.

It should be noted that peroxisome elongation is usually not observed under suboptimal growth conditions and is rather a hallmark of cell growth and proliferation (Schrader *et al.*, 1999).

Interestingly, elongated and tubular or constricted peroxisomes are also observed after partial hepatectomy in rat liver (Fahimi *et al.*, 1979). These heterogenous peroxisome morphologies were as well interpreted as an indicator of peroxisome proliferation by elongation/growth and division under conditions of cellular growth and proliferation (Schrader & Fahimi, 2008). TGFβ has a crucial role in hepatocytes, which is critical for the control of liver mass (Karkampouna *et al.*, 2012). The TGFβ pathway has activator and repressor effects on other pathways such as cell growth, and a balanced TGFβ signaling in terms of both dosage and spatiotemporal activity is crucial to control hepatic gene expression (Karkampouna *et al.*, 2012). TGFβ acts in the process of differentiation of hepatoblasts to hepatocytes during liver regeneration after partial hepatectomy (Karkampouna *et al.*, 2012). The TGFβ pathway is required at different stages of the process, to allow hepatocyte proliferation at the inductive phase followed by an efficient termination of the regenerative response afterwards.

HepG2 cells are hepatoblastoma cells, which can differentiate in culture and form bile canaliculi-like structures (Bokhari *et al.*, 2007). Like hepatocytes, they secrete the major serum proteins and do not form tumours in nude mice (Wu *et al.*, 1994). We therefore assume that similar to regenerating liver, TGFβ induces peroxisome proliferation in HepG2 cells under proliferative culture conditions via upregulation of PEX11β, a key regulator of peroxisomal growth and proliferation. As discussed before (see chapter 3), under standard culture conditions, peroxisome elongation has a maximum after 24-48 hours, before the elongated peroxisomes divide

resulting in the formation of numerous spherical peroxisomes. Peroxisomes do not massively elongate at later time points in culture, when the cells are more confluent (Schrader *et al.*, 1998b). Ultrastructural studies have shown that the peroxisomes in HepG2 cells are now larger and form little groups of organelles, possibly changing from a proliferative to a metabolic state (Grabenbauer *et al.*, 2000). As PEX11β mediates peroxisome elongation and proliferation, it represents an ideal target to adapt peroxisome number and function according to cellular needs. TGFβ signalling may foster peroxisome proliferation in an inductive phase of HepG2/hepatocyte proliferation and differentiation, but may also reduce proliferation of peroxisomes afterwards, when the inductive stage is completed, and cell growth needs to be terminated (**Figure 5.9**). As TGFβ signalling is complex, it is likely that other, non-canonical signalling pathways as well as TGFβ secretion, the concentration of active TGFβ, and the amount of TGFβ receptors and spatiotemporal activity contribute to the overall cellular response. It should be noted that SMAD transcription factor binding sites were only detected in PEX11β and PEX11γ, and not in other peroxisomal genes examined, indicating that the PEX11 proteins might be specifically targeted by TGFβ signalling (**Figure 5.8**).

TGFβ signalling has also been linked to carcinogenesis (Chaudhury, 2009), as it mediates growth arrest and induces apoptosis preventing tumour formation, but also excerts tumour promoting effects at later stages of tumour progression. Peroxisomes have been reported to be essential for the viability of liver cancer cells, probably through altering metabolism and signaling pathways ((M. Cai *et al.*, 2018). Peroxins and associated signalling pathways may thus be potential targets of therapeutics against liver cancer.

Chapter 6 – Results

The VAP–ACBD5 tether is required for peroxisome growth

Costello, J., Castro, I., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., Azadi, A. S., Godinho, L. F., Costina, V., Findeisen, P., Manner, A., Islinger, M., and M. Schrader: (2017) ACBD5 and VAPB mediate membrane associations between peroxisomes and the ER. *J Cell Biol, 216*(2), 331-342. doi:10.1083/jcb.201607055

6.1 Overview

It has been shown that the ER has a role in peroxisome biogenesis by providing peroxisomal proteins such as PEX16, but also for their lipid composition (Hettema (Hettema *et al.*, 2014; Hua *et al.*, 2017). Several models have been proposed for the lipid/protein exchange between ER and peroxisomes. One of these mechanisms includes carnitine transporters, shuttling specific lipids to peroxisomes through the cytosol (Lodhi & Semenkovich, 2014). The latest suggested mechanism is through ER/peroxisome contact sites (Costello *et al.*, 2017a; Raychaudhuri & Prinz, 2008; Schrader *et al.*, 2015). Our group identified peroxisomal acyl-CoA binding domain protein 5 (ACBD5) and VABP as a molecular linker between peroxisomes and the ER (Costello *et al.*, 2017b). Absence of ACBD5 or VAPB increased peroxisome motility; this implies that the ACBD5/VAPB complex acts as a tether between peroxisomal and ER membranes (Costello et al., 2017a). In another study, Hua et al (2017) showed that loss of this tether affects cellular plasmalogen and cholesterol levels (Hua *et al.*, 2017). There was a decrease in phosphatidylethanolamine (PE) plasmalogens and total cholesterol levels in Hela cells treated with ACBD5 or VAPB siRNA (Hua *et al.*, 2017). ACBD4 is a member of the ACBD family and like ACBD, can also act as a molecular tether, physically linking peroxisomes and the ER making this the second protein involved in peroxisome-ER contacts in mammals (Costello *et al.*, 2017b). Peroxisomes can form by growth and division of pre-existing organelles (Schrader *et al.*, 2015). A key protein in this process is Pex11β, which deforms and elongates the PO membrane and activates the GTPase DRP1 for membrane scission (Delille *et al.*, 2010; J. Koch *et al.*, 2010; Williams *et al.*, 2015). As

mentioned in the introduction (**See section 1.4.3**) peroxisome formation by growth and division follows a multistep maturation process involving peroxisomal membrane remodelling and elongation, membrane constriction and final scission. DRP1 is recruited to peroxisomes by the membrane adaptors Mff and Fis1 (Gandre-Babbe & van der Bliek, 2008; A. Koch *et al.*, 2005; Otera *et al.*, 2010). Peroxisomes in cells with no Mff lose the ability to divide and have highly elongated peroxisomes (and mitochondria) (J. Koch & Brocard, 2012; J. Koch *et al.*, 2016; Shamseldin *et al.*, 2012). The elongation step requires membrane expansion. Peroxisome-ER contacts are crucial for lipid transfer generating a membrane compartment which imports newly synthesised PMPs and matrix proteins.

