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Protein import into peroxisomes and oxidative stress: a study to elucidate the potential functional role of the conserved cysteine in Pex5p

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Abstract

The oxidation status of a cell plays a crucial role in aging. As cells get aged, their redox state gets increased. Pex5p is a peroxisomal recycling receptor which binds to newly synthesized cargo proteins in the cytosol and imports them across the peroxisomal membrane. During this transport event, Pex5p gets monoubiquitinated at a conserved cysteine (C11) residue. This C11 is very essential for the recycling of Pex5p from the peroxisomal membrane to back into the cytosol. If the cysteine is replaced by serine, Pex5p does not get recycled back to the cytosol and accumulates on the peroxisomal membrane. In the present study, we have investigated whether the C11 in Pex5p could act as a redox switch. We measured the redox state of the cytosol and the peroxisomal matrix as well as the subcellular localization of catalase in aging cells. We found that an increase in the redox state of peroxisomes (in WT) leads to an increase in the redox state of the cytosol, which ultimately results in the impairment of PTS1 import. Interestingly, in the C11K condition, we did not see an impairment of PTS1 import. These observations support our hypothesis that C11 may act as a redox switch. We also performed some challenging experiments with H_2O_2. The results of these experiments show that a) import of catalase into peroxisomes sensitizes the cytosol and b) catalase overexpression does have a protective effect against oxidative stress caused by H_2O_2. In summary the results of our experiments support our hypothesis. However, further evaluation is needed to reveal the precise role of C11 in Pex5p function during cellular aging.

Keywords: Peroxisomes, Pex5p, PTS1 import, ROS, Catalase, H_2O_2.

Contents

Abbreviations

ROS: Reactive oxygen species

MAVS: Mitochondrial antiviral signalling protein

ER: Endoplasmic reticulum

PPPs: Pex11-type peroxisome proliferators

DRPs: Dynamin-related proteins

PMPs: Peroxisomal membrane proteins

PTS: Peroxisomal targeting signal

mPTS: Membrane protein targeting signal

CP: Cargo protein

DTM: Docking/translocation machinery

RING: Really interesting new gene

AAA: ATPases associated with various cellular activities

RNS: Reactive nitrogen species

H_2O_2: Hydrogen peroxide

PEDs: Single peroxisomal enzyme (transporter) deficiency disorders

PBDs: Peroxisome biogenesis disorders

NALD: Neonatal adrenoleukodystrophy

ZS: Zellweger syndrome

IRD: Infantile Refsum disease

RCDP: Type 1 rhizomelic chondrodysplasia punctate

RoGFP: Reduction-oxidation-sensitive GFP

CTAB: Cetyl trimethyl ammonium bromide

MEFs: Mouse embryonic fibroblasts

DAPI: 4', 6-diamidino-2-phenylindole

RFIs: Relative fluorescence intensities

I. Literature overview

1. Introduction

Peroxisomes, also called microbodies, are rounded, small (0.1-1µm in diameter) single membrane-bound cell organelles present in all eukaryotic cells with the exception of a few unicellular eukaryotic organisms (Adam et al., 2001; Wolf et al., 2010). Peroxisomes are considered to be oxidative organelles as they play a very important role in the generation and decomposition of reactive oxygen species (ROS) inside a cell (Schrader et al., 2006). An average human cell contains around 500 peroxisomes, except mature red blood cells which lack all intracellular organelles (Chang et al., 1999). Mammalian peroxisomes contain a set of oxidative enzymes including: acyl-CoA oxidases, uric acid oxidase, D-amino acid oxidase, D-aspartate oxidase, L-pipecolic acid oxidase, L-α-hydroxyacid oxidase, polyamine oxidase, and xanthine oxidase (Fransen et al., 2012). However, uric acid oxidase is not found in human peroxisomes, thus explaining the disease gout, which starts by the build-up of uric acid (Anzai and Endou, 2011). Peroxisomes are involved in a number of metabolic functions such as lipid biosynthesis, amino acid metabolism, ROS metabolism, plasmalogen synthesis and lipid breakdown via ß-oxidation and α-oxidation (Antonenkov et al., 2010). They are also involved in an extensive range of other reactions such as the oxidation of purines and polyamines, as well as in the synthesis of ketones and wax bodies (Wanders et al., 2006). Very long chain fatty acids (i.e. more than C-22) are primarily shortened in peroxisomes. The corresponding breakdown products are shuttled to mitochondria for further degradation. Mammalian peroxisomes contain at least 50 types of proteins and enzymes involved in a variety of biochemical pathways depending on the cell type (Wiese et al., 2007). Peroxisomes also play a role in antiviral innate immunity in collaboration with mitochondria. Upon viral infection, rapid interferon-independent expression of defense factors is induced by peroxisomal MAVS (mitochondrial antiviral signalling protein), which provide temporary protection against viral infection. Peroxisomal MAVS-dependent signalling is regulated by interferon regulatory factor IRF1 (Dixit et al., 2010). In all we can say is that the role of peroxisomes in human physiology is indispensable (Schrader et al., 2008).

2. Biogenesis of peroxisomes

Peroxisome formation and maintenance are regulated by a group of proteins, called peroxins, which are encoded by *PEX* genes (Distel et al., 1996). So far, researchers have identified 32 peroxins which participate in the peroxisome biogenesis (Rucktaschel et al., 2011). Peroxisomal proteins are encoded in the nucleus and synthesized on free polyribosomes in the cytosol. Unlike chloroplasts and mitochondrial proteins, peroxisomal proteins do not have to be unfolded to get imported into peroxisomes (Ma et al., 2009). The peroxisomal

population of a cell is possibly expanded by a *de novo* biogenesis from the ER (endoplasmic reticulum) or by division of pre-existing organelles (Huybrechts et al., 2009; Ma et al., 2011). There are numerous factors involved in the division of peroxisomes: the Pex11-type peroxisome proliferators (PPPs), dynamin-related proteins (DRPs), and the fission factors Fis1 and Mff (Mast et al., 2010; Fidaleo, 2010). Several models have been proposed for peroxisome biogenesis: a) the growth and division model (Lazarow and Fujiki, 1985), b) the non-symmetrical growth and division model (Delille et al., 2010), and c) the maturation model (Hettema et al., 2009).

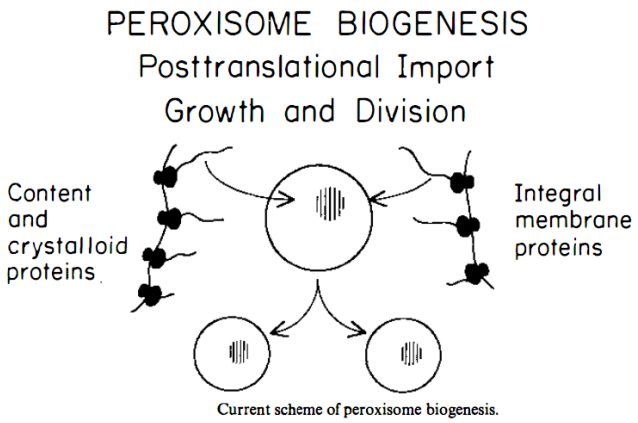


Figure 1. Growth and division model

[Source: Lazarow and Fujiki, 1985]

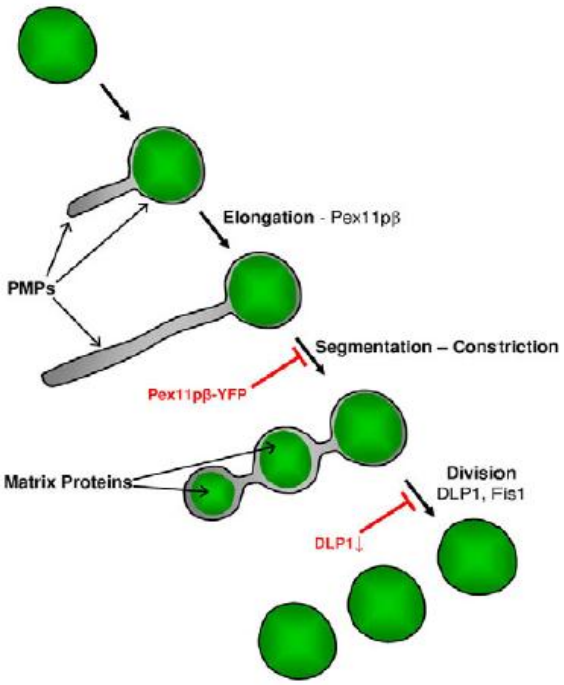


Figure 2. Non-symmetrical growth and division model

[Source: Delille et al., 2010]

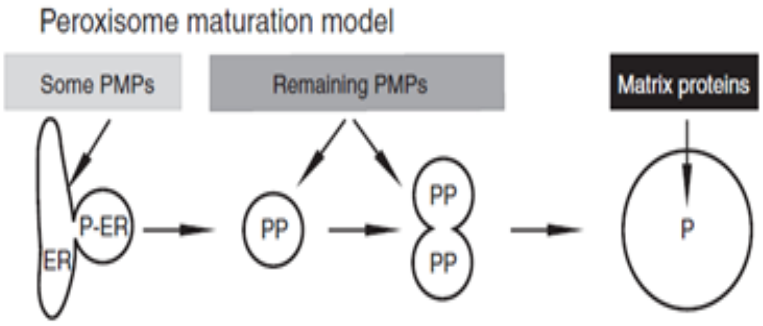


Figure 3. Maturation model

[Source: Hettema et al., 2009]

P-ER: Peroxisomal ER

PP: Pre-peroxisome

P: Peroxisome

PMPs: Peroxisomal membrane proteins

In accordance with all models, peroxisomes are directly or indirectly banked on the ER for their growth and division. Recent studies have revealed a role for the ER in peroxisome biogenesis (Fidaleo et al., 2010; Mast et al., 2010). According to the classical "growth and division" model, new peroxisomes are formed by the symmetrical fission of the pre-existing peroxisomes, after newly synthesized proteins are imported from the cytosol into peroxisomes (Fig. 1) (Lazarow and Fujiki, 1985). According to the non-symmetrical growth and division model, Pex11p initiates the formation of tubular membrane extensions that emerge from one side of spherical, mature peroxisomes. These membrane extensions get segmented and constricted. After the import of PMPs and matrix proteins, they are divided into several spherical peroxisomes by DLP1, Fis1 and Mff (Fig. 2). In the absence of DLP1, fission of the tubule is inhibited, resulting in a constricted peroxisomal tubule with PMPs and matrix proteins (Delille et al, 2010). According to the maturation model, the ER buds to form the peroxisomal ER (P-ER) which fuses homotypically to produce large membrane structures. Additional matrix proteins and PMPs are imported into the pre-peroxisome (PP) from cytosol, resulting in the formation of a mature metabolically active peroxisome (Fig. 3) (Hettema et al., 2009). In mammals, *de novo* biogenesis of peroxisomes is regulated by three different peroxins i.e. Pex3p, Pex16p, and Pex19p (Kim et al., 2007).

