Trs85 (Gsg1), a component of the TRAPP complexes is required for the organization of the preautophagosomal structure during selective autophagy via the Cvt pathway

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Autophagosomes and Cvt vesicles are limited by two membrane layers. The biogenesis of these unconventional vesicles and the origin of their membranes is hardly understood. We here identify in S. cerevisiae Trs85, a non-essential component of the TRAPP complexes to be required for the biogenesis of Cvt vesicles. The TRAPP complexes function in ER-to-Golgi and Golgi trafficking. Growing trs85Δ cells show a defect in the organization of the preautophagosomal structure. While proaminopeptidase I is normally recruited to the preautophagosomal structure, the recruitment of GFP-Atg8 depends on Trs85. Autophagy proceeds in the absence of Trs85, albeit at a reduced rate. Our electron microscopic analysis demonstrate that the reduced autophagic rate of trs85Δ cells does not result from a reduced size of the autophagosomes. Growing and starved cells lacking Trs85 did not show defects in vacuolar biogenesis, mature vacuolar proteinase B and carboxypeptidase Y were present. Also vacuolar acidification was normal in these cells.

It is known that mutations impairing the integrity of the ER or Golgi block both autophagy and the Cvt pathway. But the phenotypes of trs85Δ cells show striking differences to those seen in mutants with defects in the early secretory pathway. This suggests that Trs85 might play a direct role in the Cvt pathway and autophagy.

Starvation induced autophagy is an unselective, degradative pathway, which delivers cytosolic material to the lysosome (vacuole) (1),(2),(3). It is well conserved between eukaryotes such as fungi, plants and mammals. During the last decade work initially using the model eukaryote Saccharomyces cerevisiae led to the identification of a set of more than 20 ATG genes essential for the autophagic process (4),(5),(6),(7). Studies on the mammalian counterparts of the yeast ATG genes uncovered the importance of autophagy in the development of severe diseases such as cancer, cardiomyopathy, Huntington´s and Parkinson´s disease (8). Autophagy also plays an important role in the removal of intracellular pathogens (9).
and increasing evidence points to a relationship between autophagy and ageing (10), (11).

Autophagy starts at the preautophagosomal structure (PAS), a perivacuolar organelle, where numerous Atg proteins colocalize (12), (13). Out of the PAS double membrane-layered transport vesicles, the autophagosomes, are formed (14), (15). The outer membrane of the autophagosome then fuses with the vacular membrane and the inner part of the vesicle is released as a still membrane-limited autophagic body into the vacuole. Within the vacuole the autophagic bodies are lysed dependent on the putative lipase Atg15 (16) and the cytosolic material is degraded by the various vacuolar hydrolases.

The use of transport vesicles limited by two membrane layers distinguishes autophagy from other transport pathways. Consistently, also the molecular mechanisms used for the biogenesis of these vesicles are unconventional. For example the homotypic membrane fusion event during the sealing of autophagosomes does not involve the action of the yeast NSF Sec18, also none of the yeast t-SNAREs has been localized to the PAS (17), (18).

In S. cerevisiae the Cvt (cytoplasm to vacuole targeting) pathway was discovered as a variant of the starvation induced unselective autophagy. In contrast to the degradative autophagic pathway, the constitutive Cvt pathway acts under nutrient-rich conditions as a biosynthetic route selectively delivering specific cargo proteins such as proaminopeptidase I to the vacuole. The Cvt pathway and autophagy share most of their molecular components, albeit the function of some Atg proteins is restricted to one of the pathways. The most striking difference between autophagy and the Cvt pathway is the size of their transport intermediates. Cvt vesicles are smaller than autophagosomes and do not enclose cytosolic material.

Pexophagy, the selective autophagic degradation of dispensable peroxisomes is another variant of autophagy. It takes place when yeast cells are shifted from a medium inducing the proliferation of peroxisomes to a medium containing glucose (19).

Biogenesis of Cvt vesicles and autophagosomes requires the sorting of large amounts of membranes to the PAS. The origin of these membranes remains elusive, but recent work demonstrated that a functional ER and Golgi is essential for both the biogenesis of Cvt vesicles and autophagosomes (17), (18). Accordingly, mutations affecting the early secretory pathway block both autophagy and the Cvt pathway.

We here identify Trs85 as an essential component for the biogenesis of Cvt vesicles. Trs85 is a subunit of the TRAPP complexes, which act in ER-to Golgi and Golgi trafficking (20). One might therefore expect that defects at the ER or Golgi in trs85Δ cells might be responsible for the autophagic defects. Our experiments however point to differences in the phenotypes between trs85Δ cells and cells with defects in the early secretory pathway. First of all in contrast to components of the early secretory pathway Trs85 is not needed for the viability of yeast cells. Furthermore our experiments did not show defects in vacuolar biogenesis in trs85Δ cells. Early secretory mutants are blocked in autophagy and they fail to recruit proaminopeptidase I to their PAS (17). Here we show that autophagy proceeds in trs85Δ cells with half of the wild-type rate. Furthermore trs85Δ cells can recruit proaminopeptidase I to their PAS, but fail to recruit GFP-Atg8 in rich media. This differences support the idea that Trs85 might play a specific role during the Cvt pathway and autophagy.

**Experimental Procedures**

**Strains, media, antibodies and reagents**

Standard media were used (21). Starvation medium was either SD(-N) (1.7% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose) or 1% potassium acetate, as indicated. Antibodies were anti-3-phosphoglycerate kinase, anti-carboxypeptidase Y, anti-green fluorescent protein (Molecular Probes, Leiden, The Netherlands); horseradish peroxidase (HRPO)-conjugated goat anti-rabbit (Medac, Hamburg, Germany) and HRPO-conjugated goat anti-mouse (Dianova, Hamburg, Germany); anti-proaminopeptidase I (22) and anti-Atg8 (23). Fox3 antibodies were supplied by R. Erdmann, Bochum, Germany.