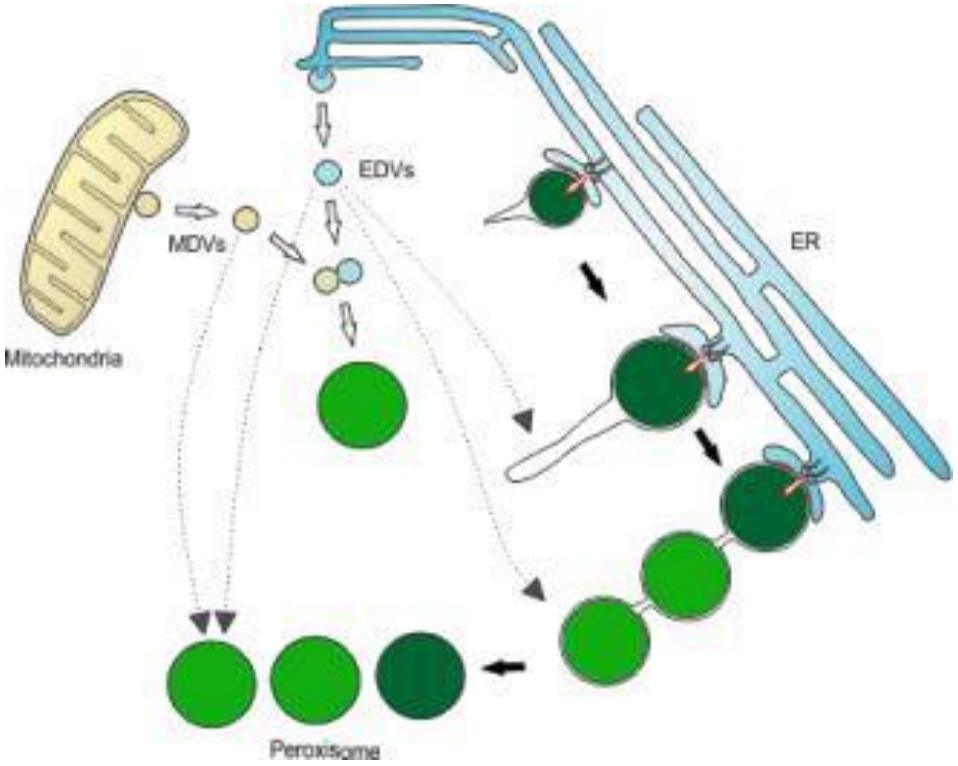


Figure 6.1. **Schematic view of peroxisome formation in mammalian cells**: Peroxisomes form by growth and division of pre-existing peroxisomes. The ER delivers lipids for membrane growth via membrane contact sites (red arrows). In the absence of pre-existing peroxisomes, peroxisomal vesicles can be generated at the ER (EDV) and mitochondria (MDV), which may fuse and mature into new import-competent peroxisomes. These newly formed peroxisomes then multiply by growth and division. In the presence of peroxisomes, pre-peroxisomal vesicles may fuse with growing or existing peroxisomes to supply certain proteins and lipids. EDV, ER-derived vesicles; MDV, mitochondria-derived vesicles; newly formed peroxisomes are coloured in light green (Costello & Schrader, 2018).

6.2 Disrupting peroxisome-ER contacts reduces peroxisome elongation

As elongation and growth of the peroxisomal membrane requires lipids, likely provided by the ER in a non-vesicular pathway (Raychaudhuri & Prinz, 2008), we suggested that the pronounced peroxisome elongation observed in patient fibroblasts after loss of Mff reflects a constant transfer of lipids from the ER to peroxisome. To investigate a role for ACBD5–VAPB in this process, ACBD5 or VAPB were silenced in patient fibroblasts deficient in Mff (**Figure. 6.2D**-**E-G**). Expression of Mff into control cells (no siRNA) resulted in the formation of spherical peroxisomes, restoring the normal organelle phenotype (**Figure. 6.2 B**). Knockdown of Pex11β had no effect on peroxisome morphology, however, silencing of ACBD5, and to a lesser extent of VAPB, reduced membrane expansion, resulting in the formation of shorter peroxisome membrane tubules and spherical organelles (**Figure. 6.2, C–F**). These findings indicate that

peroxisomal membrane growth depends on ER-peroxisome membrane contacts mediated by ACBD5 and VAPB.

