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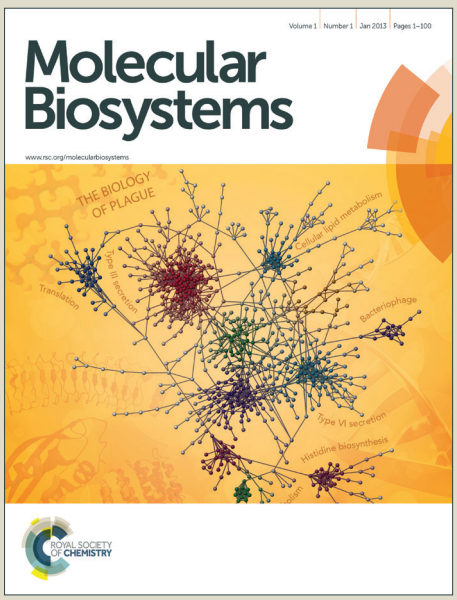

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Peroxisomes are juxtaposed to strategic sites on mitochondria

Yifat Cohen[1]*, Yoel Alexander Klug[2]*, Lazar Dimitrov[3], Zohar Erez[1], Silvia G. Chuartzman[1], Dalia Elinger[1], Ido Yofe[1], Kareem Soliman[4], Jutta Gärtner[4], Sven Thoms[4], Randy Schekman[3], Yael Elbaz-Alon[1], Einat Zalckvar[1], Maya Schuldiner[1]

[1] Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 7610001, Israel

[2] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 7610001, Israel

[3] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

[4] Department of Pediatrics and Adolescent Medicine, University Medical Center, University of Göttingen, Göttingen 37075, Germany

* These authors contributed equally to this work

Correspondence should be addressed to:

yael.elbaz@weizmann.ac.il; einat.zalckvar@weizmann.ac.il; maya.schuldiner@weizmann.ac.il

Abstract

Peroxisomes are ubiquitous and dynamic organelles that house many important pathways of cellular metabolism. In recent years it has been demonstrated that mitochondria are tightly connected with peroxisomes and are defected in several peroxisomal diseases. Indeed, these two organelles share metabolic routes as well as resident proteins and, at least in mammals, are connected via a vesicular transport pathway. However the exact extent of cross-talk between peroxisomes and mitochondria remains unclear. Here we used a combination of high throughput genetic manipulations of yeast libraries alongside high content screens to systematically unravel proteins that affect transport of peroxisomal proteins and peroxisome biogenesis. Follow up work on the effector proteins that were identified revealed that peroxisomes are not randomly distributed in cells but are rather localized to specific mitochondrial subdomains such as mitochondria/ER junctions and sites of acetyl-CoA synthesis. Our approach highlights the intricate geography of the cell and suggests an additional layer of organization as a possible way to enable efficient metabolism. Our findings pave the way to further study the machinery aligning mitochondria and peroxisomes, the role of the juxtaposition, as well as its regulation during various metabolic conditions. More broadly, the approaches used here can be easily applied to study any organelle of choice, facilitating the discovery of new aspects in cell biology.

Introduction

Peroxisomes are ubiquitous, single-membrane-enclosed, organelles that participate in various pathways of cellular metabolism such as **β**-oxidation of fatty acids, amino acid catabolism and detoxification of reactive oxygen species (ROS)[1]. Due to the central functions carried out by peroxisomes, their disruption ensues severe peroxisome biogenesis disorders (PBD)[2]. Peroxisomes are remarkably diverse in size, number, and the enzymes that they contain depending on the cell type and the environmental condition. Moreover, all of these characteristics can be rapidly regulated in response to metabolic signals[3].

