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CELLULAR MECHANISMS OF PEROXISOMAL BIOGENESIS AND MAINTENANCE

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ACADEMIC DISSERTATION

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"Are those who know equal to those who do not know?"
Quran 39:9

ABSTRACT

Peroxisomes are single membrane-bound organelles essential for cellular lipid metabolism, detoxification of reactive oxygen species, and maintenance of metabolic homeostasis. Defects in peroxisome formation or activity leads to severe human disorders, including Zellweger spectrum disorders, emphasizing the need to better understand the molecular mechanisms that govern peroxisomal biogenesis. Although the core components of peroxisomal biogenesis have been identified, the broader cellular mechanisms that coordinate peroxisome assembly and protein trafficking remain incompletely understood.

In this study, a systematic double-screening approach was applied to uncover novel regulators of peroxisomal biogenesis in human cells. By integrating proximity-labeling mass spectrometry with functional CRISPR-based genetic screens, we identified ribosome-binding protein 1 (RRBP1) as a previously uncharacterized factor required for peroxisome biogenesis. Functional analyses in HEK293T cells revealed that knockout of RRBP1 led to a striking reduction in peroxisome number, accompanied by mislocalization of peroxisomal proteins and decreased overall peroxisomal protein abundance. In addition, processing of peroxisomal matrix proteins such as acyl-CoA oxidase 1 and 3-ketoacyl-CoA thiolase was impaired in RRBP1-deficient cells, consistent with a defect in peroxisomal protein import or maturation. The absence of RRBP1 did not affect cell proliferation or protein translation, highlighting its selective role in regulating peroxisomal biogenesis. Further investigation revealed that RRBP1 depletion did not disrupt peroxisome-endoplasmic reticulum contact sites, and the reduction in peroxisome number was not due to enhanced pexophagy. Instead, in the absence of RRBP1, peroxisomal proteins were subject to proteasomal degradation, suggesting that RRBP1 plays a selective role in supporting early stages of peroxisome biogenesis, likely by facilitating the proper insertion or stabilization of peroxisomal proteins at the endoplasmic reticulum. These findings align with the established role of the ER as a platform for peroxisomal membrane formation and highlight the importance of inter-organelle communication in orchestrating organelle assembly.

In parallel, we investigated the trafficking route of tail-anchored (TA) proteins characterized by a single C-terminal transmembrane domain. Their accurate targeting to peroxisomal membranes depends on PEX19 and PEX3 and can proceed either directly from the cytosol or via the ER. Using a proximity biotinylation assay, we observed biotinylation patterns indicating that peroxisomal TA proteins interact with mitochondria before reaching peroxisomes. In particular, YgiM (TA)-AviTag was biotinylated in cells expressing mitochondrial or peroxisomal BirA, but not in cells expressing ER-BirA, supporting mitochondrial involvement in TA protein trafficking. Glycosylation assays also showed that peroxisomal TA proteins do not enter the ER, as they were not glycosylated even in cells lacking peroxisomes. Confocal microscope colocalization analysis and high-resolution microscopy showed that peroxisomal TA proteins localize

exclusively to peroxisomes in wild-type cells but shift predominantly to mitochondria when peroxisomes are absent. These results suggest that peroxisomal TA proteins might first be directed to mitochondria prior to their final delivery to peroxisomes, challenging assumptions that emphasized only direct cytosolic or ER-dependent routes.

Together, this work provides new insights into the complex dynamics of peroxisomal biogenesis by establishing RRBP1 as a key ER-associated determinant of peroxisome maintenance and by revealing mitochondrial contributions to TA protein routing. More broadly, these findings highlight the importance of coordinated communication between the ER, mitochondria, and peroxisomes for maintaining organelle homeostasis and cellular metabolic balance.

TIIVISTELMÄ

Peroksisomit ovat yksikalvoisia soluelimiä, jotka ovat välttämättömiä solun lipidien aineenvaihdunnalle, reaktiivisten happiyhdisteiden detoksifikaatiolle sekä aineenvaihdunnalliselle homeostaasille. Peroksisomien muodostumisen tai toiminnan häiriöt johtavat vakaviin sairauksiin, kuten Zellwegerin kirjon häiriöihin, mikä korostaa peroksisomien biogeneesiä säätelevien molekyylimekanismien ymmärryksen tärkeyttä. Vaikka peroksisomien biogeneesin keskeiset komponentit on tunnistettu, peroksisomien kokoamista ja proteiinien kuljetusta koordinoivia solutason mekanismeja ei tunneta hyvin.

Tässä tutkimuksessa sovellettiin systemaattista kaksoisseulontamenetelmää, jolla tunnistettiin uusia peroksisomien biogeneesiä sääteleviä tekijöitä ihmisen soluissa. Yhdistämällä läheisyysmerkintään perustuva massaspektrometria ja toiminnallinen CRISPR-pohjainen geneettinen seulonta, havaitsimme että ribosomisidontaproteiini 1 (RRBP1) on uusi peroksisomien biogeneesiin vaadittava tekijä. Funktionaaliset analyysit HEK293T-soluissa osoittivat, että RRBP1:n poistaminen (knockout) johti huomattavaan peroksisomien määrän laskuun, johon liittyi peroksisomaalisten proteiinien virheellinen paikantuminen, sekä niiden kokonaispitoisuuden aleneminen. Lisäksi RRBP1:n poisto johti peroksisomaalisten matriksiproteiinien, kuten asyylikoentsyymi-A-oksidaasi 1:n ja 3-ketoasyylikoentsyymi-A-tiolaasin, heikentyneeseen muokkaukseen soluissa, mikä vastaa häiriötä peroksisomaalisessa proteiinituonnissa tai kypsymisessä. RRBP1:n puuttuminen ei vaikuttanut solujen lisääntymiseen tai proteiinien yleiseen synteesiin, mikä korostaa sen erityisroolia peroksisomien biogeneesin säätelyssä. Lisätutkimukset osoittivat, ettei RRBP1:n poisto häirinnyt peroksisomien ja endoplasmakalvoston välistä kontaktialuetta, eikä peroksisomien väheneminen johtunut tehostuneesta peksofagiasta. Sen sijaan RRBP1:n puuttuessa peroksisomaaliset proteiinit degradoitiin proteasomeissa, mikä viittaa RRBP1:n rooliin peroksisomien biogeneesin alkuvaiheissa, jolloin RRBP1 saattaa esimerkiksi tukea peroksisomaalisten proteiinien stabilointia endoplasmakalvostossa. Nämä havainnot ovat yhteydessä endoplasmakalvoston tunnettuun rooliin peroksisomikalvojen muodostumisen alustana ja korostavat soluelinten välisen viestinnän merkitystä soluelinten kokoamisprosessissa.

Tutkimme myös häntäankkuroitujen (Tail Anchor, TA) proteiinien kuljetusreittiä. Näille proteiineille on ominaista yksi C-terminaalinen kalvon läpäisevä alue, ja niiden tarkka kohdentuminen peroksisomikalvoihin riippuu PEX19- ja PEX3-proteiineista ja voi tapahtua joko suoraan sytosolista tai endoplasmakalvoston kautta. Läheisyysbiotinylaatiomenetelmää käyttäen havaitsimme biotinylaatiokuvioita, jotka viittaavat siihen, että peroksisomaaliset TA-proteiinit ovat vuorovaikutuksessa mitokondrioiden kanssa ennen peroksisomeihin saapumistaan. Erityisesti YgiM(TA)-AviTag biotinyloitui soluissa, joissa BirA-proteiini ilmennettiin mitokondrioissa tai peroksisomeissa, mutta ei soluissa, joissa se ilmentyi endoplasmakalvostossa, mikä tukee mitokondrioiden roolia TA-proteiinien kuljetuksessa. Myös glykosylaatiokokeet tukivat endoplasmakalvostosta riippumatonta reittiä, sillä peroksisomaalisia TA-proteiineja ei

glykosyloitu edes soluissa joissa peroxisomit puuttuivat kokonaan. Konfokaalimikroskoopia ja korkean resoluution elektronimikroskopia osoittivat, että peroksisomaaliset TA-proteiinit sijaitsevat yksinomaan peroksisomeissa normaaleissa soluissa, mutta siirtyvät pääasiassa mitokondrioihin peroksisomien puuttuessa. Nämä tulokset viittaavat siihen, että peroksisomaaliset TA-proteiinit ohjautuvat mahdollisesti ensin mitokondrioihin ennen lopullista toimitustaan peroksisomeihin, haastaen näin aiemmat olettamukset suorasta sytosolisesta tai ER-riippuvaisesta reitistä.

Kokonaisuutena väitöskirjatyö lisää ymmärrystä peroksisomien biogeneesistä tunnistamalla RRBP1:n tärkeäksi ER:ään liittyväksi peroksisomien ylläpidon sääteilijäksi sekä paljastamalla mitokondrioiden osuuden TA-proteiinien ohjautumisessa peroksisomeihin. Laajemmassa mittakaavassa nämä havainnot korostavat endoplasmakalvoston, mitokondrioiden ja peroksisomien välistä koordinaatiota solun aineenvaihdunnallisen tasapainon ylläpidossa.

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LIST OF ABBREVIATIONS

Endoplasmic reticulum	ER
Hydrogen peroxide	H_2O_2
Peroxisomal membrane proteins	PMPs
Ascorbate peroxidase	APEX2
ATP-binding cassette	ABC
Very long-chain fatty acid	VLCFA
Very long-chain fatty acyl-CoA	VLCFA-CoA
Reactive oxygen species	ROS
Ribosome-binding protein 1	RRBP1
Peroxisomal targeting signals	PTSs
Peroxisomal targeting signal 1	PTS1
Peroxisomal targeting signal 2	PTS2
Dihydroxyacetone phosphate	DHAP
Very long chain fatty acid	VLCFA
Acyl-CoA oxidase 1	ACOX1
3-ketoacyl-CoA thiolase	ACAA1
Tail-anchored	TA
Green Fluorescent Protein-engineered Ascorbate Peroxidase 2-enhanced Peroxisomal Targeting Signal 1	GFP-APEX2-ePTS1
1-O-alkyl glycerol	OAG
Pre-peroxisomal vesicles	ppVs
Diaminobenzidine	DAB
Proteasome inhibitor (peptide aldehyde)	MG132
Pre-peroxisomal endoplasmic reticulum	pER

Membrane contact sites	MCS
Transmembrane domain	TMD
Guide RNAs	gRNAs
Endoribonuclease-prepared small interfering RNA	esiRNA
Peroxisomal Biogenesis Factor 3	PEX3
Peroxisomal Biogenesis Factor 16	PEX16
Peroxisomal Biogenesis Factor 19	PEX19
Peroxisomal membrane protein of 70 kDa	PMP70
Peroxisomal Membrane Protein 2	PXMP2
Electron microscopy	EM
Human Embryonic Kidney 293T	HEK293T
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
CRISPR-associated protein 9	Cas9
Knockout	KO
Biotin ligase	BirA
Biotin acceptor peptide tag	AviTag
Immunoprecipitation	IP

LIST OF PUBLICATIONS

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals I and II. The original publications are reproduced at the end of the thesis with the permission of the respective copyright holders.

I. **Fatima K**, Vihinen, H., Akpinar, A., Somborac, T., Paatero, A., Jokitalo, E., Paavilainen, V., Katajisto, P., & Konovalova, S. (2025). *Ribosome binding protein 1 maintains peroxisome biogenesis*. Journal of Cell Science, 138(24), jcs264075. https://doi.org/10.1242/jcs.264075

II. Somborac, T., Lutfullahoglu Bal, G., **Fatima, K***., Vihinen, H., Paatero, A., Jokitalo, E., Paavilainen, V. O., & Konovalova, S. (2023). *A subset of peroxisomal tail anchored proteins do not reach peroxisomes via the endoplasmic reticulum, instead mitochondria can be involved*. PLOS ONE, 18(12), e0295047. https://doi.org/10.1371/journal.pone.0295047

***Author contribution:**
KF contributed to the initial optimization of the biotinylation based proximity labelling experiments.

1 LITERATURE REVIEW

1.1 Background and significance of peroxisomes

The human body is a highly organized system composed of trillions of cells, each working together to maintain life and overall health. Cells are the basic units of structure and function in the body, carrying out a wide range of processes necessary for survival. Inside eukaryotic cells, membrane-bound organelles provide specialized environments for these processes. For example, the nucleus stores genetic information, mitochondria generate energy, the endoplasmic reticulum and Golgi apparatus handle protein and lipid processing, lysosomes digest waste materials, and peroxisomes perform key oxidative and detoxification functions. Together, these organelles coordinate cellular activities, enabling the cell and ultimately the entire organism to function efficiently. The evolutionary origin of this cellular compartmentalization is not fully understood, and two main models have been proposed. One suggests endogenous formation from pre-existing structures, which is widely accepted for organelles like peroxisomes and the endoplasmic reticulum (ER). The other model highlights the role of endosymbiotic interactions between different cells, as seen in the evolutionary origins of mitochondria and chloroplasts (Martin, 2010).

Peroxisomes were first observed by Rhodin in 1954 as microbodies in rodent kidney cells. Initially considered anomalous, they were later recognized as distinct organelles across various species. They were studied extensively by Christian de Duve and Pierre Baudhuin (Bowers, 1998; Deter et al., 1967; Schrader & Fahimi, 2004), using rat liver cells. These studies provided biochemical evidence for the presence of hydrogen peroxide (H_2O_2) producing and degrading activities in these subcellular structures, leading to the name "peroxisomes". For his work on lysosomes and peroxisomes, de Duve was awarded the Nobel Prize in Physiology or Medicine in 1974 (Sabatini & Adesnik, 2013). Peroxisomes are highly dynamic organelles that play critical roles in numerous cellular processes and are present in nearly all eukaryotic cells, including animals, plants, fungi, and protozoa.

Although they are membrane-bound organelles, peroxisomes differ from mitochondria and chloroplasts in several aspects. They are enclosed by a single membrane and lack

DNA and ribosomes (Figure 1). Similar to mitochondria and chloroplasts, peroxisomes are believed to obtain their proteins via selective cytosolic import (Demarquoy, 2015). In this respect, peroxisomes resemble the endoplasmic reticulum, as the ER as self-replicating organelles enclosed by a membrane and existing without their own genomes (Schlüter et al., 2006).

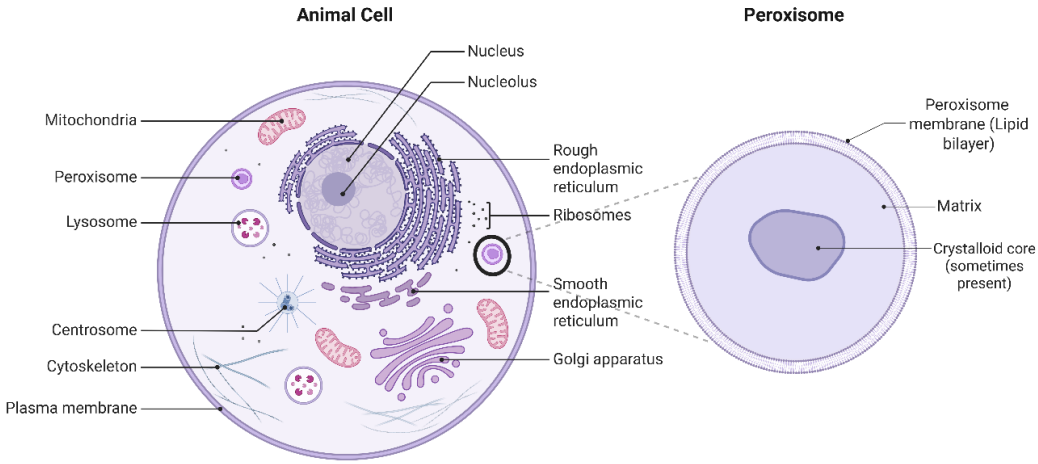

Figure 1. Overview of an animal cell and a peroxisome. *The schematic illustrates a typical animal cell containing major membrane-bound organelles. The peroxisome: highlighted and enlarged on the right shown as a single membrane-bounded organelle composed of a lipid bilayer surrounding an enzyme-rich matrix. Some peroxisomes also contain a central crystalloid core, depending on cell type and metabolic state.*

Peroxisomes in multicellular eukaryotes, including humans and plants, exhibit remarkable heterogeneity in both morphology and enzymatic content. Their size ranges from 0.1 to 1.5 µm, with forms varying from spherical to tubular or even interconnected structures, often found in clusters (Masters & Holmes, 1977). This diversity extends to their enzyme composition, which can differ not only between species but also across tissues within the same organism. Peroxisomes adapt dynamically to environmental and metabolic conditions; for example, in methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris*, they accumulate methanol-metabolizing enzymes when grown on methanol but not on glucose or ethanol (Bryers & Yeh, 1990). Similarly, in mammals, peroxisome abundance and enzyme content are strongly influenced by developmental, hormonal, nutritional, and environmental factors (Schrader & Fahimi,

2004). Specialized peroxisomes such as glycosomes in trypanosomes compartmentalize glycolytic pathways, with enzyme content shifting dramatically depending on whether the parasite resides in the mammalian host or the insect vector (Parsons, 2004).

Interest in peroxisomes expanded further in the 1970s, when research into a group of human genetic diseases collectively known as peroxisome biogenesis disorders, including Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, revealed that these conditions were due to peroxisome dysfunction. These conditions, marked by neurological, hepatic, and renal abnormalities, underscore the vital role of peroxisomes in human physiology (Thieringer et al., 2003). Peroxisomes are similarly essential in plants, where they required for embryogenesis and seedling germination, and perform diverse functions depending on developmental stage, tissue type, and environmental conditions (Hu et al., 2012; Pan et al., 2020).

1.2 Peroxisome functions and metabolic roles

Peroxisomes are multifunctional metabolic organelles best known for their role in cellular redox balance and lipid metabolism. They contain oxidases that generate H_2O_2 as they remove hydrogen from specific organic substrates, and catalase subsequently degrading H_2O_2 to prevent oxidative damage (Antonenkov et al., 2010; Lismont et al., 2019; Schrader & Fahimi, 2004). In addition, peroxisomes specialize in degrading very long-chain and branched-chain fatty acids, as well as bile acid intermediates, many of which are poorly metabolized by mitochondria (Okumoto et al., 2020). Fatty acids are transported into peroxisomes as CoA esters via ATP-binding cassette (ABC) transporters and undergo a sequence of enzymatic steps analogous to mitochondrial β-oxidation. The first reaction, catalyzed by acyl-CoA oxidases, transfers electrons to oxygen rather than the respiratory chain, producing H_2O_2 as a byproduct. Catalase, a hallmark peroxisomal enzyme, then detoxifies the H_2O_2 (R. J. A. Wanders et al., 2007). Subsequent reactions are carried out by multifunctional proteins that act as hydratases and dehydrogenases, followed by thiolases that release acetyl-CoA and chain-shortened acyl-CoAs. The products of peroxisomal β-oxidation are typically shuttled to mitochondria for complete oxidation, indicating the metabolic cooperation between these two organelles. Unlike mitochondria, peroxisomes do not generate ATP directly from fatty acid oxidation,

instead contributing to biosynthetic pathways and cellular lipid homeostasis (Arent et al., 2010).

In yeast and filamentous fungi, peroxisomes are also involved in the primary metabolism of unusual carbon and nitrogen sources, such as fatty acids, methanol, and primary amines, as well as in the biosynthesis of secondary metabolites like biotin and antibiotics (Bartoszewska et al., 2011; Stehlik et al., 2014). In Ascomycetes (a class of filamentous fungi), there are two distinct types of peroxisomes: one performs regular peroxisomal functions such as β-oxidation and oxidative defense, while the other, known as the Woronin body, seals septal pores in response to cell lysis to prevent cell death by bleeding (Bartoszewska et al., 2011; Jedd, 2011).

In plants, peroxisomes support lipid metabolism, photorespiration, nitrogen metabolism, ROS detoxification, and the synthesis of certain plant hormones (Del Río et al., 2003; Pan et al., 2020). Recent research has also linked peroxisomes to photomorphogenesis and plant-pathogen interactions (Hu et al., 2002; Kaur et al., 2013; Kubo, 2013). The essential roles of peroxisomes in embryogenesis and seedling germination highlight their critical importance, as their absence results in lethal plant phenotypes (Hu et al., 2012; Pan et al., 2019; Schumann et al., 2003). In plants, peroxisomes can be classified into three main types based on their functions and developmental stages: glyoxysomes in germinating seeds, leaf peroxisomes involved in photorespiration, and unspecialized peroxisomes in other plant parts (Hu et al., 2012; Pan et al., 2020).

In animal cells, peroxisomes participate in α- and β-oxidation of fatty acids, including very long-chain fatty acids, phytanic acid, bile acid intermediates, and pristanic acid, as well as in purine degradation and the biosynthesis of bile acids and ether lipids such as plasmalogens, major phospholipids of the myelin sheath in the brain (Poirier et al., 2006; R. J. A. Wanders et al., 2001, 2023; R. J. Wanders & Brites, 2010). Peroxisomes also detoxify harmful metabolites like glyoxylate and xenobiotics and play a central role in redox balance by producing and degrading H_2O_2 through numerous oxidases and catalase (Lazarow & Fujiki, 1985; Subramani et al., 2000) (Figure 2). Because many of these pathways are particularly important in the brain, defects in peroxisomes have profound neurological consequences. Impaired β-oxidation leads to the accumulation of

toxic fatty acids, while deficits in plasmalogen synthesis compromise myelin integrity and neuronal signaling. Peroxisomal dysfunction also disturbs redox balance, increases oxidative stress, and affects lipid-derived signaling pathways involved in inflammation and cell survival. As a result, inherited peroxisomal disorders such as Zellweger spectrum disorders cause severe neurodevelopmental defects, demyelination, and neuronal degeneration. More subtle impairments in peroxisomal metabolism are also linked to common brain diseases such as Alzheimer's, Parkinson's, and ALS. These impairments can disturb normal lipid processing, increase harmful reactive oxygen species, and change the levels of certain brain signaling molecules like D-serine and D-aspartate. Collectively, peroxisomes are indispensable for maintaining nervous system health, and their dysfunction contributes to both early-onset peroxisomal diseases and age-related neurological disorders (Islinger et al., 2012). Peroxisome biogenesis disorders (PBDs) range from severe Zellweger spectrum disorders to milder conditions such as Heimler syndrome, which typically presents sensorineural hearing loss, enamel defects, and retinal abnormalities (Ratbi et al., 2015). Recently, peroxisomes in mammals have been identified to play roles in non-metabolic functions like antiviral innate immunity and antiviral signaling (Islinger et al., 2018), inflammation (Zmijewski et al., 2009), apoptosis (Li et al., 2002), cellular aging (Beach et al., 2012), diabetes (Elsner et al., 2011) and cancer (Frederiks et al., 2010), largely through their regulation of lipid-derived and redox-dependent signaling molecules (Schrader et al., 2020).

Many metabolic processes in peroxisomes depend on the cooperative exchange of metabolites with other subcellular compartments, particularly plastids and mitochondria, evidenced by the close physical association of peroxisomes with these organelles (Hua et al., 2017; Igamberdiev & Lea, 2002; R. J. A. Wanders et al., 2016).

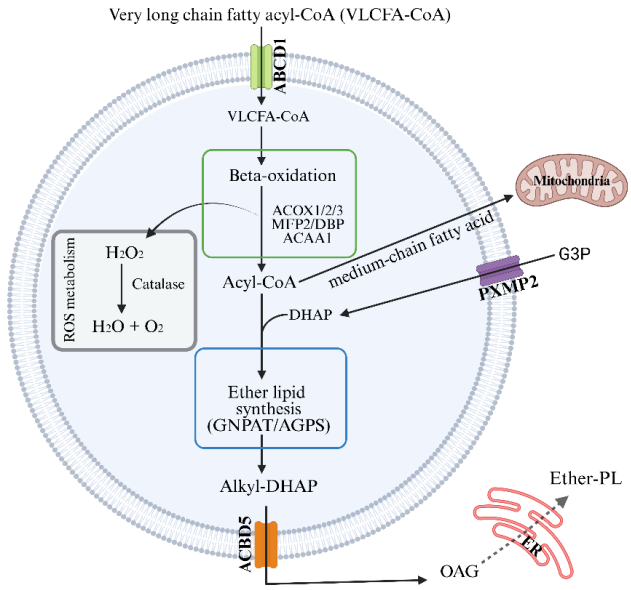

Figure 2: Overview of key metabolic pathways occurring in mammalian peroxisomes. *Peroxisomes carry out multiple essential metabolic functions, including β-oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA), ether lipid biosynthesis, and reactive oxygen species (ROS) detoxification. VLCFA-CoAs are transported into the peroxisomal lumen via the ABCD1 transporter, where they undergo β-oxidation mediated by ACOX1/2/3, MFP2/DBP, and ACAA1, generating acyl-CoA intermediates and hydrogen peroxide (H_2O_2). Peroxisome-derived H_2O_2 is subsequently degraded by catalase to water and oxygen. Acyl-CoA and dihydroxyacetone phosphate (DHAP) feed into the ether lipid synthesis pathway, initiated by GNPAT and AGPS to produce alkyl-DHAP. The peroxisomal membrane protein ACBD5 facilitates alkyl-DHAP (or the precursor 1-O-alkyl glycerol (OAG)) lipid transfer toward the ER for plasmalogen (ether-PL) production. PXMP2 mediates transport of small metabolites, including glycerol-3-phosphate (G3P). Medium-chain fatty acids generated during β-oxidation are shuttled to mitochondria for further oxidation. Collectively, the diagram highlights the central metabolic functions of peroxisomes and their metabolic crosstalk with mitochondria and the endoplasmic reticulum.*

1.3 Mechanisms of peroxisomal biogenesis

Peroxisomal biogenesis is a multistep process that depends on the coordinated action of peroxins, and other cellular components involved in organelle formation, protein import, and membrane dynamics.

1.3.1 Peroxins - key proteins in peroxisomal biogenesis

The proteins involved in peroxisome biogenesis are known as peroxins. Their identification come from genetic screens in model organisms including peroxisome assembly-defective mutants of mammalian somatic cells, such as Chinese hamster ovary cells (CHO cells) and several yeast species, including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, and *Yarrowia lipolytica* (Fujiki et al., 2014; Hu et al., 2012; Steinberg et al., 2006) (Hu et al., 2012). Additional insights have been gained from genetic studies of patients with peroxisome biogenesis disorders (Fujiki et al., 2014; Jansen et al., 2021). In the beginning, these proteins were named differently depending on the organism, which caused confusion. A standardized nomenclature was later established, where these proteins are named peroxins, abbreviated as PEX in mammals and Pex in yeast, followed by a number indicating their order of discovery (Distel et al., 1996). So far, 39 peroxins have been identified across a variety of species and 16 in human beings, each playing an important role in the process of biogenesis.

Peroxins are encoded by nuclear genes (PEX genes) and translated on free polyribosomes in the cytosol. Peroxins include cytosolic receptors and peroxisomal membrane proteins that mediate protein targeting and membrane assembly, as well as a subset of peroxins that localize to the peroxisomal matrix. These peroxins are highly conserved across eukaryotes and are crucial for the *de novo* synthesis of peroxisomes, the import of proteins into peroxisomes, and peroxisome division (Dahan et al., 2021; Jansen et al., 2021; Smith & Aitchison, 2013; R. J. A. Wanders et al., 2023). Unlike many peroxisomal matrix proteins, most peroxins do not contain classical C-terminal or N-terminal peroxisomal targeting sequences. Instead, their targeting routes are diverse; several membrane peroxins rely on PEX19-mediated targeting, while others may reach peroxisomes through vesicle-mediated pathways involving proteins such as PEX3, PEX16, and PEX26 (Kim et al., 2006; Kumar et al., 2024).

For decades, scientists have recognized two main models for how peroxisomes are created in cells: through the growth and division of pre-existing organelles and *de novo* peroxisomal biogenesis. In the following sections we will consider each of these models in detail. However, it is now recognized that although cells can regenerate peroxisomes

de novo when none are present, under normal physiological conditions peroxisomes usually proliferate by growth and division of existing organelles (Agrawal & Subramani, 2016; Islinger et al., 2018).

1.3.2 Growth and division model of peroxisomal biogenesis

The first and older model of peroxisomal biogenesis is called the growth and division model. In this model, peroxisomes, like mitochondria and chloroplasts, originate from already existing peroxisomes. These existing peroxisomes grow by incorporating peroxisomal membrane proteins (PMPs) and matrix proteins, which are synthesized in the cytosol and imported into the peroxisome. The necessary lipids for the peroxisome membrane are most likely supplied by the ER through contact sites between the organelles (Farré et al., 2019a; Motley & Hettema, 2007). Once the peroxisomes reach a certain size, they undergo a fission process, which includes elongation, constriction, and ultimately scission, resulting in the formation of daughter peroxisomes (Carmichael et al., 2022; Farré et al., 2019a). This process is mediated by peroxins such as PEX11β, which promotes membrane elongation, together with shared fission factors including FIS1 and DRP1. These daughter peroxisomes then repeat the cycle of growth and division. The growth and division model struggled to account for the formation of peroxisomes in biogenesis mutants that showed no signs of pre-existing peroxisomes, but could still produce new peroxisomes when the missing gene was introduced (Hettema et al., 2014; Hu et al., 2012; Motley & Hettema, 2007).

1.3.3 *De novo* model

The second model of peroxisomal biogenesis, known as *de novo* peroxisome biogenesis, was developed to address gaps in the growth and division model. In this model, some PMPs are first integrated into the membrane of the ER and then sorted to a specific region called the pre-peroxisomal ER (pER). From this region, pre-peroxisomal vesicles (ppVs) bud off, containing the necessary PMPs. Recent research in mammals indicates that some of these ppVs might also originate from mitochondria (Sugiura et al., 2017). These vesicles, which carry different sets of PMPs, either fuse with each other in a process called heterotypic fusion or merge with existing peroxisomes to form mature or larger peroxisomes. The *de novo* model also considers various nuances, such as whether

multiple types of ppVs can bud from different membranous compartments and whether other organelles like mitochondria play a role in peroxisome formation. Additionally, there is interest in understanding where the lipids for peroxisome membranes come from within the cell. The interaction and transfer of lipids and metabolites between peroxisomes and other organelles have highlighted the importance of inter-organelle membrane contact sites (MCS), where peroxisomes closely interact with other organelles (Hettema et al., 2014; Hu et al., 2012).

Currently, it is believed that peroxisomes can arise *de novo* from the ER as pre-peroxisome vesicles with additional contributions from mitochondria in mammalian cells. During the fusion of these vesicles and the posttranslational transport of peroxisomal membrane and matrix proteins, these pre-peroxisomes grow into mature peroxisomes and eventually divide. This aligns with the *de novo* peroxisome biogenesis model. However, it should be noted that when peroxisomes already exist, newly synthesized PMPs are directly transported to these existing peroxisomes, following the "growth and division" model (Agrawal & Subramani, 2013; Dimitrov et al., 2013).

Peroxisome biogenesis involves three sequential steps: (i) formation of the peroxisomal membrane, (ii) import of matrix proteins, and (iii) growth and proliferation of the organelle (Brown & Baker, 2003; Farré et al., 2019a).

1.3.3.1 Formation of peroxisomal membranes

Studies in mammalian cells and yeast have identified two key peroxins, PEX3 and PEX19, as essential factors in peroxisome membrane assembly (Fujiki et al., 2014; Jansen & Van Der Klei, 2019). Loss of any of these proteins results in cells completely lacking morphologically recognizable peroxisomes. Restoration of peroxisomes upon reintroduction of wild-type versions of these peroxins has been used as strong evidence for the possibility of *de novo* peroxisome formation, particularly from ER-derived membrane precursors (Kim et al., 2006; Schmidt et al., 2010). For instance, newly synthesized PEX3 has been shown to initially integrate into the ER in both yeast and mammalian systems, suggesting that peroxisomes are at least partly ER-derived organelles. In yeast, observations that certain peroxisomal membrane proteins first appear in the ER before being trafficked to nascent peroxisomes further strengthen the

ER-origin hypothesis (Agrawal & Subramani, 2016; Hoepfner et al., 2005; Mayerhofer et al., 2016).

In addition to PEX3 and PEX19, PEX16 plays an important role (with its requirement varying across organisms) in peroxisomal membrane protein assembly (Yagita et al., 2022). PEX16, an integral membrane protein present in most organisms except *Saccharomyces cerevisiae*, is targeted to the ER and is thought to establish a scaffold that recruits PEX3 and other PMPs to specialized ER subdomains, often termed the peroxisomal ER (Farré et al., 2019a; Schmidt et al., 2010). Then, PEX19, a predominantly cytoplasmic protein, binds to newly synthesized PMPs and stabilizes their hydrophobic domains, preventing their degradation. It functions both as a chaperone and receptor by recognizing conserved membrane peroxisomal targeting signals (mPTSs) on PMPs and delivering them to the peroxisome membrane (Jones et al., 2004; Pinto et al., 2006). Once docked, the PMP-PEX19 complex interacts with PEX3, which anchors the PMP into the membrane (Jones et al., 2004; Schmidt et al., 2010). This concerted action of PEX16, PEX3, and PEX19 ensures that the organelle acquires the correct complement of membrane proteins necessary for its biogenesis and maintenance (as shown in Figure 3). Mutants lacking any of these peroxins exhibit profound defects, often resulting in peroxisome absence, while other Pex mutants retain membrane ghosts that contain PMPs but lack matrix proteins, illustrating that membrane assembly and matrix import are mechanistically separable processes (R. J. A. Wanders et al., 2023).

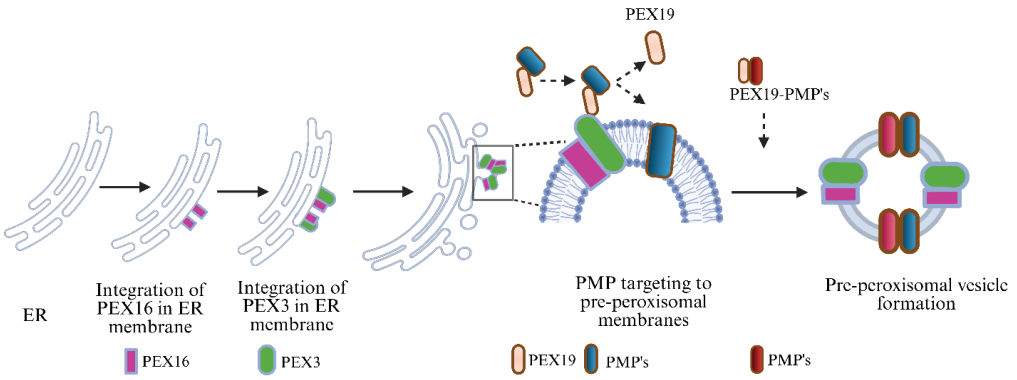

***Figure 3: De novo peroxisome biogenesis from the ER**. The schematic illustrates stepwise formation of a peroxisome originating from a specialized ER subdomain. PEX16 integrates into the ER membrane and helps establish a pre-peroxisomal membrane domain.*

PEX3 subsequently recruited to this site and incorporated into the membrane. Membrane remodeling leads to formation of pre-peroxisomal vesicles containing early membrane components. Cytosolic PEX19 binds newly synthesized peroxisomal membrane proteins (PMPs) and facilitates their targeting to pre-peroxisomal membranes through transient interaction with membrane peroxins such as PEX3. Following PMP insertion, PEX19 dissociates and returns to the cytosol. This process results in a nascent pre-peroxisomal vesicle that can mature into an import-competent peroxisome. Dashed arrows indicate proposed PMP trafficking routes.

Peroxisomal membrane proteins can be sorted by either direct or indirect pathways (Mayerhofer, 2016). In the direct pathway, PEX19 recognizes and binds PMPs in the cytosol, shielding them until they are inserted into the peroxisome membrane in a PEX3-dependent manner. This mechanism accounts for the majority of PMP imports and is sometimes referred to as the Class I pathway (Jones et al., 2004; Matsuzaki & Fujiki, 2008; Mayerhofer, 2016). The indirect pathway, or Class II, involves trafficking of certain PMPs via the ER. For example, mammalian PEX3 can reach peroxisomes either through direct import or by first integrating into the ER and later trafficking to peroxisomes (Mayerhofer et al., 2016). This flexibility provides redundancy to ensure proper membrane protein delivery. In yeast, tail-anchored proteins, defined by short C-terminal targeting sequences adjacent to their transmembrane domains, illustrate this adaptability, as they can be inserted into peroxisomal membranes either directly or via ER intermediates. Together, these pathways ensure that peroxisomes acquire the correct structural and functional components to establish their membranes and support further organelle development (Jones et al., 2004; Matsuzaki & Fujiki, 2008; Mayerhofer, 2016).