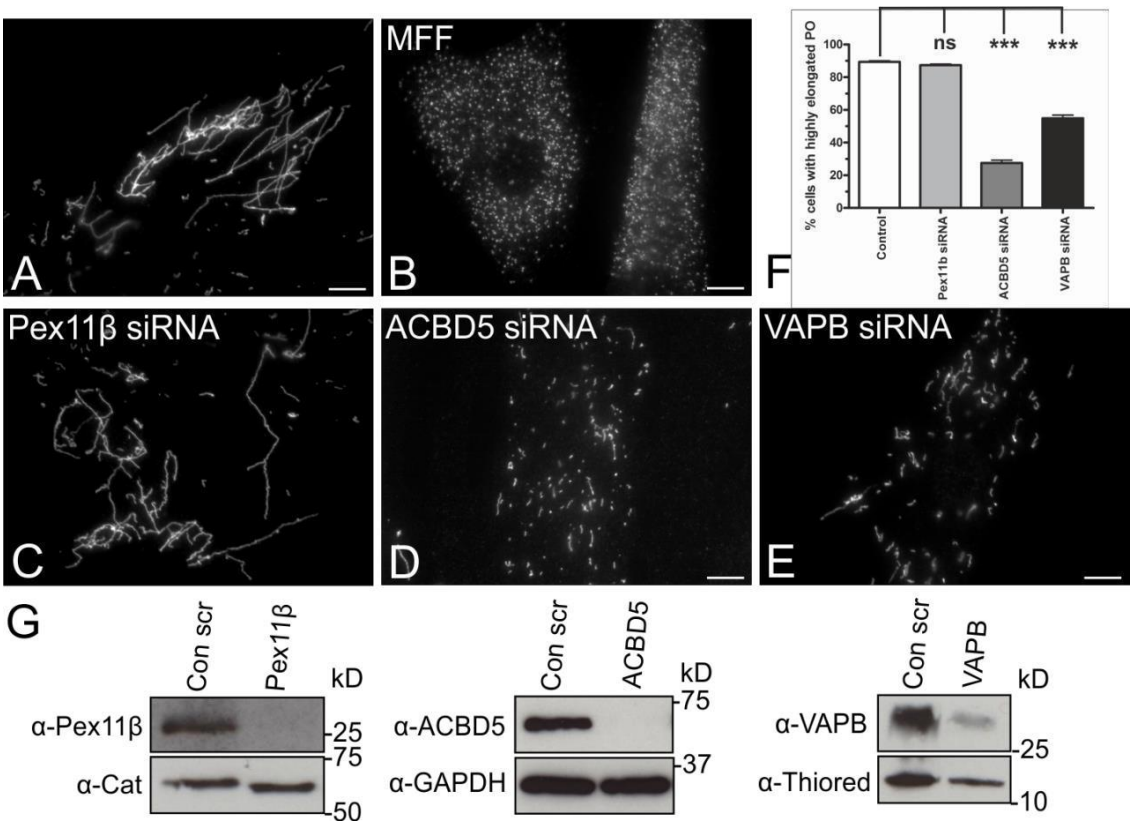


Figure 6.2. Loss of ACBD5 or VAPB reduces peroxisomal membrane expansion in Mff-deficient fibroblasts. PO morphology in Mff-deficient fibroblasts (control; A) after reintroduction of Mff (B) or silencing of Pex11β (C), ACBD5 (D), or VAPB (E). Fixed cells were labelled with anti-Pex14 antibodies. (F) Quantification of peroxisome morphology in controls and silenced cells (n = 2,500, from three independent experiments). Data are presented as mean ± SEM. ***, P < 0.001; ns, not significant. (G) Immunoblots of cell lysates. Loading controls used were catalase (Cat), GAPDH, and thioredoxin (Thiored). (H–M) Localization of endogenous ACBD5 in Mff-deficient fibroblasts. Fixed cells labelled with anti-ACBD5 and anti-catalase antibodies. Arrowheads denote ACBD5 concentrated at globular peroxisomes that give rise to tubular membranes. Bars, 10 µm (Costello *et al.*, 2017a).

6.3 Transcription factor binding site analysis of ACBD4 and ACBD5

ER-peroxisome contacts appeared to be important for peroxisome elongation, and besides ACBD5 we also identified ACBD4, which is as well involved in ER-peroxisome membrane contacts (Costello *et al.*, 2017b). To understand how ACBD5 and ACBD4 gene expression is regulated, I used my established motif search pipeline (Chapter 4) and searched for regulatory elements within the promoter regions of these genes. The most prominent transcription binding factors in the promoter regions of these genes are summarized in Figure 6.3.

As discussed in Chapter 4, PEX11β, the key modulator of peroxisome dynamics, has a SMAD2/3 binding motif, which based on our results (Chapter 5), is directly regulated by the TGFβ pathway. However, ACBD4 and ACBD5 do not have this binding motif or any other binding sites shared with PEX11β, suggesting they are not co-regulated.

An interesting binding motif, present in the ACBD4 promoter, is a glucocorticoid receptor binding site (**Figure 6.3**). Glucocorticoids (are a class of steroid hormones that are secreted by cortical cells in adrenal glands. Glucocorticoids modify the immune responses to diverse stimuli. They produce their effects through their actions on the intracellular glucocorticoid receptors (GR). The glucocorticoid receptor is a nuclear transcription factor and can activate or repress gene expression either through direct binding to glucocorticoid response elements or by binding to other transcription factors, e.g., activator protein1 (AP-1), nuclear factor (NF)-κB, tumour necrosis factor (TNF)-α (Rao et al., 2011).

Interestingly, the ACBD4 promoter also contains an NFkb motif, further suggesting a role for GR in modulating ACBD4 gene expression.

In our study, we found potential glucocorticoid response elements (GRE) in other peroxisomal genes such as PPARα, PEX5l, AGXT, EHHADH, ISOC1, ACOT4, ACAD11, and ACAA1a. This may suggest an important role for GR in activating expression of peroxisomal genes resulting in the stimulation of fatty acid breakdown and energy production.

For the ACBD5 promoter, E4BP4, PAX-3 and POU2F1 (activates the transcription of genes involved in viral immediate response) motifs were found in proximity of the transcription start site. These factors have no obvious link to peroxisome function or lipid metabolism. This suggests that ACBD5 expression at the mRNA level is not controlled by the same signalling cascades as ACBD4 or other peroxisomal genes.

