Using Pull Down Strategies to Analyze the Interactome of Peroxisomal Membrane Proteins in Human Cells

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Abstract Different pull-down strategies were successfully applied to gain novel insight into the interactome of human membrane-associated proteins. Here, we compare the outcome, efficiency and potential of pull-down strategies applied to human peroxisomal membrane proteins. Stable membrane-bound protein complexes can be affinity-purified from genetically engineered human cells or subfractions thereof after detergent solubilization, followed by size exclusion chromatography and analysis by mass spectrometry (MS). As exemplified for Protein A-tagged human PEX14, one of the central constituents of the peroxisomal matrix protein import machinery, MS analyses of the affinity-purified complexes revealed an unexpected association of PEX14 with other protein assemblies like the microtubular network or the insertion apparatus for peroxisomal membrane proteins comprising PEX3, PEX16 and PEX19. The latter association was recently supported by using a different pull-down strategy following in vivo proximity labeling with biotin, named BioID, which enabled the identification of various membrane proteins in close proximity of PEX16 in living cells.

Keywords Peroxisomal membrane proteins · BioID · AP-MS
PEX14 · PEX16

Abbreviations

AP	Affinity purification
BioID	Biotin identification
DDM	N-dodecyl-β-D-maltoside
ER	Endoplasmic reticulum

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IP Immunoprecipitation
LC Liquid chromatography
MS Mass spectrometry
PEX Peroxin
PMP Peroxisomal membrane protein
PPI Protein-protein interaction

1 Membrane Protein Complexes in Human Peroxisomes

The knowledge about the protein composition of the human peroxisomal membrane expanded over the last few years. Up to now, 31 integral peroxisomal membrane proteins have been identified with a remarkable diversity of functions, including membrane and matrix protein import into peroxisomes, metabolite transport, peroxisome maintenance, proliferation, and inheritance, innate immunity, signal transduction, and the formation of contact zones of peroxisomes with other organelles (Fig. 1).

1.1 Biogenesis I—de Novo Formation, Proliferation and Division

De novo formation of peroxisomes can exclusively originate from the ER (Kim et al. 2006), or, as recently suggested, by fusion of mitochondrial derived vesicles and ER-derived pre-peroxisomes (Sugiura et al. 2017). Alternatively, peroxisomes proliferate via growth and division (Motley and Hettema 2007; Delille et al. 2010). The initial steps of peroxisome biosynthesis involve PEX3 (Soukupova et al. 1999), PEX16 (Honsho et al. 1998) and PEX19 (Matsuzono et al. 1999b) representing together the peroxisomal membrane protein (PMP) import machinery. All three peroxins display at least a dual localization at the ER and the peroxisomal membrane (Dimitrov et al. 2013). Absence or dysfunction of one of these proteins leads to a complete loss of peroxisomal membrane remnants ('ghosts') (Shimozawa et al. 2000; Honsho et al. 1998; Matsuzono et al. 1999a).

Division of peroxisomes is at least a three-step process, consisting of membrane elongation, membrane constriction and final fission of the peroxisome (Delille et al. 2010). Members of the PEX11 family (PEX11α (PMP28)/PEX11β/PEX11γ) (Abe and Fujiki 1998; Tanaka et al. 2003) function as membrane elongation factors and ectopic expression leads to formation of juxtaposed elongated peroxisomes (Koch et al. 2010). Conversely, loss of murine PEX11β causes a reduced number of peroxisomes (Li and Gould 2003).

Upstream of the elongation process, mitochondria and peroxisomes share the same fission machinery. The mitochondrial fission factor [MFF (Gandre-Babbe and