3. Peroxisomal protein import

All peroxisomal proteins are synthesized on cytosolic ribosomes and post-translationally directed to the organelle. Import of peroxisomal matrix proteins into peroxisomes is controlled by two types of peroxisomal targeting signals (PTS) i.e. PTS1 and PTS2. Most of the peroxisomal matrix proteins are imported into peroxisomes via PTS1. PTS1 is present at the extreme COOH-terminus of proteins (Bharti et al., 2011). This PTS consists of a consensus sequence of (S/A/C)-(K/R/H/Q)-(L/M) (Antonenkov et al., 2010). Only few peroxisomal matrix proteins use PTS2. PTS2 is a degenerate nonapeptide located at the NH_2-terminus of a small number of matrix proteins of the consensus sequence R-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A) (Rucktaschel et al., 2011). Some peroxisomal matrix

proteins do not have any PTS and use a ‘piggyback’ mechanism for import into the peroxisomes (Fig. 4) (Antonenkov D et al., 2010).

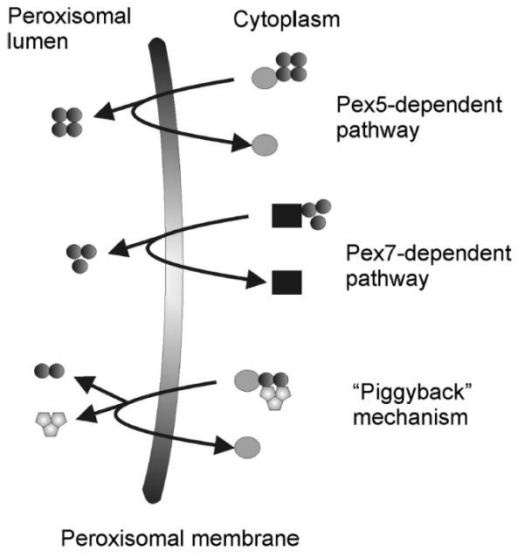


Figure 4. Different modes of protein import in peroxisomes. *Light grey circles*: Pex5p receptor. *Dark grey circles*: fully assembled and folded matrix proteins. *Black square*: Pex7p receptor. *Light grey pentagons*: proteins delivered by ‘piggyback mechanism’. [Source: Antonenkov D et al., 2010]

In the ‘piggyback’ mechanism, proteins get oligomerized in the cytosol with other proteins comprising a PTS. Import of proteins via PTS1 and PTS2 need around 12 peroxins (Hogg et al., 2010). PTS1 and PTS2 proteins are recognized in the cytosol by Pex5p and Pex7p receptors respectively, which in turn transfer their cargoes to the peroxisomal membrane. In mammals, PTS2 also needs a long isoform of Pex5p (Pex5pL); this Pex5pL binds to Pex7p and transports the Pex7p-PTS2 complex to peroxisomes. In fungi, the function of Pex5pL is taken over by accessory proteins such as Pex18p, Pex20p, and Pex21p (Hogg et al., 2010).

Peroxisomal membrane protein import is mediated by a cis-acting peroxisomal membrane protein targeting signal (mPTS). The mPTS is recognized by Pex19p, a predominantly cytosolic protein. Pex19p has multiple functions: it shields the hydrophobic domain of its cargo protein from the hydrophobic environment of the cytosol and transports the cargo proteins to peroxisomes. Pex19p binds the mPTS in the cytosol and the Pex19p-cargo complex docks at the peroxisomal membrane via Pex3p. Finally, cargo proteins (CPs) get translocated through the peroxisomal membrane with the help of Pex16p and the Pex19p molecules is recycled back to the cytosol for the next cycle (Rucktaschel et al., 2011; Ma et al., 2011).

4. Pexophagy

The population of peroxisomes inside a cell can be quickly declined by selective autophagic degradation, a process known as pexophagy (Farre et al., 2009; Iwata et. al., 2006). Peroxisomes are dynamic organelles that multiply and degrade in response to a number of

environmental stimuli (Camoes et. al., 2009). There are around 30 genes (15 autophagic and 15 other genes) involved in the process of pexophagy (Oku and Sakai, 2010). Peroxisomal degradation can occur via macropexophagy, micropexophagy, and 15-lipoxygenase-mediated autolysis. In macropexophagy a new isolation membrane is synthesized which selectively sequesters peroxisomes one after the other and forms a double or multi membrane layered structure around them, called pexophagosome. This pexophagosome is then transported to the lysosome, where the lysosomal membrane fuses with the outer membrane of the pexophagosome. This results in the hydrolysis of the sequestered peroxisomes by enzymes present inside the lysosome (Sakai et al., 2006). Micropexophagy is a process in which peroxisomes are directly sequesters by the lysosomal membrane. In case of 15-lipoxygenase-mediated autolysis, 15-lipoxygenase integrates into the membrane of the organelle causing membrane disruptions, thereby exposing the peroxisomal content to cytosolic proteases for its degradation (Huybrechts et al., 2009). In mammals, peroxisomes are thought to be degraded predominantly by macroautophagy (Sakai et al., 2006). There are some evidences, which show that Pex3p and Pex14p play an important role in this process (Tanida, 2011; Yu et al., 2008).

5. The PTS1 receptor, Pex5p

5.1 The receptor shuttling model

Translocation of peroxisomal matrix proteins across the peroxisomal membrane needs the combined action of several peroxins. The majority of newly formed peroxisomal matrix proteins are transported to peroxisomes by Pex5p, a peroxisomal cycling receptor (Freitas et al., 2011). Pex5p is a primarily cytosolic, partially peroxisomal protein which cycles between the peroxisomes and cytosol. Peroxisomal matrix protein import is a complex pathway which involves complex interactions of cytosolic and peroxisomal events. According to recent models, the protein import pathway mediated by Pex5p can be divided into 5 stages (0-4) (Fig. 5). Free Pex5p (stage 0) binds with the CP in the cytosol (stage 1a). This cytosolic Pex5p-CP complex gets docked to the docking/translocation machinery (DTM) (stage 1b). DTM comprises of Pex13p, Pex14p, and three RING (really interesting new gene) peroxins Pex2p, Pex10p, and Pex12p (Grou et al., 2009a). The attachment of the Pex5p-CP complex with the DTM eventually results in the insertion of Pex5p into the DTM (stage 2) with the translocation of the CP through the peroxisomal membrane and the monoubiquitination of Pex5p at the conserved cysteine residue presented near the NH_2-terminal region (stage 3) (Carvalho et al., 2007; Williams et al., 2007). This monoubiquitinated Pex5p is dislocated from DTM in an ATP-dependent process by the members of the ATPases associated with various cellular activities (AAA) family i.e. Pex1p and Pex6p, forming a soluble Ub-Pex5p complex (stage 4) (Grou et al., 2009b).

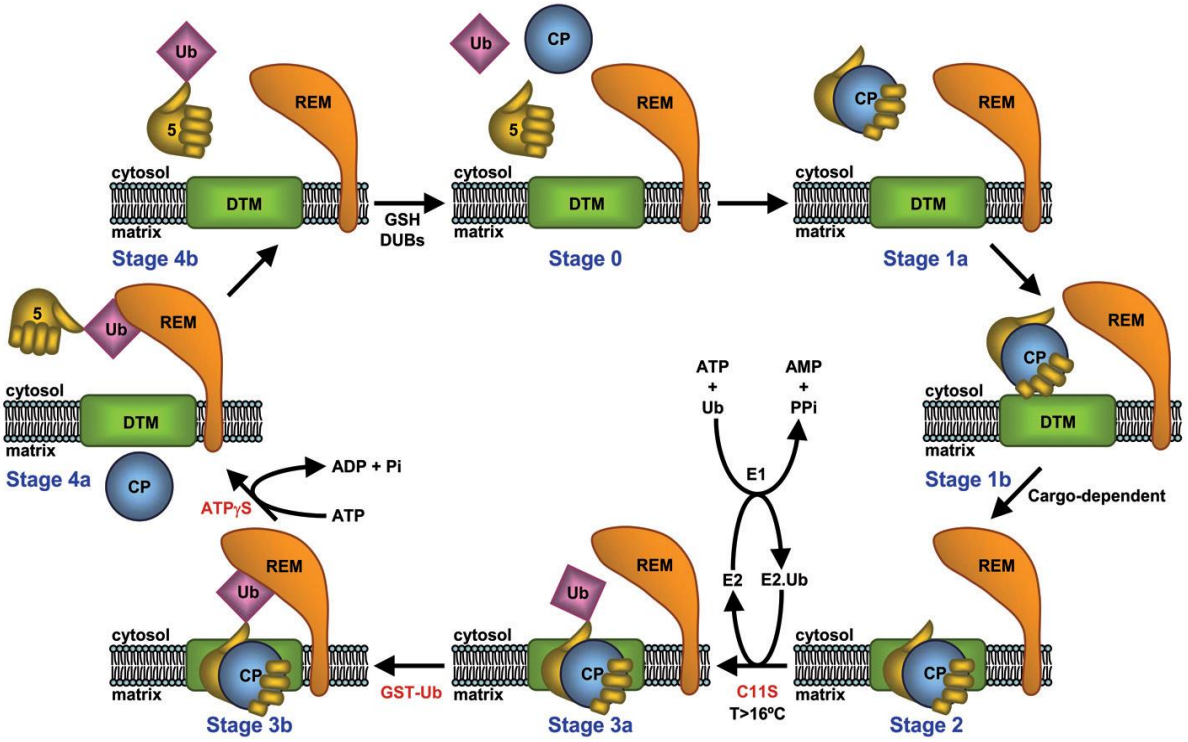


Figure 5. Model of the Pex5p-mediated peroxisomal protein import pathway

[Source: Grou et al., 2009b]

Finally, the Ub-Pex5p complex gets deubiquitinated by a combination of non-enzymatic and enzymatic reactions in the cytosol, thus regenerating stage 0 (Grou et al., 2009a).

