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REVIEW

Mechanisms of Autophagy and Pexophagy in Yeasts

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Abstract—Autophagy is a process of recycling of the intracellular constituents using vacuoles (lysosomes). General autophagy occurs due to involvement of highly conservative components found in all eukaryotes, from yeasts to higher plants and humans. Autophagy also could be a selective process and be involved in regulation of the cellular number of organelles, including that of peroxisomes. The process of specific autophagic peroxisome degradation is known as pexophagy. Yeasts appear to be convenient model for studying molecular mechanisms of pexophagy, and most known *ATG* genes (from the term AuTophaGy) were identified in yeast studies. This review examines characteristics of general autophagy, other types of autophagy as well as pexophagy, in particular, functions of Atg proteins in general autophagy and in macro- and micropexophagy. Special attention is given to mechanisms of phagophore assembly, the role of phosphatidylinositol-3-phosphate in pexophagy, the role of peroxines (proteins involved in peroxisome biogenesis) in pexophagy, as well as properties of Atg proteins specifically involved in micropexophagy.

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GENERAL (NON-SELECTIVE) AUTOPHAGY

Autophagy is a process of recycling of intracellular constituents using vacuoles (lysosomes) [1]. Non-selective autophagy (or macroautophagy) is a process of nonspecific engulfing of some cytoplasm or organelles by a vacuole for recycling [2]. This process is important for cell survival under nutritional deficit. In many eukaryotes, in particular, yeasts, macroautophagy is also necessary for differentiation (yeast sporulation). During autophagy a double membrane covers a portion of the cytoplasm forming a structure called an autophagosome. Further, the outer layer of the autophagosome merges with vacuolar membrane, and the remaining single-membrane structure, called autophagic body, moves inside the vacuole where it is degraded by vacuolar hydrolases, with the formation of repeatedly used components (Fig. 1). Proteins taking part in autophagy are marked as Atg, and genes – as *ATG* [3]. Aside from macroautophagy, accidental engulfing by the vacuoles of cell components can occur by microautophagy, a direct invagination of a vacuolar membrane (Fig. 1) [4]. Atg proteins were first identified in yeasts, and then their homologs were found in the genomes of other eukaryotes. On the whole, general autophagy is performed by highly conservative components represented in all eukaryotes [5].

Basic steps and components of general (nonspecific) autophagy in yeasts can be presented as follows [6].

1. Signaling proteins required for autophagy induction: protein kinase Tor1, protein kinase A, Sch9, Tap42, and phosphatase type 2A.

2. Packaging of protein or organelle transported for degradation (Atg19, Atg11, and Atg8).

3. Formation of preautophagosomal structure (Atg1, Atg11, Atg13, Atg17, Atg29, and Atg31).

4. Vesicle nucleation (Atg6, Atg9, and phosphatidylinositol 3-kinase).

5. Vesicle expansion and completion (Atg3-5, Atg6, Atg7, Atg8, Atg10, Atg12, Atg14, and Atg16).

6. Protein retrieval (Atg1, Atg2, Atg18, Atg23, and Atg27).

7. Homotypical fusion of isolation membrane (Tlg2).

8. Transport and heterotypical fusion of autophagosome and vacuoles (v- and t-SNAREs, Ccz1, Mon1, and HOPS complex).

Abbreviations: Cvt, cytoplasm to vacuole targeting pathway; MIPA, micropexophagy apparatus; PAS, preautophagosomal structure (or phagophore assemblage site); phagophore, isolation membrane; PNS, perinuclear structure of nuclear membrane; VSM, vacuolar sequestering membranes.