Emerging new evidence shows that regulation of peroxisomal function seems to also occur through communication pathways between peroxisomes and other organelles [3]. One type of organelle that exhibits a far greater co-dependent relationship than previously anticipated is mitochondria[4, 5]. In the last years it is becoming apparent that peroxisomes and mitochondria are metabolically linked organelles that cooperate and cross talk especially in maintenance of ROS homeostasis and **β**-oxidation of fatty acids. The product of **β**-oxidation is acetyl-CoA, the entry point into the mitochondrial TCA cycle for energy production. It is interesting to note that although in mammals **β**-oxidation can occur in both mitochondria and peroxisomes, in the bakers yeast *Saccharomyces cerevisiae,* this task is undertaken solely by peroxisomes. Since energy yielding catabolic processes take place in both peroxisomes (**β**-oxidation) and mitochondria (TCA cycle), these process should be co-regulated in order to meet cellular energy demands. In light of this close interaction, it is not surprising therefore that these two organelles have been shown to share several proteins including the fission machinery, and different metabolic enzymes[6]. Strikingly a vesicular transport pathway from mitochondria to peroxisomes was identified in mammalian cells [7]and it is well documented that mitochondria are defected in several PBDs[4]. Despite the fragmented pieces of information about the tight connection between these two organelles, little is known regarding the exact extent of cooperation and interactions and how much this relies on their relative distribution in the cell.

Classical mutagenesis screens alongside complementation assays and proteomic approaches were used to identify peroxisomal biogenesis proteins (peroxins), as well as other metabolic enzymes that are localized to peroxisomes in yeast and mammalian cells[3]. In recent years several high throughput experiments were performed in yeast [8-10] adding to the accumulating knowledge of proteins required for peroxisome formation and function. Intriguingly, each screen yielded different proteins adding to the global understanding of peroxisomal biogenesis processes. However, none of the screens uncovered genes required for the cross-talk between mitochondria and peroxisomes. The fact that new peroxisomal proteins are still being discovered is a demonstration of the diversity and complexity of peroxisomes, and alerts to the necessity of more experimental approaches that will

enable us to decipher the biogenesis, maintenance and the diverse functions of this fascinating organelle.

Here we utilized a high throughput imaging-based screen in the yeast *S. cerevisiae* looking for the first time in parallel at a peroxisomal membrane protein (PMP) and a matrix protein to uncover new regulators of these two peroxisomal constituents. We show how a combination of high-content screens can be used to systematically unravel biological functions that were not discovered before. We then focus on results demonstrating that peroxisomes are not randomly distributed in cells but rather localize to specific mitochondrial sites such as mitochondria/ER junctions and sites of shared metabolism. Our data demonstrates a topological constraint underlying peroxisomal function not previously appreciated.

Results

A dual readout high content screen can be used for differentiating complex peroxisomal phenotypes

Several visual screens have been performed in the past to uncover novel proteins playing a role in peroxisome biogenesis[9, 10]. We decided to build on these efforts to rescreen the yeast deletion and hypomorphic allele libraries utilizing a dual reporter system (Figure 1a). The first reporter, a soluble Cherry whose last three C terminal amino acids encode a serine-lysine-leucine (SKL) peroxisomal targeting signal (PTS) type 1 (Cherry-PTS1) would report on the ability of peroxisomes to import PTS1 containing proteins into their matrix. The second reporter, adenine nucleotide transporter 1 (Ant1) tagged with GFP at its N terminus (GFP-Ant1), is a peroxisomal membrane protein (PMP). Ant1 has a relatively high expression level and it requires proper trafficking of PMPs to reach peroxisomes but is not required for peroxisome biogenesis. Hence GFP-Ant1 would optimally report on the ability of peroxisomes to form and transport PMPs. This dual reporter system was designed to enable us to find mutants where peroxisomal membranes are generated but matrix protein import is hampered alongside more classical mutants affecting number and size of the organelle.

To integrate the two reporters into the collections of mutants in a systematic fashion, we created a query strain expressing both markers that is also compatible with the synthetic genetic array (SGA) automated mating procedure [11, 12] and crossed it into the yeast deletion[13] and hypomorphic allele collections[14] (Figure 1a). The result of the cross was a library of nearly 6000 haploid yeast strains each carrying both marker proteins and a mutation in one yeast gene. Once we had the yeast mutant collection we used an automated microscopy set up[15] to visualize all strains in synthetic medium containing dextrose as a carbon source and during stationary phase of the culture where peroxisomes are most pronounced and the matrix Cherry-PTS1 could be easily visualized

overlaying the GFP-Ant1 tagged membrane (Figure 1b, left image). We then manually categorized the strains into three phenotypic bins: Reduced Cherry-PTS1 in peroxisomes, Reduced/No peroxisomes, Aberrant (usually several small) peroxisomes (Figure 1b and Supplementary Table 1). We identified 56 strains that had one of these three phenotypes from which only 17 were previously identified as being important for peroxisomal function or structure (*PEX* genes+*DJP1* [3, 16, 17]) (Figure 1c).