5.2 Mutated variants of Pex5p and the receptor export cycle

In Pex5p, the first 17 amino acid residues are very important for the ATP-dependent transfer of the Pex5p molecule back to the cytosol (Costa-Rodrigues et al., 2004). The Pex5p domain consists of a cysteine-containing motive which is conserved in all known Pex5p sequences. Substituting this cysteine with lysine results in the formation of a mutant form of Pex5p(C11K). This mutant Pex5p behaves similar to the WT variant of Pex5p i.e. this variant of Pex5p enters the DTM complex and is actively recycled back to the cytosol after translocating cargo protein inside the peroxisomal matrix. This suggests that Pex5p(C11K) is as functional as the Pex5p WT, at least under standard laboratory conditions (Grou et al., 2009). However, substituting the cysteine by a serine residue, results in the formation of a dominant negative Pex5p variant (C11S) which interferes with the ATP-dependent export step of Pex5p. This can be explained by the fact that serine cannot be ubiquitinated. Due to this, Pex5p does not get recycled back to the cytosol and accumulates on the peroxisomal membrane (Carvalho et al., 2007).

6. Peroxisomes and oxidative stress

Peroxisomes are capable of generating ROS within a cell. These molecules also act as intracellular signalling molecules at controlled levels (Fialkow et al., 2007). When the balance between ROS production and elimination gets disturbed, it results in the alteration of redox state of the cell and is usually considered as a risk factor which may lead to the development of various diseases (Salmon et al., 2010). According to earlier studies, PTS1 import gets disturbed in the late passage cells, a condition linked to an increased redox status of the cytosol (Ivaschenko et al., 2011). Moreover, import and the redox balance are restored in these late passage cells by overexpressing the catalase enzyme (Terlecky et al., 2006).

7. Enzymes involved in peroxisomal redox metabolism

7.1 ROS generating enzymes

D-amino acid oxidase, uric acid oxidase (Bonekamp et al., 2009), xanthine oxidase, nitric oxide synthase (Loughran et al., 2005), acyl-CoA oxidases are the main oxidative enzymes present in peroxisomes. These enzymes generate reactive nitrogen species (RNS) or reactive oxygen species (ROS) as a consequence of their normal catalytic function (Antonenkov et al., 2010). H_2O_2, $O_2^{\cdot -}$, and $NO^{\bullet}$ are the main ROS and RNS produced by the peroxisomal enzymes.

7.2 Antioxidant defense enzymes

ROS generated inside peroxisomes are removed by the antioxidant defense enzymes. Catalase, Cu,Zn-superoxide dismutase (SOD1) (Islinger et al., 2009), peroxiredoxin V, and epoxide hydrolase are the main peroxisomal antioxidant enzymes.

Catalase is an antioxidant enzyme which is present both in the cytosol and peroxisomes. In its native form, the enzyme is a heme-containing homo-tetrameric protein (Freitas et al., 2011). Catalase is responsible for the decomposition of H_2O_2 produced by the oxidases into water and oxygen in a peroxidatic ($H_2O_2 + AH_2 \rightarrow A + 2\ H_2O$) or catalatic ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) way, where AH_2 may be formaldehyde, phenols, formic acid or short-chain alcohols such as ethanol and methanol (Fransen et al., 2012; Klei et al., 2006). Catalase is involved in the oxidation of ethanol especially in heart and brain, as these organs have low alcohol dehydrogenase activity (Lieber, 2005).

8. Peroxisomal disorders (PDs)

PDs are divided into two groups: single peroxisomal enzyme (transporter) deficiency disorders (PEDs) and peroxisome biogenesis disorders (PBDs). In PEDs, peroxisomes are intact and functional, but one of the enzyme processes is anomalous, which leads to a primary biochemical abnormality in the cell (Fidaleo et al., 2010). Clinical onset of PEDs is late, and the prototypic example of this kind of disorder is X-linked adrenoleukodystrophy (1:20,000 males). PBDs comprise neonatal adrenoleukodystrophy (NALD), Zellweger syndrome (ZS), infantile Refsum disease (IRD), and type 1 rhizomelic chondrodysplasia punctate (RCDP). In these diseases all peroxisomal functions are compromised. Mutation in the same gene can result in the development of any of the first three conditions. So they are collectively known as Zellweger spectrum disorders (ZSDs) (Wanders et al., 2006). Liver disease, retinopathy, perceptive deafness, and variable neurodevelopmental delay are the common symptoms of the ZSDs (Wanders et al., 2006). These symptoms develop in the early months of the onset. Main symptoms of ZS are reduced plasmalogen levels and elevated plasma levels of very-long chain fatty acids. Patients suffering from this disorder are weak from birth, have distinct facial features, and abnormal looking peroxisomes. Children suffering from this disease usually die within the first year of their life.

9. Reduction-oxidation-sensitive GFP (roGFP)

Green fluorescent protein (GFP) is a 238 amino acid residues long protein (approx. 26 kDa) (Cannon and Remington, 2008). This protein was first isolated from the jellyfish *Aequorea victoria* and it emits green fluorescence when exposed to blue light. This protein is highly resilient to protease activity and remains functional under unusually extreme environments. The wild type (WT) variant of GFP has dual excitation peaks: a minor peak around 480 nm and a major peak around 400 nm. Excitation at either peak results in the emission of green light at 510 nm. Excitation peaks at dual wavelengths suggests that equilibrium is present in the protein sandwiched by the two different chromophore states with comparable emission wavelengths. RoGFP is a ratiometric redox-sensitive variant of GFP. This variant is developed by inserting couples of cysteine residues on the surface of GFP on neighbouring strands in such a way that it favours the formation of disulphide linkages (Cannon and Remington, 2008). This GFP has two excitation maxima at around 400 and 490 nm. The working principle of roGFP is that it shows quick and reversible ratiometric changes in fluorescence with respect to the variations in ambient redox potential in both *in vivo* and *in vitro* conditions (Hanson et al., 2004).

II. Materials and methods

Vector	Modified pIRES-EGFP vector encoding roGFP2-PTS1
Genes/Inserts	HsPex5p (WT, C11K, C11S)
Restriction enzymes	Bgl II and Sal I
Cell types	Immortalized WT and primary and immortalized Pex5p deficient MEFs

Table1. Vectors, inserts and restriction enzymes used in the study

1. Construction of bicistronic plasmids

In this study, we constructed 3 bicistronic plasmids, each encoding roGFP2-PTS1 and a variant of Pex5p (*in casu* Pex5p (WT), Pex5p (C11K), and Pex5p (C11S)). A schematic overview of the employed strategy is presented in Fig. 6.

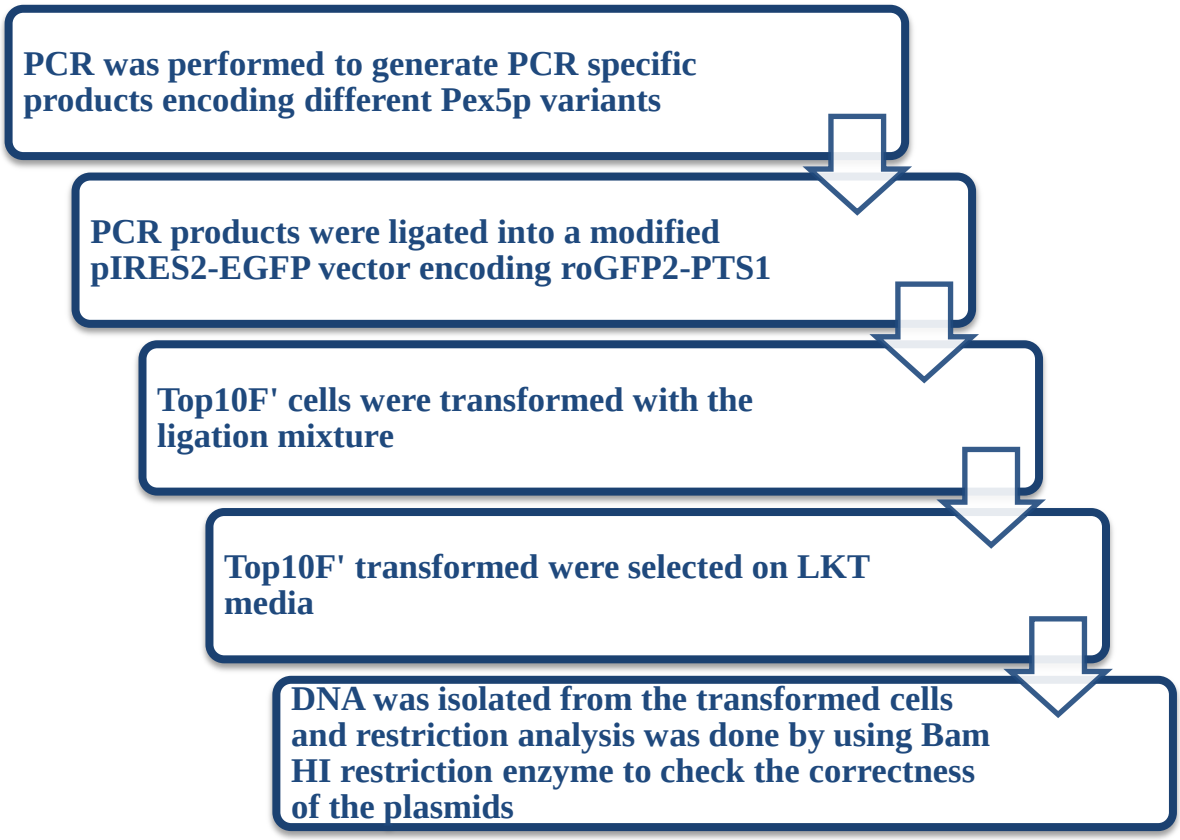


Figure 6. Schematic overview of the employed strategy used for constructing bicistronic plasmids

