Quantitative proteomics of Saccharomyces cerevisiae vacuoles and stress responses in Lactococcus lactis

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Galileo: How would it be if your Highness were now to observe these impossible as well as unnecessary stars through this telescope?

The mathematician: One might be tempted to reply that your telescope, showing something which cannot exist, may not be a very reliable telescope, eh?
Abstract

In the previous Chapter III, 22 novel vacuolar proteins were identified in a quantitative proteomics study. This approach however was not able to discriminate between vacuolar residents that function in the vacuole and proteins that are targeted to the vacuole for degradation. As validation for the proteomics study, the subcellular localization of eleven proteins was evaluated using GFP-tagging and fluorescence microscopy imaging. The vacuolar localization of nine target proteins was confirmed.
**Introduction**

In eukaryotic cells knowledge about subcellular (organelle) localization of proteins is required for understanding their physiological functions. The vacuole is the largest organelle in yeast and is involved in recycling of macromolecules, and storage and detoxification of small and macromolecular compounds. Vacuolar function has been well studied in *Saccharomyces cerevisiae*, but the exact protein composition still remains unsettled. The vacuolar protein content has been studied using high-throughput proteomics, which has greatly extended our knowledge of the proteins in the vacuolar membrane and the lumen (Sarry et al., 2007; Wiederhold et al., 2009). In vacuolar membranes, 22 novel proteins were detected which had not been assigned to any subcellular location by any study before (Chapter III, Table 1) (Wiederhold et al., 2009). Quantitative proteomics was used to demonstrate that the 22 proteins were indeed co-enriched with the vacuoles during the organelle preparation. But co-enrichment with vacuoles may occur not only for true vacuolar residents, which fulfill their cellular function in the organelle, but also e.g. for proteins that fulfill their functions elsewhere but are degraded in the vacuole. In the present study, we used GFP-fusions to confirm the vacuolar localization of a subset of the novel vacuolar proteins. GFP-fusions are widely used for determining or confirming the subcellular localization of proteins in eukaryotes, such as in yeast (Niedenthal et al., 1996). The intracellular location of 4,156 yeast proteins (Huh et al., 2003) has been determined with the GFP-tagging technique in a high-throughput study. To validate the vacuolar localization of proteins found by our proteomics approach, we selected 9 of the 22 newly identified proteins (Table 1) to be tagged with GFP. In previous GFP tagging studies, the 9 proteins had not been assigned to any subcellular location. Two additional proteins, YCR023C and YBR287W were also taken along. YBR287W was found to co-enrich with the vacuolar membrane (Wiederhold et al., 2009) although in the high-throughput global GFP-tagging study it was assigned to the ER (Huh et al., 2003). The YCR023C was previously shown to localize to the vacuolar membrane as GFP-fusion (Albertsen et al., 2003) and was used as a positive control in the present study. Thus, in total eleven proteins were used to validate their vacuolar localization by GFP-tagging and imaging (Table 1).

The GFP tag may be positioned at the N- or C-terminus of the proteins. The presence of a (cleavable) N-terminal signal peptide could affect targeting of a protein with an N-terminal GFP fusion. It is also known that C-terminal tags such as GFP may affect targeting (Huh et al., 2003) and often proteins with C-terminal GFP fail to reach their intracellular destinations and instead are trapped within the ER. The orientation of the termini (cytoplasmic or vacuolar lumen) might also affect the targeting of the chimera or, in case where the tagged terminus is located inside the vacuolar lumen, the signal might not be detectable due to low pH, or the tag can be cleaved off by vacuolar proteases. The membrane topology of the proteins used in this study was analyzed using three different topology prediction tools, TMHMM (Sonnhammer et al., 1998; Krogh et al., 2001), SignalP (Nielsen et al., 1997; Bendtsen et al., 2004) and Spoctopus (Viklund et al., 2008). Of the 11 proteins selected, two were without predicted transmembrane domains (YBL050W and YGR141W) and nine were predicted to have multiple TMD’s (Table 1). For five of the eleven proteins SignalP predicted N-terminal signal sequences, whereas Spoctopus predicted an N-terminal signal peptide for YCR011C/ADP1 only. Because the topology predictions were inconsistent in predicting the orientation of the termini and the presence of N-terminal signal peptides, we decided to tag each protein twice, at the N- and the C-terminus, and to compare the localization of both GFP-fusions. Enhanced yeast GFP developed for *Candida albicans* was used because it is an efficient expression reporter in *S. cerevisiae* (Cormack et al., 1996; Cormack et al., 1997). Both the C-terminal and N-terminal fusions were expressed from a low-copy number plasmid under the control of the methionine-repressible promoter. This promoter is switched on in the absence of methionine in the growth medium (Mumberg et al., 1994). Therefore, the yeast strain used for expression must not be auxotroph for methionine.
We chose to use two different strains, the BY4742 strain which is the parental strain for the S. cerevisiae chromosomal deletion collection (Brachmann et al., 1998), and Y258 which is the host strain for the Yeast ORF collection (Gelperin et al., 2005). The Y258 has reduced Pep4 protease activity which is the major protease of the yeast vacuoles and is responsible for the maturation of other vacuolar proteases (Jones et al., 1982). The reduced Pep4-activity of the Y258 strain prevents degradation of the heterologous (membrane) proteins that are targeted to the vacuolar membrane (Inoue et al., 1997). Although we overexpressed endogenous yeast proteins with expected destination at the vacuolar membrane, the Y258 strain could be advantageous in case the overexpressed proteins would be degraded in the BY4742 strain. Thus, for each gene of interest (except for YAL022C) four yeast strains were obtained resulting in 43 different strains for imaging (Supplemental Table 1).