Chemicals: PMSF (Sigma, Deisenhofen, Germany); oligonucleotides (MWG-Biotech, Ebersberg, Germany and Operon, Germany), other analytical grade chemicals were from Sigma or Merck (Darmstadt, Germany). For immunoblots the ECL detection kit (Amersham, Braunschweig,
Germany) was used.

**Strains** used are listed in Table 1.

**GSG1/TRS85 chromosomal deletion**

A PCR fragment with the kanamycin resistance gene flanked with TRS85 sequences was generated from a-GSG1: 5'- CTTTATTACG TCGGCTTTAC AGATACTGAGG TAACT TATA cagctgaagcttcgtacgc -3', as-GSG1: 5'- TACGTATAATTTATACTCA AAACATGAA TTTTCCATAAAAGgcataaggccacagtggatcg -3' and the plasmid pUG6 carrying the kanamycin resistance marker (24). Chromosomal replacement of TRS85 with this fragment in WCG4a yielded YKMW1 and in SEY6211 the strain YKMW28. Deletions were confirmed with Southern blot analysis (not shown).

**YPT7 Chromosomal Deletion**

YPT7 deletion strains were constructed using the plasmid pBSKS+ ypt7::HIS3 (D. Gallwitz, Goettingen), which was digested with XhoI and PacI and transformed into YKMW1 yielding YKMW18 (gsg1∆::KAN ypt7∆::HIS3). The chromosomal replacement of the YPT7 gene was confirmed by Southern blotting (not shown).

**PHO8 Chromosomal Deletion**

The PHO8 gene in the following strains was replaced with the LEU2 gene using the deletion plasmid pGF10 (pho8∆::LEU2) (25). In YKMW1 (trs85∆) the deletion yielded YKMW21 (trs85∆::KAN pho8∆::LEU2).

**Plasmids**

Plasmid pJH1 (pRS313-API-RFP) was generated by homologue recombination in yeast. A PCR fragment containing mRFP was amplified using primers API-RFP up (GGAGATCAGTCTACGAT GAATCCGCGAGTTGT CCCGGGTAgcctcctcc gagac gtcat), RFP-Vector down (TCGACGGTATCGACTGAA TTCTAGA GTCGCttag cgcgggttagggc) and pmRFP-KanMX (26) as template. pKMW13 was cut with AgeI/NotI and the resulting 6,8 kb fragment was cotransformed with the mRFP containing PCR fragment in the yeast strain Y36953 (Euroscarf). The recombinant plasmid was rescued from transformants able to grow on SC medium lacking histidine, and the correct recombination was confirmed. pKMW13 (Ape1-YFP): The eYFP-fluorescent protein was excised from pEYFP (BD Clontech) with XmaI and EcoRI and inserted into the pRS313 vector at the same sites. This vector (pKMW1) was cut with KspI and XmaI and combined with the PCR fragment, containing APE1 with its native promoter and the added KspI and XmaI incision sites, yielding the plasmid pKMW13. The PCR Fragment was constructed with the primer KSPl-APEI (AGGGCC GCGGCTACTTTAGGTATAGGTTG) and XMAI-APEI (AGGGCCCGG GACAACTCGCC GAATTCATCG) and the plasmid pRN1 (27).

The following plasmids were described elsewhere: pGFP-Atg8 (12), pMet25::GFP-Atg9 (28), and pGFP-Atg19 (29).

**Cell lysis, SDS-PAGE and immunoblotting** was done as described previously (16).

**Alkaline Phosphatase Assay**

The pho8∆::LEU2 deletion strains were transformed with the Pho8∆60-expression plasmid pCC5 (30). Enzymatic activity was measured as described (31) with the following modifications. Logarithmically grown cells were washed with water and resuspended in SD(-N) medium. One OD600 unit of cells was harvested at each time point and washed once with water. The cells were then suspended in 0.2 ml of assay buffer (250 mM Tris/HCl, pH 9.0, 10 mM MgSO4, 10 µM ZnSO4) and disrupted by vortexing with glass beads. After centrifugation, 50 µl of the supernatant was added to 0.5 ml of assay buffer and 50 µl of 55 mM potassium naphtylphosphate. After incubation for 15 min at 30°C, 0.5 ml of 2M glycine/NaOH (pH 11.0) was added to stop the reaction. Fluorescence intensity was measured with excitation at 345 nm and emission at 472 nm. Protein concentration was determined with the BCA method (Pierce, Rockford, IL, USA).

**Measurement of Pexophagy**

Following the protocol of (19), logarithmically growing cells were shifted to synthetic glycerol medium (0.67% YNB without amino acids, 50 mM 2-(N-morpholino) ethanesulfonic acid, 50 mM 3-(N-morpholino) propanesulfonic acid, 3% glycerol, 0.1% glucose, pH 5.5) for 12h at 30°C. Then a 10xYP solution was added to a final
concentration of 1% yeast extract and 2% peptone and the cells were incubated for 4h. The cells were then washed and transferred to YTO (0.67% YNB without amino acids, 0.1% Tween-40, 0.1 % oleic acid) for 19h for peroxisome induction. To induce peroxisome degradation cells were shifted to SD-(N). Aliquots were taken at the indicated times and prepared for immunoblot analysis using antibodies against Fox3p (R.Erdmann, Bochum, Germany).