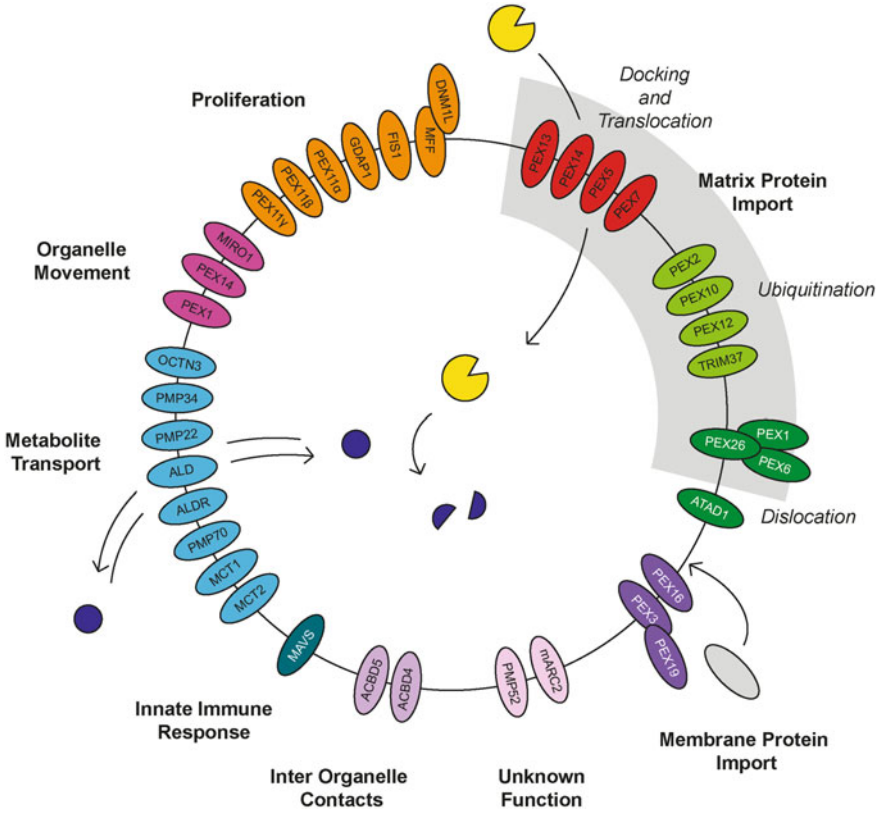

Fig. 1 Functional organization of peroxisomal membrane proteins. The peroxisomal membrane harbours at least 31 integral membrane proteins, which can be arranged in eight functional groups visualized by color. The membrane protein import machinery consists of PEX16, PEX3 and PEX19. Matrix protein import is divided in three steps: translocation and docking (PEX14, PEX13, PEX5, PEX7), ubiquitination (PEX2, PEX10, PEX12, TRIM37) and dislocation (PEX26, PEX1, PEX6). The dislocase ATAD1 does not seem to be related to matrix protein import but quality control of mistargeted tail-anchored membrane proteins. Additional import and export of metabolites and cofactors is crucial for peroxisomal functionality. For this purpose, several transporters namely OCTN3, PMP34, PXMP2, ALD (ABCD1), ALDR (ABCD2), PMP70 (ABCD3), MCT1 and MCT2 have been identified. Mature peroxisomes can divide which requires the proliferation machinery consisting of PEX11α, PEX11β, PEX11γ, GDAP1, FIS1, MFF and DNM1L. Additional proteins enable peroxisomes to maintain inter-organelle contacts (ABCD4, ABCD5, PEX11), function in innate immune response (MAVS) and move along the tubulin network (PEX14, PEX1, MIRO1). It has been shown that PMP52 and mARC2 localize to peroxisomes, but their function still has to be studied. Abbreviations: ATP-binding cassette sub-family D member (ABCD), acyl-CoA binding domain protein (ACBD), Adrenoleukodystrophy protein (ALD), Adrenoleukodystrophy-related protein (ALDR), ATPase family AAA (ATPase associated with various cellular activities) domain-containing protein 1 (ATAD1), dynamin-1-like protein (DNM1L), Ganglioside-induced differentiation-associated protein 1 (GDAP1), mitochondrial amidoxime reducing component 2 (mARC2), monocarboxylate transporters (MCT), mitochondrial fission 1 protein (FIS1), mitochondrial Rho GTPase 1 (MIRO1), mitochondrial antiviral-signaling protein (MAVS), mitochondrial fission factor (MFF), organic cation/carnitine transporter 3 (OCTN3), peroxin (PEX), peroxisomal membrane protein (PMP), Tripartite motif-containing protein 37 (TRIM37)

van der Bliek 2008)] recruits the dynamin-1-like protein [DNM1L (Koch et al. 2003)] to the peroxisomal membrane. Other proteins involved in peroxisomal fission are the mitochondrial fission 1 protein [FIS1 (Koch et al. 2005)] and ganglioside-induced differentiation associated protein 1 [GDAP1 (Huber et al. 2013)]. However, the mechanism and chronology of the fission initiation process is poorly understood. Membrane constriction and fission is then triggered by self-oligomerization of DNM1L into spirals that resemble collar structures encircling the peroxisome (Shin et al. 1999). DNM1L catalyzes membrane fission and vesicle release in a GTP hydrolysis-dependent manner, relying on the GTPase-activating function of PEX11 (Williams et al. 2015).

1.2 Biogenesis II—Membrane and Matrix Protein Import

The insertion of the majority of PMPs into peroxisomal membranes depends on the cytosolic receptor protein PEX19 (class I PMPs), which interacts at the peroxisomal membrane with its docking factor PEX3 (Fang et al. 2004). Alternatively, PMPs can be imported in a PEX19-independent pathway via the ER (class II PMPs). As shown for PEX3 and PMP34, this pathway requires direct interaction with ER-associated PEX16 (Aranovich et al. 2014), which is inserted co-translationally into the ER membrane (Kim et al. 2006).

The core of the peroxisomal matrix protein import machinery consists of the docking complex PEX13/PEX14 and a set of cytosolic receptors (PEX5, PEX7), which recognize proteins destined for the peroxisomal lumen by specific targeting sequences. Both receptors are cycling between the peroxisomal membrane and the cytosol. In mammalian cells, an isoform of PEX5 targets cargo-loaded PEX7 to the membrane (Braverman et al. 1998; Otera et al. 2000). PEX5 associates with the peroxisomal membrane during the import cycle (Gouveia et al. 2003), either becoming an integral constituent of a translocation pore, as shown for yeast (Meinecke et al. 2010), or by transient interaction with a large cavity-forming membrane complex of unknown identity (Dias et al. 2017).

Prior to export into the cytosol, the cycling receptor PEX5 becomes ubiquitinated. Therefore a set of peroxins with ubiquitin-protein isopeptide ligase activity, the so-called RING-Finger complex [PEX2, PEX10 and PEX12 (Biermanns et al. 2003; Okumoto et al. 1998; Okumoto and Fujiki 1997)] functions together with the cytosolic ubiquitin-conjugating enzymes E2D1/2/3 [UBCH5a/b/c (Grou et al. 2008)]. Recently, an additional E3 ligase called TRIM37, required for stabilization of PEX5, was discovered (Wang et al. 2017). Receptor dislocation into the cytosol is facilitated by the AAA^+ ATPases PEX1 and PEX6, anchored to the peroxisomal membrane via PEX26 (Matsumoto et al. 2003). USP9X has been identified as a cytosolic enzyme with PEX5 de-ubiquitinating activity (Grou et al. 2012). However, it is not clear whether de-ubiquitination takes place at the membrane or at a later step of the receptor cycle.

1.3 PMPs with Other Functions

1.3.1 Metabolite Transport

Since peroxisomes are involved in a plethora of metabolic functions, metabolite transporters are needed to regulate exchange of metabolites, cofactors and inorganic ions. Molecules smaller than 300 Da, like the known metabolites glyoxalate, pyruvate and 2-ketoglutarate, can diffuse freely across the peroxisomal membrane (Rokka et al. 2009), probably via the PXMP2 channel [SLC25A17 (Wylin et al. 1998)]. Bulky cofactors such as ATP, CoA, FAD and NAD^+, which are synthesized in the cytosol and exceed the size limit of the PXMP2 channel, have been shown to be transported into the peroxisomal lumen via PMP34 (Agrimi et al. 2012; Visser et al. 2002). The monocarboxylate transporters MCT1 (SLC16A1) and MCT2 (SLC16A7) were predicted to function as lactate/pyruvate shuttles (Valença et al. 2015). A human orthologue of the mouse peroxisome-located carnitine transporter OCTN3 [SLC22A21 (Lamhonwah et al. 2003)] has been identified.

The transport of fatty acids and bile acid precursors is performed via members of the ATP-binding cassette subclass D (ABCD) transporters. The peroxisomal membrane is known to harbor three of these transporters: ABCD1 [ALDP (Mosser et al. 1993)], ABCD2 [ALDRP (Lombard-Platet et al. 1996)] and ABCD3 [PMP70 (Kamijo et al. 1990)]. The localization of ABCD4 (PMP69) is still controversially discussed. It has been previously annotated as peroxisomal but actually seems to be localized to the ER (Kashiwayama et al. 2009).

