Intravacuolar Membrane Lysis in *Saccharomyces cerevisiae*

DOES VACUOLAR TARGETING OF Cvt17/Aut5p AFFECT ITS FUNCTION?

The integral membrane protein Cvt17/Aut5p is a putative lipase essential for intravacuolar lysis of autophagic bodies. It is localized at the endoplasmic reticulum, from which it is targeted via the multivesicular body (MVB) pathway to intravacuolar MVB vesicles. Proteinase protection experiments now demonstrate that the Aut5p amino terminus is located in the cytosol, and the carboxy terminus is located inside the ER lumen. In contrast to procarboxypeptidase S, targeting of Cvt17/Aut5p to MVB vesicles is not blocked in cells lacking the ubiquitin ligase Tul1p or the deubiquitinating enzyme Doa4p. Also, truncation of the amino-terminal cytosolic Cvt17/Aut5p domain does not inhibit its targeting to MVB vesicles. These findings suggest that similar to Sna3p sorting of Cvt17/Aut5p to MVB vesicles is independent of ubiquitination. By fusing the ER retention/retrieval signal HDEL to the carboxy terminus of Cvt17/Aut5p, we generated a construct that is held back at the ER. Detailed analysis of this construct suggests an essential role of vacuolar targeting of Cvt17/Aut5p for its function. Consistently, aut5Δ cells are found impaired in vacuolar degradation of autophagocytosed peroxisomes. Importantly, biochemical and morphological data further suggest involvement of Cvt17/Aut5p in disintegration of intravacuolar MVB vesicles. This points to a general function of Cvt17/Aut5p in intravacuolar membrane breakdown.

Autophagy is a starvation-induced transport pathway delivering intracellular material for degradation to the lysosome (vacuole) (for review see Refs. 1 and 2). Three independent approaches in the model eukaryote *Saccharomyces cerevisiae* identify numerous autophagic proteins termed Apg (3), Aut (4), and Cvt proteins (5).

In starving cells proaminopeptidase I is specifically targeted to the vacuole via otherwise unspecific autophagy. Proaminopeptidase I is proteolytically matured in the vacuole; this opens a convenient way to monitor autophagy. In non-starved cells proaminopeptidase I transport is taken over by the Cvt pathway. The Cvt pathway and autophagy are morphologically very similar and use many common components (5); however, the Cvt pathway does not transport cytosolic material (7).

Autophagy starts at the preautophagosomal (perivacuolar) structure (8–10) with the formation of transport vesicles (autophagosomes), which nonspecifically enclose parts of the cytosol. Autophagy differs from other protein transport pathways by using transport intermediates (autophagosomes) surrounded by two membrane layers. Consequently, after fusion with the vacuolar membrane, their cytosolic content is not released into the vacuolar lumen, but instead released as membrane-enclosed autophagic bodies. Therefore, before vacuolar breakdown of the autophagocytosed material the membrane of autophagic bodies has to be lysed. Clearly, this lysis of membranes must be strictly limited to the membranes of autophagic bodies and must not affect the integrity of the vacuolar limiting membrane. Specific intracellular membrane lysis is a fascinating feature of eukaryotic cells, which is also of medical interest, since it is involved in the pathogenesis of some microorganisms (11).

In yeast, VacA proteinases A (encoded by the *PEP4* gene) and B (PRB1 gene) are required for lysis of autophagic bodies (12), but their molecular function in disintegrating lipid membranes remains enigmatic. Further components of the lysis machinery, Aut4p (13) and Cvt17/Aut5p (14, 15), were recently uncovered. Importantly, Cvt17/Aut5p contains a lipase (or esterase) active site motif, which by site-directed mutagenesis of the active site serine was shown to be essential for its activity (14, 15). Our previous work demonstrated that the integral membrane protein Cvt17/Aut5p is targeted from the ER, where a significant steady state pool is detectable via the multivesicular body (MVB) pathway to ∼50-nm intravacuolar MVB vesicles, which in wild-type cells are degraded dependent on vacuolar protease A (15). The MVB pathway starts at the prevacuolar compartment (late endosome) (16, 17). Here, dependent on several Vps class E proteins, some membrane proteins are sorted to membrane regions of the prevacuolar compartment, which afterward invaginate and bud off as ∼50-nm MVB vesicles into the interior of the prevacuolar compartment. This process results in formation of a prevacuolar compartment filled with vesicles, a structure termed the multivesicular body. After its fusion with the vacuole the MVB vesicles are released into the vacuolar lumen and degraded. Two different modes have been described for sorting of membrane proteins to MVB vesicles. Procarboxypeptidase S sorting requires ubiquitin conjugation at its lysine residue at position 8 by the ubiquitin ligase Tul1p and the presence of Doa4p, which releases ubiquitin from ubiquitin-protein conjugates (16, 18). In contrast, sorting of Sna3p to MVB vesicles is independent of ubiquitination (19).

