The impact of ERAD on recombinant protein secretion in *Pichia pastoris* (syn *Komagataella* spp.)

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**Abstract**

The yeast *Pichia pastoris* (syn. *Komagataella* spp.) is a popular cell factory for recombinant protein production. Yeasts in general provide a good starting point for cell factory engineering. They are intrinsically robust and easy to manipulate and cultivate. However, their secretory pathway is not evolutionarily adapted to high loads of secretory protein. In particular, more complex proteins, like the antibody fragment (Fab) used in this study, overwhelm the folding and secretion capacity. This triggers cellular stress responses, which may cause excessive intracellular degradation. Previous results have shown that, in fact, about 60% of the newly synthesized Fab is intracellularly degraded. Endoplasmic reticulum-associated protein degradation (ERAD) is one possible intracellular degradation pathway for proteins aimed for secretion. We therefore targeted ERAD for cell factory engineering and investigated the impact on recombinant protein secretion in *P. pastoris*. Three components of the ERAD-L complex, which is involved in the degradation of luminal proteins, and a protein involved in proteasomal degradation, were successfully disrupted in Fab-secreting *P. pastoris*. Contrary to expectation, the effect on secretion was marginal. In the course of more detailed investigation of the impact of ERAD, we took a closer look at the intracellular variants of the recombinant protein. This enabled us to further zero in on the issue of intracellular Fab degradation and exclude an overshooting ER quality control. We propose that a major fraction of the Fab is actually degraded before entering the secretory pathway.

**INTRODUCTION**

Recombinant protein production is an expanding branch of biotechnology of increasing economic importance [1]. Besides other expression systems, yeasts are frequently applied for the production of recombinant enzymes and therapeutic proteins [2, 3]. Some proteins are efficiently secreted by yeast systems, reaching product titres in the g l−1 range. However, more complex proteins, like the antibody fragment (Fab) used in this study, overwhelm the folding and secretion capacity of the cell [4]. The methylotrophic yeast *Pichia pastoris* (syn. *Komagataella* spp.) is among the most favoured microbial eukaryotic expression systems for biopharmaceutical or industrial proteins [5, 6], most of which are produced in a secreted form [7].

In this work we challenged the *P. pastoris* expression system with an antibody fragment (Fab), which is a dimeric human protein. It consists of two chains which are linked covalently in the endoplasmic reticulum (ER) by the formation of one inter-chain disulfide bond. The α-mating factor prepropeptide (MFα secretion leader) facilitates secretion of the recombinant Fab. The first step on the classical secretion pathway is the translocation of the polypeptide into the ER. In the ER, ideally the Fab folds, dimerizes and passes the ER quality control to be further transported to the Golgi and then secreted. However, Pfeffer *et al.* found that about 60% of the newly synthesized Fab is intracellularly degraded instead of being secreted [8]. Subsequently the authors showed that the recombinant Fab interacts with proteasome components (Rpn1 and Rpt6) and that inhibition of the proteasome by MG-132 leads to enhanced levels of secreted product, thus indicating that the recombinant Fab was actually functional and not misfolded [9]. Thus, they hypothesized that the ER quality control is overshooting and triggering ER-associated protein degradation (ERAD).

In general, ERAD removes misfolded proteins from the ER by retro-translocating them back to the cytosol, where they are ubiquitinated and finally degraded by the proteasome [10]. Three different ERAD pathways (ERAD-L, -M and -C) exist for protein removal from the ER, depending on the location of the misfolded domain and solubility of the protein. It consists of two chains which are linked covalently in the endoplasmic reticulum (ER) by the formation of one inter-chain disulfide bond. The α-mating factor prepropeptide (MFα secretion leader) facilitates secretion of the recombinant Fab. The first step on the classical secretion pathway is the translocation of the polypeptide into the ER. In the ER, ideally the Fab folds, dimerizes and passes the ER quality control to be further transported to the Golgi and then secreted. However, Pfeffer *et al.* found that about 60% of the newly synthesized Fab is intracellularly degraded instead of being secreted [8]. Subsequently the authors showed that the recombinant Fab interacts with proteasome components (Rpn1 and Rpt6) and that inhibition of the proteasome by MG-132 leads to enhanced levels of secreted product, thus indicating that the recombinant Fab was actually functional and not misfolded [9]. Thus, they hypothesized that the ER quality control is overshooting and triggering ER-associated protein degradation (ERAD).