1.3.2 Inter-Organelle Contact Sites

In recent years, there has been an increasing interest in inter-organelle membrane contact sites. However, most of the work has been done in yeast and the knowledge about inter-organelle contacts, especially peroxisomal ones, in mammals is still in its infancy. For a detailed review, see (Islinger et al. 2015). The peroxisome/ER and the peroxisome/mitochondria connections seem to be mediated via protein-protein interactions (PPIs), whereas peroxisome/lysosome contacts are mediated by phospholipids in the peroxisomal membrane, which are bound by lysosomal synaptotagmin (Chu et al. 2015). Recently, the two tail-anchored acyl-CoA-binding domain-containing proteins ACBD4 and ACBD5 have been reported to function as peroxisome/ER tethers. ACBD4/5 interact with the ER-localized VAPA and VAPB (Costello et al. 2017a, b).

Peroxisomes and mitochondria share not only their fission machinery (FIS1, DNM1L, GDAP1, MFF) and metabolic pathways like β-oxidation but also some other membrane proteins, like ATAD1 and MAVS (see below). A close physical connection of peroxisomes and mitochondria has so far only been revealed in yeast. There, Pex11p could be identified to act as a tether and interact with Mdm34p,

a member of the ERMES complex (Mattiazzi Ušaj et al. 2015). Whether PEX11 fulfills such a function also in human cells has to be investigated.

Peroxisomes also contact the cytoskeleton, which contributes to their homogeneous distribution throughout the cytoplasm and is important for their inheritance. In human cells, peroxisomal motility comes in two flavors: vibrational movement and long range directional movement. Interestingly, the vibrational mode is increased by silencing of the ER tethering factor ACBD5 (Costello et al. 2017a). Peroxisomal long range movements occur along the microtubular network (Rapp et al. 1996). An involvement in organelle motility has been attributed to PEX14, PEX1 and the mitochondrial Rho GTPase 1 (MIRO1). Accordingly, PEX14 binds directly to β-tubulin (Bharti et al. 2011), PEX1 is indirectly attached to microtubules by interacting with KifC3, an adaptor protein belonging to the family of kinesin binding proteins (Dietrich et al. 2013). Only recently a function for peroxisomal MIRO1 as an adaptor for microtubule-based peroxisomal motility was proposed (Castro Inês et al. 2018; Okumoto et al. 2018).

1.3.3 Novel Functions of PMPs

Based on the observation that viral proteins can associate with peroxisomes, a role of the organelle in innate immune response has been anticipated. In support of this notion, it has been shown that MAVS, a RIG-I-like receptor adaptor protein, is localized at peroxisomes and promotes the rapid antiviral response pathway (Dixit et al. 2010).

A peroxisome-associated membrane protein with unknown function is a molybdenum-containing enzyme called mitochondrial amidoxime reducing component (mARC) that comes in two isoforms, mARC1 and mARC2. mARC2 (synonymous MOSC2) has been shown to be dually localized at mitochondria (Wahl et al. 2010) and peroxisomes (Wiese et al. 2007). In the latter study, also PMP52 and the ATPase family AAA^+ domain-containing protein 1 (ATAD1) were identified as novel peroxisomal membrane proteins. Whereas the AAA^+ ATPase has been characterized as a dislocase for mistargeted tail-anchored proteins (Wohlever et al. 2017), the function of PMP52 is still unknown.

2 Proteomic Strategies Applied to the Characterization of PMPs from Human Cells

PMPs are located at the interface between peroxisomes and the surrounding cellular milieu. Consequently, they play crucial roles in mediating peroxisomal protein import/export, the exchange of metabolites as well as interactions and communication with other cellular compartments. To fully understand a PMP's function, knowledge about its protein-protein interactions (PPIs), i.e. the "interactome" of the

PMP, the molecular organization of PMP (sub)complexes, the dynamics of their composition as well as condition- or disease-dependent changes is of great importance. Such knowledge will ultimately improve our understanding of molecular mechanisms underlying peroxisome-related processes on the organellar as well as the cellular level.

For the analysis of PMPs from human cells, three main strategies using pull-down techniques have been employed to date: (i) co-immunoprecipitation (Co-IP) combined with Western blot analysis, (ii) affinity purification of an epitope-tagged protein of interest ("bait") combined with high-resolution MS (AP-MS), and (iii) proximity-dependent biotin identification (BioID). For future studies, a combination of protein pull-down techniques with cross-linking MS may present a promising strategy to enable the identification of transient and weak interactions as well as interaction partners of low stoichiometry. In addition, this approach allows for identifying intermolecular contact sites in native PMP complexes. Furthermore, as an alternative to BioID, proximity labeling using a modified ascorbate peroxidase, referred to as APEX (Rhee et al. 2013) or APEX2 (Lam et al. 2014), can be used to explore the microenvironment of a PMP of interest.

The traditional biochemical method for the identification of PPIs, Co-IP, relies on the use of antibodies specifically recognizing the protein of interest and Western blot analysis to detect co-precipitated proteins. This method has successfully been employed to many peroxins such as PEX26 (Tamura et al. 2014) (see also the Chapter by Fujiki and colleagues in this volume). Co-IP enables to target the native, unmodified protein expressed at endogenous levels (as opposed to epitope tag-based pull-down strategies). Thus, proteins and their interaction partners are purified from in vivo conditions. For low abundant PMPs, however, this may result in low yield. A critical step for Co-IPs of membrane proteins is the solubilization of the target protein and associated interaction partners. The choice of detergent has a crucial influence on the integrity of the interactome that is pulled down. Even mild detergents such as digitonin may interfere with weak PPIs and, thus, impair the identification of transient interaction partners. Furthermore, the success of the Co-IP method strictly depends on the availability of antibodies specifically binding to the protein of interest without exhibiting cross-reactivity to other proteins. In particular, for integral PMPs, the generation of specific antibodies can be very difficult. From the conceptual point of view, the identification of PPIs by Co-IP and Western blotting is generally a hypothesis-driven approach and requires assumptions about putative interaction partners as well as a selection of suitable antibodies to detect these proteins.

AP-MS is recognized as a most powerful strategy to identify PPIs (Gingras et al. 2007; Oeljeklaus et al. 2009; Meyer and Selbach 2015). The use of MS for the identification of proteins associated with the bait represents an unbiased approach allowing for unanticipated discoveries. AP-MS-based studies of PMPs have greatly contributed to the characterization of distinct PMPs and to an improved understanding of peroxisome-associated processes in yeast [e.g., (Agne et al. 2003;

Grunau et al. 2009; Oeljeklaus et al. 2012; Chan et al. 2016; Wróblewska et al. 2017; David et al. 2013)]. An alternative strategy for the study of PPIs is proximity-dependent biotin identification (BioID). This method relies on the expression of the protein of interest fused to a biotin ligase and biotinylation of proteins in close proximity to the fusion protein (Roux et al. 2012). AP-MS and BioID have successfully been used to decipher the interactomes of PEX14 (Bharti et al. 2011) and PEX16 (Hua et al. 2017), respectively, in human cells.