We here show that Cvt17/Aut5p has a membrane topology...
similar to procarboxypeptidase S, with its amino terminus located in the cytosol and the carboxyl terminus in the ER lumen. However, in contrast to procarboxypeptidase S, the sorting of Cvt17p to MVB vesicles takes place in the cytosol and the carboxyl terminus in the ER lumen. These findings suggest that Cvt17/Aut5p to MVB vesicles takes place in a manner similar to Sna3p.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Media**—Media were prepared according to Ausubel et al. (20). If not otherwise mentioned cells were grown in synthetic complete (SC) medium containing 2% glucose. For induction of the GAL1 promoter cells were grown overnight in SC medium containing 2% galactose. Starvation was done in 1% potassium acetate.

**Materials**—DNA-modifying enzymes, restriction/modifying enzymes, and probes were from New England Biolabs. pGEM (Promega Corp.) and pUC vectors were from Stratagene. Zymolyase-100T was from Seikagaku (Tokyo, Japan). All other chemicals were from Sigma.

In Table I, the constructs were confirmed by sequencing. To introduce HDEL at the carboxyl terminus of Aut5p for lysis of autophagic bodies, this point to a function of Aut5p at or after the prevacuolar compartment. After this finding, we further tested Aut5p for a function in lysing other intravacuolar vesicles. Indeed, Aut5p cells harboring a truncation/retrieval signal HDEL at its carboxyl terminus pro-

**TABLE I**

| Strains | Genotype | Source |
|---------|----------|--------|
| WCG4a   | MATa his3–11, 15 leu2–3,112 ura3 | (4) |
| YM1TA   | WCG4a MATa pep4::HIS3 | (4) |
| YMTB    | WCG4a prb1::URA3 | (15) |
| YIS4    | WCG4a aut5::KAN pep4::HIS3 | (15) |
| YIS8    | WCG4a aut5::KAN pep4::HIS3 | (15) |
| YUE37   | WCG4a aut5::KAN pep4::HIS3 | This study |
| YUE40   | WCG4a aut5::KAN pep4::HIS3 | This study |
| YUE59   | WCG4a prb1::KAN | This study |
| YUE87   | WCG4a pep28::KAN | This study |
| BY4741  | MATa his3–11 leu2Δ met 15Δ ura3Δ | Euroscarf |
| BY4742  | MATa his3–11 leu2Δ lys2Δ ura3Δ | Euroscarf |
| BY4743  | MATa his3–11 leu2Δ lys2Δ lys2ΔLYS | Euroscarf |
| BY4743  | MATa his3–11 leu2Δ lys2Δ lys2ΔLYS | Euroscarf |
| Y14883  | BY4742 tdl1::kanMX4 | Euroscarf |
| Y12763  | BY4742 pep28::kanMX4 | Euroscarf |
| Y05799  | BY4742 pep28::kanMX4 | Euroscarf |
| Y35799  | BY4742 pep28::kanMX4 | Euroscarf |
| Y36576  | BY4742 pep28::kanMX4 | Euroscarf |
| Y52098  | BY4742 pep28::kanMX4 | Euroscarf |
| Y30302  | BY4742 pep28::kanMX4 | Euroscarf |
| Y00575  | BY4742 pep28::kanMX4 | Euroscarf |
| Y1629  | BY4743 pep28::kanMX4 | Euroscarf |
| Y3171  | BY4743 pep28::kanMX4 | Euroscarf |
| Y37088  | BY4743 pep28::kanMX4 | Euroscarf |
| Y34893  | BY4743 pep28::kanMX4 | Euroscarf |
| Y52316  | BY4743 pep28::kanMX4 | Euroscarf |
| Y35381  | BY4743 pep28::kanMX4 | Euroscarf |
| Y32763  | BY4743 pep28::kanMX4 | Euroscarf |
| Y52730  | BY4743 pep28::kanMX4 | Euroscarf |
| YUE777  | BY4742 pep28::kanMX4 | Euroscarf |
| YUE90  | BY4742 pep28::kanMX4 | Euroscarf |
| SEY6210 | MATa his3–11 leu2–3,112 ura3Δтоп1–3901 lys2–801 suc2–59 | (5) |
| TVY614  | SEY6210 pep4::LEU2 pep1::HIS3 prb1::HIS3 | (38) |
| DKY51   | TVY614 pep4::HIS3 | (16) |
| DKY61   | TVY614 pep4::HIS3 | (16) |
The molecular function of vacuolar proteinase A and B in lysing the lipidous membranes of autophagic bodies is enigmatic. It is unclear at the moment and must be the subject of further detailed investigations. Briefly, logarithmically growing cells were shifted to synthetic glycerol medium (0.67% yeast nitrogen base without amino acids, 50 mM MES, 50 mM MOPS, 3% glycerol, 0.1% glucose, pH 5.5) for 12 h at 30 °C. After the addition of a 10× yeast extract/peptone solution to a final concentration of 1% yeast extract and 2% peptone, the cells were incubated for additional 4 h. For peroxisome induction cells were then washed and transferred to YTO (0.67% yeast nitrogen base without amino acids, 0.1% Tween 40, 0.1% oleic acid) for 19 h. To induce peroxisome degradation cells were shifted to SD-N (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose). Aliquots were taken at the indicated times and either prepared for immunoblot analysis using antibodies against Foxp3 or directly analyzed in fluorescence microscopy with GFP-SKL.