1.3.3.1.1 Tail-anchored (TA) proteins targeting to peroxisomes

TA proteins are unique among integral membrane proteins due to their single transmembrane domain (TMD) positioned near the C-terminal end, a characteristic that excludes the presence of an N-terminal signal peptide. The TMD anchors these proteins to the membrane, while the N-terminus performs various roles specific to each protein (Craney et al., 2011; Mehlhorn et al., 2021). The orientation of the N-terminal region towards the cytosol, combined with the hydrophobic and charged nature of the TMD, is essential for accurate targeting and functional integration into organelles. The C-terminal region of TA proteins harbors the targeting information necessary for their post-translational insertion into membranes. This unique mechanism ensures that TA

proteins, which constitute a minor but crucial subset of cellular proteins, are accurately localized and integrated into appropriate cellular compartments after synthesis (Borgese et al., 2003; Borgese & Fasana, 2011; Costello, Castro, Camões, et al., 2017).

Studies on yeast and mammalian models showed that peroxisomal TA proteins can be routed through two pathways: directly from the cytosol or via the ER (Kim & Hettema, 2015; Liu et al., 2016). In the direct pathway, PEX19 recognizes the TA protein in the cytosol and guides it to PEX3 on the peroxisomal membrane, where it is inserted. The presence of conserved leucines and aromatic residues in the PEX19 N-terminal domain is critical for PEX3 binding and thus for the biogenesis of peroxisomes (Kim & Hettema, 2015). Moreover, hydrogen/deuterium exchange mass spectrometry (HDX-MS) experiments have demonstrated structural transformations at the C-terminus of PEX19 upon its association with PEX3, suggesting a potential regulatory role in substrate binding (Hattula et al., 2014). In the ER-dependent pathway, the TA protein first integrates into the ER membrane and subsequently traffics to the peroxisome, a process that involves PEX3 and PEX19, as well as additional cytosolic components. Studies in yeast have demonstrated that a subset of peroxisomal TA proteins, such as Pex15, initially target the ER via the GET complex and then sort to peroxisomes. This process relies on the presence of specialized vesicles that bud off from the ER and merge with pre-existing peroxisomes. In vitro budding assays and imaging studies have shown that the formation of pre-peroxisomal vesicles from the ER requires PEX3, PEX19, ATP, and other yet unidentified cytosolic components (Lisenbee et al., 2003; Mullen & Trelease, 2000; Schuldiner et al., 2008; van der Zand et al., 2012).In mammals, only a few peroxisomal TA proteins have been identified, but they perform important cellular functions. For example, ACBD5, a mammalian peroxisomal TA protein, mediates peroxisome-ER contact sites that are critical for lipid exchange and homeostasis (Costello, Castro, Hacker, et al., 2017). Mutations in ACBD5 cause neurodegenerative disorders. Another well characterized TA protein, FALDH-PO, involved in fatty acid metabolism, and its dysfunction linked to Sjögren-Larsson syndrome, a genetic disorder characterized by ichthyosis, intellectual disability, and spasticity (C. T. Chen et al., 2022; Rizzo, 2007). Understanding the mechanisms underlying the targeting and insertion of TA proteins to peroxisomes is therefore crucial to elucidate their role in cellular physiology and pathology.

1.3.3.2 Import of matrix proteins in peroxisomes

Once the membrane has been established, the next critical stage of peroxisome biogenesis is the import of matrix proteins. Unlike mitochondria or chloroplasts, peroxisomes are capable of importing fully folded or oligomeric proteins, a feature that makes their import machinery unique among eukaryotic organelles (Gould & Collins, 2002; Ma & Subramani, 2009). The matrix protein import process can be broken down into several distinct steps (Hasan et al., 2013) (Figure 4):

(a) Recognition of the protein by a receptor

(b) Docking of the receptor-protein complex onto the membrane

(c) Translocation of the receptor-protein complex across the membrane

(d) Recycling of the receptor.

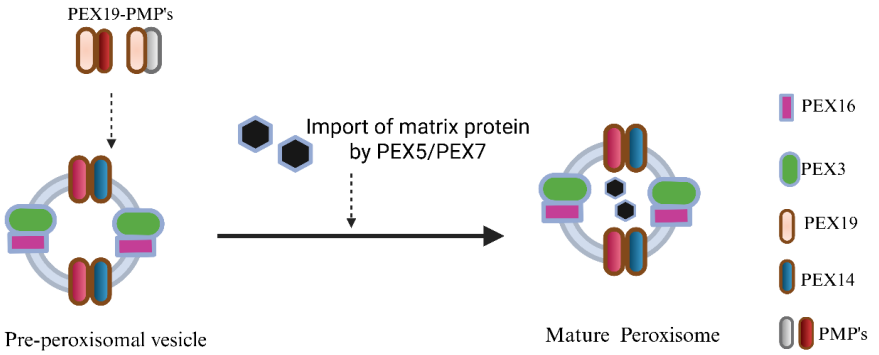

Figure 4: Maturation of peroxisomes through matrix protein import. *Following the formation of a pre-peroxisomal vesicle containing key membrane proteins, PEX19 delivers additional peroxisomal membrane proteins to the peroxisomal membrane. Once the matrix protein import machinery is assembled, matrix proteins carrying peroxisomal targeting signals (PTS1 or PTS2) are recognized by the cytosolic receptors PEX5 and PEX7, respectively. These receptor-cargo complexes mediate translocation of matrix proteins into the vesicle lumen, resulting in the formation of a mature peroxisome.*

1.3.3.2.1 Recognition of the protein by a receptor

The process begins with the recognition of peroxisomal targeting signals (PTSs) within matrix proteins. Two major classes of PTSs exist: PTS1, a C-terminal tripeptide or extended motif that directs the majority of matrix proteins, and PTS2, a nonapeptide

sequence near the N-terminus found in a subset of proteins (Hasan et al., 2013; Scott & Olpin, 2011). These targeting signals are recognized by soluble receptors, PEX5 for PTS1 and PEX7 for PTS2. PEX5 contains tetratricopeptide repeat motifs that bind PTS1 sequences, while its N-terminal domain interacts with membrane docking proteins (Bhogal et al., 2016). PEX7 adopts a β-propeller structure that recognizes PTS2 sequences, and in many organisms it requires auxiliary proteins to form an import-competent complex (Kunze et al., 2015). Recent studies have also identified PEX39 as an additional auxiliary factor involved in PTS2 protein import, further highlighting the complexity of receptor-assisted matrix protein targeting (Chen et al., 2025). Interestingly, species-specific variations exist; for example, Caenorhabditis elegans lacks PEX7 altogether and imports all matrix proteins via PEX5, while in mammals PEX5 has isoforms with distinct roles in PTS1 and PTS2 import (Ramón & Bartel, 2010).

1.3.3.2.2 Docking of receptor-protein complex

After receptor binds in the cytosol, the cargo-receptor complexes dock at the peroxisomal membrane. According to Albertini and coworkers (Albertini et al., 1997) this docking site is primarily composed of PEX14 and PEX13. PEX14 interacts strongly with PEX5 through conserved motifs, while also associating with PEX13, which contains an SH3 domain that facilitates protein-protein interactions (Wang et al., 2019). In the PTS2 pathway, PEX7 also directly interacts with this docking complex (Singh et al., 2009).

1.3.3.2.3 Translocation across the peroxisomal membrane

Translocation of the receptor-protein complex across the membrane. Following docking, the receptor–cargo complex must translocate across the peroxisomal membrane. This step is mediated by a set of integral membrane proteins, including PEX2, PEX10, and PEX12, which form a translocation subcomplex with cytosol-exposed RING finger domains (Farré et al., 2019a; Kim & Hettema, 2015; Platta et al., 2009) . These proteins work sequentially to facilitate protein import, assisted by PEX8 on the matrix side, which bridges docking and translocation subcomplexes and promotes the assembly of the transient import machinery (Agne et al., 2003). Importantly, these steps enable folded, functional proteins to enter the peroxisome matrix, a feature that distinguishes peroxisomal import from other organelle systems.

However, the exact mechanism of protein translocation across the peroxisomal membrane remains unresolved and is actively debated. One model proposes that PEX5, together with PEX14, forms a transient pore that allows receptor-cargo complexes to cross the membrane (Meinecke et al., 2010). An alternative hypothesis suggests that PEX13 may contribute to a nuclear pore like translocation channel, possibly involving liquid-liquid phase separation (Gao et al., 2022). Both models are supported by recent studies and continue to shape current discussions on peroxisomal matrix protein import.

1.3.3.2.4 Receptor recycling

Receptor recycling is a final critical step in matrix protein import. Initial models proposed that receptors merely released cargo at the membrane, but more recent evidence supports the extended shuttle model, in which receptors such as PEX5 transiently enter the peroxisome, release their cargo, and are then recycled back to the cytosol (Skowyra et al., 2024). This recycling is ATP-dependent and involves the AAA ATPases PEX1 and PEX6, which are recruited to the membrane by proteins such as PEX26 in mammals or PEX15 in yeast (Hasan et al., 2013). These ATPases mediate the dislocation of PEX5 from the membrane, allowing it to participate in further rounds of import (Thoms & Erdmann, 2006). This recycling mechanism ensures the efficiency and sustainability of the import process, while also preventing receptor accumulation at the membrane.

1.3.3.2.5 Import of proteins without consensus targeting motifs (piggy-backing transport)

Not all peroxisomal proteins contain canonical PTS1 or PTS2 signals. Some rely on alternative mechanisms, including internal targeting sequences or piggybacking on other proteins with functional PTS's. For instance, certain enzymes in yeast and mammals lacking clear targeting motifs are imported into peroxisomes through interactions with partner proteins that do contain PTS1 or PTS2 signals such as superoxide dismutase 1 and lactate dehydrogenase. This piggybacking mechanism demonstrate the flexibility of the peroxisomal import system and reflects the evolutionary diversity of peroxisomal proteomes across species (Ast et al., 2013; Kempiński et al., 2020).

1.3.3.3 Mechanism of peroxisome growth and division

Following membrane assembly and matrix protein import, mature peroxisomes undergo growth and division to increase organelle number. The final step of peroxisome biogenesis is organelle growth and proliferation. Peroxisomes are highly dynamic and can increase in number either in coordination with the cell cycle or in response to environmental stimuli, such as changes in nutrient availability or exposure to peroxisome proliferators. Organelle division generally proceeds through three steps: elongation of the peroxisomal membrane, constriction, and fission (Koch & Brocard, 2011). Proteins of the PEX11 family play central roles in elongation, with PEX11β in mammals (Figure 5) and Pex11, Pex25, and Pex27 in yeast acting to remodel membranes and promote peroxisome elongation (Koch et al., 2010). Constriction mechanisms remain less well understood, though studies in *Yarrowia lipolytica* suggest a role for Pex16 and localized lipid remodeling events (Kim & Mullen, 2013). Peroxisomal fission is mediated by dynamin-related proteins, such as Vps1 and Dnm1 in yeast and DLP1 in mammals, which assemble at construction sites and drive membrane scission. Additional factors, including Fis1 and other PMPs, act as adaptors linking dynamin-related proteins to peroxisome membranes. Beyond these core components, accessory proteins such as Pex28, Pex29, and regulators of peroxisome abundance further fine-tune division processes. These pathways ensure that peroxisomes can proliferate rapidly in response to cellular demands, providing metabolic flexibility and resilience (Aufschnaiter & Büttner, 2015; Ekal et al., 2023; Thoms & Erdmann, 2005).

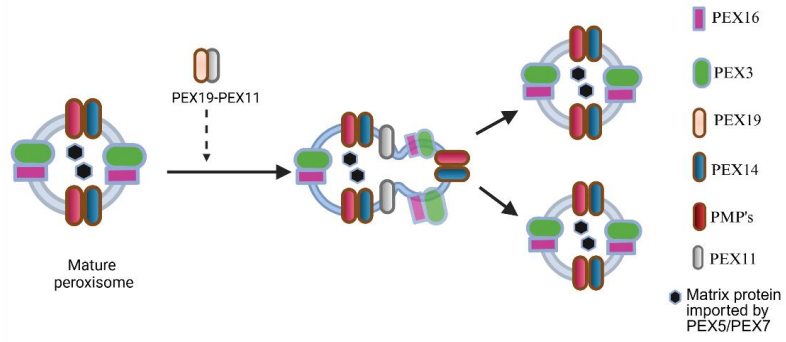

Figure 5: Growth and division of peroxisome. *A mature peroxisome receives peroxisomal membrane proteins, including PEX11, via cytosolic PEX19 complexes that deliver*

them to the peroxisomal membrane for insertion. PEX11 promotes membrane remodeling and elongation of the peroxisome, leading to a tubular intermediate. The elongated peroxisome then undergoes fission to produce two daughter peroxisomes. During this process, matrix protein import continues through PEX5 and PEX7, which recognize PTS1 and PTS2 targeting signals, maintaining metabolic functionality.

1.4 Link between ER and peroxisomes

Beyond the involvement of peroxins such as PEX3 and PEX16 in pre-peroxisomal vesicle formation, early electron microscopy studies revealed close proximity and apparent continuity between the ER and peroxisomes, suggesting that ER is a primary source of membranes and pre-peroxisomal compartments for *de novo* biogenesis (Agrawal & Subramani, 2013). For instance, the protein Pex15p was found in both peroxisomal membranes and ER derived membrane structures called karmellae (Hettema et al., 2000). However, it was later discovered that this ER localization of Pex15p was due to protein overexpression rather than a natural process (Elgersma, 1997). Proper localization of Pex15p to peroxisomal membranes depends on the proteins Pex3p and Pex19p, making it unlikely that Pex15p naturally passes through the ER membrane (Hettema et al., 2000).

Further research has shown that certain peroxisomal proteins can mis-localize to the ER under specific conditions, suggesting some level of ER involvement in peroxisome formation. However, blocking traditional ER-to-peroxisome transport pathways does not prevent peroxisome biogenesis. Thus, studies on temperature effects and vesicular transport inhibitors have shown that proteins like Pex16p still reach peroxisomes, even when ER-Golgi transport is blocked, implying the existence of alternate pathways (Toro et al., 2007). Notably, research in the yeast *Yarrowia lipolytica* demonstrated that some PMPs could be processed in the ER before reaching peroxisomes. Mutations affecting peroxisome biogenesis in this yeast led to the accumulation of PMPs in the ER and changes in ER membrane structure, providing further evidence for a connection (Van Der Zand et al., 2010).

Rather than acting solely as a membrane source, the ER is now recognized to engage peroxisomes through specialized membrane contact sites that enable direct inter-organelle communication without membrane fusion (Prinz et al., 2020; Silva et al., 2020).

In mammalian cells, a key tethering mechanism involves the peroxisomal acyl-CoA-binding protein ACBD5 interacting with the ER-resident proteins VAPA and VAPB through a FFAT-like motif, thereby stabilizing physical contacts between the two membranes (Kors et al., 2022). Similar tethering mechanisms have also been described in yeast, highlighting the evolutionary conservation of ER-peroxisome communication (Joshi, 2021). Peroxisomes lack the ability to synthesize membrane lipids *de novo*, these contact sites are thought to play a central role in supplying lipids required for peroxisomal membrane growth, division, and functional integrity (Farré et al., 2019a).

Recent studies have revealed that ER-peroxisome contact sites act as active platforms for non-vesicular lipid exchange rather than passive structural links (Castro et al., 2018). Lipid transfer at these sites supports the delivery of phospholipids necessary for peroxisomal membrane expansion and may also facilitate the exchange of specific lipid species, including cholesterol and lipid precursors involved in plasmalogen biosynthesis (Islinger et al., 2018). Emerging evidence suggests that ACBD5 not only functions as a tether but may also contribute to the local coordination of acyl-CoA pools, thereby coupling lipid transfer with peroxisomal fatty acid metabolism (Dawes et al., 2025; Hua et al., 2017). Moreover, regulatory mechanisms such as phosphorylation of tethering proteins have been shown to modulate ER-peroxisome contact formation, indicating that lipid exchange at these sites is dynamically controlled in response to cellular metabolic demands (Kors et al., 2022). Together, these findings establish ER-peroxisome contact sites as critical hubs for lipid trafficking and metabolic coordination, with their disruption leading to altered peroxisome morphology and impaired lipid homeostasis.

1.5 Link between mitochondria and peroxisomes

Mitochondria have recently been recognized as key contributors to *de novo* peroxisome biogenesis in mammalian cells, complementing the well-established role of the ER (Farré et al., 2019a; Mohanty & McBride, 2013; Schrader & Pellegrini, 2017). In the absence of functional peroxisomes, key peroxisomal membrane proteins such as PEX3 and PEX14 are first directed to the mitochondrial outer membrane. At mitochondria, these proteins contribute to the formation of mitochondria-derived pre-peroxisomal vesicles (MDppVs). The MDppVs then fuse with ER-derived vesicles containing PEX16, forming hybrid

intermediates that mature into fully import-competent peroxisomes (Farré et al., 2019a; Hua & Kim, 2016). Beyond this vesicular contribution, mitochondria also supply phospholipids required for peroxisomal membrane expansion through coordinated lipid-transfer pathways operating across the ER-mitochondria-peroxisome network (Mohanty & McBride, 2013). Moreover, mitochondria and peroxisomes share deep evolutionary and functional similarities, including roles in fatty acid oxidation, reactive oxygen species detoxification, and regulation by common nuclear transcription factors (Demarquoy, 2015). These interconnected processes position mitochondria as a central hub that provides membrane components, metabolic cues, and regulatory machinery essential for peroxisome formation, proliferation, and homeostasis. Although studies in yeast have provided much of our current understanding of peroxisomal biogenesis, the mechanisms governing mitochondrial contributions, including mitochondria-peroxisome contact sites and vesicle exchange, remain less well understood in mammalian systems and are an active area of investigation (Farré et al., 2019b; Hua & Kim, 2016).

2 AIM OF THIS STUDY

The aim of my dissertation was to investigate the mechanisms and pathways of peroxisomal biogenesis. The specific objectives of my study were:

1. To identify novel peroxisome biogenesis factors using double screening approach combining proximity labeling and CRISPR/Cas9 knockout microscopy screening.

2. To characterize the role of RRBP1 in peroxisomal maintenance by examining the effects of RRBP1 depletion in HEK293T cells.

3. To investigate the trafficking pathway by which TA proteins are delivered to peroxisomes in mammalian cells.

3 METHODOLOGY

The original article provides a detailed description of the methods and approaches used in this dissertation.

Methods	Published in
Cell culture	I, II
Generation of knockout cell lines	I, II
Plasmid construction	I
Stable cell line generation	I
APEX2-proximity labeling	I
Mass spectrometry analysis of proteins	I
esiRNA gene silencing	I
CRISPR/Cas9 microscopy screen	I
Live-cell fluorescence microscopy	I, II
Western blotting	I, II
Immunofluorescence	I, II
Quantitative analysis of immunofluorescence images	I, II
Peroxisome isolation by PEROXO-Tag IP	I
Pexophagy assay	I
Proteasomal inhibition assay	I
Translation assay	I
Cell proliferation assay	I
Transmission electron microscopy	I
PO-ER contact site analysis	I
Biotinylation assay	II
Statistical Analysis	I, II

4 RESULTS AND DISCUSSION

4.1 Systematic screening for novel peroxisome biogenesis factors

To systematically identify factors required for protein import into the peroxisomal matrix, a proximity-labeling approach followed by mass spectrometry was utilized (Lam et al., 2015). Ascorbate peroxidase (APEX2) was fused to the ePTS1 peroxisomal targeting sequence, which is recognized by PEX5 and directs the construct to the peroxisomal matrix (I, Fig. 1A). The GFP-APEX2-ePTS1 construct was stably expressed in HEK293T control cells as well as in the cells lacking essential factors for peroxisomal biogenesis, PEX3 KO and PEX19 KO cells (I, Fig. 1A). In control cells, the GFP-APEX2-ePTS1 construct is recognized by peroxisomal protein import factors and delivered to the peroxisomal matrix. However, in PEX3 KO and PEX19 KO cells, the construct, although recognized by peroxisomal import machinery, remains in the cytosol (I, Supplementary Fig. 1B). Under these conditions, factors involved in recognizing and trafficking of ePTS1-containing proteins are expected to accumulate near the GFP-APEX2-ePTS1 construct. This setup enabled the identification of factors required for the delivery of ePTS1-containing proteins to peroxisomes by analyzing proteins found in close proximity to the GFP-APEX2-ePTS1 construct in PEX3 KO and PEX19 KO cells. To exclude non-specifically labeled proteins we used negative control samples that included parental cells without the construct and cells stably expressing non-targeted GFP-APEX2.

Biotinylated proteins were extracted using streptavidin beads and analyzed by mass spectrometry. Proteins identified in two replicates for each PEX3 KO and PEX19 KO cells were considered significant hits. To identify highly specific interactors of GFP-APEX2-ePTS1 in cells lacking peroxisomes, any proteins identified in background (negative control) samples were excluded (detailed in Supplementary Table 1). Essential proteins were also excluded (I, listed in Supplementary Table 2). This strategy ensured that only those proteins essential for peroxisomal biogenesis and maintenance were selected, eliminating common background proteins and essential proteins not specific to peroxisomes. After this filtering process, a pathway analysis of the remaining candidates

led to the identification of 44 proteins potentially involved in peroxisomal biogenesis (I, refer to Fig 1B, Supplementary Fig. 1C, and Supplementary Table 3). Surprisingly, we did not detect PEX5 among the identified proteins, despite its role as the key factor recognizing PTS1. Notably, PEX5 was not identified as a specific interactor of GFP-APEX2-ePTS1 even in parental cells, suggesting that a combination of technical limitations in the labeling reaction and the unique biological properties of PEX5 may have prevented its detection in the APEX2 proximity labeling assay.

To further investigate the involvement of these 44 candidate proteins in peroxisomal biogenesis, a secondary screen was conducted using CRISPR/Cas9-mediated gene editing and live-cell fluorescence microscopy (I, Fig. 1C). Two guide RNAs (gRNAs) were designed to target each of the 44 candidate genes. HEK293T cells stably expressing GFP-APEX2-ePTS1 served as the reporter cell line for these experiments. The HEK293T cells were co-transfected with the Cas9 plasmid and individual gRNAs. The localization of the GFP signal in transfected cells was monitored using live-cell fluorescent microscopy. The key indicator of peroxisomal biogenesis disruption was the re-localization of GFP from peroxisomes to the cytosol. The cells were manually classified into four categories based on GFP localization: “no” (GFP localized exclusively to peroxisomes, no mislocalization), “mild” (some mis-localized GFP), “strong” (substantial mis-localized GFP), and “very strong” (extensive mis-localized GFP). Each gRNA was tested through two rounds of transfection, with cells analyzed at three time points: 7-, 10-, and 14-days post-transfection. As a positive control, gRNAs targeting known essential peroxisomal assembly factors such as PEX5 and PEX19 were used (I, shown in Fig. 1E and supplementary Fig. 1D). This dual-round screening approach identified several key factors likely involved in peroxisomal biogenesis. One of the top candidates highlighted was ribosome-binding protein 1 (RRBP1) (I, shown in Fig. 1D).

This candidate showed significant mislocalization of GFP-APEX2-ePTS1 into the cytoplasm indicating its potential role in the proper assembly and function of peroxisomes (Fig. 1E and supplementary Fig. 1G).

4.2 Identification of RRBP1

A comprehensive double-screening approach identified RRBP1 as a factor potentially involved in peroxisomal biogenesis. RRBP1, also known as p180/ribosome receptor, is a transmembrane ER protein. RRBP1 was originally identified as a ribosome receptor for the ER (Savitz & Meyer, 1990); it was also proposed that RRBP1 can act as the ribosome receptor that regulates local translation in axons (Koppers et al., 2024). Recent studies suggest that RRBP1 plays a role in tethering ER with mitochondria (Anastasia et al., 2021; Hung et al., 2017) and regulates mitophagy (Killackey et al., 2023). However, RRBP1 has never been linked to peroxisomal function before.

To explore the role of RRBP1 in maintaining peroxisomes, an RRBP1 KO HEK293T clonal cell line expressing the GFP-APEX2-ePTS1 construct was generated through CRISPR/Cas9-mediated gene editing. A single guide RNA (gRNA) targeting exon 2, common to all isoforms of RRBP1, was utilized to achieve a complete knockout. The absence of RRBP1 protein in the KO cell line was confirmed via immunoblotting (I, Fig. 2A, D).

4.3 Validation of RRBP1's role in peroxisome biogenesis in RRBP1 KO cells

The RRBP1 KO HEK293T cells expressing GFP-APEX2-ePTS1 were analyzed and compared to their parental cell line. Additionally, cells lacking essential peroxisomal biogenesis factors, PEX3 and PEX19, as generated in our previous study (Somborac et al., 2023), were used as positive controls. Immunoblotting results indicated a decrease in the steady-state protein levels of peroxisomal matrix protein, catalase, and peroxisomal membrane proteins, PEX3, PEX14, PEX19 and PXMP2 in RRBP1 KO cells compared to the parental line (I, Fig. 2A, B and supplementary Fig. 3D, E). Interestingly, the level of PEX5, an essential peroxisomal matrix protein import factor, was upregulated in RRBP1 KO cells (I, Fig. 2A, B). PEX5 is a cytosolic peroxin essential for protein delivery into the peroxisomal matrix. Its induction in RRBP1 KO cells indicates a cellular response aimed at compensating for peroxisomal deficiency by upregulating peroxisomal biogenesis factors.

To assess peroxisomal function in RRBP1 KO cells, the import of peroxisomal matrix proteins was analyzed. While most peroxisomal proteins are translated in the cytosol in their mature form, some undergo processing upon import into peroxisomes. For instance, the 72 kDa cytosolic form of acyl-CoA oxidase 1 (ACOX1), a PTS1-targeted peroxisomal matrix protein, is cleaved into 50 kDa and 22 kDa forms upon import (Kurochkin et al., 2007). Similarly, the 44 kDa unprocessed form of 3-ketoacyl-CoA thiolase (ACAA1), a PTS2-targeted protein, is processed to 42 kDa upon import (Miura et al., 1984). Using immunoblotting, the processing of ACOX1 and ACAA1 was shown to be eliminated in cells lacking peroxisomes (PEX3 KO and PEX19 KO cells), validating the effectiveness of this assay in analyzing peroxisomal function (Klouwer et al., 2021; Kurochkin et al., 2007). In RRBP1 KO cells, a significant decrease in the processing of both ACOX1 and ACAA1 was observed (I, Fig. 2A, B, C).

It was also observed that PEX3 KO or PEX19 KO cells exhibit significantly lower RRBP1 protein levels compared to their parental cells suggesting that RRBP1 expression is responsive to the absence of peroxisomes (I, Fig 2A, D). Furthermore, the levels of calnexin and VAPB, ER proteins involved in the ER stress response, were slightly decreased in RRBP1 KO cells, indicating that RRBP1 KO affects the ER but does not induce ER stress (I, Fig. 2A, D). The steady-state level of the mitochondrial protein TOM20 remained unaffected in RRBP1 KO cells, suggesting that mitochondrial mass is not compromised by the absence of RRBP1 (I, Fig. 2A, E).

To determine whether the number of peroxisomes is affected by the knockout of RRBP1, RRBP1 KO HEK293T cells stably expressing GFP-APEX2-ePTS1 were analyzed alongside their parental cell line as a negative control. Live-cell fluorescent microscopy revealed that RRBP1 KO cells exhibited fewer GFP-positive dots representing peroxisomes compared to the parental cells (I, supplementary Fig. 1G). In addition, GFP-APEX2-ePTS1 displayed partial localization to the cytosol in cells lacking RRBP1, whereas in parental HEK293T cells, the construct was correctly localized to peroxisomes. This finding verifies the results from the previous CRISPR screen (I, Fig. 1D, E).

To further explore the impact of RRBP1 KO on peroxisome numbers, high-resolution immunofluorescence confocal microscopy was performed. We used PEX14 or PMP70 as

membrane markers and catalase as a matrix marker of peroxisomal structures. Cells lacking peroxisomes, such as PEX3 KO or PEX19 KO HEK293T cells, served as positive controls. The numbers of PEX14-, PMP70- or catalase-positive structures per cell was significantly reduced in RRBP1 KO cells compared to the parental cells (I, Fig. 4A- E). Notably, the cell volume was not affected by RRBP1 KO (I, Fig. 4F). These results confirmed that RRBP1 is indeed required for peroxisomal maintenance. The cell volume was not affected by RRBP1 KO (I, Fig. 4F), indicating that the reduction in peroxisomal structures was not due to changes in cell size.

The reduced number of peroxisomal structures, coupled with their partial mislocalization of GFP-APEX2-ePTS1 to the cytosol, and the impaired import of peroxisomal matrix proteins highlights a failure in the biogenesis process. These observations indicate that the import of peroxisomal matrix proteins and membrane proteins is compromised in the absence of RRBP1.

4.4 Validation of RRBP1 KO findings through silencing

To validate the findings obtained from RRBP1 KO cells, RRBP1 was knocked down using endoribonuclease-prepared small interfering RNA (esiRNA) in HEK293T cells stably expressing GFP-APEX2-ePTS1. GFP esiRNA was employed as a negative control, while esiRNA targeting a peroxisomal import factor essential for the assembly of functional peroxisomes, PEX5, served as a positive control. Immunoblotting analysis revealed that protein levels of ACAA1 and catalase were reduced upon RRBP1 silencing (I, Fig. 3A-D). The processing of ACOX1 was significantly decreased in cells transfected with RRBP1 esiRNA similar to PEX5 silencing (I, Fig. 3C). This finding reinforces the conclusion that RRBP1 is specifically involved in the maintenance of peroxisomes in human cells.

Live-cell fluorescent microscopy imaging demonstrated mislocalization of the GFP-APEX2-ePTS1 construct to the cytosol in cells transfected with RRBP1 esiRNA, compared to proper peroxisomal localization in control cells (I, Fig. 3E). This mislocalization is consistent with the observations made in RRBP1 KO cells. These findings indicate that acute depletion of RRBP1 by esiRNA is sufficient to disrupt peroxisomal protein import.

Altogether, these results show that RRBP1 depletion reduces key peroxisomal protein levels, disrupts matrix protein processing, and decreases peroxisome number, demonstrating that RRBP1 is a crucial factor involved in the maintenance of peroxisomes in human cells. However, RRBP1 loss does not cause complete absence of peroxisomes as seen in cells lacking essential peroxisomal biogenesis factors such as PEX3 and PEX19. This suggests that while RRBP1 contributes to peroxisomal biogenesis, it is not an essential factor.

In this study, we show that RRBP1 is required for maintaining peroxisome number and import competence, but its loss does not result in complete peroxisome absence. This distinguishes RRBP1 from core peroxins such as PEX3 and PEX19 and suggests that RRBP1 functions as an auxiliary factor that supports peroxisomal biogenesis rather than serving as an essential assembly component. Such partial defects in peroxisomal biogenesis may reflect mechanisms that allow cells to retain residual peroxisomal function under conditions where core biogenesis pathways remain intact.

4.5 RRBP1 KO does not induce pexophagy

The steady-state number of peroxisomes is significantly decreased upon RRBP1 KO. Since peroxisomal homeostasis is regulated by the balance between peroxisomal biogenesis and selective autophagy of peroxisomes, known as pexophagy (Demers et al., 2023; Germain & Kim, 2020; Sakai et al., 2006), the observed reduction in peroxisome number in RRBP1 KO cells could result from either impaired peroxisomal biogenesis or induced pexophagy leading to peroxisome degradation. To determine if pexophagy is induced in RRBP1 KO cells, the levels of autophagy markers LC3B and ATG5 were analyzed. The steady-state levels of these markers were found to be reduced in RRBP1 KO cells compared to the parental cell line (I, Fig. 7A, B). This reduction suggests that autophagy is not upregulated in the absence of RRBP1.

To further investigate the role of pexophagy, RRBP1 KO cells were treated with chloroquine (100 μM for 3 hours), an inhibitor that prevents the fusion of autophagosomes with lysosomes. The inhibition of autophagy was confirmed by the accumulation of LC3B-II, indicating the buildup of autophagosomes. Despite this treatment, there was no rescue of PEX3 or PMP70 protein levels in RRBP1 KO cells (I,

Fig. 7A, B). This indicates that blocking pexophagy does not restore peroxisome numbers in the absence of RRBP1. These findings suggest that pexophagy is not induced in RRBP1 KO cells and, therefore, does not contribute to the decreased number of peroxisomes. The reduction in peroxisome numbers in cells lacking RRBP1 is likely due to its crucial role in peroxisomal biogenesis rather than an increase in pexophagy.

4.6 RRBP1 does not localize to peroxisome

Our data showed that RRBP1 play role in peroxisome biogenesis. Previously, RRBP1 was described as an ER transmembrane protein (Ogawa-Goto et al., 2007; Savitz & Meyer, 1990; Shibata et al., 2010). Thus, the role of RRBP1 in peroxisomal biogenesis could be mediated through the ER. To investigate if RRBP1 is also localized to peroxisomes, a colocalization analysis was performed using confocal microscopy with antibodies labeling peroxisomes, ER, or mitochondria (I, Fig. 5A). Results showed that RRBP1 primarily localized to the ER, with some localization to mitochondria, and very weak colocalization with peroxisomes (I, Fig. 5A, B). To further confirm that RRBP1 does not localize to peroxisomes, we analyzed RRBP1 presence in peroxisomes fractionated by immunoprecipitation (IP) as described by (Ray et al., 2020). We generated clonal HEK293T cell lines stably expressing 3×HA-EGFP-PEX26 at either low or high levels and verified peroxisomal targeting of 3×HA-EGFP-PEX26 by confocal microscopy (I, Supplementary Fig. 2A). The IP procedure yielded highly purified peroxisomes, confirmed by the absence of ER (calnexin) and cytosolic (GAPDH) contamination (I, Fig. 5C). Importantly, RRBP1 was not detected in the peroxisomal fraction (I, Fig. 5C). Together, these results demonstrate that RRBP1 does not localize to peroxisomes; instead, its function in peroxisome biogenesis is mediated at the ER.

According to the classical model of peroxisome biogenesis, peroxisomal membranes originate from the ER as budding pre-peroxisomal vesicles (S. K. Lam et al., 2010). While peroxisomal matrix proteins are not detected in the ER (Geuze et al., 2003; Van Der Zand et al., 2010), APEX2-based proximity labeling suggests that RRBP1 can interact with PTS1-containing cargo proteins, implying a potential role in recruiting factors required for protein import. However, since RRBP1 does not localize to peroxisomes, it is unlikely that it directly mediate matrix protein import. Instead, RRBP1 may function indirectly by

facilitating the recruitment of other factors to the peroxisomal membrane that are essential for protein import.

4.7 RRBP1 KO causes peroxisomal deficiency independent of ER stress

Since RRBP1 is an ER protein that may influence protein translation and cell proliferation, we tested whether RRBP1 KO cells exhibited altered proliferation. RRBP1 KO did not affect cell proliferation (I, Supplementary Fig. 3A), and the protein translation rate measured by puromycin incorporation into newly synthesized proteins also remained unchanged (I, Supplementary Fig. 3B, C). However, we observed a reduction in specific ER proteins, including VAPB, calnexin, IRE1α, and BiP in RRBP1 KO cells compared to the parental cell line (I, Supplementary Fig. 3D, E; Fig.2A, D). This suggests a mild disruption of ER homeostasis. However, the levels of SEC61 and RPL7 were unaltered (I, Supplementary Fig. 3D, E) indicating that the overall machinery for protein synthesis and translocation as well as ribosomal assembly remains unaffected.

4.8 Peroxisome-ER contact sites are not affected in RRBP1-depleted cells

Recent studies suggest that RRBP1 can act as a tether in contact sites between ER and mitochondrial membranes ensuring metabolic communication between two organelles (Anastasia et al., 2021; Hung et al., 2017). To investigate the specific role of RRBP1 in maintaining peroxisome-ER contacts, electron microscopy was employed. APEX2, a powerful tool for labeling intracellular structures in electron microscopy studies (Lam et al., 2015; Martell et al., 2017; Rae et al., 2021; Sengupta et al., 2019), was used for DAB staining to label peroxisomes in RRBP1 KO HEK293T cells stably expressing GFP-APEX2-ePTS1 and their parental cell line (I, Fig. 6A). The occurrence of contact sites between peroxisomes and ER was assessed at distance 10-100 nm. We observed that neither RRBP1 silencing by esiRNA nor RRBP1 KO affected the number of contact sites between the ER and peroxisomes (I, Fig. 6C-D).

In addition, using electron microscopy images, peroxisomal morphology was analyzed upon RRBP1 KO. Although the size and shape of peroxisomes varied, there were no

changes in peroxisomal size or shape upon RRBP1 KO (I, Fig. 6F, G). It suggests that while RRBP1 is important for peroxisome number and protein import, it does not directly influence peroxisomal morphology, indicating its role is likely restricted to biogenesis or maintenance rather than structural organization.