**Probing Vacuolar Acidification**
Cells were resuspended in 1ml quinacrine buffer (10mM HEPES, 2% Glucose, pH 7.4). 1µl [1mM] quinacrine stock was added and the mixture was incubated for 10min at RT. The cells were washed twice with buffer and observed with a Zeiss Axioscope2 microscope equipped with an Axiocam digital camera.

**Protease Protection Assay**
The protease protection experiment was done according to (32) with the following modifications. Forty OD$_{600}$ units of early stationary or starved cells were harvested, washed twice with water and incubated for 20 min in 4 ml buffer A (100 mM Tris/H$_2$SO$_4$, pH 9.4) containing 20 mM dithiothreitol. The cells were then pelleted, resuspended in 4 ml oxalyticase buffer (1 M Sorbitol, 50 mM NaH$_2$PO$_4$, pH 7.4) containing 50 µg/ml oxalyticase and spheroplasted at 30°C for 30 min at 30°C. The spheroplasts were harvested and hypotonically lysed in PS200 buffer (200 mM Sorbitol 20 mM potassium-PIPES, pH 6.8 with 5 mM MgCl$_2$) containing 1 mg/ml leupeptin, 1 mg/ml chymostatin, 1 mg/ml antipain, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and Complete® protease inhibitor mix (Roche Diagnostics). Cell debris was removed by centrifugation at 1,000 xg and the supernatant was transferred to a fresh tube three times. 300 µl supernatant was taken for total and the proteins precipitated with trichloroacetic acid (TCA) on ice. 700 µl was transferred to a fresh tube and centrifuged for 20 min at 4 °C and 10,000 xg. 300 µl supernatant was kept as S13 and 400 µl of the supernatant was centrifuged for 1 h, at 4 °C and 100,000xg. The pellet fraction (P13) was resuspended in Laemmlli buffer with 1 % β-mercaptoethanol. After high speed centrifugation 300 µl supernatant (S100) was precipitated with TCA and the pellet (P100) was resuspended in Laemmlli buffer with 1 % β-mercaptoethanol. The TCA precipitated proteins were centrifuged and resuspended in Laemmlli buffer with 1 % β-mercaptoethanol.

**Accumulation of autophagic bodies**
Cells grown to the stationary phase were washed twice with water and then shifted to SD(-N) with and without 10mM phenylmethylsulfonyl fluoride. Photos were taken using Nomarski optics and a Zeiss Axioscope2 microscope.

**Electron Microscopy**
For electron microscopy the cells were fixed with permanganate and Epon embedded as described previously (16). A Zeiss EM 900 transmission electron microscope was used to take photographs at 12,000x magnification. The area of autophagosome profiles was determined by point counting from these photographs.

**Cell Fractionation**
Eighty OD$_{600}$ units of late stationary cells were harvested, washed once with water and incubated at 30°C for 15 min in buffer A (100 mM Tris/H$_2$SO$_4$, pH 9.4) containing 20 mM dithiothreitol. Cells were resuspended in oxalyticase buffer (1 M Sorbitol, 50 mM NaH$_2$PO$_4$, pH 7.4) containing 50 µg/ml oxalyticase, spheroplasted at 30°C for 30 min and then hypotonically lysed in ice cold PS200 buffer (200 mM Sorbitol 20 mM potassium-PIPES, pH 6.8 with 5 mM MgCl$_2$) containing 1 mg/ml leupeptin, 1 mg/ml chymostatin, 1 mg/ml antipain, 1 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and Complete® protease inhibitor mix (Roche Diagnostics). Cell debris was removed by centrifugation at 1,000 xg and the supernatant was transferred to a fresh tube three times. 300 µl supernatant was taken for total and the proteins precipitated with trichloroacetic acid (TCA) on ice. 700 µl was transferred to a fresh tube and centrifuged for 20 min at 4 °C and 10,000 xg. 300 µl supernatant was kept as S13 and 400 µl of the supernatant was centrifuged for 1 h, at 4 °C and 100,000xg. The pellet fraction (P13) was resuspended in Laemmlli buffer with 1 % β-mercaptoethanol. After high speed centrifugation 300 µl supernatant (S100) was precipitated with TCA and the pellet (P100) was resuspended in Laemmlli buffer with 1 % β-mercaptoethanol. The TCA precipitated proteins were centrifuged and resuspended in Laemmlli buffer with 1 % β-mercaptoethanol.

**Results**

Growing trs85Δ (gsg1Δ) cells are defective in the Cvt pathway

In the yeast deletion project each non-essential yeast gene has been chromosomally deleted, resulting in a collection of ~5000 yeast deletion strains. To identify novel components of the autophagic machinery, we screened this strain collection for mutants sensitive to nitrogen-limitation, a phenotype common to autophagy mutants. Starvation-sensitive mutants can easily be scored by incubating colonies for some days on Phloxin plates lacking a nitrogen source (5),(33). Phloxin is a red dye, which stains dead cells, but is unable to enter living cells. Since on these plates colonies of starvation sensitive mutants contain more dead cells they appear dark red. This initial screen identified more than 1300 strains, which were further analyzed in Western blots for their ability to mature proaminopeptidase I. Here we report the identification of trs85Δ (gsg1Δ) in this screen. Trs85 is a 85 kDa component of both the TRAPP I and TRAPP II complex (20,34). The TRAPP complexes function in ER-to-Golgi and Golgi transport (20). Diploid cells lacking Trs85 fail to sporulate, based on this phenotype TRS85 has also been termed GSG1 (general sporulation gene) (35).