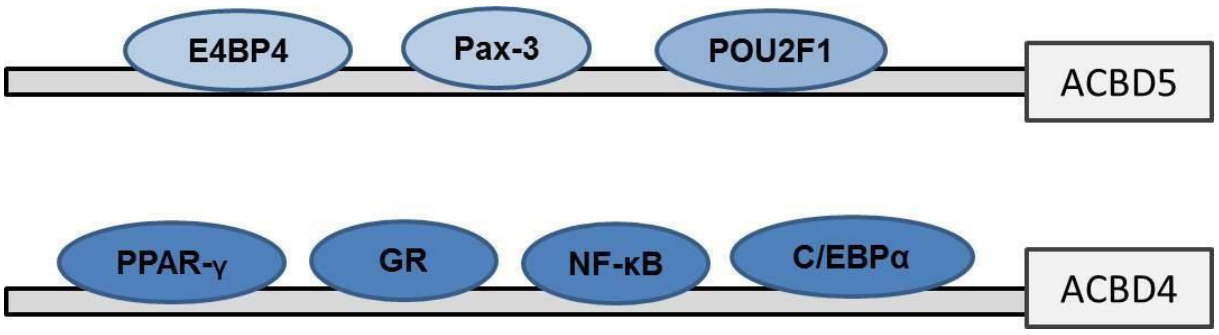


Figure 6.3. Summary of potential transcription factor binding sites in the promoter regions of ACBD5 and ACBD4. In promoter of ACBD5, E4BP4, PAX-3 and POU2F1 (activates the transcription of genes involved in viral immediate response) motifs were found in proximity of the transcription start site. Factors in ACBD4 includes PPARγ, GR, NF-κB and C/EBPα.

Chapter 7 – General Discussion

The mechanism of peroxisome proliferation is well studied, however, there are still questions to be answered about how this process is regulated and which regulatory cascades are involved in this process. In this study, a cell-based assay (HepG2 cells) has been established which allows us to assess changes in peroxisome morphology/ proliferation and the expression profile of peroxisomal genes involved in regulation of peroxisome proliferation or metabolic function after application of a well-defined stimulus.

A well-known pathway for regulating peroxisomal genes (such as ACOX1, PEX11α) in mammals is through activation of PPAR (H. Lee & Yoon, 2014; Shimizu *et al.*, 2004). However, most of the transcriptomics studies with PPAR agonists are in mice (Barish *et al.*, 2008; Guri & Hall, 2016). Despite a large body of evidence linking PPARα signalling with a variety of other signalling pathways there are currently few reports on how this links to peroxisome homeostasis in humans.

Moreover, an increase in peroxisome number in mammals can be induced by various compounds in the absence of PPAR such as, endosulfan, cyclophosphamid, acetyl salicylic acid, 4-phenylbutyrate, and BM 15,766 (Abraham & Isaac, 2011; Baumgart *et al.*, 1990; Braunbeck & Volkl, 1991; Y. Cai *et al.*, 1994; Gondcaille *et al.*, 2005; Ortiz-Zarragoitia & Cajaraville, 2005). Interestingly, peroxisome proliferation induced by BM 15,766 does not change peroxisomal β-oxidation implying that both processes can be regulated independently. Other environmental factors such as UV light and reactive oxygen species (ROS) have been described to induce peroxisome elongation which leads to peroxisome division and increase in number in HepG2 cells (Schrader *et al.*, 1998).

Also, in our HepG2 cell-based assay, growth factors (**Chapter 3. Section 3.3.1**), and arachidonic acid (**Chapter3, Section 3.3.4.1**) can induce peroxisome proliferation. This suggests that there are other factors independent of PPAR regulating peroxisome abundance.

There is currently a gap in understanding of precisely how this induction of proliferation with these tested stimuli is linked to elevation of peroxisomal related genes expression (McMullen et al., 2014).

Many studies have examined the effect of PPARα activation or inhibition on hepatic gene regulation using PPAR agonists and antagonists followed by transcriptomics (Berger & Moller, 2002; Kersten *et al.*, 1999; van der Meer *et al.*, 2010). Comparison of the effect of PPARα activation in primary mouse and human hepatocytes using a whole genome transcriptomics approach showed the role of PPARα as a master regulator of hepatic lipid metabolism in both mouse and human liver cells (Bility *et al.*, 2004; L. Guo *et al.*, 2006). However, in recent years, the role of PPARα in human liver has been questioned based on the low levels of PPARα mRNA expression in human liver compared with mouse liver, indicating the involvement of other pathways (Rakhshandehroo, 2009). This conclusion has been further supported by the low response to PPARα agonists on expression of lipid metabolism genes in HepG2 cells. Based on the lower expression level of PPARα in human liver compared to mouse liver, here, we focused on not only PPARα binding sites but other PPAR forms (PPARβ and γ). Based on our results, PPARα appears to have potential binding sites in genes involved in β-oxidation such as ACOX1 and ACAA1, however, 10 peroxins have a PPARγ binding site. (**Chapter 4. Section 4.1**). This highlights the potential role

of PPARγ in peroxisomal gene expression encoding proteins involved in peroxisome comportment.

Further, we looked for other regulatory factors in the promoters of the peroxisomal and peroxisomal-related genes (**Chapter 4. Section 4.2**). Binding of a transcription factor to DNA at a particular location is influenced by a variety of factors. The first factor that was considered in our study is the binding sequence
– i.e. the sequence of bases that appear at a putative binding site. Also, we considered the proximity of nucleosomes and the methylation, phosphorylation or acetylation states of their constituent histones (Putiri & Robertson, 2011).

Another important factor which can considered for further investigating in predicted regulatory motif sites in our system is the distant of each motif to transcription start site. The same holds for other transcription co -factors that may be bound near these binding sites whose presence can either inhibit binding of the transcription factors which we found, or enhance binding by modifying its conformation, or the DNA (Aerts *et al.*, 2003; Elkon *et al.*, 2003; Elnitski *et al.*, 2006).

