Identification of novel peroxisome functions in yeast

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SUMMARY

Eukaryotic cells are characterized by the presence of distinct membrane-bound compartments, called organelles. Each organelle has its own characteristics. The peroxisome is one of the cellular organelles and displays a wide variety of metabolic and non-metabolic functions, which depend on cellular requirements. Two of the conserved functions of peroxisomes include fatty acid β-oxidation and the metabolism of reactive oxygen species (ROS) such as hydrogen peroxide. Along with mitochondria peroxisomes also contribute to the generation of intracellular ROS. As yet two mechanisms of peroxisome biogenesis were described: growth and division of pre-existing peroxisomes or *de novo* formation from the endoplasmic reticulum (ER). For their growth peroxisomes require proper insertion of membrane lipids and proteins and translocation of matrix proteins. In human defects in peroxisome function or assembly lead to diseases that are associated with multiple severe clinical symptoms and are often lethal. Improper peroxisome functioning also contributes to aging and age-related diseases. Therefore, the interest in peroxisome biology has significantly expanded in recent years. Peroxisomes are not essential in yeast, which makes them good model systems for studying peroxisomes.

The research described in this thesis focuses on the identification and characterization of novel peroxisome proteins and functions in yeast.

Chapter 1 presents an overview of our current knowledge of peroxisomes. Special emphasis has been given to peroxisome function and the processes that are required for redox-regulation, and proliferation in yeast.

Chapter 2 discusses our research on the identification of novel stress-related proteins in *Hansenula polymorpha* peroxisomes. By using mass spectrometry analysis of peroxisomal fractions from cells exposed to ethanol stress, we identified 6 putative peroxisomal peroxiredoxins. Two out of the identified putative peroxiredoxins, named C8BNF3 and C8BNF4, contain a putative peroxisomal targeting signal (PTS1). Peroxiredoxins are thiol-specific proteins important for defense against oxidative stress. Their absence causes accumulation of ROS. Fluorescence microscopy analysis using fusion constructs with green fluorescent protein (GFP) showed that C8BNF3 is a mitochondrial protein despite the presence of a PTS1, whereas C8BNF4 partially localizes to peroxisomes in glucose-grown cells. The latter protein, however, does not accumulate in these organelles when cells are grown on methanol. Using a *H. polymorpha C8BNF4* deletion strain, called *c8bnf4*, we found that the absence of this protein does not affect sensitivity to any of the stress conditions tested. Possibly this is due to redundancy because studies in *Saccharomyces cerevisae* revealed that mutants lacking multiple peroxiredoxins were more sensitive to ROS stress when compared to a single deletion mutant [1].

Peroxisomes are not only important during conditions of oxidative stress, because in yeast peroxisomes also enzymes occur that are induced upon exposure of cells to other stress conditions. Glycerol phosphate dehydrogenase 1 (Gpd1) and nicotinamidase (Pnc1) are two stress-related proteins in *S. cerevisae,* which were reported to be are targeted to peroxisomes at normal growth conditions, but mislocalize to the cytosol and nucleus upon exposure of cells to osmotic stress. Moreover, earlier studies in *S. cerevisae* demonstrated that import of Gpd1 and Pnc1 depend on the Peroxisomal Targeting Signal 2 (PTS2) receptor Pex7 [2,3]. Indeed, Gpd1 contains a PTS2, however, Pnc1 lacks a PTS. We show in **Chapter 3** that Pnc1 physically interacts with Gpd1 allowing its piggyback import to peroxisomes. Western blotting revealed

that both proteins are not present at constant ratios in the cell, suggesting that they do not form a stable complex with fixed stoichiometry. Our attempts to demonstrate a stable physical interaction between both proteins using a variety of *in vitro* approaches proved unsuccessful. This may indicate that the interaction between these two proteins is transient or additional factors, such as post-translational modifications or other proteins, may be required for the interaction. Although Pnc1 and Gpd1 can form a transient complex, the stability of one protein is unaffected by the absence of the other, suggesting that both proteins interact mainly for targeting purposes.