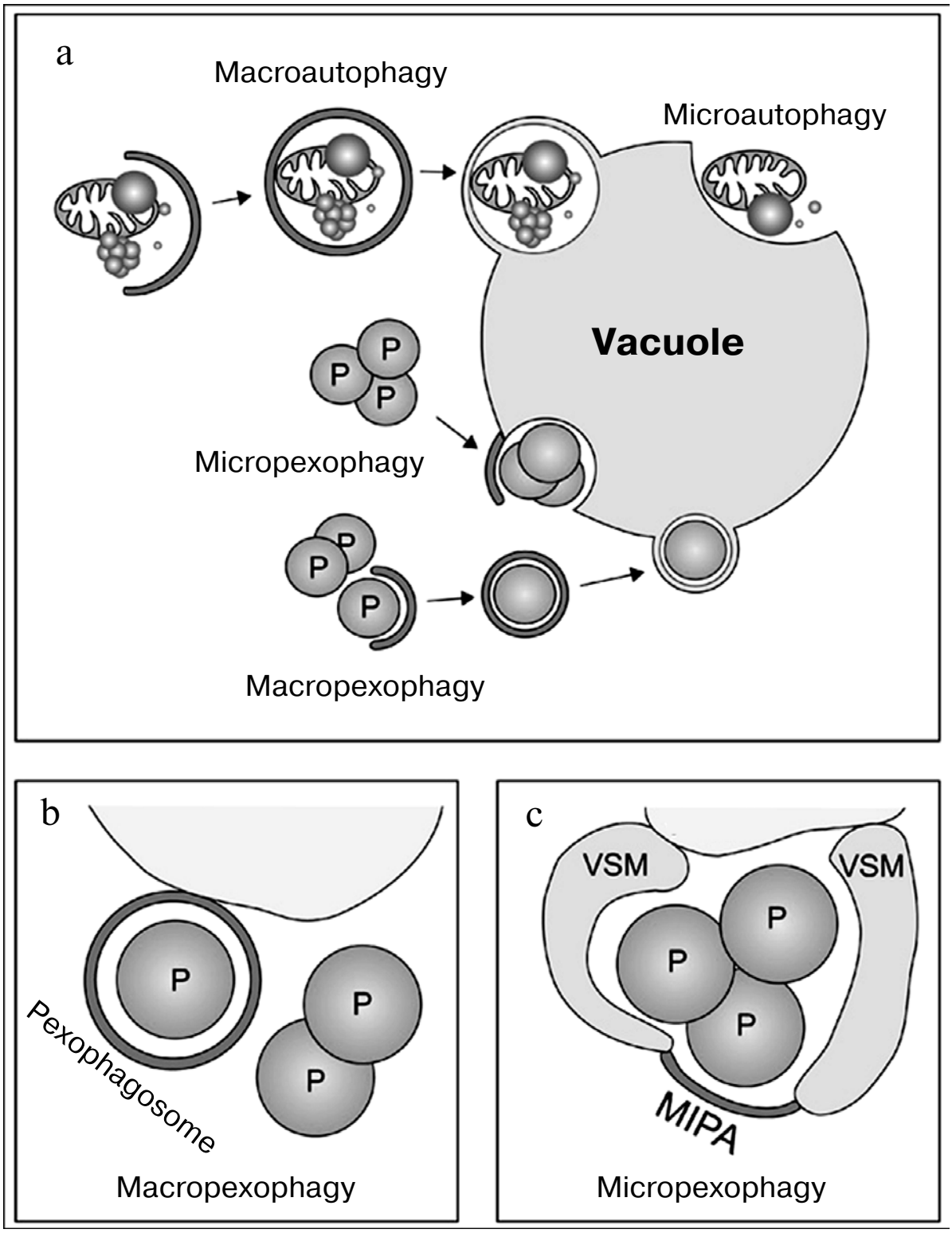

Fig. 1. Schemes of autophagy (a), macropexophagy (b) and micropexophagy (c) (after [6]). a) General (nonspecific) autophagy – nonspecific degradation of cytosol proteins and organelles through their engulfing by autophagosomes (macroautophagy) or invagination of vacuolar membranes around proteins and organelles meant for degradation (microautophagy). Thus pexophagy includes peroxisome (P) engulfing by means of macro- or micropexophagy. The formation of a pexophagosome around a peroxisome is a distinguishing feature of macropexophagy (b); vacuolar sequestering membrane (VSM) and cup-shaped micropexophagy apparatus (MIPA) engulfing a peroxisome cluster characterize micropexophagy (c).

9. Intravacuolar vesicle degradation (Atg15, proteinase A, and proteinase B).

From 35 currently known Atg proteins only 17 are necessary for all types of autophagy (selective and nonselective), whereas the other 18 are specific: either used in special pathways of selective autophagy or representing species-specific modifications.

Atg proteins participating in the first steps of autophagy. In eukaryotes the regulation of cell growth in response to nutrition in the medium and stress factors depends on protein kinase Tor [7]. In nutritionally rich medium Tor-containing complex (TORC1) inhibits macroautophagy, but it allows for a selective autophagy-related pathway for specific transport from cytoplasm to vacuole (Cvt, cytoplasm to vacuole targeting). The main effectors of Tor are most probably proteins of the phosphatase 2A family that regulate both pathways by phosphorylation–dephosphorylation of so-called Atg1 complex [8]. This complex is necessary for all types of autophagy, and it is mainly formed by Ser/Thr protein kinases Atg1 and Atg13. In sufficiently nutritious medium TORC1 blocks autophagy and Atg13 is hyperphosphorylated. Under limitation of nutrients the level of Atg13 phosphorylation is decreased, which increases its affinity to Atg1. The formed Atg1/Atg13 lowly phosphorylated complex initiates autophagy. Atg1 complex also performs as an important switch between macroautophagy and the selective Cvt pathway. Recent data show that kinase activity of Atg1 is necessary for both pathways [9]. Unfortunately, the physiological protein substrate for protein kinase Atg1 has not been identified. Atg1 and Atg13 are not the only components of the Atg1 complex. Many other Atg proteins can directly or indirectly associate with the complex (Atg11, Atg17, Atg19, Atg20, Atg24, Atg29, Atg31, and Vac8). Some of these participate only in macroautophagy, while others are required only for the Cvt pathway. It is not likely that all these components interact with Atg1/Atg13 complex simultaneously, but isolated complexes with different proteins have not been separated. Atg11 protein probably functions as a backbone connecting transported proteins with Atg1 [8]. However, it should be noted that Atg11 has been identified so far only in fungi [5].

Formation of double-membrane vesicles. Many autophagy pathways depend on processes of double membrane formation (phagophore) that sequesters proteins and organelles from the cytosol. The process starts with the transport of proteins and lipids to the membrane structure known as PAS, preautophagosomal structure [10, 11]. The complex that controls initial autophagy steps contains Ser/Thr protein kinase Vps15, phosphoinositol-3 kinase class III Vps34, and also Atg6 and Atg14 [12]. The activity of the complex on phosphatidylinositol localized on the membrane involves the binding of proteins Atg18 and Atg21, previously mentioned Atg20 and Atg24, and integral membrane protein Atg27 [13-15]. For the second step of vesicle formation two sets of proteins are necessary for ubiquitin-dependent conjugation reactions [16]. During the first reaction Atg4 protease processes ubiquitin-like Atg8 protein so that it covalently binds through glycine at the C-terminal to phosphatidylethanolamine in the preautophagosomal structure. The activity of E1 (Atg7) and E2 (Atg3) enzymes are necessary for this conjugation. Moreover, Atg8 binding to phosphatidylethanolamine also depends on the product of the second Atg12 reaction, which covalently binds with Atg5 through a conservative lysine residue. This step of conjugation is catalyzed by the same E1 (Atg7) enzyme, but a second enzyme is also required – E2 (Atg10). Conjugation using Atg5 and Atg12 is analogous to the action of E3 enzyme in conjugation of phosphatidylethanolamine with Atg8 [17]. Finally, coiled-coil Atg16 protein noncovalently binds to Atg5–Atg12 conjugate. It was shown that for vesicle growth the binding of phosphatidylethanolamine with Atg8 on the membrane and the formation of Atg5–Atg12/Atg16 complex are required. This complex probably forms a temporary cover giving shape to the phagophore membrane. Later integral membrane proteins Atg9 and Atg27 and membrane-associated protein Atg23 [18, 19] are transported as small membrane vesicles to the phagophore and included in it. During the growth of the membrane Atg9, Atg23, and Atg27 constantly move between phagophore and other membrane structures that are not yet identified.

Retrieval of autophagosome components. Not all the proteins participating in phagophore growth are localized on double-membrane structures. Many proteins including Atg8 and Atg5–Atg12/Atg16 complex are released after phagophore formation. Proteins Atg9, Atg23, and Atg27 are also continuously released in a process dependent on Atg1, Atg2, and Atg18 [18]. The release of covalently bound Atg8 from a formed phagophore includes detaching of phosphatidylethanolamine by Atg4 protease. However, a substantial part of Atg8 remains inside the vesicle and accompanies transport of proteins inside the vacuole where it undergoes degradation.