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protein. These pathways use two distinct membrane protein complexes. The Hrd1 complex removes proteins with misfolded luminal domains (ERAD-L) and proteins with disrupted transmembrane domains (ERAD-M). The Doa10 (Ssm4) complex removes membrane proteins misfolded in their cytosolic domains (ERAD-C) [10]. Both complexes are linked via Ubx2 to Cdc48-Npl4-Usf1 complexes, which extract the substrate from the membrane, translocating the polypeptide through its central pore [11, 12]. It was suggested that ERAD-L (Hrd1 complex) might play a major role in degrading recombinant protein, given that they are soluble proteins. The Hrd1 complex consists of Yos9 and Hrd3, which are involved in substrate selection; Der1, which probably helps to insert luminal substrates into the Hrd1 retro-translocation channel; and Usa1, serving as a scaffold [13].

ERAD also seems to be linked to the unfolded protein response (UPR) [14, 15], which is generally upregulated during the expression of a recombinant protein destined for secretion [16]. ERAD may be a major vent for the cell to decrease the protein load in the ER during the high overexpression of a recombinant protein, overwhelming the secretory capacity of the cell. Since the expression of Fab triggers the UPR [16, 17], possibly stimulating ERAD [14], and given that ERAD may present a major bottleneck in Fab secretion [9], we decided to investigate disruption of this pathway at a molecular level. Based on the soluble nature of the antibody fragment (Fab), we decided to focus on the ERAD-L complex and investigated the impact of its disruption on Fab secretion in *P. pastoris*.

**METHODS**

**Strains**

The *P. pastoris* host strain used in this study derives from the wild-type CBS7435 (Centraalbureau voor Schimmelcultures, NL). The host strain (P<sub>GAP</sub>Fab#34, referred to as control) contains the expression cassettes for the antibody fragment HyHEL-Fab, consisting of a light and a heavy chain under control of the *glyceraldehyde-3-phosphate dehydrogenase* promoter P<sub>GAP</sub>, fused to the *S. cerevisiae* MPα secretion leader for secretion and integrated into the GAP promoter region of the *P. pastoris* genome using a Zeocin resistance marker, as described previously [18]. Likewise, P<sub>AOX1</sub>-Fab#8 expresses the HyHEL-Fab light and heavy chains under control of the methanol-inducible alcohol oxidase promoter P<sub>AOX1</sub>. The disruption strains were generated using the split-marker system and electroporation [19, 20]. The transformed host strain was plated onto selective YPD plates containing the respective amounts of antibiotics (50 µg ml<sup>-1</sup> Zeocin+500 µg ml<sup>-1</sup> G418). After re-streaking on selective YPD plates, positively growing transformants were further confirmed by PCR. In order to verify the gene disruptions, genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen) and PCRs were performed with a detection primer pair designed to bind the native gene loci outside the split-marker cassette. Successful gene disruptions were confirmed by the altered length of PCR products in these detection PCRs. All primers used for generation of the gene disruption cassettes of *NPL4* (PP7435_Chr1-1561), *HRD3* (PP7435_Chr3-0152), *RPT6* (PP7435_Chr1-0353), *RPN4* (PP7435_Chr3-0511), *DER1* (PP7435_Chr1-0322) and *DOA1* (PP7435_Chr1-1428), as well as the primers to detect positive transformants, are summarized in Table S1 (available in the online version of this article).

