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Pichia pastoris Rppa09976 is a Peroxisomal Membrane-associated ACBP Domain-containing Protein, Delivered to Peroxisomes from the ER, and is Required for Their Selective Degradation

A Thesis submitted in partial satisfaction of the
Requirements for the degree of Master of Science

in

Biology

by

Katharine Ozeki

Committee in charge:

Professor Suresh Subramani, Chair
Professor Douglass Forbes
Professor Immo Scheffler

2009

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Chair

University of California, San Diego

2009

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ACKNOWLEDGEMENTS

Without the aid and support from a number of people, this thesis would be a bunch of blank pages. First and foremost, I am *very* grateful for the guidance and support from my mentor, Dr. Taras Nazarko. Thanks for your patience in answering my questions, showing me new techniques/equipment, improving my analytical skills, designing experiments where I would have to stay in lab for 15 hours, and offering me bananas. I have come a long way and I appreciate the trust you have had in me to run experiments that did not involve me counting thousands of colonies or making hundreds of YPD plates. I would also like to especially thank Dr. Suresh Subramani for inviting me into his lab, allowing me to learn various molecular and cellular biology techniques, and revising my thesis and presentation. Additionally, I would like to thank the past and present Subramani lab members for identifying Rppa09976, making plasmids and strains I've used, running some preliminary experiments on Rppa09976 (Figures 2-4, 2-5, 3-2) and offering encouragement and suggestions to my project.

Last but not least, I would like to thank my family and friends for the endless support and understanding. To my parents, Ted and Jean Ozeki, and my brother, Michael, thank you for always believing in me. To Jason Chen, thank you for your constant enthusiasm and willingness to read my thesis.

This thesis, in full, is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

ABSTRACT OF THE THESIS

Pichia pastoris Rppa09976 is a Peroxisomal Membrane-associated ACBP Domain-containing Protein, Delivered to Peroxisomes from the ER, and is Required for Their Selective Degradation

by

Katharine Ozeki

Master of Science in Biology

University of California, San Diego, 2009

Professor Suresh Subramani, Chair

It is imperative for organisms to find a way to recycle and degrade cytoplasmic constituents and eliminate unnecessary organelles. Autophagy is a tightly-regulated, non-selective degradation process that plays an integral role in cellular homeostasis by sequestering ubiquitous proteins or organelles, and delivers such cargo to the vacuole so

that they may be degraded and their constituents recycled. For instance, peroxisomes are single membrane-bound organelles required for certain metabolic pathways including both α- and β-oxidation of fatty acids, decomposition of harmful hydrogen peroxide molecules, the production of penicillin in certain fungi and photorespiration in plants. When peroxisomes become redundant, they are selectively delivered to the vacuole in an autophagy-related pathway, termed pexophagy. While most proteins required for autophagy are also required for pexophagy, only two proteins, Atg26 and Atg30, are known to be solely required for pexophagy, but not autophagy-related pathways. Here, we identified a novel peroxisomal membrane-associated acyl-CoA binding protein (ACBP) domain-containing protein, Rppa09976, also required for the selective degradation of peroxisomes, but dispensable for other autophagy-related pathways. Unlike most peroxisomal membrane proteins, Rppa09976 is a type II PMP, trafficking from the ER to peroxisomes, and degrades independent of pexophagy, autophagy and vacuolar proteolysis. Furthermore, we have identified a putative mammalian homolog, the murine peroxisomal MmAcbd5 protein, which, like Rppa09976, contains an N-terminal acyl-CoA binding protein (ACBP) domain. This discovery has led to a deeper understanding of the mechanism underlying the selective sequestration and degradation of peroxisomes, a process that may apply to higher organisms.

Chapter 1: Introduction

Autophagy

Organisms must find a way to degrade and recycle redundant cytoplasmic constituents and eliminate unnecessary organelles to maintain cell viability. Autophagy literally describes a lysosomal/vacuolar degradation process, 'phagy' of the cells own components, 'auto.' Christian de Duve, considered to be the founding father of autophagy, described the lysosomal degradation process using electron microscopy studies showing single- or double-membrane vesicles containing portions of the cytoplasm, including organelles (de Duve and Wattiaux, 1966). Autophagy is a tightly-regulated, non-selective process that allows cells to adapt to environmental or developmental changes by reallocating nutrients from redundant processes to essential ones (Klionsky and Ohsumi, 1999). Cells can sequester superfluous cellular components by two mechanisms, macroautophagy and microautophagy (Figure 1-1). Macroautophagy involves the sequestration of cytoplasmic constituents via a double-membrane vesicle, called the autophagosome, which then fuses with the lysosome/vacuole, where cargo is released and degraded by resident hydrolases (Nair and Klionsky, 2005). Microautophagy, on the other hand, is a process where the vacuolar/lysosomal membrane invaginates and directly engulfs cytosolic components, which are then degraded by the hydrolytic enzymes (Klionsky, 2005).

Autophagy is implicated in human disease

Autophagy was first implicated with human disease in the laboratory of Beth Levine; mice with monoallelic deletions of *BECN1/ATG6* were more susceptible for spontaneous tumor formation (Liang et al., 1999). Soon after, the number of studies showing a correlation between dysfunctional autophagy processes and pathophysiological conditions increased significantly. For instance, autophagy plays a role in the neonatal starvation period of newborn mice. Once mice are born, the only way to obtain nutrients is from the uptake of the mother's milk, but preventing this forces newborns to induce autophagy and recycle cytoplasmic components. *atg5*$^{-/-}$ null mice appear almost normal at birth, but die within 12 hours after birth, whereas wildtype mice are able to survive longer during starvation (Kuma et al., 2004). Furthermore, studies indicated a role for autophagy as a tumor suppressor, a protective mechanism against DNA damage that would otherwise progress towards tumorigenesis (Mathew et al., 2007). In addition, researchers linked a requirement for a basal level of autophagy to clear out protein aggregates in the prevention of a number of neurodegenerative diseases, including Huntington's chorea and Alzheimer's disease (Rubinsztein et al., 2005), (Ravikumar et al., 2002). Finally, autophagy has also been suggested to be coordinated with apoptosis by functioning as a cytoprotective mechanism; however, upon extensive cellular damage or when apoptosis is compromised, autophagy can be used for type II programmed cell death (Boya et al., 2005), (Yu et al., 2004). In summary, autophagy has been implicated to be important for the immune system, cell growth and survival, tumor suppression, apoptosis and

reduction of protein buildup; any defect in this process may have widespread developmental effects.

Autophagy – steps and regulation

The proteins involved in autophagy-related processes were reclassified as *ATG* genes (listed in Table 1-1), and a majority of these *ATG* genes were shown to be conserved from yeast to man (Klionsky et al., 2003), (Meijer et al., 2007), indicating its importance for cell survival and viability. Studies using *Saccharomyces cerevisiae* as a model organism revealed the multiple, complex steps involved in autophagy. To date, using multiple model systems, scientists have isolated and functionally characterized 31 *ATG* genes involved at the various stages in autophagy and autophagy-related pathways. The steps involved in macroautophagy are:

i) Signaling: carbon, nitrogen, phosphate or sulfate starvation induces autophagy. The protein kinase, Tor (target of rapamycin), regulates cell growth in response to nutrient availability and cellular stress (Abeliovich et al., 2000). When nutrients are abundant, Tor hyperphosphorylates Atg13, which, when hyperphosphorylated, has low affinity for Atg1, blocking autophagy (Nair and Klionsky, 2005). Upon nutrient starvation or the addition of rapamycin (an inhibitor of Tor), the Tor signaling pathway is inactivated, resulting in partial dephosphorylation of Atg13, increasing its affinity for Atg1. (Blommaart et al., 1995).

ii) Nucleation of vesicle formation: proteins and lipids start to aggregate at a small, dot-like membranous structure, the pre-autophagosomal

structure or phagohore assembly site (PAS), where membrane expansion and recruitment of cargo proceed. The recruitment of Atg proteins from the cytosol or other compartments to the PAS marks the nucleation event. Residing at the PAS in hierarchical order are: Atg1 and its regulators, the Atg2-Atg18 complex, the Atg8 system, the Atg12 system, the phosphatidylinositol 3-kinase (PI 3-K) complex, and Atg9 (Suzuki and Ohsumi, 2007), (Klionsky, 2005).

iii) Vesicle expansion and completion: most of the Atg proteins are involved in vesicle formation, and as a result, it is probably the least understood step. Two ubiquitin-like proteins, Atg8 and Atg12 become conjugated to phosphatidylethanolamine (PE) and Atg5, respectively. In the absence of Atg8 the size of autophagosomes are significantly reduced (Abeliovich et al., 2000)

iv) Retrieval: Like most targeting pathways, certain components of the autophagy pathway are recovered for reuse. Only Atg8-PE and Atg19 are known to remain associated with autophagosomes, thus most of the Atg proteins are soluble and able to cycle off of the vesicle. Atg9, Atg23 and Atg27 are unusual in that they cycle between the PAS and an additional punctate structure, the non-PAS structure (NPS).

v) Vesicle docking and fusion to the vacuole: the timing of vesicle fusion must be regulated so that cargo can efficiently be released for degradation. Atg8-PE conjugation is thought to prevent premature fusion events; inability to cleave off Atg8 from PE causes a partial

defect in autophagy (Kirisako et al., 2000). The machinery required for other vacuole-targeting pathways are also required for this step. The SNARE proteins, the class C Vps/HOPS complex and many other proteins play a role in the fusion process.

vi) Breakdown of the intraluminal vesicle and its cargo and recycling of the macromolecular constituents: in order to degrade and recycle cargo, the single-membrane subvacuolar vesicles (called autophagic bodies), formed after the outer autophagosomal membrane fuses to the vacuolar membrane, must be degraded as well. Both the acidity of the vacuole and Atg15, a putative lipase, are thought to aid in the breakdown of this membrane. Once cargo is released into the vacuolar lumen, resident proteases, proteinase A (Pep4) and proteinase B (Prb1) degrade cargo into its constituents (Klionsky, 2005), (Farre and Subramani, 2004), (Takeshige et al., 1992). Finally, Atg22 and other vacuolar amino acid transporters allow recycling of amino acids to the cytosol (Yang et al., 2006).

Autophagy-related pathways

Christian de Duve suggested autophagy could selectively sequester specific organelles in 1966, however not until 1973 did researchers prove specific sequestration of an organelle (the smooth endoplasmic reticulum) via an autophagy-related pathway (Bolender and Weibel, 1973). Soon after, sequestration of mitochondria and peroxisomes were also observed (Beaulaton and Lockshin, 1977), (Veenhuis et al.,

1983). In addition, in nutrient rich conditions, the constitutive cytosol-to-vacuole targeting (Cvt) pathway provides another selective, autophagy-related example in which small vesicles resembling autophagosomes, called Cvt vesicles, transport precursors of two vacuolar hydrolases, aminopeptidase 1 (Ape1) and α-mannosidase (Ams1), (Scott et al., 1996). While it is not surprising that the Cvt pathway shares many of the same proteins as the autophagy pathway, proteins required specifically for selective autophagy-related pathways, however, are unique and show little homology across species. In the cytosol, the precursor form of Ape1 forms dodecamers and then aggregates into a larger ApeI complex. The Cvt-specific receptor protein, Atg19, recognizes this complex and binds to the N terminus of Ape1, forming a Cvt complex. Atg11 then acts as an adaptor protein, recruiting the Cvt complex to the PAS (Kim et al., 2001). At the PAS, Atg19 then binds to Atg8-PE, inducing membrane expansion and incorporation of the cargo into a Cvt vesicle. Upon delivery to the vacuole, the Ape1 and Ams1 are processed to their functional, mature forms.

As stated above, autophagy can regulate organelle homeostasis by selectively sequestering and degrading redundant organelles including the mitochondria (mitophagy), ER (ER-phagy), peroxisomes (pexophagy), ribosomes (ribophagy), and parts of the nucleus (piecemeal microautophagy of the nucleus, PMN or micronucleophagy). The specific degradation of peroxisomes by autophagy-related pathways, called pexophagy, occurs in a wide array of organisms, from yeast to man. Like autophagy, pexophagy occurs by either macropexophagy or micropexophagy, similar to macroautophagy and microautophagy, respectively (Subramani, 1998). As an

autophagy-related process, it is not surprising that many genes involved in pexophagy are also involved in autophagy and Cvt pathways (Dunn et al., 2005).

Physiological role of peroxisomes

Found in nearly all eukaryotic cells, peroxisomes are subcellular organelles that participate in many catabolic and anabolic pathways, including the α- and β-oxidation of fatty acids, decomposition of harmful hydrogen peroxide molecules, the production of penicillin in certain fungi, and photorespiration in plants. Peroxisomes range from about 0.1 to 1.0 μm and contain at least one hydrogen-peroxide producing oxidase, and catalase to decompose the hydrogen peroxide (Lazarow and Fujiki, 1985). Recent interest in peroxisomes originates from an expanding list of genetic disorders resulting from peroxisome biogenesis or enzyme deficiencies. Dysfunctional peroxisome assembly caused by defects in *PEX* genes and their corresponding peroxin proteins can lead to disorders such as Zellweger's syndrome, rhizomelic chondrodysplasia punctata and adrenoleukodystrophy (Subramani, 1998).

Unlike the mitochondria, peroxisomes do not contain their own DNA or protein synthesis machinery, thus virtually all peroxisomal proteins are encoded by nuclear *PEX* genes. To date, 32 *PEX* genes and their corresponding peroxins are known to participate in peroxisome biogenesis. Most peroxisomal matrix proteins are synthesized on free polysomes in the cytosol and transported post-translationally to the peroxisome via peroxisomal targeting signals (PTS). Pex5 and Pex7 recognize the PTS1 and PTS2 signals, respectively, and bring these peroxisomal matrix proteins to the peroxisome translocation machinery for transport into the matrix (Hettema et al., 2000),

(Subramani, 1998). Unlike the trafficking of matrix proteins, peroxisomal membrane protein (PMP) trafficking is less well understood. While most integral PMPs are synthesized on free polysomes and directly inserted into the peroxisomal membrane (Lazarow and Fujiki, 1985), a small subset of PMPs, however might be targeted to peroxisomes indirectly, through the ER. In *Saccharomyces cerevisiae*, the predominantly cytosolic Pex19 protein interacts with PMPs and is thought to act as a chaperone, keeping newly synthesized PMPs in a conformation ready for integration into the peroxisomal membrane. In the absence of Pex19 in both *S. cerevisiae* and *H. sapiens*, most PMPs are either rapidly degraded or mislocalized to the mitochondria or ER (Sacksteder et al., 2000). Peroxisome assembly involves a multistep process involving the selective uptake of different matrix and membrane proteins leading to the formation of mature and metabolically active peroxisomes.

Model organisms for pexophagy

Depending on the environmental condition the cell experiences, the size, number and distribution of peroxisomes can vary (Yan et al., 2005). Cells are equipped with the genetic information to generate peroxisomes in time of need and also, to quickly and selectively degrade peroxisomes when they become redundant. The methylotrophic yeast *Hansenula polymorpha* and *Pichia pastoris* are two ideal model organisms used to study peroxisome proliferation and degradation by manipulation of nutrient environments. These yeast species are able to utilize methanol as a carbon and energy source, and under methanol adaptation, peroxisomes and peroxisomal matrix enzymes like alcohol oxidase (AOX) are induced. When methanol-induced cells are shifted to

ethanol- or glucose-containing media, peroxisomes become redundant and are selectively degraded via macro- or micropexophagy.

Macro- and Micropexophagy

Many of the same *ATG* genes involved in autophagy are also involved in macro- and micropexophagy. Macro- and micropexophagy processes are morphologically similar to macro- and microautophagy, respectively, with the exception that peroxisomes are selectively sequestered and degraded. In methylotrophic yeast species, both modes of pexophagy occur, but the environmental trigger inducing the different modes of pexophagy is species-specific. For instance, the transfer of methanol-induced cells to glucose induces macropexophagy in *H. polymorpha* and micropexophagy in *P. pastoris*. Transfer to ethanol, however, induces macropexophagy in both species. Recently, studies indicate that the trigger for macro- and micropexophagy depends on the intracellular ATP level in *P. pastoris*; high ATP levels induce micropexophagy while low ATP levels induce macropexophagy (Ano et al., 2005).

Macro- and micropexophagy alike require further membranous structures to selectively sequester peroxisomes from the cytosol. In macropexophagy, individual peroxisomes tagged for degradation are enclosed by a double-membrane pexophagosome, morphologically similar to autophagosomes. Alternatively, in micropexophagy, vacuolar sequestering membranes (VSM) engulf a cluster of methanol-induced peroxisomes. Fusion events of the VSM require the micropexophagic membrane apparatus (MIPA), formed between the extended membrane tips of an engulfing vacuole. The MIPA and pexophagosome membranes are thought to derive

from the PAS, the dot-like structure required for all autophagy-related processes (Dunn et al., 2005), (Farre and Subramani, 2004), (Kiel et al., 2003). The peroxisome specific receptor, Atg30, analogous to the Cvt-specific receptor, Atg19, was only recently identified as the protein required for peroxisome-specific degradation (Farre et al., 2008).

Pexophagy-specific proteins

Selective degradation of peroxisomes depends on the peroxisome receptor phosphoprotein, Atg30, that bridges the peroxisomal membrane with the autophagic machinery after the transfer of *P. pastoris* cells from methanol to glucose or ethanol medium (Farre et al., 2008). Atg26 and Atg30 are the only two proteins known to be required for both micro- and macropexophagy but dispensable for the Cvt and autophagy pathways (Farre et al., 2008). Overexpression of Atg30 in peroxisome proliferation conditions induces pexophagy, further suggesting its role as the peroxisome receptor protein. On the peroxisomal membrane, Atg30 interacts with peroxins, Pex3 and Pex14, as well as autophagy-related proteins, Atg11 and Atg17. Atg30 phosphorylation at serine 112 is required for interaction with Atg11, suggesting that Atg30 phosphorylation at this residue specifically tags these peroxisomes for degradation through the interaction with autophagic machinery (Farre et al., 2008).

Recently, we found a novel *P. pastoris* peroxisomal membrane protein, Rppa09976, which, like Atg30, is required specifically for both macro- and micropexophagy but not for autophagy or the Cvt pathways. Δ*rppa09976* cells showed a significant delay in pexophagy when methanol-induced cells were shifted to either

glucose or ethanol media. Δ*rppa09976* and Δ*atg30* showed similar phenotypes, in that the MIPA formation during micropexophagy and pexophagosome formation during macropexophagy were blocked. Unlike Atg30, BLAST analysis indicated a mammalian homolog of Rppa09976, the murine peroxisomal membrane protein, MmAcbd5 (Wiese et al., 2007). While Atg30 is directly incorporated into the peroxisomal membrane from the cytosol, Rppa09976 showed an ER-to-peroxisome trafficking mechanism. Furthermore, instead of being degraded along with peroxisomes like Atg30, Rppa09976 was degraded independent of pexophagy, autophagy, and vacuolar proteolysis. The goal of this study was to determine the physiological role of the Rppa09976 using molecular, biochemical and cell biological methods. Here we have developed biochemical assays and used fluorescence microscopy to follow the localization, trafficking, degradation and function of Rppa09976.

Chapter 1 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

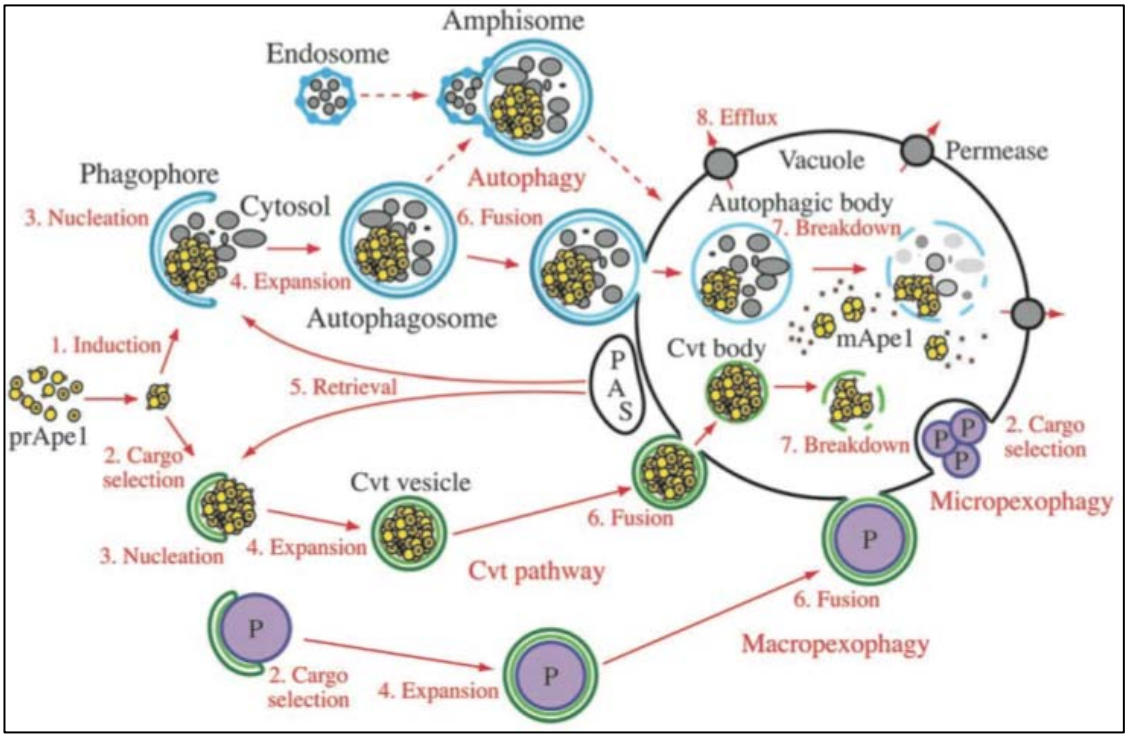

Figure 1-1: Autophagy and autophagy-related pathways. (Klionsky et al., 2007). Redundant or damaged organelles and cytosolic proteins are non-selectively sequestered into autophagosomes and transported to the vacuole by macro- and microautophagy pathways for recycling. Also shown are autophagy-related pathways, including the Cvtoplasm-to-vacuole targeting (Cvt) pathway and pexophagy pathways, both of which involve the selective sequestration and degradation of proteins (Ape1 and Ams1) and peroxisomes, respectively.