Table 1. SUMMARY FOR PROTEINS USED IN THE STUDY

For eleven proteins used in this study, the membrane topology and signal peptide predictions from TMHMM, SignalP and Spoctopus, annotated subcellular localizations from the Yeast Genome Database (SGD), and the subcellular localizations as determined in the present study are summarized. Abbreviations: TMD: transmembrane domain(s); S/N/C: signal peptide/N-terminus/C-terminus; + or -: with or without signal peptide; in or out: orientation of termini in respect to the cytosol, inside or outside; ER: endoplasmic reticulum; VM: vacuolar membrane; VP: vacuolar periphery.

<table>
<thead>
<tr>
<th>ACCESSION NAME</th>
<th>Membrane TOPOLOGY</th>
<th>LOCALISATION</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TMHMM</td>
<td>SignalP</td>
</tr>
<tr>
<td></td>
<td>#TMD</td>
<td>S/N/C</td>
</tr>
<tr>
<td>YAL022C (Fun26)</td>
<td>11</td>
<td>-/in/out</td>
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<tr>
<td>YAR028W</td>
<td>2</td>
<td>-/out/out</td>
</tr>
<tr>
<td>YBL050W (Sec17)</td>
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<td>-/out</td>
</tr>
<tr>
<td>YBR074W</td>
<td>9</td>
<td>+/in/out</td>
</tr>
<tr>
<td>YBR287W</td>
<td>6</td>
<td>+/in/in</td>
</tr>
<tr>
<td>YCR011C (Adp1)</td>
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<td>+/in/in</td>
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<tr>
<td>YCR023C</td>
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<td>-/in/in</td>
</tr>
<tr>
<td>YGL114W</td>
<td>12</td>
<td>-/in/in</td>
</tr>
<tr>
<td>YGR141W (Vps62)</td>
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<td>+/in/out</td>
</tr>
<tr>
<td>YJR124C</td>
<td>10</td>
<td>+/in/in</td>
</tr>
<tr>
<td>YLR047C (Fre8)</td>
<td>5</td>
<td>-/in/out</td>
</tr>
</tbody>
</table>

**Results and Discussion**

For eleven target genes we constructed C- and N-terminal GFP-fusions, which were expressed in the two different yeast strains each, except for YAL022C. For the latter, the N-terminal GFP-fusion was successfully transformed to Y258 strain only, whereas the C-terminal construct was transformed into both the wild-type strain INVSc and Y258. Because the expression of proteins was under control of the methionine-repressible promoter, all cultures were grown on complete drop-out medium devoid of methionine. The small scale yeast cultures were grown for at least 16 hrs to allow the cells to reach the stationary phase. Subsequently, the stationary phase cultures were diluted 1:10 into a fresh medium and grown for 4-6 h until the mid-exponential phase (OD_{600}=0.4-0.6). The cells were harvested and, prior to imaging, labeled with the lipophilic fluorescent dye FM4-64, which partitions into the vacuolar membrane under conditions that promote endo-
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cytosis, i.e. in the presence of Mg$^{2+}$ and/or Ca$^{2+}$ at 30°C for at least 15 min (Vida & Emr, 1995). Because the emission spectra of GFP and FM-64 do not overlap, the FM-labeling allowed for a dual imaging of the same cells, using the green emission channel for GFP (between 500 and 580 nm) and the red emission channel for FM-64 (between 580 and 680 nm). The signals detected in the ‘green’ channel indicate the presence of GFP-tagged protein of interest (Fig. 1, left images). The signals detected in the ‘red’ channel indicate the presence of FM4-64 dye and therefore the location of the vacuolar membrane (Fig. 1, middle images). Merging of the images corresponding to the GFP and FM4-64 signals provided an indication about the localization of the GFP-fusion products. The right-hand side images show overlap of the left and middle images: the GFP signals are shown in green, FM4-64 signals are shown in red resulting in yellow-orange color when co-localizing.