4.9 Proteasomal degradation of peroxisomal proteins in RRBP1 KO

To investigate whether peroxisomal proteins undergo proteasomal degradation in the absence of RRBP1, we inhibited the proteasomal degradation pathway using MG132 treatment. Our results revealed that PEX3 and PEX16 were significantly restored in RRBP1 KO cells upon MG132 treatment (I, Fig 7C, D), suggesting that their reduction in RRBP1 KO cells is due to proteasomal degradation. Since both PEX3 and PEX16 play crucial roles in initiating peroxisomal *de novo* biogenesis via the ER (Kim et al., 2006), their recovery upon proteasomal inhibition indicates that RRBP1 may be involved in the insertion of these proteins into ER membranes. Furthermore, in the parental cell line, MG132 treatment resulted in a significant induction of RRBP1, suggesting that RRBP1 itself has a high turnover rate and may be subject to proteasomal degradation.

These findings highlight a potential regulatory mechanism in which RRBP1 helps maintain peroxisomal membrane protein stability, and its absence leads to increased degradation of key peroxisomal biogenesis factors. Our findings suggest that proteasomal degradation of peroxisomal membrane proteins represents a critical regulatory step in peroxisomal biogenesis. In the absence of RRBP1, key biogenesis factors such as PEX3 and PEX16 are destabilized and degraded, indicating that RRBP1 contributes to peroxisome maintenance by supporting the stabilization or membrane insertion of these proteins at the ER. This mechanism highlights an additional layer of regulation beyond transcriptional control and suggests that impaired proteostasis at the ER may contribute to peroxisomal dysfunction without directly affecting core peroxin expression.

4.10 Mitochondrial involvement in peroxisomal tail-anchored protein targeting

To complement the RRBP1 focused analysis above and to address the trafficking routes of peroxisomal membrane proteins, we conducted a proximity biotinylation assay to investigate the targeting pathways of tail-anchored (TA) proteins within mammalian cells. While TA protein targeting to peroxisomes is classically mediated by the PEX19-dependent pathway, we asked whether alternative routes, including ER-mediated trafficking, also contribute to their delivery in healthy cells. This method leverages the enzymatic activity of biotin ligase (BirA) to selectively biotinylate an acceptor protein sequence (AviTag) within the same cellular compartment. We generated cells stably expressing doxycycline-inducible BirA constructs specific to selected organelles (ER lumen, mitochondrial intermembrane space, and peroxisomal lumen) and AviTag-fused EGFP TA proteins localized to peroxisome or ER. The AviTag constructs were transiently transfected into Flp-In T-REx™ HEK293 cells stably expressing different organelle-targeted BirA constructs, with controlled expression levels achieved via the weak UBC promoter. By utilizing the leakiness of the doxycycline-inducible system and omitting doxycycline, we ensured the selectivity of the approach. Confocal immunofluorescence microscopy confirmed the organelle-specific localization of BirA and AviTag constructs.

Using streptavidin immunoblotting we showed that ER targeted AviTag was biotinylated exclusively in cells expressing BirA construct in ER but not in cells expressing BirA in mitochondria or peroxisomes (II, Fig 4E), validating the assay as a reliable tool to analyze the route of targeting TA proteins. Biotinylation of YgiM(TA)-AviTag, a mitochondrial outer-membrane tail-anchored reporter used here as a model substrate, occurred only in cells expressing either PO-BirA or IMS-BirA, supporting the hypothesis that this TA reporter reaches peroxisomes via a mitochondria associated route rather than through the ER in cells with functional.

These findings are supported by several other complementary approaches that we used in this study. Thus, glycosylation assays showed that peroxisomal TA proteins do not acquire ER-specific modifications, indicating that they do not transit through the ER, even when peroxisomes are absent in the cells. Confocal colocalization analysis also

revealed that in wild-type cells, peroxisomal TA proteins localize predominantly to peroxisomes, whereas in cells lacking peroxisomes, they shift primarily to mitochondria, with little or no ER overlap. Immunogold electron microscopy provided high-resolution confirmation of this mitochondrial targeting in peroxisome-deficient cells. These results suggest that peroxisomal TA proteins exhibit mitochondrial targeting regardless of peroxisome presence, indicating a potential role for mitochondria in their routing. We demonstrated that in mammalian cells with functional peroxisomes, exogenous peroxisomal TA proteins interact with mitochondria, suggesting that what was previously thought to be mislocalization to mitochondria might be part of the native targeting pathway for peroxisomes. This aligns with reports suggesting a dual origin for newly assembled peroxisomes from both ER and mitochondria (Sugiura et al., 2017). However, alternative interpretations should be considered, such as dual localization to both peroxisomes and mitochondria, indicating a non-linear pathway. This dual localization concept adds complexity to our understanding of peroxisomal TA protein routing and challenges the conventional perspective on organelle-specific targeting.

5 CONCLUSION AND PROSPECTS

This study reveals the critical role of RRBP1 (ribosome receptor, p180) in peroxisomal biogenesis and its maintenance in human cells. RRBP1 was previously characterized as an endoplasmic reticulum transmembrane protein with ribosome-binding and microtubule interacting functions. However, the research carried out in my thesis demonstrates that RRBP1 is also involved in the biogenesis and maintenance of peroxisomes, uncovering a previously unrecognized function of this protein.

The identification of RRBP1 as a key player in peroxisomal biogenesis opens several avenues for future research. One hypothesis emerging from this work is that RRBP1 functions at an early stage of peroxisomal membrane formation at the endoplasmic reticulum by spatially coupling ER associated ribosomes to the handling of newly synthesized peroxisomal membrane proteins. Rather than affecting global protein synthesis, RRBP1 may facilitate the efficient membrane engagement, stabilization, or maturation of selected peroxisomal membrane proteins at ER subdomains where pre-peroxisomal membrane structures are formed. In the absence of RRBP1, these nascent pre-peroxisomal intermediates may initiate at the ER but fail to properly mature or detach, leading to impaired protein entry into the peroxisomal biogenesis pathway and increased susceptibility of peroxisomal membrane proteins to proteasomal degradation. However, mechanistic studies are still needed to elucidate the precise molecular mechanisms by which RRBP1 facilitates peroxisomal biogenesis, and to understand how RRBP1 interacts with other proteins and lipids at ER-peroxisome contact sites. Investigating RRBP1 in context of peroxisomal disorders could also reveal new therapeutic targets for diseases related to peroxisomal dysfunction. Exploring how RRBP1 coordinates communication and metabolic exchange between the ER, peroxisomes, and other organelles like mitochondria could uncover broader aspects of cellular organization and homeostasis. Studying potential compensatory mechanisms and redundant pathways that might mitigate the effects of RRBP1 loss could provide insights into cellular resilience and adaptation mechanisms. High-resolution structural studies of RRBP1 and its complexes at the ER-peroxisome interface could reveal detailed conformational changes and interaction sites critical for its function.

The proximity biotinylation-based approach demonstrated mitochondrial involvement in the trafficking of peroxisomal TA proteins. The biotinylation patterns indicated that peroxisomal TA proteins target both peroxisomes and mitochondria in cells with functional peroxisomes. These findings suggest a potential role for mitochondria in the routing of peroxisomal TA proteins, challenging prior assumptions regarding their targeting routes. Our study highlights the intricate pathways involved in the trafficking of peroxisomal TA proteins, revealing a significant role for mitochondria. This mitochondrial involvement suggests that the traditional view of peroxisomal TA protein targeting, which primarily considered direct cytosolic routes and ER pathways, may be incomplete. These results necessitate a reconsideration of the mechanisms governing peroxisomal TA protein trafficking and emphasize the need for further exploration of mitochondrial contributions to this process.

Building on the TA-protein findings, future research should focus on several key areas to build on these findings. The role of the ER in peroxisomal TA protein trafficking in mammalian cells remains a subject of debate. Beyond mitochondria and the ER, other cellular compartments and signaling pathways may play roles in TA protein trafficking. Exploring these additional pathways could offer a more comprehensive understanding of the process. Detailed mechanistic studies are needed to understand how mitochondria facilitate the transfer of TA proteins to peroxisomes. This could involve investigating the specific molecular interactions and signaling events that mediate this process. Understanding the functional implications of dual localization to both peroxisomes and mitochondria could provide insights into the physiological roles of TA proteins and how their mislocalization might contribute to disease. Employing advanced imaging techniques, such as live-cell fluorescence microscopy of genomically tagged proteins, could help visualize the dynamic trafficking pathways of TA proteins in real-time, providing further clarity on their intracellular routing.
Overall, this work reveals previously unrecognized aspects of peroxisomal biology and highlights the intricate connections between peroxisomes, ER, and mitochondria. It is hoped that these findings, together with future studies in the field, will contribute to a deeper understanding of peroxisome related disorders and the cellular mechanisms that maintain organelle homeostasis.

6 REFERENCES

Agne, B., Meindl, N. M., Niederhoff, K., Einwächter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W., & Kunau, W.-H. (2003). Pex8p: An Intraperoxisomal Organizer of the Peroxisomal Import Machinery. *Molecular Cell*, *11*(3), 635–646. https://doi.org/10.1016/S1097-2765(03)00062-5

Agrawal, G., & Subramani, S. (2013). Emerging role of the endoplasmic reticulum in peroxisome biogenesis. *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00286

Agrawal, G., & Subramani, S. (2016). De novo peroxisome biogenesis: Evolving concepts and conundrums. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(5), 892–901. https://doi.org/10.1016/j.bbamcr.2015.09.014

Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A. K. W., Veenhuis, M., & Kunau, W.-H. (1997). Pex14p, a Peroxisomal Membrane Protein Binding Both Receptors of the Two PTS-Dependent Import Pathways. *Cell*, *89*(1), 83–92. https://doi.org/10.1016/S0092-8674(00)80185-3

Anastasia, I., Ilacqua, N., Raimondi, A., Lemieux, P., Ghandehari-Alavijeh, R., Faure, G., Mekhedov, S. L., Williams, K. J., Caicci, F., Valle, G., Giacomello, M., Quiroga, A. D., Lehner, R., Miksis, M. J., Toth, K., De Aguiar Vallim, T. Q., Koonin, E. V., Scorrano, L., & Pellegrini, L. (2021). Mitochondria-rough-ER contacts in the liver regulate systemic lipid homeostasis. *Cell Reports*, *34*(11), 108873. https://doi.org/10.1016/j.celrep.2021.108873

Antonenkov, V. D., Grunau, S., Ohlmeier, S., & Hiltunen, J. K. (2010). Peroxisomes Are Oxidative Organelles. *Antioxidants & Redox Signaling*, *13*(4), 525–537. https://doi.org/10.1089/ars.2009.2996

Arent, S., Christensen, C. E., Pye, V. E., Nørgaard, A., & Henriksen, A. (2010). The Multifunctional Protein in Peroxisomal β-Oxidation. *Journal of Biological Chemistry*, *285*(31), 24066–24077. https://doi.org/10.1074/jbc.M110.106005

Ast, J., Stiebler, A. C., Freitag, J., & Bölker, M. (2013). Dual targeting of peroxisomal proteins. *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00297

Aufschnaiter, A., & Büttner, S. (2015). Peroxisomal fission controls yeast life span. *Cell Cycle*, *14*(15), 2389–2390. https://doi.org/10.1080/15384101.2015.1063303

Bartoszewska, M., Opaliński, Ł., Veenhuis, M., & Van Der Klei, I. J. (2011). The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotechnology Letters*, *33*(10), 1921–1931. https://doi.org/10.1007/s10529-011-0664-y

Beach, A., Burstein, M. T., Richard, V. R., Leonov, A., Levy, S., & Titorenko, V. I. (2012). Integration of peroxisomes into an endomembrane system that governs cellular aging. *Frontiers in Physiology*, *3*. https://doi.org/10.3389/fphys.2012.00283

Bhogal, M. S., Lanyon-Hogg, T., Johnston, K. A., Warriner, S. L., & Baker, A. (2016). Covalent Label Transfer between Peroxisomal Importomer Components Reveals Export-driven Import Interactions. *Journal of Biological Chemistry*, *291*(5), 2460–2468. https://doi.org/10.1074/jbc.M115.686501

Borgese, N., Colombo, S., & Pedrazzini, E. (2003). The tale of tail-anchored proteins. *The Journal of Cell Biology*, *161*(6), 1013–1019. https://doi.org/10.1083/jcb.200303069

Borgese, N., & Fasana, E. (2011). Targeting pathways of C-tail-anchored proteins. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(3), 937–946. https://doi.org/10.1016/j.bbamem.2010.07.010

Bowers, W. E. (1998). Christian de Duve and the discovery of lysosomes and peroxisomes. *Trends in Cell Biology*, *8*(8), 330–333. https://doi.org/10.1016/S0962-8924(98)01314-2

Brown, L., & Baker, A. (2003). Peroxisome biogenesis and the role of protein import. *Journal of Cellular and Molecular Medicine*, *7*(4), 388–400. https://doi.org/10.1111/j.1582-4934.2003.tb00241.x

Bryers, J. D., & Yeh, T. (1990). Diauxic Metabolism of *Hansenula polymorpha*: Steady- and Unsteady-State Considerations. *Annals of the New York Academy of Sciences*, *589*(1), 315–332. https://doi.org/10.1111/j.1749-6632.1990.tb24255.x

Carmichael, R. E., Islinger, M., & Schrader, M. (2022). Fission Impossible (?)—New Insights into Disorders of Peroxisome Dynamics. *Cells*, *11*(12), 1922. https://doi.org/10.3390/cells11121922

Castro, I. G., Schuldiner, M., & Zalckvar, E. (2018). Mind the Organelle Gap – Peroxisome Contact Sites in Disease. *Trends in Biochemical Sciences*, *43*(3), 199–210. https://doi.org/10.1016/j.tibs.2018.01.001

Chen, C. T., Shao, Z., & Fu, Z. (2022). Dysfunctional peroxisomal lipid metabolisms and their ocular manifestations. *Frontiers in Cell and Developmental Biology*, *10*, 982564. https://doi.org/10.3389/fcell.2022.982564

Chen, W. W., Rodrigues, T. A., Wendscheck, D., Pedrosa, A. G., Yang, C., Francisco, T., Möcklinghoff, T., Zografakis, A., Nunes-Silva, B., Avraham, R. E., Silva, A. R., Ferreira, M. J., Das, H., Koster, J., Neuwirth, S., Bender, J., Oeljeklaus, S., Sondhi, V., Gatsogiannis, C., … Warscheid, B. (2025). PEX39 facilitates the peroxisomal import of PTS2-containing proteins. *Nature Cell Biology*, *27*(8), 1256–1271. https://doi.org/10.1038/s41556-025-01711-z

Costello, J. L., Castro, I. G., Camões, F., Schrader, T. A., McNeall, D., Yang, J., Giannopoulou, E.-A., Gomes, S., Pogenberg, V., Bonekamp, N. A., Ribeiro, D., Wilmanns, M., Jedd, G., Islinger, M., & Schrader, M. (2017). Predicting the targeting of tail-anchored proteins to subcellular compartments in mammalian cells. *Journal of Cell Science*, *130*(9), 1675–1687. https://doi.org/10.1242/jcs.200204

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

Craney, A., Tahlan, K., Andrews, D., & Nodwell, J. (2011). Bacterial Transmembrane Proteins that Lack N-Terminal Signal Sequences. *PLoS ONE*, *6*(5), e19421. https://doi.org/10.1371/journal.pone.0019421

Dahan, N., Francisco, T., Falter, C., Rodrigues, T., Kalel, V., Kunze, M., Hansen, T., Schliebs, W., & Erdmann, R. (2021). Current advances in the function and biogenesis of peroxisomes and their roles in health and disease. *Histochemistry and Cell Biology*, *155*(4), 513–524. https://doi.org/10.1007/s00418-021-01982-1

Dawes, M. L., Haberlander, J. P., Islinger, M., & Schrader, M. (2025). The neurological pathology of peroxisomal ACBD5 deficiency – lessons from patients and mouse models. *Frontiers in Molecular Neuroscience*, *18*, 1602343. https://doi.org/10.3389/fnmol.2025.1602343

Del Río, L. A., Corpas, F. J., Sandalio, L. M., Palma, J. M., & Barroso, J. B. (2003). Plant Peroxisomes, Reactive Oxygen Metabolism and Nitric Oxide. *IUBMB Life*, *55*(2), 71–81. https://doi.org/10.1002/tbmb.718540875

Demarquoy, J. (2015). Crosstalk between mitochondria and peroxisomes. *World Journal of Biological Chemistry*, *6*(4), 301. https://doi.org/10.4331/wjbc.v6.i4.301

Demers, N. D., Riccio, V., Jo, D. S., Bhandari, S., Law, K. B., Liao, W., Kim, C., McQuibban, G. A., Choe, S.-K., Cho, D.-H., & Kim, P. K. (2023). PEX13 prevents pexophagy by regulating ubiquitinated PEX5 and peroxisomal ROS. *Autophagy*, *19*(6), 1781–1802. https://doi.org/10.1080/15548627.2022.2160566

Deter, R. L., Baudhuin, P., & De Duve, C. (1967). PARTICIPATION OF LYSOSOMES IN CELLULAR AUTOPHAGY INDUCED IN RAT LIVER BY GLUCAGON. *The Journal of Cell Biology*, *35*(2), C11–C16. https://doi.org/10.1083/jcb.35.2.C11

Dimitrov, L., Lam, S. K., & Schekman, R. (2013). The Role of the Endoplasmic Reticulum in Peroxisome Biogenesis. *Cold Spring Harbor Perspectives in Biology*, *5*(5), a013243–a013243. https://doi.org/10.1101/cshperspect.a013243

Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A., Kunau, W. H., Lazarow, P. B., Mannaerts, G. P., Moser, H. W., Osumi, T., Rachubinski, R. A., Roscher, A., Subramani, S., … Veenhuis, M. (1996). A unified nomenclature for peroxisome biogenesis factors. *The Journal of Cell Biology*, *135*(1), 1–3. https://doi.org/10.1083/jcb.135.1.1

Ekal, L., Alqahtani, A. M. S., & Hettema, E. H. (2023). The dynamin-related protein Vps1 and the peroxisomal membrane protein Pex27 function together during peroxisome fission. *Journal of Cell Science*, *136*(6), jcs246348. https://doi.org/10.1242/jcs.246348

Elgersma, Y. (1997). Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in S.cerevisiae, causes proliferation of the endoplasmic reticulum membrane. *The EMBO Journal*, *16*(24), 7326–7341. https://doi.org/10.1093/emboj/16.24.7326

Elsner, M., Gehrmann, W., & Lenzen, S. (2011). Peroxisome-Generated Hydrogen Peroxide as Important Mediator of Lipotoxicity in Insulin-Producing Cells. *Diabetes*, *60*(1), 200–208. https://doi.org/10.2337/db09-1401

Farré, J., Mahalingam, S. S., Proietto, M., & Subramani, S. (2019a). Peroxisome biogenesis, membrane contact sites, and quality control. *EMBO Reports*, *20*(1), e46864. https://doi.org/10.15252/embr.201846864

Farré, J., Mahalingam, S. S., Proietto, M., & Subramani, S. (2019b). Peroxisome biogenesis, membrane contact sites, and quality control. *EMBO Reports*, *20*(1), e46864. https://doi.org/10.15252/embr.201846864

Frederiks, W. M., Bosch, K. S., Hoeben, K. A., Van Marle, J., & Langbein, S. (2010). Renal cell carcinoma and oxidative stress: The lack of peroxisomes. *Acta Histochemica*, *112*(4), 364–371. https://doi.org/10.1016/j.acthis.2009.03.003

Fujiki, Y., Okumoto, K., Mukai, S., Honsho, M., & Tamura, S. (2014). Peroxisome biogenesis in mammalian cells. *Frontiers in Physiology*, *5*. https://doi.org/10.3389/fphys.2014.00307

Gao, Y., Skowyra, M. L., Feng, P., & Rapoport, T. A. (2022). Protein import into peroxisomes occurs through a nuclear pore–like phase. *Science*, *378*(6625), eadf3971. https://doi.org/10.1126/science.adf3971

Germain, K., & Kim, P. K. (2020). Pexophagy: A Model for Selective Autophagy. *International Journal of Molecular Sciences*, *21*(2), 578. https://doi.org/10.3390/ijms21020578

Gould, S. J., & Collins, C. S. (2002). Peroxisomal-protein import: Is it really that complex? *Nature Reviews Molecular Cell Biology*, *3*(5), 382–389. https://doi.org/10.1038/nrm807

Hasan, S., Platta, H. W., & Erdmann, R. (2013). Import of proteins into the peroxisomal matrix. *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00261

Hattula, K., Hirschberg, D., Kalkkinen, N., Butcher, S. J., & Ora, A. (2014). Association between the Intrinsically Disordered Protein PEX19 and PEX3. *PLoS ONE*, *9*(7), e103101. https://doi.org/10.1371/journal.pone.0103101

Hettema, E. H., Erdmann, R., Van Der Klei, I., & Veenhuis, M. (2014). Evolving models for peroxisome biogenesis. *Current Opinion in Cell Biology*, *29*, 25–30. https://doi.org/10.1016/j.ceb.2014.02.002

Hettema, E. H., Girzalsky, W., Van Den Berg, M., Erdmann, R., & Distel, B. (2000). Saccharomyces cerevisiae Pex3p and Pex19p are required for proper localization and stability of peroxisomal membrane proteins. *The EMBO Journal*, *19*(2), 223–233. https://doi.org/10.1093/emboj/19.2.223

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., & Tabak, H. F. (2005). Contribution of the Endoplasmic Reticulum to Peroxisome Formation. *Cell*, *122*(1), 85–95. https://doi.org/10.1016/j.cell.2005.04.025

Hu, J., Aguirre, M., Peto, C., Alonso, J., Ecker, J., & Chory, J. (2002). A Role for Peroxisomes in Photomorphogenesis and Development of *Arabidopsis*. *Science*, *297*(5580), 405–409. https://doi.org/10.1126/science.1073633

Hu, J., Baker, A., Bartel, B., Linka, N., Mullen, R. T., Reumann, S., & Zolman, B. K. (2012). Plant Peroxisomes: Biogenesis and Function. *The Plant Cell*, *24*(6), 2279–2303. https://doi.org/10.1105/tpc.112.096586

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., Vissa, A., Yip, C. M., Fairn, G. D., Braverman, N., Brumell, J. H., Trimble, W. S., Raught, B., & Kim, P. K. (2017). VAPs and ACBD5 tether peroxisomes to the ER for peroxisome maintenance and lipid homeostasis. *Journal of Cell Biology*, *216*(2), 367–377. https://doi.org/10.1083/jcb.201608128

Hua, R., & Kim, P. K. (2016). Multiple paths to peroxisomes: Mechanism of peroxisome maintenance in mammals. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(5), 881–891. https://doi.org/10.1016/j.bbamcr.2015.09.026

Hung, V., Lam, S. S., Udeshi, N. D., Svinkina, T., Guzman, G., Mootha, V. K., Carr, S. A., & Ting, A. Y. (2017). Proteomic mapping of cytosol-facing outer mitochondrial and ER membranes in living human cells by proximity biotinylation. *eLife*, *6*, e24463. https://doi.org/10.7554/eLife.24463

Igamberdiev, A. U., & Lea, P. J. (2002). The role of peroxisomes in the integration of metabolism and evolutionary diversity of photosynthetic organisms. *Phytochemistry*, *60*(7), 651–674. https://doi.org/10.1016/S0031-9422(02)00179-6

Islinger, M., Grille, S., Fahimi, H. D., & Schrader, M. (2012). The peroxisome: An update on mysteries. *Histochemistry and Cell Biology*, *137*(5), 547–574. https://doi.org/10.1007/s00418-012-0941-4

Islinger, M., Voelkl, A., Fahimi, H. D., & Schrader, M. (2018). The peroxisome: An update on mysteries 2.0. *Histochemistry and Cell Biology*, *150*(5), 443–471. https://doi.org/10.1007/s00418-018-1722-5

Jansen, R. L. M., Santana-Molina, C., Van Den Noort, M., Devos, D. P., & Van Der Klei, I. J. (2021). Comparative Genomics of Peroxisome Biogenesis Proteins: Making Sense of the PEX Proteins.

Frontiers in Cell and Developmental Biology, *9*, 654163. https://doi.org/10.3389/fcell.2021.654163

Jansen, R. L. M., & Van Der Klei, I. J. (2019). The peroxisome biogenesis factors Pex3 and Pex19: Multitasking proteins with disputed functions. *FEBS Letters*, *593*(5), 457–474. https://doi.org/10.1002/1873-3468.13340

Jedd, G. (2011). Fungal evo–devo: Organelles and multicellular complexity. *Trends in Cell Biology*, *21*(1), 12–19. https://doi.org/10.1016/j.tcb.2010.09.001

Jones, J. M., Morrell, J. C., & Gould, S. J. (2004). PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. *The Journal of Cell Biology*, *164*(1), 57–67. https://doi.org/10.1083/jcb.200304111

Joshi, A. S. (2021). Peroxisomal Membrane Contact Sites in Yeasts. *Frontiers in Cell and Developmental Biology*, *9*, 735031. https://doi.org/10.3389/fcell.2021.735031

Kaur, N., Li, J., & Hu, J. (2013). Peroxisomes and Photomorphogenesis. In L. A. Del Río (Ed.), *Peroxisomes and their Key Role in Cellular Signaling and Metabolism* (Vol. 69, pp. 195–211). Springer Netherlands. https://doi.org/10.1007/978-94-007-6889-5_11

Kempiński, B., Chełstowska, A., Poznański, J., Król, K., Rymer, Ł., Frydzińska, Z., Girzalsky, W., Skoneczna, A., Erdmann, R., & Skoneczny, M. (2020). The Peroxisomal Targeting Signal 3 (PTS3) of the Budding Yeast Acyl-CoA Oxidase Is a Signal Patch. *Frontiers in Cell and Developmental Biology*, *8*, 198. https://doi.org/10.3389/fcell.2020.00198

Killackey, S. A., Bi, Y., Philpott, D. J., Arnoult, D., & Girardin, S. E. (2023). Mitochondria-ER cooperation: NLRX1 detects mitochondrial protein import stress and promotes mitophagy through the ER protein RRBP1. *Autophagy*, *19*(5), 1601–1603. https://doi.org/10.1080/15548627.2022.2129763

Kim, P. K., & Hettema, E. H. (2015). Multiple Pathways for Protein Transport to Peroxisomes. *Journal of Molecular Biology*, *427*(6), 1176–1190. https://doi.org/10.1016/j.jmb.2015.02.005

Kim, P. K., & Mullen, R. T. (2013). PEX16: A multifaceted regulator of peroxisome biogenesis. *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00241

Kim, P. K., Mullen, R. T., Schumann, U., & Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *The Journal of Cell Biology*, *173*(4), 521–532. https://doi.org/10.1083/jcb.200601036

Klouwer, F. C. C., Falkenberg, K. D., Ofman, R., Koster, J., Van Gent, D., Ferdinandusse, S., Wanders, R. J. A., & Waterham, H. R. (2021). Autophagy Inhibitors Do Not Restore Peroxisomal Functions in Cells With the Most Common Peroxisome Biogenesis Defect. *Frontiers in Cell and Developmental Biology*, *9*, 661298. https://doi.org/10.3389/fcell.2021.661298

Koch, J., & Brocard, C. (2011). Membrane elongation factors in organelle maintenance: The case of peroxisome proliferation. *BioMolecular Concepts*, *2*(5), 353–364. https://doi.org/10.1515/BMC.2011.031

Koch, J., Pranjic, K., Huber, A., Ellinger, A., Hartig, A., Kragler, F., & Brocard, C. (2010). PEX11 family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance. *Journal of Cell Science*, *123*(19), 3389–3400. https://doi.org/10.1242/jcs.064907

Koppers, M., Özkan, N., Nguyen, H. H., Jurriens, D., McCaughey, J., Nguyen, D. T. M., Li, C. H., Stucchi, R., Altelaar, M., MacGillavry, H. D., Kapitein, L. C., Hoogenraad, C. C., & Farías, G. G. (2024). Axonal endoplasmic reticulum tubules control local translation via P180/RRBP1-mediated ribosome interactions. *Developmental Cell*, *59*(16), 2053-2068.e9. https://doi.org/10.1016/j.devcel.2024.05.005

Kors, S., Hacker, C., Bolton, C., Maier, R., Reimann, L., Kitchener, E. J. A., Warscheid, B., Costello, J. L., & Schrader, M. (2022). Regulating peroxisome–ER contacts via the ACBD5-VAPB tether by FFAT motif phosphorylation and GSK3β. *Journal of Cell Biology*, *221*(3), e202003143. https://doi.org/10.1083/jcb.202003143

Kubo, Y. (2013). Function of Peroxisomes in Plant-Pathogen Interactions. In L. A. Del Río (Ed.), *Peroxisomes and their Key Role in Cellular Signaling and Metabolism* (Vol. 69, pp. 329–345). Springer Netherlands. https://doi.org/10.1007/978-94-007-6889-5_18

Kumar, R., Islinger, M., Worthy, H., Carmichael, R., & Schrader, M. (2024). The peroxisome: An update on mysteries 3.0. *Histochemistry and Cell Biology*, *161*(2), 99–132. https://doi.org/10.1007/s00418-023-02259-5

Kunze, M., Malkani, N., Maurer-Stroh, S., Wiesinger, C., Schmid, J. A., & Berger, J. (2015). Mechanistic Insights into PTS2-mediated Peroxisomal Protein Import. *Journal of Biological Chemistry*, *290*(8), 4928–4940. https://doi.org/10.1074/jbc.M114.601575

Kurochkin, I. V., Mizuno, Y., Konagaya, A., Sakaki, Y., Schönbach, C., & Okazaki, Y. (2007). Novel peroxisomal protease Tysnd1 processes PTS1- and PTS2-containing enzymes involved in β-oxidation of fatty acids. *The EMBO Journal*, *26*(3), 835–845. https://doi.org/10.1038/sj.emboj.7601525

Lam, S. S., Martell, J. D., Kamer, K. J., Deerinck, T. J., Ellisman, M. H., Mootha, V. K., & Ting, A. Y. (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature Methods*, *12*(1), 51–54. https://doi.org/10.1038/nmeth.3179

Lazarow, P. B., & Fujiki, Y. (1985). Biogenesis of Peroxisomes. *Annual Review of Cell Biology*, *1*(1), 489–530. https://doi.org/10.1146/annurev.cb.01.110185.002421

Li, X., Baumgart, E., Morrell, J. C., Jimenez-Sanchez, G., Valle, D., & Gould, S. J. (2002). PEX11β Deficiency Is Lethal and Impairs Neuronal Migration but Does Not Abrogate Peroxisome Function. *Molecular and Cellular Biology*, *22*(12), 4358–4365. https://doi.org/10.1128/MCB.22.12.4358-4365.2002

Lisenbee, C. S., Heinze, M., & Trelease, R. N. (2003). Peroxisomal Ascorbate Peroxidase Resides within a Subdomain of Rough Endoplasmic Reticulum in Wild-Type Arabidopsis Cells. *Plant Physiology*, *132*(2), 870–882. https://doi.org/10.1104/pp.103.019976

Lismont, C., Revenco, I., & Fransen, M. (2019). Peroxisomal Hydrogen Peroxide Metabolism and Signaling in Health and Disease. *International Journal of Molecular Sciences*, *20*(15), 3673. https://doi.org/10.3390/ijms20153673

Liu, Y., Yagita, Y., & Fujiki, Y. (2016). Assembly of Peroxisomal Membrane Proteins via the Direct Pex19p-Pex3p Pathway. *Traffic*, *17*(4), 433–455. https://doi.org/10.1111/tra.12376

Ma, C., & Subramani, S. (2009). Peroxisome matrix and membrane protein biogenesis. *IUBMB Life*, *61*(7), 713–722. https://doi.org/10.1002/iub.196

Martell, J. D., Deerinck, T. J., Lam, S. S., Ellisman, M. H., & Ting, A. Y. (2017). Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. *Nature Protocols*, *12*(9), 1792–1816. https://doi.org/10.1038/nprot.2017.065

Martin, W. (2010). Evolutionary origins of metabolic compartmentalization in eukaryotes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1541), 847–855. https://doi.org/10.1098/rstb.2009.0252

Masters, C., & Holmes, R. (1977). Peroxisomes: New aspects of cell physiology and biochemistry. *Physiological Reviews*, *57*(4), 816–882. https://doi.org/10.1152/physrev.1977.57.4.816

Matsuzaki, T., & Fujiki, Y. (2008). The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway. *The Journal of Cell Biology*, *183*(7), 1275–1286. https://doi.org/10.1083/jcb.200806062

Mayerhofer, P. U. (2016). Targeting and insertion of peroxisomal membrane proteins: ER trafficking versus direct delivery to peroxisomes. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(5), 870–880. https://doi.org/10.1016/j.bbamcr.2015.09.021

Mayerhofer, P. U., Bañó-Polo, M., Mingarro, I., & Johnson, A. E. (2016). Human Peroxin PEX3 Is Co-translationally Integrated into the ER and Exits the ER in Budding Vesicles. *Traffic*, *17*(2), 117–130. https://doi.org/10.1111/tra.12350

Mehlhorn, D. G., Asseck, L. Y., & Grefen, C. (2021). Looking for a safe haven: Tail-anchored proteins and their membrane insertion pathways. *Plant Physiology*, *187*(4), 1916–1928. https://doi.org/10.1093/plphys/kiab298

Meinecke, M., Cizmowski, C., Schliebs, W., Krüger, V., Beck, S., Wagner, R., & Erdmann, R. (2010). The peroxisomal importomer constitutes a large and highly dynamic pore. *Nature Cell Biology*, *12*(3), 273–277. https://doi.org/10.1038/ncb2027

Miura, S., Mori, M., Takiguchi, M., Tatibana, M., Furuta, S., Miyazawa, S., & Hashimoto, T. (1984). Biosynthesis and intracellular transport of enzymes of peroxisomal beta-oxidation. *Journal of Biological Chemistry*, *259*(10), 6397–6402. https://doi.org/10.1016/S0021-9258(20)82155-3

Mohanty, A., & McBride, H. M. (2013). Emerging roles of mitochondria in the evolution, biogenesis, and function of peroxisomes. *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00268

Motley, A. M., & Hettema, E. H. (2007). Yeast peroxisomes multiply by growth and division. *The Journal of Cell Biology*, *178*(3), 399–410. https://doi.org/10.1083/jcb.200702167

Mullen, R. T., & Trelease, R. N. (2000). The Sorting Signals for Peroxisomal Membrane-bound Ascorbate Peroxidase Are within Its C-terminal Tail. *Journal of Biological Chemistry*, *275*(21), 16337–16344. https://doi.org/10.1074/jbc.M001266200

Ogawa-Goto, K., Tanaka, K., Ueno, T., Tanaka, K., Kurata, T., Sata, T., & Irie, S. (2007). P180 Is Involved in the Interaction between the Endoplasmic Reticulum and Microtubules through a Novel Microtubule-binding and Bundling Domain. *Molecular Biology of the Cell*, *18*(10), 3741–3751. https://doi.org/10.1091/mbc.e06-12-1125

Okumoto, K., Tamura, S., Honsho, M., & Fujiki, Y. (2020). Peroxisome: Metabolic Functions and Biogenesis. In G. Lizard (Ed.), *Peroxisome Biology: Experimental Models, Peroxisomal Disorders and Neurological Diseases* (Vol. 1299, pp. 3–17). Springer International Publishing. https://doi.org/10.1007/978-3-030-60204-8_1

Pan, R., Liu, J., & Hu, J. (2019). Peroxisomes in plant reproduction and seed-related development. *Journal of Integrative Plant Biology*, *61*(7), 784–802. https://doi.org/10.1111/jipb.12765

Pan, R., Liu, J., Wang, S., & Hu, J. (2020). Peroxisomes: Versatile organelles with diverse roles in plants. *New Phytologist*, *225*(4), 1410–1427. https://doi.org/10.1111/nph.16134

Parsons, M. (2004). Glycosomes: Parasites and the divergence of peroxisomal purpose. *Molecular Microbiology*, *53*(3), 717–724. https://doi.org/10.1111/j.1365-2958.2004.04203.x

Pinto, M. P., Grou, C. P., Alencastre, I. S., Oliveira, M. E., Sá-Miranda, C., Fransen, M., & Azevedo, J. E. (2006). The Import Competence of a Peroxisomal Membrane Protein Is Determined by Pex19p before the Docking Step. *Journal of Biological Chemistry*, *281*(45), 34492–34502. https://doi.org/10.1074/jbc.M607183200

Platta, H. W., El Magraoui, F., Bäumer, B. E., Schlee, D., Girzalsky, W., & Erdmann, R. (2009). Pex2 and Pex12 Function as Protein-Ubiquitin Ligases in Peroxisomal Protein Import. *Molecular and Cellular Biology*, *29*(20), 5505–5516. https://doi.org/10.1128/MCB.00388-09