Degradation of captured material in a vacuole and release of degradation products. After formation of a double-membrane autophagosome, it merges with the outer vacuolar membrane. Components that participate in homotypical membrane fusion – like SNARE (attachment receptor of the soluble N-ethylmaleimide-sensitive factor) and HOPS (homotypic fusion and vacuole protein sorting complex) – are used at this stage [20]. Such a mechanism of homotypical fusion probably exists in *Saccharomyces cerevisiae* and other yeast species, in particular, *Pichia pastoris* [21]. The corresponding proteins are not called Atg because they also participate in membrane interaction other than autophagy. Such fusion completes with introduction of transported material into vacuolar matrix where it degrades. Yeast vacuoles contain many proteinases (proteinase A, proteinase B, car-

boxypeptidase S, and carboxypeptidase Y). Moreover, two Atg proteins necessary for degradation of specific proteins in vacuoles have been identified. Integral membrane proteins Atg15 and Atg22 probably take part in export of regenerated material from vacuole into cytosol [22-24].

The Cvt pathway of transport of resident hydrolases into the vacuole takes place under conditions of sufficient cell nutrition and includes transport of aminopeptidase I (Ape1) and α-mannosidase (Ams1) [25]. The Cvt pathway has been identified so far only in *S. cerevisiae* and *P. pastoris* [26, 27]. In this pathway Ape1 precursors form dodecamers, later assembled in larger Ape1 complexes. Later Cvt-specific Atg19 receptor binds to the Ape1 N-terminus forming the Cvt complex. It also contains α-mannosidase Ams1, a second protein that penetrates inside the vacuole using the Cvt pathway. Atg11 acts as an adaptor protein between Atg19 and the preautophagosomal structure allowing for compact packing of transported molecules in the double-membrane structure called a Cvt vesicle. This process includes activity of the basic autophagy pathway as well as of cytoskeleton actin [28]. Subsequently the outer layer of the Cvt vesicle merges with vacuolar membrane, and Cvt-transported protein is released inside the vacuole. It is interesting that Cvt vesicles formation from the autophagosomal structure is not initiated in the absence of transferred Atg11 and Atg19 proteins, evidencing the necessity of binding of transferred hydrolases with the preautophagosomal structure [29]. Moreover, Atg19 undergoes ubiquitination that increases its binding to the Cvt-transferred proteins [30].

Selective forms of autophagy. Many processes connected with autophagy that require Atg proteins have been discovered in recent years. Apart from transport of some resident vacuolar proteins from cytoplasm to vacuole (Cvt-pathway), there are degradation pathways: pexophagy, mitophagy, autophagy of endoplasmic reticulum, ribophagy, and partial nucleus microautophagy [31]. In all of these processes the recognition of transferred protein or organelle is a necessary stage initiating action of a set of proteins. Moreover, cytoskeleton actin also takes part in this process, which does not take place during nonspecific macroautophagy. For instance, the function and morphology of yeast mitochondria alter depending on growing conditions. Mitophagy (mitochondrial degradation using an autophagy mechanism) in yeast is induced after cells are transferred from medium with respiratory substrates into glucose medium or in medium with respiratory substrates in stationary phase during nitrogen deficiency. The main function of mitophagy is elimination of mitochondria containing increased levels of reactive oxygen species. Mitophagy is performed through nonselective macroautophagy, as well as selective processes of micro- and macromitophagy [32]. A few proteins participating in mitophagy have been identified in *S. cerevisiae*, but the mechanism of their action is still unknown [33]. Investigation of the role of *ATG* genes in mitophagy revealed a new protein, Atg32 receptor, participating exclusively in mitophagy [34, 35].

Apart from degradation using general autophagy mechanism, parts of endoplasmic reticulum (ER) during the process of general autophagy can be selectively packed in autophagosomes either in response to unfolded or lacking secondary structure proteins (unfolded-protein response, UPR), or during starvation [36, 37]. ER autophagy as unfolded-protein response depends on Atg proteins, while during starvation this process is only partly Atg-dependent [36, 38]. Possibly, only some part of the phagophore assembly apparatus takes part in the absorption of ER parts by autophagosomes. The participation of actin in this process suggests its selectivity [36].

For a long time ribosomes were assumed to degrade through general autophagy, but in some cases the rate of ribosomal degradation exceeds the rate of degradation of cytosolic proteins, which indirectly suggests the presence of a selective mechanism [39]. In ribophagy of 60S and 40S ribosomal subunits both main Atg proteins of general autophagy and specific proteins are active. It is known that specific degradation of 60S subunit, but not 40S subunit, did not occur in the absence of ubiquitin protease Ubp3 or its cofactor Bre3. Both proteins interact with Cvt receptor Atg19 [30]. Ubiquitin conjugation is probably required for effective transfer of proteins and organelles into the vacuole.

Even the nucleus undergoes vacuolar degradation. In yeasts, starvation leads to "detachment" and degradation of nuclear fragments in vacuoles during the process of so-called Piecemeal Microautophagy of the Nucleus (PMN). This process depends on the main Atg proteins participating in general autophagy, and also Atg11, indicating selectivity of the process [40]. During piecemeal microautophagy of the nucleus nuclear–vacuolar junctions, or NVJs, are formed through the interaction of vacuolar membrane protein Vac8 and outer nuclear membrane protein Nvj1 [41]. Starvation increases Nvj1 level and initiates its binding with ankyrin repeats of Osh1 (oxysterol-binding protein homolog) and enoyl reductase Tsc13 (temperature-sensitive suppressors of Csg2 mutants) [42, 43]. As a result part of Nvj1 grows into the vacuole forming a vesicle that detaches into the vacuole lumen where it subsequently degrades.

PEXOPHAGY

The number of peroxisomes in a cell is strictly regulated not only through the regulation of peroxisome growth and division but also though their specific degradation by the autophagy mechanism known as pexophagy [44, 45]. Methylotrophic yeasts are probably the most convenient object for studying molecular mechanisms of pexophagy because simple transfer of cells from methanol

medium to glucose or ethanol medium causes degradation of about half of the cell contents within a few hours.