Studies have shown a high percentage of functional motifs are found within the first 100 base pair (bp) and 2000bp upstream of the TSS (Buckland *et al.*, 2005; Cooper *et al.*, 2006; Elkon *et al.*, 2003; Hoogendoorn *et al.*, 2003). However, more recent studies have showed that regulatory regions, such as enhancers may extend many thousands of bp from the TSS creating complicated 3D structures that can influence gene expression by physical interaction with the proximal promoter (Angelini & Costa, 2014; Gandolfi & Tramontano, 2017). Although in our study these factors were not taken into consideration, our analysis still

represents a major advancement in our understanding of regulatory elements in promoter of peroxisomal genes in HepG2 cells.

The data obtained from our bioinformatics approach revealed that, unlike regulatory systems used by other organelles (e.g. lysosomal biogenesis with TFEB transcription factor as its master regulator), peroxisomal gene expression in humans appears to complex with no single dominant factor present at the majority of peroxisomal genes. Whilst for lysosomes it has been shown that TFEB coordinates expression of genes encoding lysosomal hydrolases, membrane proteins and genes involved autophagy (Settembre & Ballabio, 2014). Here, we found different transcription factors regulating expression of metabolic genes and peroxins. As discussed before (**Introduction, section 1.1**) peroxisomes respond to external stimuli by increase in number or enzymatic activity and based on our results we suggest these are controlled by different pathways. Transcription factors such as CEBP and FOXA1 were found in 42, and 32 peroxisomal genes, respectively. Both factors are linked to various signalling pathways regulating liver development or immune response by cross talk with NFκB, further highlighting the role of peroxisomes in these processes (Di Cara *et al.*, 2017; Jin *et al.*, 2015; Moya, 2012). Previous studies have demonstrated the importance of peroxins in postnatal development especially the development of nerve systems. Disruption of these signaling functions of peroxisomes can be directly or indirectly linked to a number of diseases, such as diabetes, neurodegenerative disease, and cancer (Marcus Nordgren *et al.*, 2013).

In a recent study, it has been shown that PEX2 and other peroxins are essential for liver cancer cells viability, probably through altering metabolism and signaling pathways (M. Cai *et al.*, 2018). Based on this study, peroxins may be potential

targets of therapeutics against liver cancer (M. Cai *et al.*, 2018). Whilst the available epidemiologic and clinical studies on the effect of peroxisome proliferators in human are inconclusive they still provide evidence that classical peroxisome proliferators used as drugs can promote cancer cell growth (Kristiansen *et al.*, 2006; Takeuchi *et al.*, 2016; Youssef & Badr, 2011).

Hence, identification of other signalling pathways regulating one single or a group of peroxisomal genes will provide more information of other therapeutical targets.

Alteration in expression of just a single gene or a set of peroxisomal genes has an effect on peroxisome abundance. It has been showed that overexpression of PEX11β alone can efficiently induce peroxisome proliferation demonstrating that, even in the absence of extracellular stimuli, altered expression of only PEX11β can induce peroxisome proliferation (Schrader *et al*., 1998). Hence, we focused on regulatory elements present in PEX11 promoters. Based on our results, PEX11β possess a SMAD2/3 binding site on its promoter, 200 bp away from the transcription start site. (**Chapter 4, Section 4.2.5**). As discussed in Chapter 5, activated TGFβ type I receptor phosphorylates Smads2/3 and initiate the cascade which leads to its target gene expression. Hence, we tested the TGFβ in our cell-based assay to elucidate its ability to induce peroxisome proliferation. As predicted, TGFβ induced peroxisome elongation (cells with tubular peroxisomes), followed by an increase in peroxisome number compared to non-treated control cells. TGFβ also activates the non-Smad pathways, which include Ras, phosphatidylinositol 3-kinase (PI3K), and Par 6 (X. Guo & Wang, 2009). These molecules regulate the activities of glycogen synthase kinase 3 (GSK3) and RhoA, which can increase the cancer metastasis in primary liver cancer cells. In order to link TGFβ activation to SMAD2/3 activation and PEX11β

promoter activity, a functional assay was performed using luciferase vectors and co-transfection with Renillla vectors as internal control. As expected, TGFβ treatment induce the promoter activity of PEX11β^{proWT}, however, this induction was disrupted by a mutation in the SMAD2/3 binding site in the PEX11β promoter (PEX11β^{ProMut}). Here, we introduced a novel regulatory element regulating abundance by direct effect on peroxisomal gene expression.

In order to gain more insight on expression of peroxisomal genes, other functional studies can be used, e,g by performing ChIP-qPCR, we will be able to test some of the transcription factors candidates (such as PPARγ, GRC and FOXA1) , to see if they directly are associated with our gene(s) of interest. Future studies using large scale expression profiling in this system will contributes to the identification of more novel components regulating peroxisome morphology and dynamics.

In conclusion, in this study we provide a data set of regulatory elements in peroxisomal genes We introduced a new signalling pathway controlling peroxisome proliferation by regulating PEX11b gene expression this opens a possibility for therapeutical approaches controlling regulation of PEX11β in patient where peroxisome abundance / activity reduced.