Poirier, Y., Antonenkov, V. D., Glumoff, T., & Hiltunen, J. K. (2006). Peroxisomal β-oxidation—A metabolic pathway with multiple functions. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1763*(12), 1413–1426. https://doi.org/10.1016/j.bbamcr.2006.08.034

Prinz, W. A., Toulmay, A., & Balla, T. (2020). The functional universe of membrane contact sites. *Nature Reviews Molecular Cell Biology*, *21*(1), 7–24. https://doi.org/10.1038/s41580-019-0180-9

Rae, J., Ferguson, C., Ariotti, N., Webb, R. I., Cheng, H.-H., Mead, J. L., Riches, J. D., Hunter, D. J., Martel, N., Baltos, J., Christopoulos, A., Bryce, N. S., Cagigas, M. L., Fonseka, S., Sayre, M. E., Hardeman, E. C., Gunning, P. W., Gambin, Y., Hall, T. E., & Parton, R. G. (2021). A robust method for particulate detection of a genetic tag for 3D electron microscopy. *eLife*, *10*, e64630. https://doi.org/10.7554/eLife.64630

Ramón, N. M., & Bartel, B. (2010). Interdependence of the Peroxisome-targeting Receptors in *Arabidopsis thaliana*: PEX7 Facilitates PEX5 Accumulation and Import of PTS1 Cargo into Peroxisomes. *Molecular Biology of the Cell*, *21*(7), 1263–1271. https://doi.org/10.1091/mbc.e09-08-0672

Ratbi, I., Falkenberg, K. D., Sommen, M., Al-Sheqaih, N., Guaoua, S., Vandeweyer, G., Urquhart, J. E., Chandler, K. E., Williams, S. G., Roberts, N. A., El Alloussi, M., Black, G. C., Ferdinandusse, S., Ramdi, H., Heimler, A., Fryer, A., Lynch, S.-A., Cooper, N., Ong, K. R., … Van Camp, G. (2015). Heimler Syndrome Is Caused by Hypomorphic Mutations in the Peroxisome-Biogenesis Genes PEX1 and PEX6. *The American Journal of Human Genetics*, *97*(4), 535–545. https://doi.org/10.1016/j.ajhg.2015.08.011

Ray, G. J., Boydston, E. A., Shortt, E., Wyant, G. A., Lourido, S., Chen, W. W., & Sabatini, D. M. (2020). A PEROXO-Tag Enables Rapid Isolation of Peroxisomes from Human Cells. *iScience*, *23*(5), 101109. https://doi.org/10.1016/j.isci.2020.101109

Rizzo, W. B. (2007). Sjögren–Larsson syndrome: Molecular genetics and biochemical pathogenesis of fatty aldehyde dehydrogenase deficiency. *Molecular Genetics and Metabolism*, *90*(1), 1–9. https://doi.org/10.1016/j.ymgme.2006.08.006

Sabatini, D. D., & Adesnik, M. (2013). Christian de Duve: Explorer of the cell who discovered new organelles by using a centrifuge. *Proceedings of the National Academy of Sciences*, *110*(33), 13234–13235. https://doi.org/10.1073/pnas.1312084110

Sakai, Y., Oku, M., Van Der Klei, I. J., & Kiel, J. A. K. W. (2006). Pexophagy: Autophagic degradation of peroxisomes. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1763*(12), 1767–1775. https://doi.org/10.1016/j.bbamcr.2006.08.023

Savitz, A. J., & Meyer, D. I. (1990). Identification of a ribosome receptor in the rough endoplasmic reticulum. *Nature*, *346*(6284), 540–544. https://doi.org/10.1038/346540a0

Schlüter, A., Fourcade, S., Ripp, R., Mandel, J. L., Poch, O., & Pujol, A. (2006). The Evolutionary Origin of Peroxisomes: An ER-Peroxisome Connection. *Molecular Biology and Evolution*, *23*(4), 838–845. https://doi.org/10.1093/molbev/msj103

Schmidt, F., Treiber, N., Zocher, G., Bjelic, S., Steinmetz, M. O., Kalbacher, H., Stehle, T., & Dodt, G. (2010). Insights into Peroxisome Function from the Structure of PEX3 in Complex with a Soluble Fragment of PEX19. *Journal of Biological Chemistry*, *285*(33), 25410–25417. https://doi.org/10.1074/jbc.M110.138503

Schrader, M., & Fahimi, H. D. (2004a). Mammalian peroxisomes and reactive oxygen species. *Histochemistry and Cell Biology*, *122*(4), 383–393. https://doi.org/10.1007/s00418-004-0673-1

Schrader, M., & Fahimi, H. D. (2004b). Mammalian peroxisomes and reactive oxygen species. *Histochemistry and Cell Biology*, *122*(4), 383–393. https://doi.org/10.1007/s00418-004-0673-1

Schrader, M., Kamoshita, M., & Islinger, M. (2020). Organelle interplay—Peroxisome interactions in health and disease. *Journal of Inherited Metabolic Disease*, *43*(1), 71–89. https://doi.org/10.1002/jimd.12083

Schrader, M., & Pellegrini, L. (2017). The making of a mammalian peroxisome, version 2.0: Mitochondria get into the mix. *Cell Death & Differentiation*, *24*(7), 1148–1152. https://doi.org/10.1038/cdd.2017.23

Schuldiner, M., Metz, J., Schmid, V., Denic, V., Rakwalska, M., Schmitt, H. D., Schwappach, B., & Weissman, J. S. (2008). The GET Complex Mediates Insertion of Tail-Anchored Proteins into the ER Membrane. *Cell*, *134*(4), 634–645. https://doi.org/10.1016/j.cell.2008.06.025

Schumann, U., Wanner, G., Veenhuis, M., Schmid, M., & Gietl, C. (2003). AthPEX10, a nuclear gene essential for peroxisome and storage organelle formation during *Arabidopsis* embryogenesis. *Proceedings of the National Academy of Sciences*, *100*(16), 9626–9631. https://doi.org/10.1073/pnas.1633697100

Scott, C., & Olpin, S. (2011). Peroxisomal disorders. *Paediatrics and Child Health*, *21*(2), 71–75. https://doi.org/10.1016/j.paed.2010.08.013

Sengupta, R., Poderycki, M. J., & Mattoo, S. (2019). CryoAPEX – an electron tomography tool for subcellular localization of membrane proteins. *Journal of Cell Science*, *132*(6), jcs222315. https://doi.org/10.1242/jcs.222315

Shibata, Y., Shemesh, T., Prinz, W. A., Palazzo, A. F., Kozlov, M. M., & Rapoport, T. A. (2010). Mechanisms Determining the Morphology of the Peripheral ER. *Cell*, *143*(5), 774–788. https://doi.org/10.1016/j.cell.2010.11.007

Silva, B. S. C., DiGiovanni, L., Kumar, R., Carmichael, R. E., Kim, P. K., & Schrader, M. (2020). Maintaining social contacts: The physiological relevance of organelle interactions. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1867*(11), 118800. https://doi.org/10.1016/j.bbamcr.2020.118800

Singh, T., Hayashi, M., Mano, S., Arai, Y., Goto, S., & Nishimura, M. (2009). Molecular components required for the targeting of PEX7 to peroxisomes in *Arabidopsis thaliana*. *The Plant Journal*, *60*(3), 488–498. https://doi.org/10.1111/j.1365-313X.2009.03970.x

Skowyra, M. L., Feng, P., & Rapoport, T. A. (2024). Towards solving the mystery of peroxisomal matrix protein import. *Trends in Cell Biology*, *34*(5), 388–405. https://doi.org/10.1016/j.tcb.2023.08.005

Smith, J. J., & Aitchison, J. D. (2013). Peroxisomes take shape. *Nature Reviews Molecular Cell Biology*, *14*(12), 803–817. https://doi.org/10.1038/nrm3700

Somborac, T., Lutfullahoglu Bal, G., Fatima, K., Vihinen, H., Paatero, A., Jokitalo, E., Paavilainen, V. O., & Konovalova, S. (2023). The subset of peroxisomal tail-anchored proteins do not reach peroxisomes via ER, instead mitochondria can be involved. *PLOS ONE*, *18*(12), e0295047. https://doi.org/10.1371/journal.pone.0295047

Stehlik, T., Sandrock, B., Ast, J., & Freitag, J. (2014). Fungal peroxisomes as biosynthetic organelles. *Current Opinion in Microbiology*, *22*, 8–14. https://doi.org/10.1016/j.mib.2014.09.011

Steinberg, S. J., Dodt, G., Raymond, G. V., Braverman, N. E., Moser, A. B., & Moser, H. W. (2006). Peroxisome biogenesis disorders. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1763*(12), 1733–1748. https://doi.org/10.1016/j.bbamcr.2006.09.010

Subramani, S., Koller, A., & Snyder, W. B. (2000). Import of Peroxisomal Matrix and Membrane Proteins. *Annual Review of Biochemistry*, *69*(1), 399–418. https://doi.org/10.1146/annurev.biochem.69.1.399

Sugiura, A., Mattie, S., Prudent, J., & McBride, H. M. (2017). Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature*, *542*(7640), 251–254. https://doi.org/10.1038/nature21375

Thieringer, H., Moellers, B., Dodt, G., Kunau, W.-H., & Driscoll, M. (2003). Modeling human peroxisome biogenesis disorders in the nematode *Caenorhabditis elegans*. *Journal of Cell Science*, *116*(9), 1797–1804. https://doi.org/10.1242/jcs.00380

Thoms, S., & Erdmann, R. (2005). Dynamin-related proteins and Pex11 proteins in peroxisome division and proliferation. *The FEBS Journal*, *272*(20), 5169–5181. https://doi.org/10.1111/j.1742-4658.2005.04939.x

Thoms, S., & Erdmann, R. (2006). Peroxisomal matrix protein receptor ubiquitination and recycling. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1763*(12), 1620–1628. https://doi.org/10.1016/j.bbamcr.2006.08.046

Toro, A., Arredondo, C., Cordova, G., Araya, C., Palacios, J. L., Venegas, A., Morita, M., Imanaka, T., & Santos, M. J. (2007). Evaluation of the role of the Endoplasmic Reticulum-Golgi transit in the biogenesis of peroxisomal membrane proteins in wild type and peroxisome biogenesis mutant CHO cells. *Biological Research*, *40*(2). https://doi.org/10.4067/S0716-97602007000200014

Van Der Zand, A., Braakman, I., & Tabak, H. F. (2010). Peroxisomal Membrane Proteins Insert into the Endoplasmic Reticulum. *Molecular Biology of the Cell*, *21*(12), 2057–2065. https://doi.org/10.1091/mbc.e10-02-0082

van der Zand, A., Gent, J., Braakman, I., & Tabak, H. F. (2012). Biochemically Distinct Vesicles from the Endoplasmic Reticulum Fuse to Form Peroxisomes. *Cell*, *149*(2), 397–409. https://doi.org/10.1016/j.cell.2012.01.054

Wanders, R. J. A., Baes, M., Ribeiro, D., Ferdinandusse, S., & Waterham, H. R. (2023). The physiological functions of human peroxisomes. *Physiological Reviews*, *103*(1), 957–1024. https://doi.org/10.1152/physrev.00051.2021

Wanders, R. J. A., Visser, W. F., Van Roermund, C. W. T., Kemp, S., & Waterham, H. R. (2007). The peroxisomal ABC transporter family. *Pflügers Archiv - European Journal of Physiology*, *453*(5), 719–734. https://doi.org/10.1007/s00424-006-0142-x

Wanders, R. J. A., Vreken, P., Ferdinandusse, S., Jansen, G. A., Waterham, H. R., Van Roermund, C. W. T., & Van Grunsven, E. G. (2001). Peroxisomal fatty acid α- and β -oxidation in humans: Enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochemical Society Transactions*, *29*(2), 250–267. https://doi.org/10.1042/bst0290250

Wanders, R. J. A., Waterham, H. R., & Ferdinandusse, S. (2016). Metabolic Interplay between Peroxisomes and Other Subcellular Organelles Including Mitochondria and the Endoplasmic Reticulum. *Frontiers in Cell and Developmental Biology*, *3*. https://doi.org/10.3389/fcell.2015.00083

Wanders, R. J., & Brites, P. (2010). Biosynthesis of ether-phospholipids including plasmalogens, peroxisomes and human disease: New insights into an old problem. *Clinical Lipidology*, *5*(3), 379–386. https://doi.org/10.2217/clp.10.16

Wang, J.-Y., Li, L., Chai, R.-Y., Qiu, H.-P., Zhang, Z., Wang, Y.-L., Liu, X.-H., Lin, F.-C., & Sun, G.-C. (2019). Pex13 and Pex14, the key components of the peroxisomal docking complex, are required for peroxisome formation, host infection and pathogenicity-related morphogenesis in *Magnaporthe oryzae*. *Virulence*, *10*(1), 292–314. https://doi.org/10.1080/21505594.2019.1598172

Yagita, Y., Abe, Y., & Fujiki, Y. (2022). *De novo* formation and maintenance of mammalian peroxisomes in the absence of PEX16. *Journal of Cell Science*, jcs.258377. https://doi.org/10.1242/jcs.258377

Zmijewski, J. W., Lorne, E., Zhao, X., Tsuruta, Y., Sha, Y., Liu, G., & Abraham, E. (2009). Antiinflammatory Effects of Hydrogen Peroxide in Neutrophil Activation and Acute Lung Injury. *American Journal of Respiratory and Critical Care Medicine*, *179*(8), 694–704. https://doi.org/10.1164/rccm.200806-851OC

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Kaneez Fatima

PUBLICATIONS

I

Publication I

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RESEARCH ARTICLE

Ribosome-binding protein 1 maintains peroxisome biogenesis

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ABSTRACT

Peroxisomes are single-membrane-bound organelles essential for human health, yet the mechanisms of peroxisome biogenesis are not fully understood. Here using a systematic double screening approach, we identified ribosome-binding protein 1 (RRBP1) as a novel peroxisome biogenesis factor in human cells. Deletion of RRBP1 in HEK293T cells led to a reduction in both peroxisome number and peroxisomal protein levels as well as in defects in processing of peroxisomal matrix proteins, such as ACOX1 and thiolase. However, cell proliferation and protein translation were not altered in cells lacking RRBP1. RRBP1 depletion did not affect peroxisome–endoplasmic reticulum (ER) contact sites, and pexophagy did not contribute to the reduction of peroxisomes in RRBP1 knockout cells. Instead, in the absence of RRBP1, peroxisomal proteins were processed by proteasomal degradation, suggesting that RRBP1 plays a role in the insertion of these proteins into ER membranes and their stabilization. Altogether, our results show that RRBP1 promotes peroxisome biogenesis in human cells, highlighting the power of systematic approaches in discovering novel factors of organellar biogenesis.

KEY WORDS: Peroxisome, RRBP1, CRISPR/Cas9, Peroxisome biogenesis

INTRODUCTION

Peroxisomes are single-membrane-bound organelles, essential for lipid breakdown and β-oxidation of very long-chain fatty acids. Peroxisomes can originate through two distinct mechanisms: *de novo* biogenesis and fission. *De novo* peroxisome biogenesis begins with the assembly of peroxisomal membrane receptors PEX3 and PEX16 at the endoplasmic reticulum (ER) and mitochondria, creating pre-peroxisomal vesicles that fuse. Following fusion, the newly formed peroxisome continues to import peroxisomal membrane proteins via the PEX19-mediated pathway. Once the import machinery is established on the membrane, peroxisomes can import matrix proteins, which are translated on free ribosomes and transported post-translationally from the cytosol. This process leads to the maturation of pre-peroxisomes into fully functional peroxisomes (Sugiura et al., 2017; Kim et al., 2006; Farré et al., 2019; Goldman and Blobel, 1978; Hasan et al., 2013; Rachubinski et al., 1984).

Most of the peroxisomal matrix proteins contain PTS1 or PTS2 targeting sequences, which are recognized by the cytosolic peroxins PEX5 and PEX7, respectively (Walter and Erdmann, 2019; Okumoto et al., 2018; Farré et al., 2019). The translocation of substrate proteins into the peroxisomal matrix is mediated by factors localized on the peroxisomal membrane such as PEX14 and PEX13 (Schliebs et al., 1999; Albertini et al., 1997; Distel et al., 1996; Matsumoto et al., 2003). Although studies in yeast (Akşit and van der Klei, 2018; Erdmann et al., 1989) and mammalian cells (Fang et al., 2004; Fujiki et al., 2014) have identified core components required for peroxisomal matrix import, the discovery of additional factors in recent studies (Baldwin et al., 2021; Guillén-Samander et al., 2021) suggests that more remain to be identified.

To systematically identify factors involved in biogenesis of peroxisomes in mammalian cells we first utilized APEX2 proximity labeling followed by mass spectrometry as a powerful tool to study protein–protein interactions (Lam et al., 2015). Using this approach, we identified factors that specifically interact with peroxisomal matrix cargo. Secondly, we tested these factors by CRISPR/Cas9 knockout (KO) microscopy screening. Using this double screening approach we revealed ribosome-binding protein 1 (RRBP1, also known as p180/ribosome receptor), as a novel peroxisome biogenesis factor.

Using KO and knock-down models, we demonstrated that RRBP1 depletion results in peroxisome loss. Notably, RRBP1 KO did not affect cell proliferation or protein translation, and we confirmed that pexophagy was not responsible for peroxisome loss in these cells. Thus, we established that RRBP1 is essential for maintaining peroxisome number in human cells. Although RRBP1 is an ER membrane protein, its depletion did not significantly alter the abundance of the contacts between ER and peroxisomes. However, the inhibition of the proteolytic activity with the proteasome inhibitor MG132 revealed that peroxisomal proteins undergo enhanced proteasomal degradation in RRBP1 KO cells. These findings suggest that RRBP1 is involved in early induction of peroxisome biogenesis by regulating the initial membrane insertion of key peroxisomal components into the ER membrane.

RESULTS

Systematic identification of peroxisome biogenesis factors using a double screening approach

To systematically identify factors required for protein import into the peroxisomal matrix, we first used a proximity-labeling approach followed by mass spectrometry (Lam et al., 2015) (Fig. 1A). Ascorbate peroxidase (APEX2) was fused to ePTS1 (DeLoache et al., 2016), the enhanced peroxisomal targeting signal type 1 recognized by PEX5, directing the construct to the peroxisomal matrix. The GFP–APEX2–ePTS1 construct was stably expressed in HEK293T control cells or cells lacking peroxisomes (PEX3 KO or PEX19 KO cells; Fig. S1A). As expected, the GFP–APEX2–ePTS1 construct was recognized by the peroxisomal protein import

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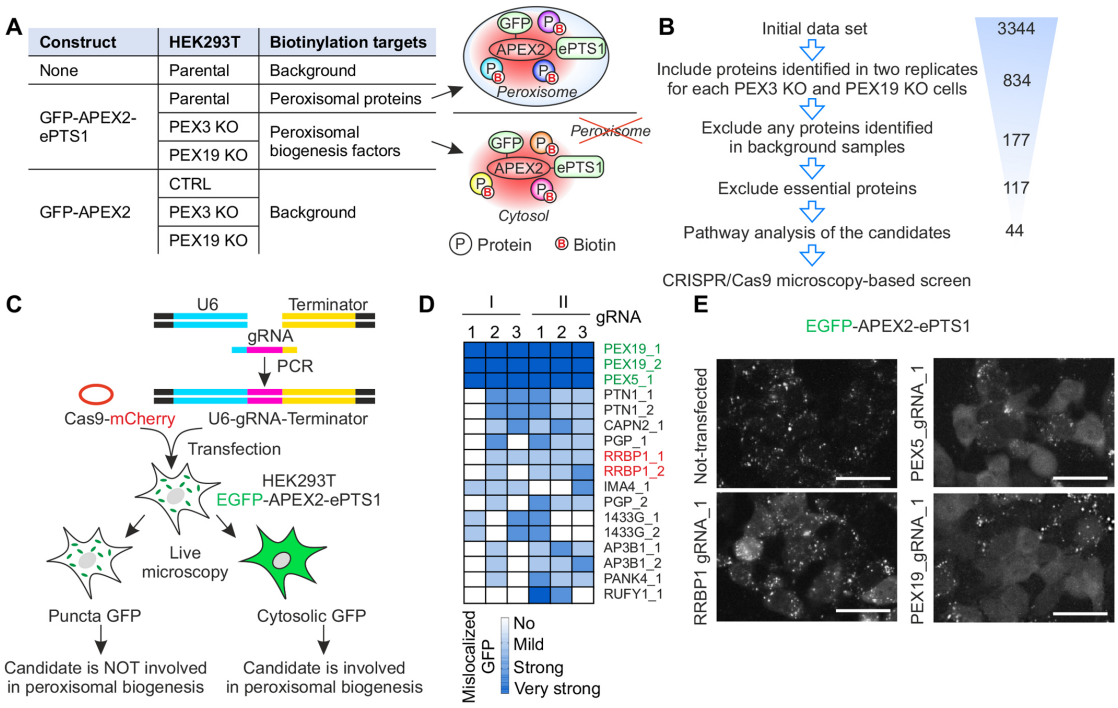

Fig. 1. Systematic identification of peroxisome biogenesis factors using proximity labeling and CRISPR/Cas9 microscopy-based screen. (A) Overview of the APEX2-based proximity labeling approach to identify peroxisome biogenesis factors. B, biotin; P, protein. (B) Steps in filtering of the mass spectrometry data to identify peroxisome biogenesis factors with high confidence. Number of candidates at each step is indicated. (C) Overview of the CRISPR/Cas9 microscopy-based screen. (D) Heat map of CRISPR/Cas9 microscopy assessing the phenotype of HEK293T cells. HEK293T cells stably expressing GFP–APEX2–ePTS1 were transiently transfected with gRNAs and Cas9–mCherry plasmid as shown in C. Two rounds of transfection were performed (I and II). Each round the phenotype of the cells was assessed three times at 7, 10 and 14 days after transfection using live-cell fluorescence microscopy. Cells were manually classified based on GFP localization into 'no' (GFP localized exclusively to peroxisomes, no mislocalization), 'mild' (some mislocalized GFP), 'strong' (substantial mislocalized GFP) and 'very strong' (extensive mislocalized GFP) categories. gRNAs targeting PEX5 or PEX19 were used as positive controls (in green). The full list of the screened gRNAs is in Fig. S1D. (E) Live-cell fluorescence microscopy images of HEK293T cells stably expressing GFP–APEX2–ePTS1 before (not-transfected) and after 13 days of transfection with Cas9–mCherry plasmid and gRNA targeting RRBP1, PEX5 or PEX19. The localization of peroxisomal targeted construct GFP–APEX2–ePTS1 was visualized using the GFP channel. Scale bars: 50 µm. Images are representative of five experiments.

machinery and delivered to the peroxisomal matrix in control cells. In contrast, in PEX3 KO or PEX19 KO cells, which lack peroxisomes, the construct remained in the cytosol (Fig. S1B). Under these conditions, factors involved in recognizing and trafficking ePTS1-containing proteins would be expected to accumulate near the GFP–APEX2–ePTS1 construct. Therefore, proteins specifically enriched in proximity to GFP–APEX2–ePTS1 in PEX3 KO or PEX19 KO cells represent strong candidates for peroxisome biogenesis factors. To exclude non-specifically labeled proteins we used parental cells not expressing GFP–APEX2–ePTS1 and cells stably expressing non-targeted GFP–APEX2 as negative control samples.

Biotinylated proteins were extracted from cell lysates using streptavidin beads and analyzed by mass spectrometry. Proteins identified in both of the two replicates performed for each of the two KO cell lines, PEX3 KO and PEX19 KO, were considered as significant hits. To identify highly specific interactors of GFP–APEX2–ePTS1 in cells lacking peroxisomes, we excluded any protein identified in the negative control samples (Table S1). Next, we excluded essential proteins (Table S2). Following pathway analysis of the remaining candidates, we identified 44 proteins that are potentially involved in peroxisome biogenesis (Fig. 1B; Fig. S1C; Table S3). Surprisingly, we did not detect PEX5 among the identified proteins, despite its role as the key factor recognizing PTS1. Notably, PEX5 was not identified as a specific interactor of GFP–APEX2–ePTS1 even in parental cells, suggesting that a combination of technical limitations in the labeling reaction and the unique biological properties of PEX5 may have prevented its detection in the APEX2 proximity labeling assay.

To ensure that only factors with a functional role in peroxisome biogenesis were identified, we performed a secondary CRISPR/Cas9-based microscopy screen of the candidates from the proximity labeling assay (Fig. 1C). For each candidate, two guide RNAs (gRNAs) were designed and co-transfected with Cas9 into HEK293T cells stably expressing GFP–APEX2–ePTS1, which served as a reporter cell line for peroxisome biogenesis. Live-cell fluorescence microscopy was performed to examine the localization of GFP in the transfected cells, in which redistribution of GFP from peroxisomes to the cytosol indicated disruption of peroxisome biogenesis (Fig. 1C). The phenotype of the cells was analyzed using live-cell microscopy, and

the cells were manually classified into four categories based on GFP localization: 'no' (GFP localized exclusively to peroxisomes, no mislocalization), 'mild' (some mislocalized GFP), 'strong' (substantial mislocalized GFP) and 'very strong' (extensive mislocalized GFP). Each gRNA was tested in two independent rounds of transfection, with analyses performed at days 7, 10 and 14 after transfection. As positive controls, gRNAs targeting known peroxisome assembly factors, such as PEX5 and PEX19, produced robust mislocalization of GFP–APEX2–ePTS1 (Fig. 1D,E; Fig. S1D) without altering its overall expression level (Fig. S1E,F). Among the hits, KO of ribosome-binding protein 1 (RRBP1) caused strong mislocalization of GFP–APEX2–ePTS1 into the cytoplasm (Fig. 1D,E), suggesting its role in peroxisomal biogenesis.

RRBP1, also known as p180/ribosome receptor, is a transmembrane ER protein. RRBP1 was originally identified as a ribosome receptor for the ER (Savitz and Meyer, 1990) and has also been proposed to regulate local translation in axons (Koppers et al., 2024). Recent studies suggest that RRBP1 plays a role in tethering ER with mitochondria (Anastasia et al., 2021; Cardoen et al., 2024; Hung et al., 2017) and regulates mitophagy (Killackey et al., 2023). However, RRBP1 has not been linked to peroxisomal function previously. Therefore, RRBP1 emerged as a particularly promising candidate due to its ER localization and established role in ribosome binding and membrane protein translocation – processes closely tied to organelle biogenesis. Together with the strong phenotype observed in our screen (Fig. 1D,E), these features provided the rationale for selecting RRBP1 for detailed functional characterization as a potential peroxisome biogenesis factor.

RRBP1 is required for the maintenance of peroxisome number in human cells

To investigate the involvement of RRBP1 in the maintenance of peroxisomes we generated a clonal RRBP1 KO HEK293T cell line expressing the GFP–APEX2–ePTS1 construct by CRISPR/Cas9 gene editing. To achieve a complete KO of the RRBP1 gene we used a single-guide RNA (sgRNA) targeting exon 2, which is common to all isoforms of RRBP1. We confirmed the complete depletion of the RRBP1 protein in the generated RRBP1 KO cell line using immunoblotting (Fig. 2A). We analyzed RRBP1 KO HEK293T cells expressing GFP–APEX2–ePTS1 and its parental cell line as a control. In addition, as positive controls we used cells lacking essential peroxisome biogenesis factors, PEX3 or PEX19, generated in our previous study (Somborac et al., 2023). We observed that the RRBP1 KO cells have significantly lower steady-state protein levels of the peroxisomal matrix protein catalase, as well as the peroxisomal membrane proteins PEX3, PEX19, PEX14 and PXMP2, compared to the parental cell line (Fig. 2A,B; Fig. S3D). In contrast, the protein level of PEX5, an essential peroxisomal matrix protein factor, was increased upon RRBP1 KO (Fig. 2A,B).

Next, we analyzed peroxisomal function in RRBP1 KO cells by assessing peroxisomal matrix protein import. Although most of the peroxisomal proteins are translated in the cytosol in their mature form, some undergo processing upon import to peroxisomes. Once imported to the peroxisomal matrix, the 72 kDa cytosolic unprocessed acyl-CoA oxidase 1 (ACOX1), a PTS1-targeted peroxisomal matrix protein, is cleaved into its 50 kDa and 22 kDa mature forms (Kurochkin et al., 2007). Similarly, the 44 kDa unprocessed 3-ketoacyl-CoA thiolase (ACAA1), a PTS2-targeted peroxisomal matrix protein, is processed to 42 kDa upon impot to peroxisomes (Miura et al., 1984). Using immunoblotting, we showed that the processing of both ACOX1 and ACAA1 was largely abolished in PEX3 KO cells and PEX19 KO cells, which lack peroxisomes (Fig. 2A–C), confirming that this assay can be efficiently used to analyze peroxisomal function, as shown previously (Klouwer et al., 2021; Kurochkin et al., 2007). Interestingly, a significant decrease in the processing of ACOX1 and ACAA1 was observed in the RRBP1 KO cells (Fig. 2A–C).

Notably, the PEX3 KO cells and PEX19 KO cells had significantly lower RRBP1 protein levels compared to their parental cells, suggesting that RRBP1 protein is responsive to the absence of peroxisomes. We also observed a slight decrease in the protein levels of the ER proteins calnexin and VAPB in RRBP1 KO cells compared to parental cells (Fig. 2D), suggesting that RRBP1 KO might have a mild impact on the ER. We observed no effect of RRBP1 KO on the steady-state level of the mitochondrial protein TOM20 (also known as TOMM20; Fig. 2E), suggesting that mitochondrial mass is not affected in cells lacking RRBP1.

To validate our findings on RRBP1 KO cells we performed transient knock down of RRBP1 using endoribonuclease-prepared short interfering RNA (esiRNA) in HEK293T cells stably expressing GFP–APEX2–ePTS1. GFP esiRNA was used as a negative control, whereas esiRNA targeting a peroxisomal import factor essential for the assembly of functional peroxisomes, PEX5, was used as a positive control. In cells silenced for RRBP1, protein levels of ACAA1 and catalase were reduced (Fig. 3A,B,D) and the processing of ACOX1 was significantly decreased, similar to the effect seen upon PEX5 silencing (Fig. 3C). Importantly, similar to RRBP1 KO cells, we showed mislocalization of GFP–APEX2–ePTS1 construct to the cytosol in cells with acute silencing of RRBP1 (Fig. 3E). Thus, we demonstrated that loss of RRBP1 leads to peroxisomal deficiency, indicating that RRBP1 plays a specific role in peroxisome maintenance.

RRBP1 KO causes a decreased number of peroxisomes in human cells

Next, we investigated whether peroxisome number is affected by the loss of RRBP1. We used RRBP1 KO HEK293T cells stably expressing GFP–APEX2–ePTS1 and its parental cell line as a negative control. Live-cell fluorescence microscopy confirmed the absence of RRBP1 in KO cells and revealed a reduced number of GFP-positive puncta representing mature peroxisomes compared to the parental cells (Fig. S1G). In addition, GFP–APEX2–ePTS1 was partially localized to cytosol in cells lacking RRBP1, whereas in parental HEK293T cells the construct was correctly localized to peroxisomes, confirming our result from the CRISPR/Cas9 screen (Fig. 1D). These data collectively show that the import of peroxisomal matrix proteins is compromised in RRBP1 KO cells.

To study whether RRBP1 KO also affects peroxisome number, we performed high-resolution immunofluorescence confocal microscopy of RRBP1 KO cells using PMP70 (also known as ABCD3) and PEX14 as a membrane markers and catalase as a matrix marker of peroxisomal structures. As a positive control we used PEX3 KO or PEX19 KO HEK293T cells, which lack peroxisomes. The numbers of PMP70-, PEX14- or catalase-positive structures per cell were reduced in RRBP1 KO cells compared to the parental cells (Fig. 4A–E), confirming that RRBP1 is required for maintaining peroxisome number. Notably, cell volume was unaffected by RRBP1 KO, excluding this as a factor contributing to the reduced peroxisome abundance (Fig. 4F). Altogether these results show that RRBP1 is an important factor involved in the maintenance of peroxisome number in human cells.

RRBP1 does not localize to peroxisomes

Our data indicate that RRBP1 maintains peroxisome biogenesis. Previously, RRBP1 has been described as an ER transmembrane

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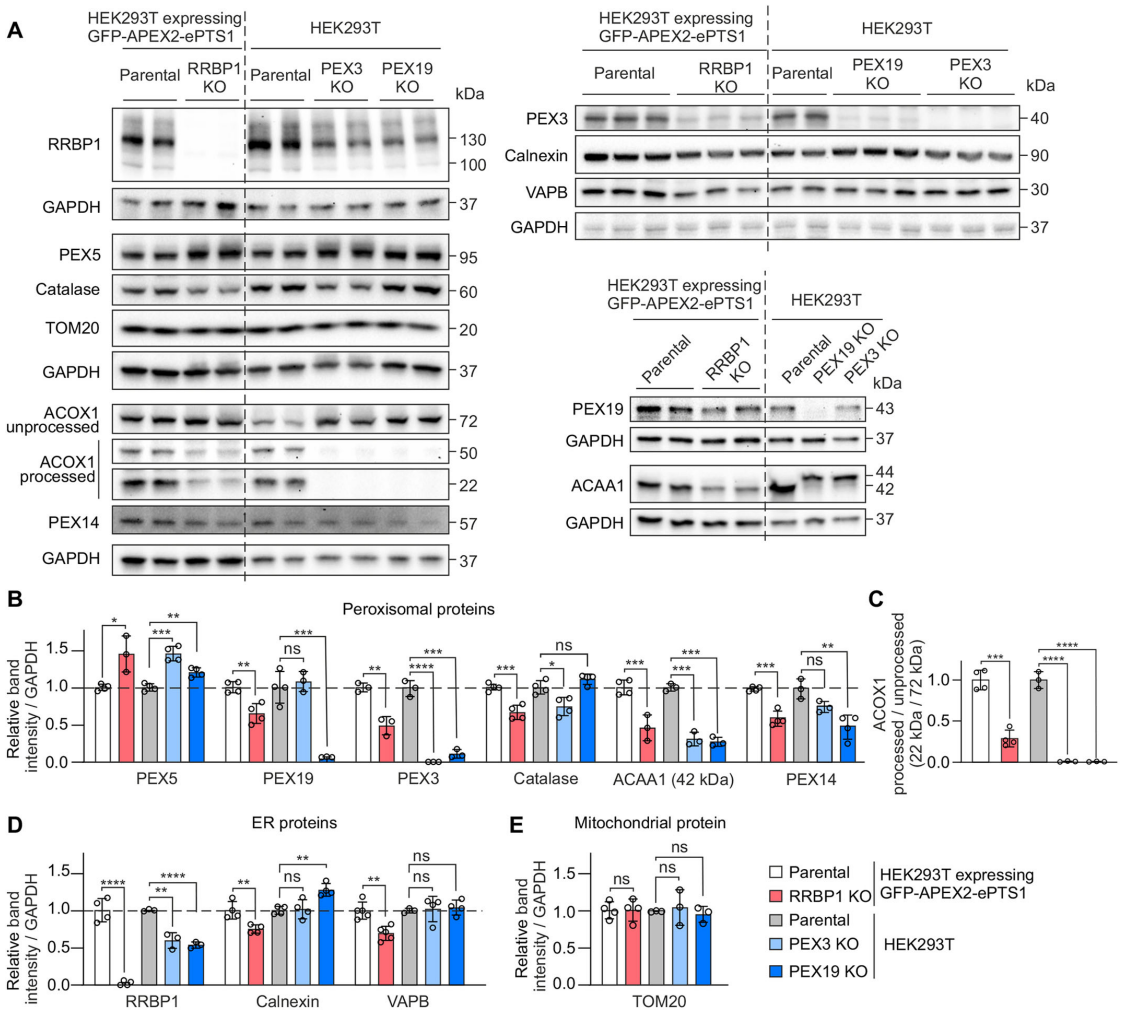

Fig. 2. RRBP1 is required for normal peroxisome maintenance in human cells. (A) Western blot analysis of the indicated proteins in RRBP1 KO HEK293T cells stably expressing GFP–APEX2–ePTS1. Parental, PEX3 and PEX19 KO HEK293T cells not expressing the peroxisomal-targeted construct were used as controls. GAPDH was used as a loading control. (B) Quantification of peroxisomal protein levels using western blot images as represented in A (n=3–4). (C) Quantification of ACOX1 processing using western blot images as represented in A (n=3–4). (D) Quantification of ER protein levels using western blot images as represented in A (n=3–5). (E) Quantification of TOM20 protein level using western blot images as represented in A (n=3–4). Each western blot analysis was performed at least three times. Values are normalized to the corresponding parental cell line, set to 1. In all graphs, data are presented as mean±s.d. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, not significant as compared to the parental cell line (unpaired two-tailed t-tests).

protein (Shibata et al., 2010; Ogawa-Goto et al., 2007; Savitz and Meyer, 1990). Thus, the role of RRBP1 in peroxisome biogenesis could be mediated through the ER. To analyze whether in addition to the ER, RRBP1 is also localized to peroxisomes, we performed colocalization analysis using confocal microscopy and corresponding antibodies labeling peroxisomes, ER or mitochondria (Fig. 5A). RRBP1 mainly localized to the ER (overlay of RRBP1 with calnexin) and showed some localization to mitochondria [ATPIF1 (ATP5IF1) colocalization with RRBP1], whereas we observed only weak colocalization of RRBP1 with peroxisomes (catalase with RRBP1) (Fig. 5A,B).