A systematic secondary screen uncovers the effect of Spf1 on peroxisomes.

Under the specific screening conditions used we noticed that in several strains GFP-Ant1 localizes to mitochondria in some cells. Indeed, it was previously demonstrated that when peroxisomes have various defects, some PMPs localize to mitochondria[18-21]. Strikingly, in one strain, Δ*spf1,* GFP-Ant1 localization to mitochondria was highly pronounced (Figure 2A). Spf1 is a P-type ATPase localized to the membranes of the endoplasmic reticulum (ER). We have recently suggested that Spf1 is important for regulating manganese homeostasis [22] and have shown that its loss causes mistargeting of mitochondrial tail anchored proteins to ER membranes [23]. However neither its newly suggested role nor any of the many previously demonstrated phenotypes explain the GFP-Ant1 mistargeting phenotype. Nevertheless, Spf1 was previously found in other peroxisomal biogenesis screens [8, 24] and cannot grow on oleic acid as the sole carbon source [24] poising it as a true effector of peroxisomal structure and function.

A challenging aspect of analyzing the results of high content screens is that it is often very difficult to unequivocally determine the direct cause underlying an interesting phenotype observed in a mutant strain. We therefore decided to check whether an unbiased approach can be used to mechanistically explain the role of Spf1 in protein targeting to peroxisomes. To this end, we used again the SGA approach described above, this time to cross a deletion of *spf1* into the GFP library (Figure 2b). The GFP library is a collection of nearly 6000 yeast strains each expressing one yeast gene fused to a green fluorescent protein (GFP) at its C terminus and under its natural promoter in its endogenous chromosomal site[25]. Characterizing protein localization in control and Δ*spf1* cells would therefore allow us to systematically gauge the effect of losing Spf1 on the yeast proteome. We visualized the GFP libraries in logarithmic growth and manually categorized each protein that changed localization in the mutant Δ*spf1* strain relative to a control strain.

Interestingly, mutating Spf1 did not have a general effect on all peroxisomal transmembrane proteins. Rather, out of all proteins that changed localization (Supplementary Table 2) we could only find one peroxisomal protein, Pex3-GFP, which lost its uniform distribution and did not reside on peroxisomes in many of the cells (Figure 2C). Since Pex3 is required for proper localization and

stability of PMPs, it is not surprising the GFP-Ant1 could not get to peroxisomes in this background. It is, however, surprising that other PMPs were not affected by loss of Spf1. One explanation may be that the ability to see the phenotype for other PMPs is more quantitative than qualitative, and our manual analysis of the data could therefore not uncover it. Another option is that GFP-Ant1 is more dependent on Pex3 than other PMPs and cannot use alternate, backup, routes. Notably although Ant1-GFP was included in the GFP library, we could not detect an effect of the loss of Spf1 on its localization. Hence we think that the effect of the loss of Spf1 on Ant1 localization is prevented by the GFP tag at the C terminus of Ant1. This may also be the reason why we did not see an effect on other PMPs, also tagged with GFP at the C terminus. However, more generally, our results suggest that a systematic high content screen can be an effective way to trace back the cause of an observed phenotype in a specific mutant strain.

Peroxisomes are adjacent to mitochondria/ER junctions.