Appendix 1

Table S 3.1. Human primers for quantitative real-time RT-PCR

h18S_F1	CCATCCAATCGGTAGTAGCG	21		
h18S_R1	GTAACCCGTTGAACCCCATT	20		
hCyclophilin_F	CCCACCGTGTTCTTCGACATT	21	275	NM_021130
hCyclophilin_R	GGACCCGTATGCTTTAGGATGA	22		
hPEX11a_F	GAGAAGGTGGTAATGAAGCTCAA	23	108	NM_003847
hPEX11a_R	GCTCTGCTCAGTTGCCTGT	19		
hPEX11b_F	CGCCCAGTATGCTTGCTCTC	20	220	NM_003846
hPEX11b_R	TCGATTGAGGTGACTAACAGTGA	23		
hPEX11g_F	GGGGACACGTCTGTTGGTG	19	184	NM_080662
hPEX11g_R	ACAGGGGTAGTAGAGCTGGTC	21		
hPEX1_F	GGGGACAGGTATTTCTCAAGC	21	243	NM_000466
hPEX1_R	CCAGCCTTCCATAAGAGGCAG	21		
hPEX6_F	CCCTTTCCGACCGAGACAC	19	205	NM_000287
hPEX6_R	CGCGGCTAACCAGTAGCTG	19		
hPEX26_F	GTGCTCCCTGTGTGTTGTG	19	93	NM_001199319
hPEX26_R	GGGACCTGGTAATACTGAAGGAC	23		
hPEX5_F	AAGCCTTTGGGAGTAGCTTCT	21	75	NM_001131024
hPEX5_R	GGACACAAGGGGTGCATTC	19		
hPEX2_F1	GAGAATGCGAAGAGTGCAAACA	22	148	NM_000318
hPEX2_R1	CTGGCTCAAAGCGAGCTAACA	21		
hPEX14_F	GCCACGGCAGTGAAGTTTCTA	21	119	NM_004565
hPEX14_R	TGCTGGAAGGCCATATCAATCT	22		
hPEX3_F	TGGCTGAGTTCTTTCGACCTA	21	131	NM_003630
hPEX3_R	ACTGCAAACTGAATGGATCTGTC	23		
hPEX13_F	ACCTGGACAACCAGCACTTAC	21	187	NM_002618
hPEX13_R	GCCCAGCCCATTATATCCATAAC	23		
hPEX19_F	GCTGAGGAAGGCTGTAGTGTC	21	157	NM_002857
hPEX19_R	CTGGCGATCTCTTCTGGGG	19		
hPEX16_F	GTGCGGGGCTTCAGTTACC	19	82	NM_004813
hPEX16_R	GGTTAGAGGCAGAGTACACCA	21		
hPGC1a_F	TCTGAGTCTGTATGGAGTGACAT	23	112	NM_013261
hPGC1a_R	CCAAGTCGTTCACATCTAGTTCA	23		
hPGC1b_F	GATGCCAGCGACTTTGACTC	20	186	NM_001172698
hPGC1b_R	ACCCACGTCATCTTCAGGGA	20		
hPex10_F	TCTGCTGGGAGTGCATCAC	19	94	NM_153818
hPex10_R	CGAAGGTAGATGAGCTTCTGGG	22		
hPex12_F	TTGAGGTGGTAGCACAGGACA	21	88	NM_000286
hPex12_R	GTGGGATTTGATTCTGCAAGAAC	23		

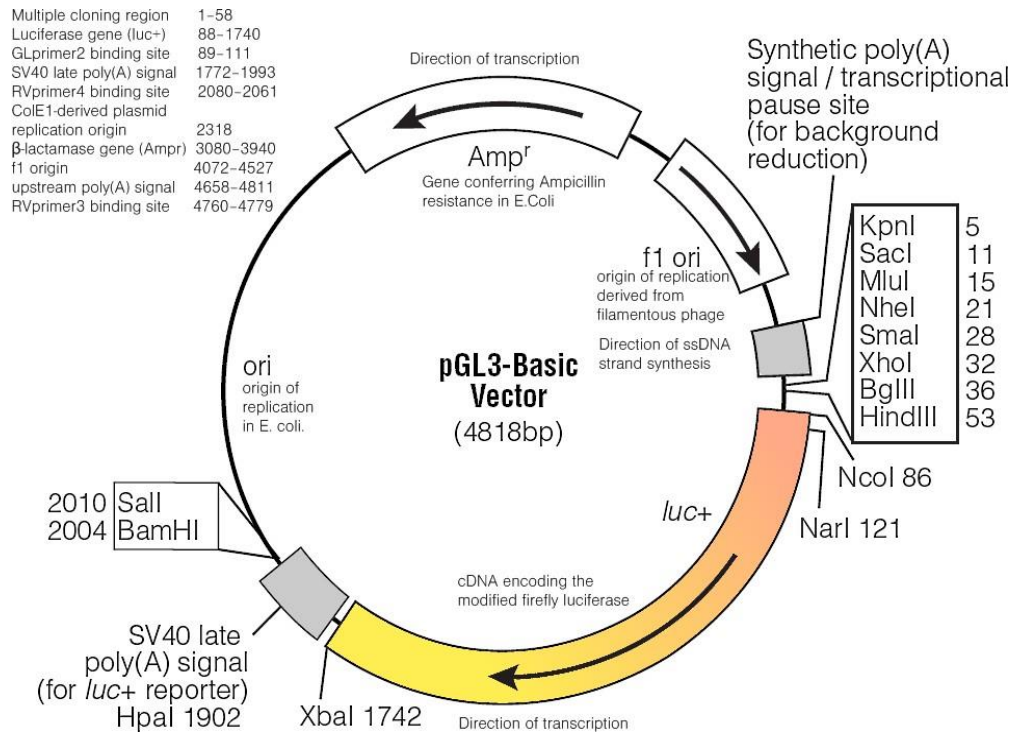
hPPARa_F1	ACACTGTGTATGGCTGAGAAGA	22	117	NM_005036
hPPARa_R1	GACGGTCTCCACTGACGTG	19		
hPPARg1_F	AAAGAAGCCGACACTAAACC	20		
hPPARg1_R	CTTCCATTACGGAGAGATCC	20		
hABCD1_F	GCTGGCATGAACCGGGTATT	20	143	NM_000033
hABCD1_R	GCCACATACACCGACAGGAA	20		
hABCD3_F	GCCTGCACGGTAAGAAAAGTG	21	100	BT006644
hABCD3_R	AGCCTTGAGAAAAACACCTTGTC	23		
hAcox1a_F1	CCTGAACGACCCAGACTTCC	20	227	NM_004035
hAcox1a_R1	TTGCCTGGTGAAGCAAGGTG	20		
hAcox1b_F1	GGGCCTCAATTACTCCATGTTT	22	114	NM_007292
hAcox1b_R1	TGGGCGTAGGTGCCAATTATC	21		
hMFP1_F1	GGTCAACGCGATCAGTACGAC	21	104	NM_001966
hMFP1_R1	CCTCTGCTCCACAAATCACAATG	23		
hACAA1_F	GCGGTTCTCAAGGACGTGAAT	21	128	NM_001607
hACAA1_R	GTCTCCGGGATGTCACTCAGA	21		