To further confirm that RRBP1 does not localize to peroxisomes, we examined RRBP1 protein in isolated peroxisomes using immunoprecipitation (IP), as described by Ray et al. (2020). We generated clonal HEK293T cell lines stably expressing 3×HA–EGFP–PEX26 at either low or high levels and verified peroxisomal localization of tagged PEX26 by confocal microscopy (Fig. S2A,B). The IP procedure yielded highly purified peroxisomes, confirmed by the absence of ER (calnexin) and cytosolic (GAPDH) contamination (Fig. 5C). Importantly, RRBP1 was not detected in the peroxisomal fraction (Fig. 5C). Thus, our data clearly show that RRBP1 does not localize to peroxisomes, instead its role in peroxisome biogenesis is mediated at the level of ER.

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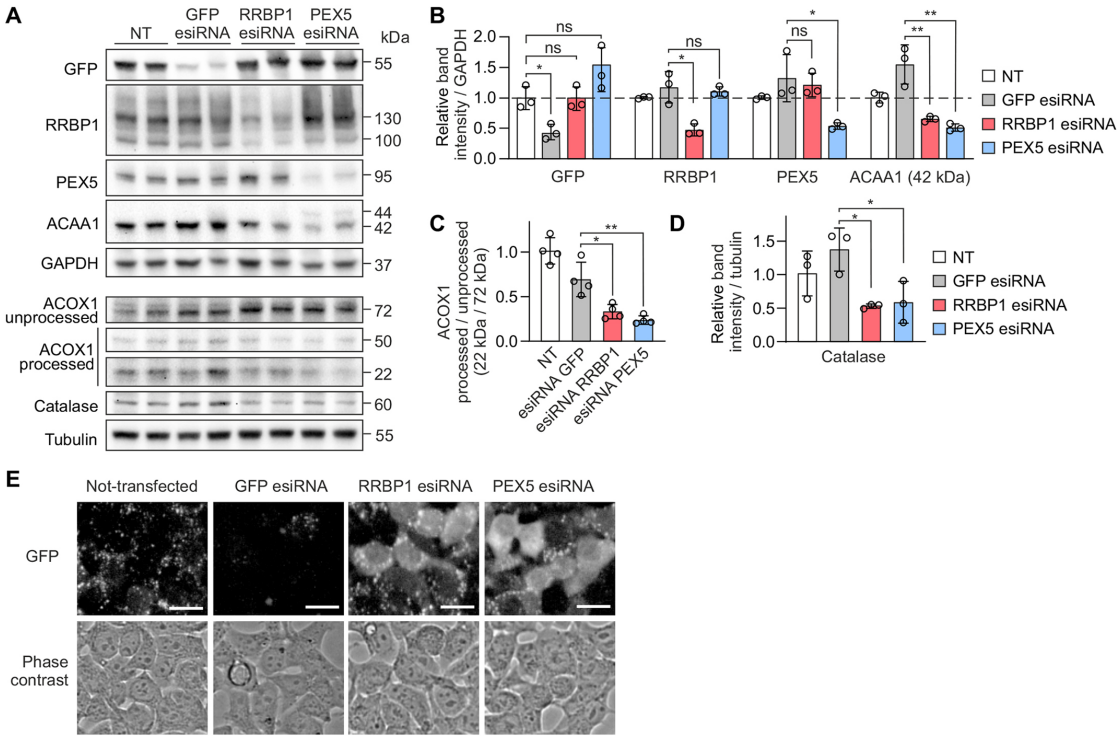

Fig. 3. RRBP1 silencing causes peroxisomal deficiency in human cells. (A) Western blot analysis showing the effect of RRBP1 silencing on levels of the indicated proteins in HEK293T cells stably expressing GFP–APEX2–ePTS1. The cells were transfected with corresponding esiRNAs for 72 h. GFP esiRNA was used as a negative control, PEX5 esiRNA was used as a positive control. NT, non-treated cells. (B–D) Quantification of western blot images as presented in A (n=3 in B and D, n=4 in C). GAPDH or tubulin was used as a loading control. Each western blot analysis was performed at least three times. Values are normalized to the non-treated cells, set at 1. In all graphs data are presented as mean±s.d. *P<0.05; **P<0.01; ns, not significant (unpaired two-tailed t-tests). (E) Live-cell fluorescence microscopy images of HEK293T cells stably expressing GFP–APEX2–ePTS1 before (not-transfected) and after transfection with the indicated esiRNAs. The localization of peroxisomal targeted construct GFP–APEX2–ePTS1 was visualized using the GFP channel. GFP esiRNA was used as a negative control, PEX5 esiRNA was used as a positive control. Scale bars: 25 µm. Images are representative of two independent transfection experiments.

RRBP1 KO causes peroxisomal deficiency independent of ER stress

Since RRBP1 is an ER protein that might influence protein translation and cell proliferation, we tested whether RRBP1 KO cells exhibited altered proliferation. RRBP1 KO did not affect cell proliferation (Fig. S3A), and the protein translation rate measured by puromycin incorporation into a newly synthesized proteins also remained unchanged (Fig. S3B,C). However, we observed a reduction in specific ER proteins, including VAPB, calnexin, IRE1α and BiP (HSPA5), in RRBP1 KO cells compared to the parental cell line (Fig. S3D,E; Fig. 2A,D). This suggests a mild disruption of ER homeostasis. However, the levels of SEC61 and RPL7 were unaltered, indicating that the overall machinery for protein synthesis and translocation, as well as ribosomal assembly, remains unaffected.

Peroxisome–ER contact sites are not affected in RRBP1-depleted cells

Recent studies suggest that RRBP1 can act as a tether in contact sites between ER and mitochondrial membranes ensuring metabolic communication between two organelles (Hung et al., 2017; Anastasia et al., 2021). To test whether RRBP1 has a role in maintaining peroxisome–ER contacts we used electron microscopy. We took advantage of APEX2, a well-characterized powerful tool for labeling intracellular structures in electron microscopy studies (Martell et al., 2017; Rae et al., 2021; Sengupta et al., 2019; Lam et al., 2015). Using DAB staining, we labeled peroxisomes in RRBP1 KO HEK293T cells stably expressing GFP–APEX2–ePTS1 and in the cells transfected with esiRNA targeting RRBP1. Parental cells and cells transfected with esiRNA targeting luciferase were used as controls. We observed that neither RRBP1 silencing by esiRNA nor RRBP1 KO affected the number of contact sites between the ER and peroxisomes (Fig. 6A–E). Additionally, electron microscopy analysis of peroxisomal morphology in RRBP1 KO cells revealed no changes in peroxisomal size or shape (Fig. 6F,G).

The decreased number of peroxisomes in RRBP1 KO cells is independent of pexophagy but is associated with increased proteasomal degradation of peroxisomal membrane proteins

We showed that the steady-state number of peroxisomes is decreased upon RRBP1 KO (Fig. 4A–E). Peroxisomal homeostasis is regulated by the interplay between peroxisome biogenesis and the selective

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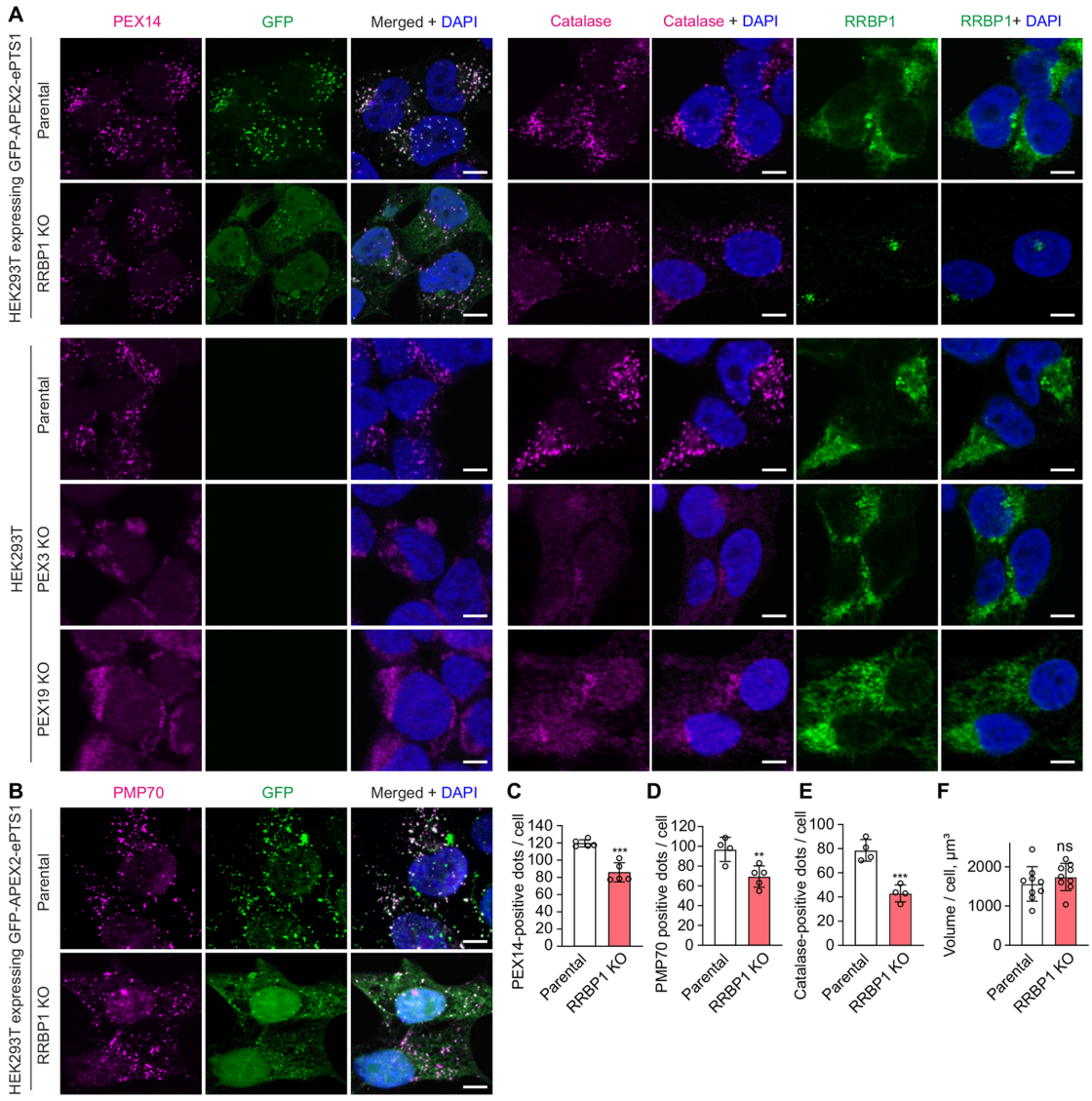

Fig. 4. The number of peroxisomes is decreased in RRBP1 KO cells. (A) Confocal microscopy images of RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1. Parental, PEX3 KO and PEX19 KO cells are shown as controls. Immunostaining was performed using anti-PEX14, anti-GFP, anti-catalase or anti-RRBP1 antibody, as indicated. Scale bars: 5 µm. (B) Confocal microscopy images of RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1. Immunostaining was performed using anti-PMP70 and anti-GFP antibody. Scale bars: 5 µm. (C–E) Quantification of (C) PEX14-, (D) PMP70- or (E) catalase-positive puncta in RRBP1 KO and parental HEK293T cells stably expressing GFP–APEX2–ePTS1. *Z*-stacks of confocal microscopy images were processed and reconstructed in a 3D model using Imaris software. Each value represents analysis of ∼100 cells (n=4–5). (F) The volume of RRBP1 KO and parental HEK293T cells stably expressing GFP–APEX2–ePTS1 was analyzed using confocal microscopy images. *Z*-stacks of confocal microscopy images were processed and reconstructed in a 3D model using Imaris software. Immunostaining for each protein was performed in at least three independent experiments. Each value represents analysis of ∼100 cells (n=9). PEX14 or catalase signal was used for the analysis. In all graphs data are presented as mean±s.d. **P<0.01; ***P<0.001; ns, not significant as compared to the parental cell line (unpaired two-tailed t-tests).

autophagy of peroxisomes, pexophagy (Demers et al., 2023; Germain and Kim, 2020; Sakai et al., 2006). Therefore, the decreased peroxisome number upon RRBP1 KO could be the result of affected peroxisome biogenesis, where fewer peroxisomes were formed, or induced pexophagy leading to the loss of peroxisomes. To distinguish between these two possibilities, we tested whether pexophagy is induced in RRBP1 KO cells. First, we assessed the steady-state levels of autophagy markers in RRBP1 KO cells and observed a reduction in LC3B (also known as MAP1LC3B) and ATG5 levels compared to the parental cell line (Fig. 7A,B). However, steady-state levels of

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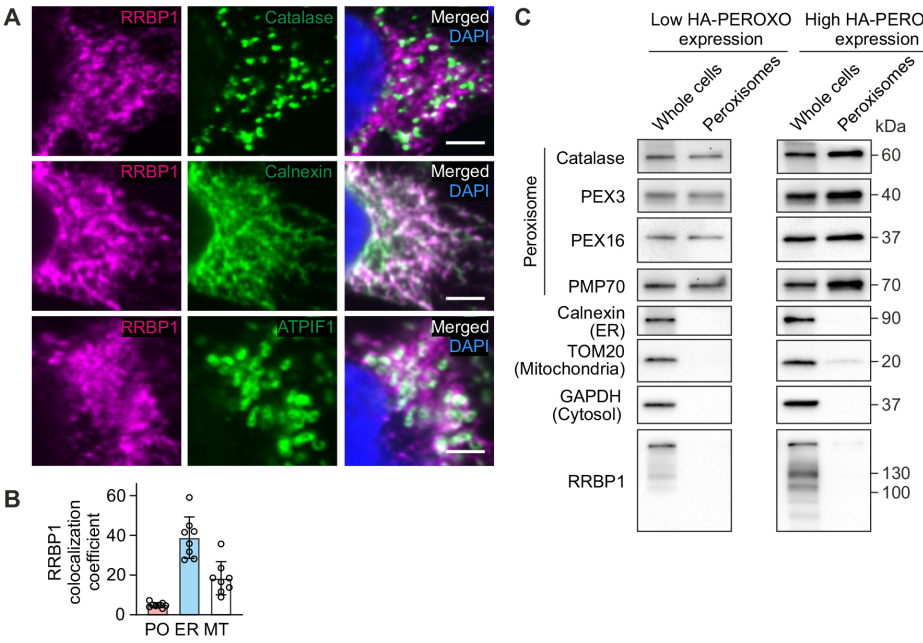

Fig. 5. RRBP1 does not localize to peroxisomes. (A) Intracellular localization of RRBP1 was analyzed in HEK293T cells using immunofluorescence confocal microscopy. Catalase was used to label peroxisomes, ATPIF1 was used to visualize mitochondria, calnexin was used to label ER. Scale bars: 3 µm. Immunostaining for each protein was performed in at least three independent experiments. (B) Colocalization analysis of confocal microscopy images as represented in A by Manders' overlap coefficient. The data are presented as mean±s.d. Immunostaining for each protein was performed in at least three independent experiments (n=8). MT, mitochondria; PO, peroxisomes. (C) Western blot of the indicated proteins in HEK293T cells stably expressing 3×HA–EGFP–PEX26 (HA–PEROXO) at either low or high levels and immunopurified peroxisomes. Each western blot analysis was performed at least two times.

autophagy markers such as LC3B or ATG5 cannot reliably measure autophagic activity, since autophagy is a dynamic process in which intermediates are continuously generated and degraded. For this reason, we used chloroquine to inhibit pexophagy and assessed whether blocking pexophagy could restore the levels of peroxisomal proteins in RRBP1 KO cells. We confirmed the inhibition of autophagy upon chloroquine treatment by induction of lipidated LC3B (LC3B-II), indicating the accumulation of autophagosomes (Fig. 7A,B). However, chloroquine treatment did not rescue the levels of PEX3 or PMP70 proteins in RRBP1 KO cells (Fig. 7A,B). These results conclusively suggest that pexophagy is not induced in RRBP1 KO cells. Consequently, the reduced number of peroxisomes in cells lacking RRBP1 is likely due to its involvement in peroxisome biogenesis.

To investigate whether peroxisomal proteins undergo proteasomal degradation in the absence of RRBP1, we inhibited the proteasomal degradation pathway using MG132 treatment. Our results revealed that PEX3 and PEX16 were significantly restored in RRBP1 KO cells upon MG132 treatment (Fig. 7C,D), suggesting that their reduction in RRBP1 KO cells is due to proteasomal degradation. Since both PEX3 and PEX16 play crucial roles in initiating peroxisome *de novo* biogenesis via the ER (Aranovich et al., 2014; Kim et al., 2006), their recovery upon proteasomal inhibition indicates that RRBP1 might be involved in the insertion of these proteins into ER membranes. Furthermore, in the parental cell line, MG132 treatment resulted in a significant induction of RRBP1, suggesting that RRBP1 itself has a high turnover rate and might be subject to proteasomal degradation. These findings highlight a potential regulatory mechanism in which RRBP1 helps maintain peroxisomal membrane protein stability, and its absence leads to increased degradation of key peroxisome biogenesis factors.

DISCUSSION

In this study, we identified and characterized RRBP1, an ER transmembrane protein, as a novel factor involved in peroxisome biogenesis. RRBP1 has been previously implicated in ribosome binding (Savitz and Meyer, 1990), ER–mitochondria tethering (Anastasia et al., 2021; Hung et al., 2017; Cardoen et al., 2024), mitophagy regulation (Killackey et al., 2023) and local axonal translation (Koppers et al., 2024), but its role in peroxisomal function has not been identified.

We showed that cells lacking RRBP1 lose peroxisomes. However, in contrast to cells lacking known essential components of peroxisome biogenesis machinery, like PEX3 and PEX19, RRBP1 KO did not result in complete absence of peroxisomes, indicating that although RRBP1 plays a role in peroxisome biogenesis it is not an essential factor.

Interestingly, in RRBP1 KO cells, as well as in the PEX3 KO cells and PEX19 KO cells completely lacking peroxisomes, we observed induction of PEX5 protein level, a cytosolic peroxin essential for protein delivery into the peroxisomal matrix. These data indicate a complex role for RRBP1 in peroxisomal protein homeostasis. Notably, in cells lacking peroxisomes (PEX3 KO and PEX19 KO cells), the RRBP1 protein level was reduced, suggesting that RRBP1 is responsive to the absence of peroxisomes.

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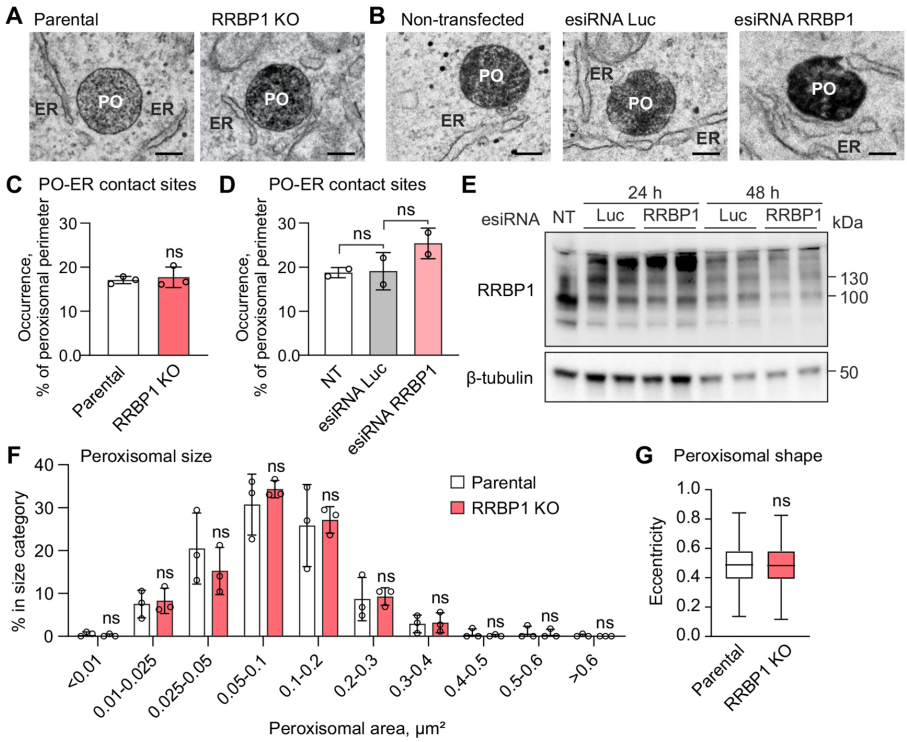

Fig. 6. Peroxisome–ER contact sites are not affected in RRBP1-depleted cells. (A) Representative electron microscopy images of RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1. Peroxisomes were labeled by DAB staining. Scale bars: 200 nm. Experiments were performed in at least two independent replicates for each cell line and condition. (B) Representative electron microscopy images of non-transfected HEK293T cells stably expressing GFP–APEX2–ePTS1 (non-transfected) or the same cell line transfected for 48 h with esiRNAs targeting RRBP1 or luciferase (Luc) as a negative control. Peroxisomes were labeled by DAB staining. Scale bars: 200 nm. (C) Relative occurrence of peroxisome–ER contact sites at 10–100 nm distance in RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1 analyzed using electron microscopy images. In each experiment for each cell line, 13–18 cells having peroxisomal profiles were selected using systematic random sampling. All the peroxisomes in the selected cells were imaged (n=105–175 for each replicate of parental cell line, n=84–168 for each replicate of RRBP1 KO cell line, from n=3 experiments), and the peroxisome–ER distances were analyzed using Microscopy Image Browser (MIB; Belevich et al., 2016). (D) Relative occurrence of peroxisome–ER contact sites at 10–100 nm distance in non-transfected HEK293T cells stably expressing GFP–APEX2–ePTS1 (NT) or in cells transfected for 48 h with esiRNAs targeting RRBP1 or luciferase (Luc) as a negative control. In each experiment for each cell line, 13–15 cells having peroxisomal profiles were selected using systematic random sampling. All the peroxisomes in the selected cells were imaged, n=60–90 for each replicate from n=2 experiments, and the peroxisome–ER distances were analyzed using the Microscopy Image Browser (MIB; Belevich et al., 2016). (E) Western blot analysis showing the effect of RRBP1 silencing on levels of the indicated proteins in HEK293T cells stably expressing GFP–APEX2–ePTS1. The cells were transfected with corresponding esiRNAs for 24 or 48 h. Luciferase (Luc) esiRNA was used as a negative control. Each western blot analysis was performed at least two times. (F,G) The distribution of the peroxisomal area (peroxisomal size) analyzed in RRBP1 KO (n=437) or parental HEK293T cells (n=403) stably expressing GFP–APEX2–ePTS1. Electron microscopy images and Microscopy Image Browser were used for the analysis. Data are from n=3 experiments. (G) Eccentricity (peroxisomal shape) was analyzed in RRBP1 KO (n=437) or parental HEK293T cells (n=403) stably expressing GFP–APEX2–ePTS1 using electron microscopy images and Microscopy Image Browser. Data are from n=3 experiments. In C,D and F, data are presented as mean±s.d. ns, not significant as compared to the parental cell line (unpaired two-tailed t-tests). In G, line represents the median, box represents the values from the 25th to 75th percentiles and whiskers represent the minimum and maximum values. PO, peroxisome.

To determine whether peroxisome loss in RRBP1 KO cells was due to enhanced peroxisomal degradation, we tested whether blocking pexophagy could restore peroxisomal protein levels. However, inhibition of pexophagy did not rescue peroxisomal protein levels in RRBP1 KO cells. Interestingly, we observed a significant reduction in steady-state LC3B levels in RRBP1 KO cells, which remained lower than in treated parental cells even after pexophagy inhibition. These findings suggest that pexophagy does not contribute to peroxisome loss in RRBP1 KO cells. Instead, the reduced pexophagy rate in RRBP1 KO cells is likely a consequence of the overall decrease in peroxisome abundance.

It is interesting to find that peroxisome biogenesis in human cells requires RRBP1, an ER membrane protein. Notably, RRBP1 KO did not affect cell proliferation or protein translation. Moreover, SEC61 and RPL7 levels remained unchanged in cells lacking RRBP1, suggesting that RRBP1 does not affect translation or translocation machinery.

The classical model of peroxisome biogenesis suggests that peroxisomal membranes originate from the ER in the form of budding pre-peroxisomal vesicles (Lam et al., 2010). However, our APEX2-based proximity labeling suggests that RRBP1 interacts with cargo proteins containing the PTS1 peroxisomal targeting signal,

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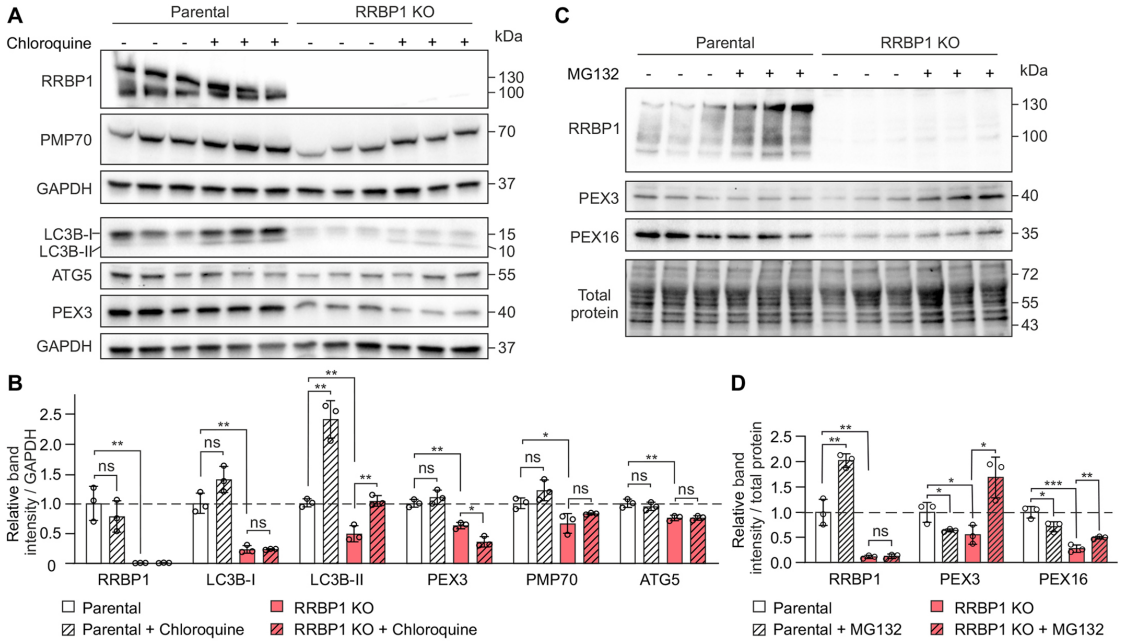

Fig. 7. The decreased number of peroxisomes in RRBP1 KO cells is independent of pexophagy but is associated with increased proteasomal degradation of peroxisomal membrane proteins. (A) Western blot analysis of the indicated proteins in RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1 construct before and after treatment with 100 µM chloroquine for 3 h. GAPDH was used as a loading control. LC3B-I, unlipidated LC3B; LC3B-II, lipidated LC3B. (B) Quantification of western blot images as presented in A (n=3). (C) Western blot analysis of the indicated proteins in RRBP1 KO or parental HEK293T cells stably expressing GFP–APEX2–ePTS1 construct before and after treatment with 10 µM MG132 for 4 h. Stain-Free total protein staining was used as a loading control. (D) Quantification of western blot images as presented in C (n=3). Each western blot analysis was performed at least two times. Values in B and D are normalized to the parental cell line, set to 1. In all graphs data are presented as mean±s.d. *P<0.05; **P<0.01; ***P<0.001; ns, not significant (unpaired two-tailed t-tests).

indicating that RRBP1 has a role in the import of peroxisomal matrix proteins. Importantly, in contrast to peroxisomal membrane proteins, peroxisomal matrix proteins are not detected in the ER (Geuze et al., 2003; Van Der Zand et al., 2010). We also showed that RRBP1 is not localized to peroxisomes, thus making it unlikely that RRBP1 is directly involved in peroxisomal protein import. Instead, our data suggest that RRBP1 might play an indirect role by facilitating the recruitment of other factors to peroxisomal membranes that are required for matrix protein import.

How can an ER protein be involved in peroxisome biogenesis? Recent studies indicate that peroxisomes interact closely with the ER through membrane contact sites, which are crucial for peroxisome dynamics, biogenesis and metabolic signaling (Costello et al., 2017; Hua et al., 2017; Guillén-Samander et al., 2021; Baldwin et al., 2021). However, we did not observe significant changes in the abundance of peroxisome–ER contact sites in RRBP1-depleted cells. However, it is important to note that the DAB staining method used in our study detects only mature peroxisomes, therefore, our data cannot exclude a potential role of RRBP1 in regulating contacts between the ER and pre-peroxisomes.

Our findings indicate a reduction in both peroxisomal membrane and matrix proteins, along with a decreased peroxisome number, suggesting a potential disruption in *de novo* peroxisome biogenesis. We demonstrated that the decreased levels of peroxisomal proteins, such as PEX3 and PEX16, in RRBP1 KO cells result from enhanced proteasomal degradation. Therefore, a potential mechanism by which RRBP1 contributes to peroxisome biogenesis is through the integration of PEX16 into the ER membrane and its stabilization. PEX16 is known to initiate peroxisome formation by recruiting peroxisomal membrane proteins (Kim et al., 2006; Aranovich et al., 2014; Hua et al., 2015).

Previous studies have shown that RRBP1 promotes the localization of specific mRNAs to the ER surface (Koppers et al., 2024; Cui et al., 2012; Cui and Palazzo, 2014), and a number of mRNAs encoding peroxisomal proteins are enriched on the ER (Haimovich et al., 2016). Additional evidence indicates that targeted mRNA trafficking and local translation contribute to peroxisome biogenesis (Dahan et al., 2022; Zipor et al., 2009). Taken together, these findings support the idea that RRBP1 facilitates the efficient translation of critical peroxisomal proteins, such as PEX16, by directing their mRNAs to the ER. However, the precise mechanism, including whether RRBP1 directly assists in the insertion of peroxisomal proteins or indirectly regulates their recruitment by influencing mRNA localization, remains unclear.

Interestingly, RRBP1 appears to be a metazoan-specific protein: its homologs are not found in yeast. Therefore, despite the conservation of general protein targeting mechanisms in eukaryotic cells, the role of RRBP1 in peroxisome biogenesis could represent a specific pathway of peroxisomal maintenance in higher eukaryotes.

In conclusion, the identification and characterization of RRBP1 as a factor involved in peroxisomal formation advances

our understanding of organelle biogenesis, a complex process involving multiple steps and diverse intracellular cooperation.

MATERIALS AND METHODS

Cell culture

HEK293T cells (ATCC, CRL-11268) were cultured (37°C, 5% CO_2) in DMEM (Pan-Biotech, P04-03600) supplemented with 10% fetal bovine serum (GIBCO, 10270106), L-glutamine (GIBCO, 25030081) and penicillin-streptomycin (GIBCO, 15140122). The cells were regularly tested for mycoplasma contamination. TransIT-X2 (MirusBio, MIR 6000) or TransIT2020 (Mirus, MIR 5400) were used for cell transfection according to manufacturer's instruction.

Generation of knockout cell lines

PEX3 KO and PEX19 KO HEK293T cells were generated in our previous study (Somborac et al., 2023) using CRISPR/Cas9 genome editing. Cells were co-transfected with two gRNAs and pSpCas9n(BB)-2A-Puro (PX462) V2.0 (Addgene plasmid 62987; Ran et al., 2013). PEX3 gRNAs were 5′-ACGTGCTTGAAAGGGGGCAT-3′ and 5′-CACCTCCAAGGA-CCGTGCCC-3′. PEX19 gRNA were 5′-GGGCCCCAGAAGAGAT-CGCC-3′ and 5′-GGGGCCGTGGTGGTAGAAGG-3′. After 24 h, the cells were selected with puromycin and expanded. A T7 assay was used to confirm genome editing. Single-cell clones were obtained and validated using immunoblotting analysis.

To generate RRBP1 KO HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1, an sgRNA transcriptional cassette prepared by PCR and pCAS9-mCherry empty (Addgene plasmid 80975; Schmid-Burgk et al., 2016) were co-transfected into HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1. After 24 h of transfection, mCherry-positive cells were sorted by fluorescence-activated cell sorting (FACS) onto a 96-well plate containing DMEM medium supplemented with 15% fetal bovine serum (GIBCO, 10270106), L-glutamine (GIBCO, 25030081) and penicillin-streptomycin (GIBCO, 15140122), and single-cell clones were generated. The RRBP1 KO cells were validated using immunoblotting analysis. Exon 1 in RRBP1 is non-coding, therefore we used gRNA targeting the first coding exon common to all isoforms of RRBP1, exon 2. The sequence of the sgRNA was 5′-CGAAGGAGGACAGTCACATT-3′.

Plasmid construction

To generate GFP–APEX2–ePTS1 (pLL3.7-GFP-FLAG-APEX2-ePTS1) we used PCR overlap extension. A FLAG–APEX2 fragment was amplified from pcDNA3 APEX2-NES (Addgene plasmid 49386; Lam et al., 2015), ePTS1 was generated using corresponding primers. The resulting fused construct was inserted into pLL3.7 vector (Addgene plasmid 11795; Rubinson et al., 2003) using EcoRI and NheI restriction sites. To generate GFP–APEX2 (pLL3.7-GFP-FLAG-APEX2), a GFP–FLAG–APEX2 fragment was amplified from pLL3.7-GFP-FLAG-APEX2-ePTS1 plasmid and ligated into pLL3.7 vector. All plasmids were confirmed by Sanger sequencing. Primers used for the cloning are in Table S4.

Stable cell line generation

HEK293T cells stably expressing GFP–APEX2–ePTS1 or GFP–APEX2 were generated by using standard lentiviral cell line generation protocol. Briefly, wild-type, PEX3 KO or PEX19 KO cells were co-transfected with pMD2.G (Addgene plasmid 12259), psPAX2 (Addgene plasmid 12260) and pLL3.7-GFP-FLAG-APEX2-ePTS1 or pLL3.7-GFP-FLAG-APEX2 using TransIT-2020 (MirusBio, MIR 5400) following the manufacturer's instructions. Single-cell clones were obtained by FACS. The expression of the constructs was confirmed by immunoblotting.

To generate HEK293T cells stably expressing HA–PEROXO (3×HA–EGFP–PEX26), the cells were co-transfected with lentiviral construct pLJC5-3XHA-EGFP-PEX26 (Addgene plasmid 139054; Ray et al., 2020), together with pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) using TransIT-2020 (MirusBio, MIR 5400) following the manufacturer's instructions. Cell populations with low or high GFP expression were subsequently isolated by FACS to establish cell lines.

APEX2 proximity labeling

APEX2 proximity labeling was performed using a previously described protocol (Hung et al., 2016) with few modifications. HEK293T cells were grown on T75 flasks. The medium was changed and 500 µM biotin–phenol (Iris Biotech, LS-3500) was added to the cell culture medium for 30 min (37°C, 5% CO_2). Proximity labeling was induced by adding 10 mM H_2O_2 (Fisher Scientific, BP2633-500). The reaction was stopped after 1 min, by removing the medium and washing the cells with quencher solution (10 mM sodium ascorbate, 5 mM Trolox and 10 mM sodium azide solution in PBS). Cells were collected in 15 ml quencher solution and pelleted at 3000 ***g*** at 4°C for 10 min. Cell pellets were flash frozen in liquid nitrogen. When processing all cell lines was complete, pellets were lysed in RIPA lysis buffer (Cell Signaling Technology, 9806) supplemented with protease inhibitor cocktail (Thermo Scientific, 10320015), 1 mM PMSF, 5 mM Trolox, 10 mM sodium azide and 10 mM sodium ascorbate. Cell lysates were incubated on ice for ∼2 min and then centrifuged at 15,000 ***g*** at 4°C for 10 min. Streptavidin magnetic beads (Thermo Scientific, 88816) were washed twice with RIPA lysis buffer supplemented with protease inhibitor cocktail (Thermo Scientific, 10320015), 1 mM PMSF, 5 mM Trolox, 10 mM sodium azide and 10 mM sodium ascorbate. Then, clarified cell lysates were added to the beads and incubated overnight with rotation at 4°C. Biotinylated proteins were eluted by boiling the beads in elution buffer [3× protein loading buffer (NEB, P7712S) supplemented with 2 mM biotin and 20 mM dithiothreitol (DTT)] for 10 min. Tubes were briefly centrifuged and, using a magnetic rack, samples were collected, flash frozen in liquid nitrogen and stored at −80°C.