For further analysis we chromosomally deleted TRS85 in our lab wild-type strain WCG (see Experimental procedures). In contrast to wild-type cells non-starved trs85Δ cells fail to mature proaminopeptidase I (Fig.1A, lanes 20,21). Starvation induction of autophagy rescues this maturation defect (Fig.1A lanes 22 to 26). This suggests that Trs85 is required for the selective Cvt pathway, but not for autophagy. To confirm that a defect in the targeting of proaminopeptidase I to the vacuole is responsible for the maturation defect, we generated a pApe1-RFP fusion protein of proaminopeptidase I with the red fluorescent protein (26). Fluorescence microscopy confirmed vacuolar targeting of this fusion protein via the Cvt pathway in non-starved wild-type cells (Fig.1B). In non-starved trs85Δ cells pApe1-RFP is retained in the cytosol (Fig.1B), demonstrating a vacuolar targeting defect. As a control we included atg19Δ cells, which are defective in vacuolar targeting of proaminopeptidase I.

The two TRAPP complexes consist of ten proteins, three of them (Trs33, Trs65 and Trs85) are dispensable for the vitality of yeast cells (20). As shown in Fig.1C cells lacking Trs33 or Trs65 do not show defects in the Cvt pathway or autophagy.

Autophagy takes place in starved trs85Δ cells, but with a reduced rate

Maturation of proaminopeptidase I in starved trs85Δ cells suggests that autophagy may not be affected. To further address this question, we checked cells starved in the presence of the proteinase B inhibitor PMSF under the light microscope. PMSF is known to inhibit the intravacuolar breakdown of autophagic bodies (36) allowing their direct visualization. trs85Δ cells clearly showed the accumulation of autophagic bodies within their vacuoles after 2 hours in nitrogen-free medium (Fig.2A). Compared to wild-type cells the number of autophagic bodies seemed reduced. To estimate the autophagic capacity more quantitatively we then followed the generation of GFP from the GFP-Atg8 fusion protein. During formation of autophagosomes Atg8 as well as GFP-Atg8, is specifically enclosed in these vesicles and transported to the vacuole, where it is degraded. Since GFP is rather resistant against proteolytic attack in the vacuole, the amount of generated GFP correlates with the autophagic rate. As shown in Fig.2B compared to wild-type cells generation of GFP is significantly reduced, but not completely blocked in starving trs85Δ cells. As a control atg18Δ cells, which are completely blocked in autophagy are included. As an alternative method to quantify autophagy, we used Pho8Δ60, a truncated version of the vacuolar alkaline phosphatase Pho8 (31). The Pho8Δ60 protein does not contain a membrane domain and is therefore retained in the cytosol. After autophagic transport of Pho8Δ60 to the vacuole, the protein is proteolytically matured yielding an enzymatically active phosphatase. We chromosomally deleted PHO8 in trs85Δ cells and expressed Pho8Δ60 from a plasmid. Measurement of the phosphatase activity in lysates of cells starved for nitrogen demonstrated that the autophagic rate of trs85Δ cells is approximately half of the wild-type rate (Fig.2C).

Pexophagy is significantly retarded in trs85Δ cells

We were next interested, whether Trs85 also plays a role during pexophagy, a selective variant of
autophagy. Growth of *S. cerevisiae* cells in media containing oleic acid leads to a massive proliferation of peroxisomes. A subsequent shift of these cells to SD(-N) medium containing glucose, but lacking a nitrogen source induces the selective removal of peroxisomes in an autophagic process termed pexophagy (19). As a marker protein to score peroxisomal degradation we used the 3-ketoacyl-CoA thiolase Fox3. Autophagy deficient *atg1Δ* cells were included as a control in our analysis. As shown in Fig.3A and B peroxisomal breakdown was severely affected in the absence of Trs85.

**The autophagic defects in the absence of Trs85 are not due to defects in vacuolar biogenesis**

The function of the TRAPP complexes is needed for normal vacuolar biogenesis (20). One might therefore speculate that the observed reduced rate in autophagy and pexophagy might be caused by a lack of proteolytic capacity of the vacuole. To address this possibility we analyzed the steady state levels of vacuolar proteinase B and vacuolar carboxypeptidase Y in *trs85Δ* cells grown in rich medium and starved for nitrogen. Under both conditions significant amounts of mature proteinase B and carboxypeptidase Y were detectable (Fig.4A), unprocessed precursor forms of the proteinases were almost completely absent. This argues against a reduced proteolytic capacity of *trs85Δ* vacuoles. Impaired vacuolar acidification has also been reported to affect breakdown of autophagic bodies inside the vacuole thus mimicking autophagic defects (37). We confirmed vacuolar acidification of growing (Fig.4B, left) and starved *trs85Δ* cells (Fig.4B, right) using quinacrine staining. Quinacrine is a fluorescent dye, which is known to accumulate only inside acidic vacuoles (38). *vma1Δ* cells exhibiting a defect in vacuolar acidification were included as a control. To further demonstrate that starving *trs85Δ* cells indeed show defects in the autophagic transport from the cytosol to the vacuole rather than a defect in vacuolar breakdown, we checked in fluorescence microscopy the localization of GFP-Atg8, which is specifically targeted to the vacuole via autophagy (39). As expected wild-type cells showed a green fluorescent vacuole due to the transport of GFP-Atg8 to the vacuole and the subsequent release of proteolysis-resistant GFP (Fig.4C; compare with Fig.2B). In autophagy-deficient *atg1Δ* cells GFP-Atg8 and thus GFP is absent from the vacuolar lumen. Consistent with a reduced autophagic rate starved *trs85Δ* cells showed no significant green fluorescence within their vacuoles (Fig.4C), since the amounts of GFP-Atg8, which are targeted by the residual autophagic rate (compare Fig.2B) are hard to visualize. Altogether our experiments demonstrate that the reduced autophagic rate in *trs85Δ* cells is caused by a slower autophagic transport.