### RESULTS

**Vacuolar Targeting of Cvt17p**

Aut5p is targeted from the ER via the Golgi and the prevacuolar compartment (late endosome) to the vacuolar lumen at 50-nm MVB vesicles (15). In wild-type vacuoles the MVB vesicles carrying Aut5p are broken down, resulting in a half-life of Aut5p of 50–70 min (14, 15). Our previous indirect immunofluorescence microscopy indicated a significant steady state level of Aut5p at the ER (15). We therefore speculated that Aut5p might act at the ER probably by modifying specific lipids, which after transport to autophagosomes render them competent for intravacuolar lysis. If this is true, vacuolar transport would only reflect the turnover of the protein and would be dispensable for its function. Alternatively, Aut5p might function at the prevacuolar compartment or inside the vacuole; in this scenario its vacuolar targeting would be essential. To distinguish between these possibilities, we wanted here to block the vacuolar targeting of Aut5p. One idea was to look for vacuolar-targeting sequences within Aut5p. Site-directed mutagenesis of such targeting sequences should then prevent its vacuolar entry. We started our search for vacuolar-targeting sequences with an evaluation of the Aut5p membrane topology.

**At the ER the Amino Terminus of Aut5p Is Exposed to the Cytosol and Its Carboxy Terminus Points Inside the Lumen**

The molecular function of vacuolar proteinase A and B in lysing the lipidous membranes of autophagic bodies is enigmatic. It is tempting to speculate that they might proteolytically activate Aut5p. Our previous analysis of biologically active carboxyb-terminally HA-tagged Aut5p (Aut5-HAp) did not suggest an amino-terminal processing of Aut5p (15). We generated here an amino-terminally HA-tagged Aut5p (HA-Aut5p) and expressed this under control of the inducible GAL1 promoter to check for carboxyl-terminal processing. Complementation of the proaminopeptidase I maturation defect of aut5Δ cells indicated biological activity of HA-Aut5p (Fig. 1A, lane 3). Interestingly, in immunoblots HA antibodies detected bands with lower molecular mass than Aut5-HAp (Fig. 1A, lanes 3, 4, and 6), which were absent in glucose-grown cells (Fig. 1A, lane 7). Identical bands were detected with a polyclonal antibody against Aut5p (not shown), confirming their identity with Aut5p species. After immunoprecipitation with HA antibodies and subsequent deglycosylation with endoglycosidase H in aut5Δ cells and in cells either deficient in vacuolar proteinase A (pep4Δ) or B (prb1Δ), HA-Aut5p did not show unambiguously different mobilities in Western blots (Fig. 1B, lanes 2–5). In pep4Δ cells the HA-Aut5p band appeared broader, however, suggesting the presence of higher molecular mass species (Fig. 1B, lane 4). If this might indicate proteolytic processing at the carboxyl terminus is unclear at the moment and must be the subject of further detailed studies.

**HA-Aut5p was pelletable in lysed spheroplasts (Fig. 1C), indicating it was membrane-associated.** We further confirmed in indirect immunofluorescence the localization of HA-Aut5p to the ER in aut5Δ cells (Fig. 1D). As expected the typical ring-

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**Measurement of Pexophagy**—The induction of peroxisomes was done according to Hutchins et al. (28). Briefly, logarithmically growing cells were shifted to synthetic glycerol medium (0.67% yeast nitrogen base without amino acids, 50 mM MES, 50 mM MOPS, 3% glycerol, 0.1% glucose, pH 5.5) for 12 h at 30 °C. After the addition of a 10× yeast extract/peptone solution to a final concentration of 1% yeast extract and 2% peptone, the cells were incubated for additional 4 h. For peroxisome induction cells were then washed and transferred to YTO (0.67% yeast nitrogen base without amino acids, 0.1% Tween 40, 0.1% oleic acid) for 19 h. To induce peroxisome degradation cells were shifted to SD-N (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose). Aliquots were taken at the indicated times and either prepared for immunoblot analysis using antibodies against Foxp3 or directly analyzed in fluorescence microscopy with GFP-SKL.