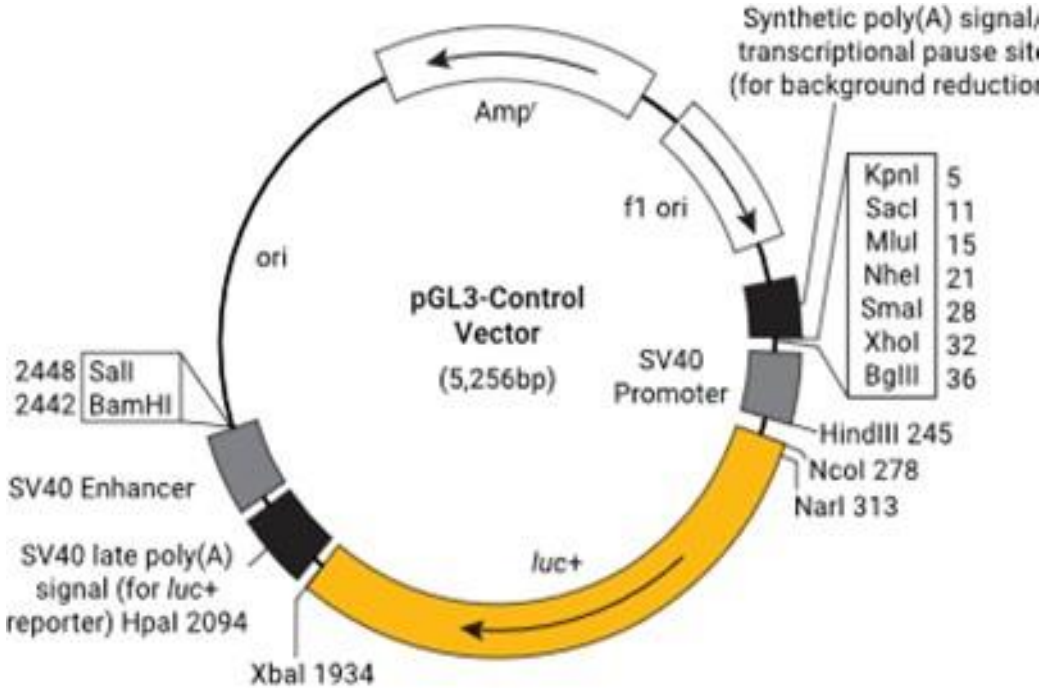
Table S 4.1 List of genes encoding metabolic enzymes

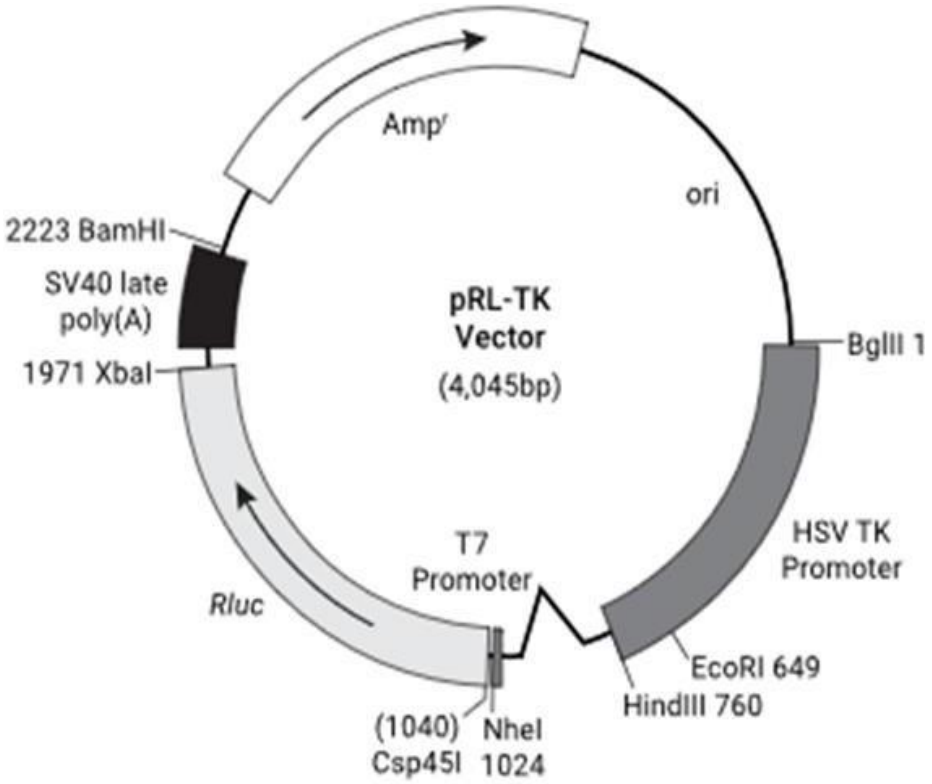
Gene Name	Gene Description
ACOT1	acyl-CoA thioesterase 1
ACOT2	acyl-CoA thioesterase 2
ACOT4	acyl-CoA thioesterase 4
ACOT8	peroxisomal acyl-CoA thioesterase 1
ACOX1	acyl-Coenzyme A oxidase 1
ACOX2	acyl-Coenzyme A oxidase 2
ACOX3	acyl-Coenzyme A oxidase 3
ACSF3	acyl-CoA synthetase family member 3
ACSL1	acyl-CoA synthetase long-chain family member 1
ACSL3	acyl-CoA synthetase long-chain family member 3
ACSL4	acyl-CoA synthetase long-chain family member 4
ACSL5	acyl-CoA synthetase long-chain family member 5
ACSL6	acyl-CoA synthetase long-chain family member 6
AGPS	alkyldihydroxyacetone phosphate synthase
AGTX	alanine-glyoxylate aminotransferase
ALDH3A2	aldehyde dehydrogenase 3A2