Pexophagy is a special case of general autophagy, which is a nonspecific degradation of cytosol contents. Special methods for selection of mutants with pexophagy defects have been developed [46-52]. All of them belong to negative selection methods when a few mutant colonies grow on plates among a huge number of wild type colonies, and mutants are identified afterwards directly in colonies using peroxisome enzyme analysis [52]. Apart from mutagenesis under standard mutagen treatment, insertion mutagenesis using DNA fragments was proposed, which substantially facilitates further cloning of mutant genes [49]. Studying pexophagy in the methylotrophic yeast *P. pastoris* revealed two morphologically diverse pexophagy processes, called macropexophagy and micropexophagy [6, 44, 53, 54] (Fig. 1). During macropexophagy initiated by transferring cells from methanol medium to ethanol medium, individual peroxisomes are gathered in double-membrane structures called pexophagosomes that merge with vacuoles leading to degradation and repeated usage of pexophagosomal contents. During micropexophagy (occurring after transferring methylotrophically grown cells to glucose medium) peroxisome clusters are engulfed by vacuolar sequestering membranes (VSM) and specific micropexophagy apparatus (MIPA) [55], which forms a cap above a cup-shaped vacuolar sequestering membrane surrounding a peroxisome [56]. Heterotypical fusion between vacuolar sequestering membranes and the specific micropexophagy apparatus transports peroxisomes inside the vacuole for degradation and repeated use of its components. The specific micropexophagy apparatus and pexophagosome originate from the preautophagosomal structure PAS. Glucose and ethanol were shown to be specific inducers of micro- and macropexophagy, correspondingly. Micropexophagy turned out to be more sensitive to decrease of intracellular ATP compared to macropexophagy; in other words, intracellular ATP pool plays a more important role in defining the pexophagy pathway than the nature of the carbon substrate [57]. However, this work has not received independent confirmation.

In *S. cerevisiae* pexophagy can be experimentally initiated by transferring cells from medium containing oleate, a peroxisome biogenesis inducer, to glucose medium lacking a nitrogen source [58]. In *P. pastoris* peroxisome biogenesis is induced by methanol, oleate, or amines. Transferring cells from methanol medium to ethanol medium, or from medium containing oleate or methylamine to glucose medium, induces macropexophagy [59, 60]. However, transferring cells from methanol medium to glucose medium induces micropexophagy [53]. It is interesting that in *Hansenula polymorpha* such adaptation to nutritional medium (transfer from methanol to glucose medium) induces macropexophagy [61]. Transfer of *H. polymorpha* cells to medium with nitrogen deficiency causes peroxisome degradation using the micropexophagy pathway [61, 62].

Sensing and signaling during specific pexophagy. The questions of cell recognition of pexophagy effectors (ethanol and glucose) and transmission of signals initiating sequence of events ending up with peroxisome degradation in vacuoles remain largely unknown. On the model of the methylotrophic yeast *Pichia methanolica* the nature of effector inducing pexophagy after transferring methylotrophically grown cells to ethanol medium was identified. Mutants with defects of each step of ethanol catabolism were obtained for this purpose. Mutants unable to grow in medium with ethanol and acetate as the only source of carbon were isolated. Mutants were divided into four complementation groups, *icl1*, *mls1*, *pck1*, and *mdd1*, which were lacking isocitrate lyase, malate synthase, phosphoenolpyruvate carboxykinase, and malate enzyme, respectively [63-66]. Mutants tolerant to 2-fluoroacetate that could not use ethanol and acetate as carbon sources were also isolated. Such mutants were divided into three complementation groups, *acs1*, *acs2*, and *acs3*, where mutants of each group were all lacking activity of the same enzyme – acetyl-CoA synthetase. Mutant *adh1* tolerant to allyl alcohol, whose alcohol dehydrogenase activity was decreased 30-40-fold, and also double mutant *adh1 adh2* totally lacking alcohol dehydrogenase activity were obtained [65, 67]. Mutant *aldX* capable of growing on acetate, but not ethanol, medium was also obtained [65, 66]. Studying the influence of ethanol and acetate on inactivation of alcohol oxidase and catalase and peroxisome degradation showed that these processes are disturbed in *acs1*, *acs2*, *acs3*, *icl1*, *adh1 adh2*, and *aldX* mutants in ethanol medium and only in *acs1*, *acs2*, and *icl1* mutants in acetate medium [65-67]. Glyoxylate was assumed to be direct pexophagy effector in ethanol medium in *P. methanolica* as the synthesis of only this substance is disturbed in mutants defective in acetyl-CoA synthetase, isocitrate lyase, and malate synthase [3, 65, 66].

The possible glucose intermediates initiating pexophagy remain unknown. Besides glucose itself, these substances may include glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and other intermediates of glycolysis and the pentose phosphate pathway. *Saccharomyces cerevisiae* is reported to have three partially independent pathways for glucose sensing and signaling. These are pathways that use membrane glucose receptors – the Gpr1/Gpa2 pathway (participates in cell response to stress) and the Snf3/Rgt2 pathway (participates in induction of genes of hexose transporters and a number of intracellular enzymes), and also a pathway using intracellular glucose sensor hexokinase 2 Hxk2 (participates in glucose catabolic repression) (reviews [68-70]). The role of first two signal pathways in pexophagy is partly analyzed. It turned out that in *S. cerevisiae* defects of Gpr1 receptor or Gpa2 G-protein inhibit pexo-

phagy [71]. Recently the role of Slt2, mitogen-activated protein kinase (MAPK), was revealed in pexophagy [72]. Defects of individual glucose sensors, Snf3 (high affinity sensor) or Rgt2 (low affinity sensor), had slight influence on pexophagy, but lack of both sensors Snf3 and Rgt2 significantly retarded pexophagy [73]. It was concluded that for initiation of pexophagy in glucose medium both functioning of the Gpr1/Gpa2 signaling pathway and glucose transport inside the cell (totally absent in mutants with defects of Snf3 and Rgt2 sensors) are required. The possible role of a signaling pathway using Hxk2 intracellular sensor remains unknown. It is interesting that in methylotrophic yeast *P. pastoris* deletions of *GPR1* and *GPA2* gene orthologs did not disturb pexophagy, indicating differences in mechanisms of glucose-induced signal transduction in pexophagy in different species of yeasts. One of the signal molecules participating in micropexophagy in *P. pastoris* is α-subunit of phosphofructokinase Pfk1, whose catalytic activity is not necessary for glucose-induced micropexophagy [74].