**Media and cultivation conditions**

After verification of the gene disruptions, as mentioned above, the strains were cultivated in 24-deep well plates. Each pre-culture, 2 ml of YPD medium (20 g l<sup>-1</sup> soy peptone, 10 g l<sup>-1</sup> yeast extract and 20 g l<sup>-1</sup> glucose set to pH 7.5 with NaOH) containing 50 µg ml<sup>-1</sup> Zeocin+500 µg ml<sup>-1</sup> G418 was inoculated and incubated overnight at 25 °C with shaking at 280 r.p.m. In the morning, the cells were harvested and washed with minimal M2 medium [per liter: 3.15 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.49 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.80 g KCl, 0.0268 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.47 ml PTM1 trace metals, 4 mg biotin; pH was set to 5.0 with KOH tablets]. The main culture containing 2 ml M2 medium supplemented with glucose feed beads (12 mm, Kuhner) was inoculated with a starting OD<sub>600</sub> of 0.1. In the case of methanol cultivations with P<sub>AOX1</sub>, no glucose feed beads were used and the starting OD<sub>600</sub> was 4. The main culture was incubated for 48 h at 25 °C with shaking at 280 r.p.m. The methanol cultivations were additionally fed by adding methanol in the following manner: 0.5, 1, 1, 1% after 0, ~6, ~16 and ~24 h, respectively. The cells were then harvested by centrifugation for 5 min at full speed and the supernatant was collected for further analyses [18].

**Relative transcript level analysis by quantitative PCR**

Cell pellets were harvested from the glucose-limited main culture 44 h after inoculation. Total RNA was extracted by mechanical cell lysis (glass beads at 5.5 m s<sup>-1</sup> with FastPrep cell homogenizer) and isolated with 1 ml of TRI Reagent Solution (Life Technologies). After DNA-free (Ambion) treatment, 1 µg of RNA per sample was reverse-transcribed using the DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific) and an oligo d(T)<sub>23</sub> VN primer (New England Biolabs). The qPCR reaction was made up of 0.25 µl of forward and 0.25 µl of reverse primer (Table S2), 5 µl of 2× SensiMix SYBR Hi-ROX Kit (Bioline), 3.5 µl of nuclease-free water and 1 µl of the sample. The qPCRs were performed under the following conditions: 10 min hot start at 95 °C followed by 45 cycles of 15 s at 95 °C, 20 s at 60 °C and 15 s at 72 °C [9]. The relative transcription levels were determined by normalizing the fluorescence signal of the respective sample to the chosen control sample using the Rotor-Gene software comparative quantification method. This ratio was further normalized to the ACT1 signal to compensate for initial concentration differences.

**Quantification of Fab in culture supernatants**

The amount of secreted Fab was quantified in the supernatant at the end of cultivation by ELISA. The anti-human IgG antibody (2A11) (anti-hinge; Abcam, ab7497) was used...
for coating. The anti-human IgG (Fab-specific)-AP antibody (Sigma-Aldrich, A8542) was used to generate a signal, which was normalized to a Fab standard (starting concentration 100 ng ml⁻¹; Bethyl Laboratories, P80-115). The phosphatase substrate pNPP was used, and was read spectrophotometrically at 405 nm. In between, the immunosorbent plates (Nunc, Maxisorp) were washed with PBS containing 0.1 % Tween 20 at pH adjusted to 7.2. Yield values are defined as µg Fab per gram wet cell weight [18].

Separation of cell lysates into soluble and insoluble fractions

Membrane-associated proteins in the insoluble fraction were separated from cytosolic and vacuolar proteins in the soluble protein fraction. First the cell pellets were harvested from 1 ml of screening culture, washed with PBS and resuspended in 500 µl of disintegration solution (SigmaFast Protease Inhibitor S8820 dissolved in 50 ml PBS and 10 nM N-ethylmaleimide). The cells were disrupted with glass beads (0.5 mm) using a MP FastPrep (3 × 15 s, 4 m s⁻¹ with cooling of the samples on ice in between). The liquid phase was transferred to a fresh tube and was then centrifuged for 30 min at 4 °C and full speed (approximately 15 000 g). After centrifugation, the supernatant containing soluble cytosolic and vacuolar proteins was stored at −20 °C. The pellet (containing membrane-associated proteins and organelles) was further treated with 600 µl of solubilization solution (disintegration solution with 2 % SDS and 1 % Triton X100) to extract the insoluble protein fraction. After centrifugation at 2300 g for 5 min at 4 °C, the supernatant containing the membrane proteins was stored at −20 °C.