The initial screen for mutants in peroxisomal structure and import flagged another interesting mutant, Δ*mdm10* (Mitochondrial Distribution and Morphology), that was not previously studied in the context of peroxisomes. We focused on this specific hit as Mdm10 was recently identified as part of a four-protein complex, termed ERMES (ER/Mitochondria Encounter Structure), which holds the membranes of mitochondria and ER in close proximity [26]. In this mutant we saw that most cells displayed aberrant peroxisomal structure as several small peroxisomes could be seen instead of the typical one large peroxisome of the control strain under these conditions (Figure 1b, aberrant peroxisomes). Importantly, mutant strains of three ERMES complex components displayed the same phenotype demonstrating that aberrant peroxisomes are indeed the result of losing the ERMES complex (Figure S1). Since the connection of peroxisomes with mitochondria is only now beginning to be appreciated[4, 5] as is the fact that peroxisomes can bud directly from the ER[27], we wondered whether there might be a topological benefit for peroxisomes to combine these two connections and reside in close proximity to both organelles by juxtaposing to mitochondrial/ER junctions.

To test this we co-localized peroxisomes with ERMES junctions and found that during growth in logarithmic phase 33% of the peroxisomes (about one per cell) were adjacent to an ERMES focus (Figure 3a, 3b, 3c). The proximity was also observed under stationary phase where 22% of the peroxisomes were adjacent to an ERMES focus (Figure S2), and did not depend on whether the marker we used for the ERMES complex was on the mitochondrial (Mdm34) or ER (Mmm1) side. The proximity was also not influenced by whether the peroxisomal marker was a PMP or a matrix

protein. In fact, all three combinations of markers used (Mdm34-Cherry/GFP-PMP, Mdm34-GFP/Cherry-PTS1 or Mmm1-GFP/Cherry-PTS1) displayed the exact same phenotype (Figure 3a, 3b, 3c). Hence, our results suggest that peroxisomes can have a distinct geographical distribution in the cell, leading to the question whether this contact site represents a more general strategic point on mitochondrial membranes.

Systematic screening of proteins that reside in punctate structures demonstrates that peroxisomes align with sites of mitochondrial acetyl-CoA synthesis.

To uncover additional strategic mitochondrial subdomains that could align with peroxisomes we used the yeast GFP library again. From the GFP library we picked a collection of 96 strains in which the tagged protein displayed a punctate and a clearly visible GFP signal (Supplementary Table 3) and, using SGA, crossed it against a query strain containing a mitochondrial marker (Aco2-Cherry) (Figure 4a). We then screened the collection of resulting haploids containing both markers for mitochondrial proteins that do not have a distribution over the entire surface of the organelle but rather are localized in subdomains during logarithmic growth. Interestingly we found that the strongest hit was Pda1, a subunit of the pyruvate dehydrogenase (PDH) complex (Figure 4b). Co-localizing Pda1 with another subunit of the complex, Lpd1, demonstrated that the entire complex most probably resides in a mitochondrial subdomain both in logarithmic growth (Figure 4c) and in stationary phase (Figure 4d, see Pda1 localization). Although this complex has been intensively studied biochemically [28-30] it has never before been studied for its spatial sub localization in the mitochondrial matrix. Hence, this is the first demonstration that this complex has a distinct geographical subdomain within the mitochondrial matrix.

Notably, we observed that Pda1-GFP and Mdm34-RFP are often also juxtaposed in the cell (Figure S3) suggesting that indeed these foci represent a higher level of organization within the mitochondria membrane. Hence as expected, when co-localizing the complex with peroxisomes we found that ~50% of peroxisomes were indeed adjacent to the PDH containing subdomains (Figure 4d) (similar results were obtained for Lpd1-GFP puncta, data not shown). Since both β oxidation of lipids occurring in peroxisomes, and dehydrogenation of pyruvate occurring by the PDH complex in mitochondria, produce acetyl-CoA, this proximity may serve to either provide an alternate acetyl-CoA source for mitochondria during reduced cytosolic glycolysis (stationary phase) or else to concentrate acetyl-CoA in specific mitochondrial domains in order to maximize entry into the TCA cycle [30] (Figure 4e). Regardless of the biochemical reason underlying this proximity, our results demonstrate a remarkable geographical regulation of substrate availability and shed new light on

how this biochemical pathway retains effectiveness in face of diffusion in the large yeast cell. Further work is required to examine if peroxisomes are localized adjacent to the PDH complex and the ERMES complex simultaneously. However, these findings suggest a higher order organization of peroxisomes, PDH complex and mito/ER junctions to one cellular locale.