**Electron Microscopy**—Electron microscopy after permanganate fixation and Epon embedding was done as described (27).
like staining around the nucleus and staining near the plasma membrane was seen. Accordingly, in cells lacking vacuolar proteinase A (pep4Δ/H9004) HA-Aut5p was detectable inside the vacuole (Fig. 1E,left), whose position is easily visible in Nomarski optics (Fig. 1E,middle). To determine the topology of Aut5p we made proteinase protection experiments using the amino- and carboxyl-terminally HA-tagged Aut5p. In aut5Δ cells, where Aut5p is located at the ER, HA-Aut5p was proteinase-accessible even in the absence of the detergent Triton X-100 (Fig. 1F, lanes 8–10), whereas Aut5-HA p was proteinase-protected (Fig.
F, lanes 3–5). This suggests that the Aut5 amino terminus is located in the cytosol, and the carboxyl terminus is located in the ER lumen.

Sorting of Aut5 via the MVB Pathway Does Not Depend on Ubiquitination—Sorting of procarboxypeptidase S (proCPS) via the MVB pathway requires the ubiquitin ligase Tul1p. Tul1p ubiquitinates the lysine residue 8 of proCPS, which is located in a 19-amino acid amino-terminal domain in the cytosol just preceding the proCPS transmembrane domain (16, 18). A lack of ubiquitination in tul1Δ cells leads to missorting of proCPS to the vacuolar-limiting membrane (18) (Fig. 2B). Our proteinase protection experiments now demonstrate a similar topology for Aut5p, which exposes a 14-amino acid amino-terminal stretch to the cytosol followed by a transmembrane domain (Fig. 3A). As in the case of proCPS the amino-terminal cytosolic domain of Aut5p contains two lysine residues at positions 4 and 9 (Fig. 3A). We therefore checked in indirect immunofluorescence whether sorting of Aut5-HAp to the vacuolar lumen via the MVB pathway depends on Tul1p. Because Aut5-HAp is rapidly degraded in the vacuole, we used tul1Δ pep4Δ cells lacking vacuolar proteinase A for this experiment. Interestingly, in TUL1-deficient cells a significant vacuolar pool of Aut5-HAp was detectable (Fig. 2A). As a control we confirmed mislocalization of GFP-CPS to the vacuolar-limiting membrane (G and B). When indicated vacuolar membranes were additionally stained with the fluorescent dye FM4–64. Bar, 10 μm.

Fig. 2. Vacuolar sorting of Aut5-HAp proceeds in cells lacking the ubiquitin ligase Tul1p or the deubiquitinating enzyme Doa4p. Indirect immunofluorescence microscopy of strains TVY614 (pepΔ prb1Δ prc1Δ, lacking vacuolar proteinases A, B, and Y) (D), YUE90 (tul1Δ pep4Δ) (A), and DKY51 (doa4Δ pepΔ prb1Δ prc1Δ) (E) expressing Aut5-HA from a centromeric plasmid (pJUE13). Cells were processed as described in Fig. 1D. From left to right, immunofluorescence (Aut5-HAp), Nomarski optics (NOM) indicating the vacuole, and nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) is shown. The GFP fluorescence of cells expressing GFP-CPS or Sna3-GFP was checked to make sure these proteins are localized to the vacuole lumen (F and C) or the prevacuolar compartment and the vacuolar limiting membrane (G and B). When indicated vacuolar membranes were additionally stained with the fluorescent dye FM4–64. Bar, 10 μm.
ubiquitin pool of the cells. A lack of Doa4p therefore affects all ubiquitin-dependent processes (28). Consistently, missorting of GFP-CPS to the vacuolar-limiting membrane occurred in doa4/H9004 cells (Fig. 2G) (16, 19). To prevent vacuolar degradation of Aut5-HAp we used cells deficient in the vacuolar proteinases A, B, and Y (pep4/H9004 prb1/H9004 prc1/H9004). Indirect immunofluorescence microscopy indicated that Aut5-HAp is targeted to the vacuole lumen irrespective of presence of Doa4p (Fig. 2, D and E).

In proCPS the first lysine (position 8) of the cytosolic amino-terminal stretch is the target site for ubiquitin conjugation (16) and, thus, essential for proCPS targeting to MVB vesicles. Using site-directed mutagenesis we replaced lysines 4 and 9 of Aut5-HAp with arginine. To further evaluate, if there is any sorting signal within the 14 amino acids of the amino-terminal cytosolic domain, we generated a truncated Aut5-HA(del2–12)-p lacking amino acids 2–12. We expressed these constructs in aut5/H9004 cells. Indirect immunofluorescence microscopy confirmed the normal ER localization of all these Aut5-HAp species (not shown). In aut5/pep4/H9004 cells significant amounts of these mutant proteins were detectable in the vacuole in addition to the ER localization (Fig. 3, B–D). These findings further argue against a ubiquitination-dependent sorting of Aut5p as well as against the presence of sorting determinants in the amino-terminal cytosolic domain. Interestingly, these constructs complemented the proaminopeptidase I maturation defect in aut5/pep4 cells both under non-starvation conditions, where the Cvt pathway is active (Fig. 3E) or after starvation induction of autophagy (Fig. 3F). The constructs also complemented the defect in lysis of autophagic bodies in aut5/H9004 cells (Fig. 3G). Some aut5/H9004 cells expressing Aut5(K9R)-HAp exhibited few autophagic bodies in their vacuoles (Fig. 3G), indicating slightly retarded degradation. This might indicate a slightly reduced activity of the mutated protein, since accumulation of autophagic bodies is more sensitive in monitoring autophagy than proaminopeptidase I maturation. Taken together our findings suggest that the sorting of Aut5p is independent of ubiquitination and that the Aut5p amino-terminal cytosolic domain contains no sorting information nor is it essential for activity.