SDS-PAGE and Western blot

The protein lysate samples were quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) in order to normalize the samples. In the case of sample reduction, samples were treated with PNGase F according to the supplier’s protocol (New England Biolabs). Afterwards, the samples were treated with precipitation solution (SigmaFast Protease Inhibitor S8820 dissolved in 50 ml PBS and 10 nM N-ethylmaleimide). The cells were disrupted with glass beads (0.5 mm) using a MP FastPrep (3 × 15 s, 4 m s⁻¹ with cooling of the samples on ice in between). The liquid phase was transferred to a fresh tube and was then centrifuged for 30 min at 4 °C and full speed (approximately 15 000 g). After centrifugation, the supernatant containing soluble cytosolic and vacuolar proteins was stored at −20 °C. The pellet (containing membrane-associated proteins and organelles) was further treated with 600 µl of solubilization solution (disintegration solution with 2 % SDS and 1 % Triton X100) to extract the insoluble protein fraction. After centrifugation at 2300 g for 5 min at 4 °C, the supernatant containing the membrane proteins was stored at −20 °C.

RESULTS

Disruption of ERAD genes

To investigate the impact of ERAD on Fab secretion in P. pastoris, we targeted the genes listed in Table 1 in order to reduce ERAD activity [21].

The split-marker system [19] was used to disrupt these components in two Fab-producing P. pastoris strains (P GAP-HyHEL-Fab#34 and P AOX1-HyHEL-Fab#8). Positive gene disruptions (confirmed by PCR) were obtained for HDR3, DER1, DOA1 and RPN4, but we did not find any viable clones with the disrupted target genes NPL4 and RPT6. While RPT6 was reported to be essential also in S. cerevisiae, NPL4 disruption in S. cerevisiae only exhibited decreased fitness according to a large-scale survey [22].

First, we investigated the influence on growth and Fab secretion. To do this, three clones of each gene disruption, along with the control clone, were cultivated in biological

Table 1. Gene disruption targets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Process</th>
<th>Function of protein (in S. cerevisiae)</th>
<th>Clones obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPL4</td>
<td>ERAD</td>
<td>Npl4 is part of the Cdc48-Npl4-Ufd1 complex and aids in retro-translocation [11].</td>
<td>No</td>
</tr>
<tr>
<td>HRD3</td>
<td>ERAD</td>
<td>Hrd3 is part of the Hrd1 complex and recognizes misfolded substrates in the ER [24]. Hrd3 also prevents excessive degradation of Hrd1 [48].</td>
<td>Yes</td>
</tr>
<tr>
<td>DER1</td>
<td>ERAD</td>
<td>Der1 helps to insert luminal substrates, and its disruption strongly affects ERAD [14].</td>
<td>Yes</td>
</tr>
<tr>
<td>DOA1</td>
<td>ERAD</td>
<td>Doa1 plays an important role in ubiquitin-dependent protein degradation by direct interaction with Cdc48; the disruption of its homologue in A. niger caused slower growth [23]</td>
<td>Yes</td>
</tr>
<tr>
<td>RPT6</td>
<td>Proteasome</td>
<td>Rp6 is an ATPase subunit of the proteasome, which has been found to interact with Fab [9]</td>
<td>No</td>
</tr>
<tr>
<td>RPN4</td>
<td>Proteasome</td>
<td>Rpn4 is a transcription factor that stimulates expression of proteasomal genes [49]</td>
<td>Yes</td>
</tr>
</tbody>
</table>
The Fab concentrations in the supernatant of the small-scale screenings were measured with ELISA. A summary of the effects on biomass, titre and product yield (mg secreted Fab per gram wet cell weight) of the disruption clones is listed in Table 2. The effect was measured by comparing those absolute values of the engineered clones versus the non-engineered producer strain (control) and calculating the fold-changes thereof. It is important to mention that the absolute product yields were several-fold higher for \( \text{P}_\text{GAP} \)-HyHEL-Fab#8 (cultivated on methanol) than for \( \text{P}_\text{GAP} \)-HyHEL-Fab#34 (cultivated on glucose). This shows the greater production capacity of the methanol-induced expression system. As regards the disruption clones, we did not observe any major increases in Fab secretion. Contrary to expectation, Fab secretion even decreased in the case of \( \Delta \text{hrd}3 \) and \( \Delta \text{rpn}4 \) in both glucose and methanol cultivations. In the case of \( \Delta \text{der1} \) and \( \Delta \text{doa1} \), Fab secretion remained virtually unchanged in glucose cultivations, but Fab titres were increased in the methanol cultivations. It is interesting to mention that \( \Delta \text{doa1} \) caused increased growth in the methanol cultivation. This is in contrast to the observation in \( \text{A. niger} \), where \( \Delta \text{doa1} \) (\( \text{doaA} \)) caused a phenotype of slower growth [23]. Owing to this increase in biomass, there was an actual decrease in biomass-specific secretion. This can be seen by the yield fold-change (FC) of 0.85, which is the titre normalized to the increased biomass.