Initially, PCR was performed using a PCR primer set (forward primer: HsPex5.1; reverse primer: Pex5.2) and the polymerase enzyme *Pfx* (Appendix C) for constructing PCR products encoding different Pex5p variants. The MinElute PCR purification kit (QIAGEN) was used

to purify the PCR products and the DNA was eluted with 30 µl of water (Appendix B). Purified PCR products were ligated into the modified pIRES-EGFP vector encoding roGFP2-PTS1 by digesting both vector and PCR products with Bgl II and Sal I restriction enzymes (Appendix D a), b)) and ligating them with T4-DNA ligase (Appendix E a), b)). Already prepared competent cells (*E.coli.* strain Top10F') were used for transformation. Competent cells were transformed with the ligation mixture by heat shock treatment at 42 °C for 1 minute and were later grown overnight on agar plates (Appendix F) containing tetracycline (25 µg/ml) and kanamycin (50 µg/ml) in an incubator at 37 °C. The next day, 10 individual colonies were selected for each condition and grown overnight in LKT medium (Appendix F) in an incubator shaker at 37 °C. The next day the plasmids were isolated from each individual colony by using the CTAB method (Appendix A) and the restriction analysis was performed on the isolated plasmid from each colony by using Bam HI restriction enzyme and was run on agarose gel to establish whether the plasmids prepared were correct or not (Appendix G). One colony from each construct was grown overnight in 50 ml of LKT media (Appendix F) in an incubator shaker at 37 °C. The next day, plasmids were isolated by using the CTAB method and the isolated plasmids were purified by using MinElute purification kit (QIAGEN) and eluted with 30 µl of water (Appendix B). The purity of eluted DNA was checked by using a spectrophotometer. Glycerol stock was prepared in an eppendorf tube by adding 700 µl of cell culture and 300 µl of glycerol and preserved at -80 °C for future storage. DNA sequencing was performed on the constructs to verify the correct sequence of the plasmids.

2. Cell culture

Immortalized (T) WT and both primary and immortalized Pex5p deficient mouse embryonic fibroblasts (MEFs) were cultured in a humidified 5% CO_2 incubator at 37 °C in MEM Eagle alpha (BioWhittaker; catalogue no. 12-169; Lonza, Verviers, Belgium) supplemented with 2 mM Glutamax (Invitrogen), 10% (v/v) heat-inactivated South American fetal calf serum (Invitrogen), and Mycozap (Lonza).

3. Transfection and immunofluorescence microscopy

The Neon Transfection System was employed to transfect the cells (Invitrogen; MEFs: 1350 V, 30-ms pulse width, 1 pulse) (Appendix I). In order to perform live-cell imaging cells were seeded in the FD-35 Fluorodish cell culture dishes (World Precision Instruments, Hertfordshire, England). Fluorescence was evaluated on a motorized inverted IX-81 microscope, controlled by Cell-M software and equipped with 1) temperature, humidity, and CO_2 controlled incubation chamber; 2) BP360-370, BP470-495, BP545-580, and D405/20x excitation filters; 3) a 100X Super Apochromat oil immersion objective; 4) BA420-460, BA510-550, and BA610IF emission filters; and 5) a CCD-FV2T digital black and white camera (Olympus, Aartselaar, Belgium).

4. Transfection efficiency

Transfection efficiency was checked by individually transfecting immortalized Pex5p-deficient MEFs with all three construct. One day post transfection, cells were visualized under bright field and green light illumination. The numbers of the cells giving green fluorescence signals were recorded (at least 200 cells from each condition were counted) and the percentage of transfection efficiency was calculated.

5. Redox measurements

Redox measurements in the cytosol and peroxisomes were done by visualizing transfected cells three days post transfection under the fluorescence microscope in the green light illumination. Images of the individual cells were captured; camera exposure time was set to 100 and 500 ms to get roGFP2 images at 480- and 400-nm excitation wavelengths, respectively. Quantative image analysis was done by using the Olympus image analysis and particle detection software. To quantify the relative fluorescence intensities (RFIs) of individual peroxisome or in the cytosol, the area was selected by employing the circle region-of-interest tool.

6. Immunostaining

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized in 1% Triton X-100. After permeabilization, nuclei were stained by DAPI and later were incubated by primary (mouse anti-catalase) and secondary (α-mouse Texas Red) antibodies. At the end the samples were mounted on the glass slides by using n-propyl-gallat (Sigma, P-3130) and Mowiol 4-88 (Vel, 17951) (1:3) (Appendix H). Samples were visualized on an Olympus IX-81 inverted fluorescence microscope in three different light illuminations i.e. blue for DAPI, red for Texas Red and green for roGFP2. Images were captured for the localization of catalase.

7. Challenging cells with hydrogen peroxide (H_2O_2)

Immortalized Pex5p-deficient MEFs were transfected with cytosolic roGFP2 (c-roGFP2) or with non-tags WT and C11S variant of Pex5p co-transfected with cytosolic-roGFP2 and with or without overexpressing catalase (Appendix I). Three days post transfection cell culture

medium was replaced by 200 µl of PBS and cells were visualized under the fluorescence microscope; ten different cells were selected from each condition and were treated with 1 ml solution of 25 µM H_2O_2 in PBS. Simultaneously images of the individual cells were captured after every five minutes for 60 minutes; camera exposure time was set to 100 and 500 ms to get roGFP2 images at 480- and 400-nm excitation wavelengths, respectively. Quantitative image analysis was done by using the Olympus image analysis and particle detection software. To quantify the relative fluorescence intensities (RFIs) in the cytosol, the area was selected by employing the circle region-of-interest tool.

8. Statistical analysis

Statistical analysis was performed by using a website for statistical computation, VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). Differences among independent groups of numerical values were determined by using one-way analysis of variance and Students's t test were used for further exploring the individual differences. Level of significance was chosen to be 0.05.

III. Results and discussion

1. Functional characterization of bicistronic plasmids encoding roGFP2-PTS1 and Pex5p variants

Three bicistronic plasmids encoding roGFP2-PTS1 and one of the Pex5p variants were constructed as described in Materials and methods. The correctness of the plasmids was verified as follows: ten individual colonies from each condition were selected and grown overnight in LKT medium; the next day, the plasmids were isolated from each individual colony by using the CTAB method; and the restriction analysis by Bam HI was performed (see Materials and methods). One clone from each condition having the fragment of +/- 1500 bp after digesting with Bam HI was selected. As shown in Fig. 7, clones number 2 for C11K, 14 for WT and 26 for C11S Pex5p variant have been chosen as potentially correct and verified by DNA sequencing (LGC Genomics, Berlin, Germany).

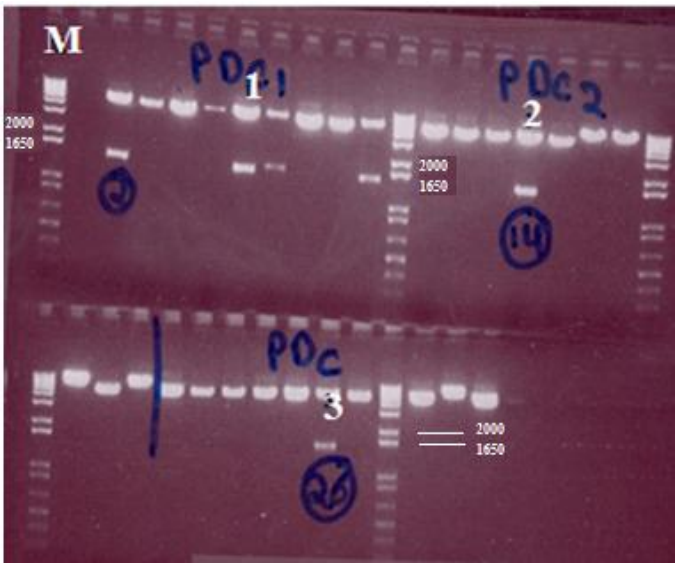


Figure 7. Restriction analysis of 10 clones from each plasmid extracted by CTAB method and digested with Bam HI (expected band size +/- 1500 bp).

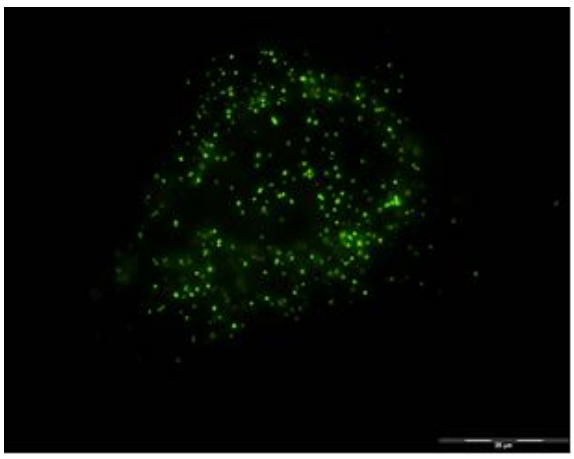
M: 1kb DNA ladder (100 bp-12,000 bp) relevant markers are indicated

pDC1 (for C11K variant)

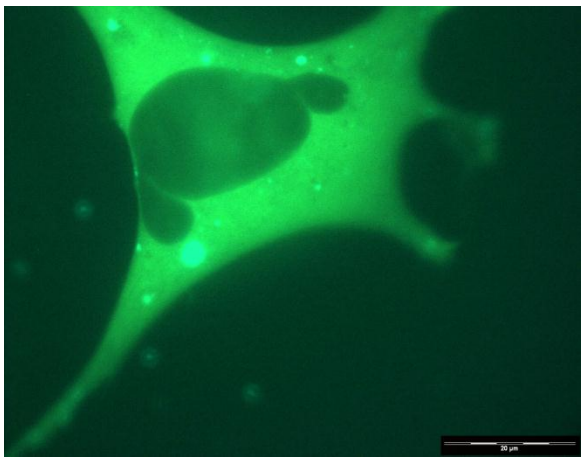
pDC2 (for WT variant)

pDC3 (for C11S variant)

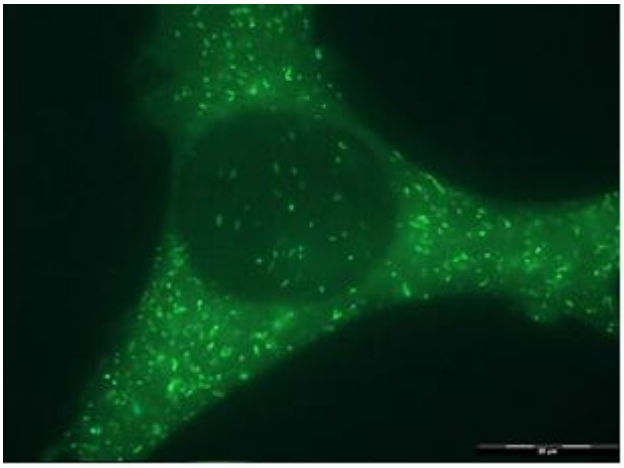
A functional characterization of the bicistronic plasmids was done by transfection of immortalized wild type (WT) and Pex5p-deficient MEFs with bicistronic plasmids encoding a Pex5p variant and roGFP2-PTS1 (Appendix I). The subcellular localization of roGFP2-PTS1 in the cells was determined in order to see whether it displayed a peroxisomal (P), cytosolic (C), or mixed (P/C) distribution pattern.