AMACR	alpha-methylacyl-CoA racemase
BAAT	bile acid Coenzyme A: amino acid N-acyltransferase
CAT	Catalyse
CRAT	carnitine acetyltransferase
CROT	peroxisomal carnitine O-octanoyltransferase
DAO	D-amino-acid oxidase
DDO	D-aspartate oxidase
EHHADH	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
EPHX2	epoxide hydrolase 2, cytoplasmic
FAR1	fatty acyl CoA reductase 1
FAR2	fatty acyl CoA reductase 2
HACL1	2-hydroxyphytanoyl-CoA lyase
HAO1	hydroxyacid oxidase 1
HAO2	hydroxyacid oxidase 2
HMGCL	3-hydroxy-3-methylglutaryl CoA lyase
HSD17B4	hydroxysteroid (17-beta) dehydrogenase 4
IDE	insulin-degrading enzyme
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble
IDI1	isopentenyl-diphosphate delta isomerase
IDI2	isopentenyl-diphosphate delta isomerase 2
LONP2	peroxisomal LON protease-like
MLYCD	malonyl-CoA decarboxylase
MVK	mevalonate kinase
NOS2	nitric oxide synthase 2A
NUDT12	nudix-type motif 12
PAOX	polyamine oxidase
PECI	peroxisomal D3,D2-enoyl-CoA isomerase
PECR	peroxisomal trans-2-enoyl-CoA reductase
SOD1	superoxide dismutase 1,
SOD2	superoxide dismutase 2, mitochondrial
XDH	xanthine dehydrogenase

Figure S 5.1. Map of DRL assay vectors. pGL3-basic vector (#E1751, Promega), and pGL3-control vector (#E1741) and pRL-TK vector (#E2231, Promega).






Amp^r
ori
2223 BamHI
SV40 late
poly(A)
1971 XbaI
pRL-TK
Vector
(4,045bp)
BglII 1
HSV TK
Promoter
T7
Promoter
Rluc
EcoRI 649
HindIII 760
(1040)
Csp45I
NheI
1024

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Portfolio

Publications

- Costello, J., Castro, I., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., Azadi, A. S., Godinho, L. F., Costina, V., Findeisen, P., Manner, A., Islinger, M., and M. Schrader: (2017) ACBD5 and VAPB mediate membrane associations between peroxisomes and the ER. *J Cell Biol, 216*(2), 331-342. doi:10.1083/jcb.201607055

- Schrader, M., Costello, J. L., Godinho, L. F., Azadi, A. S., & Islinger, M. (2016). Proliferation and fission of peroxisomes - An update. *Biochim Biophys Acta, 1863*(5), 971-983. doi:10.016/j. bbamcr.2015.09.024

- Azadi, A. S et al (2018). The canonical TGFβ pathway induces peroxisome proliferation in HepG2 cells via PEX11β expression (Manuscript in preparation)

Courses & workshops

Scientific and laboratory skills

- PerFuMe Workshop I ‘Systems Biology of metabolic pathways’, Wageningen, NL 2014

- PerFuMe Workshop II ‘Peroxisomes in Health and Disease’, Exeter, UK 2014

- PerFuMe Workshop III ‘Science in Industry’, Potsdam 2015

- RNAseq Workshop, Edinburgh, Scotland 2015

Transferable skills

- Project Management
- Scientific Writing
- Learning and Teaching Stage I;
- Researcher Development Induction;
- Presentation Skills: Learn the basics
- Health and Safety training

Conferences

- Oral Presentation, PERFUME 2nd Conference (Jul 2016, Hamburgh, Germany)
- Elevator Pitch, ESOF 2016 (EuroScience Open Forum) (Modern science & Business Session, July 2016, Manchester, UK)
- Coordinator of Organising Committee of BioCon-2016, PhD student Conference in Biosciences (Jun 2016, Exeter, United Kingdom)
- Oral Presentation, Progress Meetings for PERFUME Network (Sep 2013 Groningen, Netherlands; March 2014 Brussels, Belgium; Dec 2014, Exeter, United Kingdom; Jun 2015 Sevilla, Spain; Dec 2015, Berlin, Germany)
- Coordinator of Organising Committee of BioCon-2015, 1st PhD student Conference in Biosciences (Jun 2015, Exeter, United Kingdom)

- Half time PhD Upgrade (March 2015, University of Exeter)
- Poster Presentation, Health and Medical showcase 2014 (Jun 2014, Exeter, UK)
- Oral Presentation, 6-months assessment for University of Exeter (March 2014, University of Exeter) Poster presentation in PERFUME-ITN Kick off conference (Dec 2013)

Activities and Societies

- Founder of Bio-PGR-Network: This committee is organising events targeted to postgraduate students in Biosciences at University of Exeter. Postgraduate Researcher Liaison Forum Representative: Bringing issues in the department to the meeting with CLES (College of Life and Environmental Sciences) admin staff and with the Director of Postgraduate Research at University of Exeter.
- Committee member of Early Career Research Network: This network has recently been established in Biosciences to help support postdocs and PhD students in their career development

Awards and Grants

- Best Presentation and Business idea Award Entrepreneurship and career Workshop (Dec 2015, Berlin, Germany)

- Biosciences Research Strategy Grant BioPGR Committee has been granted £2000 per year (2015-2016, 2016-2017)
- Researcher led-initiative Award, Funding has been received for organising an event for PhD students career development: Beyond Academia: what your PhD can do for you? (May 2015)

Other PhD training

- PerFuMe Progress meetings
- Weekly Biosciences seminars at University of Exeter
- Journal clubs at University of Exeter, Schrader lab
- Secondment I and II, University of Sevilla,
- Secondment III, Amsterdam Medical center