Table 1-1: List and description of autophagy-related genes. (Klionsky et al., 2003)

Gene Designation								
Current	Former							
ATG	*APG*	*AUT*	*CVT*	*GSA*	*PAZ*	*PDD*	Reference	Protein Characteristics
1	1	3	10	10	1	7	Matsuura et al., 1997; Straub et al., 1997; Komduur et al., 2003; Mukaiyama et al., 2002; Harding et al., 1995; Stromhaug et al., 2001	Protein kinase
2	2	8	–	11	7	–	Shintani et al., 2001; Wang et al., 2001a; Barth and Thumm, 2001; Mukaiyama et al., 2004; Stromhaug et al., 2001	Peripheral membrane interacts with protein Atg9
3	3	1	–	20	–	–	Schlumpberger et al., 1997; Ichimura et al., 2000; Habibzadegah-Tari and Dunn, 2003	E2-like enzyme conjugates Atg8 to phosphatidylethanolamine (PE)
4	4	2	–	–	8	–	Lang et al., 1998; Kirisako et al., 2000; Mukaiyama et al., 2004	Cysteine protease; cleaves C-terminal extension or PE from Atg8
5	5	–	–	–	–	–	Kametaka et al., 1996	Conjugated to Atg12 through internal lysine
(6)[a]	6	–	–	–	–	–	Kametaka et al., 1998; Kihara et al., 2001	Component of PtdIns 3-kinase complexes I and II
7	7	–	2	7	12	–	Kim et al., 1999; Yuan et al., 1999; Tanida et al., 1999; Mukaiyama et al., 2004	E1-like enzyme activates Atg8 and Atg12
8	8	7	5	–	2	–	Lang et al., 1998; Kirisako et al., 2000; Harding et al., 1995; Mukaiyama et al., 2002	Ubiquitin-like protein conju gated to PE via C-terminal glycine
9	9	9	7	14	9	–	Noda et al., 2000; Lang et al., 2000; Mukaiyama et al., 2002; Stromhaug et al., 2001	Integral membrane protein
10	10	–	–	–	–	–	Shintani et al., 1999	E2-like enzyme; conjugates Atg12 to Atg5
11	–	–	9	9	6	18	Kim et al., 2001; Mukaiyama et al., 2002	Specific component involved in cargo recognition
12	12	–	–	–	–	–	Mizushima et al., 1998a	Ubiquitin-like protein; conjugated to Atg5 via C-terminal glycine
13	13	–	–	–	–	–	Funakoshi et al., 1997; Scott et al., 2000	Modifier of Atg1 activity; hyperphosphorylated in rich media
14	14	–	12	–	–	–	Kametaka et al., 1998; Kihara et al., 2001	Component of PtdIns 3-kinase complex I
15	–	5	17	–	–	–	Epple et al., 2001; Teter et al., 2001	Putative lipase required for breakdown of intravacuolar vesicles
16	16	–	11	–	3	–	Mizushima et al., 1999; Mukaiyama et al., 2002	Component of Atg12-Atg5 complex
17	17	–	–	–	–	–	Kamada et al., 2000	Modifier of Atg1 activity
18	–	10	18	12	–	–	Barth et al., 2001; Guan et al., 2001	Peripheral membrane protein; required for localization of Atg2
19	–	–	19	–	–	–	Scott et al., 2001; Leber et al., 2001	Cargo receptor for the Cvt pathway
20	–	–	20	–	–	–	Nice et al., 2002	PX domain protein needed for the Cvt pathway
21	–	–[b]	21	–	–	–	Barth et al., 2002	Specific to the Cvt pathway
22	–	4	–	–	–	–	Suriapranata et al., 2000	Integral membrane protein; involved in autophagic body breakdown
23	–	–[c]	23	–	–	–	Tucker et al., 2003	Needed for Cvt vesicle completion
(24)[d]	–	–	13	–	16	–	Nice et al., 2002; Y. Ano and Y.S., unpublished	Sorting nexin; PX domain-containing protein involved in the Cvt pathway and pexophagy
25	–	–	–	–	–	4	I.L. Monastyrska, J.A.K.W. Kiel, and M.V., unpublished	Coiled-coil protein involved in macropexophagy
26[e]	–	–	–	–	4	–	Mukaiyama et al., 2002; Oku et al., 2003; Stasyk et al., 2003	UDP-glucose:sterol glucosyltransferase-containing GRAM domain
27[f]	–	–	24	–	–	–	Wurmser and Emr, 2002	PtdIns(3)P binding protein required for the Cvt pathway

[a] The standard name for this gene is *VPS30*.
[b] This gene was originally named *MAI1*.
[c] This gene was also named *MAI2*.
[d] The standard name for this gene is *SNX4*.
[e] This gene was originally named *UGT51*.
[f] This gene was originally named *ETF1*.

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Chapter 2: Rppa09976 is required for pexophagy but not for autophagy or the Cvt pathways

Abstract

The use of a tandem affinity purification (TAP) tag linked to the peroxisomal membrane protein, Pex3, led to the identification of an unknown ORF, *RPPA09976*, which encodes a 44 kDa protein with an N-terminal acyl-CoA binding (ACBP) domain. To determine the function of this protein, *Pichia pastoris* strains deleted for *RPPA09976* were constructed and verified. Δ*rppa09976* strains were shown to have normal peroxisome biogenesis in oleate or methanol medium. Similarly, Δ*rppa09976* strains were not affected in autophagy or Cvt pathways. However, cells without *RPPA09976* show a significant delay in the selective degradation of peroxisomes by autophagic mechanism (pexophagy). Along with Atg26 and Atg30, Rppa09976 is third protein known to be specifically required for pexophagy, yet dispensable in other selective or non-selective autophagy-related pathways.

Introduction

In order to survive, organisms must find a way to degrade and recycle redundant cytoplasmic constituents and eliminate unnecessary organelles. The two major degradative processes within eukaryotic cells are proteasomal degradation and vacuolar degradation. Proteasomes are multi-protein complexes, located in the cytosol or nucleus that coordinate the regulated breakdown of proteins (Peters *et al*., 1994). On the other hand, vacuoles are membrane-bound organelles containing acidic hydrolases and proteolytic enzymes capable of degrading virtually any subcellular constituent, including entire organelles. Under starvation conditions, this process is up-regulated in

a process known as autophagy. Dysfunction in the autophagy pathway can lead to severe human disorders (Huang and Klionsky, 2007). Therefore monitoring autophagy and autophagy-related pathways will provide researchers with better tools for the treatment and prevention of such disorders.

Atg8 and its mammalian homologue, LC3, is the most widely used marker to follow the autophagy pathway. Atg8/LC3 is required for phagophore expansion and the formation of large autophagosomes; its absence severely affects autophagy and all autophagy-related processes (Xie et al., 2008), (Abeliovich and Klionsky, 2001). Atg8 conjugated to phosphitidylethanolamine (Atg8-PE) localizes to the phagophore assembly site/pre-autophagosomal structure (PAS) and, unlike many Atg proteins, some of the Atg8-PE remains associated with the completed autophagosome. Furthermore, upon delivery to the vacuole, Atg8 is degraded along with the cargo. In *P. pastoris*, the N-terminal GFP fusion to Atg8, GFP-Atg8, allows researchers to monitor the delivery of the autophagosomal membrane to the vacuole for degradation. Transfer of cells grown in medium containing nitrogen (SD) to nitrogen starvation medium (SD-N) induces autophagy, corresponding to the disappearance of Atg8-GFP and the appearance of GFP, which remains relatively stable inside the vacuole due to its relatively high resistance to vacuolar proteases (Shintani and Klionsky, 2004).

In contrast to autophagy, the cytoplasm to vacuole targeting (Cvt) pathway selectively sequesters hydrolases Ape1 and Ams1 for delivery to the vacuole (Shintani et al., 2002). Although first discovered in *Saccharomyces cerevisiae,* a *P. pastoris* Cvt pathway has recently been identified (Farre et al., 2007). Unlike autophagy, which is induced during both extracellular and intracellular stress conditions, the Cvt pathway is

constitutively activated, even in nutrient rich conditions. In the cytosol, Ape1 is synthesized as a precursor (prApe1) where it aggregates forming a large Ape1 complex (Kim et al., 2001). prApe1 is recognized by the Cvt-specific receptor protein, Atg19, and forms a Cvt complex. Atg11, the adaptor protein directing the Cvt complex to the PAS, is involved specifically in the Cvt pathway, but not in general autophagy (Yorimitsu and Klionsky, 2005). Upon delivery to the vacuole, the Ape1 pro-peptide is removed and the mature form of Ape1 (mApe1) is formed. Ape1 processing can therefore be used to monitor the kinetics of the Cvt pathway.

Likewise, during pexophagy, redundant or damaged peroxisomes are specifically sequestered and delivered to the vacuole. Since alcohol oxidase (AOX) constitutes the bulk of the matrix proteins in methanol-induced peroxisomes in *P. pastoris*, AOX serves as an ideal protein to monitor pexophagy. In methylotrophic yeast, AOX is maximally induced under methanol adaptation but strongly repressed during ethanol or glucose adaptation (van der Klei et al., 1991). Synthesized on free ribosomes in the cytosol, AOX is post-translationally transported into the peroxisomal matrix (Roa and Blobel, 1983). Once inside the peroxisome, AOX aggregates into a homoctameric flavoprotein consisting of eight identical subunits of approximately 74 kDa, each containing a flavin adenine dinucleotide (van der Klei et al., 1991). AOX catalyzes the oxidation of methanol to formaldehyde and hydrogen peroxide. Wild-type cells that are pexophagy-proficient degrade all their AOX when switched from peroxisome-induction conditions to pexophagy medium. In contrast, most of the *atg* mutants unable to degrade peroxisomes show unchanging levels of AOX in a given time-course.

Here we identified a novel protein, Rppa09976, using TAP-tag purification to the peroxisomal membrane protein, Pex3. Pex3 is essential for the proper localization and stability of peroxisomal membrane proteins, and is required early in peroxisome biogenesis. Yeast mutants deficient in Pex3 lack peroxisomes and are unable to grow in peroxisome biogenesis conditions (Hettema et al., 2000) , (Hazra et al., 2002). Despite its association with Pex3, Rppa09976 is dispensable for peroxisome biogenesis. Rppa09976 is, however, required for pexophagy, but not autophagy or the Cvt pathway. To date, Rppa09976 is the third protein (besides Atg26 and Atg30) known to be solely required for pexophagy, but not autophagy or any other autophagy-related pathways.

Materials and Methods

Yeast strains, plasmids and transformations

The *P. pastoris* strains and plasmids used in this study are listed in Table 2-1. Cells were transformed via electroporation (Cregg and Russell, 1998). His^+-transformants were selected on SD (1.7 g/l yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 2% w/v dextrose, 0.5% w/v ammonium sulfate, 2% w/v agar) plates without histidine. Arginine (50 mg/l) was added, when needed. Geneticin-, zeocin- or hygromycin-resistant transformants were selected on YPD (1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v dextrose, 2% w/v agar) plates with 0.25 mg/ml of geneticin, 0.1 mg/ml of zeocin, or 0.8 mg/ml of hygromycin, respectively. Two independent transformants of each strain with each plasmid were examined in parallel by each functional assay.

Peroxisome- and pexophagy-induction conditions

Cells were pre-grown to the late exponential-stationary phase in the first YPD culture, diluted 25-50 fold with fresh YPD medium and pre-grown to the early-mid exponential phase in the second YPD culture. Cells were washed twice with YNB solution (1.7 g/l YNB without amino acids and ammonium sulfate) and inoculated into peroxisome-induction medium at an OD_{600} of 0.3-0.5 (for PPY12h cells) or 1.0 (for PPY12m cells, which grow more slowly than PPY12h cells for unknown reasons) for 15 h. Cells were washed twice with YNB solution and inoculated into pexophagy-induction medium, containing either 0.5% ethanol or 2% glucose. All peroxisome- and pexophagy-induction media were prepared in YNB solution. Incubation times are shown in the

figures. Histidine (50 mg/l) and/or arginine (50 mg/l) were added, when needed. All peroxisome-, but not pexophagy-induction media contained 0.05% w/v yeast extract. The concentration of carbon sources were: 0.5% v/v methanol, 0.5% v/v oleate, 0.5% v/v ethanol or 2% w/v glucose. The oleate stock emulsion contained 20% v/v oleate and 0.5% v/v Tween-80. The concentration of nitrogen sources was 0.25 or 0.5% w/v ammonium sulfate.

Biochemical studies of pexophagy

Peroxisomes were induced as described above in peroxisome-induction medium, cells were washed twice with YNB solution transferred to fresh glucose/(-N) or ethanol/(-N) medium at an OD_{600} of 1 or 2, respectively, to induce pexophagy. Cells from 1 ml culture samples were collected by centrifugation after 0, 6, 12 and 24 h of adaptation. Crude extracts were prepared in the presence of TCA (Baerends et al., 2000). SDS-PAGE and immunoblotting were performed as described previously (Laemmli, 1970) (Kyhse-Andersen, 1984). Antigen-antibody complexes were detected by enhanced chemiluminescence.

GFP-Atg8 processing assay

Cells were pre-grown twice in YPD media as described for pexophagy studies, washed twice with YNB solution and transferred to glucose/ammonia medium at an OD_{600} of 0.1 for 13.5 h. Cells were then washed twice with YNB solution and transferred to glucose/(-N) medium. Cells from 1 ml culture samples were collected by centrifugation after 0, 1, 2, and 4 h of nitrogen starvation.

Ape1 maturation assay

Cells were pre-grown twice in YPD as described for pexophagy studies, washed twice with YNB solution and transferred to SD media for 17 and 24 h. 0.5 OD_{600} of cells from each culture were collected by centrifugation.

Alcohol Oxidase plate assay for pexophagy

Cells were pre-grown on YPD for two days. YPD plates were then replica plated onto a 0.5% methanol plate (1.7 g/l YNB without amino acids and ammonium sulfate, 0.5% methanol, 0.25% w/v ammonium sulfate, 50 mg/l arginine, 50 mg/l histidine, and 2% w/v agar) plate for 48 h. These strains were then replica-plated onto SE plates (1.7 g/l YNB without amino acids and ammonium sulfate, 0.5% ethanol, 0.25% w/v ammonium sulfate, 50 mg/l arginine, 50 mg/l histidine, and 2% w/v agar for 15 h in order to induce macropexophagy.

For a 100 ml of AOX plate assay solution, 50 mM Tris buffer pH 7 was added to 50 mg o-dianisidine and 300 mg of low melt agarose. This solution was brought to a boil and then 200 mg of hexadecyltrimethyl ammonium bromide was added. When the solution became warm to the touch, 6 mg of peroxidase was added, along with 1 ml of methanol. The 10-12 ml of solution was poured onto plates and left at room temperature for approximately 30 min and then placed into the 30 ^{o}C incubator for approximately 2 h (Nazarko *et al*., 2002).

Table 2-1: Strains used in this study. All strains are shown below along with genotype.

Strain Name	Genotype	References
PPY12h	WT *his4 arg4*	(Gould et al., 1992)
PPY12m	WT *his4 arg4*	Subramani laboratory
R12	GS115 *atg1-1*::Zeocin^R *his4*	(Stromhaug et al., 2001)
SEW1	PPY12h Δ*pex3*::ARG4 *his4 arg4*	(Wiemer et al., 1996)
SMD1163	*his4 pep4 prb1*	(Tuttle and Dunn, 1995)
SJCF925	PPY12h Δ*atg8*::Geneticin^R *his4 arg4*	This study
SJCF936	PPY12h Δ*atg30*::Zeocin^R *his4 arg4*	This study
SMY432	PPY12m Δ*rppa09976*::Zeocin^R *his4 arg4*	This study
SMY443	SMY432 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
SMY458	SMY432 pr*RPPA09976*-ΔACBP-GFP::HIS4 *his4 arg4*	This study
SMY459	SMY432 pr*RPPA09976*-ACBP-GFP::HIS4 *his4 arg4*	This study
SMY460	SMY432 pr*RPPA09976*-ACBP-GFP-Pex11::HIS4 *his4 arg4*	This study
SMY461	PPY12m pr*RPPA09976*-ACBP-GFP-Pex11::HIS4 *his4 arg4*	This study
STN52	SMY432 pr*AOX1*-BFP-SKL::Hygromycin^R *his4 arg4*	This study
STN54	SMY443 pr*AOX1*-BFP-SKL::Hygromycin^R *his4 arg4*	This study
STN55	SMY458 pr*AOX1*-BFP-SKL::Hygromycin^R *his4 arg4*	This study
STN57	SMY459 pr*AOX1*-BFP-SKL::Hygromycin^R *his4 arg4*	This study
STN59	SMY460 pr*AOX1*-BFP-SKL::Hygromycin^R *his4 arg4*	This study
STN66	R12 GFP-Atg8::HIS4 *his4 arg4*	This study
STN95	SJCF936 Δ*rppa09976*::Geneticin^R *his4 arg4*	This study
STN96	PPY12h Δ*rppa09976*::Geneticin^R *his4 arg4*	This study
STN126	SJCF936 pr*ATG30*-Atg30-GFP::HIS4 *his4 arg4*	This study
STN128	STN96 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN130	SJCF936 pr*GAPDH*-Atg30-GFP::HIS4 *his4 arg4*	This study
STN132	STN96 pr*GAPDH*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN133	STN96 pr*GAPDH*-6xHA-MmAcbd5::HIS4 *his4 arg4*	This study
STN135	STN96 pr*GAPDH*-6xHA-Rppa09976::HIS4 *his4 arg4*	This study
STN138	STN96 pr*GAPDH*-GFP-MmAcbd5::HIS4 *his4 arg4*	This study
STN139	STN96 pr*GAPDH*-GFP-Rppa09976::HIS4 *his4 arg4*	This study
STN146	STN96 pr*GAPDH*-MmAcbd5::HIS4 *his4 arg4*	This study
STN148	STN95 pr*GAPDH*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN149	STN95 pr*GAPDH*-Atg30-GFP::HIS4 *his4 arg4*	This study
STN151	STN128 pr*GAPDH*-BFP-SKL::ARG4	This study
STN153	STN132 pr*GAPDH*BFP-SKL::ARG4	This study
STN155	STN95 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN156	STN151 pr*GAPDH*-Sec61-mCherry::Hygromycin^R	This study
STN158	STN153 pr*GAPDH*-Sec61-mCherry::Hygromycin^R	This study
STN161	STN128 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study
STN163	STN155 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study
STN164	STN96 pr*RPPA09976*-ΔACBP-GFP::HIS4 *his4 arg4*	This study
STN166	STN164 pr*GAPDH*-BFP-SKL::ARG4	This study
STN168	PPY12h pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN170	SJCF936 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN172	SJCF925 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN174	SMD1163 pr*RPPA09976*-Rppa09976-GFP::HIS4 *his4 arg4*	This study
STN176	STN166 pr*GAPDH*-Sec61-mCherry::Hygromycin^R	This study
STN195	STN96 pr*RPPA09976*-ACBP-GFP::HIS4 *his4 arg4*	This study

Table 2-1: (continued)

Strain Name	Genotype	References
STN197	STN96 pr*RPPA09976*-ACBP-GFP-Pex11::HIS4 *his4 arg4*	This study
STN199	STN126 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study
STN201	STN95 pr*ATG30*-Atg30-GFP::HIS4 *his4 arg4*	This study
STN203	STN201 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study
STN205	STN138 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study
STN208	STN138 pr*AOX1*-BFP-SKL::Hygromycin^R *arg4*	This study

Results

Identification of Rppa09976

An unknown ORF, designated *RPPA09976* hereafter, was identified using mass spectrometric analysis following a two step purification of the TAP-tagged peroxin, Pex3. *RPPA09976* encodes a 44 kDa protein containing an N-terminal acyl-CoA binding domain (ACBP) and two transmembrane domains.

Rppa09976 is not involved in peroxisome biogenesis

Pex3 functions early in peroxisome biogenesis, incorporating various peroxisomal membrane proteins into the peroxisomal membrane. Due to its interaction with Pex3, Rppa09976 may also play a role in peroxisome biogenesis. To test this, wild-type (PPY12h), Δ*rppa09976*, and Δ*pex3* strains were grown in either 0.5% methanol medium or 0.5% oleate medium to induce peroxisome biogenesis. Most *PEX* mutants are deficient in peroxisome biogenesis and show reduced growth rates. Thus, following growth curves is an indirect way of determining whether or not proteins are involved in peroxisome biogenesis. Growth rates between Δ*rppa09976* cells and the wild-type strain were comparable, suggesting that Rppa09976 does not play a role in peroxisome biogenesis (Figure 2-1).

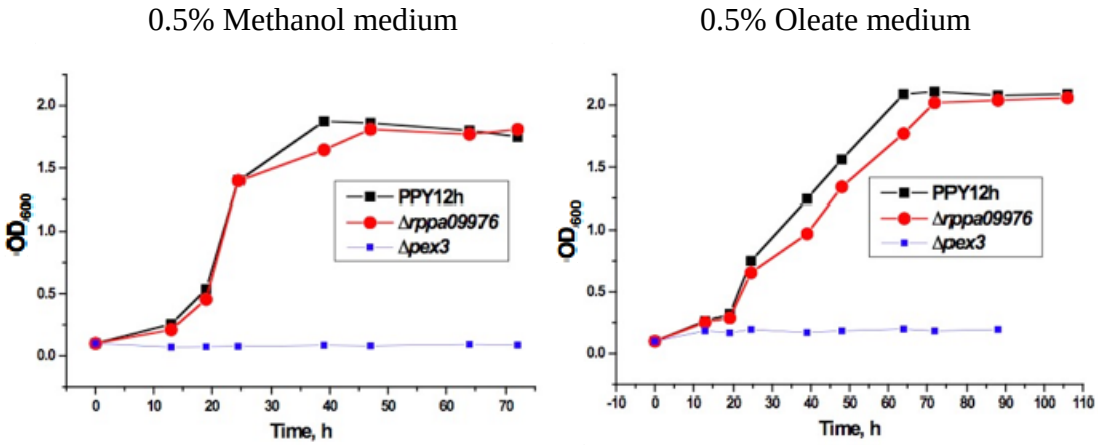

Figure 2-1: Rppa09976 is not required for peroxisome biogenesis of methanol or oleate-induced cells. The left panel shows cell growth in methanol medium while the right panel shows growth in oleate medium. Strains were grown in YPD medium and transferred to methanol or oleate medium after two washes in YNB at 0.1 OD/ml.

Rppa09976 is required for pexophagy

Since peroxisome biogenesis is unaffected in the absence of Rppa09976, we considered the possibility that Rppa09976 plays a role in the selective degradation of peroxisomes. An AOX plate assay was used to verify whether Rppa09976 served a function in pexophagy. The presence of a brown color in the AOX plate assay indicates the presence of alcohol oxidase, and consequently, peroxisomes. Despite the induction of macropexophagy, Δ*rppa09976* cells contained comparable levels of AOX as Δ*atg30* cells, indicating that Rppa09976 is indeed required for macropexophagy (Figure 2-2).