P

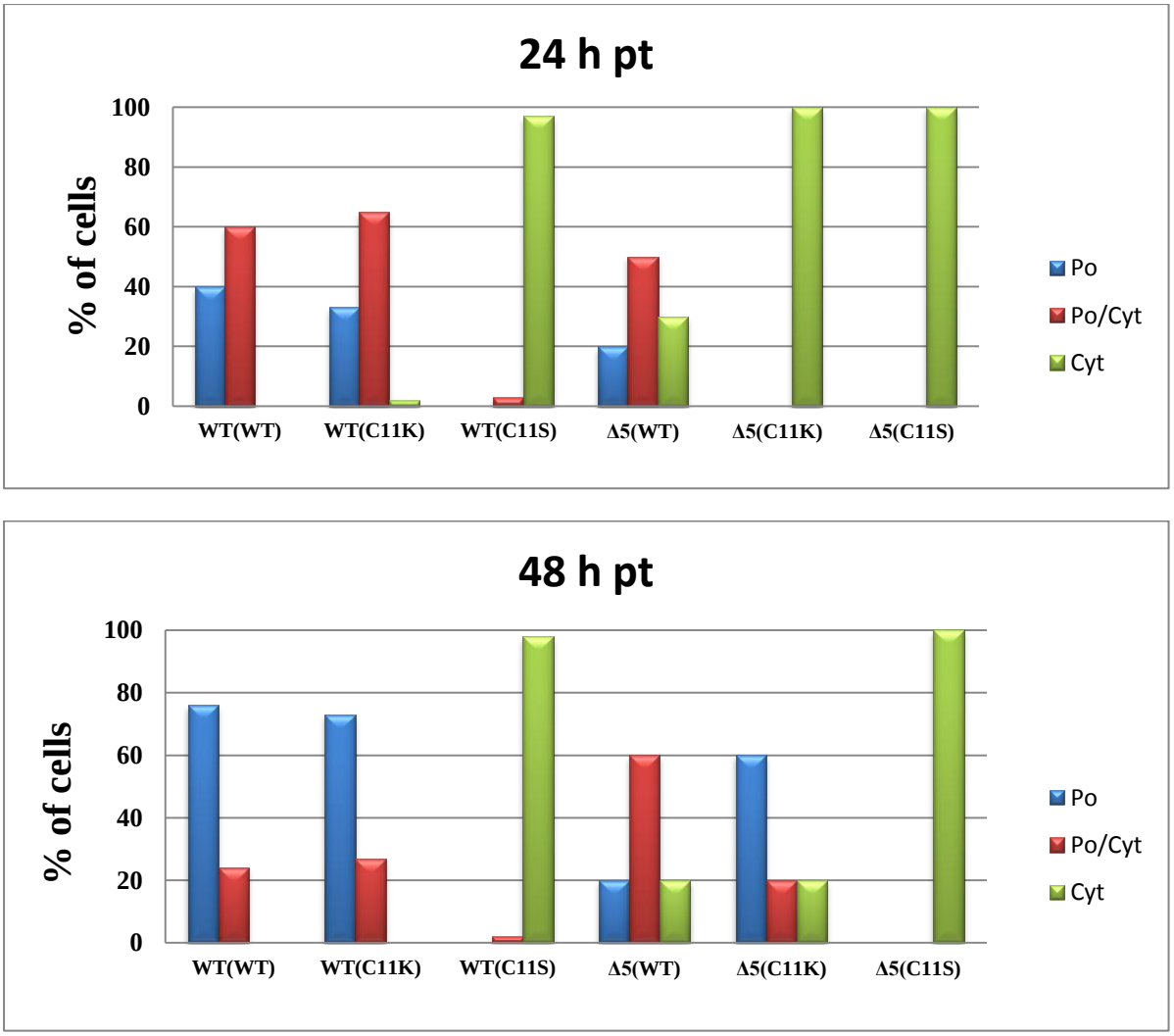


C



Mixed (P/C)

Figure 8. Sub-cellular localization of roGFP2-PTS1 in Pex5p-deficient MEFs-T representing punctate (peroxisomal, P), diffuse (cytosolic, C) or bimodal (peroxisomal/cytosolic, P/C) pattern. Scale bar, 20 µm.



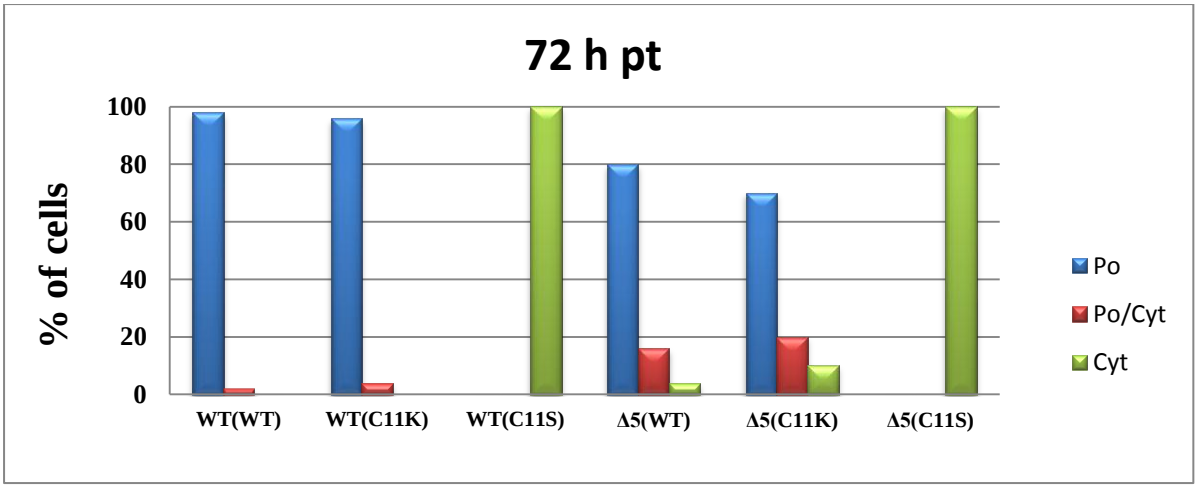


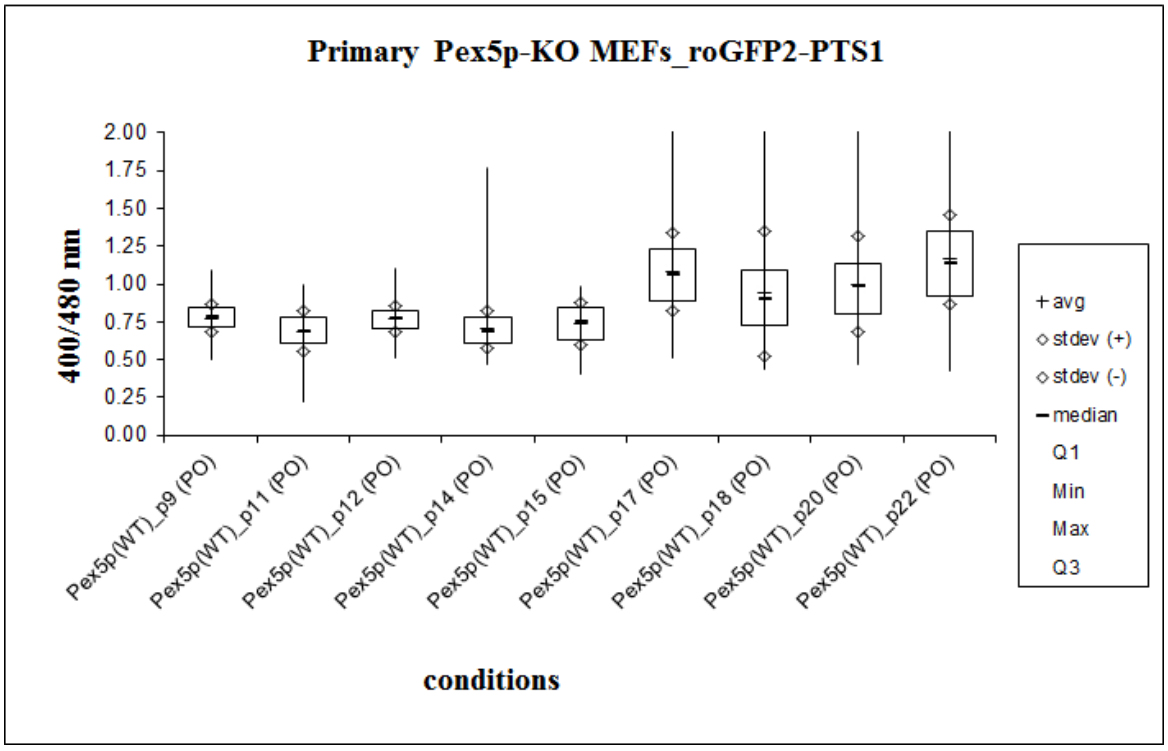
Figure 9. Percentage of the cells displaying different distributions of roGFP2-PTS1 in WT and Pex5$^{-/-}$ (Δ5) MEFs in function of time upon expression of Pex5p(WT), Pex5p(C11K), or Pex5p(C11S) plasmids encoding roGFP2-PTS1. The distribution pattern was scored in function of time by visualizing the cells under green illumination and the results were enumerated (at least 200 cells from each condition).