Frozen eluates were thawed on ice and run on 10% separating gel at 200 V for 9 min. Samples were cut from the gel, stained with Coomassie, and washed twice with H_2O. Then individual lanes were cut out and stored at −20°C in separate microfuge tubes. Scalpels used to cut the gel were single use, and the samples were loaded with one empty well between.

Mass spectrometry analysis of proteins

Protein bands were cut out of the SDS-PAGE gel and 'in-gel' digested. Cysteine bonds were reduced with 0.045 M DTT (Sigma-Aldrich, D0632) for 20 min at 37°C and alkylated with 0.1 M iodoacetamide (Fluka, Sigma-Aldrich, 57670) at room temperature in the dark for 30 min. Proteins were digested with 0.75 µg trypsin (Sequencing Grade Modified Trypsin, V5111, Promega) overnight at 37°C. After digestion, peptides were purified with C-18 Micro SpinColumns (The Nest Group Inc. USA) according to the manufacturer's instructions. The dried peptides were reconstituted in 30 µl 0.1% trifluoroacetic acid (TFA) and 1% acetonitrile (ACN) in liquid chromatography–mass spectrometry (LC-MS)-grade water. The mass spectrometry analysis was performed on an Orbitrap Elite and Q Exactive ESI-quadrupole-orbitrap mass spectrometer coupled to an EASY-nLC 1000 nanoflow LC (Thermo Fisher Scientific), using the Xcalibur version 3.1.66.10 (Thermo Scientific) as described previously (Liu et al., 2018). Acquired spectral data files were searched against the human component of UniProtKB/SwissProt database using SEQUEST search engine in the Proteome Discoverer 1.4 (Thermo Scientific). The following parameters were applied: only fully tryptic peptides were allowed with maximum of two missed cleavages, precursor mass tolerance and fragment mass tolerance were set to ±15 ppm and 0.05 Da, respectively. Carbamidomethylation of cysteine was defined as static modification, and oxidation of methionine was set as variable modification. The mass spectrometry data were filtered and presented as peptide spectrum matches.

esiRNA gene silencing

HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated in 6-well plate 24 h before transfection. The cells were transfected with 25 nM esiRNA targeting GFP (Merck, EHUEGFP), RRBP1 (Merck, EHU073471) or PEX5 (Merck, EHU090061) using TransIT-X2 (MirusBio, MIR 6000). After 72 h the cells were collected for immunoblotting analysis.

For electron microscopy, HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated in 6-well plate containing small 13 mm glass coverslips 24 h before transfection. The cells were transfected with 25 nM esiRNA targeting LUC (Merck, EHURLUC) or RRBP1 (Merck, EHU073471), using FuGENE HD Transfection reagent (Promega, E2311). After 72 h, the coverslips were fixed, and samples were prepared for

electron microscopic analysis. The esiRNA cDNA target sequences are in Table S5.

CRISPR/Cas9 microscopy screen

To perform the CRISPR/Cas9 microscopy screen, for each of the 44 selected candidates and two positive controls (PEX5 and PEX19) two gRNAs were designed, except for CSK21, for which only one target sequence was found with sufficient specificity. gRNAs with U6 overhang at the 5′ end and terminator overhang at the 3′ end were purchased as oligonucleotides (Table S6). U6 was cloned from PX335-dCas9-VP192-PGKpuro using PCR. Obtained U6 was 5'-tailed and amplified using PCR. 3'-tailed terminator was generated using PCR. The gRNA transcriptional cassette was generated by PCR using 5'-tailed U6, 3'-tailed terminator and U6 overhang-gRNA-terminator overhang as described and utilized previously (Balboa et al., 2015; Konovalova et al., 2018, 2023). The sequences of used oligonucleotides are in Table S4.

HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated in 24-well plates 24 h before the transfection. The cells were co-transfected with a single gRNA and pCAS9-mCherry empty (Addgene plasmid 80975; Schmid-Burgk et al., 2016) using TransIT-2020 (Mirus, MIR 5400). The cells were passaged when needed. Two rounds of transfections were performed for each gRNA, each time the cells were analyzed three times at 7, 10 and 14 days after transfection using an EVOS FLoid Imaging System (Thermo Fisher Scientific). The phenotype of the cells was manually classified based on GFP localization: 'no' (GFP localized exclusively to peroxisomes, no mislocalization), 'mild' (some mislocalized GFP), 'strong' (substantial mislocalized GFP), and 'very strong' (extensive mislocalized GFP). The investigator was not aware of the group allocation during the cell analysis.

Live-cell fluorescence microscopy

Live-cell microscopy imaging was performed on cells in 6-well or 24-well plates using an EVOS FLoid Imaging System (Thermo Fisher Scientific) equipped with 20× objective or Eclipse TS100 (Nikon) equipped with 40× objective.

Western blotting

HEK293T cells were lysed in RIPA buffer [Cell Signaling Technology (CST), 9806S] supplemented with protease inhibitor cocktail (Thermo Scientific, 78429). Following 15 min incubation on ice, the samples were centrifuged at 14,000 ***g*** for 10 min at 4°C. Protein concentration was measured by bicinchoninic acid (BCA) assay (Thermo Fisher, 23228). Protein lysates were supplemented with a Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% Bromophenol Blue), boiled for 5 min at 95° and resolved on 10% or 7.5% Mini-PROTEAN TGX Precast Gels (Bio-Rad, 4561033, 4561036, 4561023, 4561026). The SDS-PAGE was performed at 100 V for 5 min and then 120 V for 1 h 15 min using SDS running buffer (30 g Tris, 144 g glycine, 10 g SDS to 1 l distilled water). Then the proteins were transferred to a 0.2 µM PVDF membrane (Bio-Rad, 1704150) by Trans-Blot Turbo Transfer System (Bio-Rad) using pre-installed settings (2.5 A for 7 min) for transferring proteins of mixed molecular weight. The membranes were blocked in 5% milk or 5% BSA in TBS with 0.1% Tween 20 (TBST) for 1 h at room temperature. Proteins were incubated with the indicated primary antibodies in TBST containing 1% or 5% BSA overnight at 4°C. The following primary antibodies were used: anti-catalase, 1:1000 (CST, 12980S); anti-RRBP1, 1:1000 (Proteintech, 22015-1-AP); anti-PEX3, 1:1000 (Schmidt et al., 2012); anti-PEX19, 1:1000 (Abcam, 137072); anti-PEX14, 1:1000 (Abcam, 183885); anti-PEX16, 1:1000 (Proteintech, 14816-1-AP); anti-PEX5, 1:1000 (Invitrogen, PA5-58717); anti-calnexin, 1:1000 (Abcam, 22595); anti-PMP70, 1:800 (Sigma, SAB4200181); anti-GFP, 1:1000 (Aves, GFP-1020); anti-TOM20, 1:500 (CST, 42406); anti-ACOX1, 1:1000 (Proteintech, 10957-1-AP); anti-ACAA1, 1:2000 (Sigma-MERCK, HPA007244); anti-acetylated tubulin, 1:1000 (Sigma-MERCK, T7451); anti-GAPDH, 1:10,000 (Sigma-MERCK, G9545); anti-ATG5, 1:2500 (Proteintech, 10181-2-AP); anti-LC3B 1:1000 (Novus biologicals, 600-1384); anti-VAPB, 1:5000 (Proteintech, 14477-1-AP); anti-BiP, 1:1000 (CST, 3177); anti-IRE1α, 1:1000 (CST, 3294); anti-SEC61A, 1:1000 (Abcam, 183046); anti-puromycin antibody, 1:10,000 (Sigma, MABE343); anti-RPL7, 1:1000 (Proteintech, 14583-1-AP); and anti-PXMP2, 1:1000 (Proteintech, 24801-1-AP). Next, the membranes were washed with TBST quickly three times and then three times for 15 min at room temperature on shaker. Then the membranes were incubated with HRP-conjugated secondary antibodies against mouse IgG 1:5000 (CST 7076S), rabbit IgG (CST 7074S) or chicken IgY (Promega G135A) in TBST containing 1% BSA for 1 h at room temperature on a shaker. Then the membranes were washed with TBST quickly three times and then three times for 15 min at room temperature on a shaker. ECL western blotting substrate (Thermo Scientific, 32106) was applied to the membrane, and chemiluminescence was captured by Chemidoc imaging system (Bio-Rad). Quantification of the bands was performed by Image Lab Software (Bio-Rad). Full uncropped blot images are presented in Fig. S4.

Immunofluorescence

HEK293T cells were cultured on 13 mm coverslips 24 h before fixation. Then the medium was removed, and the cells were washed three times with PBS. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed three times with PBS. Then the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature and washed with PBS. Next DAPI (1:1000 in PBS) was added for 10 min at room temperature. The cells were washed with 0.1% Tween 20 in PBS (PBST) and blocked with 5% BSA in PBST for 2 h at room temperature. The cells were then incubated with corresponding primary antibodies diluted in 5% BSA in PBST overnight at 4°C. The following primary antibodies were used: anti-catalase, 1:400 (CST, 12980S); anti-RRBP1, 1:500 (GeneTex GT5610); anti-calnexin, 1:1000 (Abcam, 22595); anti-PEX14, 1:250 (Abcam, 183885); anti-PMP70, 1:150 (Sigma, SAB4200181); anti-GFP, 1:10,000 (Aves, GFP-1020); and anti-ATPIF, 1:100 (CST 8528). Then the cells were washed with PBST three times for 15 min. The cells were incubated with secondary antibodies diluted 1:1000 in 5% BSA in PBST (anti-mouse IgG Alexa Fluor 555, CST 4409S; anti-mouse IgG Alexa Fluor 647, Invitrogen, A-21235; anti-rabbit IgG Alexa Fluor 555, CST, 4413S; anti-rabbit IgG Alexa Fluor 647, CST, 4414S; anti-chicken IgY Alexa Fluor 488, Invitrogen, A-11039) for 1 h at room temperature and washed with PBST three times for 5 min. Finally, the cells were mounted using antifade mounting medium (Vector Laboratories, H-1700-10). The slides were left to dry overnight at room temperature and then stored at 4°C until analysis. The cells were imaged using an upright SP8 confocal microscope (Leica) with a 63× oil immersion objective.

Quantitative analysis of immunofluorescence images

Imaris Software was used for the analysis of confocal immunofluorescence images. For colocalization analysis Coloc tool within the Surpass system (version 9.5.1) was used. The images were carefully adjusted manually to distinguish between background and signal. The Manders' overlap coefficient was used to quantify the percentage of overlap. In each sample, 5–8 fields of view were analyzed, with each field capturing ∼60–120 cells.

To quantify PMP70-, PEX14- or catalase-positive dots, counting cell number or volume, the confocal microscopy images were processed and reconstructed in a three-dimensional (3D) model using Imaris software.

PMP70-, PEX14- or catalase-positive dots were counted using the 'Dots' function. PMP70, PEX14 or catalase signal was adjusted for the optimal dot detection: X4 diameter (0.5 µm) and model PSF (1.5 µm). The number of PMP70-, PEX14- or catalase-positive dots was normalized to the number of the cells in the specific field of view.

The cell number was counted based on DAPI nuclear staining. Parameters were set with an *XY* diameter of 7 µm to optimize nucleus detection. To analyze the cell volume, PMP70, PEX14 or catalase signal was adjusted to cover the cytosolic area and eliminate any redundant covered regions and debris. Within the Surpass system, a surface was added with default settings, excluding the shortest distance calculations.

Peroxisome isolation by PEROXO-tag IP

Peroxisomes were isolated as described previously (Ray et al., 2020) with slight modifications. HEK293T cells stably expressing 3×HA–EGFP–PEX26 were cultured in two 10 cm dishes for 24 h. Plates were placed on ice and washed twice with cold PBS, after which cells were scraped into 1 ml cold potassium phosphate-buffered saline (KPBS) and pelleted at 1000 ***g*** for

2 min at 4°C. Pellets were resuspended in 1 ml KPBS. For whole-cell lysates, 5 µl of the resuspended pellet was mixed with 50 µl lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% IGEPAL (Sigma-Aldrich, I3021) in ultrapure water] containing protease inhibitors (Thermo Fisher Scientific, 78429). The remaining 995 µl was homogenized on ice using a 2 ml Dounce homogenizer (25 strokes). The homogenate was centrifuged at 1000 ***g*** for 2 min at 4°C, and the supernatant was collected. The supernatant was incubated with 200 µl anti-HA magnetic beads (Pierce Anti-HA Magnetic Beads, Thermo Scientific, 88836) on a rotator at 4°C for 3.5 min. Beads were washed 10 times with cold KPBS, with tubes replaced after the first and final washes. Beads were resuspended in 50 µl lysis buffer containing protease inhibitors, incubated on ice for 10 min, and removed using a magnetic stand. The supernatant was centrifuged at 21,000 ***g*** for 10 min at 4°C, and the resulting clarified fraction was collected as the purified peroxisome protein sample. Prior to immunoblot analysis, both whole-cell lysates and purified peroxisomes were sonicated in a water bath sonicator for three 30 s cycles. Protein concentrations were determined by BCA assay, and proteins were analyzed by western blotting.

Pexophagy assay

RRBP1 KO HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 or the parental cell line were plated on 6-well plates 24 h before the treatment. 100 µM chloroquine was added to the cells cultured in complete DMEM medium. The cells were incubated for 3 h, and after the incubation the cells were collected and used for western blotting analysis.

Proteosomal inhibition assay

Parental cell line or RRBP1 KO HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated on 12-well plates 24 h before the treatment. The cells were treated with 10 µM MG132 for 4 h, then the cells were collected and used for western blotting analysis.

Translation assay

Protein translation rate was analyzed by SUrface SEnsing of Translation (SUnSET) method, which involves puromycin labeling of nascent proteins followed by immunoblotting to detect the labeled proteins (Schmidt et al., 2009). HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 or RRBP1 KO HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated in 12-well plates. After 24 h, the cells were treated with 10 µg/ml puromycin and incubated for 20 min in a 37°C, 5% CO_2 incubator. Afterwards, the cells were collected and lysed, followed by immunoblot analysis.

Cell proliferation assay

HEK293T, HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 or RRBP1 KO HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were plated in a 48-well plate at a starting density of 23,000 cells per well. After 24 h, the cells were resuspended and mixed with Trypan Blue stain (1:1). Cell counting was performed using the Bio-Rad TC20 automated cell counter.

Transmission electron microscopy

HEK293T cells expressing EGFP–FLAG–APEX2–ePTS1 were grown on glass coverslips (thickness #1) and fixed with 2% glutaraldehyde (EM-grade) in 0.1 M sodium cacodylate buffer, pH 7.4, supplemented with 2 mM $CaCl_2$. After fixation for 30 min at room temperature, the samples were washed with sodium cacodylate buffer followed by washing with 50 mM glycine–sodium hydroxide buffer, pH 10.5. For identification of peroxisomes, the cells expressing APEX2 were labeled with 5 mM 3,3′-diaminobenzidine (DAB) and 0.15% H_2O_2 in glycine–sodium hydroxide buffer for 20–25 min, on ice, in the dark. The reaction was stopped by washing the samples with glycine–sodium hydroxide buffer, after which the cells were post-fixed with 1% reduced osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h on ice. After washing, the samples were gradually dehydrated in ethanol and acetone prior to flat embedding (Jokitalo et al., 2001) in epoxy (TAAB 812, Aldermaston, UK). Sections of 60 nm were cut using an Ultracut UC7 ultramicrotome (Leica, Vienna, Austria), post-stained with uranyl acetate and lead citrate, and examined at 100 kV using an HT7800 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan). Images were acquired using a Rio9 CMOS-camera (AMETEK Gatan Inc., Pleasanton, CA, USA).

Peroxisome–ER contact site analysis

In each experiment for each cell line, 13–18 cells having peroxisomal profiles were selected using systematic random sampling for the analysis of peroxisomes and peroxisome–ER contact sites. All the peroxisomes in the selected cells were imaged at 8000× nominal magnification with a pixel size of 1.4 nm. Images were contrast normalized and converted to 8-bit using Microscopy Image Browser (MIB; Belevich et al., 2016). For unbiased analysis, the images were anonymized before manually segmenting peroxisomes and ER using MIB. The contact sites were analyzed using the MCcalc plug-in (Lak et al., 2021) in MIB software, with probing distance of 150 nm, smoothing 15 and accepting gaps of 7 pixels. The peroxisomal area (peroxisomal size) and eccentricity (peroxisomal shape) were analyzed using MIB software (Belevich et al., 2016).

Statistical analysis

All data are presented as mean±s.d. For the statistical analyses with two samples, Student's unpaired two-tailed *t*-tests was performed using Graph Pad Prism Software. All the graphs and heat maps were prepared with Graph Pad Prism Software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.K.; Data curation: S.K.; Formal analysis: K.F., S.K.; Funding acquisition: K.F.; Investigation: K.F., H.V., B.A.A., T.S., A.P., E.J., S.K.; Methodology: K.F., H.V., B.A.A., E.J., S.K.; Project administration: S.K.; Supervision: S.K.; Visualization: K.F., S.K.; Writing – original draft: K.F., S.K.; Writing – review & editing: K.F., V.P., P.K., S.K.

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Data and resource availability

All relevant data and details of resources can be found within the article and its supplementary information.

First Person

This article has an associated First Person interview with the first author of the paper.

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References

Akşit, A. and van der Klei, I. J. (2018). Yeast peroxisomes: how are they formed and how do they grow? *Int. J. Biochem. Cell Biol.* **105**, 24-34. doi:10.1016/j.biocel.2018.09.019

Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A. K. W., Veenhuis, M. and Kunau, W.-H. (1997). Pex14p, a peroxisomal membrane protein binding both receptors of the two PTS-dependent import pathways. *Cell* **89**, 83-92. doi:10.1016/S0092-8674(00)80185-3

Anastasia, I., Ilacqua, N., Raimondi, A., Lemieux, P., Ghandehari-Alavijeh, R., Faure, G., Mekhedov, S. L., Williams, K. J., Caicci, F., Valle, G. et al. (2021). Mitochondria-rough-ER contacts in the liver regulate systemic lipid homeostasis. *Cell Rep.* **34**, 108873. doi:10.1016/j.celrep.2021.108873

Aranovich, A., Hua, R., Rutenberg, A. D. and Kim, P. K. (2014). PEX16 contributes to peroxisome maintenance by constantly trafficking PEX3 via the ER. *J. Cell Sci.* **127**, 3675-3686. doi:10.1242/jcs.146282

Balboa, D., Weltner, J., Eurola, S., Trokovic, R., Wartiovaara, K. and Otonkoski, T. (2015). Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. *Stem Cell Rep.* **5**, 448-459. doi:10.1016/j.stemcr.2015.08.001

Baldwin, H. A., Wang, C., Kanfer, G., Shah, H. V., Velayos-Baeza, A., Dulovic-Mahlow, M., Brüggemann, N., Anding, A., Baehrecke, E. H., Maric, D. et al. (2021). VPS13D promotes peroxisome biogenesis. *J. Cell Biol.* **220**, e202001188. doi:10.1083/JCB.202001188

Belevich, I., Joensuu, M., Kumar, D., Vihinen, H. and Jokitalo, E. (2016). Microscopy image browser: a platform for segmentation and analysis of multidimensional datasets. *PLoS Biol.* **14**, e1002340. doi:10.1371/journal.pbio.1002340

Cardoen, B., Vandevoorde, K. R., Gao, G., Ortiz-Silva, M., Alan, P., Liu, W., Tiliakou, E., Vogl, A. W., Hamarneh, G. and Nabi, I. R. (2024). Membrane contact site detection (MCS-DETECT) reveals dual control of rough mitochondria-ER contacts. *J. Cell Biol.* **223**, e202206109. doi:10.1083/jcb.202206109

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

Cui, X. A. and Palazzo, A. F. (2014). Localization of mRNAs to the endoplasmic reticulum. *Wiley Interdiscip. Rev. RNA* **5**, 481-492. doi:10.1002/wrna.1225

Cui, X. A., Zhang, H. and Palazzo, A. F. (2012). P180 promotes the ribosome-independent localization of a subset of mRNA to the endoplasmic reticulum. *PLoS Biol.* **10**, e1001336. doi:10.1371/journal.pbio.1001336

Dahan, N., Bykov, Y. S., Boydston, E. A., Fadel, A., Gazi, Z., Hochberg-Laufer, H., Martenson, J., Denic, V., Shav-Tal, Y., Weissman, J. S. et al. (2022). Peroxisome function relies on organelle-associated mRNA translation. *Sci. Adv.* **8**, eabk2141. doi:10.1126/sciadv.abk2141

DeLoache, W. C., Russ, Z. N. and Dueber, J. E. (2016). Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways. *Nat. Commun.* **7**, 11152. doi:10.1038/NCOMMS11152

Demers, N. D., Riccio, V., Jo, D. S., Bhandari, S., Law, K. B., Liao, W., Kim, C., McQuibban, G. A., Choe, S.-K., Cho, D.-H. et al. (2023). PEX13 prevents pexophagy by regulating ubiquitinated PEX5 and peroxisomal ROS. *Autophagy* **19**, 1781-1802. doi:10.1080/15548627.2022.2160566

Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W. et al. (1996). A unified nomenclature for peroxisome biogenesis factors. *J. Cell Biol.* **135**, 1-3. doi:10.1083/jcb.135.1.1

Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W. H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. *Proc. Natl Acad. Sci. USA* **86**, 5419-5423. doi:10.1073/pnas.86.14.5419

Fang, Y., Morrell, J. C., Jones, J. M. and Gould, S. J. (2004). PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. *J. Cell Biol.* **164**, 863-875. doi:10.1083/jcb.200311131

Farré, J. C., Mahalingam, S. S., Proietto, M. and Subramani, S. (2019). Peroxisome biogenesis, membrane contact sites, and quality control. *EMBO Rep.* **20**, e46864. doi:10.15252/embr.201846864

Fujiki, Y., Okumoto, K., Mukai, S., Honsho, M. and Tamura, S. (2014). Peroxisome biogenesis in mammalian cells. *Front. Physiol.* **5**, 102311. doi:10.3389/fphys.2014.00307

Germain, K. and Kim, P. K. (2020). Pexophagy: a model for selective autophagy. *Int. J. Mol. Sci.* **21**, 578. doi:10.3390/ijms21020578

Geuze, H. J., Murk, J. L., Stroobants, A. K., Griffith, J. M., Kleijmeer, M. J., Koster, A. J., Verkleij, A. J., Distel, B. and Tabak, H. F. (2003). Involvement of the endoplasmic reticulum in peroxisome formation. *Mol. Biol. Cell* **14**, 2900-2907. doi:10.1091/mbc.e02-11-0734

Goldman, B. M. and Blobel, G. (1978). Biogenesis of peroxisomes: intracellular site of synthesis of catalase and uricase. *Proc. Natl. Acad. Sci. USA* **75**, 5066-5070. doi:10.1073/pnas.75.10.5066

Guillén-Samander, A., Leonzino, M., Hanna, M. G., Tang, N., Shen, H. and De Camilli, P. (2021). VPS13D bridges the ER to mitochondria and peroxisomes via Miro. *J. Cell. Biol.* **220**, e202010004. doi:10.1083/jcb.202010004

Haimovich, G., Cohen-Zontag, O. and Gerst, J. E. (2016). A role for mRNA trafficking and localized translation in peroxisome biogenesis and function? *Bioch. Biophys. Acta Mol. Cell Res.* **1863**, 911-921. doi:10.1016/j.bbamcr.2015.09.007

Hasan, S., Platta, H. W. and Erdmann, R. (2013). Import of proteins into the peroxisomal matrix. *Front. Physiol.* **4**, 261. doi:10.3389/fphys.2013.00261

Hua, R., Gidda, S. K., Aranovich, A., Mullen, R. T. and Kim, P. K. (2015). Multiple domains in PEX16 mediate its trafficking and recruitment of peroxisomal proteins to the ER. *Traffic* **16**, 832-852. doi:10.1111/TRA.12292

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., Vissa, A., Yip, C. M., Fairn, G. D., Braverman, N. et al. (2017). VAPs and ACBD5 tether peroxisomes to the ER for peroxisome maintenance and lipid homeostasis. *J. Cell Biol.* **216**, 367-377. doi:10.1083/jcb.201608128

Hung, V., Udeshi, N. D., Lam, S. S., Loh, K. H., Cox, K. J., Pedram, K., Carr, S. A. and Ting, A. Y. (2016). Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nat. Protoc.* **11**, 456-475. doi:10.1038/nprot.2016.018

Hung, V., Lam, S. S., Udeshi, N. D., Svinkina, T., Guzman, G., Mootha, V. K., Carr, S. A. and Ting, A. Y. (2017). Proteomic mapping of cytosol-facing outer mitochondrial and ER membranes in living human cells by proximity biotinylation. *eLife* **6**, e24463. doi:10.7554/eLife.24463

Jokitalo, E., Cabrera-Poch, N., Warren, G. and Shima, D. T. (2001). Golgi clusters and vesicles mediate mitotic inheritance independently of the endoplasmic reticulum. *J. Cell Biol.* **154**, 317. doi:10.1083/jcb.200104073

Killackey, S. A., Bi, Y., Philpott, D. J., Arnoult, D. and Girardin, S. E. (2023). Mitochondria-ER cooperation: NLRX1 detects mitochondrial protein import stress and promotes mitophagy through the ER protein RRBP1. *Autophagy* **19**, 1601. doi:10.1080/15548627.2022.2129763

Kim, P. K., Mullen, R. T., Schumann, U. and Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J. Cell Biol.* **173**, 521-532. doi:10.1083/jcb.200601036

Klouwer, F. C. C., Falkenberg, K. D., Ofman, R., Koster, J., van Gent, D., Ferdinandusse, S., Wanders, R. J. A. and Waterham, H. R. (2021). Autophagy inhibitors do not restore peroxisomal functions in cells with the most common peroxisome biogenesis defect. *Front. Cell Dev. Biol.* **9**, 661298. doi:10.3389/fcell.2021.661298

Konovalova, S., Liu, X., Manjunath, P., Baral, S., Neupane, N., Hilander, T., Yang, Y., Balboa, D., Terzioglu, M., Euro, L. et al. (2018). Redox regulation of GRPEL2 nucleotide exchange factor for mitochondrial HSP70 chaperone. *Redox. Biol.* **19**, 37. doi:10.1016/j.redox.2018.07.024

Konovalova, S., Torregrosa-Muñumer, R., Manjunath, P., Liu, X., Baral, S., Fatima, K., Holopainen, M., Kvist, J., Rajendran, J., Yang, Y. et al. (2023). Small mitochondrial protein NERCLIN regulates cardiolipin homeostasis and mitochondrial ultrastructure. *Proc. Natl. Acad. Sci. USA* **120**, e2210599120. doi:10.1073/pnas.2210599120

Koppers, M., Özkan, N., Nguyen, H. H., Jurriens, D., McCaughey, J., Nguyen, D. T. M., Li, C. H., Stucchi, R., Altelaar, M., MacGillavry, H. D. et al. (2024). Axonal endoplasmic reticulum tubules control local translation via P180/RRBP1-mediated ribosome interactions. *Dev. Cell* **59**, 2053-2068.e9. doi:10.1016/j.devcel.2024.05.005

Kurochkin, I. V., Mizuno, Y., Konagaya, A., Sakaki, Y., Schönbach, C. and Okazaki, Y. (2007). Novel peroxisomal protease Tysnd1 processes PTS1- and PTS2-containing enzymes involved in β-oxidation of fatty acids. *EMBO J.* **26**, 835. doi:10.1038/sj.emboj.7601525

Lak, B., Li, S., Belevich, I., Sree, S., Butkovic, R., Ikonen, E. and Jokitalo, E. (2021). Specific subdomain localization of ER resident proteins and membrane contact sites resolved by electron microscopy. *Eur. J. Cell Biol.* **100**, 151180. doi:10.1016/j.ejcb.2021.151180

Lam, S. K., Yoda, N. and Schekman, R. (2010). A vesicle carrier that mediates peroxisome protein traffic from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **107**, 21523-21528. doi:10.1073/pnas.1013397107

Lam, S. S., Martell, J. D., Kamer, K. J., Deerinck, T. J., Ellisman, M. H., Mootha, V. K. and Ting, A. Y. (2015). Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nat. Methods* **12**, 51-54. doi:10.1038/nmeth.3179

Liu, X., Salokas, K., Tamene, F., Jiu, Y., Weldatsadik, R. G., Öhman, T. and Varjosalo, M. (2018). An AP-MS- and BioID-compatible MAC-tag enables comprehensive mapping of protein interactions and subcellular localizations. *Nat. Commun.* **9**, 1188. doi:10.1038/s41467-018-03523-2

Martell, J. D., Deerinck, T. J., Lam, S. S., Ellisman, M. H. and Ting, A. Y. (2017). Electron microscopy using the genetically encoded APEX2 tag in cultured mammalian cells. *Nat. Protoc.* **12**, 1792. doi:10.1038/nprot.2017.065

Matsumoto, N., Tamura, S. and Fujiki, Y. (2003). The pathogenic peroxin Pex26p recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes. *Nat. Cell Biol.* **5**, 454-460. doi:10.1038/ncb982

Miura, S., Mori, M., Takiguchi, M., Tatibana, M., Furuta, S., Miyazawa, S. and Hashimoto, T. (1984). Biosynthesis and intracellular transport of enzymes of peroxisomal beta-oxidation. *J. Biol. Chem.* **259**, 6397-6402. doi:10.1016/S0021-9258(20)82155-3

Ogawa-Goto, K., Tanaka, K., Ueno, T., Tanaka, K., Kurata, T., Sata, T. and Irie, S. (2007). p180 is involved in the interaction between the endoplasmic reticulum and microtubules through a novel microtubule-binding and bundling domain. *Mol. Biol. Cell* **18**, 3741. doi:10.1091/mbc.e06-12-1125

Okumoto, K., Miyata, N. and Fujiki, Y. (2018). Identification of peroxisomal protein complexes with PTS receptors, pex5 and pex7, in mammalian cells. *Subcell. Biochem.* **89**, 287-298. doi:10.1007/978-981-13-2233-4_12

Rachubinski, R. A., Fujiki, Y., Mortensen, R. M. and Lazarow, P. B. (1984). Acyl-Coa oxidase and hydratase-dehydrogenase, two enzymes of the peroxisomal beta-oxidation system, are synthesized on free polysomes of clofibrate-treated rat liver. *J. Cell Biol.* **99**, 2241-2246. doi:10.1083/jcb.99.6.2241

Rae, J., Ferguson, C., Ariotti, N., Webb, R. I., Cheng, H.-H., Mead, J. L., Riches, J. D., Hunter, D. J. B., Martel, N., Baltos, J. et al. (2021). A robust method for particulate detection of a genetic tag for 3D electron microscopy. *eLife* **10**, e64630. doi:10.7554/eLife.64630

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281. doi:10.1038/nprot.2013.143

Ray, G. J., Boydston, E. A., Shortt, E., Wyant, G. A., Lourido, S., Chen, W. W. and Sabatini, D. M. (2020). A PEROXO-tag enables rapid isolation of peroxisomes from human cells. *iScience* **23**, 101109. doi:10.1016/j.isci.2020.101109

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Zhang, M., McManus, M. T., Gertler, F. B. et al. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33**, 401-406. doi:10.1038/ng1117

Sakai, Y., Oku, M., van der Klei, I. J. and Kiel, J. A. K. W. (2006). Pexophagy: autophagic degradation of peroxisomes. *Biochim. Biophys. Acta Mol. Cell Res.* **1763**, 1767-1775. doi:10.1016/j.bbamcr.2006.08.023

Savitz, A. J. and Meyer, D. I. (1990). Identification of a ribosome receptor in the rough endoplasmic reticulum. *Nature* **346**, 540-544. doi:10.1038/346540a0

Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W. and Kunau, W.-H. (1999). Recombinant human peroxisomal targeting signal receptor PEX5. *J. Biol. Chem.* **274**, 5666-5673. doi:10.1074/jbc.274.9.5666

Schmid-Burgk, J. L., Höning, K., Ebert, T. S. and Hornung, V. (2016). CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat. Commun.* **7**, 12338. doi:10.1038/ncomms12338

Schmidt, E. K., Clavarino, G., Ceppi, M. and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods* **6**, 275-277. doi:10.1038/nmeth.1314

Schmidt, F., Dietrich, D., Eylenstein, R., Groemping, Y., Stehle, T. and Dodt, G. (2012). The role of conserved PEX3 regions in PEX19-binding and peroxisome biogenesis. *Traffic* **13**, 1244-1260. doi:10.1111/j.1600-0854.2012.01380.x

Sengupta, R., Poderycki, M. J. and Mattoo, S. (2019). CryoAPEX - an electron tomography tool for subcellular localization of membrane proteins. *J. Cell Sci.* **132**, jcs222315. doi:10.1242/jcs.222315

Shibata, Y., Shemesh, T., Prinz, W. A., Palazzo, A. F., Kozlov, M. M. and Rapoport, T. A. (2010). Mechanisms determining the morphology of the peripheral ER. *Cell* **143**, 774. doi:10.1016/j.cell.2010.11.007

Somborac, T., Lutfullahoglu Bal, G., Fatima, K., Vihinen, H., Paatero, A., Jokitalo, E., Paavilainen, V. O. and Konovalova, S. (2023). The subset of peroxisomal tail-anchored proteins do not reach peroxisomes via ER, instead mitochondria can be involved. *PLoS ONE* **18**, e0295047. doi:10.1371/journal.pone.0295047

Sugiura, A., Mattie, S., Prudent, J. and McBride, H. M. (2017). Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* **542**, 251-254. doi:10.1038/nature21375

van der Zand, A., Braakman, I. and Tabak, H. F. (2010). Peroxisomal membrane proteins insert into the endoplasmic reticulum. *Mol. Biol. Cell* **21**, 2057-2065. doi:10.1091/mbc.e10-02-0082

Walter, T. and Erdmann, R. (2019). Current advances in protein import into peroxisomes. *Protein J.* **38**, 351-362. doi:10.1007/s10930-019-09835-6

Zipor, G., Haim-Vilmovsky, L., Gelin-Licht, R., Gadir, N., Brocard, C. and Gerst, J. E. (2009). Localization of mRNAs coding for peroxisomal proteins in the yeast, Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **106**, 19848-19853. doi:10.1073/pnas.0910754106

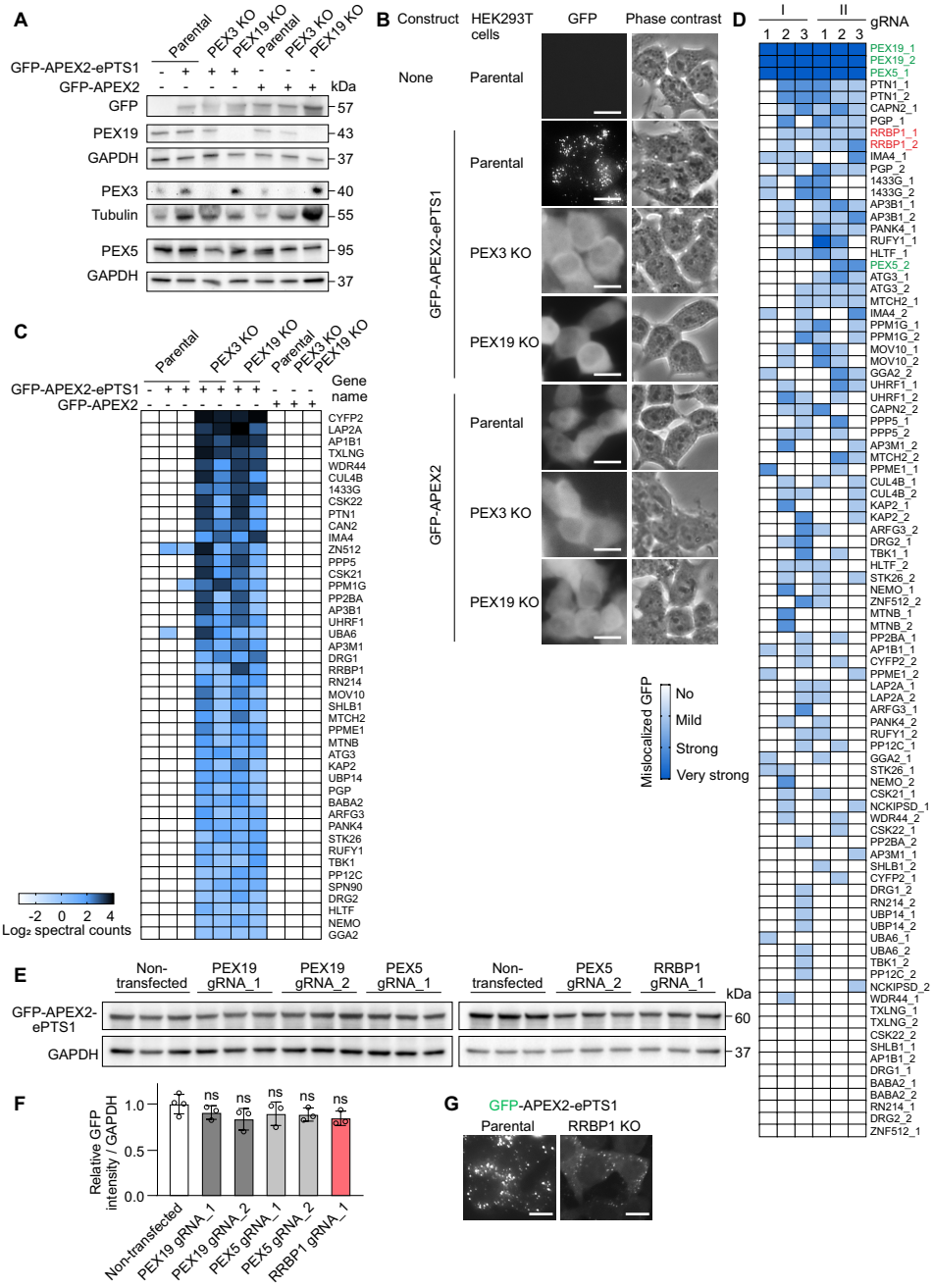

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Fig. S1. Systematic identification of peroxisome biogenesis factors using proximity labeling and CRISPR/Cas9 microscopy-based screen.