An Aut5-HA-HDEL Fusion Protein Suggests That Aut5p Does Not Function at the ER—To determine, if Aut5p functions at the ER or if its vacuolar targeting via the MVB pathway is...
Vacuolar Targeting of Cvt17p

Aut5p Is Essential for Pexophagy—Because our findings suggest a function of Aut5p at the prevacuolar compartment or the vacuole, we next analyzed whether breakdown of other vesicular intermediates in the vacuole also depends on Aut5p. Growth of S. cerevisiae cells in medium containing oleic acid as the sole carbon source induces proliferation of peroxisomes. When these cells are shifted to nitrogen starvation, peroxisomes are specifically targeted to and degraded in the vacuole in a process called pexophagy (26). For morphological analysis of pexophagy we used a plasmid encoded GFP-SKL fusion protein (31). The carboxyl-terminal peroxisomal targeting signal, light microscopic evaluation of autophagic body lysis is more sensitive than proaminopeptidase I maturation. Because in our wild-type strain vacuoles of starved cells are more readily visible in Nomarski optics, we deleted VPS23 and VPS28 in WCG4a. Consistent with proaminopeptidase I maturation vps23Δ cells showed no vacuolar accumulation of autophagic bodies after starvation (Fig. 4E). Also, most of the vps28Δ cells accumulated no autophagic bodies. Some cells accumulated few autophagic bodies in each cell less than observed in aut5Δ cells (Fig. 4E). Also in vps28Δ pepΔ cells indirect immunofluorescence microscopy confirmed that the bulk transport of Aut5-HA to the vacuolar lumen is inhibited (not shown). However, this does not exclude that the Aut5-HA mislocalized to the vacuolar-limiting membrane or the small amounts that might still reach the vacuole lumen are sufficient for lysis of almost all autophagic bodies.

For a more detailed analysis, we therefore generated an Aut5-HA species carrying a HDEL motif at its carboxyl terminus (see “Experimental Procedures”). The HDEL motif in yeast functions as an ER retention/retrieval signal, i.e. HDEL-proteins leaking to the early Golgi are continuously retrieved back to the ER (29). Indirect immunofluorescence microscopy indeed confirmed ER localization of Aut5-HA-HDEL in aut5Δ pepΔ cells and, if any, detected only minor amounts in the vacuolar lumen (Fig. 5B). In yeast after linkage of core N-glycan within the ER, further glycosylation in the Golgi includes the addition of α-1,6-mannose residues (30). Accordingly, Aut5-HA-HDEL exhibits compared with Aut5-HA a significantly enhanced α-1,6-mannose glycosylation pattern due to its repeated retrieval from the Golgi (Fig. 5C). As a control the samples were further treated with N-glycosidase F and endoglycosidase H (Fig. 5C); both enzymes are widely used to release N-glycans from proteins. Taken together the findings confirm ER retention/retrieval of Aut5-HA-HDEL. Interestingly, Aut5-HA-HDEL expressed from a centromeric plasmid with its native promoter only partly complemented the proaminopeptidase I maturation defect in aut5Δ cells (Fig. 5D, lane 3). Overexpression of Aut5-HA-HDEL from a two micron plasmid however lead to a more complete proaminopeptidase I maturation (Fig. 5D, lane 4). This dosage dependent complementation is in agreement with the idea, that a small amount of Aut5-HA-HDEL, which might still reach the vacuole is sufficient for lysis of autophagic bodies. The MVB-sorting defect in vps28Δ cells should further reduce the amount of Aut5-HA-HDEL reaching the vacuolar lumen. Indeed, vps28Δ aut5Δ cells expressing Aut5-HA-HDEL from a centromeric plasmid contained almost exclusively proaminopeptidase I (Fig. 5D, lane 6), and overexpression from a 2-μm plasmid led to only partial proaminopeptidase I maturation (Fig. 5D, lane 7). The enhancement of the proaminopeptidase I maturation defect in vps28Δ cells further supports the idea that small amounts of Aut5-HA-HDEL are sufficient to lyse autophagic bodies. Taken together our results do not suggest a function of Aut5p at the ER, but at later stages of its sorting pathway, namely at the prevacuolar compartment or the vacuole.
Fig. 5. Aut5-HA-HDEL expressing cells suggest that Aut5p does not function at the ER. To retain/retrieve Aut5p at the ER, a HDEL sequence was fused to the carboxyl terminus of Aut5-HA. A and B, indirect immunofluorescence microscopy of starved (4 h in 1% potassium acetate) aut5A pep4Δ cells expressing Aut5-HA (A) or Aut5-HA-HDEL (B) from a centromeric plasmid. Cells were treated as in Fig. 1E and analyzed with antibodies against HA. Note the localization of Aut5-HA-HDEL to the ER (ring-like staining around the nucleus and near the plasma membrane), whereas no significant labeling is seen in the vacuole. Bar, 10 µm. DAPI, 4',6-diamidino-2-phenylindole; NOM, Nomarski optics. C, α-1,6-mannose linkages were monitored in immunoblots with specific antibodies. Crude extracts of stationary cells expressing Aut5-HA (pUE35), Aut5-HA-HDEL (pUE37), or an empty vector (pRS425) were immunoblotted and probed with antibodies against α-1,6-mannose linkages (upper panel) and HA (lower panel). Samples were immunoprecipitated with HA antibodies and either deglycosylated with endoglycosidase H or N-glycosidase F or mock-treated. An asterisk marks cross-reacting material. Aut5p* corresponds to deglycosylated Aut5p species. D, Aut5-HA-HDEL shows a dosage-dependent complementation of the proaminopeptidase I maturation defect of aut5A cells. pep4Δ aut5A cells and aut5A cells expressing Aut5-HA or Aut5-HA-HDEL from a centromeric (CEN) or 2-µm plasmid (2µ) were grown to stationary phase and analyzed in immunoblots with antibodies against proaminopeptidase I (upper panel), against HA (middle), and cytosolic phosphoglycerate kinase (PGK) (lower panel). An asterisk marks cross-reacting material. After quantification using ImageQuant, the amount of mature aminopeptidase I was expressed as the percentage of the total amount of mature and proaminopeptidase I present in the sample. mAPI, proaminopeptidase I; mAPI*, mature aminopeptidase I.