2.1 MS-Based Methods for the Characterization of Human PMP Complexes

2.1.1 Affinity Purification Combined with High-Resolution Mass Spectrometry (AP-MS)

A generic AP-MS workflow for PMPs comprises the affinity purification of an epitope-tagged version of the PMP of interest and associated proteins from detergent-treated crude membrane fractions, proteolytic digestion of purified proteins, liquid chromatography (LC)-MS analysis of the resulting peptide mixture, and computational data analysis to identify proteins co-purified with the bait. For higher sensitivity, subfractionation on protein or peptide level may be included. As control, a "mock" purification using cells expressing the native, non-tagged version of the PMP is performed and analyzed in parallel. Since protein complexes cannot be purified to homogeneity and due to the high sensitivity of modern MS instruments that enable the detection of minute amounts of protein, the discrimination between specific interaction partners and co-purified background proteins can be a challenge. This task is facilitated by incorporating quantitative MS techniques into the workflow, which include label-free as well as metabolic or chemical stable isotope labeling approaches (Bantscheff et al. 2012) and allow to determine differences in the abundance of individual proteins between the purified PMP complex and control sample with high reliability and accuracy (Oeljeklaus et al. 2012). True interaction partners are specifically enriched with the bait, resulting in complex-to-control abundance ratios significantly higher than 1. Non-specifically co-purified background proteins, which are equally present in the purified complex and the control sample, exhibit abundance ratios of approximately 1.

The mere identification of a protein by AP-MS as part of a protein complex does not provide information about the nature of the interaction, which can be stable or transient and dynamic. This knowledge, however, may be essential to fully understand the protein's function within the complex. Warscheid and co-workers performed dual-track quantitative AP-MS studies using stable isotope labeling by amino acids in cell culture [SILAC (Ong et al. 2002)] that allowed the discrimination between stable core components and transient interaction partners of Pex14p and Pex30p in yeast (Oeljeklaus et al. 2012; David et al. 2013), a strategy that is

also applicable to study PMP interactomes in human cells. Cells expressing epitope-tagged Pex14p or Pex30p were grown in the presence of unlabeled "light" arginine and lysine, while control cells expressing the untagged, native version of the proteins were metabolically labeled by growth in medium containing the stable isotope-coded "heavy" counterparts. Protein affinity purification was either performed prior to mixing ("AP-PM") or after mixing of differentially labeled samples ("AP-AM"). Following the AP-PM track, all purification steps were carried out separately for the differentially labeled cell populations before the eluates were combined for joint LC-MS analysis, and interaction partners were identified based on their light-to-heavy protein abundance ratios. In contrast, in AP-AM experiments, equal amounts of light and heavy labeled cells were mixed directly after harvesting and subsequent purification and analysis steps were performed together for differentially labeled protein complexes. This workflow results in the exchange of labeled and unlabeled interaction partners that only transiently interact with the protein complex during the process of the affinity purification. As a consequence, transient interaction partners may exhibit complex-to-control abundance ratios of approximately 1 and, thus, are misclassified as co-purified contaminants. To exemplify, the cytosolic receptor Pex5p that cycles between cytosol and peroxisomal membrane where it associates with Pex14p was identified as specific Pex14p interaction partner in AP-PM experiments but classified as "contaminant" in AP-AM experiments (Oeljeklaus et al. 2012). Thus, the integration of interaction data obtained by AP-AM and AP-PM ultimately enables to discriminate between stable core components of a protein complex and transient or dynamic interaction partners.

2.1.2 Proximity-Dependent Biotin Identification (BioID)

The proximity labeling strategy BioID requires the expression of the PMP of interest fused to a mutant form of the biotin ligase BirA from *Escherichia coli* [BirA* (Roux et al. 2012)]. In the presence of biotin, BirA* promotes the biotinylation of accessible lysine side chains in proteins that are located in close proximity of the PMP in vivo [labeling radius of approx. 10 nm (Kim et al. 2014)]. These include direct interaction partners as well as proteins present in the nano-environment of the PMP but not directly interacting with it. Biotinylated proteins can be affinity-purified from cell lysates using streptavidin and subsequently analyzed by LC-MS. Inclusion of appropriate control experiments (e.g., cells expressing PMP-BirA* not treated with biotin, cells not expressing the BirA* fusion protein treated with biotin) and quantitative MS facilitates the discrimination between "true" PMP-proximal proteins and nonspecifically co-purified background proteins as described above for the identification of true interaction partners by AP-MS. Commonly co-purified contaminants in BioID experiments are carboxylases that contain biotin as cofactor and are biotinylated by endogenous

protein-biotin ligases. An improved BioID method using a smaller promiscuous biotin ligase, BioID2 (27 vs. 35 kDa of BirA*), with enhanced labeling efficiency has recently been reported (Kim et al. 2016).

The strength of the BioID method lies in the in vivo-labeling of proteins present in the nano-environment of the target protein. As a consequence, the detection of interaction partners and other proximal proteins does not depend on maintaining the integrity of protein complexes or entire interactomes during the purification, which makes this approach amenable for stringent lysis and purification procedures. This is particularly advantageous for the study of membrane proteins that need to be extracted from the membrane for a comprehensive analysis. Furthermore, BioID enables to capture transient or week interactions, which are often lost during conventional affinity purification processes, and it provides information about the spatial environment of the target protein, which is also lost when proteins are affinity-purified from crude membrane fractions but may be a crucial aspect to reveal a protein's biological function.

2.2 Critical Aspects to Consider for Experimental Design

The introduction of a stably expressed tagged protein of interest is the first step to enable large scale affinity purification of membrane complexes out of mammalian cells. Today several techniques exist to generate stably expressing cell lines. A common method is to integrate fusion genes coding for tagged bait proteins into the human genome. The Flp-In™ System (Invitrogen) is an efficient tool to create isogenic cell lines by making use of Flp recombinase-mediated DNA recombination at the Flp Recombination Target (FRT) site. The correct integration of the target gene can be verified by a set of growth tests using hygromycine selection, zeocine sensitivity, lack of β-galactosidase activity and protein expression. For both pull-down approaches used so far for human PMPs, Protein A-tag affinity purification and BioID, the Flp-In™ methodology has been used to isolate cell lines expressing the bait proteins PEX14 and PEX16. In contrast to Co-IP, the additional expression of a marker gene, which is required to monitor the genomic integration, and the control of the gene of interest by a foreign promotor can cause artefacts such as mislocalization and non-physiological posttranslational modifications and, thus, must be controlled carefully. In the case of tagged PEX14, patient cell lines defective in the respective genes could be used to verify functional complementation and peroxisomal localization of the bait proteins.