The inability of Δ*rppa09976* cells to efficiently degrade peroxisomes was further verified by following the disappearance of AOX in a 24-h time course that now monitored micropexopagy. In wild-type cells, but not in the Δ*atg1* (deficient in all autophagy-related processes) or Δ*atg30* (deficient only in pexophagy) strains, a significant loss of AOX was seen after 6 h. Δ*rppa09976* cells, however, showed an intermediate phenotype (Figure 2-3). AOX levels decreased only after a significant delay of 12 h, implying Rppa09976 affects the kinetics of micropexophagy. Both the AOX plate assay and biochemical assays suggest that Rppa09976 is involved in the selective degradation of peroxisomes by macropexophagy and micropexophagy.

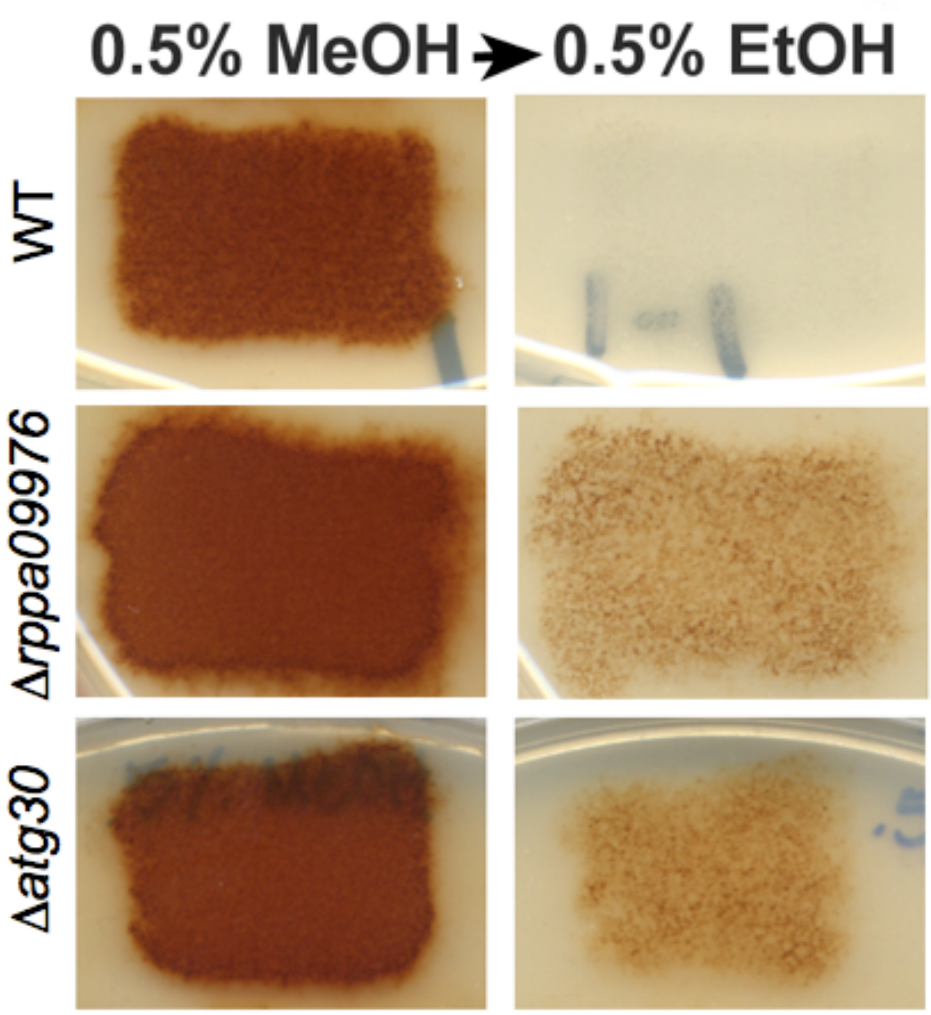

Figure 2-2: Rppa09976 is required for macropexophagy. Visualization of alcohol oxidase activity after ethanol adaptation for 18 h (macropexophagy) in wild-type, Δ*atg30*, and Δ*rppa09976* cells. Staining on methanol plates serves as an internal control to verify the induction of peroxisome biogenesis and the intensity should not be compared to the staining intensity on ethanol plates.

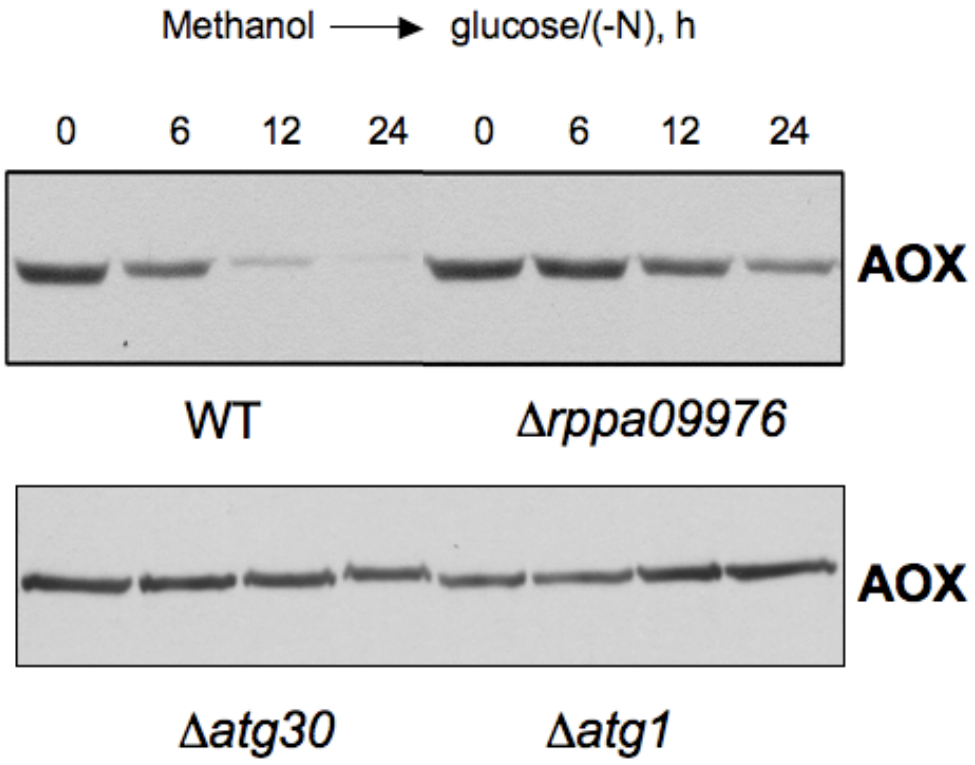

Figure 2-3: Rppa09976 is required for micropexophagy. Cells were grown in methanol medium overnight and shifted to glucose medium. 1 ml of cells were lysed and analyzed. Alcohol oxidase (AOX), detected by immunoblotting, was used to follow peroxisome degradation.

Autophagy and the Cvt pathways are unaffected in the absence of Rppa09976

Because Rppa09976 plays a role in pexophagy, it could play a role in the autophagy and Cvt pathways, as well. Atg8, a ubiquitin-like protein required for all autophagy-related pathways is also degraded in the vacuole by resident proteases (Shintani and Klionsky, 2004). The GFP-Atg8 processing assay is a useful technique to monitor autophagy in which the disappearance of GFP-Atg8 corresponds to the appearance of free, protease-resistant GFP in the vacuole (Shintani and Klionsky, 2004). Similar to the wild-type, upon nitrogen starvation, Δ*rppa09976* cells processed GFP-Atg8, but the autophagy-deficient control strain, Δ*atg1,* did not (Figure 2-4), suggesting that Rppa09976 is not required for autophagy.

Only recently has a Cvt pathway been found in *P. pastoris* (Farre et al., 2007). As in *S. cerevisiae*, the Cvt pathway can be monitored by following Ape1 processing to its mature form. Since the Cvt pathway is a constitutive process, under nutrient rich conditions at any given time, about half of the Ape1 is in its mature form while the other half is in its precursor form. Ape1 processing in Δ*rppa09976* cells was comparable to the wild-type, while the Δ*atg8* strain, deficient in all autophagy-related pathways, could not process prApe1 into its mature form (Figure 2-5). Rppa09976 therefore is only required for pexophagy, but not for the Cvt or autophagy pathways.

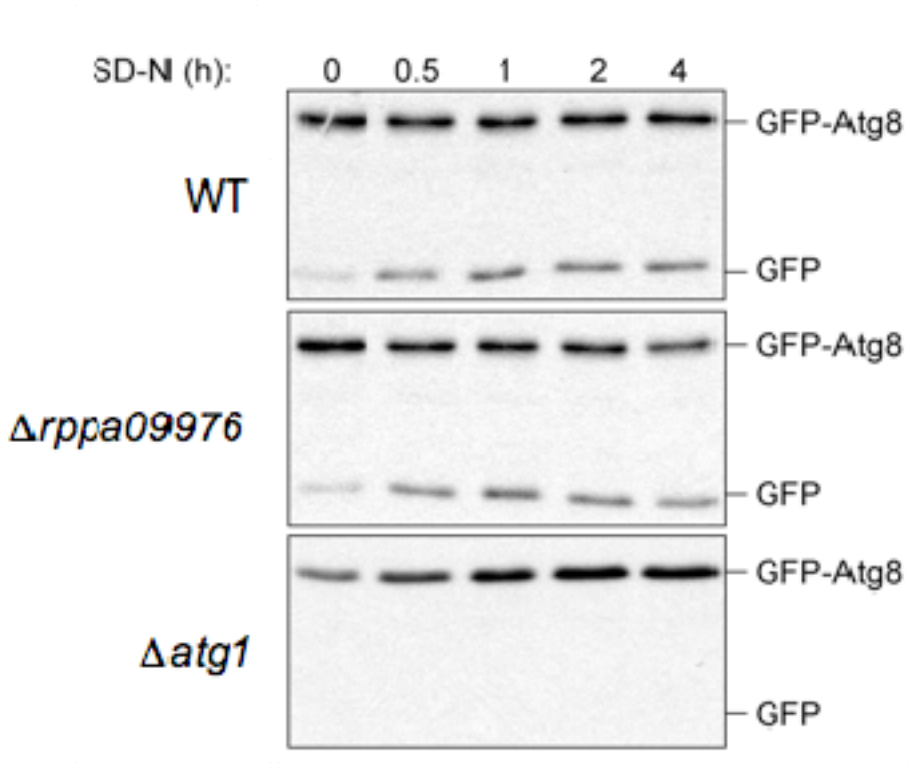

Figure 2-4: Rppa09976 is not essential for autophagy. Wild-type, Δ*rpp09976* and Δ*atg1* cells expressing GFP-Atg8 were grown in glucose medium and shifted to nitrogen starvation conditions for 4 h to induce autophagy. Protein extracts were immunoblotted with anti-GFP antibody.

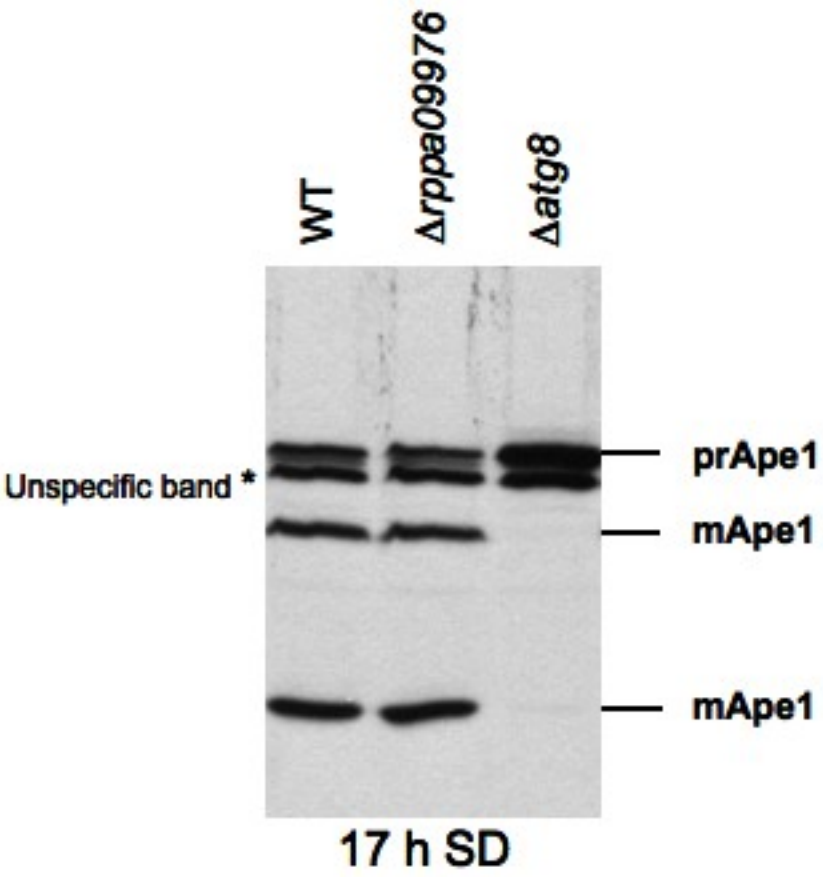

Figure 2-5: Rppa09976 is not required for the Cvt pathway. Cells were grown in glucose medium (SD) for 17 h. Both precursor (pr) and mature (m) forms of Ape-1 are shown. * indicates bands unspecific to Ape1.

Discussion

The identification of Rppa09976 through its interaction with Pex3 suggested that Rppa09976 might be required for peroxisome biogenesis. This hypothesis was negated by the normal growth of Δ*rppa09976* cells in methanol- or oleate-containing media. Despite its lack of requirement in peroxisome biogenesis, we tested the hypothesis that Rppa09976 functions in pexophagy. The AOX plate assay and biochemical assays showed that Rppa09976 is in fact involved in the selective degradation of peroxisomes, specifically affecting the kinetics of pexophagy. Since most genes required for pexophagy are also involved in autophagy-related pathways, it was important to test the involvement of Rppa09976 in autophagy and the Cvt pathways. Our findings show that Rppa09976 is specifically required for pexophagy, but not other autophagy-related pathway.

Researchers are just starting to understand how the nonselective autophagic machinery is recruited for the selective degradation of peroxisomes. The Δ*rppa09976* mutant shows a strong delay in pexophagy, but completely normal Cvt and autophagy pathways, making it one of three proteins, together with Atg26 and Atg30, known to be specifically required for pexophagy.

It is interesting that two (Atg30 and Rppa09976) of three proteins selectively required for pexophagy, interact with the heart of the peroxisome biogenesis machinery, Pex3 (Farre et al., 2008). This peroxin is essential for the assembly of peroxisome membrane proteins, and also indirectly for the import of peroxisomal matrix proteins via its interactions with components of the importomer (Hazra et al., 2002). It will be

interesting to explore the mechanistic significance of this interaction because it seems plausible that a decision to target peroxisomes for degradaton might somehow be coupled with the inhibition of peroxisome biogenesis.

Chapter 2 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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Chapter 3: Rppa09976 and its mammalian homolog, MmAcbd5, localize to the peroxisomal membrane in *P. pastoris*

Abstract

The interaction of Rppa09976 with the peroxisomal membrane protein (PMP) Pex3 and its requirement for pexophagy (but not other autophagy-related pathways) suggested that Rppa09976 might localize to the peroxisomal membrane. Additionally, to test whether the N-terminal acyl-CoA binding protein (ACBP) domain is required for proper localization and pexophagy, we constructed different truncated versions of Rppa09976. These included the full-length protein, both the ACBP domain and a deletion lacking the ACBP domain, and an ACBP-GFP-Pex11 fusion. The full-length Rppa09976, the ACBP-deleted version (Rppa09976(ΔACBP)-GFP) and ACBP-GFP-Pex11 localized to the peroxisomal membrane. However, just the ACBP domain of Rppa09976 mislocalized to the cytosol. Furthermore, only the full-length Rppa09976-GFP fusion protein was able to complement Δ*rppa09976*, suggesting that the entire protein is required for proper function. In addition, we found a putative mouse homolog, MmAcbd5, 473 amino acids in length, that also contains an N-terminal ACBP domain. Although unable to restore pexophagy in the Δ*rppa09976* mutant, GFP-MmAcbd5 was still able to localize to the *P. pastoris* peroxisomal membrane, in a similar fashion as GFP-Rppa09976.

Introduction

The approximately 10 kDa acyl-CoA binding protein (ACBP) is highly conserved across eukaryotes (Burton et al., 2005). ACBP was first discovered in mammals as a neuropeptide, inhibiting diazepam binding to the GABA (γ-aminobutyric

acid) receptor (Guidotti et al., 1983). Further studies indicate that ACBP preferentially binds to long-chain acyl-CoA esters (C_{12}-C_{22}) in a one-to-one ratio. Long-chain acyl-CoA esters are amphipathic molecules known to play a role in the budding or fusion of membranes, the synthesis and remodeling of lipids, and are substrates for β-oxidation and protein acylation reactions. It is thought that the majority of long-chain acyl-CoA esters are sequestered by ACBP in vivo. The ability of ACBP to bind to long chain acyl-CoA esters protects them from potent cytosolic acyl-CoA hydrolases, allowing the maintenance of a long-chain acyl-CoA pool available for specific purposes (Kragelund et al., 1999). Furthermore, the ability of ACBP to mediate intermembrane acyl-CoA transport in vitro suggests its involvement in intracellular acyl-CoA trafficking (Rasmussen et al., 1994).

Like the mammalian ACBP, the *Saccharomyces cerevisiae* homologue, Acb1p, is a cytosolic 86 amino acid protein that binds to long-chain acyl-CoAs. Acb1p is the only known protein in *S. cerevisiae* to contain an ACBP domain. In addition, while the absence of Acb1p is not lethal, defects include a significant reduction in growth rate, membrane perturbations, vacuolar fragmentation and decreased ceramide synthesis.

Recently a number of larger proteins with an acyl-CoA binding domain were found in higher eukaryotes including *Arabidopsis thaliana* (Chye, 1998), *Caenorhabditis elegans* (Larsen et al., 2006), and *Cryptosporidium parvum* (Zeng et al., 2006). In *A. thaliana*, six genes encoding acyl-Coa binding proteins (ACBPs) were identified (Xiao and Chye, 2009). Each of the six proteins were a different size and showed varying affinities for acyl-CoA esters, implying that each may have a different role in plant lipid metabolism. Of the six proteins, 2 are membrane-associated, one is

extracellularly targeted, and the other 3 are localized to the cytosol. Furthermore, in *C. elegans* a membrane-associated ACBP-containing protein, Maa-1, was identified and shown to modulate vesicle trafficking via the recruitment of long-chain acyl-CoAs to membranous organelles in the endosomal and Golgi systems, promoting vesicle biogenesis and fusion (Larsen et al., 2006).

Likewise, mass spectrometry studies of rat liver and mouse kidney peroxisomes also revealed the identification of novel proteins containing an N-terminal ACBP-domain that localized to peroxisomes (Kikuchi et al., 2004), (Wiese et al., 2007). Using immunocytochemistry experiments, researchers showed that the mouse ACBP-containing protein, MmAcbd5, localized to peroxisomes (Wiese et al., 2007).

Similar to the aforementioned proteins, the 409-residue *Pichia pastoris* Rppa09976 protein also contains an N-terminal ACBP domain. Previously, we demonstrated that Rppa09976 is required for pexophagy but is dispensable for the autophagy and Cvt pathways. Identifying the localization of Rppa09976 and requirement of its ACBP domain may provide further insights as to how peroxisomes are selectively sequestered and degraded.

Materials and Methods:

Yeast strains, plasmids and transformation

The *P. pastoris* strains and plasmids used in this study are listed in Table 2-1. Transformation methods are described in detail in Chapter 2.

Complementation studies of pexophagy

Cells were pre-grown in two pre-cultures of YPD. Cells were washed once with YNB solution and transferred to 25 ml of 0.5% methanol medium with a starting OD_{600} equal to 0.3/ml for 15 h to induce peroxisome biogenesis. After 15 h, cells were washed twice with YNB solution and transferred to 25 ml of 0.5% ethanol medium for 0, 6, 9, 12, and 15 h. At 12 h, 125 µl of ethanol was added to each flask. Cells (1 ml) were collected and pelleted for each strain at each timepoint. Crude extracts were prepared in the presence of TCA (Baerends et al., 2000). SDS-PAGE and immunoblotting was performed as described previously (Kyhse-Andersen, 1984), (Laemmli, 1970). Antigen-antibody complexes were detected by enhanced chemiluminescence.

Fluorescence Microscopy

Cells were grown in two precultures of YPD, washed twice with YNB and transferred to methanol medium to an OD_{600} of 0.3 (for PPY12h cells) or 1.0 (for PPY12m cells, which grow more slowly than PPY12h cells for unknown reasons) for 15 h. To monitor localization by fluorescence microscopy, 100 µl of methanol-induced cells were collected and kept on ice before observation. Optimal exposition time for both the GFP and BFP fluorophores were taken for each picture. Images were captured using a

motorized fluorescence microscope (Axioskop 2 MOT; Carl Zeiss MicroImaging, Jena, Germany), coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Alcohol Oxidase plate assay for pexophagy

Cells were pre-grown on YPD for two days. YPD plates were then replica plated onto a 0.5% methanol plate (1.7 g/l YNB without amino acids and ammonium sulfate, 0.5% methanol, 0.5% w/v ammonium sulfate, 50 mg/l arginine, 50 mg/l histidine, and 2% w/v agar) plate for 48 h. These strains were then replica-plated onto SE plates (1.7 g/l YNB without amino acids and ammonium sulfate, 0.5% ethanol, 0.5% w/v ammonium sulfate, 50 mg/l arginine, 50 mg/l histidine, and 2% w/v agar for 15 h in order to induce macropexophagy.

For a 100 ml of AOX plate assay solution, 50 mM Tris-HCl buffer (pH 8.0) was added to 100 mg of digitonin, 50 mg *o*-dianisidine and 300 mg of low melt agarose. This solution was brought to a boil and then 50 mg of 2,2'-azinodi[3-ethylbenzthiazoline-6-sulfonic acid], or ABTS was added. When the solution became warm to the touch, 6 mg of peroxidase was added, along with 1 ml of methanol. The 10-12 ml of solution was poured onto plates and left at room temperature for approximately 30 min and then placed into the 30° C incubator for approximately 2 h (Nazarko et al., 2002).