Cells were analysed in function of time by immuno-fluorescence microscopy. As shown in graph (Fig. 9), 72 h post transfection roGFP2-PTS1 was localized in peroxisomes in majority of both WT and Pex5p-deficient MEFs transfected with WT and C11K variant of Pex5p. In contrast, all the roGFP2-PTS1 was localized in the cytosol of the cells in both WT and Pex5p-deficient MEFs transfected with a plasmid encoding Pex5p(C11S). It might be possible that Pex5p(C11S) was trapped at the DTM after one cycle of the protein transport event and thus further blocking it (Grou et al., 2009). These data favour the interpretation that Pex5p(C11K) variant displays similar phenotype to WT Pex5p under standard laboratory conditions while the Pex5p(C11S) variant shows a dominant negative phenotype, as was previously shown (Grou et al., 2009). Transfection efficiency was checked by individually transfecting immortalized Pex5p-deficient MEFs with all three construct. Transfection efficiency was around 75.66 % (± 3.511) (±SD).

2. Effect of cellular aging on intraperoxisomal and cytosolic redox balance in primary MEFs

It has been well accepted that a general increase of oxidative stress is associated with cellular aging (Muller, 2009). Recent studies showed that the intraperoxisomal redox state was slightly but significantly higher ($p < 0.0001$) in the late passage compared to the young passage cells of primary human fibroblasts. The redox state of the cytosol was intensely increased (Ivashchenko et al., 2011). Here, we investigated the redox state of peroxisomal matrix and cytosol in primary Pex5p-deficient MEFs in function of passage number. The redox status of the cytosol (for C11S) and peroxisomal matrix (for WT and C11K) was

checked. Primary Pex5p-deficient MEFs were individually transfected with all three Pex5p variants encoding roGFP2-PTS1. Three days post transfection cells were visualized under green light illumination and the images of the individual cells were captured and analyzed as described in Materials and methods. After analyzing of the primary cells during several passage numbers (starting from the passage number 7 and up to 22 included) (these experiments were performed by my supervisor Oksana Apanasets) we have seen changes in the redox state. In WT condition, there was a significant increase ($p<0.0001$) in the redox state of peroxisomal matrix, similar results were obtained for C11K condition. Moreover, there was a slight but significant ($p<0.0001$) increase in the redox state of the cytosol in C11S condition. We also observed that in passage number 22 PTS1 import was impaired in 53 % of the cells (per 47 cells counted) transfected with a WT variant of Pex5p i.e. in these cells roGFP2-PTS1 was localized both in peroxisomes and the cytosol because of the disturbed PTS1 import. We measured the redox state of the cytosol in those cells and found out that the cytosolic redox state of those cells was slightly, but significantly higher ($p<0.0001$) in comparison with the cells transfected with c-roGFP2. While in the cells overexpressing C11K variant, no such change was observed. These observations suggest that an increase in the redox state of peroxisomes (in WT) leads to the increase in the redox state of the cytosol which ultimately results in the impairment of the PTS1 import. In C11K condition, we have not seen any impairment of the PTS1 import. This observation supports our hypothesis that conserved cysteine (C11) may act as a redox switch. Moreover, the cytosol of cells without any functional peroxisomes (in C11S) is more protected against oxidative stress during cellular aging.



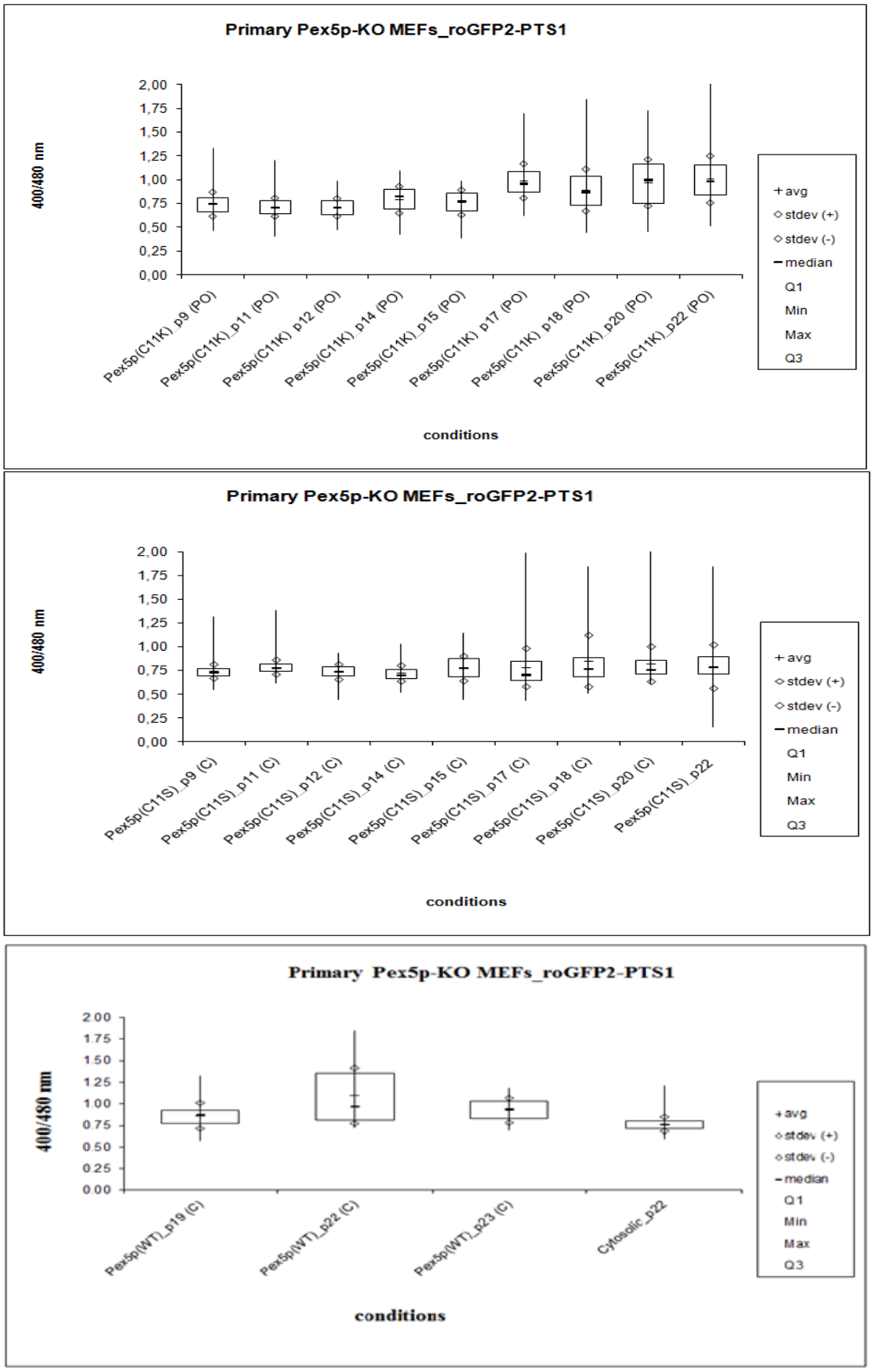


Figure 10. Redox state of roGFP2-PTS1 in primary Pex5p-deficient MEFs during cellular aging. RoGFP2 is a redox sensitive probe which can be used to monitor redox changes in the cytosol and peroxisomal matrix. Primary Pex5p-deficient MEFs were transiently transfected with three different

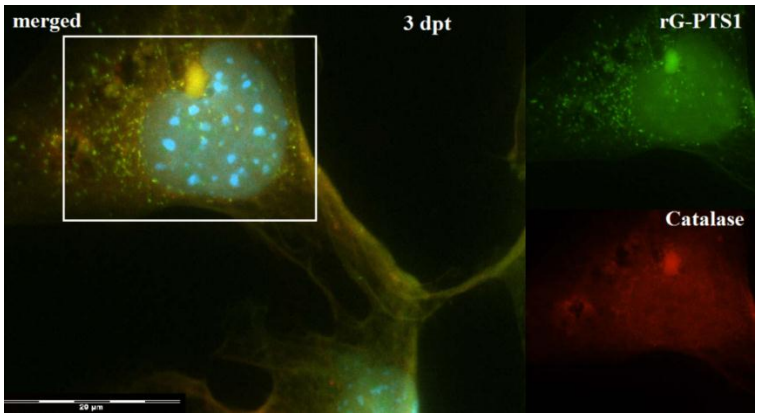
plasmids individually encoding a Pex5p variant and roGFP2-PTS1 and were grown in standard growth medium for 3 days and were analyzed as described in Materials and methods.

As it has been earlier reported primary MEFs have the tendency to rapid replicative senescence i.e. they get immortalized after continuous passaging. This might be because of the *in vivo* culture shock or oxygen sensitivity (Odell et al. 2010; Parrinello et al., 2003). That is why we decided to try an alternative way to senesce the primary cells with an approach used by Chen and co-workers. They were able to prematurely senesce the primary cells successfully treating young human diploid fibroblasts (HDFs) with 75 µM H_2O_2 twice or 150 µM H_2O_2 once in two weeks (Chen et al., 2001). In order to induce artificial/premature senescence in the primary MEFs, we performed some challenging experiments with H_2O_2 by treating them with 25 µM of H_2O_2 in PBS as described in Materials and methods. The experiments of section 2 were performed in collaboration with Oksana Apanasets.

3. Subcellular localization of catalase in primary MEFs during cellular aging

Here, we have checked the subcellular localization of catalase in primary Pex5p-deficient MEFs in function of passage number (till passage 15). As shown in Fig. 11, expression of Pex5p(WT) or Pex5p(C11K) resulted in a peroxisomal localization of catalase. However, upon expression of a dominant negative variant of Pex5p(C11S), the protein was mis-localized to the cytosol of the cell. This suggests that like any other peroxisomal matrix protein, import of catalase inside peroxisomes requires a functional Pex5p.

We have only analyzed the cells for the subcellular localization of the catalase till passage number 15. Until this passage number no mislocalization of catalase could be observed neither with the Pex5p(WT) nor with the Pex5p(C11K) constructs. In addition, all conditions with Pex5p(C11S) resulted in a cytosolic staining pattern for catalase.