Another critical parameter for affinity purification of membrane complexes concerns the solubilization procedure, in particular the choice of detergent has a significant impact on the amount, composition and integrity of isolated protein complexes. Frequently, non-ionic and zwitter-ionic detergents are chosen to solubilize membrane proteins with retention of function (Seddon et al. 2004). For the isolation of Protein A-tagged peroxisomal membrane complexes from yeast, the nonionic detergents DDM, CYMAL-4, Triton X-100, or digitonin were compared

(Agne et al. 2003). Only Pex14p extraction by 1% digitonin preserved association with all known binding partners at the peroxisomal membrane. Reguenga et al. solubilized human peroxisomal membranes using either 1% digitonin or a mixture of non-ionic NP40 and anionic sodium deoxycholate and determined the molecular size of PMP assemblies by Blue Native gel electrophoresis (Reguenga et al. 2001). Using the mild detergent digitonin, the core constituents of the matrix protein import machinery PEX5, PEX14, PEX10 and PEX12 retain as subunits of a membrane complex larger than 750 kDa. Using the harsh detergent mixture, the same peroxins were detected at a molecular size of about 250 kDa, suggesting that subunits of the heteromeric complex were lost during the preparation. Similar sizes for PEX5 containing membrane complexes were obtained in other studies using 1% Triton X-100 (Itoh and Fujiki 2006). Thus, 1% digitonin is recommended for affinity purification aimed to characterize the size and function of native complexes. For BioID approaches, the detergent can be chosen based on solubilization efficacy of PMPs.

In addition, one should be aware that AP-MS and BioID coupled to MS are complementary strategies to characterize a protein's interactome and subcellular environment (Lambert et al. 2015; Hesketh et al. 2017). While AP-MS in clever combination with metabolic labeling has the potential to discriminate between core components and transient interaction partners of a protein assembly, weakly interacting or low abundance proteins may be lost during the purification process and elude detection by MS. In addition, AP-MS is prone to artifacts originating from abundant cellular proteins or from pooling proteins from different subcellular localizations during cell lysis resulting in artificial interactions. This kind of erroneous data is prevented in BioID experiments since labeling of protein interaction partners and proteins of the nano-environment with biotin occurs prior to cell lysis in living cells. Results of BioID studies, however, do not allow the discrimination whether proteins are part of a distinct interactome or just located in close proximity. Thus, the integration of data obtained in AP-MS and BioID experiments enables a most comprehensive characterization of proteins. It should further be noted that neither AP-MS nor BioID provides information about direct or indirect PPIs. Further studies are required to ascertain direct interactions between individual proteins (e.g. by yeast two-hybrid/split-ubiquitin assay, in vitro binding studies, fluorescence-based microscopy techniques).

3 Towards a PMP Interactome

3.1 The PEX14 Complex Analysis Revisited

One of the best studied interactomes at the peroxisome membrane refers to Protein A-tagged PEX14, which could be isolated together with associated binding partners from yeast and man (Bharti et al. 2011; Oeljeklaus et al. 2012).

In the proteomic approach by Bharti et al., a C-terminally Protein A-tagged version of PEX14 was integrated into the genome of Flp-In^{TM}-293 cells (Invitrogen) and affinity-purified from 1% digitonin-solubilized membranes. In total, more than 200 associated proteins were identified by MS. Among these were 22 proteins with annotated peroxisomal localization, including 14 annotated membrane-associated proteins (Fig. 1). All PEX14-associated peroxins are involved in protein import into peroxisomes: PEX5, PEX13, and the RING-finger peroxins PEX2, PEX10, PEX12 and the AAA^{+}-peroxin PEX1 are required for matrix protein import, while PEX19, PEX3 and PEX16 accomplish membrane protein targeting. Noteworthy, other highly abundant membrane-bound peroxins like the proliferation factor PEX11ß were not detected, thereby demonstrating the specificity of the pull-down approach.

Several of the identified peroxins are supposed to interact directly with PEX14, in particular the soluble receptor for matrix enzymes, PEX5, and the PMP receptor PEX19 (Will et al. 1999; Schliebs et al. 1999; Neufeld et al. 2009; Fransen et al. 2002). Physical interaction of mammalian PEX13 and PEX14 had been reported (Fransen et al. 1998; Will et al. 1999; Itoh and Fujiki 2006). However, other studies, i.e. two hybrid analyses, failed to detect complex formation between human PEX14 and PEX13 (Fransen et al. 2002; Will et al. 1999).

Surprisingly, ß-tubulin could be identified as an additional interacting partner of the conserved N-terminal domain of PEX14 by Bharti et al. (2011). Independent binding assays with recombinant PEX14 variants and purified tubulin supported a direct protein-protein interaction between the N-terminal domain of PEX14 and microtubular filaments (Bharti et al. 2011; Theiss et al. 2012).

The fact that human PEX5, PEX19 and ß-tubulin bind to the same domain of PEX14, and they even compete with each other for in vitro interaction (Bharti et al. 2011), suggests that distinct PEX14 subcomplexes may exist at the peroxisomal membrane. Accordingly, Bharti et al. showed by size exclusion chromatography that PEX14-complexes cover a broad range of molecular masses ranging from the size exclusion limit (about 2 MDa) to 150 kDa (Bharti et al. 2011). Only minor amounts of PEX14 were detected at lower molecular weight regions, suggesting that PEX14 does not primarily functions as a monomeric protein. Within the high-molecular weight range, the peaks of tubulin, PEX5, and PEX19 were clearly separated, suggesting that the peroxisomal membrane harbors at least three heteromeric PEX14 subcomplexes with different composition and function. The complex containing PEX5 and PEX14 displays a molecular mass between 800 and 1000 kDa. The size of this complex correlates with a previous finding by Reguenga et al. using Blue Native gel electrophoresis from digitonin-solubilized rat liver peroxisomes (Reguenga et al. 2001). Here, it was demonstrated that PEX5, PEX14, PEX10 and PEX12 are subunits of a membrane complex with a size larger than 750 kDa. Bharti et al. identified another PEX14 assembly with a molecular mass between 600 and 800 kDa, which co-fractionated with PEX19 (Bharti et al. 2011). It is still an open question whether the PEX14/PEX19 complex represents a PMP in transit or if PEX14 even fulfills another not yet identified function in conjunction with PEX19, PEX3 and PEX16 (see Sect. 3.3). The largest PEX14-containing