Results

Both the ACBP and C-terminal part of Rppa09976 protein are required for pexophagy

Structural analysis of Rppa09976 revealed the presence of an N-terminal ACBP domain (Figure 3-1). Only a handful of membrane-associated proteins containing an N-terminal ACBP domain have been identified. Of these proteins, the *M. musculus* Acbd5 and *C. elegans* Maa-1 proteins seem most similar to Rppa09976. BLAST analysis between the three proteins indicated the conservation of the N-terminal ACBP domain (Figure 3-1). Studies indicate a role of membrane-associated ACBP-containing proteins in the recruitment of acyl-CoA esters to specific subcellular compartments within a cell. To determine if the ACBP domain of Rppa09976 is required for pexophagy, we deleted the ACBP domain of Rppa09976, which like the full-length protein, localizes to the peroxisomal membrane (Figure 3-3). We also deleted the C-terminal end of the protein by fusing the ACBP domain to GFP, which was found to mislocalize to the cytoplasm (Figure 3-3). To determine if the ACBP region functions in pexophagy, we brought the ACBP domain back to the peroxisomal membrane using an ACBP-GFP-Pex11 fusion. An AOX plate assay was used to verify which region of Rppa09976 is for pexophagy. The presence of a green color indicates the presence of AOX, and consequently, peroxisomes. Despite the induction of both macro- and micropexophagy, only Δ*rppa09976* cells complemented with the full length Rppa09976-GFP protein were able to exhibit pexophagy (Figure 3-2). Neither the C-terminal region alone nor the ACBP domain was able to restore pexophagy, suggesting that both parts of the Rppa09976 protein are required for pexophagy.

PS51228 **ACB_2** *Acyl-CoA-binding (ACB) domain profile* :

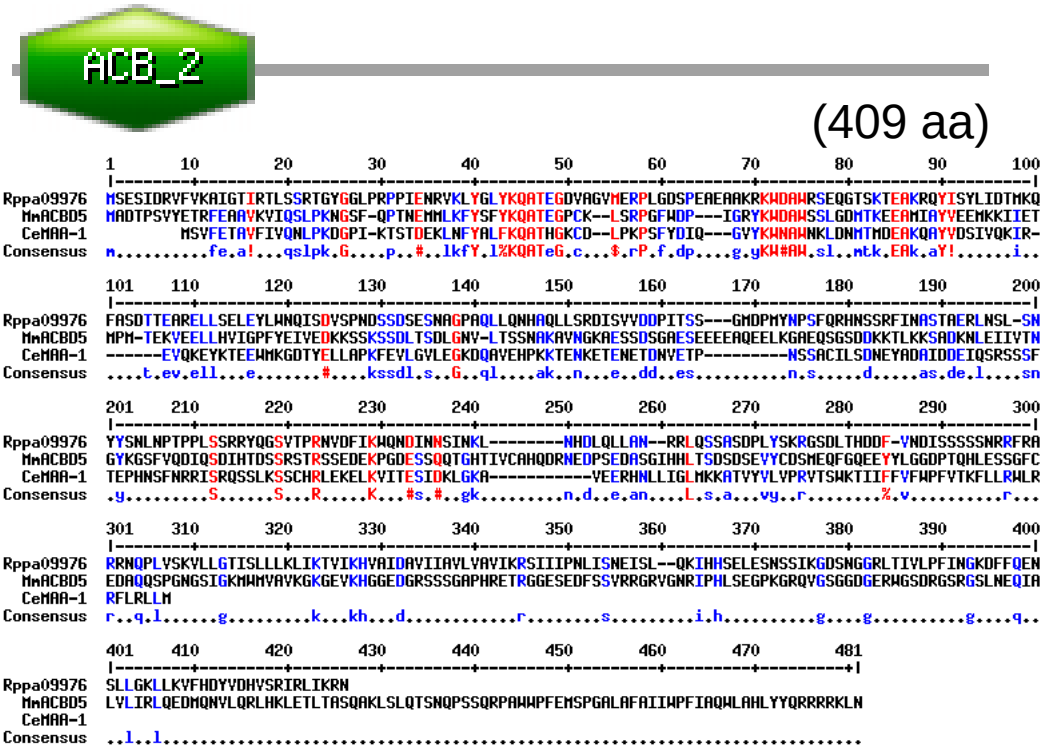

Figure 3-1: Rppa09976 contains an N-terminal ACBP domain, similar to MmAcbd5 and CeMaa-1. The 409-residue Rppa09976 contains an N-terminal ACBP domain, also found in the mouse peroxisomal Acbd5 protein and *C. elegans* membrane-associated Maa-1 protein involved in endosomal vesicle trafficking.

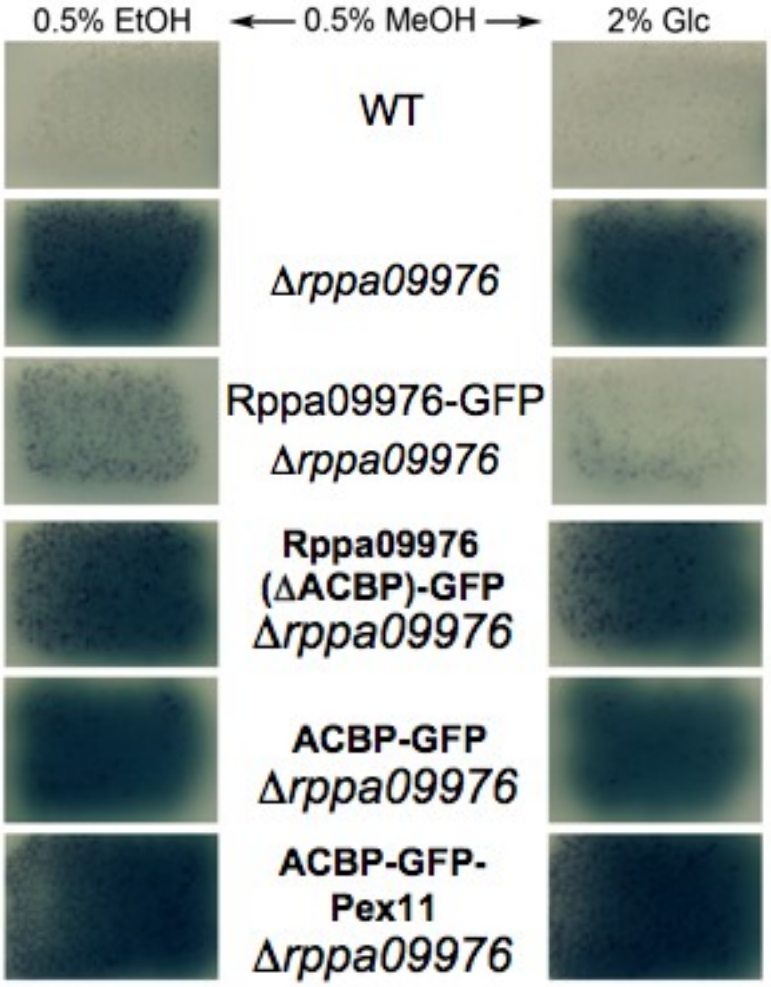

Figure 3-2: Complementation studies of different truncations of Rppa09976 using the AOX plate assay. Visualization of AOX activity after both ethanol and glucose adaptation (to induce macro- and micropexophagy, respectively) for 18 h in the strains listed above are shown.

Rppa09976 localization to the peroxisomal membrane is not dependent on its ACBP domain

Rppa09976 association with the PMP Pex3 and its role in pexophagy, but not other autophagy-related pathways suggest that Rppa09976 localizes to peroxisomes. Furthermore, to determine if Rppa09976 was a membrane or matrix protein, we used the SOSUI algorithm (Hirokaya et al., 1998), which predicted two putative transmembrane domains from residues 290-312 and 317-339 (Figure 3-3). Unlike the targeting of matrix proteins, PMPs are recognized by the peroxisomal membrane protein receptor, Pex19 (Rottensteiner et al., 2004). We used the BLOCKS Pex19BS algorithm to look for possible Pex19 binding sites. BLOCKS predicted two putative sites between residues 297-306 and 318-327, both of which are well within to the two predicted transmembrane domains (Figure 3-3). To visualize Rppa09976 localization, we used fluorescence microscopy to follow the localization of the full-length and truncated versions of Rppa09976 (Figure 3-4). Both the full length Rppa09976-GFP and Rppa09976(ΔACBP)-GFP proteins colocalized with BFP fused to the peroxisomal targeting signal, SKL. Furthermore, Rppa09976 localized to portions of the peroxisomal membrane in a cluster of peroxisomes. Since the absence of the ACBP domain did not affect its localization, this domain is not required in proper Rppa09976 localization. Similarly, since the localization of ACBP-GFP was cytosolic, the C-terminal portion of Rppa09976 must be essential for proper localization to the peroxisomal membrane. Interestingly, bringing the ACBP domain back to the peroxisomal membrane via an ACBP-GFP-Pex11 fusion did not restore pexophagy in Δ*rppa09976* cells, suggesting that the entire protein is required for its role in pexophagy.

SOSUI Result

Query title : Rppa

Total length : 409 A. A.

Average of hydrophobicity : -0.457457

This amino acid sequence is of a MEMBRANE PROTEIN which have 2 transmembrane helices.

No.	N terminal	transmembrane region	C terminal	type	length
1	290	PLVSKVLLGTISLLLKLIKTVIK	312	PRIMARY	23
2	317	DAVIIAVLVAVIKRSIIIPNLIS	339	PRIMARY	23

```
================================================================================
Here are your search results from the BLOCKS searcher.
================================================================================

Query=Unknown Unknown
Size=409 Amino Acids
Blocks Searched=1
Alignments Done=              418
Cutoff combined expected value for hits= 10
Cutoff block expected value for repeats/other= 10
================================================================================
                                                              Combined
Family                                              Strand  Blocks   E-value
x12847xbl  ../tmp/Pex19.blin                            1   1 of 1       0.1

================================================================================
>x12847xbl 1/1 blocks Combined E-value=     0.1: ../tmp/Pex19.blin
Block    Frame    Location (aa)      Block E-value
x12847xblA  0        297-306               0.1
Other reported alignments:
x12847xblA  0        318-327              0.27
```

Figure 3-3: Bioinformatics analyses of Rppa09976 reveal 2 putative transmembrane domains and 2 putative Pex19 binding sites. The SOSUI algorithm revealed two putative transmembrane domains in Rppa09976 between residues 290-312 and 317-339 (top). The BLOCKS algorithm revealed two putative Pex19 binding sites in Rppa09976 between residues 297-306 and 318-327, both of which are within the two predicted transmembrane domains.

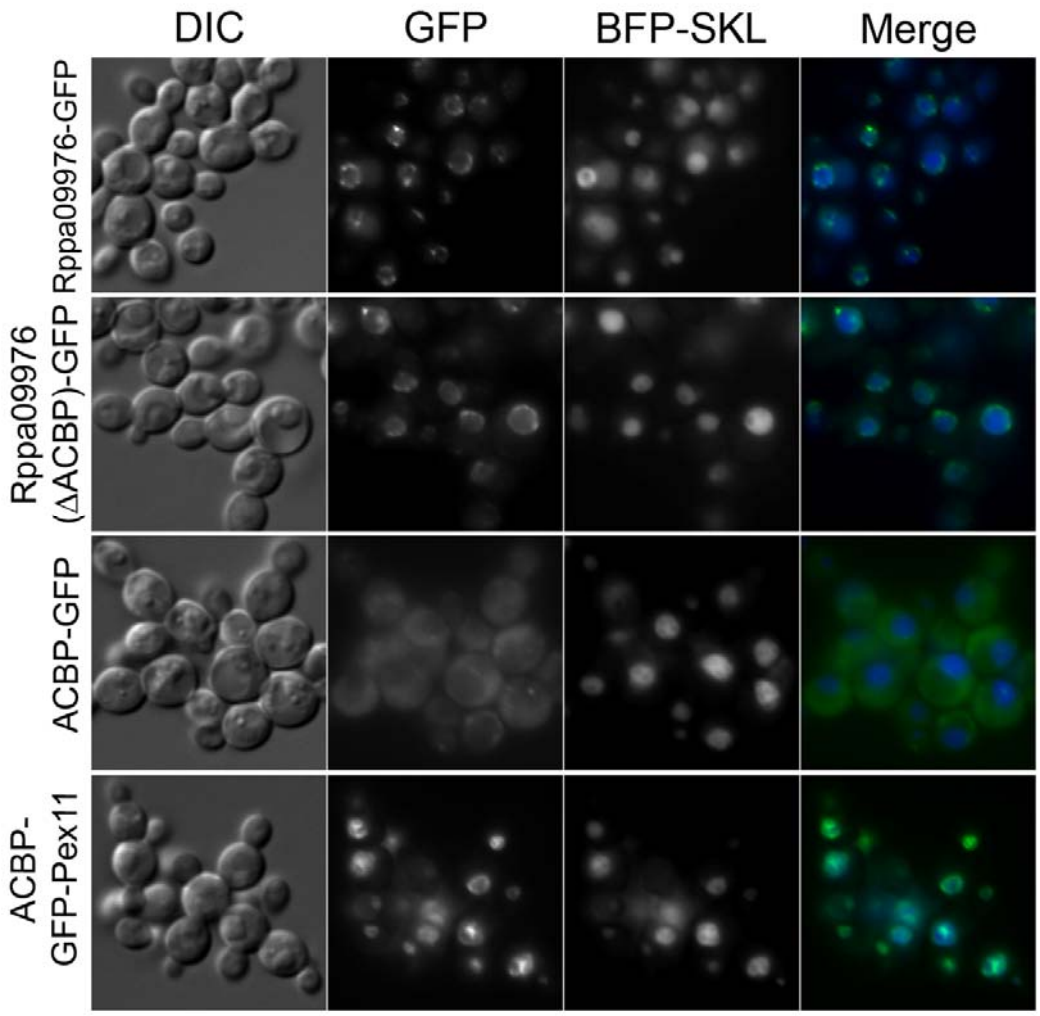

Figure 3-4: Localization of truncated versions of Rppa09976 in methanol adaptation. Δ*rppa09976* cells were complemented with Rppa09976-GFP, Rppa09976(ΔACBP)-GFP, ACBP-GFP, and ACBP-GFP-Pex11 and transformed with BFP-SKL. Cells were grown in 0.5% methanol medium for 15 h.

MmAcbd5 localizes to the peroxisomal membrane in P. pastoris

Proteomic studies of mouse kidney peroxisomes revealed a novel 473-residue peroxisomal protein containing an N-terminal ACBP domain, called MmAcbd5, similar to the 402-residue PMP Rppa09976. Unlike Rppa09976, the SOSUI program did not predict any transmembrane domains for MmAcbd5, however the BLOCKS Pex19BS algorithm predicted one putative Pex19 binding site between amino acids 388-397. Thus, it may still be possible that MmAcbd5 localizes to the peroxisomal membrane in *P. pastoris*. To test MmAcbd5 localization, we compared the localization of 15 h methanol-induced cells constitutively overexpressing either GFP-MmAcbd5 or GFP-Rppa09976 fusion proteins under the *GAPDH* promoter. In cells overexpressing GFP-Rppa09976, colocalization with BFP-SKL showed that the majority of GFP-Rppa09976 localized to the peroxisomal membrane (Figure 3-5). However, after 15 h of methanol adaptation, GFP-Rppa09976 was also found on structures resembling the nuclear envelope and along the cell periphery, presumably the endoplasmic reticulum (ER), suggesting that Rppa09976 may traffic to peroxisomes through the ER. Similar to GFP-Rppa09976 localization, in cells overexpressing the mouse protein, GFP-MmAcbd5 localized to the peroxisomal membrane. In addition, some GFP-MmAcbd5 also localized to punctate structures along the cell periphery, however no localization to structures resembling the nuclear envelope was found, indicating that MmAcbd5 may use a different trafficking mechanism to peroxisomes.

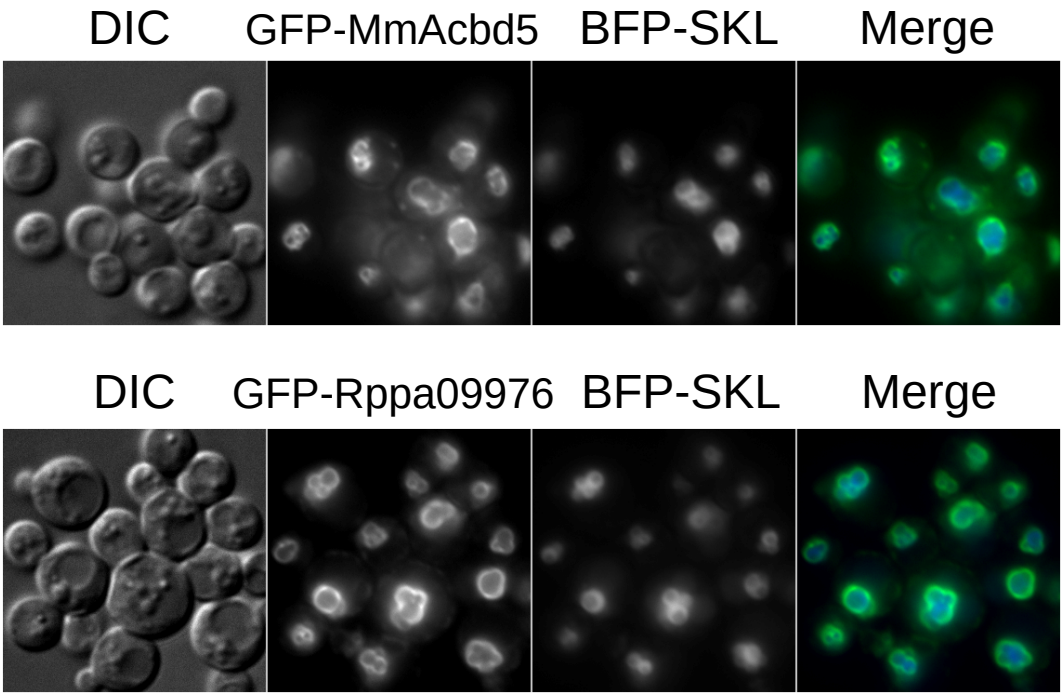

Figure 3-5: Localization of MmAcbd5 under methanol adaptation. Δ*rppa09976* cells complemented with GFP-MmAcbd5 or GFP-Rppa09976 and expressing BFP-SKL were grown in 0.5% methanol medium for 15 h.

MmAcbd5 does not restore pexophagy in the Δrppa09976 mutant

Since the peroxisomal localization of MmAcbd5 is preserved in *P. pastoris* cells, its function of MmAcbd5 may be conserved, as well. To test this, we followed the levels of AOX and Pex12 during ethanol adaptation of methanol-grown cells to quantitatively measure any restoration of macropexophagy. Within 6 h, levels of both AOX and Pex12 were significantly reduced in the wild-type and Δ*rppa09976* cells complemented with the full-length GFP-Rppa09976 (Figure 3-6). By 9 h, most of the AOX and Pex12 had degraded, indicating that the majority of peroxisomes had degraded as well. It is interesting to note that whereas levels of AOX and Pex12 remain constant in Δ*atg30* cells, in Δ*rppa09976* cells, AOX and Pex12 levels remain relatively constant until 12 h. Only after 12 h did levels drop and peroxisome degradation occur, suggesting that Rppa09976 might affect the kinetics of peroxisome degradation. The Δ*rppa09976* cells complemented with GFP-MmAcbd5 showed similar degradation kinetics as Δ*rppa09976* cells. However, unlike Rppa09976, there was no modification on GFP-MmAcbd5, and perhaps an unmodified version renders the protein unable to function in pexophagy. Therefore, while MmAcbd5 peroxisomal localization is conserved in *P. pastoris*, MmAcbd5 may be too divergent to be functionally homologous.

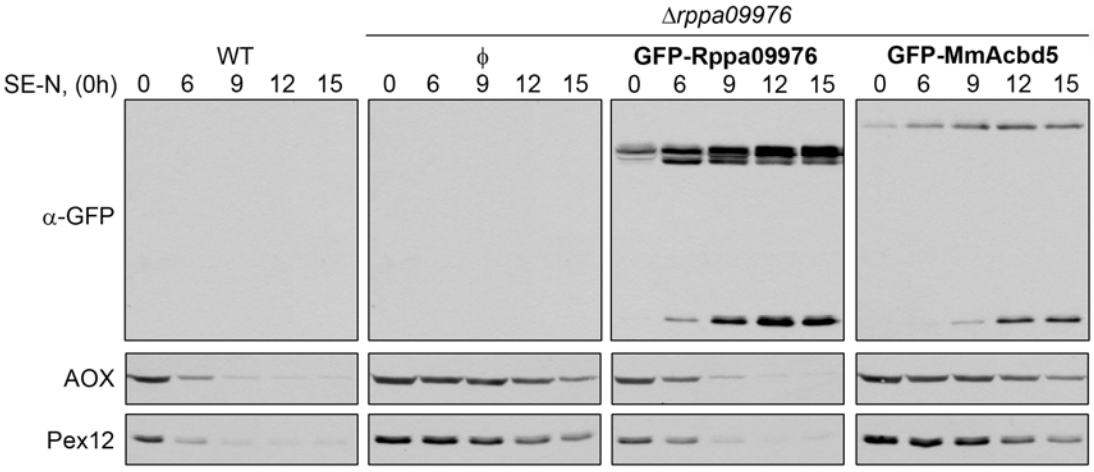

Figure 3-6: MmAcbd5 does not complement the pexophagy defect of Δ*rppa09976*. 15 h methanol-induced cells were transferred to 0.5% ethanol medium for 15 h to induce macropexophagy in wild-type (PPY12h), Δ*rppa09976* and Δ*rppa09976* cells complemented with P_{GAPDH}-GFP-MmAcbd5 and P_{GAPDH}-GFP-Rppa09976 cells. AOX and Pex12 were used as peroxisomal matrix and membrane protein markers to measure the extent of pexophagy.

Discussion

Rppa09976 interaction with the PMP Pex3, its two putative transmembrane domains and its putative Pex19-binding sites makes it very likely that Rppa09976 localizes to the peroxisomal membrane. Colocalization of Rppa09976-GFP with BFP-SKL verified Rppa09976 localization to the peroxisomal membrane and revealed that while just the C-terminal part of Rppa09976 is required for proper localization, both the ACBP and the C-terminal portions of Rppa09976 are required for function in pexophagy.

Recently, studies revealed a novel 473-residue mouse peroxisomal protein with an N-terminal ACBP domain, MmAcbd5. While GFP-MmAcbd5 failed to complement the pexophagy defect of Δ*rppa09976* cells, its localization remained conserved. Interestingly, constitutive overexpression of GFP-Rppa09976 also revealed localization to the cell periphery and nuclear envelope-like structures, suggesting that unlike most PMPs, which are directly inserted into the peroxisomal membrane post-translationally, Rppa09976 might traffic to the peroxisomal membrane through the endoplasmic reticulum.