Discussion

Our work demonstrates how a series of systematic screens that complement each other can be rapidly used to uncover rich layers of data not mined before. First we screened for novel regulators of peroxisome biogenesis and import of peroxisomal proteins and uncovered several new mutants that affect these processes and have not previously been described. We then demonstrate how a systematic secondary follow up screen can rapidly suggest an underlying cause for a phenotype displayed by a mutant strain. Finally, by focusing on hits that shed light on the connection between mitochondria and peroxisomes we reveal that peroxisomes are juxtaposed to very specific sites on mitochondria.

It has not been well documented that mitochondria have metabolically distinct subdomains and hence our work, at its very basis, is about identifying an additional layer of topological organization in the cell. In this organization, enzymatic processes are concentrated, potentially allowing efficient utilization for future steps. Interestingly, this happens not only within an organelle (such as the entire PDH complex being in a subdomain) but also in a coordinated manner between mitochondria and peroxisomes. The proximity between peroxisomes and sites of pyruvate decarboxylation in mitochondria may therefore reflect a way to enhance metabolism by creating a short distance for efficient transport of metabolites from one organelle to another. The observation that peroxisomes and mitochondria can be found in proximity also in mammalian cells (Figure S4) might imply evolutionary conservation of this phenomenon.

Our work opens up the road to uncovering the protein-tethers that aligns peroxisomes with sites of PDH concentration and/or ERMES foci on mitochondria as well as the regulation of such tethers during the various metabolic requirements of the cell.

More generally, the approaches and methodologies brought forward here demonstrate how the flexibility to easily and rapidly create genetically tailored, “mix and match”, libraries has the potential to uncover new biology. We hope that such approaches will provide a basis to integrate functional genomics of the peroxisome into the existing cell biology of this organelle. Moreover, these approaches are by no way restricted to studying peroxisomes, but rather could be easily used to attack any important question of choice or be adapted to other organelles.

Experimental

Yeast strains and strain construction

All yeast strains in this study are based on the BY4741 laboratory strain [31]. Libraries used were the yeast deletion library[13], the DAmP (Decreased Abundance by mRNA Perturbation) library [14] and the GFP library[25]. N terminal GFP tag for Ant1 at its endogenous chromosomal location was created with a PFA6 based vector with NAT selection and a Tef2 promoter [14]. TDH3pr-mCherry-SKL was integrated as a pop-in into the endogenous TDH3 locus with URA selection. The Δ*spf1* strain was taken from the deletion library [13] and verified to be a true deletion. GFP tagged Mdm34, Mmm1 and Pda1 as well as RFP/Cherry tagged Aco2, Lpd1 and Pex3 were created by PCR mediated homologous recombination [32].

Robotic library manipulations

All genetic manipulations were performed using Synthetic Genetic Array (SGA) techniques to allow efficient introduction of the markers into systematic yeast libraries. SGA was performed as previously described [11, 12]. Briefly, using a RoToR bench top colony arrayer (Singer Instruments, UK) to manipulate libraries in 1536-colony high-density formats, haploid strains from opposing mating types, each harboring a different genomic alteration, were mated on rich media plates. Diploid cells were selected on plates containing all selection markers found on both parent haploid strains. Sporulation was then induced by transferring cells to nitrogen starvation plates. Haploid cells containing all desired mutations were selected for by transferring cells to plates containing all selection markers alongside the toxic amino acid derivatives Canavanine and Thialysine (Sigma-Aldrich) to select against remaining diploids. Each SGA procedure was validated by inspecting representative strains for the presence of the encoded fluorophores and for the correct genotype using check PCR.