Proteins participating in pexophagy. Many Atg proteins participating in general autophagy and Cvt pathways of transfer of some resident vacuolar hydrolases also take part in selective pexophagy. Unique proteins of selective autophagy include Atg19 receptor participating in the Cvt pathway, and Atg11 protein, which is selective factor integrating receptor protein with basic proteins of general autophagy [75]. Receptor Atg19 has double function: binding transported structure and interaction with one or more components of the autophagy apparatus on the preautophagosomal structure [1, 75]. In *S. cerevisiae* Atg19 interacts with Atg11, which is an organizing component of the preautophagosomal structure. Additionally, Atg19 interacts with Atg8, a key protein for the formation of isolation membrane from the preautophagosomal structure. Such receptors transferring peroxisomes and components initiating the growth of isolation membrane from the preautophagosomal structure are also present during pexophagy. Thus, peroxisomal receptor Atg30 from *P. pastoris* interacts with peroxisomes using two peroxisomal membrane proteins Pex3 and Pex14, and also with the components of the autophagy mechanism through Atg11 and Atg17, which organize the preautophagosomal structure [76]. Unlike Atg32 participating in the Cvt pathway, Atg30 probably does not interact with Atg8. Three selectivity factors (Atg19, Atg30, and Atg32) interact directly with adaptor protein Atg11; this suggests that the latter is a common component of all known autophagy pathways. During pexophagy, Atg30 phosphorylation is a necessary condition for interaction with Atg11 and this interaction probably takes place on the preautophagosomal structure [76]. Phosphorylation of another autophagy receptor, Atg19, is also described, but whether it has any physiological importance remains unknown [77]. Thus, pexophagy includes the following steps: sensing and signaling; receptor modification; receptor interaction with adaptor protein Atg11; Atg11-dependent organization of the preautophagosomal structure; interaction with Atg8 for control of isolation membrane elongation around the peroxisome. It is interesting that three receptors described for the Cvt pathway, mitophagy, and pexophagy are species-specific.

There are proteins that participate in autophagy and determine peroxisome size [60]. In the case of macropexophagy, Atg11 and Atg26 (sterol glucosyl transferase) are used for degradation of large size or large and medium size peroxisomes respectively, but not for small peroxisomes. In *S. cerevisiae* the amount of Atg8 on the surface of the preautophagosomal structure determines autophagosome size [78]. During pexophagy the amount of Atg8 changes insignificantly, but the amount of Atg8 conjugated with phosphatidylethanolamine is doubled [6]. If Atg11 and Atg26 regulate the efficiency of Atg8–phosphatidylethanolamine complex formation, they can also participate in regulation of larger size pexophagosome formation. Moreover, interaction between Atg30 on the peroxisomal surface and Atg11 can also form matrix increasing the surface of the isolation membrane. Additional proteins required for pexophagy are phosphatidylinositol-3-phosphate binding proteins such as Atg24, which binds phosphatidylinositol-3-phosphate through specific PX (or Phox, first found in p40phox and p47phox domains of NADPH oxidase; Phox meaning Phagocytic oxidase) domain [79]. There are data about additional proteins specifically taking part in pexophagy, in particular, phosphatidylinositol-4-kinase Pik1 that generates phosphatidylinositol-4-phosphate on the preautophagosomal structure during pexophagy [80, 81]. This lipid recruits Atg26 that has a GRAM (Glucosyltransferases, Rab-like GTPase activators and Myotubularins) domain, resulting in binding with the preautophagosomal structure where it participates in the formation of the specific micropexophagy apparatus and pexophagosomes. Another example of a selective factor is the coiled-coil protein Atg25 necessary for macropexophagy in *H. polymorpha* [82]. Atg25 is present in pexophagosomes and participates in final stages of peroxisome isolation or fusion of pexophagosomes with the vacuolar membrane [83]. Because micropexophagy requires a specific micropexophagy apparatus that is not described for macroautophagy and the Cvt pathway, it is not surprising that the proteins specifically necessary for its formation are revealed.

Pexophagy-specific preautophagosomal structures. The origin and formation of isolation membranes during all autophagy processes is interesting in the respect that the only membrane protein Atg9, which can be the source of membrane for phagophore formation, is disappearing from autophagosomes at the moment of their formation [84-86]. All other components of the main autophagy proteins bind to the preautophagosomal structure in special order [87, 88]. Preautophagosomal structures specif-

ic for Cvt pathways and autophagy have been identified. Autophagy-specific preautophagosomal structure requires Atg17, Atg29, and Atg31, while Cvt-specific preautophagosomal structure requires Atg11 and Atg19 [88-90]. It is interesting that pexophagy-specific preautophagosomal structure requires Atg11, Atg17, and Atg30 [60]. In *atg29* and *atg31 S. cerevisiae* mutants, pexophagy is disturbed while the Cvt pathway remains normal [34, 91]. In *P. pastoris* species-specific protein Atg28, a homolog of Atg29 and Atg31 from *S. cerevisiae*, is required for all autophagy pathways [92]. *Pichia pastoris* Atg28 was shown to have homology not only to *S. cerevisiae* Atg29 (at the N-terminus) but also to Atg31 (at the C-terminus), and therefore it can function as both mentioned proteins [60]. This is why yeasts carrying Atg29 and Atg31 lack Atg28, and vice versa.

Role of phosphoinositols in pexophagy. It is widely known that phosphatidylinositol-3 kinases are necessary for autophagy in yeasts and mammals [93]. In yeasts synthesis of phosphatidylinositol-3-phosphate is controlled by phosphatidylinositol-3 kinase Vps34, which is specifically required for autophagy. Two complexes containing Vps34 have been revealed [12, 94]. Complex I in *S. cerevisiae* consists of Vps15, Vps34, Vps30/Atg6, and Atg14. Complex II participating in vacuolar protein sorting (VPS) carries the same first three proteins as complex I, but instead of Atg14 it contains Vps38 [12]. In *P. pastoris*, the UVRAG homolog (UV radiation resistance associated gene) was described that takes part in vacuolar protein sorting, but not in autophagy [95]. There were no Atg14 homologs revealed in *P. pastoris*. Complex I plays a role during the step of vesicle nucleation on PAS [56, 93]. The formation of the pexophagosome during macropexophagy in *P. pastoris* proceeds through the following stages (all are dependent on phosphatidylinositol-3-phosphate generation on PAS [95]): Atg6 localizes at a few perivacuolar dots; Atg8 begins to localize on PAS; isolation membrane starts growing from PAS; isolation membrane assimilates peroxisome forming the pexophagosome. Atg6 remains bound to PAS during the whole process.

Using green fluorescent protein (GFP) fused with FYVE domain (called after four cysteine-rich proteins Fab1, YOTB, Vac1, and EEA1 in which this domain has been identified) able to bind with phosphatidylinositol-3-phosphate, it was shown that during pexophagy phosphatidylinositol-3-phosphate is localized on the concave part of the isolation membrane [96]. Other phosphatidylinositol-3-phosphate-binding proteins, such as Atg18, are also localized there [97]. The way phosphatidylinositol-3-phosphates constrain their localization to the concave part of double membrane structure remains unclear. Considering that effective pexophagosome formation requires additional components, it is possible that these phosphatidylinositol-3-phosphate-binding proteins provide additional framework for assimilation of large size peroxisomes [60]. It is possible that this framework during interaction with peroxisomes can stabilize the completion of pexophagosome formation around large peroxisomes. In such cases the growth of isolation membrane during elongation may be described as "zipper" type, and the completion of pexophagosome formation may occur through homotypical fusion.