**Trs85 is essential for the formation of proaminopeptidase I-containing Cvt vesicles**

We next wanted to determine at which step the Cvt pathway is blocked in the absence of Trs85. The Rab GTPase Ypt7 is essential for the fusion of Cvt vesicles with the vacuole (40). Cells lacking Ypt7 therefore accumulate proaminopeptidase I-containing Cvt vesicles in the cytosol. We converted non-starved cells to spheroplasts and lyed them under mild hypooosmotic conditions leaving the Cvt vesicles intact. After removing non-lysed cells by low speed centrifugation, we separated the total lysate (Fig.5A, lane T) in a 13,000xg supernatant (S13) and pellet fraction (P13). Subsequent 100,000xg centrifugation of the 13,000xg supernatant fraction yielded a S100 supernatant and P100 pellet fraction. Consistent with previous work the Cvt vesicles of non-starved *ypt7Δ* cells are found in the P13 fraction (Fig.5A, lane 3). In *atg1Δ ypt7Δ* cells the biogenesis of Cvt vesicles is blocked, proaminopeptidase I is therefore absent from the P13 pellet fraction (Fig.5A, lane 8). The absence of proaminopeptidase I in the P13 fraction of non-starved *trs85Δ ypt7Δ* cells (Fig.5A, lane 13) suggests that no proaminopeptidase I-containing Cvt vesicles are formed in these cells. Absence of cytosolic 3-phosphoglycerate kinase (PGK) in the P13 fractions (Fig.5A, lanes 3, 8 and 13) excludes the possibility that unlysed whole cells interfere with the detection of Cvt vesicles.

To evaluate, whether proaminopeptidase I accumulates in membrane-enclosed form or not, we next performed a proteinase protection experiment. In lysates of spheroplasts from non-starved *ypt7Δ* cells, proaminopeptidase I was protected against exogenously added proteinase K (Fig.5B, lane 2). Treatment with proteinase K in the presence of the detergent Triton X-100 resulted in the conversion of proaminopeptidase I into a...
Trs85 is required for proper organization of the preautophagosomal structure during the Cvt pathway

The preautophagosomal structure (PAS) is believed to be the donor compartment for formation of Cvt vesicles and autophagosomes (14,15). The inability of trs85Δ cells to form Cvt vesicles therefore prompted us to analyze the organization of the PAS in these cells. In S.cerevisiae the PAS is visible in fluorescence microscopy as a dot near the vacuolar membrane, where many of the Atg-proteins colocalize (12),(13). Typically, only a fraction of the cells in a culture show a clearly visible PAS. We determined under the fluorescence microscope in logarithmically growing and starved cells the percentage of cells with a PAS-like punctate structure using biologically active fusion proteins of Atg-proteins with the green fluorescent protein (GFP). In trs85Δ cells expressing GFP-Atg19 or GFP-Atg9 the percentage of cells with a PAS was not altered compared to wild-type cells (Fig.6A). Most interestingly, logarithmically growing trs85Δ cells failed almost completely to recruit GFP-Atg8 to the PAS (Fig.6A). Starvation induction of autophagy rescued this phenotype, resulting in a wild-type-like number of cells exhibiting a PAS (Fig.6A).

During autophagy and the Cvt pathway Atg8 is covalently coupled via a ubiquitin-like system to the membrane lipid phosphatidylethanolamine (42). Our Western blot analyses suggest that in growing trs85Δ cells the lipidation reaction is not blocked (Fig.6B). During starvation the Atg8 level is induced, but our analysis did not point to a significantly altered level of Atg8 in trs85Δ cells compared to wild-type cells.

In starved trs85Δ cells autophagosomes are normally sized

The reduced autophagic rate in trs85Δ cells might be attributed to a decreased size of the autophagosomes. In wild-type cells autophagosomes are rarely detectable in the cytosol due to their rapid fusion with the vacuole. Ypt7 is essential for the vacuolar fusion of autophagosomes. To determine the size of autophagosomes we therefore used trs85Δ ypt7Δ cells. We prepared cells starved for nitrogen for electron microscopy and estimated the mean area of the autophagosome profiles. Quantifying the area of autophagosome profiles is more accurate than measuring their diameter, since all autophagosomes are not round. The autophagosomes in trs85Δ ypt7Δ cells had an average area of 0.152 µm² (S.D. = 0.094), (Fig.7A,C) which is similar to autophagosomes in ypt7Δ cells (0.164 µm² ; S.D. = 0.079), (Fig.8A,B). Assuming a spherical shape for autophagosomes the measured area corresponds to ~450 nm diameter, which is in agreement with previous work on autophagosomal size (36). This finding argues against a role of Trs85 in the expansion step of autophagosomes.

Localization of Trs85-GFP

In a genome-wide approach yeast cells chromosomally expressing GFP fusion proteins were generated (43). In this study Trs85-GFP was
detected in the cytosol. We reprobed this finding and detected beside a cytosolic localization the recruitment of Trs85-GFP to a punctate structure (Fig.8). In Bet3-GFP and Trs120-GFP expressing cells multiple dot-like structures were visible, while Trs85-GFP expressing cells showed only one puncte per cell (Fig.8). This supports the idea that Trs85 does not colocalize with all Bet3 and Trs120 containing structures. Unfortunately, the fluorescence intensity of Trs85-GFP was not strong enough to allow colocalization with proaminopeptidase I-RFP, a marker of the preautophagosomal structure. Growing cells expressing Trs85-GFP were able to mature proaminopeptidase I, assuming biological activity of the fusion protein (data not shown).