Previous studies indicated that Gpd1 and Pnc1 levels were increased upon exposure of cells to osmotic stress, accompanied by Gpd1 mislocalization to the cytosol. The mislocalization was proposed to be regulated by reduced phosphorylation of two serine residues adjacent to the PTS2 of Gpd1, which decreases its affinity for Pex7 [3]. We analyzed the peroxisomal and cytosolic Gpd1 and Pnc1 levels before and after exposure of cells to different stress conditions by using quantitative fluorescence microscopy. This revealed that both proteins were mainly localized to peroxisomes at normal growth conditions. However, upon exposure of the cells to stress, the levels of both proteins increased in the cytosol as well as inside peroxisomes. This observation is not in line with the model that Gpd1 relocalizes from peroxisomes to the cytosol upon stress exposure. Indeed, we observed a similar distribution pattern when a non-stress related peroxisomal PTS2 protein (thiolase) was produced under the control of the Gpd1 promoter and exposed to stress. This suggests that the presence of cytosolic Gpd1 and Pnc1, both at normal and stress conditions, is related to the inefficiency of the PTS2 import machinery and not regulated by osmotic stress.

Damage caused by high temperatures (heat stress) can be counterbalanced by the so called heat shock proteins, such as Hsp70 proteins. *S. cerevisiae* Sym1 (for "stress-inducible yeast Mpv17") is a heat shock protein required for ethanol metabolism [4]. The human *MPV17* gene encodes for a small hydrophobic protein located in the inner mitochondrial membrane and a mutation in this gene causes hepatocerebral mtDNA depletion syndrome (MDS) [5]. The MPV17 protein belongs to the PXMP2 family of integral membrane proteins. PXMP2 is a peroxisomal protein which serves as a non-selective channel for transfer of small solutes across peroxisomal membrane [6,7]. The PXMP2 protein family comprises among others the Woronin body (WB) protein Wsc in *Neurospora crassa,* the human peroxisomal membrane protein Pxmp2, the mitochondrial inner membrane protein Sym1 in *S. cerevisiae* and its mammalian homologue MPV17. The WB is a highly specialized peroxisome, which plugs septal pores upon hyphal wounding to prevent cytoplasmic leakage in filamentous Ascomycetes [8].

In order to obtain further insights into this protein family, we studied PXMP2 family proteins in *H. polymorpha*. In **chapter 4**, we show that one of the PXMP2 proteins designated as Pex37 localizes to the peroxisomes in *H. polymorpha.* Previous studies indicated that proteins of the PXMP2 family not only play a role in solute transport but also fulfil a function in processes related to membrane shaping and organelle positioning. We first checked growth of the *H. polymorpha pex37* deletion mutant on several carbon and nitrogen sources that are metabolized by peroxisomal enzymes. We found that there was no clear growth defect in any of

the tested media, suggesting that Pex37 does not fulfil an essential function in the transport of compounds required for their metabolism across the peroxisomal membrane.

Deletion of *PEX37* did not affect peroxisome biogenesis or proliferation in cells grown at peroxisome inducing conditions (methanol), however, a peroxisomal phenotype was observed at peroxisome repressing conditions (glucose medium). Non-budding *H. polymorpha* WT cells contain a single peroxisome, whereas in budding WT cells, one peroxisome is detected in both the mother cell and bud. For proper peroxisome segregation peroxisomes have to divide during yeast budding, a process which requires Dnm1 and Pex11 [9,10]. Retention of one of the organelles in the mother depends on Inp1, whereas the motor protein Myo2 attaches to peroxisomes via its interaction with the integral peroxisomal membrane protein, Inp2, and carries them to the bud via the actin cytoskeleton [11–13]. Interestingly, in glucose-grown *pex37* cells, the peroxisomes were often distributed to the bud thereby leaving the mother cells devoid of peroxisomes. In other cells the peroxisomes stayed in the mother cell during budding, resulting in a bud without a peroxisome. Our data indicate that Pex37 is required for proper peroxisome multiplication and segregation in glucose-grown cells, but not in methanol-grown cells. Further investigations such as the identification of binding partners of Pex37 may reveal the details of such regulatory process and will help to figure out the underlying molecular mechanism.