It has been described that overexpression of heterologous proteins in the vacuolar membrane of *S. cerevisiae* is greatly affected by the strain of use, whereby the pep4-deficient strain increased the expression levels significantly. In the present study, we used a wild type strain BY4742 and a mutated strain Y258 with decreased activity of the PEP4 protease. However, no differences in subcellular localization or expression level between BY4742 and Y258 strains were found for the constructs used here. Because both strains performed equally well, only images from BY4742 are shown in Fig. 1 with the exception of YAL022C GFP-fusions.

As shown in Table 1, for only one protein, YGR141W/VPS62, no signal at 510 nm was detected in either strain and with either construct. The reason for the lack of fluorescent signal is unclear, but presumably GFP-tagged Vps62 was either not expressed or to levels below the detection limit. For the other 10 proteins we could detect fluorescent signals, indicating the expression of GFP-tagged proteins either as C-terminal (Fig. 1, G and H) or as N-terminal fusions (Fig. 1, I and J), or both (Fig. 1, A-F).

For four target proteins, we did not observe differences in cellular localization between C- and N-terminal constructs (Fig. 1, A, C, D, F). For one target protein, YBR287W, the GFP-signal was observed at the cell and nuclear periphery pointing towards ER-localization (Fig. 1, A). YBR287W was also localized to the ER in a global localization study, using C-terminal chromosomal fusion (Table 1) (Huh et al., 2003). The reason why GFP-YBR287W has a dual localization, whereas our proteomic study pointed to a unique localization in the vacuolar membrane is not clear. It is possible that GFP-tagged YBR287W is persistently mis-localized to the ER due to erroneous targeting. For YJR124C and YLR047C (Fig. 1, C and D), we could observe an overlap between the GFP-signal and the FM4-64 signal, indicating that the GFP-fusion protein was targeted to the vacuolar membrane. In the case of YBL050W/Sec17 (Fig. 1, F), the signals of GFP and FM4-64 dye did not overlap perfectly. Whereas the GFP-signal was diffuse and located rather at the vacuolar periphery, the FM4-64 stained the vacuolar membranes as sharp rings. These signal patterns point towards a soluble protein that is associated with the vacular membrane at its cytoplasmic site, which is in agreement with previous biochemical characterizations of Sec17; here the protein was found associated with intracellular membranes (Griff et al., 1992). The association of Sec17 with the vacuolar membrane appears logical in the light of Sec17 being required for the priming step in homotypic vacuole fusion (Mayer et al., 1996).

There were detectable differences between the C- and N-terminal tags for two proteins, YAR028W and YAL022C, (Fig. 1, B and E). Whereas YAR028W was detected in both the vacuolar membrane and the ER in the C-terminal fusion, it was detected at the vacuolar membrane only in the N-terminal fusion (Fig. 1, B: merged images). YAL022C was exclusively detected in the ER as C-terminal GFP-fusion (Fig. 1, E: upper) and at the vacuolar membrane as N-terminal GFP-fusion (Fig. 1, E: bottom). The data for these proteins show that some vacuolar membrane proteins with C-terminal GFP-fusions may have problems targeting to their vacuolar destination and be trapped within the ER.
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Figure 1. SUBCELLULAR LOCALIZATIONS OF TEN YEAST PROTEINS
To each protein GFP was fused either at the C- or at the N-terminus. The GFP emission signals are shown in the left-hand images of each panel (detected at 510 nm). The emission signals of the vacuolar membrane-specific dye FM4-64 are shown in the middle images. The overlap between the GFP-signals (green) and FM4-64 (red) are shown in the right-hand images. Coinciding signals (yellow-orange) indicate that the GFP-fusion is associated with the vacuolar membrane.

As mentioned above, for four target proteins, YCR023C, YCR011C/ADP1, YBR074W, YGL114W, we could detect GFP-signals only with either the C-terminal (Fig. 1, G and H) or N-terminal constructs (Fig. 1, I and J). Whereas the absence of GFP-signal for N-terminal fusion of ADP1 may be explained by the presence of a N-
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terminal signal peptide, the reason for its absence in YCR023C is unclear. On the other hand, if we would assume that the N-terminal signal peptide of ADP1 is cleaved off, we would expect to detect cytosolic diffuse GFP signals, unless the GFP is degraded after cleavage. So, we propose that ADP1 as N-terminal GFP fusion was not expressed.