WT

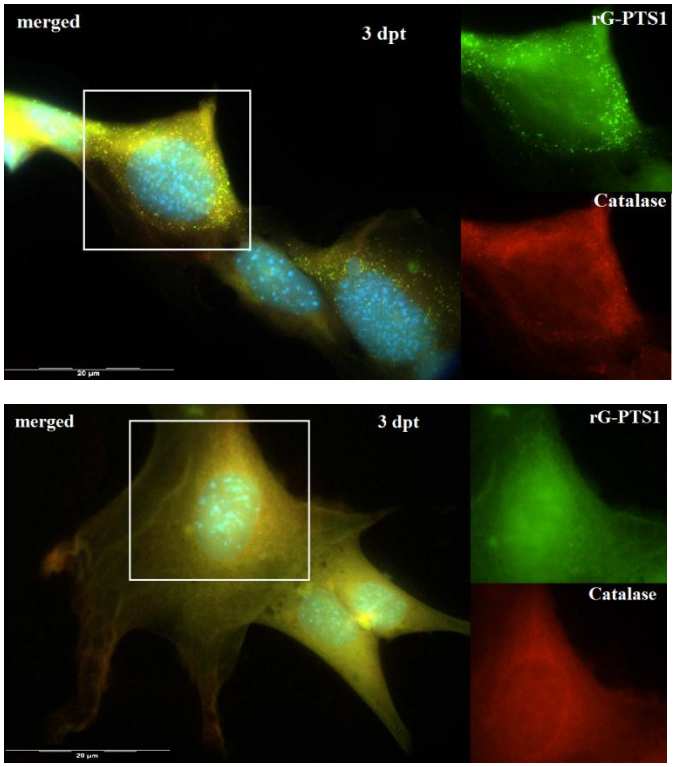


Figure 11. Subcellular localization of catalase in primary Pex5p-deficient MEFs (passage number 15) transfected with plasmids encoding different Pex5p variants and roGFP2-PTS1. Scale bar, 20 µm.

4. Effect of H_2O_2 treatment on the redox state of immortalized MEFs overexpressing Pex5p/catalase

As described above, we performed these experiments to induce premature senescence of the primary MEFs. We compared the redox state of the cytosol with the redox state of the cytosol upon catalase overexpression in immortalized Pex5p-deficient MEFs. We observed that catalase has a protective effect on the cytosol of the cell. As shown in graph 1 in Fig. 12, the redox state of the cytosol of the cells overexpressing catalase is less compared to the cells without overexpression of catalase i.e. the cytosol of the cells overexpressing catalase are better protected from the oxidative stress generated after addition of H_2O_2. To confirm this observation we co-transfected immortalized MEFs with non-tagged WT or C11S Pex5p variant with cytosolic-roGFP2. We have analysed both conditions upon catalase overexpression. As shown in graph 2 Fig. 12, we can see that cells transfected with WT Pex5p variant are more sensitive to oxidative stress generated after addition of H_2O_2 compared to the cells with C11S variant. This can be explained by the fact that in WT

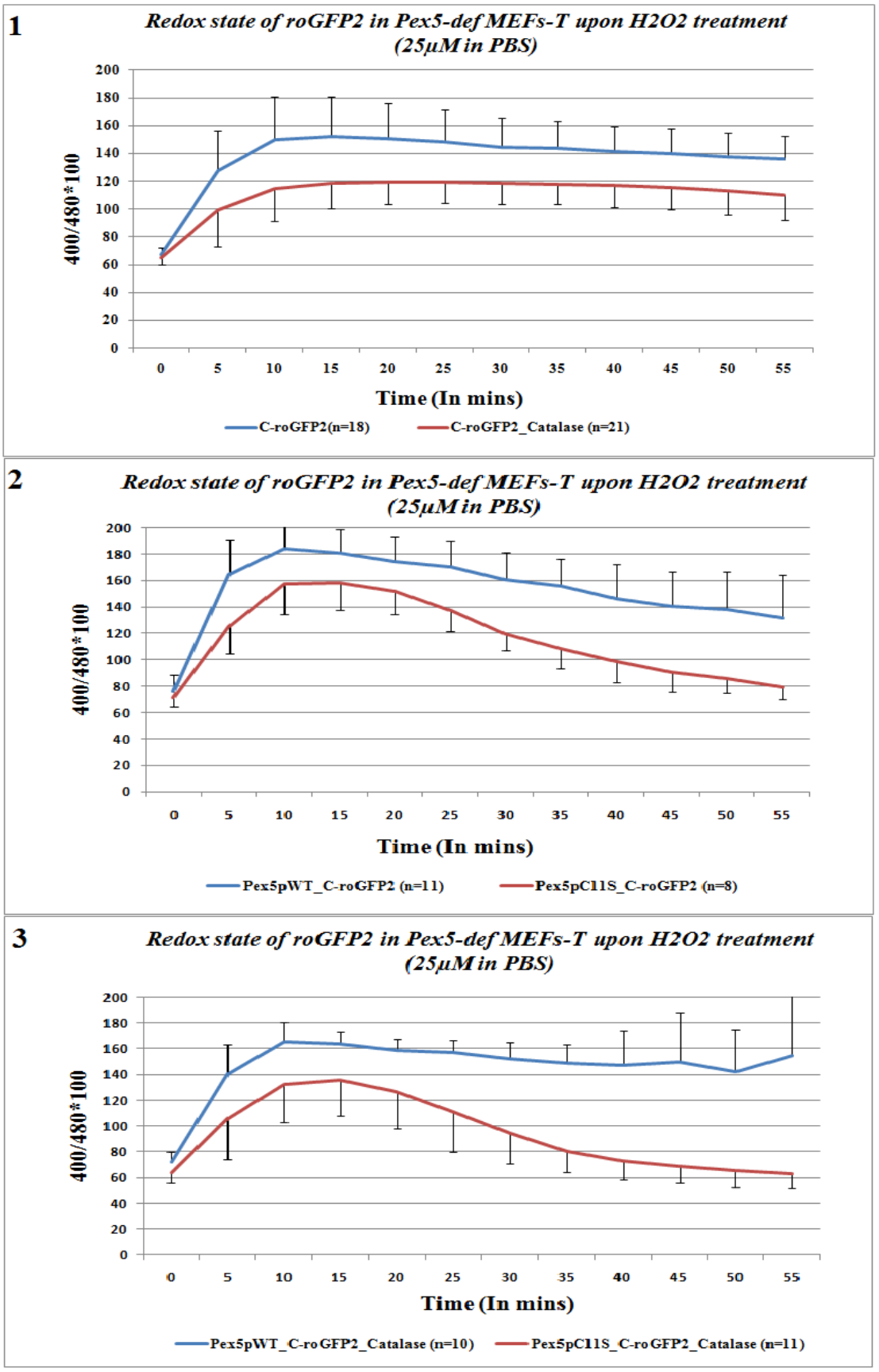


Figure 12. Redox state of cytosolic roGFP2 in immortalized Pex5p-deficient cells upon Pex5p/catalase overexpression. Immortalized Pex5p-deficient MEFs were transfected with plasmids encoding cytosolic roGFP2 (c-roGFP2) or co-transfected with non-tagged WT or C11S variant of

Pex5p and cytosolic-roGFP2 with or without catalase overexpression. Three days post transfection the cells were incubated in PBS for 5 min, afterwards the cells were treated with 25 µM H_2O_2 in PBS for the next 55 min. Images were captured every 5 min for 60 min and analysed as described in materials and methods. n= number of the cells analyzed. The ratio values signify the average ± SD.

condition there is a functional PTS1 import while in case of C11S variant PTS1 import gets impaired and there is no import of proteins in peroxisomes. If we compare these conditions upon catalase overexpression (graph 3, Fig. 12), we can see that the redox state of the cytosol was decreased in both conditions. This supports the hypothesis that catalase has some protective effect on the cytosol of the cells (Bai et al., 1999). Furthermore, we compared the cytosolic redox state of the cells transfected with cytosolic roGFP2 or with non-tagged WT or C11S Pex5p variant co-transfected with cytosolic roGFP2 (graph 1 and 2). The cytosolic redox state of the cells transfected with the WT and C11S variant was higher compared to the cells transfected with cytosolic roGFP2 without Pex5p co-expression. This might be because of binding of the monomeric catalase with Pex5p. Recent studies show that Pex5p binds catalase in the cytosol in its monomeric form forming a stable complex and leads to blocking its tetramerization this ultimately results in the inactivation of catalase activity in the cytosol (Freitas et al., 2011). Cells without active catalase are more sensitive to oxidative stress compared with the cells expressing it. These results suggest that a) import of catalase into peroxisomes sensitizes the cytosol and b) catalase overexpression does have a protective effect against oxidative stress caused by H_2O_2.

IV. Perspectives

Nowadays there is a huge interest among the researchers regarding the role of peroxisomes in cellular oxidative balance, redox signalling, and ROS levels. Results from our experiments could help to elucidate the physiological relevance of peroxisomes in cellular aging and in the beginning and development of various age-related diseases caused by oxidative stress. Observations from challenging experiments will help in determining the interaction between Pex5p and catalase. This may shed more light on the question whether catalase is imported into peroxisomes as a monomer or tetramer.

V. Acknowledgements

We thank Chantal Brees for her technical support of this work. This work is supported by grants from the “Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Onderzoeksproject G.0754.09)” and the “Bijzonder Onderzoeksfonds van de K.U.Leuven (OT/09/045).”

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APPENDIX

A. Plasmid isolation

This method is used for isolating plasmid DNA from bacteria. CTAB is a cationic detergent that binds to the negatively charged DNA and precipitates it from the mixture (Del Sal et al., 1988).