Peroxisomal proteins required for pexophagy. In *P. pastoris* Atg30 interacts with two proteins, Pex3 and Pex14, localized on the peroxisomal membrane [76]. Effective peroxisome homeostasis probably requires their biogenesis and degradation to be coordinated. It was shown that interacting partners of Atg30 are proteins participating in peroxisome biogenesis. Thus, Pex3 is important for peroxisome biogenesis, and Pex14 – for protein import to peroxisomal matrix [98]. In *H. polymorpha* Pex14, more exactly the 64 N-terminal amino acid residues (a.a.), are necessary for pexophagy [60, 61]. Also, it was shown that during macropexophagy in *H. polymorpha* Pex3 is withdrawn from peroxisomes and does not undergo degradation [99]. The way Pex3 is removed from peroxisomes is unknown. Pex3 is known to be required for stabilization of a complex of proteins with a RING-finger domain (Really Interesting New Gene, structural domain similar to protein zinc finger) of peroxisome importer [100]. Therefore, at this stage, besides inhibiting peroxisome biogenesis, also destabilization of some complexes in peroxisomal membrane occurs.

In *P. pastoris* a gene called *PDG1* (Peroxisome DeGradation) was identified whose mutations led to disturbances in peroxisome degradation ([45], O. V. Stasyk and A. A. Sibirny, unpublished data). Moreover, such mutations disturbed localization of peroxisomal proteins that, apart from peroxisomes, were also localized in cytosol, indicating disturbance in peroxisome biogenesis in *pdg1* mutants. Corresponding protein Pdg1 is a membrane peroxin, which confirms its role in peroxisome biogenesis.

Micropexophagy-specific genes in *P. pastoris*. First we shall give a list of specific Atg proteins in *P. pastoris* participating in both types of pexophagy. These are peroxisome receptor Atg30, its adaptor proteins Atg11 and Atg17, sterol glucoside transferase Atg26, and protein Atg28 [44, 60, 76, 79, 92, 101-103]. Double interaction of Atg30 with functionally active Atg11 and Atg17 organizes the preautophagosomal structure in *P. pastoris* [60, 76]. Atg17 interacts and colocalizes with Atg28, a protein of the basic autophagy mechanism participating in all autophagy pathways, an element connecting pexophagy-specific and general components of preautophagosomal structure [60, 92]. As mentioned earlier, Atg28 protein in *P. pastoris* is homologous to two proteins of *S. cerevisiae*, Atg29 and Atg31, which interact with Atg17 and, together with Atg1 and Atg13, organize preautophagosomal structure in *S. cerevisiae* [89, 104-106]. Proteins Vps15 and Vps34 also take part in macro- and macropexophagy (see above).

The presence of a specific morphological structure in the micropexophagy process, the micropexophagy apparatus MIPA in *P. pastoris*, suggests the existence of specific genes and proteins participating in this process. The earlier mentioned gene *PFK1* (PhosphoFructoKinase 1) encodes phosphofructokinase 1 α-subunit, which is required for peroxisome engulfment by vacuoles after transferring *P. pastoris* cells from methanol medium to glucose medium [74]. Participation of phosphofructokinase 1 α-subunit in micropexophagy does not depend on its ability to phosphorylate fructose-6-phosphate since a catalytically inactive form of this enzyme provides for normal pexophagy. Moreover, the *VAC8* gene (VACuole related) was identified whose product is a 60-64 kDa protein with so-called *armadillo* repeat that specifically participates in micro- but not macropexophagy [59, 107]. In mutant cells, vacuolar sequestering membrane during micropexophagy is not formed. Hybrid protein Vac8–GFP is localized on vacuolar membrane independently of Atg1, Atg9, or Atg11. Deletion of repeating *armadillo* did not alter Vac8 localization, but the protein lost its functionality. Vac8 probably participates in early (formation of sequestering membrane) and late (membrane fusion after formation of micropexophagy apparatus) micropexophagy stages. Micropexophagy and vacuole inheritance were shown to be dependent on different Vac8 domains [108].

The new micropexophagy-specific protein Atg35 was identified during the analysis of partners interacting with our earlier discovered protein Atg28 from *P. pastoris* [92]. To search for such Atg proteins, a yeast two-hybrid (YTH) screening system was used for the first time. YTH screening of the genome database of *P. pastoris* DNA was carried out in *S. cerevisiae* cells using *Pp*Atg28 as "bait". As positive control we used the interaction between human Ras (Rat sarcoma) and shortened Raf1 (Rapidly Accelerated Fibrosarcoma) protein (57-648 a.a.) [109].

Using analysis of DNA sequence of two identified positive clones two sequences were revealed encoding proteins Atg35 and Rdi1 (Rho GDP Dissociation Inhibitor). Atg35 consists of 463 a.a. and incorporates two putative domains: RING-finger (5-47 a.a.) and PHD (Plant Homeo Domain) (88-128 a.a.). The closest Atg35 homologs are hypothetical proteins from *Pichia stipitis* (PICST_60919), *P. guilliermondii* (PGUG_03993) and *Clavispora lusitaniae* (CLUG_01611).

Testing *P. pastoris* mutant *atg35*Δ for pexophagy using peroxisome alcohol oxidase immunoblotting revealed that after transferring of methanol-grown cells to ethanol medium (macropexophagy) the mutant did not differ from wild type, while after transferring of methylotrophically grown cells into glucose medium (micropexophagy is induced) the level of alcohol oxidase decreased more slowly than that in wild type cells (Fig. 2). Thus, only micropexophagy and not macropexophagy is disturbed in the *atg35*Δ mutant. Comparison of vacuolar isolating membrane formation and the micropexophagy apparatus in wild type and mutant using fluorescent microscopy with Atg8 fused with GFP on N-terminus (GFP–Atg8) for labeling the micropexophagy apparatus, blue fluorescent protein (BFP) with C-terminal tripeptide Ser-Lys-Leu (BFP–SKL) for peroxisome labeling, and *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64) for labeling vacuolar isolating membranes revealed that formation of vacuolar isolating membranes in the mutant was normal. The micropexophagy apparatus MIPA was found in *atg35*Δ cells 1.5 times less frequently than in wild type cells, while in *atg28*Δ mutant the micropexophagy apparatus was not being formed at all (Fig. 2). However, both mutants (*atg28*Δ and *atg35*Δ) exhibited normal formation of pexophagosomes during macropexophagy, which were not formed at all in *atg1*Δ mutant (Fig. 2). Studying the role of Atg35 in general autophagy and Cvt pathways revealed that this protein is not required for either process (Fig. 2). Thus, Atg35 is necessary only for micropexophagy at the stage of micropexophagy apparatus formation [110]. It is interesting that overexpression of *ATG35* as well as deletion of this gene inhibits micropexophagy but does not influence macropexophagy. With *ATG35* overexpression the formation of vacuolar sequestering membranes was not disturbed, while formation of the micropexophagy apparatus was blocked. However, overexpression of *ATG35* did not influence general (nonspecific) autophagy [110].