subcomplex with a size above 1 MDa contains tubulin and, most likely, other microtubule-associated proteins. The existence of large oligomeric PEX14 assemblies in peroxisomes of mammalian cells has previously also been shown by Itoh and Fujiki (Itoh and Fujiki 2006) (see also the Chapter by Fujiki and colleagues in this book/volume). By subjecting Triton X-100-solubilized peroxisomes to glycerol gradient centrifugation, they could detect the major fraction of PEX14 with a size above 1 MDa, clearly separated from PEX5 at 200–300 kDa and PEX19 below 66 kDa.

To analyze PEX14-associated proteins in conjunction with the microtubular network in greater detail, PEX14-Protein A affinity purification was carried out in the presence of the inhibitor nocodazole, which decreases stability of microtubules in vivo and in vitro (Bharti et al. 2011; Vasquez et al. 1997). This treatment drastically reduced the amount of tubulin and microtubule-associated proteins (Bharti et al. 2011). In addition, numerous organellar (mitochondria, ER) proteins, which were found in solubilisates of untreated cells, were not detected in the complexes from the nocodazole-treated cells, suggesting that many unspecific proteins identified in the PEX14 affinity eluate represent vesicular and organellar cargos of the microtubular network. The complete list of PEX14-associated proteins co-purified using nocodazole-treated cells is shown in Table 1. PEX5 and PEX19, the known binding partners of PEX14, were detected in the complex. However, other peroxins, especially those which are supposed to bind indirectly to PEX14, were not detected, suggesting some unknown effects of the inhibitor on PEX14 assemblies.

Most of the PEX14-interacting proteins with highest abundance are cytosolic proteins belonging to the super families of chaperones and other folding assists, especially members of the HSP70 and HSP90 class. Indeed, a role for HSP70 in PEX5 targeting to the peroxisomal membrane had been suggested previously (Harano et al. 2001). Even earlier, Walton et al. showed that microinjection of anti HSP70 antibodies into human fibroblasts impaired peroxisomal protein import (Walton et al. 1994). In this study, HSP70 molecules were localized to the outer surface of peroxisomal membranes. Another cytosolic binding partner of interest is the de-ubiquitinating hydrolase USP9X which is by far the most active cytosolic de-ubiquitinase acting on an artificial Ub-PEX5 fusion construct (Grou et al. 2012). This enzyme, which was not denoted by Bharti et al. (2011), was identified from eluate fractions of all four affinity purifications with or without nocodazole. This suggests that the process of de-ubiquitanation takes also place at the peroxisomal membrane, maybe at a state of the receptor cycle where PEX5 is still attached with PEX14.

3.2 PEX16 Interactome Defined by BioID

The group by Peter Kim used the advanced pull-down technology BioID (Roux et al. 2012) to identify proteins which are in close vicinity or potentially interact with PEX16 (Hua et al. 2017). In brief, PEX16 was fused to the prokaryotic biotin

Table 1 PEX14 interaction partners identified by AP-MS following inhibition of the PEX14-tubulin interaction using nocodazole

Gene symbol	UniProt ID	Protein	M_W (kDa)	Sequ. cov. (%)	Mascot score	Enr. factor	SC PEX14
PEX14	O75381	Peroxisomal membrane protein PEX14 (isoform 2)	36.7	50.6	1644.4	33.3	200
HSPA1A; HSPA1B	P0DMV8; P0DMV9	Heat shock 70 kDa protein 1A; Heat shock 70 kDa protein 1B	70.1	41.5	2225.9	3.4	103
HSPA8	P11142	Heat shock cognate 71 kDa protein (isoform 1)	70.9	36.8	2462.1	6.8	81
HSP90AA1	P07900	Heat shock 90 kDa protein 1, alpha (isoform 2)	98.2	26.2	1567.0	2.8	73
PEX5	P50542	Peroxisomal targeting signal 1 receptor (isoform 3)	69.8	31.4	1576,2	13.2	66
HSP90AB1	P08238	Heat shock protein HSP 90-beta	83.3	24.2	1156.3	2.6	58
CAD	P27708	Putative uncharacterized protein CAD	243.0	12.2	883.4	spec.	43
HSPA2	P54652	Heat shock-related 70 kDa protein 2	70.0	20.5	703.3	4.4	40
n/a	B4DJ30	cDNA FLJ61290, highly similar to Neutral alpha-glucosidase AB	112.9	18.2	855.8	32.0	32
RUVBL1	Q9Y265	RuvB-like 1	50.2	25.4	817.1	spec.	29
HSPA5	P11021	HSPA5 protein	72.3	24.1	786.8	4.5	27
DAW1	Q8N136	Dynein assembly factor with WDR repeat domains 1	45.8	9.9	85.3	2.2	24
DYNC1H1	Q14204	Cytoplasmic dynein 1 heavy chain 1	532.4	3.7	832.0	21.0	21
PRKCSH	P14314	Glucosidase 2 subunit beta	59.4	19.6	670.2	spec.	21
CENPJ	Q9HC77	Centromere protein J	153.0	0.9	587.4	2.1	21
RUVBL2	Q9Y230	RuvB-like 2	51.1	28.7	401.7	spec.	17
CCT2	P78371	T-complex protein 1 subunit beta	57.5	22.6	320.5	14.0	14
FASN	P49327	Fatty acid synthase	273.4	5.7	361.0	6.5	13
PEX19	P40855	Peroxisomal biogenesis factor 19	32.8	25.1	250.8	4.0	12
n/a	B7Z809	cDNA FLJ56016, highly similar to C-1-tetrahydrofolate synthase	110.5	11	231.1	5.0	10
TTC28	Q96AY4	Tetratricopeptide repeat protein 28	270.9	3.4	217.3	spec.	8

(continued)

Table 1 (continued)