Aut5p Affects Intravacuolar Lysis of MVB Vesicles—Vacuolar degradation of GFP-SKL-containing peroxisomes liberates a quite proteolysis-resistant GFP into the vacuole. In contrast, a defective vacuolar breakdown of peroxisomes in pep4Δ cells lacking vacuolar protease A results in vacuolar accumulation of green dots (Fig. 6B). After induction of pexophagy, aut5A cells expressing GFP-SKL clearly accumulated distinct green dots in their vacuoles (Fig. 6B). This indicates vacuolar uptake of peroxisomes in aut5Δ cells but a defective vacuolar breakdown. This is in agreement with the defect of aut5Δ cells in lysing autophagic bodies. We further confirmed the peroxisomal degradation defect in aut5Δ cells in immunoblots using the peroxisomal matrix protein Fox3p (3-ketoacyl-CoA thiolase) (26) as a marker. In wild-type cells Fox3p levels were reduced during starvation, but this reduction was not observed in aut5Δ or pep4Δ cells (Fig. 6C).

Aut5p affects intravacuolar lysis of MVB vesicles. In growing and nitrogen-starved cells, our analysis yielded similar results using GFP-CPS or Sna3-GFP as markers. In growing and nitrogen-starved cells, our analysis yielded similar results using GFP-CPS or Sna3-GFP as markers.
Fig. 6. During pexophagy aut5Δ cells take up peroxisomes in their vacuoles but fail to degrade them. Cells expressing the peroxosomal marker protein GFP-SKL were grown in oleic acid medium (see “Experimental Procedures”) to induce peroxisomes, and the vacuolar membrane was stained with the fluorescent dye FM4–64. Then the cells were shifted to nitrogen-free starvation medium (SD-N), and after 0 h (A) and 4.5 h (B) of starvation cells were checked by fluorescence microscopy. From left to right GFP fluorescence (GFP-SKL), vacuolar staining with FM4–64 (FM4–64), and Nomarski optics (NOM) is shown. Wild-type (WCG) and pep4Δ cells, lacking vacuolar proteinase A, are included. During starvation (panel B) intact peroxisomes, visible as distinct dots, accumulate in vacuoles of aut5Δ and pep4Δ cells, whereas in wild-type (WCG) cells the peroxisomes are degraded, leading to release of soluble GFP into the vacuole lumen. Bar, 10 μm. C, cells were treated as described in A, and after a shift to nitrogen-free medium, aliquots were taken at the indicated times and prepared for immunoblotting using antibodies against the peroxisomal marker protein Fox3p. The amounts of Fox3p were quantified using ImageQuant and expressed as the percentage of the amount present at time point 0.