DISCUSSION

The formation of double membrane-layered Cvt vesicles and autophagosomes out of the PAS seems to require novel mechanistic features, since none of the yeast t-SNAREs has been located to the PAS (17). Also the yeast NSF Sec18 is not involved in the biogenesis of these vesicles (18). To learn more about the unconventional biogenesis of Cvt vesicles and autophagosomes, we aimed to identify novel components of this process and here identify Trs85. Our experiments demonstrate that Trs85 is essential for the biogenesis of Cvt vesicles. For formation of autophagosomes Trs85 is dispensable, but its absence reduces the autophagic rate to approximately half of the wild-type rate (Fig.2). The biogenesis of Cvt vesicles and autophagosomes requires a massive membrane flux, whose origin remains elusive. Recent work demonstrated that mutations disturbing the early secretory pathway by either affecting the exit from the ER or the trafficking through the Golgi lead to a block in both the Cvt pathway and autophagy (17,18). Trs85 is a substoichiometric component of the TRAPP I complex, which is required for the ER to Golgi transport (20). Trs85 furthermore is a stoichiometric subunit of the TRAPP II complex acting in Golgi trafficking (20). Seen in the light of the studies reporting an essential function of the early secretory pathway for the Cvt pathway and autophagy (17,18), the finding of trs85Δ cells in our screen seems trivial at first glance. However our detailed analysis uncovered striking differences between the phenotypes of trs85Δ cells and mutants of the early secretory pathway. This supports the idea that a lack of Trs85 does not simply interfere with the fidelity of the ER and Golgi, but has a more specific function during the Cvt pathway and autophagy itself.

First of all proteins functioning in trafficking through the ER and Golgi typically are essential for growth. This is also true for seven of the ten components of the TRAPP complexes (20). Trs33, Trs65 and Trs85, the further three subunits of the TRAPP complexes are not essential for growth, however and their absence does not block the early secretory pathway (20). Under temperature stress conditions (37°C) trs85Δ cells, however showed slower growth and an ER to Golgi transport defect (20). Our analysis demonstrated that in trs85Δ cells grown at 30°C mature vacuolar proteinases are present and vacuolar acidification is normal (Fig.4). While early secretory mutants show a block in both the Cvt pathway and autophagy (17), trs85Δ cells are only blocked in the Cvt pathway. The autophagic rate is reduced in these cells (Fig.2), but enough autophagosomes are formed to rescue the proaminopeptidase I maturation defect during starvation (Fig.1). trs85Δ in this respect is a typical member of a subset of ATG genes such as ATG21 (23),(44) and VAC8 (45), which are only essential for the Cvt pathway. In wild-type cells proaminopeptidase I forms a large cytosolic complex termed the Cvt complex, which is then recruited to the PAS, where it is enwrapped by forming Cvt vesicles. Sec12 is a GDP/GTP exchange factor required for exit from the ER (46). Temperature sensitive sec12 mutant cells show at the non-permissive temperature a defect in the recruitment of the Cvt complex to the PAS (17). trs85Δ cells in contrast showed a normal recruitment of proaminopeptidase I to the PAS (Fig.5). Taken together, the listed differences in the phenotypes observed in mutants of the early secretory pathway and in trs85Δ cells argue against the trivial explanation that a compromised function of the ER or Golgi is responsible for the defect in the Cvt pathway. This opens the possibility that Trs85 plays a more specific role in the Cvt pathway.

Our findings demonstrate that growing trs85Δ cells are defective in the biogenesis of Cvt vesicles (Fig.5). To further dissect the molecular defects in trs85Δ cells, we analysed the organisation of the PAS. The integral membrane protein Atg9 and proaminopeptidase I colocalised...
at the PAS similar to wild-type cells (Fig.5D). GFP-Atg8, in contrast is not recruited to the PAS in growing trs85Δ cells, as it is in growing wild-type cells. Although Atg8 coupled to the lipid phosphatidylethanolamine was still detectable in trs85Δ cells (Fig.6B), this defect gives an easy explanation for the impaired biogenesis of Cvt vesicles, since previous studies have demonstrated the essential role of Atg8 during the formation of Cvt vesicles (23),(44). After starvation induction of autophagy, the failure in the PAS-recruitment of GFP-Atg8 is rescued in trs85Δ cells (Fig.6). But the autophagic rate of these cells is significantly reduced compared to wild-type cells (Fig.2,3). The reduced autophagic rate might be due to either a reduced size or a reduced number of autophagosomes. Our electron microscopic study of starved trs85Δ ypt7Δ cells showed the formation of normally-sized autophagosomes (Fig.7). This argues against an involvement of Trs85 in the membrane expansion step of autophagosomes. Trs85 instead seems to act as a helper protein increasing the rate of autophagosome formation.

Based on the absence of t-SNAREs at the PAS and the essential role of the ER and Golgi for autophagy and the Cvt pathway, Klionsky and coworkers have proposed a maturation model for the formation of the PAS (17). In this model components of the PAS travel through the ER and Golgi and are segregated in a compartment, which finally matures into the PAS by the specific retrograde transport of proteins and lipids back to the donor compartment. A block of a retrograde transport step would then result in the generation of a premature PAS, probably unable to recruit GFP-Atg8 and thus unable to fulfill its function. Since Trs85, in contrast to most components of the TRAPP complexes, is not essential for growth, one might speculate about the existence of a specific TRAPP complex acting in such a step during the generation of the PAS. This hypothesis is further supported by the observed differences in the localization of Trs85-GFP compared to Bet3-GFP and Trs120-GFP (Fig.8).