Together with Atg30, Rppa09976 is the only other peroxisomal protein dispensable for peroxisome biogenesis, yet required for pexophagy but not other autophagy-related pathways. However, unlike Atg30, which is essential for peroxisome degradation, Rppa09976 affects the kinetics of peroxisome degradation. The presence of low pexophagy activity in the Δ*rppa09976* mutant could be explained by the presence of the cytosolic 10-kDa form of ACBP, Rppa08070. Rppa09976 may serve to function in the recruitment of long-chain acyl-CoAs the peroxisomal membrane. Upon

pexophagy induction, Rppa09976 may then donate these acyl-CoAs for pexophagosome and MIPA formation during macro- and micropexophagy, respectively. In the absence of Rppa09976, however, Rppa08070, present at all times throughout the cytosol, may eventually provide enough acyl-CoAs necessary for limited rounds of membrane formation during pexophagy. Creation of a double deletion may help resolve this matter, assuming it is viable.

Chapter 3 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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Chapter 4: Atg30 affects Rppa09976 localization

Abstract

Recently, the peroxisomal peripheral membrane protein, Atg30, was discovered as the peroxisomal receptor protein responsible for tagging peroxisomes for their degradation. The overexpression of Atg30 induces pexophagy and subsequently inhibits cell growth under peroxisome biogenesis conditions in methanol medium. Since Atg30 and Rppa09976 are involved specifically in pexophagy, we wanted to test if Rppa09976 behaves like Atg30. Unlike Atg30, constitutively overexpressing Rppa09976 did not induce pexophagy nor did it cause a substantial growth defect. However, the overexpression of Atg30 in Δ*atg30* Δ*rppa09976* cells partially rescued peroxin levels and the growth defect in methanol medium, in comparison to Δ*atg30* cells overexpressing Atg30. Furthermore, we tested the effect of Rppa09976 localization in the presence or absence of Atg30. We found that without Atg30, Rppa09976 accumulates to the middle of the peroxisome cluster. Collectively, our results suggest that Atg30 might interact with Rppa09976 and influence its localization. Furthermore, we find that Rppa09976 acts downstream of Atg30 and is required for Atg30-induced pexophagy.

Introduction

Scientists are just beginning to understand the coupling process of peroxisomes and the autophagic machinery during pexophagy. Once pexophagy is induced, an unknown kinase hyperphosphorylates the peroxisomal membrane, Atg30, which then interacts with Atg11 and Atg17 to recruit the autophagy machinery to the peroxisomal

membrane. It has recently been found that constitutive overexpression of Atg30 induces pexophagy, even in peroxisome proliferation conditions (Farre et al., 2008). Cells constantly overexpressing Atg30 are unable to efficiently proliferate peroxisomes, and thus show pronounced growth defects (Farre et al., 2008).

Like Atg30, Rppa09976 is specifically required for both macro- and micropexophagy, but not other autophagy-related pathways. Furthermore, we previously demonstrated that Rppa09976 also localizes to the peroxisomal membrane. Similarly, both Atg30 and Rppa09976 interact with Pex3, a peroxin essential in both peroxisome biogenesis and macropexophagy in the methylotrophic yeast, *Hansenula polymorpha* (Bellu et al., 2002). Pex3 is not only required for the import of peroxisomal membrane proteins (PMPs), but Pex3 disappearance from the peroxisomal membrane prior to peroxisome sequestration events is also required for pexophagy (Bellu et al., 2002). Since Δ*atg30* and Δ*rppa09976* show similar phenotypes, we examined the effects of constitutively overexpressing Rppa09976. We also tested the effects of endogenous Rppa09976-GFP and Atg30-GFP localization in the presence or absence of Atg30 and Rppa09976, respectively.

Materials and Methods

Yeast strains, plasmids and transformations

The *P. pastoris* strains and plasmids used in this study are listed in Table 2-1. Transformation procedures are described in detail in Chapter 2.

Growth curve in methanol-containing media

Cells were pre-grown to the late exponential-stationary stage in YPD culture, diluted 25-50 fold with fresh YPD medium and pre-grown to the early-mid exponential phase in a second YPD culture. Cells were washed twice with YNB solution and inoculated into 0.5% methanol solution at a starting OD_{600} of 0.1/ml. Timepoints were taken at 0, 14, 18, 24, 36, 43, 48, and 62 h

Peroxisome-induction conditions

Cells were pre-grown to the late exponential-stationary phase in a YPD culture, diluted 25-50 fold with fresh YPD medium and pre-grown to the early-mid exponential phase in the second YPD culture. Cells were washed twice with YNB solution and inoculated into methanol medium at an OD_{600} of 1.0 for 6 and 12 h.

Biochemical studies of peroxisome biogenesis

Cells were pre-grown in two pre-cultures of YPD. Cells were washed twice with YNB solution and 1.0 OD_{600} cells were transferred to 0.5% methanol medium to induce peroxisomes biogenesis. A volume of cells equivalent to 1.0 OD_{600}/ml was collected by centrifugation after 6 and 12 h of methanol adaptation. Crude extracts were prepared in

the presence of TCA (Baerends et al., 2000). SDS-PAGE and immunoblotting were performed as described previously (Laemmli, 1970), (Kyhse-Andersen, 1984). Antigen-antibody complexes were detected by enhanced chemiluminescence.

Fluorescence Microscopy

To monitor the localization of endogenously expressed and overexpression Rppa09976 and Atg30 by fluorescence microscopy, 100 µl of 6 and 12 h methanol-induced cells were collected. Six pictures of each strain were taken at each timepoint. Optimal exposition times for the GFP fluorophore were used for each picture. Images were captured using a motorized fluorescence microscope (Axioskop 2 MOT; Carl Zeiss MicroImaging, Jena, Germany), coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

To monitor the localization of Rppa09976 in Δ*atg30* cells, 100 µl of 15 h methanol-induced cells were collected and put on ice. 800 ms and 12000 ms exposition times were used for cells expressing Rppa09976-GFP and Atg30-GFP, respectively. Z-stack images with 25 planes separated by a width of 0.255µm were taken. Images were captured and processed by the same equipment mentioned above.

Results

Rppa09976 overexpression does not inhibit cell growth in methanol medium

Overexpression of Atg30 induces pexophagy despite peroxisome proliferation conditions, severely affecting cell growth (Farre et al., 2008). Since the absence of Rppa09976 affects pexophagy, we considered the possibility that the overexpression of Rppa09976 might also induce pexophagy. We overexpressed Rppa09976 using the constitutive *GAPDH* promoter. Cells with Rppa09976 overexpression in the Δ*rppa09976* and Δ*atg30* Δ*rppa09976* backgrounds grew similarly to cells expressing endogenous levels of Rppa09976 (Figure 4-1). As expected, Atg30 overexpression substantially reduced cell growth compared to cells expressing Atg30 endogenously. Interestingly, even endogenous expression of Atg30 in Δ*atg30* cells caused slower growth than endogenous levels of Rpppa09976 in Δ*rppa09976* cells.

It was surprising to find, however, that growth defects in the Δ*atg30* cells overexpressing Atg30 were alleviated in the absence of Rppa09976. Initially, cells overexpressing Atg30 in the absence of Rppa09976 grew similarly to cells overexpressing Atg30 in the wild-type background, however, after 36 h, these cells continued to grow, reaching an OD_{600} similar to cells expressing endogenous levels of Atg30, suggesting that Rppa09976 plays a role in Atg30-induced pexophagy.

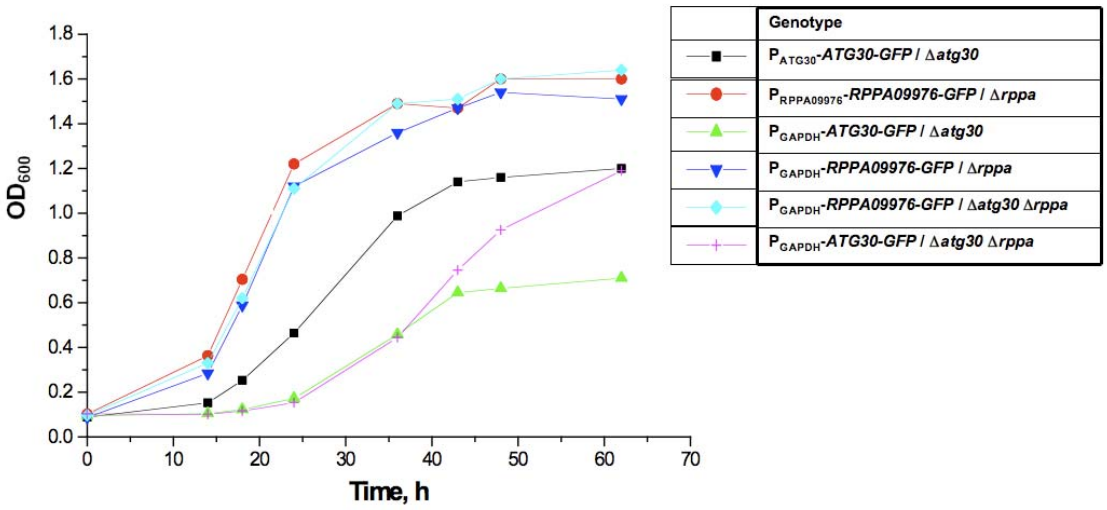

Figure 4-1: Overexpression of Rppa09976 does not inhibit cell growth in peroxisome proliferation conditions. Growth curves in methanol medium of endogenously expressed Rppa09976 in the Δ*rppa09976*, overexpressed Rppa09976 in the Δ*rppa09976* and Δ*atg30* Δ*rppa09976* backgrounds, endogenously expressed Atg30 in the Δ*atg30*, and overexpressed Atg30 in the Δ*atg30* and Δ*atg30* Δ*rppa09976* background.

Overexpression of Rppa09976 does not induce pexophagy in methanol medium

After 6 h of methanol adaptation, the levels of peroxins Pex3 and Pex17 are drastically lower in cells overexpressing Atg30 than in cells expressing endogenous levels of Atg30 due to Atg30-induced pexophagy (Farre et al., 2008). As expected, overexpression of Atg30 in the Δ*atg30* background showed significantly reduced levels of Pex3 and Pex12 compared to cells expressing endogenous levels of Atg30 (Figure 4-2). Furthermore, overexpression of Atg30 in the Δ*atg30* Δ*rppa09976* background provided higher peroxin levels, relative to those in the Δ*atg30* background. The levels of peroxins in the absence of Rppa09976 correlated to the rescued growth of this strain after 36 h in methanol media (Figure 4-1).

Similarly, we compared Pex3 and Pex12 levels in strains endogenously expressing and overexpressing Rppa09976. In methanol media, Rppa09976-GFP seemed to show multiple bands in all Rppa09976-GFP expressing strains, suggesting it may become modified during peroxisome proliferation conditions (Figure 4-2). However, despite Rppa09976 overexpression, peroxin levels seemed similar to those of cells expressing endogenous Rppa09976 levels. Results from the growth curve and peroxin levels indicate that the overexpression of Rppa09976 does not induce pexophagy nor does it inhibit cell growth in peroxisome proliferation conditions.

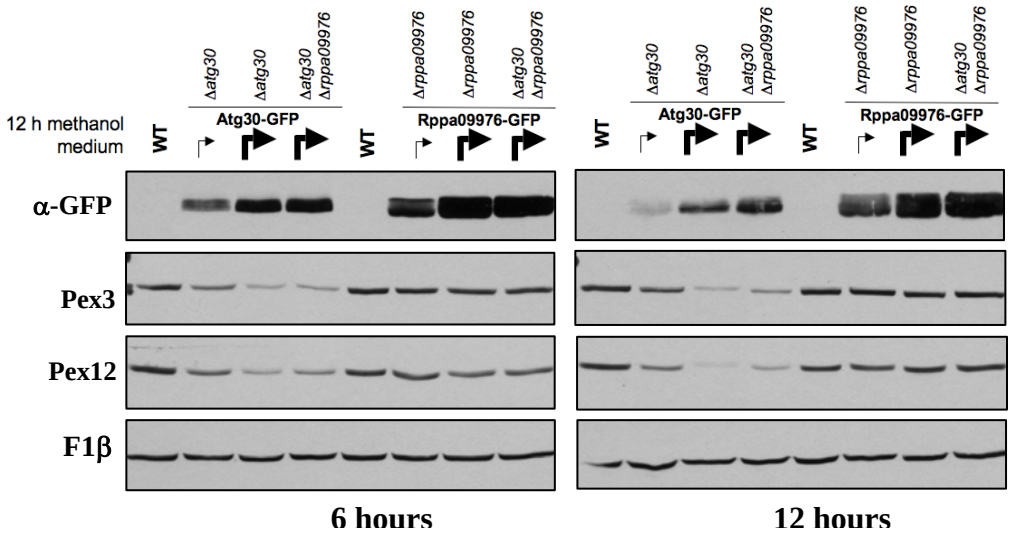

Figure 4-2: Rppa09976 overexpression does not induce pexophagy in methanol medium. Strains with endogenous levels of Rppa09976 in the Δ*rppa09976*, overexpressed Rppa09976 in the Δ*rppa09976* or Δ*atg30* Δ*rppa09976* backgrounds, endogenous levels of Atg30 in the Δ*atg30*, overexpressed Atg30 in the Δ*atg30* or Δ*atg30* Δ*rppa09976* backgrounds, and the wild-type (PPY12h) strains were grown in methanol for 6 and 12 h. The β subunit of the mitochondrial F0/F1 ATPase served as a loading control.

Overexpressed Rppa09976 localizes to peroxisomes, as well as ER-like structures

We used fluorescence microscopy to follow the localization of overexpressed and endogenously expressed Rppa09976-GFP and Atg30-GFP. The majority of both endogenously expressed and overexpressed Atg30-GFP localizes to the peroxisomal membrane and only a small amount is detected inside the vacuole (Farre et al., 2008). We followed the localization of overexpressed Atg30-GFP in Δ*atg30* Δ*rppa09976* cells and found that the localization of Atg30 did not change, suggesting that Rppa09976 does not affect Atg30 localization (Figure 4-3).

Likewise, we followed the localization of endogenously expressed and overexpressed Rppa09976-GFP in the presence or absence of Atg30. After 6 and 12 h, endogenously expressed Rppa09976-GFP localized to the peroxisomal membrane (Figure 4-4). Surprisingly, for both strains overexpressing Rppa09976-GFP, after 6 h, Rppa09976 localized to structures resembling peroxisome clusters and dot-like structures along the cell periphery, resembling that of the ER. However no GFP fluorescence was ever seen inside the vacuole at any timepoint, verifying that the overexpression of Rppa09976 does not induce pexophagy. In some instances, Rppa09976-GFP localized to membranes surrounding two distinct structures, one resembling the peroxisome cluster and the other resembling the nucleus (shown by white arrows). Similar localization patterns between the two strains indicated that the localization of overexpressed Rppa09976 did not require Atg30. In contrast to the 6-h timepoint, after 12 h, most of the Rppa09976-GFP accumulated to structures resembling the peroxisomal membrane, suggesting that Rppa09976 may arrive at the peroxisomal membrane through indirect trafficking via the ER.

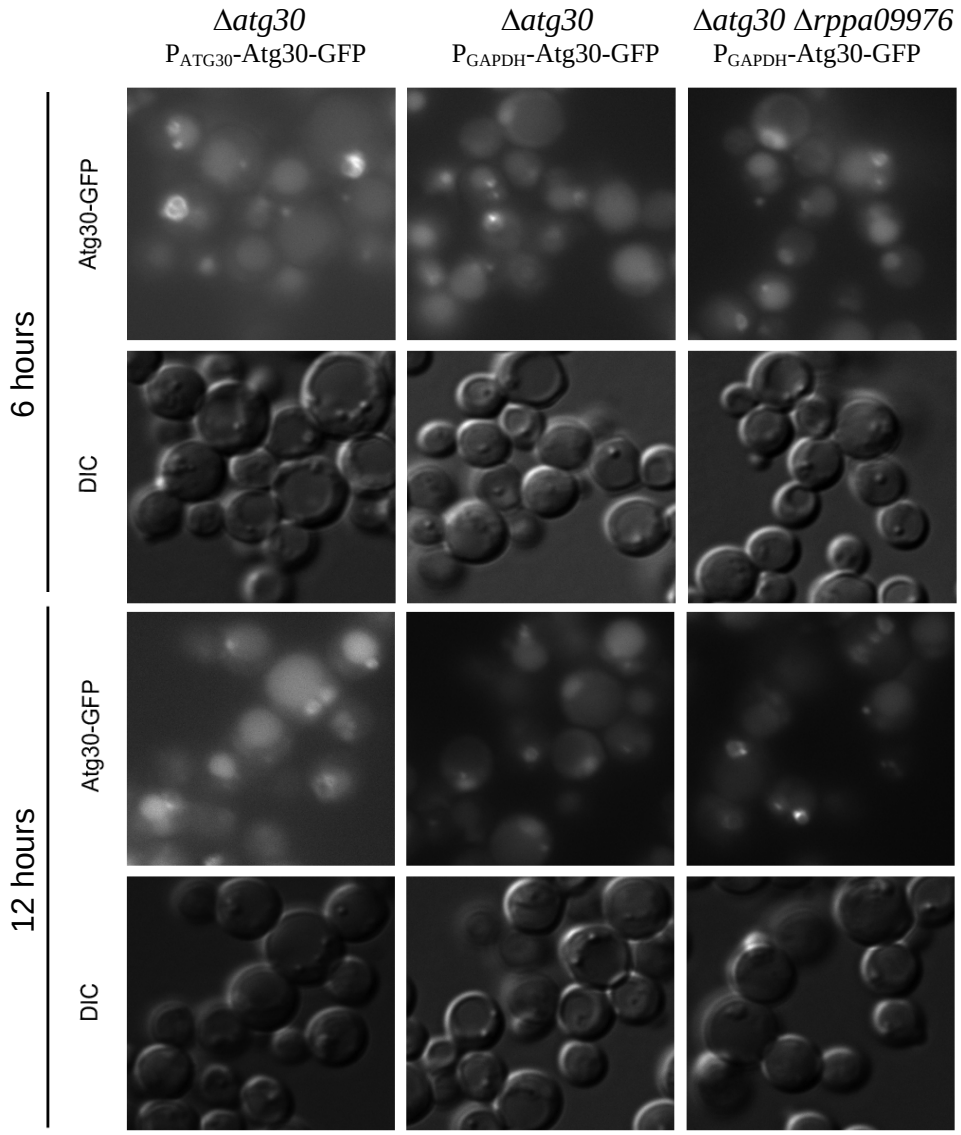

Figure 4-3: Localization of endogenously expressed and overexpressed Atg30. Endogenously expressed Atg30-GFP in Δ*atg30* and overexpresssed Atg30-GFP in Δ*atg30* and Δ*atg30* Δ*rppa09976* cells were grown for 6 and 12 h in methanol medium and examined by fluorescence microscopy.

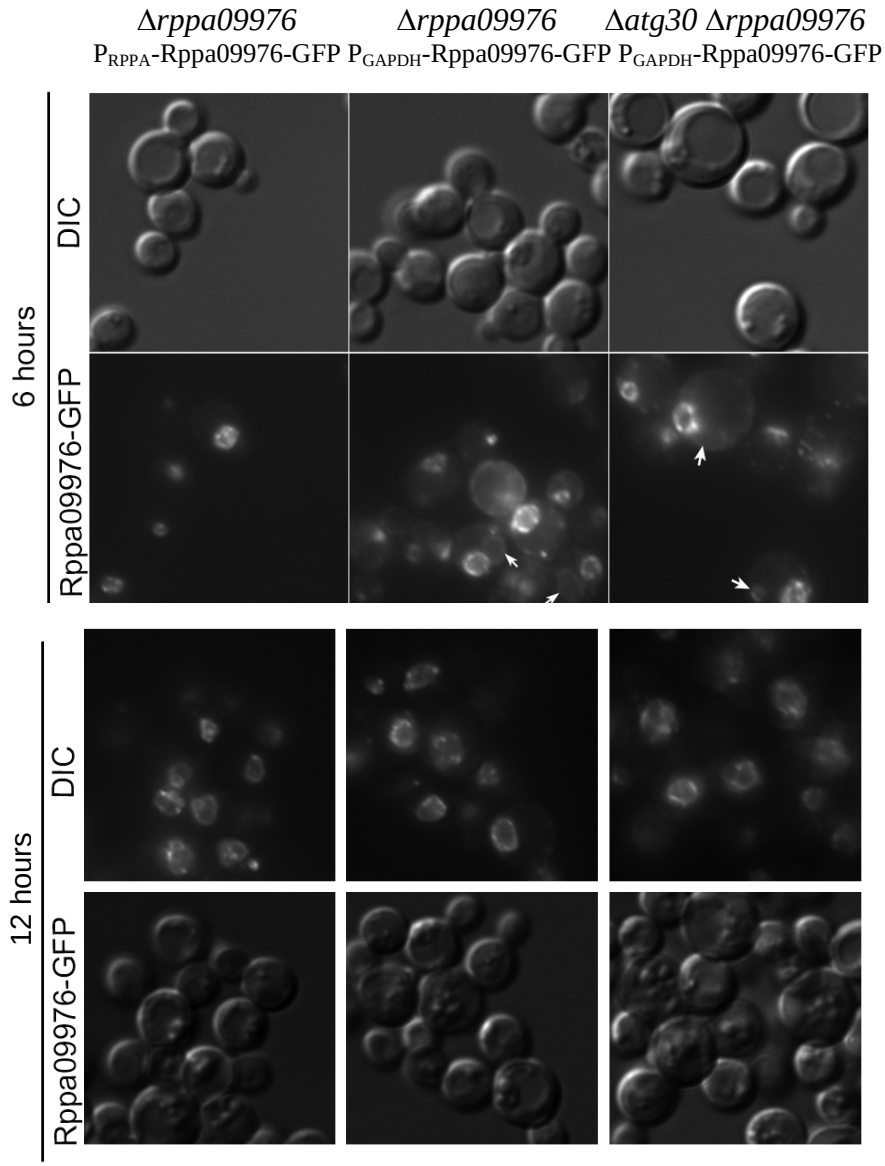

Figure 4-4: Localization of endogenously expressed and overexpressed Rppa09976. Endogenously expressed Rppa09976-GFP in Δ*rppa09976* and overexpresssed Rppa09976-GFP in Δ*rppa09976* and Δ*atg30* Δ*rppa09976* cells were grown for 6 and 12 h in methanol medium and examined by fluorescence microscopy.

Proper Rppa09976 localization depends on the presence of Atg30

Since both Rppa09976 and Atg30 localize to the peroxisomal membrane and are specifically involved in pexophagy, we considered the possibility that these two proteins may affect each other's localization. We examined the localization of endogenously expressed Atg30-GFP in the presence and absence of Rppa09976, as well as endogenously expressed Rppa09976-GFP in the presence and absence of Atg30. To confirm localization in the middle of the cluster, we created a series of images derived from different focal planes (Z-stack) and used deconvolution microscopy for analysis.