Studying expression of genes *ATG28* and *ATG35* during peroxisome proliferation and degradation revealed that corresponding proteins are almost completely absent in ethanol medium and are present in significant amounts in glucose and methanol medium.

Atg35 contains putative nuclear localization signal. Testing of localization of overexpressed hybrid protein Atg35–eYFP (enhanced Yellow Fluorescent Protein) compared to marker of peripheral endoplasmic reticulum/nuclear membrane Sec61–mCherry (hybrid protein consisting of Sec61 protein (SECretory) and red fluorescent protein mCherry, monomeric red fluorescent protein with fluorescence close to cherry color), and marker of preautophagosomal structure marked by overexpressed eCFP–Atg17 (enhanced Cyan Fluorescent Protein), revealed that in methanol-grown cells eCFP–Atg17 was localized in a few dot structures distributed across the endoplasmic reticulum, though sometimes they could also be found on nuclear membrane. After transferring cells to glucose medium there were no significant changes in eCFP–Atg17 localization observed. In methanol medium Atg35–eYFP was localized in the nucleus and single dot-like structures on nuclear membrane (perinuclear structure, PNS). Transfer of cells to glucose medium caused homogenous distribution of Atg35–eYFP in

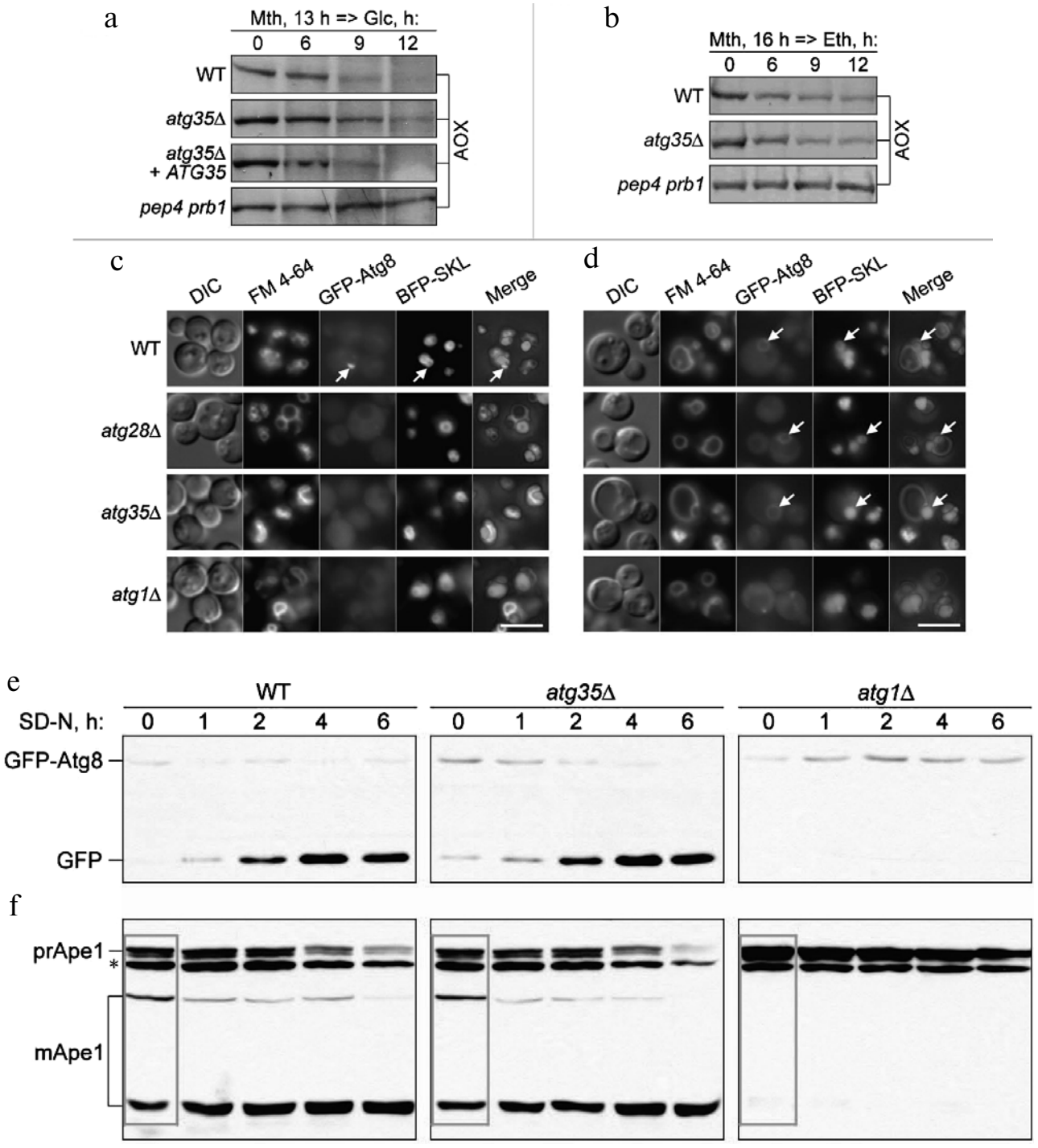

Fig. 2. Atg35 – a specific micropexophagy protein required for effective MIPA formation [110]. Cells were grown in methanol medium and transferred into glucose (a) or ethanol (b) medium. At set time intervals culture samples were analyzed for alcohol oxidase using immunoblotting. "*atg35*Δ + *ATG35*", *atg35*Δ mutant complemented by *ATG35* gene (SVN2). Atg28 and Atg35 are specifically required for MIPA formation. Wild type cells WT (STN78), *atg28*Δ (STN87), *atg35*Δ (STN179), and *atg1*Δ (STN71) expressing GFP–Atg8 and BFP–SKL were grown overnight in methanol medium with FM 4-64 strain and transferred to glucose (c) or ethanol (d) medium for 1 h. VSM was labeled with FM 4-64, MIPA and pexophagosomes – with GFP–Atg8, and peroxisomes – with BFP–SKL; arrows in (c) indicate MIPA, in (d) – pexophagosomes (line 5 μm); Atg35 is not required for general autophagy and Cvt pathway; e) GFP–Atg8 processing; f) prApe1 maturation analysis. Cells of wild type WT (STN70), *atg35*Δ (SVN3), and *atg1*Δ (STN66) expressing GFP–Atg8 were cultivated overnight in glucose medium and transferred to glucose medium lacking nitrogen. At set time intervals culture aliquots were taken and immunoblotting was carried out with GFP (e) and Ape1 antibodies (f); *, nonspecific band; in frame, prApe1 maturation under vegetative conditions.