ing wild-type and aut4Δ cells large amounts of free GFP indicated lysis of MVB vesicles (Fig. 7, A and B, lanes 1 and 3). As expected, pep4Δ cells showed no disintegration of MVB vesicles (Fig. 7, A and B, lane 2). Interestingly, growing aut5Δ cells exhibited a significantly reduced amount of free GFP (Fig. 7, A and B, lane 2), indicating a reduced lysis of MVB vesicles. Starved wild-type, aut4Δ, and pep4Δ cells showed results similar to growing cells (Fig. 7). Starved aut5Δ cells contained a significant level of undigested Sna3-GFP (Fig. 7A, lane 6) and GFPΔ (Fig. 7B, lane 6). These findings suggest a function of Aut5p in MVB vesicle lysis both in growing and starved cells. To exclude the occurrence of proteolysis during cell lysis, we confirmed the presence of proaminopeptidase I in the aut5Δ extracts analyzed in Fig. 7, A and B by immunoblotting with anti-aminopeptidase I antibodies (not shown). To further exclude that the defects in MVB vesicle lysis in aut5Δ cells are caused by missorting of the marker proteins, we checked their localization by fluorescence microscopy. Both GFP-CPS and Sna3-GFP localized to the vacuole lumen in aut5Δ cells (Fig. 7, C and D).

To further confirm that the GFP-CPS and Sna3-GFP degradation defects are due to defects in MVB vesicle lysis, we performed electron microscopy. Because in starved cells the large number of accumulating autophagic bodies interferences with detection of the 50-nm MVB vesicles, we generated mutant strains also lacking the autophagy protein Apg1/Aut3p. A lack of this serine/threonine protein kinase selectively abolishes formation of Cvts vesicles and autophagic bodies (22, 32). aut3Δ aut5Δ pep4Δ mutant cells accumulated 50-nm vesicles in their vacuoles (Fig. 8C). This shows that Aut5p is not essential for formation of 50-nm vesicles. The electron microscopic analysis corroborated our biochemical study by showing 50-nm vesicles in aut3Δ aut5Δ cells (Fig. 8A). Compared with aut3Δ pep4Δ cells (Fig. 8B) aut3Δ aut5Δ cells (Fig. 8A) showed fewer but clearly visible intravacuolar 50-nm vesicles.

**DISCUSSION**

To learn more about the site of action of Aut5p, we wanted to block its vacuolar targeting. We started with proteinase protection experiments (Fig. 1) using amino- and carboxyl-terminally HA-tagged Aut5p to evaluate the Aut5p topology. The experiments demonstrated a cytosolic localization of the Aut5p amino terminus, with the carboxyl terminus trapped in the ER lumen. This suggests a localization of the lipase active site inside the ER and is consistent with the previously observed glycosylation of this part of Aut5p (14, 15). Interestingly, our findings now demonstrate the existence of an amino-terminal 14-amino acid-long cytosolic domain of Aut5p just before the transmembrane domain. This is reminiscent to the topology of proCPS. proCPS is targeted to MVB vesicles after Tulllp-dependent ubiquitination of lysine 8. Lysine 8 is located in a short cytosolic amino-terminal stretch preceding the transmembrane domain of proCPS (16, 18). Because of the topological similarities, we analyzed the two lysines found in the cytosolic amino-terminal Aut5p domain for a vacuolar-targeting function. However, in contrast to proCPS replacement of lysines 4 and 9 of Aut5p-1Ap with arginine did not prevent its vacuolar localization in indirect immunofluorescence microscopy (Fig. 3, B and C). In addition, the complete deletion of the Aut5 amino-terminal cytosolic domain (amino acids 2–12) did not abolish its vacuolar localization nor its activity (Fig. 3, D–G). Together with vacuolar targeting in tul1Δ and in dow4Δ cells, this argues against a ubiquitin-dependent targeting of Aut5p to MVB ves-
icles. Aut5p in this respect resembles Sna3-GFP (19). This finding is highly interesting; however, the lack of a specific sorting signal in the amino-terminal cytosolic domain of Aut5p prevented us from using the mutated Aut5p species to block its vacuolar targeting.

We therefore next analyzed Vps class E mutants, where Aut5-HA is retained at the prevacuolar compartment and partly mislocalized to the vacuolar membrane (Fig. 4C). Under starvation and non-starvation conditions the tested Vps class E mutants showed in immunoblots mature aminopeptidase I, suggesting the occurrence of the Cv t and autophagic pathway. Light microscopic examination of autophagic body lysis is more sensitive to detect autophagy defects than the maturation of proaminopeptidase I. vps28Δ cells showed wild-type like lysis of autophagic bodies. Some vps28Δ cells, however, accumulated a few autophagic bodies in their vacuoles, but fewer than did aut5Δ cells (Fig. 4E). We followed this first hint for a non-ER function by generating an Aut5-HA-HDEL protein. We confirmed its retention/retrieval at the ER in indirect immunofluorescence microscopy (Fig. 5B) and by analyzing its glycosylation pattern (Fig. 5C). Most interestingly, in aut5Δ cells expression of Aut5-HA-HDEL from a centromeric plasmid only partially complemented the proaminopeptidase I maturation defect (Fig. 5D, lane 3), but overexpression from a 2-μm plasmid complemented almost completely (Fig. 5D, lane 4). Although indirect immunofluorescence microscopy did not detect a vacuolar pool of Aut5-HA-HDEL, we hypothesized that small amounts of Aut5-HA-HDEL, which might still leave the ER, might be responsible for lysis of autophagic bodies. If this is true, combining the MVB-sorting defect of Vps class E mutants with the ER retention/retrieval of Aut5-HA-HDEL should further enhance the proaminopeptidase I maturation defect. Indeed, centromeric expression of Aut5-HA-HDEL in aut5Δ vps28Δ cells resulted in almost no proaminopeptidase I maturation (Fig. 5, lane 6), and overexpression of Aut5-HA-HDEL in these cells only led to partial maturation. This suggests that a small amount of Aut5p is sufficient for vesicle lysis, consistent with the idea of an enzymatic function. Our findings further suggest that Vps28-dependent sorting of Aut5p to MVB vesicles is essential for its biological function.