Similar to overexpression studies, the localization of endogenously expressed Atg30 did not change in the absence of Rppa09976, suggesting Rppa09976 does not influence Atg30 localization (Figure 4-5). In both strains, Atg30 localized to individual peroxisomes, as well as the vacuole. In a separate experiment, we also compared the levels of PMPs Pex3 and Pex12 in the presence or absence of Rppa09976. We found that in the absence of Rppa09976, levels of Atg30-GFP, Pex3, and Pex12 were slightly higher, possibly due to the block of pexophagy.

In the presence of Atg30, Rppa09976-GFP mostly localized to portions of the peroxisomal membrane facing the cytosol (Figure 4-5). In the absence of Atg30, however, some cells not only showed Rppa09976 along the peroxisomal membrane facing the cytosol, but there was also significant Rppa09976 accumulation in the middle of the peroxisome cluster. Following the Z-stack of an individual cell, we found that the highest intensity of Rppa09976-GFP occurred in the middle of the peroxisome cluster. Furthermore, when we used biochemistry to follow PMP levels, we noticed that Rppa09976-GFP, Pex3, and Pex12 were slightly higher in the Δ*atg30* strain, most

probably due to the block in pexophagy. Furthermore, levels of Rppa09976 along the periphery of the peroxisome cluster were comparable between the two strains. Collectively, our results indicate that Atg30 directly restricts Rppa09976 localization to the peripheral membranes of the peroxisome cluster.

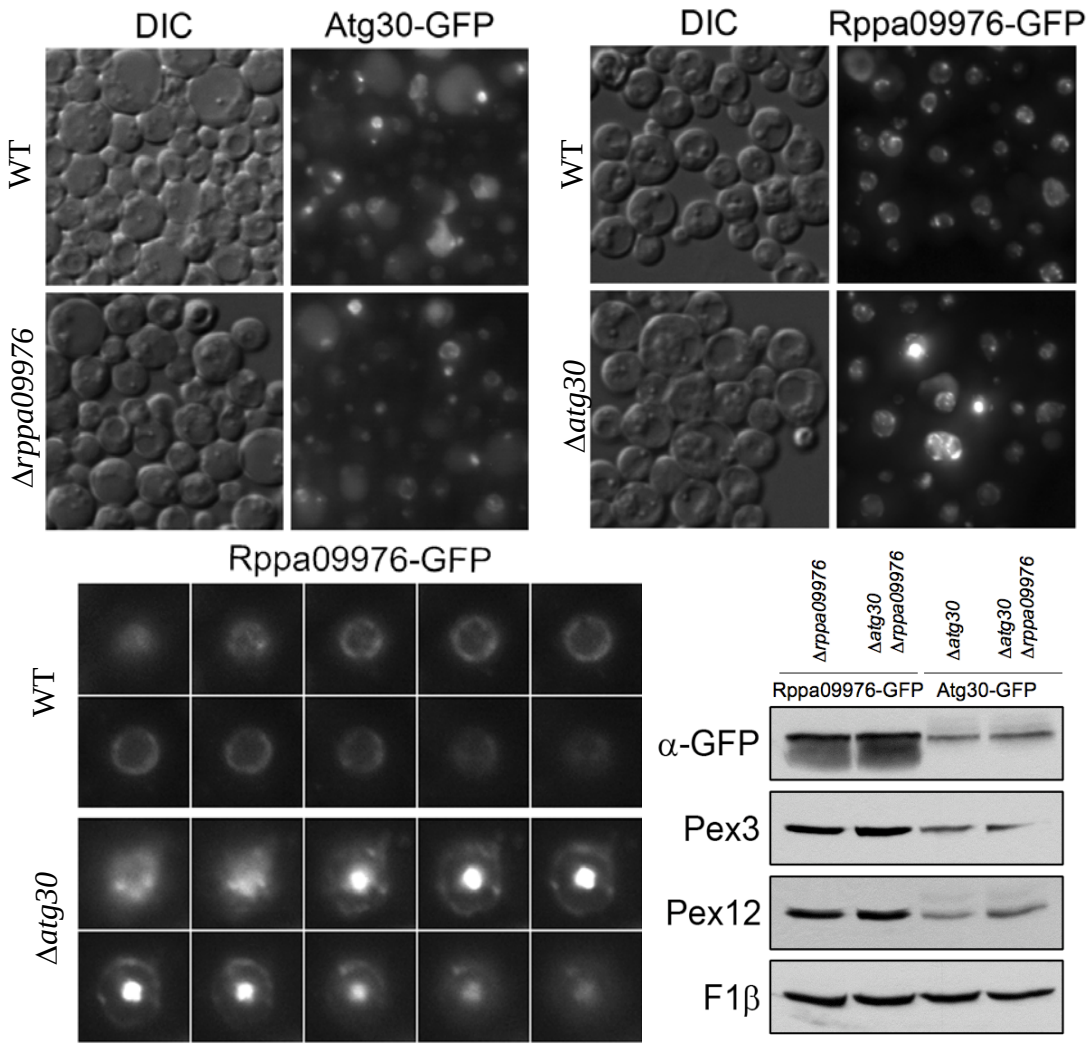

Figure 4-5: Rppa09976 accumulates to the center of the peroxisome cluster in *Δatg30* cells. The top two images are a maximum intensity projection (MIP) of deconvoluted Z-stack images. The bottom left pictures are original Z-stack images. Atg30-GFP localization was compared in the presence or absence of Rppa09976. Rppa09976-GFP localization was compared in the presence or absence of Atg30. Biochemistry experiments compared peroxin and Rppa09976-GFP in the presence or absence of Atg30 and Atg30-GFP in the presence or absence of Rppa09976, respectively. F1β was used as a loading control.

Discussion

The similar phenotypes of Δ*atg30* and Δ*rppa09976* cells suggest that these two proteins may be directly or indirectly acting together during pexophagy. Rppa09976, like Atg30, localizes (partially) to the peroxisomal membrane. Furthermore, like Atg30, Rppa09976 interacts with an essential peroxisome biogenesis factor, Pex3. Thus, we proposed that the overexpression of Rppa09976 might have a similar effect on cells as does overexpression of Atg30. Our results indicate, however, that this is not the case. Instead of inducing pexophagy under peroxisome biogenesis conditions, constitutively overexpressing Rppa09976 has no discernable phenotype.

Rppa09976 must therefore serve a different function in pexophagy than Atg30. Clues from these studies point to the function of Rppa09976. If Rppa09976 were to act upstream of Atg30, the overexpression of Rppa09976 should induce pexophagy. Since the overexpression of Rppa09976 does not induce pexophagy, we can conclude that Rppa09976 does not act upstream of Atg30, and most probably acts downstream of it. If this were the case, overexpression of Atg30 would cause increased Rppa09976 function, which would then induce pexophagy. Likewise, if Atg30 were overexpressed in the absence of Rppa09976, then Atg30-induced pexophagy would be affected. Therefore, we suggest that Rppa09976 acts downstream of Atg30 in tagging peroxisomes for selective degradation.

Furthermore, in the absence of Atg30, Rppa09976 accumulates to the middle of the peroxisome cluster, suggesting that Atg30 influences Rppa09976 localization. Both Rppa09976 and Atg30 interact with Pex3. In the absence of Atg30, one might argue that Rppa09976 has increased likelihood for Pex3 binding, however, since the levels of

Rppa09976 on the peroxisomal membrane are comparable in the presence and absence of Atg30, competition for similar binding partners is unlikely. On the other hand, our results do suggest that Atg30 directly interacts with Rppa09976 to restrict to portions of the peroxisomal membrane facing the cytosol.

Chapter 4 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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Chapter 5: Trafficking of Rppa09976 from the endoplasmic reticulum to peroxisomes

Abstract

Previous overexpression studies involving Rppa09976 showed that is modified and that Rppa09976 might indirectly localize to peroxisomes via the ER. To evaluate Rppa09976 trafficking dynamics, we followed endogenously expressed and overexpressed Rppa09976-GFP in cells co-expressing an ER marker, Sec61-mCherry and a peroxisomal marker, BFP-SKL. In a 4-h timecourse, Rppa09976 did in fact act as a class II PMP, trafficking from the ER to the peroxisomal membrane. Furthermore, to test how Rppa09976 modification corresponded with its trafficking, we followed Rppa09976-GFP and Rppa09976(ΔACBP)-GFP localization and expression in a 16-h timecourse. We found that as Rppa09976 accumulates on peroxisomes, a series of modifications occur, and that the modified form is the most stable and resistant to degradation. The absence of the ACBP domain delays trafficking to peroxisomes only slightly, however, and unlike the complex modifications of the full-length protein, Rppa09976(ΔACBP)-GFP shows limited modification upon arrival to peroxisomes. Our results suggest that Rppa09976 is the first class II PMP required for pexophagy.

Introduction

Peroxisome biogenesis requires a series of processes (most of which are not well understood) including peroxisome membrane biosynthesis, import of matrix proteins, and peroxisome growth and division (Sacksteder and Gould, 2000). Unlike the mitochondria, all peroxisome membrane proteins (PMPs) and matrix proteins are encoded by nuclear DNA and inserted into peroxisomes post-translationally.

Peroxisome membrane phospholipids originate from the endoplasmic reticulum (ER) and are delivered to the peroxisome membrane by a nonvesicular pathway (Raychaudhuri and Prinz, 2008). Currently, 32 *PEX* genes play roles in peroxisome biogenesis. Most *pex* mutants are unable to import peroxisomal matrix proteins, but still contain PMPs that localize to peroxisomal remnant structures. However in *Saccharomyces cerevisiae* and *H. sapiens,* there are a few *pex* mutants that mislocalize PMPs and lack peroxisomal remnant structures, suggesting that PMP and peroxisomal matrix protein import are two separate pathways (Subramani, 1998). Unlike peroxisomal matrix protein import, PMPs contain internal membrane targeting sequences (mPTS) (Van Ael and Fransen, 2006). mPTSs have been identified in a number of PMPs and show great variability in amino acid sequence and length. To date, PMPs are post-translationally targeted and imported to peroxisomes in one of two ways: i) class I PMPs are targeted from the cytoplasm directly to the peroxisomal membrane in a Pex19-dependent pathway, and ii) class II PMPs are sorted indirectly to peroxisomes via trafficking through the ER (Sparkes et al., 2005), (Tabak et al., 2003).

Pex19 predominantly localizes to the cytoplasm, but is also found associated with the peroxisomal membrane (Sacksteder et al., 2000). It was believed that Pex19 acted as a PMP shuttling receptor, stabilizing newly synthesized PMPs and directing them to the peroxisomal membrane. However, studies indicate Pex19 binds to a variety of peripheral and integral PMPs at the peroxisomal membrane with high affinity and functions as an assembly or disassembly factor for peroxisome membrane-associated protein complexes (Snyder et al., 2000).

S. cerevisiae Pex3 was the first PMP found to traffic from the ER to peroxisomes (Hoepfner et al., 2005). Since then, *Yarrowia lipolytica* Pex2, *Y. lipolytica* and mammalian Pex16, and *P. pastoris* Pex31 have also been shown to display ER-to-peroxisome trafficking in a Pex19-dependent fashion (Titorenko and Rachubinski, 1998), (Kim et al., 2006), (Yan et al., 2008).

Atg26, Atg30 and Rppa09976 are specifically required for pexophagy, but not for other autophagy-related pathways. Of these three proteins, only Atg30 and Rppa09976 localize to the peroxisomal membrane (Farre et al., 2008). Atg30, however, acts as a class I PMP and localizes to peroxisomes directly from the cytosol. Overexpression studies of Rppa09976 suggested that it might act as a class II PMP, indirectly localizing to the peroxisomal membrane through the ER. Using fluorescence microscopy and biochemical assays simultaneously, we followed the trafficking, expression and modification of Rppa09976.

Materials and Methods

Yeast strains, plasmids and transformations

The *P. pastoris* strains and plasmids used in this study are listed in Table 2-1. Transformation procedures are described in detail in Chapter 2.

Biochemical studies of peroxisome biogenesis

Cells were pre-grown in two pre-cultures of YPD. Cells were washed twice with YNB solution and 1.5 OD_{600} cells were transferred to pre-warmed 25 mL of 0.5% methanol medium to induce peroxisome biogenesis. 1 ml of cells were collected for the 0, 0.5, 1, 2, and 4 h timepoints. For the 8 and 16 h timepoints, a volume of cells equivalent to the OD_{600}/ml at the 4h timepoint was collected by centrifugation for each strain. Crude extracts were prepared in the presence of TCA (Baerends et al., 2000). SDS-PAGE and immunoblotting were performed as described previously (Laemmli, 1970), (Kyhse-Andersen, 1984). An 8% gel was used for α-GFP blots for better separation, indicated by a * (Figure 5-5). All other gels used were 10% unless otherwise indicated. Antigen-antibody complexes were detected by enhanced chemiluminescence.

Fluorescence Microscopy

To monitor the localization of endogenously expressed and overexpressed Rppa09976 by fluorescence microscopy, 100 µl of methanol-induced cells were collected and placed on ice at all timepoints. Six pictures of each strain were taken at each timepoint. Optimal exposition times for the BFP, GFP and RFP fluorophores were used for each picture. Images were captured using a motorized fluorescence microscope (Axioskop 2

MOT; Carl Zeiss MicroImaging, Jena, Germany), coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Results:

Rppa09976 traffics from the ER to peroxisomes

Studies comparing the localization of constitutively overexpressed Atg30 and Rppa09976 suggested that Rppa09976 might act as a class II PMP and localize to peroxisomes through the ER (Figure 3-4). To test this hypothesis, we first followed endogenously expressed and overexpressed Rppa09976-GFP localization in a 4-h timecourse using an ER marker (Sec61-mCherry) and a peroxisomal marker (BFP-SKL). Upon induction of peroxisome biogenesis, endogenously expressed Rppa09976-GFP showed a weak signal diffusely localized around the cytosol (Figure 5-1). However, after 30 min, Rppa09976 started to accumulate along the cell periphery, perhaps in specialized subcompartments of the ER. Furthermore, Rppa09976-GFP showed no colocalization with any pre-existing peroxisomes at either of these timepoints, indicating that *RPPA09976* transcription is repressed in nutrient rich conditions (YPD) and activated under peroxisome biogenesis conditions. Not until one hour did Rppa09976 start to accumulate on peroxisomes. However, a diffuse cytosolic signal was also seen, which may indicate Rppa09976 transport to peroxisomes. By 2 h, the majority of Rppa09976-GFP localized to peroxisomes and after 4 h Rppa09976 localized to the peroxisomal membrane that faces the cytosol in the peroxisome cluster.

In contrast, when Rppa09976 was constitutively overexpressed, it appeared on the peroxisomes, ER, and nuclear envelope at all timepoints (Figure 5-2). Unlike endogenous expression of Rppa09976, when Rppa09976 was constitutively overexpressed, it was found on peroxisomes at the 0 h timepoint. It is interesting to note that after 0, 0.5, and 1 h, not only did Rppa09976-GFP localize to peroxisomes, the ER,

and nuclear envelope, but there was a significant amount within the cytosol as well. However, after 2 h, Rppa09976-GFP primarily localized to peroxisomes, the ER and nuclear envelope.

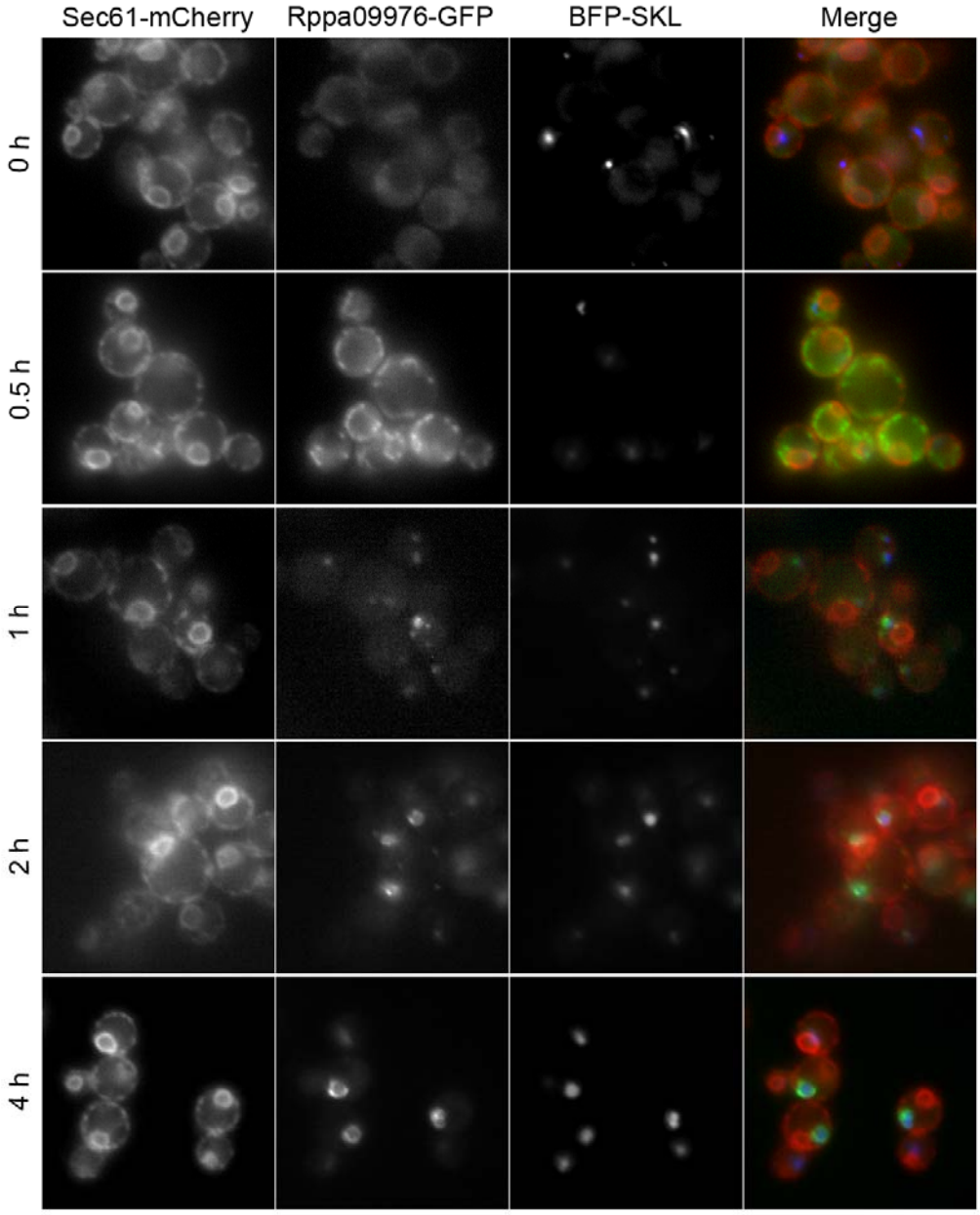

Figure 5-1: Rppa09976 traffics from the ER to peroxisomes. Endogenously expressed Rppa09976 cells were grown in methanol medium during a 4 h timecourse and examined by fluorescence microscopy. Colocalization with Sec61-mCherry (ER) and BFP-SKL (peroxisomes) is shown.

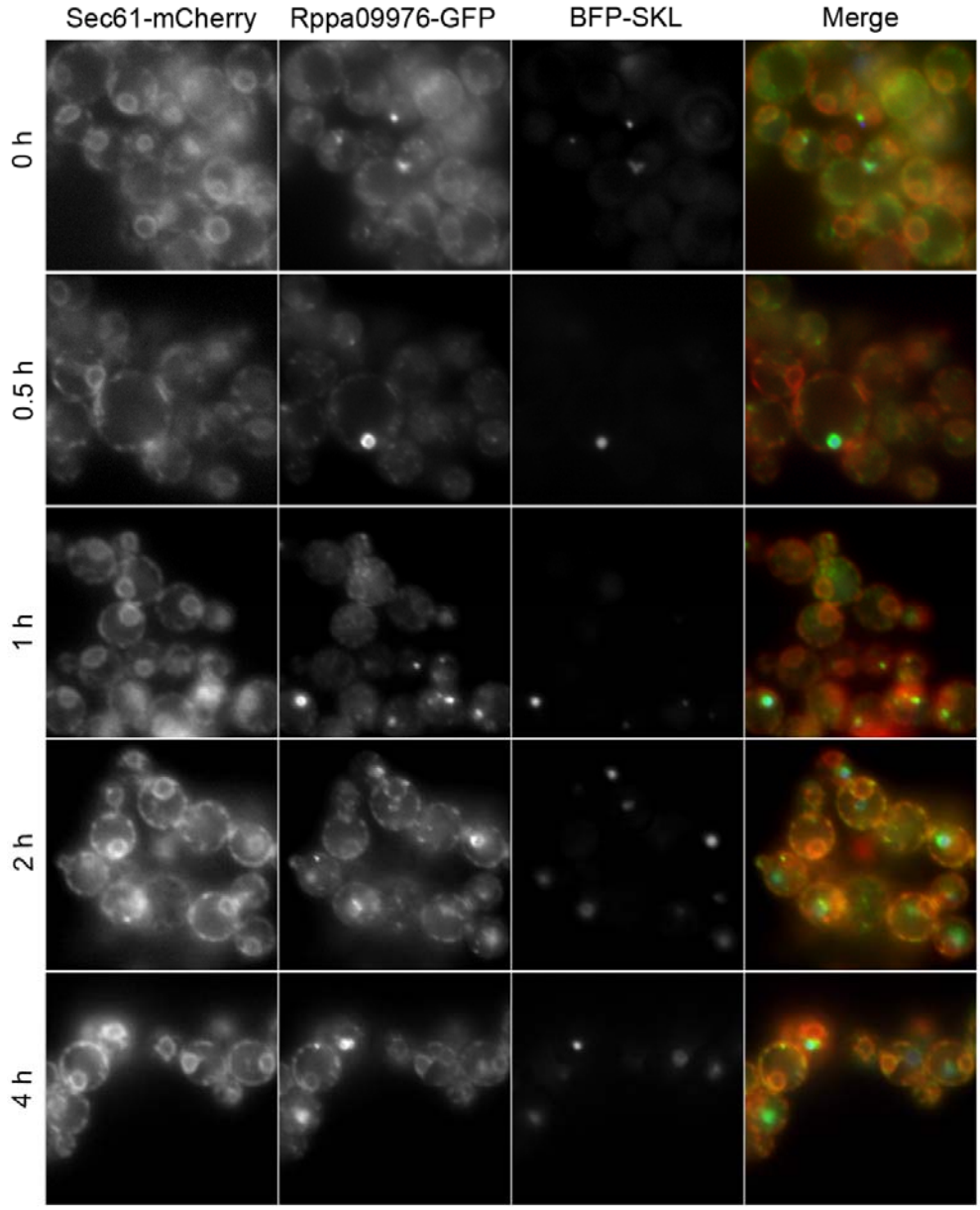

Figure 5-2: Constitutively overexpressed Rppa09976 localizes to the ER, nuclear envelope, and peroxisomes. Cells constitutively overexpressing Rppa09976 were grown in methanol medium during a 4 h time course and examined by fluorescence microscopy. The ER was labeled with Sec61-mCherry (red) and peroxisomes were labeled with BFP-SKL (blue).