Owing to the generally low impact of ERAD disruptions on Fab secretion, we questioned whether the disruptions actually had the anticipated effect. Since the \( \Delta \text{hrd}3 \) and \( \Delta \text{rpn}4 \) clones showed the most significant effects, they were selected for further investigation. In \( \text{S. cerevisiae} \), \( \Delta \text{hrd}3 \) caused a constitutive activation of the UPR [14] and decreased ERAD substrate degradation [24, 25]. We therefore investigated transcriptional levels of the two UPR marker genes \( \text{PDI1} \) and \( \text{KAR2} \). In previous experiments with \( \text{P. pastoris} \), \( \Delta \text{rpn}4 \) caused an upregulation of genes related to the heat shock response (HSR) and UPR in addition to the lower expression levels of proteasomal subunits (unpublished data). Also, in \( \text{S. cerevisiae} \), \( \Delta \text{rpn}4 \) decreases expression of proteasomal subunits and the transcriptional regulatory network overlaps with the heat shock response [26]. While proteasomal genes still showed lower expression levels, it is important to mention that in these previous \( \text{P. pastoris} \) experiments the HSR upregulation was overlain after induction of the recombinant protein expression, and the difference between the \( \Delta \text{rpn}4 \) and control clone became insignificant. Nevertheless, we selected the two marker genes, \( \text{HSF1} \) and \( \text{SSA3} \), for the heat shock response (Fig. 1).

The \( \Delta \text{hrd}3 \) disruption seems to have had the anticipated effect on the \( \text{P. pastoris} \) cells, as indicated by the slight increase in \( \text{PDI1} \) and the greater increase of \( \text{KAR2} \) transcript levels (Fig. 1). The \( \Delta \text{rpn}4 \) disruption did not seem to influence UPR and ERAD (Fig. 1). This is most likely due to the cellular stresses caused by constant strong overexpression of the Fab chains by \( \text{P}_\text{GAP} \), overlaying possible regulatory patterns of HSR and UPR in the \( \Delta \text{rpn}4 \) mutant.

### Intracellular recombinant protein

Next, we investigated whether these disruptions actually increased the intracellular levels of recombinant protein, as was the case for ERAD mutants in \( \text{A. niger} \) [23]. The cells of the production strain, the \( \Delta \text{hrd}3 \) strain and the \( \Delta \text{rpn}4 \) strain were lysed and the total protein extracts were separated into membrane-associated and soluble protein fractions. In \( \text{S. cerevisiae} \), \( \Delta \text{hrd}3 \) comes with an increased accumulation of ERAD substrates [27, 28]. In our case, we therefore expected increased levels of intracellular Fab, especially in the ER. The \( \Delta \text{rpn}4 \) disruption is expected to produce an accumulation in the cytosol due to a decrease in proteasomal gene expression (unpublished data) and a decrease in overall proteasomal activity, as observed for \( \Delta \text{rpn}4 \) in \( \text{S. cerevisiae} \) [29]. In order to test this assumption, we prepared cell lysates and separated them into soluble and insoluble fractions. In addition to soluble cytosolic proteins, in \( \text{P. pastoris} \) the soluble fraction also contains soluble vacuolar proteins such as mature CPY (Fig. 2a). The insoluble fraction contains all proteins within membrane-surrounded compartments or proteins attached to membranes. Western blots of the fractionated samples from glucose-grown \( \text{P}_\text{GAP} \)-Fab, \( \text{P}_\text{GAP} \)-Fab \( \Delta \text{hrd}3 \) and \( \text{P}_\text{GAP} \)-Fab \( \Delta \text{rpn}4 \) are shown in Fig. 2. In addition, the methanol-grown control (\( \text{P}_\text{AOX1} \)-Fab) is also shown. Engineered \( \text{P}_\text{AOX1} \)-Fab \( \Delta \text{hrd}3/\Delta \text{rpn}4 \) samples are not shown, because we did not observe any differences relative to the control \( \text{P}_\text{AOX1} \)-Fab (Fig. S1a).