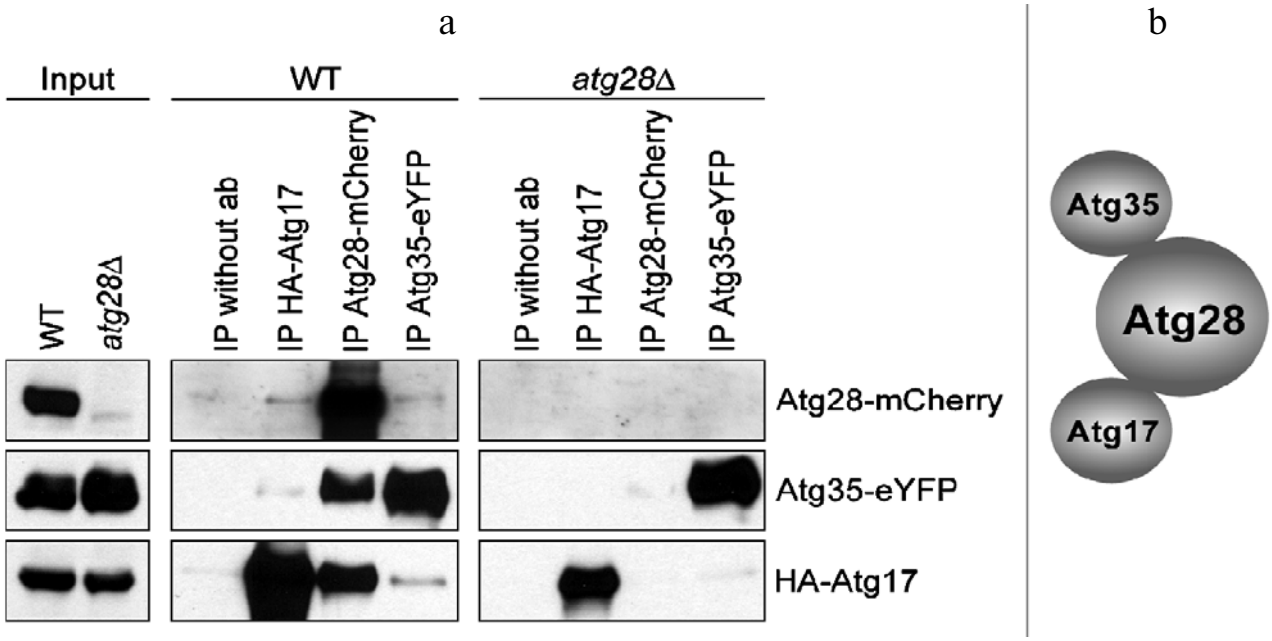

Fig. 3. Atg28 provides interaction of Atg35 with Atg17 [110]. a) Atg17, Atg28, and Atg35 coimmunoprecipitation. Wild type strain overexpressing Atg28–mCherry, Atg35–eYFP, and HA–Atg17 (STN401) and *atg28*Δ mutant overexpressing Atg35–eYFP and HA–Atg17 (STN387) were grown for 16 h in methanol medium and transferred for 1 h to glucose medium. Immunoprecipitation data is shown. b) Atg17, Atg28, and Atg35 interaction scheme.

the nucleus. However, combined overexpression of eCFP–Atg17 caused relocalization of Atg35–eYFP to single dot-like structures of nuclear membrane during micropexophagy. Moreover, in glucose medium Atg35–eYFP on dot-like structures of nuclear membrane colocalized with one of the eCFP–Atg17 dots. Obviously eCFP–Atg17 accumulation on nuclear membrane during micropexophagy is necessary for organization of single dot-like structures of nuclear membrane and involvement of Atg35–eYFP into this structure. Supposedly, Atg35–eYFP localization on single dot-like structures of nuclear membrane in glucose medium depends on eCFP–Atg17 and is significant for the micropexophagy process.

Atg28 is known to interact with Atg17 [59] and Atg35 [110]. Overexpression of Atg28–mCherry, Atg35–eYFP, and HA–Atg17 (HA, HemAgglutinin) was performed in wild type cells after transferring them from methanol medium to glucose medium for micropexophagy induction, and possible interaction of these proteins was evaluated using coimmunoprecipitation. The results showed that Atg17–Atg35 interaction may be due to Atg28 protein, as after introduction of Atg35–eYFP and HA–Atg17 in *atg28*Δ mutant no coprecipitation of Atg35–eYFP and HA–Atg17 was observed. These data suggest that Atg28 plays a central role in the interaction of Atg17 and Atg35 (Fig. 3).

Therefore, Atg35 is the first revealed nuclear Atg protein participating in autophagy in yeasts. Deletion and overexpression of this gene lead to specific disturbance of micropexophagy alone. Atg35 protein functions through interaction with Atg17 and Atg28, the latter protein playing a central role in this interaction [110].

It should be noted in the conclusion that autophagy and pexophagy occur in all eukaryotic cells, from yeasts to human, and yeasts appear to be a convenient model system for studying mechanisms of these processes. Studies on identification of morphologically distinct stages of pexophagy and genes and proteins involved in this process revealed general and specific characteristics of pexophagy compared to nonspecific autophagy. Recent studies performed in the author's lab surprisingly revealed that studying pexophagy in yeasts can have important practical significance. Defect of the *VPS34* gene in *H. polymorpha* was found to cause twofold increase of alcohol fermentation productivity [111]. Phosphatidylinositol-3 kinase encoded by *VPS34* in *S. cerevisiae* is known to be required for pexophagy [112]. Further, we have shown that deficiency of *ATG11*, *ATG13*, and *ATG25* genes in *H. polymorpha* also increases ethanol synthesis productivity by 1.5-2.0-fold (O. Gryniv, O. Kurilenko, D. Grabek-Leiko, K. Dmitruk, and A. Sibirny, unpublished data). The mechanisms of connection between pexophagy and effectiveness of alcohol fermentation are still unknown, and this unexplained interaction is a goal of our future studies.

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