ACKNOWLEDGEMENTS
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REFERENCES

**LEGENDS TO FIGURES**

Figure 1: **Trs85 is essential for the Cvt pathway.** A. When grown to the logarithmic (log) or stationary (stat) growth phase trs85Δ cells are impaired in maturation of proaminopeptidase I. Starvation for the indicated times in 1% potassium-acetate rescued this defect. Cells were harvested and processed for immunoblotting with antibodies to proaminopeptidase I. As a control wild-type cells (WCG), cells defective in autophagy (atg1Δ), and lacking vacuolar proteinase A (pep4Δ) or proaminopeptidase I (ape1Δ) are included. pApe1: proaminopeptidase I; mApe1: mature aminopeptidase I. B. Stationary grown trs85Δ cells fail to transport a fusion protein of proaminopeptidase I with the monomeric “red fluorescent protein” (proApe1-RFP) to the vacuole. Cells expressing proApe1-RFP from a centromeric plasmid were visualized with a Zeiss AxioScope 2 fluorescence microscope. Nomarski optics (upper row) and fluorescence (lower row) are shown. Bar: 10µm. atg19Δ cells are defective in vacuolar targeting of proaminopeptidase I via the Cvt pathway (29,32). proAminopeptidase I forms a large cytosolic complex (the Cvt complex) prior to vacuolar uptake, therefore fluorescent punctae are visible in the cytosol. In wild-type cells (WCG) aminopeptidase I is dispersed within the vacuole. C, trs33Δ and trs65Δ cells mature proaminopeptidase I. Cells grown to the stationary growth phase or starved for 4 hours in nitrogen-free SD(-N) medium were analysed in immunoblots with antibodies to proaminopeptidase I. pApe1: proaminopeptidase I; mApe1: mature aminopeptidase I.

Figure 2: **The autophagic rate is reduced in trs85Δ cells.** A. Lower row: Starvation for 4 hours in nitrogen-free SD(-N) medium in the presence of the proteinase B inhibitor PMSF leads to the accumulation of autophagic bodies in the vacuole. This opens an easy way to monitor autophagy under the light microscope. Autophagic bodies were detectable in the vacuoles of trs85Δ cells under these conditions. Compared to wild-type cells the number of autophagic bodies seemed to be reduced. Autophagy-deficient atg3Δ cells are included. Upper row: Starvation of the cells in the absence of PMSF did not lead to accumulation of autophagic bodies in the vacuole of trs85Δ cells, confirming their ability to breakdown autophagic bodies. B, During autophagy GFP-Atg8 is selectively enclosed in autophagosomes and targeted to the vacuole. Its vacuolar breakdown releases a rather proteolysis resistant GFP. The amount of GFP generated during starvation therefore allows the estimation of the autophagic rate. Cells expressing GFP-Atg8 from a centromeric plasmid (pGFP-Atg8) were starved in SD(-N) medium and aliquots were taken at the indicated times. Immunoblots probed with antibodies to GFP are shown. Compared to wild-type cells the amount of GFP generated during autophagy is significantly reduced in trs85Δ cells. atg18Δ cells are defective in autophagy. As a loading control the immunoblot was reprobed with antibodies against cytosolic 3-phosphoglycerate kinase (PGK). C, Autophagy can be quantitatively measured using a truncated alkaline phosphatase Pho8Δ60. Due to the lack of its membrane domain the protein stays in the cytosol and is targeted via autophagy to the vacuole, where it is proteolytically matured to an enzymatically active phosphatase. Cells chromosomally deleted for their endogenous PHO8 gene were transformed with a Pho8Δ60-expression plasmid (see Experimental Procedures). After shifting to SD(-N) starvation medium aliquots were taken and their phosphatase activity measured. The enzymatic activity of the wild-type cells after 4 hours starvation was set to 100%. mon1Δ and ccz1Δ cells are defective in autophagy.

Figure 3: **The selective pexophagic degradation of peroxisomes is retarded in trs85Δ cells.** Cells were grown in medium containing oleic acid to proliferate peroxisomes. Pexophagy was then induced by shifting the cells to SD(-N) medium. At the indicated time points aliquots were taken and analysed in immunoblots with antibodies to the peroxisomal marker protein Fox3 (A) using a Fuji LAS3000 imaging system with the AIDA program package. Quantification is shown in B. As a control autophagy deficient atg1Δ cells were included.

Figure 4: **The reduced autophagic capacity of starving trs85Δ cells is not caused by defects in vacuolar biogenesis, but due to reduced autophagic transport.** A, Mature vacuolar carboxypeptidase Y (mCPY) and mature vacuolar proteinase B (mPrB) are present in trs85Δ cells. Cells of the logarithmic (log) and stationary growth phase (stat) and cells starved for the indicated times in nitrogen-free 1%
potassium acetate were analysed in immunoblots with antibodies to proaminopeptidase I (pApe1). The blots were then reprobed with antisera against carboxypeptidase Y and proteinase B. As a loading control cytosolic 3-phosphoglycerate-kinase was detected. B, the vacuoles of trs85Δ cells are acidic. As described in Experimental Procedures cells of the stationary growth phase (left panel) or starved for 4 hours in 1% potassium acetate (right) were stained with the fluorescent dye quinacrine, which only accumulates in acidic vacuoles. Bar: 10µm. vma1Δ cells are defective in vacuolar acidification, therefore quinacrine does not accumulate in their vacuoles. C, Vacuolar targeting of GFP-Atg8 via autophagy is retarded in starved trs85Δ cells. Cells expressing GFP-Atg8 from a centromeric plasmid were starved for 4 hours in nitrogen-free SD-(N) medium and visualized with a Zeiss Axioscope2 fluorescence microscope. Bar: 10µm. In wild-type cells the selective autophagic cargo GFP-Atg8 is targeted to the vacuole, where proteolysis resistant GFP is proteolytically released (compare with Fig.2B). GFP-Atg8 is further seen at the perivacuolar PAS. atg1Δ cells are defective in autophagy.