High-throughput microscopy

Microscopic screening was performed using an automated microscopy set-up as previously described[12] . Briefly, liquid cultures were grown over night in synthetic defined (SD) medium, with appropriate auxotrophic selections, in a shaking incubator (LiCONiC Instruments) in 30ºc. Cells were then transferred onto glass bottom 384-well microscope plates (Matrical Bioscience) coated with Concanavalin A (Sigma-Aldrich). The microscope plates were conveyed to an automated inverted fluorescent microscopic ScanR system (Olympus), equipped with a cooled CCD camera. Images were acquired using a 60X air lens -GFP (excitation at 490/20 nm, emission at 535/50 nm) and mCherry (excitation at 572/35 nm, emission at 632/60 nm) channels. After acquisition, images

were manually reviewed using the ScanR analysis program. Images were processed by the Adobe Photoshop CS3 program for minimal contrast and brightness adjustments.

Manual Microscopy

Manual microscopy was performed using an Olympus IX71 microscope controlled by the Delta Vision SoftWoRx 3.5.1 software with either a X60 or X100 oil lens. Images were captured by a PhotometricsCoolsnap HQ camera with excitation at 490/20 nm and emission at 528/38 nm (GFP) or excitation at 555/28 nm and emission at 617/73 nm (mCherry/RFP). Images were transferred to Adobe Photoshop CS3 for minimal contrast and brightness adjustments.

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Figures legends

Figure 1

A dual readout high content screen reveals different peroxisomal phenotypes. (a) To uncover proteins playing a role in transport of peroxisomal proteins and peroxisome biogenesis we expressed Cherry-PTS1 and GFP-Ant1 in a query strain, and crossed it into the yeast deletion and hypomorphic allele collections. We then used automated microscopy to visualize all strains. (b) We identified 56 strains that had one of the following three phenotypes: reduced Cherry-PTS1 in peroxisomes, reduced/no peroxisomes or aberrant peroxisomes. A representative image of the control and strains from each category is shown. Below each image is the list of strains manifesting the same phenotype. Names are organized such that known *PEX* genes are listed first followed by additional mutants in alphabetical order. The representative strain of each category is marked in red. (c) Pie chart of functional distribution of genes identified in the screen.

Figure 2

Loss of Spf1 affects peroxisomes. (a) Representative images of GFP-Ant1 and Cherry-SKL in the control and Δ*spf1* strains. GFP-Ant1 is localized to mitochondria when *SPF1* is deleted. (b) To uncover the role of Spf1 in targeting peroxisomal proteins we crossed a deletion of *spf1* into the yeast GFP collection. We then used automated microscopy to gauge the effect of losing Spf1 on the yeast proteome. (c) Representative images of Pex3-GFP in the control and Δ*spf1* strains. In the Δ*spf1* strain Pex3-GFP did not reside on peroxisomes in many of the cells.

Figure 3

Peroxisomes are adjacent to mitochondria/ER junctions. Representative images of peroxisomal and mitochondria/ER junctions (ERMES) markers. (a) GFP-PMP and Mdm34-RFP. (b) Mmm1-

GFP and RFP-PTS1. (c) Mdm34-GFP and RFP-PTS1. All three combinations of markers showed that in each cell at least one peroxisome was adjacent to an ERMES focus.

Figure 4

Proxisomes are adjacent to sites of acetyl-CoA synthesis in mitochondria. (a) To uncover additional subdomains in mitochondria that could align with peroxisomes we picked 96 strains from the yeast GFP collection that displayed a punctate pattern, and crossed them against a query strain containing a mitochondrial marker (Aco2-Cherry). We then used automated microscopy to gauge proteins that were localized to subdomains in mitochondria. (b) Representative images demonstrating that Pda1, a subunit of the pyruvate dehydrogenase (PDH) complex, resides in a mitochondrial subdomain. (c) Representative images demonstrating that two subunits of the PDH complex, Pda1 and Lpd1, are co-localized. (d) Representative images demonstrating that Pda1-GFP and Pex3-RFP are adjacent to each other, suggesting that peroxisomes are localized adjacent to sites of acetyl-CoA synthesis in mitochondria. (e) A scheme demonstrating that peroxisomes are localized adjacent to PDH and ERMES sites in mitochondria. Note that acetyl-CoA may enter mitochondria as additional molecules beside acetylcarnitine.