Gene symbol	UniProt ID	Protein	M_W (kDa)	Sequ. cov. (%)	Mascot score	Enr. factor	SC PEX14
GNPAT	O15228	Dihydroxyacetone phosphate acyltransferase	77.2	8.2	200.5	spec.	8
PHGDH	O43175	D-3-Phosphoglycerate dehydrogenase	56.7	10.8	196.0	spec.	8
RPS27A; UBC; UBB	P62979 P0CG48 P0CG47	Ubiquitin-40S ribosomal protein S27a; Polyubiquitin-C; Polyubiquitin-B	9.1 (Ubiquitin)	35.4	143.8	2,7	8
PFKP	Q01813	ATP-dependent 6-phosphofructokinase, platelet type	85.6	8.5	247.2	spec.	7
PGK1	P00558	Phosphoglycerate kinase 1	44.6	12.5	181.3	3.0	6
RPL12	P30050	60S ribosomal protein L12 (isoform 1)	17.8	23.6	127.6	3.0	6
RPS13	P62277	40S ribosomal protein S13	17.2	33.8	253.9	5.0	5
ERP44	Q9BS26	Endoplasmic reticulum resident protein 44	47.0	8.6	172.0	spec.	5
GCN1	Q92616	eIF-2-alpha kinase activator GCN1	292.8	1.8	169.9	spec.	5
PCBP1	Q15365	Poly(rC)-binding protein 1	37.5	7.3	153.9	2.5	5
PHB2	Q99623	Prohibitin-2	33.3	12.7	64.4	4.0	4
RPL5	P46777	60S ribosomal protein L5	34.4	11.4	1671.9	spec.	4
LYZ	P61626	Lysozyme C	16.5	14.2	1145.4	spec.	4
PKM2	P14618	Pyruvate kinase	57.9	9.8	53.7	4.0	4
BAG6	P46379	Large proline-rich protein BAG6	119.4	6.2	615.8	spec.	4
RPS18	P62269	40S ribosomal protein S18	17.8	19.1	632.7	3.0	3
RCN2	Q14257	Reticulocalbin-2	36.9	7.3	275.4	spec.	3
UBL4A	P11441	Ubiquitin-like protein 4A	17.8	16.6	119.2	spec.	3
VPS52	Q8N1B4	Vacuolar protein sorting-associated protein 52 homolog	82.2	4.0	232.5	spec.	3
USP9X	Q93008	ubiquitin specific protease 9, X-linked (isoform 2	290.5	0.5	197.3	spec.	3
RPL11	P62913	60S ribosomal protein L11 [Ribosomal protein L11]	20.3	8.6	159.4	spec.	3
UACA	Q9BZF9	Uveal autoantigen with coiled-coil domains and ankyrin repeats	162.5	1.1	74.7	spec.	3

Abbreviations: ID Identification; *Mw* Molecular weight; *Sequ. Cov.* Sequence coverage; *Enr. factor* Enrichment factor; *SC* Spectra counts; *spec.* NOT identified in the Control pull-down

protein ligase BirA*. The fusion protein was stably expressed in Flp-In™ T-REx™ cells, which were grown in biotin-supplemented medium. Membranes were collected by sedimentation and solubilized with 1% Triton X-100. Subsequently, all biotinylated proteins were affinity-purified using streptavidin Sepharose beads and identified by MS. As demonstrated by Kim and co-workers, BioID allows the detection of PPIs between different membranes. In this case, they identified ER-located VAPA and peroxisomal ACBD5 as main constituents of the ER-peroxisome contact zone.

The validity of this method to detect proximal or interacting proteins was demonstrated by the identification of other known peroxisomal membrane proteins, like the half-transporter PMP70 and the putative channel PMP52, signal-transducing MAVS, the proliferation factors MFF, DNML1 and PEX11ß. The best hits, as based on the number of spectral counts (i.e. the number of MS/MS fragmentation spectra assigned to a given protein, which is used as a measure for protein abundance in a sample) derived from MS analysis were known peroxisomal binding partners of PEX16, in particular PEX19 and PEX3. Surprisingly, PEX14, PEX5 and PEX13 were identified as closely attached proteins with similar numbers of spectral counts as shown for PEX19 and PEX3. This is in agreement with the results obtained by PEX14-Protein A affinity purification, suggesting that a close association between the import machineries for matrix proteins and membrane proteins exists (Fig. 2). A direct interaction between PEX19 and PEX14 has been shown previously (Neufeld et al. 2009; Fransen et al. 2004; Sacksteder et al. 2000). Noteworthy, the PEX14 binding site of PEX19 is not overlapping with the PEX3- and the general PMP binding domain (Fransen et al. 2005). For this reason, it has been speculated that the PEX14 association with PEX16 and PEX3 is bridged by PEX19 in transit (Bharti et al. 2011). However, the failure to detect other highly abundant PMPs, like PEX11, that binds with higher affinity than PEX16 to the same binding region of PEX19 (Schueller et al. 2010), argues against this hypothesis. Although the functional meaning for the association of PEX16/PEX19/PEX3 complex with PEX14/PEX5 at the peroxisomal membrane is not clear, it should be noted that a specific role for PEX19 in assembly of the PTS receptor complex has been suggested previously (Fransen et al. 2004).

The BioID approach also proved that the receptor dislocation complex consisting of the AAA^+ peroxins PEX1 and PEX6, and their membrane anchor PEX26 are in close proximity to the membrane and matrix protein import machineries. Assuming that the number of spectral counts correlates with true distances, quantification could provide some insight into the structural organization of subcomplexes. Using PEX16 as bait (Hua et al. 2017), PEX1 was detected with the highest number of peptide counts. PEX6 was also detected, but with lower yield, while PEX26, the membrane anchor of AAA^+-peroxins, could not be identified at all. The dominant abundance of PEX1 correlates with findings of the PEX14 complex isolation and might reflect a direct binding of ubiquitinated PEX5 with PEX1. Indeed, a physical interaction between a homo-oligomer of PEX1 and PEX5 was reported (Tamura et al. 2014). Based on structural similarities, Shiozawa et al. reports on a putative ubiquitin-binding domain at the N-terminal region of PEX1

(Shiozawa et al. 2004). However, non-detection of PEX26 in the pull-down approaches is difficult to reconcile with another finding by Tamura et al. (2014). In their study, PEX26 directly binds PEX14 (CHO and HEK cells, 1% Triton-X) and this interaction is modulated by the AAA^+ peroxins and also by PEX5. Therefore, it seems possible that the PEX26/PEX14 interaction reflects an initial interaction, which is lost at later steady-states of protein import.