We also established conservation of function between yeast Pex37 and human PXMP2 through the complementation of the yeast *pex37* phenotype by expression of human PXMP2. Mammalian PXMP2 is proposed to be a non-selective pore in the peroxisome membrane but we show that *H. polymorpha* Pex37 may not fulfill an essential function in transport of compounds across the peroxisomal membrane.

Detailed organelle proteomic analysis reported the presence of Vac8 in the peroxisomal fractions isolated from *S. cerevisiae* [14] or *H. polymorpha* (Chapter 2). Therefore, we also explored the role of HpVac8 **(Chapter 5)**. Like the function of Inp2 in peroxisome transport to yeast buds, Vac8 is required for vacuole inheritance in *S. cerevisae*. Vac8 interacts with Myo2 via Vac17 (vacuole-related protein 17) and this complex is responsible for the bud-directed movement of vacuoles to the developing daughter cell [15]. Vac8 also plays a role in vacuole-vacuole fusion and is an important component of nucleus-vacuole junctions (NVJ) in *S. cerevisae*.

Recently, a novel peroxisome-vacuole contact site has been identified, which is formed at conditions of rapid peroxisome expansion in *H. polymorpha* [16]. We showed that HpVac8 localizes close to the peroxisome-vacuole contacts, however, it is not present at these contacts. Our data indicate that HpVac8 most likely does not play a role in peroxisome biology as deletion and overproduction of Vac8 had no effect on peroxisome abundance. Therefore, the reason why HpVac8 and ScVac8 were identified in yeast peroxisomal fractions remains obscure.

In **chapter 5** we also studied the role of HpVac8 in the formation of NVJs, vacuole inheritance and vacuole fusion. In-silico analysis of the *H. polymorpha* genome to find homologs of *S. cerevisiae* NVJ proteins identified all homologs of the NVJ-related proteins except Nvj1, a protein essential for NVJ formation in *S. cerevisae*. This implies that the NVJ composition is not conserved. In addition, we show that HpVac8 is required for NVJ formation as no close contact between nucleus and vacuole could be detected in the absence of Vac8. Also, the absence of HpVac8 resulted in a defect in vacuole inheritance. Interestingly, unlike ScVac8,

the absence of HpVac8 deletion had no effect on vacuole-vacuole fusion indicating that it does not play a role in this process in *H. polymorpha*.

Outlook/perspectives

Regardless of the considerable advances in identifying genes/proteins and defining molecular mechanisms involved in peroxisome biogenesis and function, there is still a large gap in our understanding of how peroxisomes contribute to cellular redox metabolism and aging. Finding proximal targets of peroxisomal oxidative stress, as well as the molecular mechanisms of how cellular stress affects peroxisome function, would provide a more coherent understanding of the underlying mechanisms related to peroxisomes and oxidative stress. The identification of a novel peroxisomal peroxisome peroxiredoxin, as described in this thesis, supports the view that the atlas of peroxisome function is still not complete. Quantitative mass spectrometry analysis of peroxisomal fractions isolated from cells exposed to stress and untreated controls, may lead to further information on peroxisomal stress related proteins. Also, using more sensitive proteomic approaches will help to identify yet uncharacterized peroxisomal proteins, and open doors understand novel stress related functions. This, in turn, may result in leads to new therapies to prevent aging and age-related diseases.

Despite our comprehensive knowledge on peroxisomal targeting signals, the sorting mechanism of several peroxisome proteins remain elusive. Still new examples of piggy back import (Chapter 3, [17]) and novel PTS receptors [18,19] are identified. Also, a few peroxisome proteins are localized to other subcellular compartments (e.g. the Dnm1 dependent peroxisome fission machinery). The mechanism by which their distribution over various organelles is regulated is poorly understood and requires further elucidation. Such studies will open endless research prospects to explore and identify novel peroxisome functions.