The ACBP domain of Rppa09976 becomes modified upon the arrival of the protein at peroxisomes

Previous studies showed that the absence of the ACBP domain did not affect Rppa09976 localization in 15-h methanol induced cells (Figure 3-4). To determine if the ACBP domain is required for efficient trafficking to peroxisomes, we compared Rppa09976(ΔACBP)-GFP and Rppa09976-GFP trafficking. Furthermore, previous studies showed two distinct bands for Rppa09976-GFP, suggesting that Rppa09976 was modified after 15 h of methanol induction. To test if Rppa09976 modification correlated to its trafficking, we compared Rppa09976-GFP and Rppa09976(ΔACBP)-GFP localization using fluorescence microscopy and Rppa09976-GFP and Rppa09976(ΔACBP)-GFP levels using SDS-PAGE in the same experiment. Consistent with previous results (Figure 5-1), Rppa09976 trafficking from peroxisomes to the ER was completed after 2 h (Figure 5-3). After 8 and 16 h, Rppa09976-GFP remained on the peroxisomal membrane facing the cytosol in the peroxisome cluster and distributed rather evenly.

Trafficking of Rppa09976(ΔACBP)-GFP trafficking was much like the trafficking of Rppa09976-GFP, with a one time-point delay. After 2 h, a majority of the full-length Rppa09976-GFP localized to peroxisomes, however, it was not until 4 h when the majority of Rppa09976(ΔACBP)-GFP localized to the peroxisomal membrane (Figure 5-4). Whereas the full-length Rppa09976-GFP was distributed evenly along the peroxisomal membrane after 8 and 16 h, Rppa09976(ΔACBP)-GFP accumulated to specific areas along the peroxisomal membrane in most cells.

To trace Rppa09976 modification, we examined Rppa09976-GFP and Rppa09976ΔACBP)-GFP expression in methanol medium in conjunction with the trafficking timecourse. For better resolution, we used an 8% polyacrylamide gel for α-GFP blots. Instead of the two bands seen on 10% gels in Rppa09976 overexpression studies, we saw a series of complex modification and degradation products throughout the 16-h timecourse (Figure 5-5). Furthermore, Rppa09976 seemed to be first modified at 2 h, and at 4 h, corresponding to the time when the majority of Rppa09976 reaches peroxisomes. After 16 h, Rppa09976 remained on peroxisomes, mainly in its fully modified form, while unmodified forms were degraded.

In contrast, Rppa09976(ΔACBP)-GFP did not show the complex modifications exhibited by Rppa09976-GFP, despite localization to the peroxisomal membrane (Figure 5-5). While Rppa09976(ΔACBP)-GFP did show the primary modification, the secondary modification was absent. The modified form of Rppa09976(ΔACBP)-GFP remained stable and was the prevalent form after 16 h of methanol induction. Furthermore, higher levels of Rppa09976(ΔACBP)-GFP were seen than the full-length Rppa09976-GFP from timepoints 1 to 8 h, suggesting that Rppa09976(ΔACBP)-GFP was more stable and relatively more resistant to degradation than the full-length protein under growth conditions. However, after 16 h, when cells reached stationary phase in methanol medium, Rppa09976(ΔACBP)-GFP was rapidly degraded.

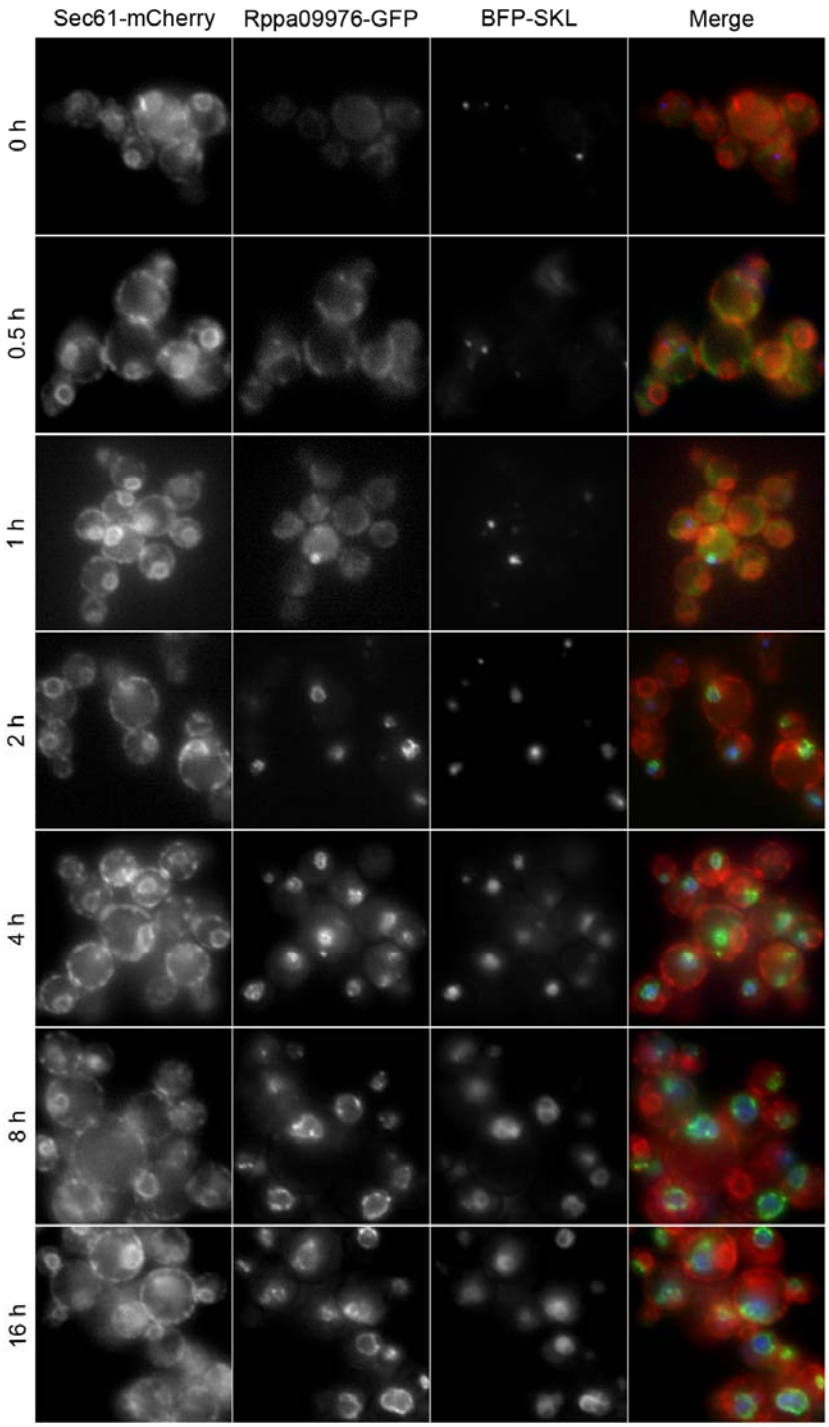

Figure 5-3: Endogenously expressed Rppa09976 trafficking. Cells expressing full-length, endogenously expressed Rppa09976 were grown in methanol medium during a 4 h timecourse and examined by fluorescence microscopy. Colocalization with Sec61-mCherry (ER) and BFP-SKL (peroxisomes) is shown.

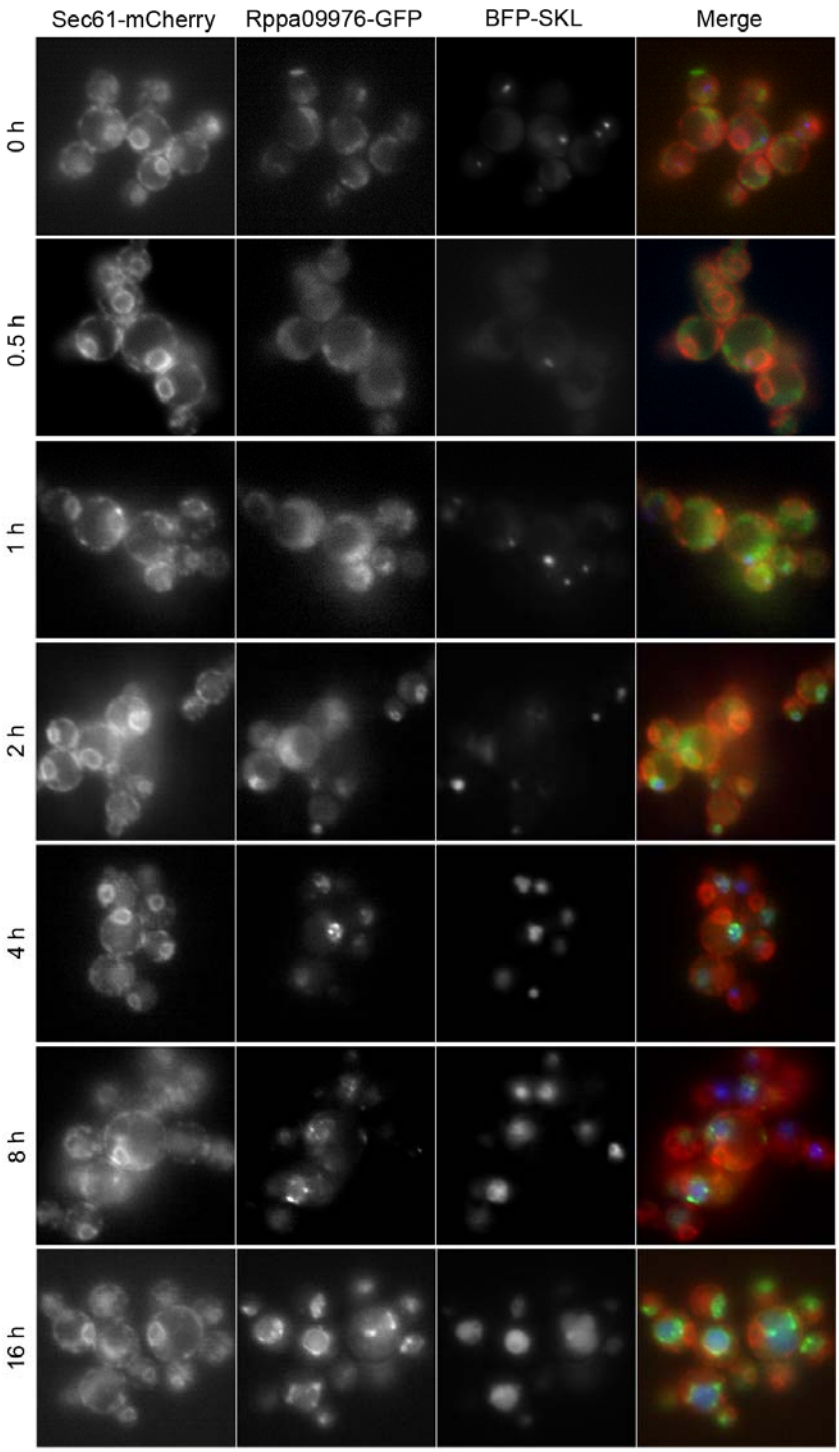

Figure 5-4: Rppa09976(ΔACBP)-GFP trafficking. Cells expressing endogenous levels of Rppa09976(ΔACBP)-GFP were grown in methanol medium during a 4 h timecourse and examined by fluorescence microscopy. Colocalization with Sec61-mCherry (ER) and BFP-SKL (peroxisomes) is shown.

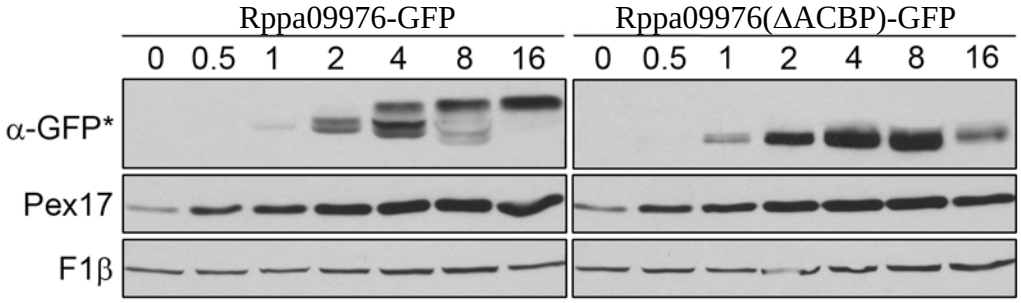

Figure 5-5: Rppa09976 becomes modified upon arrival at peroxisomes. Strains expressing the full-length Rppa09976-GFP and truncated Rppa09976(ΔACBP)-GFP were grown in methanol medium and 1 ml aliquots were taken at 0, 0.5, 1, 2, and 4 h. A volume of cells equivalent to the OD_{600} at the 4 h timepoint were taken for the 8 and 16 h timepoint for each strain. The * denotes that an 8% polyacrylamide gel was used. The β subunit of the mitochondrial F0/F1 ATPase served as a loading control.

Discussion

Studies investigating the effects of overexpressing Rppa09976 suggested that Rppa09976 might indirectly localize to peroxisomes via trafficking from the ER. Colocalization using Sec61-mCherry and BFP-SKL verified that Rppa09976 acts as a class II PMP, trafficking from the ER to peroxisomes. All known class II PMPs are involved in peroxisome biogenesis. However, Rppa09976 is the first known class II PMP that is specifically required for pexophagy but not other autophagy-related pathways.

Overexpression studies also indicated the possibility of Rppa09976 modification in methanol medium. Fluorescence microscopy and biochemistry experiments allowed us to determine that the Rppa09976 modification correlates with its arrival to peroxisomes. Better resolution of Rppa09976-GFP also showed that Rppa09976 does not contain just one modification, but rather a series of complex modification and degradation products, where the most modified form appears after 4 h and is the most stable form after 16 h of methanol adaptation.

Localization studies showed that the ACBP domain of Rppa09976 is not required for proper localization to peroxisomes. However, kinetic results indicate that the lack of the ACBP domain slightly delays the trafficking of the truncated protein to peroxisomes. Unlike full-length Rppa09976-GFP, which distributes evenly along the peroxisomal membrane, after 8 and 16 h, Rppa09976(ΔACBP)-GFP seems to be restricted to specific spots along the peroxisomal membrane. In addition, the protein without the ACBP domain did not undergo the second modification as the full-length Rppa09976 protein did upon arrival to peroxisomes, suggesting that the second

modification may occur on the ACBP domain. It is also interesting to note that the levels of Rppa09976(ΔACBP)-GFP are relatively higher than those of the full-length Rppa09976-GFP protein except after 16 h, when cells reach the stationary stage. Since Rppa09976(ΔACBP)-GFP complemented Δ*rppa09976* cells are deficient in pexophagy, this degradation is not due to some basal level of peroxisome degradation. Besides, the levels of Pex17 remain stable at 16 h. Therefore, some other mechanism may be responsible for Rppa09976(ΔACBP)-GFP degradation.

Chapter 5 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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Chapter 6: Rppa09976 is degraded independent of pexophagy, autophagy and vacuolar proteolysis

Abstract

Selective degradation of peroxisomes involves the sequestration, engulfment, and eventual vacuolar degradation of individual organelles. While most peroxisomal membrane proteins (PMPs) are degraded together with peroxisomes, in *Hansenula polymorpha*, Pex3 evades this autophagic process and is removed prior to peroxisome sequestration. The other PMP and peripheral peroxisome membrane protein required for pexophagy, but not for other autophagy-related pathways, Pex14 and Atg30 respectively, are degraded together with peroxisomes. To test whether Rppa09976 is degraded with peroxisomes or not, we followed the levels of Rppa09976-GFP biochemically and using fluorescence microscopy. Not only did Rppa09976 degrade independent of pexophagy, but its degradation was independent of autophagy and vacuolar proteolysis, as well. Furthermore, all Rppa09976 truncations tested were also degraded in the absence of peroxisome degradation. Taken together, our results suggest that Rppa09976 degradation may depend on an alternative degradation pathway.

Introduction

It is imperative for organisms to find a way to degrade and recycle unneeded or damaged proteins and/or organelles. Two major degradation systems exist in eukaryotic cells, the ubiquitin-proteasome system and autophagic degradation in vacuoles/lysosomes. Studies indicate a functional relationship between the two systems: proteasomal inhibition activates autophagy and likewise, the suppression of autophagy increases poly-ubiquitinated protein aggregates (Ding et al., 2007). In addition, studies

investigating the mono- and poly-ubiquitination of long-lived cytoplasmic substrates further suggest a coupling of the proteasomal and autophagic degradation processes.

Proteasomes are nuclear or cytoplasmic multi-protein complexes that coordinate the regulated breakdown of proteins. Protein ubiquitination is a highly conserved modification that controls a variety of cellular processes (Kerscher et al., 2006). Proteins targeted for proteasomal degradation are covalently ubiquitinated on a lysine residue by a cascade of enzymatic reactions involving activating enzymes (E1), conjugating enzymes (E2) and ligases (E3). To date, two types of proteasomes exist in the yeast, *Saccharomyces cerevisiae*, the 20S and 26S proteasomes. The 26S proteasome, the most common form, is composed of one 20S core particle structure and two 19S regulatory caps, which recognize polyubiquitinated proteins and transfers them to the catalytic core. The mechanisms targeting polyubiquitinated proteins to the proteasome are not well understood.

On the other hand, vacuoles and lysosomes are membrane-bound organelles containing acidic hydrolases and proteolytic enzymes capable of degrading virtually any subcellular constituent, including entire organelles. Under starvation conditions, this process is up-regulated and known as autophagy. Peroxisomes are versatile organelles that can rapidly proliferate in conditions requiring the oxidative metabolism of carbon compounds (oleic acid or methanol) as well as rapidly and selectively degrade when they become redundant in an autophagy-related process termed pexophagy. Upon macropexophagy induction, the whole organelle is sequestered by the pexophagosome and, upon its fusion with the vacuole, is subsequently degraded by resident hydrolases. While most proteins required for autophagy are also required for pexophagy, certain

proteins are specifically required for the degradation of peroxisomes. Namely, Atg11, Atg26, Atg28, and Atg30 may be required early in peroxisome recognition and targeting for degradation. Furthermore, in *Hansenula polymorpha*, Pex3 and Pex14, two crucial peroxins required for peroxisome biogenesis, are also essential for selective peroxisome degradation (Bellu et al., 2002) (Zutphen et al., 2008).

It is widely accepted that upon pexophagy induction, the entire organelle is degraded by vacuolar hydrolases, including the PMPs. Unlike most PMPs, in *H. polymorpha*, Pex3 is rapidly degraded, at a rate faster than the matrix and membrane proteins, alcohol oxidase and Pex14, respectively, suggesting that Pex3 may employ an alternate degradative pathway (Bellu et al., 2002). MG-132, a potent cytosolic proteasome inhibitor, blocks Pex3 degradation (Bellu et al., 2002). In *Pichia pastoris*, the pexophagy-specific Atg30 protein is associated with peroxisomes and is degraded within the vacuole together with peroxisomes (Farre et al., 2008). To determine if Rppa09976 shares a similar fate as Atg30, we followed its degradation upon pexophagy induction. To our surprise, we found that Rppa09976 is degraded independent of pexophagy. Furthermore, Rppa09976 is also degraded independent of autophagy and vacuolar proteolysis, suggesting that it uses an alternative degradation pathway.

Materials and Methods

Yeast strains, plasmids and transformations

The *P. pastoris* strains and plasmids used in this study are listed in Table 2-1. Transformation procedures are described in detail in Chapter 2.

Biochemical studies of pexophagy

Cells were pre-grown in two pre-cultures of YPD. Cells were washed once with YNB solution and 0.3 OD_{600} cells were transferred to 25 ml of 0.5% methanol medium for 15 h to induce peroxisome biogenesis. After 15 h, 2.0 OD_{600}/ml of cells were washed twice with YNB solution and transferred to 25 ml of 0.5% ethanol medium for 0, 6, 12, and 24 h. At 12 h, 125 µl of ethanol was added to each flask. Cells (1 ml) were collected and pelleted for each strain at each timepoint. Crude extracts were prepared in the presence of TCA (Baerends et al., 2000). SDS-PAGE and immunoblotting was performed as described previously (Kyhse-Andersen, 1984; Laemmli, 1970). Antigen-antibody complexes were detected by enhanced chemiluminescence.

Fluorescence Microscopy

To monitor the localization of Rppa09976 under pexophagy conditions, 100 µl of cells were collected and placed on ice at all timepoints. Six pictures of each strain were taken at each timepoint. Optimal exposition times for the BFP, GFP and RFP fluorophores were used for each picture. Averages of the optimal exposition times for BFP-SKL and Rppa09976-GFP were taken and are shown in Table 6-1. Images were captured using a motorized fluorescence microscope (Axioskop 2 MOT; Carl Zeiss MicroImaging, Jena,

Germany), coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Results

Rppa09976 degradation is independent of peroxisomal degradation

There are three known pexophagy-specific PMPs and pexophagy-specific peroxisome membrane-associated proteins: Pex3, Pex14 and Atg30. It has been found that upon pexophagy induction, unlike most PMPs including Pex14, Pex3, a protein essential for peroxisome biogenesis, escapes vacuolar delivery and is degraded prior to peroxisome sequestration events (Bellu et al., 2002). Recently, the pexophagy-specific peripheral membrane protein, Atg30, was identified. Like most PMPs and peroxisome membrane-associated proteins, upon pexophagy induction, Atg30 is degraded together with the peroxisome. To test whether Rppa09976, the only other pexophagy-specific PMP, shares a similar fate as Atg30, we followed Rppa09976-GFP levels in a 24-h time course after inducing macropexophagy. In Δ*rppa09976* cells complemented with Rppa09976-GFP (STN128), we followed levels of AOX and Pex12 to measure the extent of pexophagy (Figure 6-1). By 12 h, levels of AOX and Pex12 decreased similarly. The levels of Pex17, another PMP, decreased within 6 h, as did Rppa09976-GFP. In cells of the Δ*atg30* mutant, specifically blocked in pexophagy (STN155), AOX and Pex12 levels remained unchanged, however Pex17 was still degraded, suggesting that Pex17 is another PMP that is degraded independent of peroxisome degradation (our unpublished data). Furthermore, Rppa09976-GFP levels decreased in STN155, implying that like Pex3 in *H. polymorpha* and Pex17 in *P. pastoris*, Rppa09976 degradation is not directly associated with peroxisome degradation. Interestingly, the levels of Rppa09976-GFP at the 0 h timepoint were higher in STN155 than in STN128, indicating that Rppa09976-GFP was more stable in the absence of Atg30, however

upon ethanol adaptation, the kinetics of Rppa09976 degradation were similar between the two strains.