### Table 2. Effect of gene disruptions on the secretion of HyHEL-Fab

The effect is measured in fold-change (FC) values, giving the relative change of the respective parameter in reference to the non-engineered producer strain (control). At least three clones with the respective disruption were cultivated in biological triplicate in 24-deep-well plates. The amount of product in the supernatant was quantified by ELISA after approx. 48 h of cultivation. Fold-changes (FC) with \( p \)-values below 0.05 are marked with an asterisk (*).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Process</th>
<th>( \text{P}_\text{GAP} )-Fab (glucose)</th>
<th>( \text{P}_\text{AOX1} )-Fab (methanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Biomass FC</td>
<td>Titer FC</td>
</tr>
<tr>
<td>( \Delta \text{hrd}3 )</td>
<td>ERAD</td>
<td>0.97±0.02</td>
<td>0.69±0.09</td>
</tr>
<tr>
<td>( \Delta \text{der1} )</td>
<td>ERAD</td>
<td>1.01±0.00</td>
<td>1.02±0.03</td>
</tr>
<tr>
<td>( \Delta \text{doa1} )</td>
<td>ERAD</td>
<td>0.98±0.05</td>
<td>1.03±0.06</td>
</tr>
<tr>
<td>( \Delta \text{rpn}4 )</td>
<td>Proteasome</td>
<td>0.89±0.02</td>
<td>0.74±0.14</td>
</tr>
</tbody>
</table>
In P\textsubscript{GAP}-Fab, the detected intracellular Fab light chain (LC) seems to be either fully processed (~27 kDa; marked by [●] in Fig. 2a), not completely processed (~32 kDa; marked by [▼] in Fig. 2a), covalently linked to the heavy chain (HC; ~47 kDa; marked by [□] in Fig. 2a) or ubiquitinated (Fig. 2b, c). The P\textsubscript{GAP}-Fab \(\Delta\text{hrd3}\) disruption clones show slightly increased amounts of the LC in the soluble fraction compared to the control. This is interesting, especially in the case of the \(\Delta\text{hrd3}\) clone, because we were actually expecting increased amounts in the insoluble fraction due to a decrease in retro-translocation and therefore mainly accumulation in the ER. The expected increase in the insoluble fraction of the \(\Delta\text{hrd3}\) clone is scarcely present (Fig. 2b). The detected bands are barely more intense, especially when judging the much stronger HDEL signal (ER marker) of this clone. Based on their molecular weight, most probably these bands represent MF\textalpha-LC fusion proteins located within the ER lumen (see scheme in Fig. 3e). Strikingly, the multiple bands in the P\textsubscript{GAP}-Fab samples showed a pattern very similar to the bands of the anti-ubiquitin signal (Fig. 2c), suggesting that we were detecting membrane-associated or insoluble protein aggregates of ubiquitinated Fab LCs. Ubiquitin seems to mark the recombinant Fab LC for proteasomal degradation, which is in line with the finding that nearly 60% of Fab is degraded largely by the proteasome [8, 9]. Most probably, the Fab LC is attached to the ER membrane in P\textsubscript{GAP}-Fab. It is not clear, however, whether the detected Fab LCs ever reached the interior of the ER before undergoing degradation.

Answering this question is critical, because two different bottlenecks in the expression system are indicated. If the majority of Fab chains has been translocated into the ER and retro-translocated out of the ER again (ERAD), it would manifest the symptom of an overloaded ER with insufficient folding capacity [4]. In the other case, where the Fab chains are degraded without ever reaching the interior of the ER, we would see the symptom of a translocation bottleneck [30] due to insufficient translocation capacity or premature folding in the cytosol.