Figure 5: Trs85 is required for the biogenesis of Cvt vesicles. A, ypt7Δ cells are impaire in vacuolar fusion of Cvt vesicles and therefore accumulate these vesicles in their cytosol. Stationary grown cells were spheroplasted and hypotonically lysed, leaving Cvt vesicles intact. Centrifugation of the total cell lyasate (T) at 13,000xg yielded a pellet (P13) and supernatant fraction (S13). The S13 supernatant was further separated at 100,000xg in a P100 pellet and S100 supernatant fraction. The fractions were then analysed in immunoblots with antibodies to proaminopeptidase I (pApe1). Proaminopeptidase I-containing Cvt vesicles sedimented in the 13,000xg pellet (lane 3). atg1Δ ypt7Δ cells are impaire in Cvt vesicle formation, consistent pApe1 is absent in the P13 pellet (lane 8). As controls the blots were reprobed with antibodies to the 100kDa subunit of vacuolar membrane ATPase (vATPase) and cytosolic 3-phosphoglycerate kinase (PGK). B,C proaminopeptidase I accumulates in proteinase sensitive form in growing trs85Δ ypt7Δ (B) and trs85Δ (C) cells. As above a spheroplast lyasate of stationary cells was incubated with buffer (B), proteinase K (K) or proteinase K with the detergent triton X100 (K+T). As expected in ypt7Δ cells proaminopeptidase I accumulated in membrane-protected form (lane 2). In atg3Δ ypt7Δ cells defective in biogenesis of Cvt vesicles proaminopeptidase I was proteinase sensitive in the absence of detergent. It should be noted that proaminopeptidase I as a resident vacuolar peptidase is not broken down by proteinase K, but converted to a pseudomature form (m*Ape1). D, In trs85Δ cells proaminopeptidase I is recruited to the PAS. Cells expressing GFP-Atg9 (28) and pApe1-RFP from plasmids were visualized with a Zeiss Axioscope2 fluorescence microscope and an Axiocam camera. Bar: 10µm. Nom: Nomarski optics. 90% of wild-type cells (236 cells analyzed) and 89% of trs85Δ cells (361 cells) showed colocalization of the integral membrane PAS protein Atg9 with proaminopeptidase I. Atg19 acts as a receptor recruiting proaminopeptidase I to the PAS, consistently in atg19Δ cells only 21% of 193 cells showed colocalization.

Figure 6: In logarithmically growing cells Trs85 is needed for the recruitment of GFP-Atg8 to the PAS. A, The preautophagosomal structure appears in fluorescence microscopy as a perivacuolar dot, where many Atg proteins colocalize. Cells expressing either GFP-Atg8, GFP-Atg9 or GFP-Atg19 from a plasmid were grown to the logarithmic phase (filled bars) or starved 4 hours in SD-(N) medium (hatched bars). The cells showing a fluorescent PAS were then counted. Growing trs85Δ cells failed to recruit GFP-Atg8 to the PAS (upper row). In the lower row typical microscopic images of growing cells are shown (bar 10µm). atg1Δ cells are defective in autophagy; WCG: wild-type. B, Atg8 covalently coupled to phosphatidylethanolamine (Atg8-PE) is detectable in trs85Δ cells. Cells of the logarithmic growth phase (log) or starved four hours in SD-(N) were prepared for immunoblotting with antibodies to Atg8. To allow separation of Atg8 from its lipidated form SDS gels containing 6M urea were used. Since starvation induced Atg8 is hard to detect in growing cells, two different gentic backgrounds were used. SEY, WCG: wild-types. atg7Δ and atg3Δ cells are defective in the lipidation of Atg8.

Figure 7: Trs85 does not affect the membrane expansion step during biogenesis of autophagosomes. ypt7Δ (B) and ypt7Δtrs85Δ (C) cells were starved four hours in SD-(N) medium, fixed with permanganate and then prepared for electron microscopy. The mean area of autophagosomes was determined by point
counting (A) and found identical in both strains. The inset in C is 2.5 fold enlarged. Due to the lack of Ypt7, the vacuoles are fragmented in these cells. N: nucleus; V: vacuole; A: autophagosome. Bar: 600nm.

**Figure 8: Localization of Trs85-GFP.** Cells expressing the indicated GFP fusion proteins under control of their native promotor from the chromosome, where grown to the logarithmic growth phase and visualized under the fluorescence microscope (upper row) and with Nomarski optics (lower row). Bar 10µm. Bet3-GFP and Trs120-GFP expressing cells showed multiple dots, cells expressing Trs85-GFP a single punctate structure beside a cytosolic pool.
Table 1 Strains used

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Fig. 6

A

- GFP-Alg19
- GFP-Alg9
- GFP-Alg8

% of cells with PAs:

- trs85Δ
- alg1Δ
- WCG
Fig. 6B

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Atg8
Atg8-PE
Fig. 7

A. Av profile mean size

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B. 600 nm

C.

Fig. 8

Bet3-GFP  Trs85-GFP  Trs120-GFP
Trs85 (Gsg1), a component of the TRAPP complexes is required for the organization of the preautophagosomal structure during selective autophagy via the Cvt pathway
Khuyen Meiling-Wesse, Ulrike D. Epple, Roswitha Krick, Henning Barth, Anika Appelles, Christiane Voss, Eeva-Liisa Eskelinen and Michael Thumm

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