(A) Western blotting analysis of the indicated proteins in HEK293T clonal cell lines stably expressing GFP-APEX2-ePTS1 or GFP-APEX2 constructs used for APEX2-based proximity labeling (Fig. 1A). GAPDH or tubulin was used as a loading control. (B) Live-cell fluorescence microscopy images showing intracellular localization of GFP-APEX2-ePTS1 or GFP-APEX2 constructs in HEK293T cells used for APEX2-based proximity labeling. The localization of GFP-containing constructs was visualized using GFP channel. Scale bars: 20 μm. (C) Heat map of the potential peroxisome biogenesis factors identified by APEX2-based proximity labeling (Fig. 1B). (D) Heat map of CRISPR/Cas9 microscopy assessing the phenotype of HEK293T cells. HEK293T cells stably expressing GFP-APEX2-ePTS1 were transiently transfected with gRNAs and Cas9-mCherry plasmid as shown in (A). Two rounds of transfection were performed (I and II). Each round the phenotype of the cells was assessed three times at 7, 10 and 14 post-transfection days using live-cell fluorescence microscopy. Cells were manually classified based on GFP localization: into “no” (GFP localized exclusively to peroxisomes, no mislocalization), “mild” (some mislocalized GFP), “strong” (substantial mislocalized GFP), and “very strong” (extensive mislocalized GFP) categories. gRNAs targeting PEX5 or PEX19 were used as positive controls (in green). (E) Western blot analysis of GFP-APEX2-ePTS protein level in HEK293T cells stably expressing GFP-APEX2-ePTS1 before and after 13 days of transfection with Cas9-mCherry plasmid and gRNA targeting PEX19, PEX5 or RRBP1. Anti-GFP antibody was used to detect the GFP-APEX2-ePTS protein. GAPDH was used as a loading control. (F) Quantification of western blot images as represented in E (n = 3). Values are normal-ized to the non-transfected cells, set to 1. In all graphs data are presented as mean ± SD; ns, not significant as compared to the non-transfected cells (unpaired two-tailed t-tests). (G) Live-cell fluorescence mi-croscopy images showing intracellular localization of stably expressing GFP-APEX2-ePTS1 construct in RRBP1 KO or parental HEK293T cells. The localization of GFP-containing constructs was visualized using GFP channel. Scale bars: 10 μm.

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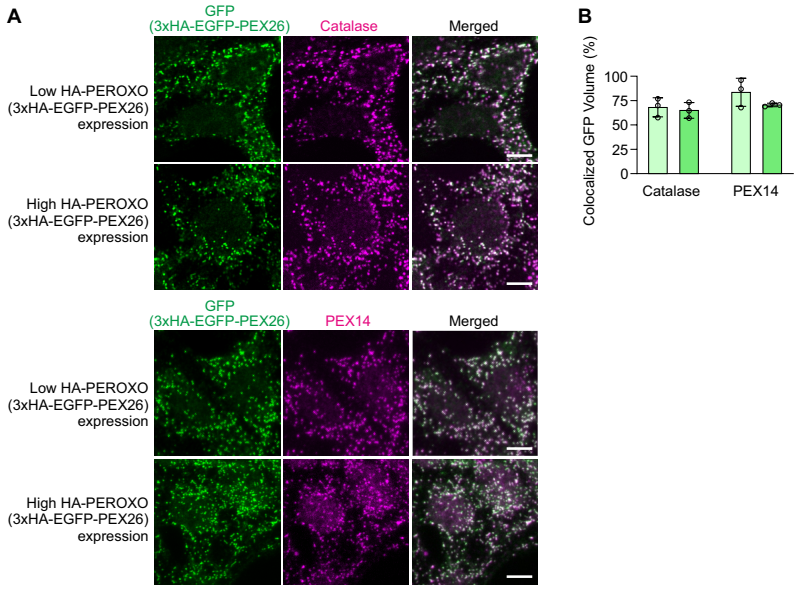

Fig. S2. HA-PEROXO (3×HA-EGFP-PEX26) construct colocalizes with peroxisomal markers.

(A) Confocal microscopy images of HEK293T cells stably expressing 3×HA-EGFP-PEX26 (HA-PEROXO). Immunostaining was performed using anti-GFP, anti-catalase or anti-PEX14 antibody. Scale bars: 10 µm. (B) Colocalization analysis of confocal microscopy images as represented in A. The data are presented as mean ± SD.

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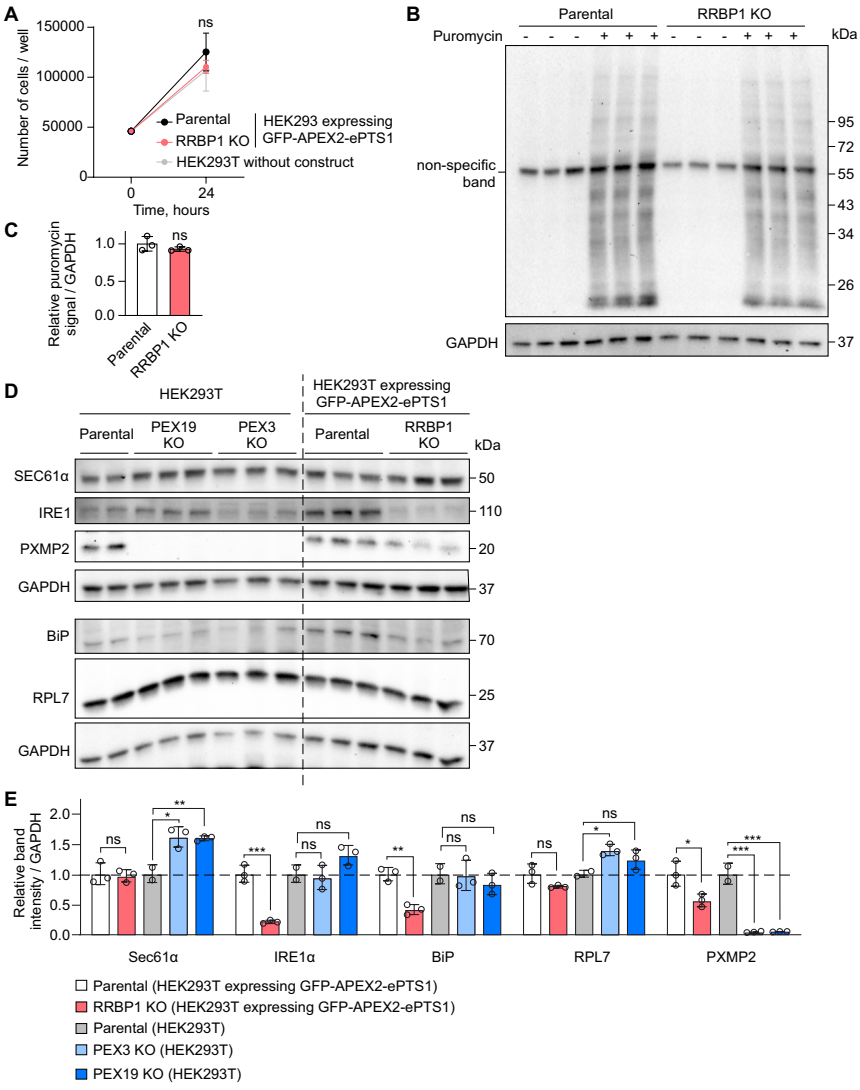

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Fig. S3. RRBP1 KO does not affect cell proliferation and does not induce ER stress. (A) Proliferation rate of HEK293T cells stably expressing GFP-APEX2-ePTS1 (parental, RRBP1 KO) or HEK293T cells not expressing peroxisomal targeted construct was analyzed using automated cell counter. (B) Protein translation rate in RRBP1 KO or parental HEK293T cells stably expressing GFP-APEX2-ePTS1 was analyzed by puromycin incorporation into nascent proteins. Cells were labeled with puromycin (10 µg/ml, 20 min) and the protein extracts were analyzed by western blotting using anti-puromycin antibody. GAPDH was used as a loading control. (C) Quantification of western blot images as represented in B (n = 3). (D) Western blot analysis of the indicated proteins in RRBP1 KO HEK293T cells stably ex-pressing GFP-APEX2-ePTS1. Parental, PEX3 and PEX19 KO HEK293T not expressing peroxisomal tar-geted construct were used as controls. GAPDH was used as a loading control. (E) Quantification of west-ern blot images as represented in D (n = 3). Each western blot analysis was performed at least two times. Values are normalized to the corresponding parental cell line, set to 1. In all graphs data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P 0.001, ns, not significant as compared to the parental cell line (unpaired t-tests).

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Table S1. Highly specific interactors of GFP-APEX2-ePTS1 in cells lacking peroxisomes

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Table S2. Non-essential highly specific interactors of GFP-APEX2-ePTS1 in cells lacking peroxisomes

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Table S3. Selected non-essential highly specific interactors of GFP-APEX2-ePTS1 in cells lacking peroxisomes

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Table S4. List of oligos used in this study

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Table S5. esiRNA target sequences

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Table S6. gRNA sequences

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II

Publication II

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The Subset of Peroxisomal Tail-Anchored Proteins Do Not Reach Peroxisomes via ER, Instead Mitochondria Can Be Involved.

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*Author contribution:

KF contributed to the initial optimization of the biotinylation based proximity labelling experiments.

PLOS ONE

RESEARCH ARTICLE

The subset of peroxisomal tail-anchored proteins do not reach peroxisomes via ER, instead mitochondria can be involved

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Abstract

Peroxisomes are membrane-enclosed organelles with important roles in fatty acid breakdown, bile acid synthesis and biosynthesis of sterols and ether lipids. Defects in peroxisomes result in severe genetic diseases, such as Zellweger syndrome and neonatal adrenoleukodystrophy. However, many aspects of peroxisomal biogenesis are not well understood. Here we investigated delivery of tail-anchored (TA) proteins to peroxisomes in mammalian cells. Using glycosylation assays we showed that peroxisomal TA proteins do not enter the endoplasmic reticulum (ER) in both wild type (WT) and peroxisome-lacking cells. We observed that in cells lacking the essential peroxisome biogenesis factor, PEX19, peroxisomal TA proteins localize mainly to mitochondria. Finally, to investigate peroxisomal TA protein targeting in cells with fully functional peroxisomes we used a proximity biotinylation approach. We showed that while ER-targeted TA construct was exclusively inserted into the ER, peroxisome-targeted TA construct was inserted to both peroxisomes and mitochondria. Thus, in contrast to previous studies, our data suggest that some peroxisomal TA proteins do not insert to the ER prior to their delivery to peroxisomes, instead, mitochondria can be involved.

Introduction

Peroxisomes are single membrane-bounded organelles with essential metabolic functions in human cells. Peroxisomes are crucial in lipid metabolism, catabolism of very long chain fatty acids, biosynthesis of sterols, and ether lipids. Defects in peroxisome biogenesis result in critical genetic diseases, such as Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and myelopathies [1]. *De novo* peroxisomal biogenesis includes assembly of peroxisomal membrane proteins and subsequent import of soluble proteins into peroxisomal matrix [2, 3]. Although the main components and pathways of peroxisomal matrix protein import have been successfully identified [4–6], how peroxisomal membrane proteins (PMPs) are targeted to their respective destinations remains unclear [7, 8].

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A small portion of PMPs is embedded in the peroxisomal membrane by their C-terminal transmembrane segment, termed the tail-anchor (TA) that can direct newly synthesized TA proteins to different subcellular compartments. A combination of hydrophobicity of transmembrane domain and tail charge of TA proteins plays a role in the specific targeting of TA proteins to peroxisomes [9]. TA proteins carry out important functions in cell metabolism [10] and several disorders are linked to defective targeting of these proteins [11, 12]. Studies on PMP targeting in mammalian cells have suggested different modes of delivery to peroxisomes: a direct delivery from cytosol to pre-existing organelles or/and ER dependent trafficking. In the direct delivery model, PMPs reach peroxisomes without ER involvement by using essential peroxisome biogenesis factors [13, 14]. On the other hand, the indirect pathway suggests that PMPs are initially inserted into the ER membrane before being sorted to peroxisomes. It has been demonstrated that in the absence of mature peroxisomes many PMPs accumulate at the ER membrane [15, 16]. One study suggests that peroxisomes can be formed from PMPs deriving from both the ER and mitochondria [8].

Several distinct mechanisms of PMP delivery were proposed. One such mechanism implicates the role of the Sec61 translocon [17], a protein-complex channel involved in protein translocation across the ER membrane. Despite the diversity of proposed mechanisms for PMP delivery, a consistent element across all these models is the involvement of a specific set of proteins known as peroxins. These peroxins are indispensable in facilitating the trafficking of TA proteins through the ER.

Two highly conserved peroxins PEX19 and PEX3, are thought to be the key elements of PMP trafficking in cells [18]. While PEX19 is mainly a cytosolic chaperone, PEX3 acts as a membrane docking factor for protein insertion [10]. Deletion of one of these peroxins results in cells devoid of mature peroxisomes.

The process by which peroxisomal TA proteins in mammals are targeted to peroxisomes is a critical element for our understanding of peroxisomal cell biology. However, the targeting mechanisms of mammalian peroxisomal TA proteins remain largely unexplored.

We studied the pathway of TA proteins targeting peroxisomes using biochemical assays and microscopy. We took advantage of YgiM(TA), a well-characterized bacteria-derived TA protein targeted specifically to peroxisomes in mammalian cells [19]. Its small size and lack of function-directed interactions within eukaryotic cells make it a versatile tool to investigate protein targeting to peroxisomal membrane. Our data suggest that peroxisomal TA proteins do not insert ER prior to their delivery to peroxisomes. Instead, mitochondria can play a role in the targeting of TA proteins to peroxisomes.

Results

Exogenous peroxisomal TA protein does not transit through the ER in mammalian cells

We rationalized that if peroxisomal TA proteins become exposed to the ER lumen at some point during the delivery to mature peroxisomes, then in the absence of peroxisomes, peroxisomal TA proteins could be detectable at the ER. Therefore, we generated HEK293T cell lines lacking peroxisomes by deletion of the essential peroxisomal biogenesis factors, PEX3 or PEX19, using CRISPR Cas9 editing. Immunoblotting of the resulting cell lines showed the specific deletion of either PEX3 or PEX19 (Fig 1A). As expected from previous research [13, 14], confocal microscopy revealed lack of peroxisomes in both cell lines as assessed by immunofluorescence labeling of catalase (Fig 1B).

To test whether peroxisomal TA proteins insert into the ER on their way to peroxisomes, we used a previously described model protein YgiM(TA), a bacterial derived sequence, shown

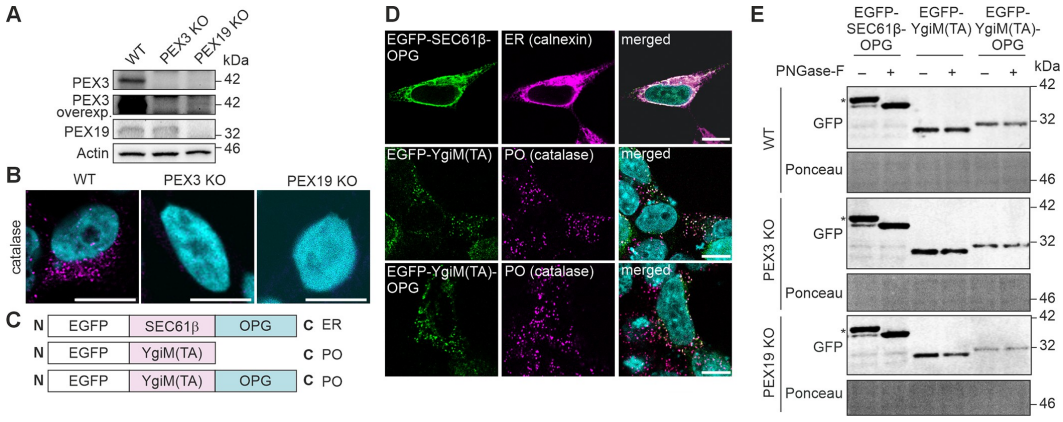

Fig 1. Exogenous peroxisomal TA protein does not transit through the ER in mammalian cells. (A) Immunoblotting analysis of PEX3 KO or PEX19 KO HEK293T cells generated by CRISPR Cas9 approach. WT, wild type, parental HEK293T cells were used as a control. Actin was used as a loading control. (B) Confocal immunofluorescence imaging showed the absence of peroxisomes in PEX3 KO or PEX19 KO HEK293T cells. WT, wild type, parental HEK293T cells were used as a control. Anti-catalase antibody was used to visualize peroxisomes (magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (C) Schematic view of the OPG-fused constructs used in glycosylation assays. EGFP-YgiM(TA) without OPG was used as a control. (D) Intracellular localization of the constructs used in glycosylation assay analyzed by confocal immunofluorescence imaging. WT HEK293T were transiently transfected with EGFP-SEC61β-OPG, EGFP-YgiM(TA) or EGFP-YgiM(TA)-OPG constructs for 24 h. Anti-GFP antibody was used to visualize the EGFP constructs (green). Calnexin was used to visualize ER (magenta), catalase was used to visualize peroxisomes (PO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (E) TA substrates with or without OPG tag were transfected to WT, PEX3 KO or PEX19 KO HEK293T cells. Cell lysates were analyzed by immunoblotting using anti-GFP antibody. Glycosylation bands are indicated by asterisk. Glycosylation was confirmed by loss of the band after addition of glycosidase, PNGase-F. Ponceau staining was used as a loading control.

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to specifically target peroxisomes in mammalian cells [20]. As a reporter for protein entry into the ER we used opsin glycosylation tag (OPG) since it contains a glycosylation site which accepts glycans if exposed to the ER lumen [21]. OPG was fused to the C-terminus of YgiM (TA), a peroxisomal TA construct, or SEC61β, a known ER-targeted TA construct (Fig 1C). Confocal microscopy coupled with specific immunostaining demonstrated that the OPG tag did not influence localization of either construct (Fig 1D), consistent with the previous work on C-terminally inserted OPG tag in TA proteins [22, 23].

WT, PEX3 KO or PEX19 KO HEK293T cells were transiently transfected with peroxisomal TA protein, YgiM(TA)-OPG, or ER TA protein, SEC61β-OPG. SEC61β-OPG expressed in control cells or cells lacking peroxisomes showed clear glycosylation as detected by a western blot band shift when treated with PNGase-F that removes oligosaccharides from glycoproteins (Fig 1E). In contrast, YgiM(TA)-OPG glycosylation was non-detectable in control cells or in cells lacking peroxisomes. Together, these results suggest that peroxisomal TA proteins do not enter ER before their insertion into the peroxisome. Also, peroxisomal TA proteins do not appear to target the ER even in the absence of mature peroxisomes (Fig 1E).

Exogenous YgiM(TA) and endogenous ACBD5 peroxisomal TA proteins are targeted to mitochondria in the absence of peroxisomes

Since peroxisomal TA proteins do not appear to enter ER in control cells or cells lacking peroxisomes, we sought to identify the localization of peroxisomal TA proteins in cells devoid of peroxisomes. For this purpose, we generated cells stably expressing peroxisomal TA protein, YgiM(TA), in WT, PEX3 KO or PEX19 KO HEK293T cells. We analyzed the colocalization of

YgiM(TA) with peroxisomes, ER or mitochondria by immunofluorescence microscopy and quantitative colocalization analysis. In WT cells YgiM(TA) localized strongly to peroxisomes (Fig 2A–2C). In PEX3 KO or PEX19 KO cells YgiM(TA) showed colocalization with both ER and mitochondria, with most notable colocalization with mitochondria (Fig 2C).

To visualize YgiM(TA) localization at superior resolution level, we used immunoelectron microscopy. Immunogold electron microscopy analysis showed that while in WT cells YgiM (TA) localized mainly to peroxisomes, in the absence of peroxisomes it was primarily targeting mitochondria (Fig 2D). We analyzed electron microscopy images using stereological analysis to identify mitochondrial relative labeling index [24, 25]. Consistent with the immunofluorescence experiments (Fig 2A–2C), we observed robust localization of YgiM(TA) to mitochondria in PEX3 KO or PEX19 KO cells (Fig 2E).

YgiM(TA) is an exogeneous peroxisomal TA protein that was overexpressed in HEK293T cells in our model. Since overexpressed proteins have the propensity to mistarget [26], we analyzed localization of an endogenous peroxisomal TA protein, ACBD5, in cells lacking peroxisomes. By immunofluorescent microscopy and quantitative colocalization analysis we showed that ACBD5 is strictly localized to peroxisomes in WT HEK293T cells (Fig 3A and 3D). In PEX19 KO cells ACBD5 was detected in mitochondria, but not in the ER (Fig 3B–3D). Notably, ACBD5 was barely detectable by immunofluorescent analysis or by immunoblotting in PEX3 KO cell (Fig 3A–3C and 3E), suggesting its degradation. Thus, the endogenous peroxisomal TA protein localized to mitochondria in the absence of peroxisomes, suggesting that mitochondria, but not ER may be involved in peroxisomal targeting of TA proteins in mammals.

Exogenous peroxisomal TA protein inserts to mitochondria in mammalian cells with intact peroxisomes

Having established that in the absence of peroxisomes, the peroxisomal TA proteins can localize to mitochondria, we sought to investigate the route by which they reach peroxisomes under normal conditions. To achieve this, we used a proximity biotinylation approach. We used wild type BirA/AviTag labeling approach [27] to reveal interactions between peroxisomal TA (AviTagged) and specific organelles containing a localized BirA protein. This approach is based on the ability of a biotin ligase (BirA) to biotinylate AviTag in a highly specific manner only if both tagged proteins are present in the same cellular compartment [28, 29]. We introduced a doxycycline-inducible BirA construct targeting ER (ER-BirA), mitochondrial intermembrane space (IMS-BirA) or peroxisomes (PO-BirA) in the Flp-In T-Rex™ HEK293 cells using corresponding targeting sequences. We then fused the AviTag sequence to the C-terminus of EGFP-tagged ER or peroxisomal TA protein (Fig 4A and 4B). These AviTag constructs were subsequently transiently transfected to the Flp-In T-Rex™ HEK293 cells stably expressing different organelle-targeted BirA constructs. To avoid overexpression of the AviTag constructs, we used the weak promoter, UBC [30]. We then confirmed the expected organelle localization of BirA and AviTag constructs using immunofluorescence microscopy (Fig 4C and 4D).

To ensure the specificity of the assay we kept the expression of BirA at a very low level by using inherent leakiness in our doxycycline system without adding additional doxycycline to the culture media. Flp-In T-Rex™ HEK293 cells expressing ER-BirA, IMS-BirA or PO-BirA were transiently transfected with ER-targeted TA construct, SEC61β-AviTag, or with peroxisome-targeted TA construct, YgiM(TA)-AviTag. Using streptavidin immunoblotting we showed that as expected SEC61β-AviTag was biotinylated in ER-BirA cells but not in IMS-BirA or PO-BirA expressing cells (Fig 4E), validating the assay as a reliable tool to analyze

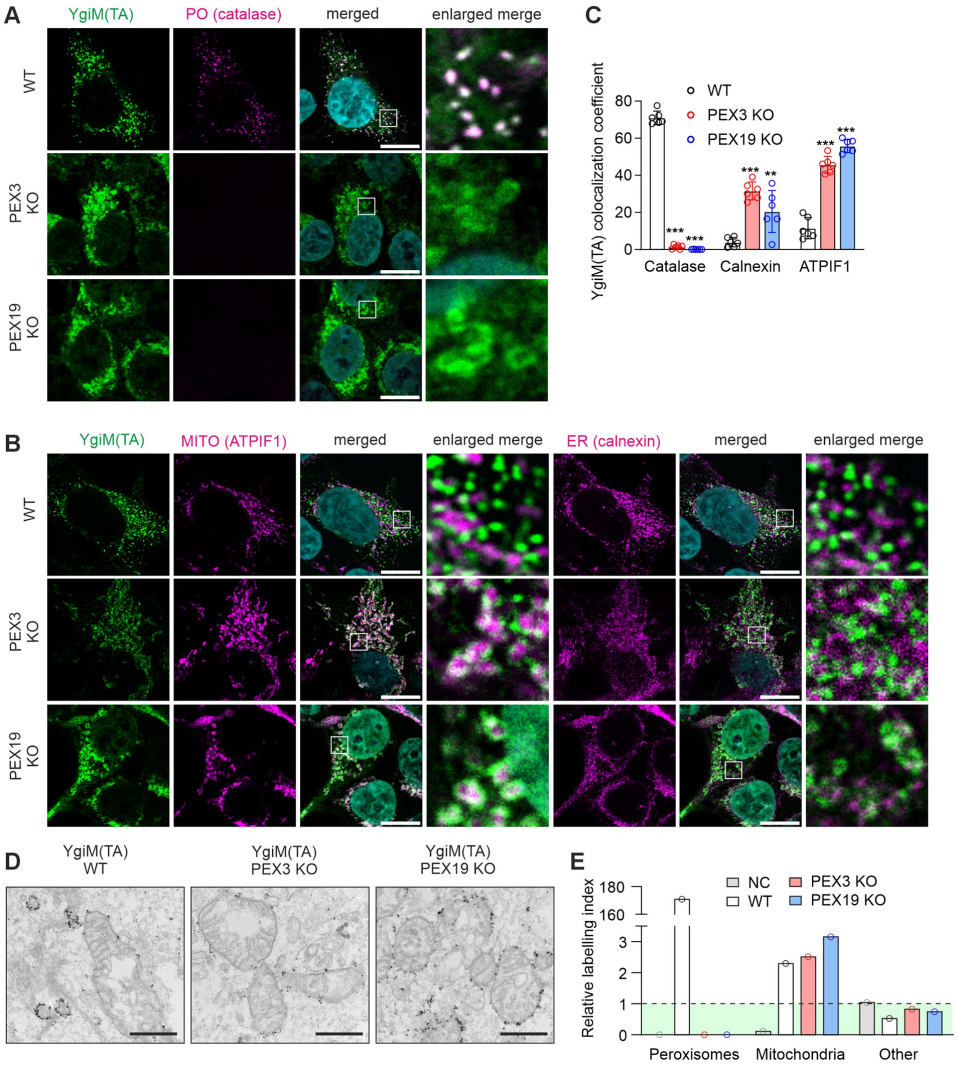

Fig 2. Exogenous peroxisomal TA YgiM(TA) is targeted to mitochondria in mammalian cells lacking peroxisomes. (A and B) Localization of EGFP-YgiM(TA) stably expressed in WT, PEX3 KO or PEX19 KO HEK293T cells. (A) Confocal immunofluorescence imaging showing EGFP-YgiM(TA) localization. Anti-GFP antibody was used to visualize EGFP-YgiM(TA) (green). Anti-catalase antibody was used to label peroxisomes (PO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (B) Confocal immunofluorescence imaging showing EGFP-YgiM (TA) localization. Anti-GFP antibody was used to visualize EGFP-YgiM(TA) (green). Anti-ATPIF1 antibody was used to label mitochondria (MITO, magenta) and anti-calnexin antibody was used to label ER (magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (C) Colocalization of EGFP-YgiM(TA) with catalase, calnexin or ATPIF1 was analyzed by Mander's overlap coefficient using confocal microscopy images. For each sample 5–6 fields of view were analyzed, each view captured approximately 50 cells. The data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to WT cells (unpaired t-tests, n = 5–6). (D) Electron microscopy imaging of WT, PEX3 KO or PEX19 KO HEK293T cells stably expressing EGFP-YgiM(TA). Anti-GFP immunogold staining was used to visualize EGFP-YgiM(TA). Scale bars, 500 nm. (E) Stereological analysis of electron microscopy images was used to identify relative labeling index of YgiM(TA) in peroxisomes or mitochondria. If no association with mitochondria or peroxisomes was observed, the gold particle label was assigned to the 'other' category. The 'other' category includes all other organelles (except mitochondria and peroxisomes) and cytosol. NC, negative control, cells not expressing

EGFP-YgiM(TA) construct used for quantitation of background signal from immunogold labeling. Area above the green surface depicts non-randomly distributed gold particles. For each cell line 15 cells were analyzed (2 images per cell with the area of 300 μm^2).

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the route of targeting of TA proteins that are ultimately delivered to different target organelles. In contrast, YgiM(TA)-AviTag biotinylation was exclusively detected in cells expressing PO-BirA or IMS-BirA (Fig 4E). Therefore, we conclude that peroxisomal TA proteins pass through mitochondria, but not through ER, in cells containing intact mature peroxisomes.

Collectively, our results showed that peroxisomal TA proteins may target mitochondria in mammalian cells both in the presence and in absence of peroxisomes, suggesting that mitochondria could be involved in the targeting of peroxisomal TA proteins in mammalian cells.

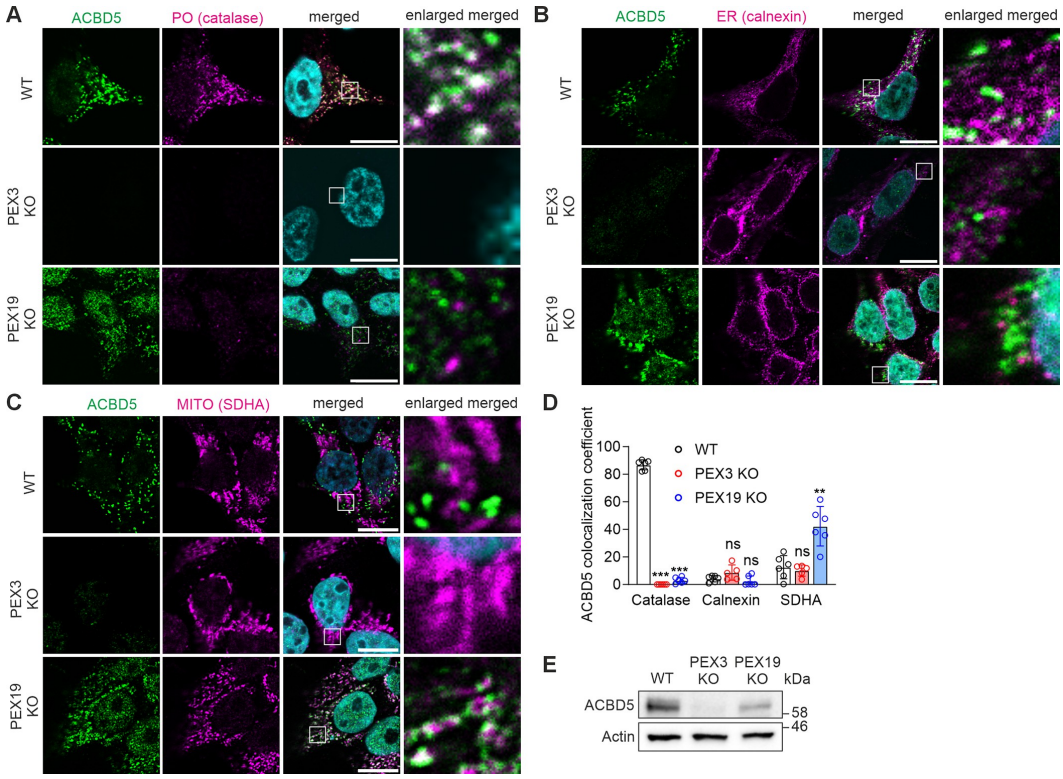

Fig 3. Endogenous peroxisomal TA protein ACBD5 localizes to mitochondria in the absence of peroxisomes in mammalian cells. (A–C) Localization of ACBD5 in WT, PEX3 KO or PEX19 KO HEK293T cells. (A) Confocal immunofluorescence imaging showing ACBD5 localization. Anti-ACBD5 antibody was used to visualize ACBD5 (green), anti-catalase antibody was used to label peroxisomes (PO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (B) Confocal immunofluorescence imaging showing ACBD5 localization. Anti-ACBD5 antibody was used to visualize ACBD5 (green), anti-calnexin antibody was used to label ER (magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (C) Confocal immunofluorescence imaging showing ACBD5 localization. Anti-ACBD5 antibody was used to visualize ACBD5 (green), anti-SDHA antibody was used to label mitochondria (MITO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (D) Colocalization of ACBD5 with catalase, calnexin or SDHA was analyzed by Mander's overlap coefficient using confocal microscopy images. For each sample 5–6 fields of view were analyzed, each view captured approximately 50 cells. The data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant as compared to WT cells (unpaired t-tests, n = 5–6). (E) Immunoblotting analysis of ACBD5 in WT, PEX3 KO or PEX19 KO HEK293T cells. Actin was used as a loading control.

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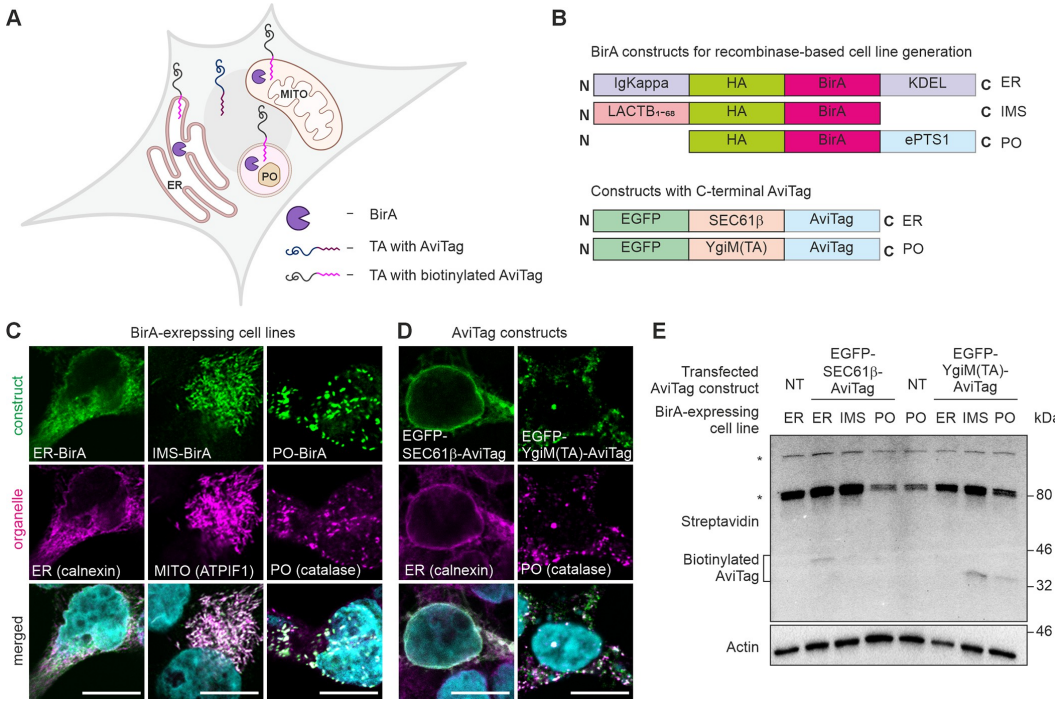

Fig 4. Exogenous peroxisomal TA protein inserts to mitochondria in mammalian cells with intact peroxisomes. (A) Schematic representation of the biotinylation experiment. TA constructs fused to AviTag are transfected to the cells expressing BirA in endoplasmic reticulum (ER), peroxisomes (PO) or mitochondria (MITO). Once TA inserts to the specific organelle labeled with BirA, biotin acceptor sequence of the TA, AviTag, is biotinylated by BirA. (B) Schematic view of the constructs used to generate BirA-expressing cell lines and TA constructs fused to AviTag. (C) Doxycycline-inducible BirA constructs targeted to ER (ER-BirA), mitochondrial intramembrane space (IMS-BirA) or peroxisomes (PO-BirA) were stably introduced into Flp-In™ T-REx™-293 cells. The expression of the constructs was induced by adding of 10 μg / mL doxycycline for 24 h. The intracellular localization of doxycycline-inducible BirA constructs targeted to the specific organelle was analyzed by confocal immunofluorescence imaging. Anti-BirA antibody was used to visualize BirA construct (green), anti-calnexin antibody was used to label ER (magenta), anti-ATPIF1 antibody was used to visualize mitochondria (magenta), anti-catalase antibody was used to label peroxisomes (PO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (D) Flp-In™ T-Rex™-293 cells were transiently transfected with EGFP-SEC61β-AviTag or EGFP-YgiM(TA)-AviTag for 24 h. The intracellular localization of AviTag constructs was analyzed by confocal immunofluorescence imaging. Anti-GFP antibody was used to visualize AviTag constructs (green), anti-calnexin antibody was used to label ER (magenta), anti-catalase antibody was used to label peroxisomes (PO, magenta). DAPI (cyan) shows nuclei. Scale bars, 10 μm. (E) Flp-In T-Rex™ HEK293 cells expressing ER-BirA, IMS-BirA or PO-BirA were transiently transfected with ER-targeted TA construct, SEC61β-AviTag, or with peroxisome-targeted TA construct, YgiM(TA)-AviTag. Cell lysates were analyzed by immunoblotting using streptavidin. Bands of ~33 kDa and 39 kDa indicate biotinylated AviTag constructs. Endogenous biotinylated mitochondrial proteins are shown by asterisks. Actin was used as a loading control. NT, non-transfected control. Fig (A) was created with BioRender.com.