3.3 Comparing the Peroxin Network of Yeast and Man

The interactome of Protein A-tagged Pex14p has been studied in the yeast *Saccharomyces cerevisiae* by affinity purification of digitonin-solubilized membrane complexes. SILAC combined with high-resolution MS led to the identification of 9 core components and 12 additional transient binding partners (Oeljeklaus et al. 2012). The results obtained resemble those of the human PEX14 affinity approach in the way that PEX14 and PEX13 together with the RING-finger peroxins are forming a core complex of the importomer. In addition, Pex5p, which is forming a proteinaceous pore together with Pex14p (Meinecke et al. 2010), could be identified in the yeast Pex14p-interactome, albeit as a transient binding partner.

The most striking differences when comparing yeast and human PEX14 interactome analyses concern the lack of distinct peroxins. While a PEX16 homolog has not yet been identified in bakers' yeast, homologs of Pex8p and Pex17p which are both constituents of the core complex in yeast do not seem to exist in mammalian cells. While the role of Pex17p is not clearly defined, Pex8p has been shown to connect the receptor docking complex to the RING-finger complex (Agne et al. 2003) in yeast cells. It is tempting to speculate that analogous proteins, maybe human peroxins themselves, perform this function in human cells.

Another remarkable difference concerns the association of AAA^+ peroxins with the translocation apparatus. In yeast cells, the AAA^+-complex anchoring Pex15p was detected as transient binding partner of Pex14p, whereas both AAA proteins, Pex1p and Pex6p, were not stably associated with the protein import machinery. In contrast, in human cells an association between the AAA^+ peroxin PEX1 and PEX5 was reported. As discussed above, this species-dependent differences could reflect different binding affinities and dynamics of association of the receptor dislocation machinery.

One remarkable feature of the human peroxin interactome is the association of the PEX5/PEX14 complex with the PEX3/PEX16/PEX19 network, which is required for membrane biogenesis (Fig. 2). In contrast, neither Pex19p nor its associated binding partner Pex3p are components of the yeast Pex14p-complex, not even as transient interactors. As discussed above, in human cells PEX19 is a suitable candidate to bridge these two assemblies. Interestingly, the high-affinity PEX14-binding motif of human PEX19, which competes with the PEX14 binding

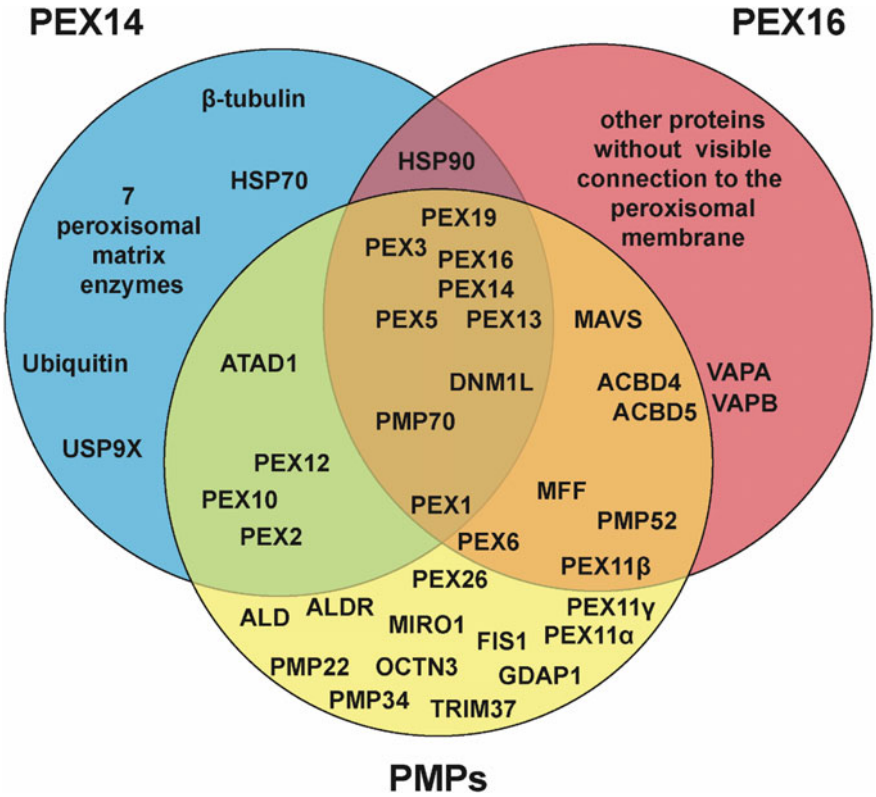

Fig. 2 Overlap of proteins copurifying with PEX14-Protein A, BirA*-PEX16 and the peroxisomal membrane proteome. Constituents of PEX14 and PEX16 complexes were compared to the human peroxisomal membrane proteome and displayed as Venn diagram. Bharti et al. used isolated membranes for affinity purification of digitonin-solubilized PEX14- Protein A complexes and mass spectrometry to identify PEX14 interaction partners (Bharti et al. 2011). A different approach called BioID was used by Hua et al. to target transient and stable interaction partners of PEX16 as well as proximal proteins (Hua et al. 2017). Abbreviations (only those not defined in Fig. 1): heat shock protein (HSP), x-linked ubiquitin specific protease 9 (USP9X), vesicle-associated membrane protein-associated protein (VAP)

sites of PEX5, is not conserved in yeast (Neufeld et al. 2009). Although highly speculative, the close association of complexes facilitating matrix protein import and insertion of membrane proteins might be correlated with an increased efficacy in biogenesis of peroxisomes.

4 Outlook

Studies of the peroxisomal interactome using pull-down approaches is a new field in molecular and cell biology of human cells. The fusion of bait proteins with either pull-down tags (Protein A) or catalytic domains allowing in vivo labeling of proximal proteins (BioID) depends on genomic engineering of human cells and methods which were not well established in the past. Novel technologies like the Flp-In™ technique allowed stable expression of bait proteins in cultured cells. However, several limitations of this method are due to the expression of additional non-tagged copies of the bait proteins under control of their own promotors which could compete for interactions. These problems can be avoided in future research using the CRISPR/Cas9 technique to either knock-out endogenous copies of the gene of interest or, even better, to manipulate target genes at authentic alleles. Use of CRISPR/Cas9 technology will also allow to distinguish between indirect and physical interaction of two proteins. To this end, a potentially bridging protein could be deleted, resulting in a different set of identified proteins. If this can be carried out in a systematic way, organization of protein-protein networks could be analyzed. Multiple PPIs are strongly regulated by post-translational modifications like phosphorylation, ubiquitylation or proteolytic processing, Thereby, the growing number of available specific compounds, like kinase or proteasome inhibitors, will help to analyze the peroxisomal interactome in even higher spatio-temporal resolution.

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