In summary, we have validated our previous proteome study, using GFP as a marker for subcellular localization of proteins in yeast. For nine of ten successfully GFP-tagged target proteins, we could confirm their vacuolar localization. No differences in localization were detected between the C- and N-terminally tagged proteins, except for YAL022C. Therefore, we conclude that targeting was largely unaffected by the tag. As four proteins were detected only with either a C-terminal or an N-terminal tag, we recommend using both for subcellular localization studies.

**Experimental procedures**

**Yeast strains**

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INVSc</td>
<td>his3Δ1/leu2Δ3,112 trp1-289/ur3-52</td>
<td>Invitrogen</td>
<td>-</td>
</tr>
<tr>
<td>BY4742</td>
<td>MAT-α his3Δ1</td>
<td>EUROSCARF</td>
<td>(Brachmann et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>leu2Δ0 lys2Δ0 ura3Δ0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y258</td>
<td>Mat a pep4Δ3,112,112 ura3Δ53</td>
<td>Thermo Scientific Open Biosystems</td>
<td>(Gelperin et al., 2005)</td>
</tr>
</tbody>
</table>

**Construction of GFP fusions**

The genes of interest were amplified by PCR from S. cerevisiae W303 (MATα ade2-1 leu2-3,112 his3-22,15 trp1-1 ura3-1 can1-100) (Lawson & Douglas, 1988) genomic DNA with Phusion-DNA polymerase (Finnzymes), using specific primers that introduced desirable restriction sites and, in case of C-terminal GFP fusions, removed the “stop” codon (Supplemental Table 2). The PCR products were ligated in-frame into pUG35 and pUG36 that were cleaved to create the corresponding overhangs for the PCR products. The low copy number plasmids pUG35 and pUG36 were used to construct C-terminal and N-terminal yeast-enhanced GFP (yEGFP) fusions, respectively. Escherichia coli DH5α was used for plasmid amplification and isolation (Nishimura et al., 1990). For subcellular localization analysis, two different yeast strains, BY4742 and Y258, were transformed with the fusion constructs according to Gietz et al., (Gietz et al., 1992) and selected on the basis of uracil auxotrophy. The fusion constructs were expressed in yeast under the control of the methionine-repressible MET25 promoter.

**Growth conditions**

All E. coli cultures were grown at 37°C on Luria Broth plus 50 mg/L ampicillin. All yeast cultures were grown at 30°C on synthetic complete drop-out medium without uracil and methionine (SCura-/met-). Cells were grown for 16 h under shaking to stationary phase. The next morning, the cultures were diluted 1:10 into fresh SCura-/met- medium and allowed to grow for 4-6 h until the mid log phase (OD600 = 0.4-0.6).

**FM staining**

To 200-250 μL culture at OD600 =0.4-0.5, 1 μL of 200 μM stock FM-64 stain in 10 mM sodium phosphate, pH 6.9, 1 mM MgCl2 plus 1 mM CaCl2 was added. Cells were incubated with the stain for 1 h at 30°C. The OD600 of the culture after incubation was around 0.5-0.7. The cells were then spun down, resuspended in 1 ml of fresh SCura-/met- medium and immediately collected by pelleting. Cells were resuspended in a small volume of fresh SCura-/met- medium and subjected to imaging.

**Fluorescence microscopy**

The images were acquired on a wide-field fluorescence microscope which was based on an inverted microscope Observer D1 (Carl Zeiss, Jena, Germany). The laser beam (488 nm, argon ion laser, Melles Griot, Carlsbad, CA, USA) was focused by a Zeiss C-Apochromat infinity-corrected 1.2 NA 63 x water immersion objective and directed to the sample. The fluorescence emission was collected through the same objective and separated from the excitation beam by a dichroic mirror (Chroma Technology, Rockingham, VT, USA) and further directed through a 488 nm cut-off filter (Notch filter, CVI, Melles Griot, Carlsbad, CA, USA). The fluorescence signal was then separated into two channels, green (500-580 nm) and red (580-680 nm), by a dual view and detected by a Cool-Snap HQ2 CCD camera (Photometrics, Tucson, AZ, USA). The merged images were obtained using the BMP-inkleurer image processing tool (http://www.bogeert.com/BMPinkleurer/publish.htm).