To verify pexophagy-independent Rppa09976 degradation, we used fluorescence microscopy to follow Rppa09976-GFP upon macropexophagy induction. Cells from both strains were induced in methanol medium for 15 h and transferred to ethanol-containing medium for 0, 6, and 12 h. In STN161, Rppa09976-GFP localized to the peroxisomal membrane of the cluster (labeled with BFP-SKL) at 0 h. Furthermore, after 6 h, most of the Rppa09976-GFP remained on the peroxisomal membrane surrounding the peroxisomal cluster. Very little BFP-SKL was localized to the vacuole labeled with FM4-64 after 6 h, however after 12 h, a majority of the BFP-SKL diffusely localized inside the vacuole, indicating peroxisomal degradation. Like BFP-SKL, after 12 h, Rppa09976-GFP localized to any remaining cytosolic peroxisomes, as well as inside the vacuole. While a relatively large amount of BFP-SKL remained inside the vacuole, the intensity of the Rppa09976-GFP signal was significantly less; cells that accumulate BFP-SKL within the vacuole do not contain much Rppa09976-GFP there, if at all. In the pexophagy-deficient Δ*atg30* strain (STN163), Rppa09976-GFP localized to the peroxisomal membrane at all timepoints. Additionally, in a few cells at each timepoint, Rppa09976-GFP accumulated at a spot in the middle of the peroxisome cluster.

Over the course of 12 h, Rppa09976-GFP appeared to remain on peroxisomes in the Δ*atg30* strain, conflicting with biochemical data depicted in Figure 6-1. To resolve this discrepancy, we also compared the exposition times of BFP-SKL and Rppa09976-GFP for each strain at each timepoint. Three pictures for each strain at the optimal

exposition time for the BFP and GFP fluorophore were taken and averaged. Table 6-1 shows the average optimal exposition times for BFP-SKL and Rppa09976-GFP. Since optimal exposition times correlate to the intensity of the signal, we can indirectly measure the relative amounts of GFP and BFP. The BFP-SKL exposition time for STN161 barely increased after 6 h, but increased about 3-fold after 12 h, indicating that some pexophagy occurred. In STN163, where pexophagy is blocked, the exposition time of BFP-SKL increased less than 2-fold over 12 h. However the Rppa09976-GFP exposition time for STN161 increased about 3-fold after 6 h, and 8-fold after 12 h. In STN163, after 6 h, when the exposition time of BFP-SKL remained about the same, the exposition time of Rppa09976-GFP increased more than 10-fold, further suggesting that Rppa09976 is degraded independent of peroxisome degradation. Comparing optimal exposition times with biochemical and fluorescence microscopy data verified that Rppa09976-GFP degraded independent of peroxisome degradation. Furthermore, the exposition time of Rppa09976-GFP at the 0 h timepoint was significantly lower in the absence of Atg30, in agreement with higher levels of Rppa09976-GFP in the biochemical experiment.

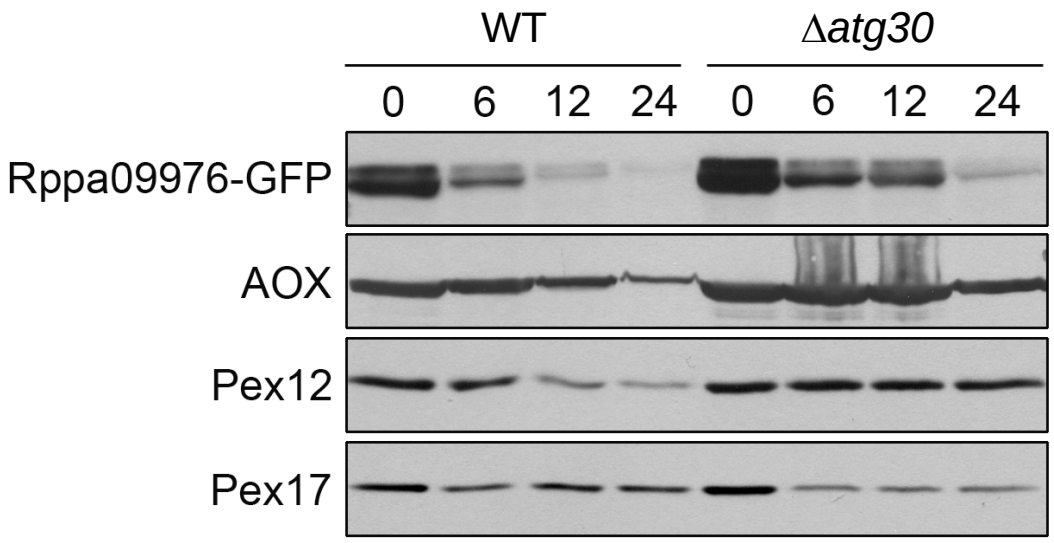

Figure 6-1: Rppa09976 is degraded independent of peroxisome degradation. 15 h methanol-induced cells were transferred to 0.5% ethanol medium to induce macropexophagy. Rppa09976-GFP in Δ*rppa09976* and Δ*atg30* Δ*rppa09976* was followed over 24 h. AOX and Pex12 were used as peroxisomal matrix and membrane protein controls that are degraded together with peroxisomes. Pex17 was degraded independent of pexophagy.

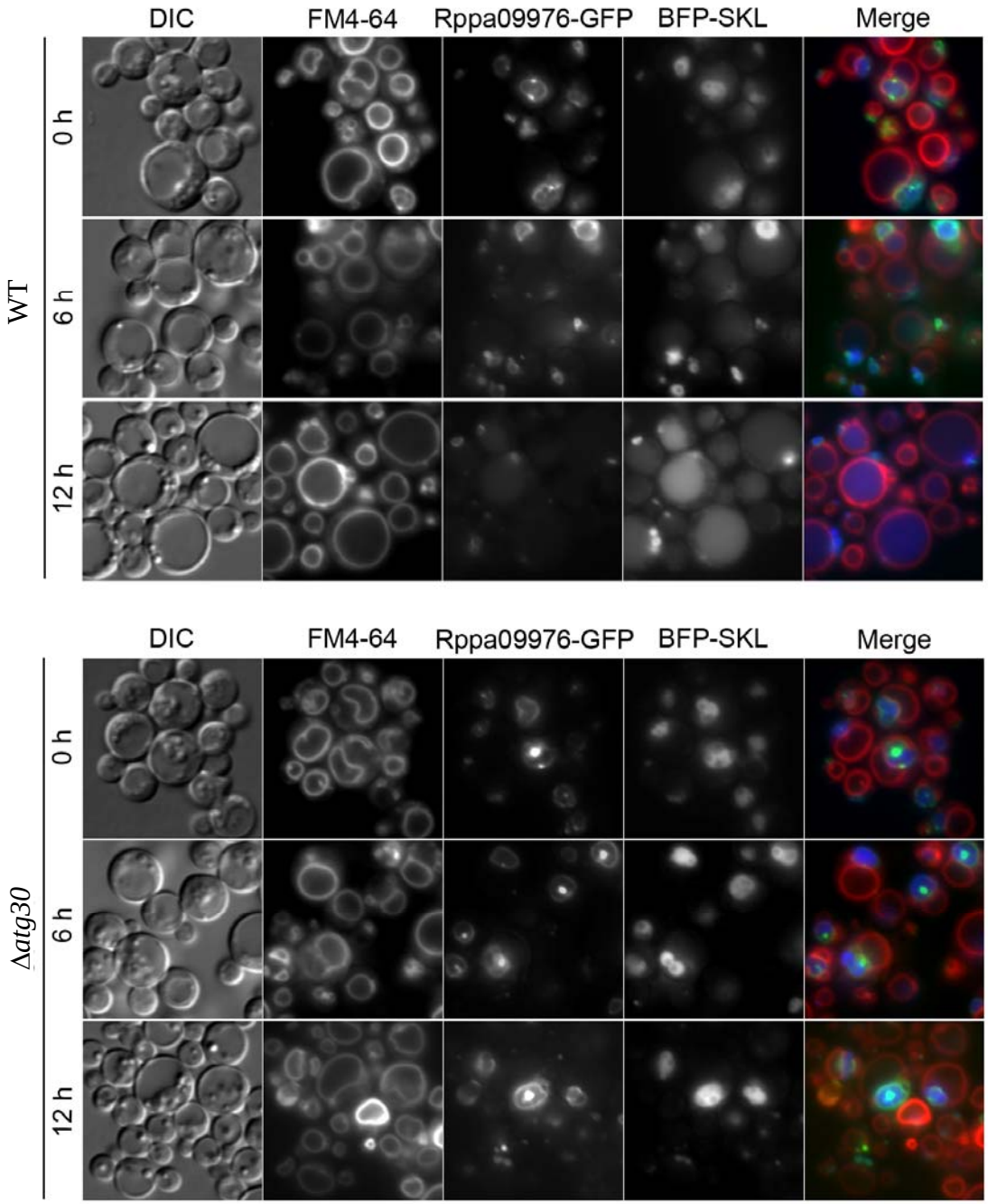

Figure 6-2: The fate of Rppa09976 upon pexophagy induction. Δ*rppa09976* and Δ*atg30* Δ*rppa09976* cells complemented with Rppa09976-GFP and expressing BFP-SKL were grown in 0.5% methanol medium for 15 h and shifted to 0.5% ethanol medium for 0, 6, and 12 h to induce macropexophagy. Vacuoles are labeled in red (FM4-64).

Table 6-1: Optimal exposition times for Rppa09976-GFP and BFP-SKL. Average optimal exposition times for Rppa09976-GFP and BFP-SKL between the three pictures taken for STN161 and STN163 at each timepoint are shown below.

Rppa09976-GFP optimal exposition times

	0h	6h	12h
WT	1,026 ms	2,710 ms	8,329 ms
Δ*atg30*	324 ms	3,816 ms	4,790 ms

BFP-SKL optimal exposition times

	0h	6h	12h
WT	487 ms	663 ms	1,466 ms
Δ*atg30*	408 ms	454 ms	713 ms

Rppa09976 degrades independent of pexophagy, autophagy, and vacuolar proteolysis

Two major degradative pathways exist in cells: the autophagic degradation system and the ubiquitin-proteasome degradation system. While Rppa09976 does not degrade together with peroxisomes, it could still be degraded through an autophagy-dependent or vacuolar proteolysis-dependent pathway. For instance, some unknown mechanism could sequester Rppa09976 from the peroxisomal membrane and transport it to the vacuole where resident proteases and hydrolases could degrade Rppa09976. To test the requirement of autophagy and vacuolar proteolysis, we inserted an extra copy of Rppa09976 fused to GFP in wild-type, Δ*atg30*, Δ*atg8* (deficient all autophagy-related pathways), and Δ*pep4* Δ*prb1* (deficient in vacuolar proteases) cells. Cells were induced in methanol medium for 15 h and then transferred to ethanol medium for 0, 6, 12, and 24 h. In all strains except the wild-type, peroxisome degradation was blocked, as judged by the constant levels of AOX and Pex12 (Figure 6-3). In the wildtype, after 6 h, AOX and Pex12 levels decreased significantly and after 12 h, almost disappeared. On the other hand, both Pex17 and Rppa09976-GFP levels decreased in all strains, verifying that Rppa09976 and Pex17 are degraded independent of pexophagy, autophagy, and vacuolar proteolysis. Rppa09976 and Pex17 then must degrade by some other pathway.

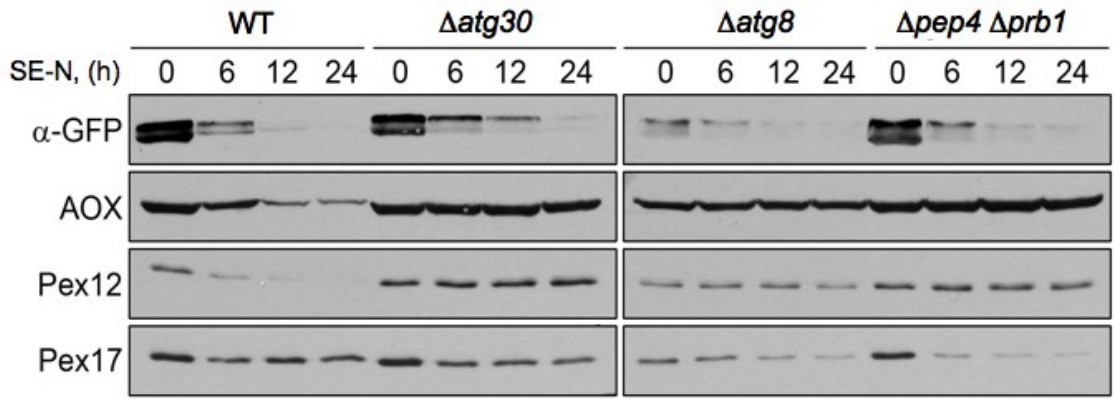

Figure 6-3: Rppa09976 is degraded independent of pexophagy, autophagy and vacuolar proteolysis. An extra copy of Rppa09976-GFP was introduced in WT, Δ*atg30*, Δ*atg8*, and Δ*pep4* Δ*prb1* cells. 15 h methanol-induced cells were transferred to 0.5% ethanol medium and Rppa09976-GFP, AOX, Pex12 and Pex17 levels were followed in each strain after 0, 6, 12, and 24 h.

Rppa09976 truncations are also degraded independent of pexophagy

Previous studies using the AOX plate assay indicated that none of the truncated versions of Rppa09976 fused to GFP were able to complement Δ*rppa09976* cells in pexophagy, indicating that the entire protein is required for peroxisome degradation. Furthermore, the possibility existed that Rppa09976 degradation may also be dependent on the entire protein. To verify a block in pexophagy quantitatively and to also test whether the truncated versions of Rppa09976 were degraded in the absence of peroxisome degradation, we transferred 15 h methanol-induced cells to ethanol medium for 0, 6, 12, and 24 h. Similar to Δ*rppa09976* cells, strains expressing truncated versions of Rppa09976: Rppa09976(ΔACBP)-GFP, ACBP-GFP, and ACBP-GFP-Pex11 were blocked in pexophagy after 12 h, indicated by levels of AOX and Pex12 (Figure 6-4). As expected, Pex17 was degraded in all strains. Surprisingly, cells complemented with different truncated versions of Rppa09976 also degraded these forms of Rppa09976 independent of peroxisome degradation. Thus, while both the ACBP domain and the C-terminal part of Rppa09976 are required for pexophagy, the ACBP domain and the C-terminal part of Rppa09976 alone are sufficient for its degradation in the absence of peroxisomal degradation. In addition, the truncated versions of Rppa09976-GFP seemed to be unmodified in methanol medium, even when cytosolic ACBP-GFP was brought to the peroxisomal membrane by Pex11 (ACBP-GFP-Pex11). However, upon ethanol-adaptation, the kinetics of their degradation was similar, except for the ACBP-GFP-Pex11 fusion protein. Interestingly, this ACBP-GFP-Pex11 fusion stabilized ACBP-GFP under pexophagy conditions.

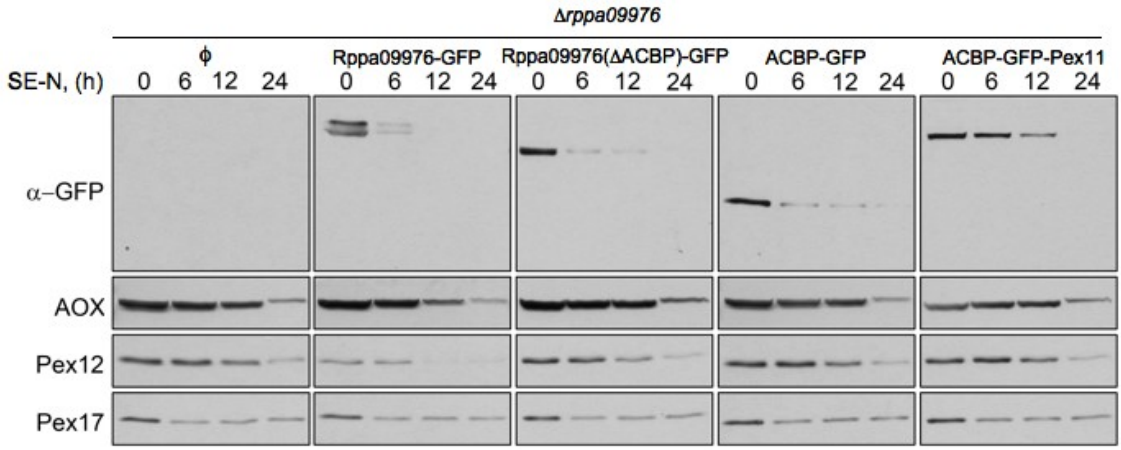

Figure 6-4: Truncations of Rppa09976 are degraded in the absence of peroxisome degradation. Rppa09976-GFP, Rppa09976(ΔACBP)-GFP, ACBP-GFP and ACBP-GFP-Pex11 was introduced in Δ*rppa09976* cells. All strains were compared to Δ*rppa09976* cells. 15 h methanol-induced cells were transferred to 0.5% ethanol medium and Rppa09976-GFP, AOX, Pex12 and Pex17 levels were followed in each strain after 0, 6, 12, and 24 h.

Discussion

Like many PMPs, as well as the pexophagy-specific peroxisome receptor Atg30, we expected Rppa09976 to degrade together with peroxisomes. Comparing both fluorescence microscopy and biochemical data, we found a different fate for Rppa09976 upon pexophagy induction. To our surprise, not only did Rppa09976 degrade independent of pexophagy, but also its degradation did not require autophagy and vacuolar proteolysis either, making Rppa09976 the only known pexophagy-specific PMP that does not degrade together with peroxisomes. Thus, Rppa09976 must degrade by an alternative degradation pathway, perhaps through some cytosolic protease pathway or the ubiquitin-proteasome pathway. However, the mechanism through which Rppa09976 is targeted for degradation is not yet known.

Furthermore, both fluorescence microscopy and biochemistry indicate that Rppa09976 is more stable in methanol-induced Δ*atg30* cells relative to wild-type cells. Both Rppa09976 and Atg30 interact with Pex3 (Farre et al., 2008). Therefore, the absence of Atg30 might increase the likelihood of Rppa09976-Pex3 interaction, and this could possibly stabilize Rppa09976. However, upon pexophagy induction, Pex3 removal from the peroxisomal membrane prior to peroxisomal degradation might disrupt this interaction, and cause the destabilization and eventual degradation of Rppa09976 in Δ*atg30* cells as in wild-type.

Surprisingly, despite the requirement of the entire Rppa09976 protein for proper modification and function, just the cytosolic ACBP domain or just the peroxisomal C-terminal part of the protein is sufficient for its degradation. The degradation of ACBP-GFP and Rppa09976(ΔACBP)-GFP show similar kinetics to the degradation of the full-

length protein (although they may be initially more stable than the full-length Rppa09976 protein). Unexpectedly, bringing the ACBP domain back to the peroxisomal membrane via an ACBP-GFP-Pex11 fusion does not restore pexophagy or modification and is more resistant to degradation. Our data suggest that Rppa09976 is the first pexophagy-specific PMP whose degradation is independent of pexophagy, autophagy, and vacuolar proteolysis.

Chapter 6 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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Chapter 7: Conclusion

Previous studies indicate Atg30 functions as the peroxisomal receptor bridging the autophagic machinery to the peroxisomal membrane during the induction of pexophagy (Farre et al., 2008). Here, we found a novel protein, Rppa09976, involved specifically in pexophagy, but not other autophagy-related processes. Although Δ*rppa09976* and Δ*atg30* cells share similar phenotypes Rppa09976 is a unique protein required for both forms of pexophagy. Like Atg30, we show that Rppa09976 also localizes to the peroxisomal membrane. Unlike Atg30, Rppa09976 shares a mammalian homolog, MmAcbd5, which also localizes to the peroxisomal membrane in *P. pastoris*. While MmAcbd5 failed to complement the pexophagy defect in Δ*rppa09976* cells, we noticed that unlike Rppa09976, the mouse protein was not modified in P. pastoris. We previously showed that modification was dependent on the ACBP domain of Rppa09976, thus the inability of P. pastoris proteins to recognize MmAcbd5 and modify it may explain why this protein is unable to complement pexophagy. Perhaps swapping the mouse ACBD domain with the Rppa09976 ACBD domain may restore both modification and the ability to carry out pexophagy.

Furthermore have also shown that unlike most PMPs, Rppa09976 both traffics to the peroxisomal membrane through the ER and also degrades independent of peroxisome degradation. Furthermore, we show that while the C-terminal of Rppa09976 is required for proper localization, both its C-terminal and ACBP domain are required for function in pexophagy.

It is interesting to note that Rppa09976 degrades independent of pexophagy, autophagy, and vacuolar proteolysis. Previous studies indicate that Pex3 removal from

the peroxisomal membrane is required for their degradation (Bellu et al., 2002). Unlike Pex3, however, Rppa09976 removal from the peroxisomal membrane is not required for peroxisome degradation, since all truncated versions are degraded, as well. It is currently unclear how Rppa09976 is degraded and why its degradation is significant.

Although interactions with other Pex and Atg proteins have yet to be determined, here we found that Rppa09976 interacts with both Pex3 and Atg30. Pex3 is an integral membrane protein that acts early in peroxisome development, required for the docking of the majority of PMPs. Atg30, on the other hand, is involved in tagging peroxisomes for degradation. Our results indicate a role of Atg30 to restrict Rppa09976 localization to portions of the peroxisomal membrane facing the cytosol. The interactions of Rppa09976 strongly suggest the maintenance of peroxisome homeostasis via the coupling between the biogenesis and degradation machinery.

Current models suggest that the pexophagosome and MIPA are formed using the peroxisome surface as a template, until the elongating structure reaches the tips of the sequestering membranes (for the MIPA) or the vacuolar membrane (for the pexophagosome). We propose that after Atg30 tags peroxisomes for degradation, through its localization to portions of the peroxisomal membrane facing the cytosol, Rppa09976 may act as the template, donating the acyl-CoAs necessary for pexophagosome and MIPA formation and elongation. In the absence of Rppa09976, the 10 kDa cytosolic ACBP (Rppa08070) might intermittently donate acyl-CoAs allowing for the eventual degradation of peroxisomes. Currently, it is not known whether a Δ*rppa08070* Δ*rppa09976* is viable in *P. pastoris*, but the deletion of the only ACBP protein in *S. cerevisiae*, Δ*acb1p*, remains viable.

Chapter 7 is currently being prepared to submit for publication of the material. Ozeki, Katharine; Nazarko, Taras Y; Lotfi, Pouya; and Subramani, Suresh. The thesis author was the primary investigator and author of this paper.

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