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Discussion

Research on targeting pathways of peroxisomal TA in mammalian cells provided data both in favor [7] and against a route involving the ER [31, 32]. It has been demonstrated that in yeast [33, 34] and plants [35] peroxisomal TA proteins insert to the ER prior to reaching peroxisome. Further, in yeast cells devoid of peroxisomes, peroxisomal TA proteins were visualized in structures described as pre-peroxisomal vesicles derived from ER [20, 36–38]. Using glycosylation assays in human cells, we tested the possibility of peroxisomal TA transiting through the ER in both WT and cells lacking peroxisomes. To generate cells lacking peroxisomes we knocked out essential membrane or cytosolic peroxisomal biogenesis factors, PEX3 or PEX19,

respectively. The absence of N-linked glycosylation provided evidence against ER-dependent targeting of peroxisomal TA proteins in mammalian cells. However, we cannot fully exclude the possibility that peroxisomal TA proteins might transiently visit the ER lumen, without becoming glycosylated. Therefore, we analyzed the localization of peroxisomal TA proteins in mammalian cells by immunofluorescence and immunoelectron microscopy imaging. We observed that in the absence of peroxisomes in PEX19 KO cells, endogenous peroxisomal TA protein ACBD5 localizes mainly to mitochondria. Mitochondrial localization of peroxisomal membrane proteins in the absence of peroxisomes has been reported previously [39, 40], although, this finding was considered as a mistargeting artifact. However, we showed that in PEX3 KO cells, which lack peroxisomes as well, ACBD5 does not target mitochondria. These data suggest that mitochondrial localization of peroxisomal membrane proteins may not represent simple passive mistargeting, but instead an active process in which PEX3 may play an important role. Interestingly, PEX3 and PEX14 were found to be targeted to mitochondria prior to their assembly into peroxisomes (Sugiura et al) [8], suggesting the involvement of these factors in the insertion of peroxisomal TA proteins to mitochondria. It is also possible that PEX19 interacts with ACBD5 and prevents its mistargeting to mitochondria, thus in PEX19 KO cells we observed ACBD5 in mitochondria, while in PEX3 KO cells ACBD5 did not target mitochondria and was degraded in the cytosol.

We observed almost complete depletion of ACBD5 in PEX3 KO cells, suggesting that at least some untargeted peroxisomal TA proteins become directed for degradation. Notably, PEX19 KO causes reduction of PEX3 protein level, suggesting that PEX3 is degraded when peroxisomes are absent. However, the residual level of PEX3 in PEX19 KO cells was sufficient to protect ACBD5 from complete degradation in PEX19 KO cells. It is possible that PEX3 is involved in the transcriptional regulation of ACBD5 and modulates its expression. Deletion of PEX3 could disrupt this regulatory mechanism, resulting in the loss of ACBD5 expression. It was demonstrated that yeast orthologue of ACBD5, Atg37, binds PEX3 at the peroxisomal membrane [41] ensuring correct localization of PEX3 to peroxisomes [42]. PEX3 may influence the stability or post-translational modifications of ACBD5 such as proper folding, stabilization, or localization within the cell. Deletion of PEX3 may disrupt this interaction, leading to the degradation or mislocalization of ACBD5. Finally, PEX3 knockout may indirectly affect ACBD5 by disrupted cellular processes or signaling pathways that are necessary for the expression or stability of ACBD5.

Further investigations are warranted to elucidate the specific molecular mechanisms underlying the relationship between PEX3 and ACBD5. These studies could involve examining the transcriptional regulation of ACBD5 in PEX3-deficient cells, assessing the stability and post-translational modifications of ACBD5 in the absence of PEX3, and investigating potential downstream signaling pathways affected by PEX3 deletion that could influence ACBD5 expression.

Using organelle-specific proximity biotinylation assays we showed that peroxisomal TA protein targets both peroxisomes and mitochondria in cells containing intact peroxisomes. This indicates that mitochondria-related targeting may be a process that occurs during peroxisomal TA trafficking under normal conditions. Overall, our data suggest that TA proteins targeting to mitochondria, previously described as mislocalization, may be part of the endogenous targeting pathway to peroxisomes. These observations are consistent with the findings by Sugiura *et al.* [8] showing that newly assembled peroxisomes arise from both ER and the mitochondria.

Taken together, we observed a subset of YgiM(TA) localizing to the mitochondria in wild-type cells (Fig 2D), and subsequent biotinylation by a mitochondrial protein tagged with BirA (Fig 4E). These observations led us to the hypothesis that YgiM(TA), and potentially other

peroxisomal tail-anchored proteins, might transit via mitochondria *en route* to peroxisomes. However, it is crucial to consider alternative interpretations. One plausible scenario is that YgiM(TA) is dually localized to both peroxisomes and mitochondria. This would suggest that the localization of YgiM(TA) is not necessarily linear, but instead might follow parallel pathways, with minor subsets of the protein targeted independently to mitochondria. This concept of dual localization could provide additional complexity to understanding peroxisomal TA protein routing, supports previous data on dual localization of some mitochondrial TA proteins in yeast [43] and mammals [44], while also challenges our current view of organelle-specific peroxisomal TA protein targeting. It is still not clear why at least some TA proteins pass mitochondria *en route* to the peroxisome. Mitochondria and peroxisomes share several functional overlaps, including fatty acid and reactive oxygen species (ROS) metabolism [45, 46]. During mitochondrial transit, TA proteins potentially can undergo specific post-translational modifications or protein-protein interactions that prime them for their peroxisomal function. This transit could also serve as a quality control step, ensuring that only correctly folded and functional TA proteins reach the peroxisome. In addition, the functional interplay between peroxisomes, mitochondria, and ER [47–49] might necessitate certain proteins to be present in multiple organelles, leading to dual targeting.

We believe that our study offers valuable insights into the localization of peroxisomal TA proteins and invites further exploration of these complex cellular mechanisms.

Materials and methods

Cell culture

Human embryonic kidney cells (HEK293T) or Flp-In T-Rex™-293 cells (ThermoFisher, R780-07) were cultured (37˚C, 5% CO2) in DMEM (Pan-Biotech, P04-03600), supplemented with 10% fetal bovine serum (GIBCO, 10270106), l-glutamine (GIBCO, 25030081) and penicillin/streptomycin (GIBCO,15140122). TransIT-X2 (MirusBio, MIR 6000) was used for cell transfection according to manufacturer's instruction.

Generation of knockout cell lines

CRISPR/Cas9 was used to generate knockout HEK293T cells. Cells were co-transfected with two gRNAs and pSpCas9n(BB)-2A-Puro (PX462) V2.0 (Addgene, 62987). PEX3 gRNAs were `ACGTGCTTGAAAGGGGGCAT` and `CACCTCCAAGGACCGTGCCC`. PEX19 gRNAs were `GGGCCCCAGAAGAGATCGCC` and `GGGGCCGTGGTGGTAGAAGG`. After 24 h the cells were selected with puromycin and expanded. T7 assay was used to confirm genome editing. Single cell clones were obtained and validated using immunoblotting analysis.

Generation of stably expressing cell lines

HEK293T cells stably expressing EGFP-YgiM(TA) were generated by using standard lentiviral cell line generation protocol. Briefly, WT, PEX3 KO or PEX19 KO cells were co-transfected with pMD2.G (Addgene, 12259), psPAX2 (Addgene,12260) and pLL3.7-EGFP-YgiM(TA) using TransIT-2020 (MirusBio, MIR 5400) following manufacturer's instructions. Single cell clones were selected for low GFP expression, just above the base autofluorescence as measured in the WT cells by FACS.

Doxycycline-inducible BirA constructs were introduced into Flp-In™ T-Rex™-293 cells by co-transfecting with pOG44 (ThermoFischer, V600520) and corresponding BirA construct using TransIT-2020 (MirusBio, MIR5400) according to manufacturer's instructions. After 48 h the selection antibiotic was added (100 μg / ml hygromycin B (ENZO, ALX-380-306-G001)).

Every 48 h the growth medium was replaced with the fresh one, containing fresh selection antibiotic. The cells were under selection for 2.5 to 4 weeks until sufficient number of colonies were formed.

Plasmid construction

OPG-tagged plasmids were constructed by homologous recombination in yeast as previously described [50]. Briefly, to generate pEGFP-SEC61β-OPG, mCherry-SEC61β was PCR amplified from mCh-Sec61 beta (a gift from Gia Voeltz (Addgene plasmid, 49155; http://n2t.net/addgene:49155; RRID:Addgene 49155)) with primers ATGGTGAGCAAGGGCGAGGA and TTACCCTGTCTTATTGCTAAATGGAACGTAAAAGTTAGGACCCGAACGAGTGTACTTGCC CCAAATGTG. SEC61β-OPG was generated by PCR amplification using primers ATCACTC TCGGCATGGACGAGCTGTACAAGAGATCTATGCCTGGTCCGACC and GGTATGGCTGATTA TGATCAGTTATCTAGATTACCCTGTCTTATTGCTAAATGGAAC and the obtained product was co-transformed with pEGFP-C1-2μ-URA3 cut with BamHI (NEB, R3136) into yeast strain BY4743 (EUROSCARF Y20000) cut. The obtained yeast plasmid DNA transformed into competent cells (NEB, C2987H).

To generate pEGFP-YgiM(TA)-OPG, YgiM(TA)-OPG sequence was produced by PCR from pRS315-mCherry-YgiM(TA) with primers ATCACTCTCGGCATGGACGAGCTGTACA AGATGGTAGAGGATAAGATCCAGAAGGAAACA and TATGGCTGATTATGATCAGTTATCT AGATTACCCTGTCTTATTGCTAAATGGAACGTAAAAGTTAGGACCGTTCATCCAGCGATC TTTG. The obtained PCR product co-transformed with pEGFP-C1-2μ-URA3, that was cut with BamHI (NEB, R3136), into yeast strain BY4743 (EUROSCARF Y20000). The obtained yeast plasmid DNA was transformed into competent cells (NEB, C2987H). Yeast strains, plasmids, and culture conditions are as described in [50].

To generate pLL3.7-EGFP-YgiM(TA), pLL3.7 (Addgene, 11795) was cut with EcoRI (NEB, R3101) and NheI (NEB, R3131) and the 6907 bp band was gel purified (Macherey-Nagel, 740609.50). EGFP-YgiM(TA) was PCR amplified from pEGFP-YgiM(TA) with primers ATATTGCTAGCGCTACCGGTCGCCACCATG and CCTACTGAATTCTTAGTTCATCCAGC GATCTTTGC. The obtained product was then cut with NheI (NEB, R3131) and EcoRI (NEB, R3101) and PCR purified (Macherey-Nagel, 740609.50). The two purified fragments were ligated using T4 DNA ligase (NEB, M0202) following manufacturer's instructions and transformed into competent cells (NEB, C2987H).

To generate BirA-constructs, pDisplay-BirA-ER (a gift from Alice Ting (Addgene plasmid # 20856; http://n2t.net/addgene:20856; RRID:Addgene_20856)) was used as a PCR template for ER-BirA or PO-BirA. The HA-BirA region was PCR amplified with primers GCGAAGCTTTGGGGATATCCACCATGGAGACAGAC/GCGGGATCCGTTCGTCGACTCACAGC TCGTCCTT for ER-BirA and TTTTAAGCTTGCCACCATGTATCCATATGATGTTCC/ TTTTGGATCCTCATAGCTTACTTCTTCTGCCACGCCCCAGTTTTTCTGCACTAC for PO-BirA. IMS-BirA ($LACTB_{1-68}$-HA-BirA) construct was purchased from Twist Bioscience (San Francisco, California, United States). The IMS sequence was as in [51]. The IMS-BirA construct and PCR products from ER-and PO-BirA products were cut with HindIII (NEB, R3104) and BamHI (NEB, R3136) and cloned into pcDNA™5/FRT/TO (TermoFischer, V652020) that was cut with the same restriction enzymes.

To generate AviTag constructs, pLJC5-3XHA-EGFP-PEX26 (Addgene, 139054) was cut with AgeI (NEB, R3552) and EcoRI(NEB, R3101). The large DNA fragment was gel purified (Macherey-Nagel, 740609.50) and used as a target backbone for cloning the AviTag constructs. AgeI-EGFP-SEC61β sequence and linker AviTag-EcoRI were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA), PCR amplified, annealed, ligated with the

cut backbone and transformed into competent cells (NEB, C2987H). EGFP-YgiM(TA) was PCR amplified from pEGFP-YgiM(TA)-noFis1(119–128) with appropriate primers, annealed with linker AviTag sequence as above and cloned to competent cells. GSGSTSGSGK was used as a linker sequence [27].

Glycosylation assay in cells

Cells were transiently transfected with pEGFP- SEC61β-OPG or pEGFP-YgiM(TA)-OPG using TransIT2020 transfection reagent (MirusBio, MIR 5400) according to manufacturer's instructions. After 24 h cells were lysed, and the lysates were treated with PNGase-F (NEB, P0704L) or water by following the manufacturer's protocol. The samples were resolved by SDS-PAGE and immunoblotted with anti-GFP antibody (described in Immunoblotting section).

Immunofluorescence analysis

HEK293T or Flp-In™ T-Rex™-293 were fixed with 4% PFA for 10 min on 13 mm coverslips and then washed with PBS three times at room temperature. Next, the cells were permeabilized with 1% TritonX-100 (CAS 9002-93-1) in PBS for 10 min, washed three times with PBS for 5 min and blocked with 1% BSA (Fisher Scientific, 11413164) in PBST (PBS containing 0.1% Tween20 (Fisher Scientific, 10485733)) for 2 h. The cells were then incubated with the corresponding primary antibody diluted in blocking buffer at 4˚C overnight. After washing with PBS for 5 min three times, the corresponding secondary antibodies were added for 1 h at room temperature. Next, DAPI (1:1000 in PBS) was added for 10 min. After washing with PBS for 5 min two times the samples were mounted with antifade mounting medium (Vector Laboratories, H-1700). The samples were imaged on Leica TCS SP8 STED 3X CW 3D microscope using HC PL APO 93x/1.30 motCORR STED WHITE (glycerol, wd 0.3mm) objective. Primary antibodies used for immunofluorescence analysis: rabbit anti-ACBD5, 1:300 (Atlas Antibodies, HPA012145), chicken anti-GFP, 1:10000 (Aves, GFP-1020), rabbit anti-catalase, 1:400 (CST, 12980S), rabbit anti-calnexin, 1:1000 (Abcam, 22595), rabbit anti-ATPIF1, 1:250 (CST, 8528), mouse anti-SDHA, 1:100 (Santa Cruz, 390381), mouse anti-calnexin, 1:500 (Abcam, 112995), mouse anti-HA, 1:400 (Santa Cruz, 2367), mouse anti-catalase 1,6 μg / mL (Biotechne, MAB3398). Secondary antibodies: Alexa Fluor 647 (Invitrogen, A-21235 or CST, 4414S), Alexa Fluor 555 (CST, 4413S or CST, 4409S), Alexa Fluor 488 (Invitrogen, A-11001 or CST, 4412S).

Colocalization analysis

Colocalization analysis was performed with the Imaris Software (Imaris File Converter 9.5.1) using the Coloc tool of the Surpass system. Images were manually adjusted to separate background from the real signal. Mander's overlap coefficient was used to determine the percentage overlap. For each sample 5–6 fields of view each captured approximately 50 cells were analyzed.

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (CST, 9806). Protein concentration was determined by BCA assay kit (Thermo Scientific, 23227). Protein lysates were supplemented with Laemmli buffer, heated at 95˚C for 5 min and separated by SDS-PAGE. Then the proteins were transferred to a PVDF membrane using Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Pack (BioRad, 1704156). The membranes were blocked in 5% non-fat milk in TBST for 1 h at

room temperature, incubated with appropriate primary antibodies diluted in 1% BSA in TBST for 1 h at RT or overnight at 4˚C. Next the membranes were washed three times in TBST followed by 1 h incubation at room temperature with appropriate secondary antibodies diluted in 1% BSA in TBST, washed trice in TBST and imaged for either chemiluminescence or IR signal. Primary antibodies: rabbit anti-GFP 1:2500 (Cell Signaling Technology, 2956), rabbit anti-PEX19 1:1000 (Abcam, 137072), rabbit anti-ACBD5 1:500 (Atlas Antibodies, HPA012145), mouse anti-HA 1:1000 (Cell Signaling Technology, 2367), mouse anti-β-actin, 1:1000 (Cell Signaling Technology, 3700), IRDye 800CW conjugated streptavidin 1:3000 (LI-COR, 926–32230). Secondary antibodies: anti-mouse IgG HRP-linked antibody 1:5000 (Cell Signaling Technology, 7076), anti-rabbit IgG HRP-linked antibody 1:5000 (Cell Signaling Technology, 7074), IRDye 800CW anti-rabbit IgG 1:15000 (LI-COR, 926–32211). The chemiluminescence was developed using ECL substrate for enhanced chemiluminescence (Thermo Scientific, 32106) and the signal was captured by Chemidoc imaging system (Bio-Rad). IR signal was detected using iBright Imaging Systems (Invitrogen).

Immunoelectron microscopy

WT, PEX3 KO or PEX19 KO HEK293T cells were fixed with paraformaldehyde-lysine-periodate [52], permeabilized with 0.01% saponin (Sigma-Aldrich, S7900) and stained for 1.5 h with anti-GFP antibody (1:500, Sigma, 290), followed by 1 h incubation with 1.4 nm nanogold-conjugated secondary antibody (1:60, RRID: AB_2631182, NY, Nanoprobes, Stony Brook, 2004). Silver enhancement of nanogold particles was performed according to manufacturer's instructions with HQ Silver Kit (Nanoprobes, Stony Brook, NY), then gold toned with 2% Na-Acetate, 0.05% HauCl4, and 0.3% Na2S2O3·5H2O. Post-fixation was done for 1 h on ice with 1% reduced osmium tetroxide in sodium cacodylate buffer pH 7.4, dehydrated by serial washing in ethanol and acetone and finally embedded in Epon (TAAB 812, Aldermaston, UK) for 2 h before 14 h polymerization at 60˚C. Sections were prepared with Leica UCT6 microtome, then post-stained with lead citrate and uranyl acetate. Samples were imaged with Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan) operated at 80 kV, with Orius CCD-camera Gatan SC 1000B, bottom mounted (Gatan Inc., Pleasanton, CA). Quantification of the relative labeling index was performed as previously described [53], software used was Microscopy Image Browser [54] to segment and calculate. In case the gold particles were associated with mitochondria or peroxisomal structures, the label was assigned correspondingly to mitochondria or to peroxisomes. If no association with mitochondria or peroxisomes was observed, the gold particle label was assigned to the 'other' category. Therefore, the 'other' category includes all other organelles (except mitochondria and peroxisomes) and cytosol. Nucleus was excluded from this analysis.

Biotinylation assay

Flp-In™ T-Rex™-293 cells were grown in 6 well-plates and transiently transfected with 250 ng of EGFP- SEC61β-AviTag or 1000 ng of EGFP-YgiM(TA)-AviTag for 24 h and processed for immunoblotting. Membranes were incubated with IRDye 800CW conjugated streptavidin.

Statistical analysis

All data are presented as mean ± standard deviation (SD). For the statistical analysis with two samples, unpaired two-tailed t-test was performed using Graph Pad Prism 9 Software. All the graphs were prepared with Graph Pad Prism 9 Software.

Supporting information

S1 Data.
(XLSX)

S1 Raw images.
(PDF)

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References

1. Wanders RJA, Baes M, Ribeiro D, Ferdinandusse S, Waterham HR. THE PHYSIOLOGICAL FUNCTIONS OF HUMAN PEROXISOMES. Physiol Rev 2023; 103:957–1024. https://doi.org/10.1152/PHYSREV.00051.2021/ASSET/IMAGES/MEDIUM/PRV-00051-2021R01.PNG. PMID: 35951481
2. Chornyi S, IJlst L, van Roermund CWT, Wanders RJA, Waterham HR. Peroxisomal Metabolite and Cofactor Transport in Humans. Front Cell Dev Biol 2020;8. https://doi.org/10.3389/FCELL.2020.613892.
3. Fujiki Y, Okumoto K, Mukai S, Honsho M, Tamura S. Peroxisome biogenesis in mammalian cells. Front Physiol 2014;5. https://doi.org/10.3389/FPHYS.2014.00307.
4. Hasan S, Platta HW, Erdmann R. Import of proteins into the peroxisomal matrix. Front Physiol 2013; 4 SEP:261. https://doi.org/10.3389/FPHYS.2013.00261/BIBTEX. PMID: 24069002
5. Platta HW, Brinkmeier R, Reidick C, Galiani S, Clausen MP, Eggeling C. Regulation of peroxisomal matrix protein import by ubiquitination. Biochimica et Biophysica Acta (BBA)—Molecular Cell Research 2016; 1863:838–49. https://doi.org/10.1016/j.bbamcr.2015.09.010 PMID: 26367801
6. Emmanouilidis L, Gopalswamy M, Passon DM, Wilmanns M, Sattler M. Structural biology of the import pathways of peroxisomal matrix proteins. Biochimica et Biophysica Acta (BBA)—Molecular Cell Research 2016; 1863:804–13. https://doi.org/10.1016/j.bbamcr.2015.09.034 PMID: 26450166
7. Buentzel J, Vilardi F, Lotz-Havla A, Gartner J, Thoms S. Conserved targeting information in mammalian and fungal peroxisomal tail-anchored proteins. Sci Rep 2015;5. https://doi.org/10.1038/srep17420 PMID: 26627908
8. Sugiura A, Mattie S, Prudent J, Mcbride HM. Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. Nature 2017; 542:251–4. https://doi.org/10.1038/nature21375 PMID: 28146471

9. Costello JL, Castro IG, Camões F, Schrader TA, McNeall D, Yang J, et al. Predicting the targeting of tail-anchored proteins to subcellular compartments in mammalian cells. J Cell Sci 2017; 130:1675. https://doi.org/10.1242/jcs.200204 PMID: 28325759

10. Borgese N, Colombo S, Pedrazzini E. The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. J Cell Biol 2003; 161:1013. https://doi.org/10.1083/jcb.200303069 PMID: 12821639

11. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nature Reviews Cancer 2002 2:9 2002; 2:647–56. https://doi.org/10.1038/nrc883 PMID: 12209154

12. Verhagen JMA, Van Den Born M, Van Der Linde HC, Gj Nikkels P, Verdijk RM, Kivlen MH, et al. Biallelic Variants in ASNA1, Encoding a Cytosolic Targeting Factor of Tail-Anchored Proteins, Cause Rapidly Progressive Pediatric Cardiomyopathy. Circ Genom Precis Med 2019; 12:e002507–e002507. https://doi.org/10.1161/CIRCGEN.119.002507 PMID: 31461301

13. Chen Y, Pieuchot L, Loh RA, Yang J, Kari TMA, Wong JY, et al. Hydrophobic handoff for direct delivery of peroxisome tail-anchored proteins. Nature Communications 2014 5:1 2014; 5:1–12. https://doi.org/10.1038/ncomms6790 PMID: 25517356

14. Matsuzaki T, Fujiki Y. The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway. J Cell Biol 2008; 183:1275–86. https://doi.org/10.1083/jcb.200806062 PMID: 19114594

15. Toro AA, Araya CA, Córdova GJ, Arredondo CA, Cárdenas HG, Moreno RE, et al. Pex3p-dependent peroxisomal biogenesis initiates in the endoplasmic reticulum of human fibroblasts. J Cell Biochem 2009; 107:1083–96. https://doi.org/10.1002/jcb.22210 PMID: 19479899

16. Kim PK, Mullen RT, Schumann U, Lippincott-Schwartz J. The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. J Cell Biol 2006; 173:521–32. https://doi.org/10.1083/jcb.200601036 PMID: 16717127

17. Mayerhofer PU, Bañó-Polo M, Mingarro I, Johnson AE. Human Peroxin PEX3 Is Co-translationally Integrated into the ER and Exits the ER in Budding Vesicles. Traffic 2016; 17:117. https://doi.org/10.1111/TRA.12350.

18. Yagita Y, Abe Y, Fujiki Y. De novo formation and maintenance of mammalian peroxisomes in cultured PEX16-knockout cells generated by CRISPR/Cas9. J Cell Sci 2022;135. https://doi.org/10.1242/jcs.258377 PMID: 35437598

19. Lutfullahoğlu-Bal G, Keskin A, Seferoğlu AB, Dunn CD. Bacterial tail anchors can target to the mitochondrial outer membrane. Biol Direct 2017;12. https://doi.org/10.1186/S13062-017-0187-0.

20. Lutfullahoğlu-Bal G, Seferoğlu AB, Keskin A, Akdoğan E, Dunn CD. A bacteria-derived tail anchor localizes to peroxisomes in yeast and mammalian cells. Sci Rep 2018; 8:16374. https://doi.org/10.1038/s41598-018-34646-7 PMID: 30401812

21. Pedrazzini E, Villa A, Longhi R, Bulbarelli A, Borgese N. Mechanism of Residence of Cytochrome B(5), a Tail-Anchored Protein, in the Endoplasmic Reticulum. J Cell Biol 2000; 148:899. https://doi.org/10.1083/jcb.148.5.899 PMID: 10704441

22. Leznicki P, Warwicker J, High S. A biochemical analysis of the constraints of tail-anchored protein biogenesis. Biochemical Journal 2011; 436:719. https://doi.org/10.1042/BJ20101737 PMID: 21466504

23. Leznicki P, Clancy A, Schwappach B, High S. Bat3 promotes the membrane integration of tail-anchored proteins. J Cell Sci 2010; 123:2170. https://doi.org/10.1242/jcs.066738 PMID: 20516149

24. Mayhew TM. Mapping the distributions and quantifying the labelling intensities of cell compartments by immunoelectron microscopy: progress towards a coherent set of methods. J Anat 2011; 219:647–60. https://doi.org/10.1111/j.1469-7580.2011.01438.x PMID: 21999926

25. Mayhew TM, Lucocq JM, Griffiths G. Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. J Microsc 2002; 205:153–64. https://doi.org/10.1046/j.0022-2720.2001.00977.x PMID: 11879430

26. Moriya H. Quantitative nature of overexpression experiments. Mol Biol Cell 2015; 26:3932. https://doi.org/10.1091/mbc.E15-07-0512 PMID: 26543202

27. Fernández-Suárez M, Chen TS, Ting AY. Protein-Protein Interaction Detection In Vitro and in Cells by Proximity Biotinylation. J Am Chem Soc 2008; 130:9251. https://doi.org/10.1021/ja801445p PMID: 18582056

28. Howarth M, Takao K, Hayashi Y, Ting AY. Targeting quantum dots to surface proteins in living cells with biotin ligase. Proc Natl Acad Sci U S A 2005; 102:7583. https://doi.org/10.1073/pnas.0503125102 PMID: 15897449

29. de Boer E, Rodriguez P, Bonte E, Krijgsveld J, Katsantoni E, Heck A, et al. Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice. Proc Natl Acad Sci U S A 2003; 100:7480–5. https://doi.org/10.1073/pnas.1332608100 PMID: 12802011

30. Qin JY, Zhang L, Clift KL, Hulur I, Xiang AP, Ren BZ, et al. Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter. PLoS One 2010; 5:10611. https://doi.org/10.1371/journal.pone.0010611 PMID: 20485554

31. Yagita Y, Hiromasa T, Fujiki Y. Tail-anchored PEX26 targets peroxisomes via a PEX19-dependent and TRC40-independent class I pathway. J Cell Biol 2013; 200:651. https://doi.org/10.1083/jcb.201211077 PMID: 23460677

32. Halbach A, Landgraf C, Lorenzen S, Rosenkranz K, Volkmer-Engert R, Erdmann R, et al. Targeting of the tail-anchored peroxisomal membrane proteins PEX26 and PEX15 occurs through C-terminal PEX19-binding sites. J Cell Sci 2006; 119:2508–17. https://doi.org/10.1242/jcs.02979 PMID: 16763195

33. Elgersma Y, Kwast L, Van Den Berg M, Snyder WB, Distel B, Subramani S, et al. Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in S.cerevisiae, causes proliferation of the endoplasmic reticulum membrane. EMBO J 1997; 16:7326. https://doi.org/10.1093/emboj/16.24.7326 PMID: 9405362

34. Schuldiner M, Metz J, Schmid V, Denic V, Rakwalska M, Schmitt HD, et al. The GET Complex Mediates Insertion of Tail-Anchored Proteins into the ER Membrane. Cell 2008; 134:634. https://doi.org/10.1016/j.cell.2008.06.025 PMID: 18724936

35. Lisenbee CS, Heinze M, Trelease RN. Peroxisomal Ascorbate Peroxidase Resides within a Subdomain of Rough Endoplasmic Reticulum in Wild-Type Arabidopsis Cells. Plant Physiol 2003; 132:870. https://doi.org/10.1104/pp.103.019976 PMID: 12805617

36. Van Der Zand A, Gent J, Braakman I, Tabak HF. Biochemically Distinct Vesicles from the Endoplasmic Reticulum Fuse to Form Peroxisomes. Cell 2012; 149:397–409. https://doi.org/10.1016/j.cell.2012.01.054 PMID: 22500805

37. Tam YYC, Fagarasanu A, Fagarasanu M, Rachubinski RA. Pex3p Initiates the Formation of a Preperoxisomal Compartment from a Subdomain of the Endoplasmic Reticulum in Saccharomyces cerevisiae. Journal of Biological Chemistry 2005; 280:34933–9. https://doi.org/10.1074/jbc.M506208200 PMID: 16087670

38. Knoops K, Manivannan S, Cepińska MN, Krikken AM, Kram AM, Veenhuis M, et al. Preperoxisomal vesicles can form in the absence of Pex3. Journal of Cell Biology 2014; 204:659–68. https://doi.org/10.1083/jcb.201310148 PMID: 24590171

39. Li L, Zheng J, Wu X, Jiang H. Mitochondrial AAA-ATPase Msp1 detects mislocalized tail-anchored proteins through a dual-recognition mechanism. EMBO Rep 2019;20. https://doi.org/10.15252/embr.201846989 PMID: 30858337

40. Sacksteder KA, Jones JM, South ST, Li X, Liu Y, Gould SJ. Pex19 Binds Multiple Peroxisomal Membrane Proteins, Is Predominantly Cytoplasmic, and Is Required for Peroxisome Membrane Synthesis. J Cell Biol 2000; 148:931. https://doi.org/10.1083/jcb.148.5.931 PMID: 10704444

41. Nazarko TY, Ozeki K, Till A, Ramakrishnan G, Lotfi P, Yan M, et al. Peroxisomal Atg37 binds Atg30 or palmitoyl-CoA to regulate phagophore formation during pexophagy. Journal of Cell Biology 2014; 204:541–57. https://doi.org/10.1083/jcb.201307050 PMID: 24535825

42. Zientara-Rytter K, Ozeki K, Nazarko TY, Subramani S. Pex3 and Atg37 compete to regulate the interaction between the pexophagy receptor, Atg30, and the Hrr25 kinase. Autophagy 2018; 14:368. https://doi.org/10.1080/15548627.2017.1413521 PMID: 29260977

43. Cichocki BA, Krumpe K, Vitali DG, Rapaport D. Pex19 is involved in importing dually targeted tail-anchored proteins to both mitochondria and peroxisomes. Traffic 2018; 19:770–85. https://doi.org/10.1111/tra.12604 PMID: 30033679

44. Koch A, Yoon Y, Bonekamp NA, McNiven MA, Schrader M. A Role for Fis1 in Both Mitochondrial and Peroxisomal Fission in Mammalian Cells. Mol Biol Cell 2005; 16:5077. https://doi.org/10.1091/mbc.e05-02-0159 PMID: 16107562

45. Schrader M, Costello J, Godinho LF, Islinger M. Peroxisome-mitochondria interplay and disease. J Inherit Metab Dis 2015; 38:681–702. https://doi.org/10.1007/s10545-015-9819-7 PMID: 25687155

46. Camões F, Islinger M, Guimarães SC, Kilaru S, Schuster M, Godinho LF, et al. New insights into the peroxisomal protein inventory: Acyl-CoA oxidases and -dehydrogenases are an ancient feature of peroxisomes. Biochimica et Biophysica Acta (BBA)—Molecular Cell Research 2015; 1853:111–25. https://doi.org/10.1016/j.bbamcr.2014.10.005 PMID: 25307522

47. Wanders RJA, Waterham HR, Ferdinandusse S. Metabolic Interplay between Peroxisomes and Other Subcellular Organelles Including Mitochondria and the Endoplasmic Reticulum. Front Cell Dev Biol 2015; 3:83. https://doi.org/10.3389/fcell.2015.00083 PMID: 26858947

48. Demarquoy J, Borgne F Le. Crosstalk between mitochondria and peroxisomes. World J Biol Chem 2015; 6:301. https://doi.org/10.4331/WJBC.V6.I4.301.

49. Shai N, Yifrach E, Van Roermund CWT, Cohen N, Bibi C, Ijlst L, et al. Systematic mapping of contact sites reveals tethers and a function for the peroxisome-mitochondria contact. Nat Commun 2018;9. https://doi.org/10.1038/S41467-018-03957-8.

50. Keskin A, Akdoğan E, Dunn CD. Evidence for amino acid snorkeling from a high-resolution, in vivo analysis of Fis1 tail-anchor insertion at the mitochondrial outer membrane. Genetics 2017; 205:691–705. https://doi.org/10.1534/genetics.116.196428 PMID: 28007883

51. Polianskyte Z, Peitsaro N, Dapkunas A, Liobikas J, Soliymani R, Lalowski M, et al. LACTB is a filament-forming protein localized in mitochondria. Proc Natl Acad Sci U S A 2009; 106:18960. https://doi.org/10.1073/pnas.0906734106 PMID: 19858488

52. McLean IW, Nakane PK. PERIODATE-LYSINE-PARAFORMALDEHYDE FIXATIVE A NEW FIXATIVE FOR IMMUNOELECTRON MICROSCOPY. 1974;22:1077–83. https://doi.org/10.1177/22.12.1077.

53. Lak B, Li S, Belevich I, Sree S, Butkovic R, Ikonen E, et al. Specific subdomain localization of ER resident proteins and membrane contact sites resolved by electron microscopy. Eur J Cell Biol 2021; 100:151180. https://doi.org/10.1016/j.ejcb.2021.151180 PMID: 34653930

54. Belevich I, Joensuu M, Kumar D, Vihinen H, Jokitalo E. Microscopy Image Browser: A Platform for Segmentation and Analysis of Multidimensional Datasets. PLoS Biol 2016;14. https://doi.org/10.1371/journal.pbio.1002340 PMID: 26727152