Buffers

- STET: 8% (w/v) sucrose, 0.1% (v/v) Triton X-100, 50mM EDTA, 50mM Tris pH 8.0, 0.1% mg/ml DNAse-free RNAse [Stock: 10 mg RNAse TE, pH 7.5 (boil for 15 minutes, aliquot and freeze)]
- CTAB: 5% (w/v) cetyl trimethyl ammonium bromide (precipitates below 20° C, reheat to dissolve)
- Lysozyme: 50 mg/ml (can be stored in water at -20° C, but once thawed it should be used the same day)
- NaCl: 1.2 M
- TE: 10 mM Tris-HCL, 1 mM EDTA, pH 8.0
- EtOH: 100% (v/v) and 70% (v/v)

Method

1. Pellet 1.5 ml of overnight bacterial culture by centrifuging at 3709 Xg for 2 minutes in a microcentrifuge.
2. Resuspend cells in 200 µl STET.
3. Add 5 µl lysozyme (lysozyme is an enzyme that lysis the cell wall).
4. Incubate tube for 5 minutes at RT and boil (heating block) for 45 seconds to deactivate the lysozyme.
5. Spin the tube in a microcentrifuge at 20196 Xg for 20 minutes.
6. Transfer supernatant into another microfuge tube.
7. Add 8 µl of 5% (w/v) CTAB to the tube to precipitate the DNA from the mixture.
8. Spin the tube in a microcentrifuge at 20196 Xg for 12 minutes.
9. Remove supernatant.
10. Resuspend pellet in 300 µl 1.2 NaCl to remove the CTAB from the mixture.
11. Add 750 µl of EtOH 100% (v/v) to the tube.
12. Spin the tube in a microcentrifuge at 20196 Xg for 12 minutes.
13. Remove supernatant.
14. Wash pellet with 500 µl EtOH 70% (v/v).
15. Spin the tube in a microcentrifuge at 20196 Xg for 10 minutes.
16. Remove supernatant.

17. Short spin
18. Remove last traces of supernatant.
19. Resuspend dry pellet in 20 µl RNAse water (water is prepared by adding 20 µl RNAse in 1 ml H_2O).
20. Incubate tube for 10 minutes at 37°C to give the optimum time and conditions for RNAse to work.

B. MinElute PCR Purification Kit Protocol using a microcentrifuge (QIAGEN kit)

This protocol is designed to purify double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA. Fragments ranging from 70 bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge. The main principle behind this method is ionic chromatography.

MinElute PCR Purification Kit from QIAGEN was used to purify the DNA after plasmid isolation and PCR (refer to MinElute handbook, available at: www.qiagen.com/literature/render.aspx?id=160).

C. PCR

1. DNAse/RNAse free 0.2 ml thin-walled microcentrifuge tubes were used.

Reagent	Volume	Final concentration
10X Reaction buffer (without $MgCl_2$)	5.0 µl	1X
10 mM dNTP mix	1.5 µl	0.3 mM each
50 mM $MgSO_4$	1.0 µl	1.0 mM
Template DNA [1]	1.0 µl	---------
Forward primer (Hs Pex5.1) [2a] (10 µM)	5.0 µl	1 µM
Reverse primer (Pex5.2) [2b] (10 µM)	5.0 µl	1 µM
Thermostable *Pfx* DNA polymerase (2.5 U/µl)	0.5 µl	1.25 U
Double distilled water to make final volume 50 µl	----------	---------

[1]Concentration of DNA:

pMF1578 (HsPex5pC11K in pIRES2-GFP-KSKL): ~2 µg/µl

pMF1678 (HsPex5pL in pIRES-GFP-KSKL): ~1 µg/µl

pMF1679 (HsPex5pL(C11S) in pIRES-GFP-SKL): ~1.3 µg/µl

[2a]Forward primer sequence:

5’ GGGA*GATCTACCATGCCAATGCCGGAGCTGGTGGAGGCCGAAGGG 3’

[2b]Reverse primer sequence:

5’ GCCCG*TCGACCTGTCACTGGGGCAGGCCAAAC 3’

2. All the reagents mentioned above are mixed in the tubes (briefly spin to ensure that all the reagents are collected at the bottom).
3. All the tubes were placed in the thermocycler and the conditions for the different steps i.e. denaturing, annealing and extension were set and the PCR was performed for 30 cycles.

   Denature: 94°C for 30 seconds

   Anneal: 55°C for 30 seconds

   Extension: 68°C for 60 seconds

4. The amplification products were later analyzed by agarose gel electrophoresis and visualized using SYBR safe (Invitrogen) staining (Appendix I) and stored at -20°C.

* Restriction sites

D. Restriction digestion by Bgl II and Sal I

a) For plasmids encoding different Pex5p variants: C11K (pMF1578), WT (pMF1678), C11S (pMF1679)

Reagent	Quantity
Template DNA (~2.5 µg/µl)	30 µl
Buffer 10X M	10 µl
Bgl II (10 U/µl)	3 µl
Sal I (12 U/µl)	3 µl
Distilled water	54 µl
Total	100 µl

Incubate for 2 hours at 37°C.

b) For pIRES2-EGFP vector with roGFP2-PTS1 sequence (pMN8)

Reagent	Quantity
Template DNA (~2 µg/µl)	5 µl
Buffer 10X M	5 µl
Bgl II (10 U/µl)	1.5 µl
Sal I (12 U/µl)	1.5 µl

Distilled water	37 µl
Total	50 µl

Incubate for 2 hours at 37°C.

E. Protocol for ligation and transformation

a) For wild type Pex5p variant

Reagent	Quantity
Vector (~2 µg/µl)	1 µl
Insert (~1.4 µg/µl)	2 µl
Ligation mix (T4-DNA ligase)	3 µl
Total	6 µl

b) For C11K and C11S Pex5p variant

Reagent	Quantity
Vector (~2 µg/µl)	1 µl
Insert (~2.1 µg/µl)	1 µl
Ligation mix (T4-DNA ligase)	2 µl
Total	4 µl

- After mixing all the reagents for each ligation in three different 1.5 ml eppendorf tubes leave the mixture for 15 minutes at room temperature.
- Add 100 µl of Top10F' cells to each from three tubes.
- Put the tubes on ice for 30 minutes.
- Keep the eppendorfs at 42°C for 1 minute for heat shock.
- Put them back on ice for few a minutes.
- Add 700 µl of YTS media (Appendix F) to all eppendorfs and put them in to the incubator shaker for 40-45 minutes at 37°C.
- Add agar media containing the appropriate amount of selective antibiotic(s) (Appendix F) in petri dishes and let it solidify.
- Spread the media containing cells to the respective agar plates and put them in the incubator at 37°C overnight.

F. YTS media composition

Reagent	Quantity
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water to make final volume 1 L and adjust final pH to 7.	----------

- Agar media was prepared by adding 2 % of agar in YTS media.

- Tetracycline (25 µg/ml) and kanamycin (50 µg/ml) were added as a selective antibiotic marker in YTS media to prepare LKT medium.

G. Restriction analysis by Bam HI

Reagent	Quantity
DNA [3]	3 µl
Buffer 10X K	2 µl
Bam HI (15 U/µl)	1 µl
Distilled water	14 µl
Total	20 µl

[3] **Concentration of DNA:**

pMF1578 (C11K): ~2.2 µg/µl

pMF1678 (WT): ~1.9 µg/µl

pMF1679 (C11S): ~2 µg/µl

H. Immunostaining of adherent mammalian cells

Formaldehyde fixation

1. Grow cells on coverslips (in 24 well plates).
2. Before use, rinse the cells 3 times with 1 ml 1X phosphate-buffered saline (PBS) pH: 7.36.
3. Immerse for 20 minutes in 1 ml 4% (w/v) paraformaldehyde (pH: 7.2)/PBS solution.
4. Aspirate and wash 3 times with 1 ml 1X PBS.

Post-fixative permeabilization

1. Immerse for 5 minutes in 1 ml 1% (w/v) Triton X-100/PBS.
2. Aspirate and wash 3 times with 1 ml 1X PBS.

DAPI staining

1. Immerse the coverslips for 1 minute in 500 µl 1X PBS containing 0.5 µg DAPI/ml.
2. Aspirate and wash 3 times with 1 ml 1X PBS.

Blocking

1. To minimize non-specific adsorption of the antibodies to the coverslips, immerse the coverslips for 15 minutes in 500 µl blocking buffer (5% (w/v) BSA/PBS).
2. Aspirate and wash 3 times with 1 ml 1X PBS.

Incubation with primary antibody

1. Dilute the primary antibody i.e. mouse anti-catalase (1:100) in 1% (w/v) BSA/PBS buffer.
2. Incubate the cells with the primary antibody dilution by using a wet chamber (pipet 30-40 µl antibody on parafilm and flip the coverslips with the cells on the drop of antibodies, the parafilm is put in a closed box with wet the tissues at the sides). Incubate for 60 minutes at room temperature.
3. Aspirate and wash 10 times with 1 ml 1X PBS.

Incubation with secondary antibody

1. Dilute the secondary antibodies i.e. anti-mouse Texas Red (1:200) in 1% (w/v) BSA/PBS.
2. Incubate the cells with secondary antibody dilution by using a wet chamber. Incubate for 60 minutes at room temperature.
3. Aspirate and wash 10 times with 1 ml 1X PBS.

Preparation for microscopy

1. Invert each coverslip onto a slide containing one drop of n-propyl-gallat (Sigma, P-3130) and Mowiol 4-88 (Vel, 17951) (1:3). Remove the excess of mounting media with a fibre free paper. Store at 4°C in dark until viewing.

I. Agarose gel (1.5%)

1. Weigh 1.5 gram of agarose.
2. Add 1X TAE buffer to the agarose to make the final volume 100 ml.
3. Add 10 µl of SYBR safe (Invitrogen) dye to the solution.
4. Warm the solution in a microwave until the agarose gets completely dissolved.
5. Cool the solution for some time and pour it on the gel plate having comb. Let it solidify for some time.
6. Remove the comb after the gel gets solidified and load the samples into the wells.
7. Turn ON the electric supply and let the samples run on the gel at 200 Volts for 10-15 minutes.
8. Turn OFF the electric supply after the samples passes half a way of gel.
9. Visualize the gel under the blue light.

J. Electroporation protocol

Electroporation is a technology used for transforming the bacterial, yeast, plant or mammalian cells by applying external electric field. In this, a pulse of voltage of specific magnitude is passed for a specific time, as a result of this membrane of the cells gets permeable and the foreign DNA can be easily transformed into the cells via pores formed after passing current.

Electroporation parameters for MEFs

Pulse voltage (V): 1,350
Pulse width (ms): 30
Pulse number: 1 (refer to Invitrogen, catalog no. MPK5000, available at: http://tools.invitrogen.com/content/sfs/manuals/neon_device_man.pdf)