Within the vacuolar lumen not only autophagic bodies but also numerous MVB vesicles are lysed. We wanted to know...
whether Aut5p is also involved in the lysis of these MVB vesicles. We used GFP-CPS (16) and Sna3-GFP (18) with similar results as marker proteins to monitor the integrity of MVB vesicles in immunoblots (Fig. 7, A and B). Electron microscopy further confirmed that the degradation defects observed with the GFP fusion proteins correspond to defects in lysing the MVB vesicles (Fig. 8). Most interestingly, in growing and starved cells lacking Aut5p a significantly reduced breakdown of MVB vesicles was detected. This points to an additional function of Aut5p in lysing these membranes. As a control we checked in aut3Δ pep4Δ cells that Aut5p is not obviously needed for biogenesis of the MVB vesicles (Fig. 8C). In agreement with the observed overlapping function of several proteins between pexophagy and autophagy (26, 33), we could further demonstrate (Fig. 6) that aut5Δ cells are able to take up peroxisomes in their vacuoles but are defective in their breakdown. Taken together our findings suggest a function of Aut5p at the prevacuolar compartment (late endosome) or at the vacuole and point to a more general role of Aut5p in lysis of intravacuolar vesicles.

Several ways that Aut5p could mediate vesicle breakdown seem conceivable. Based on the essential role of the active site motif characteristic for lipases and esterases, Aut5p might act as an unspecific hydrolase directly attacking membranes inside the vacuole. In this case it would be crucial for the cells to prevent untimely activation of Aut5p during its transit to the vacuole. Selective activation within the vacuolar lumen might be achieved by proteolytic maturation. So far, our analysis of amino-terminally (Fig. 1) and carboxyl-terminally (15) HA-tagged Aut5p did not clearly detect a matured Aut5p species; however, the observed broader band of HA-Aut5p in cells lacking vacuolar proteinase A (pep4Δ) needs further detailed studies. Alternatively, Aut5p might be activated by the acidic vacuolar pH. However, neither of these activation strategies explains how lysis of the vacuolar limiting membrane is prevented. Another possibility that explains the specificity would be an activation of Aut5p by interaction with another protein such as a colipase. To identify such a putative interacting protein, we made a high copy suppressor screen using aut5Δ cells, but under the conditions used, this did not detect any suppressors. Also, in our hands a two-hybrid screen using Aut5p as bait did not result in detection of a valuable interaction partner. Interestingly, a large scale two-hybrid approach (34) pointed to the inositol phosphosphingolipid phospholipase C Isc1p (35, 36) as a putative Aut5p-interacting protein. We chromosomally deleted ISC1, but under the conditions tested light microscopic examination did not show vacuolar accumulation of autophagic bodies during starvation (not shown). This does not support a direct involvement of Isc1p in lysis of autophagic bodies. In general, the idea of Aut5p as a hydrolase with low substrate specificity seems unlikely, since this demands sophisticated mechanisms for controlling its activity. If Aut5p acts as a hydrolase, a high specificity for molecules present only at its target membranes would significantly limit its risk for the integrity of the cell.

In an alternative scenario in the cytosol a multivesicular body, i.e. a late endosome (prevacuolar compartment) filled with MVB vesicles, might fuse with an autophagosome. Within the resulting organelle the MVB vesicles then could fuse with the inner membrane layer of autophagosomes. This would deliver...
Aut5p to the inner membrane of autophagosomes and, thus, to autphagic bodies. In this scenario Aut5p would attack in the vacuolar lumen those membranes where it is located. As discussed, a high substrate selectivity of Aut5p would also be expected in this scenario. In mammalian cells indeed fusions between endosomes and autophagosomes, resulting in the formation of amphisomes, have been reported (37). One should also take into account the possibility that Aut5p might function already at or inside the multivesicular body. Because in all scenarios a high substrate selectivity of Aut5p seems likely, it is a challenging task for future work to identify such a putative Aut5p substrate.

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Intravacuolar Membrane Lysis in *Saccharomyces cerevisiae*: DOES VACUOLAR TARGETING OF Cvt17/Aut5p AFFECT ITS FUNCTION?

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