**Does the majority of recombinant protein even reach the interior of the ER?**

The processes around the ER membrane seem to be crucial in view of the above-mentioned issues in recombinant protein secretion. For this reason, we attempted to focus on the...
Fab peptides in this area of the cell by further investigating the insoluble (membrane-associated) fractions using a fluorescent multiplex Western blot, which simultaneously detects anti-ubiquitin and anti-Fab. In this respect, it is important to mention that the \textit{S. cerevisiae} MFa secretion leader, which is the most commonly used and effective secretion leader for recombinant proteins in \textit{P. pastoris} \cite{31}, consists of a 2 kDa pre-region and a 7 kDa pro-region (Fig. 3e). Processing of this signal peptide occurs in the following manner: first, the pre-peptide is removed by signal peptidases in the ER during translocation and, second, the Kex2 endopeptidase cleaves the pro-peptide C-terminal of the Lysine-Aginine dibasic site in the Golgi \cite{31}. The pro-peptide still present on the Fab chain in the ER has three N-glycosylation sites. The presence of these N-linked oligosaccharides would indicate that the recombinant polypeptide has reached the interior of the ER \cite{32}. We therefore investigated whether the apparently ubiquitinated Fab light chain (Fig. 2) carries any N-linked oligosaccharides by treating the cell extracts with PNGase (Fig. 3).

Fig. 3(a) shows the insoluble protein fractions of \textit{P}_{GAP}\textit{-Fab}, \textit{P}_{GAP}\textit{-Fab \textDelta}hrd3 and \textit{P}_{GAP}\textit{-Fab \textDelta}rpn4 as well as the methanol-grown control (\textit{P}_{AOX1}\textit{-Fab}). This set-up shows relative protein amounts, because samples of the soluble fraction of \textit{P}_{GAP}\textit{-Fab} strains were normalized to 15 µg (\textit{P}_{AOX1}\textit{-Fab}: 5 µg) and samples of the insoluble fraction were normalized to 30 µg (\textit{P}_{AOX1}\textit{-Fab}: 10 µg) per well. (a) Fab light chain detection in the soluble (non-membrane) fraction. The symbol [■] denotes the assembled heavy- and light chain-Fab, [▲] denotes un- or semi-processed light chains and [●] denotes fully processed light chains. (b) Fab light chain detection and (c) ubiquitin detection in insoluble (membrane-associated) fraction of cell lysates. The protein fractions were also analysed with antibodies detecting HDEL (ER marker), GAPDH (cytosolic marker) and CPY (vacuolar marker). (d) Migration patterns of the secreted and therefore fully processed Fab light and heavy chains obtained from culture supernatants under either non-reducing (assembled Fab) or reducing (unassembled light or heavy chains) conditions. There is no difference in the migration pattern of Fab-LC/-HC from \textit{P}_{GAP}\textit{-Fab} or \textit{P}_{AOX1}\textit{-Fab} culture supernatants.
**Fig. 3.** Fluorescent multiplex Western blot simultaneously detecting Fab and ubiquitin in reduced and PNGase-treated insoluble fractions of *P. pastoris* cell lysates. (a) Fab light chain [red] and ubiquitin [green] detection in insoluble (membrane-associated) fraction of previously non-treated [-] and PNGase-treated samples [+]. The symbol [-] marks un- or semi-processed light chains and [►] marks fully processed light chains. (b) Supernatant with scFv-Fc known to be glycosylated [50] as control for PNGase digest. (c) Soluble cell lysate fraction of P*_{GAP}*-Fab showing unbound ubiquitin (lower band; higher band: dye front). (d) Fab heavy chain [red] and ubiquitin [green] detection in insoluble (membrane-associated) fraction of previously non-treated [-] and PNGase-treated samples [+]. The symbol [-] marks un- or semi-processed heavy chains. (e) Schematic structure with calculated sizes of secretory Fab light/heavy chain with MFα secretion leader consisting of the pre-peptide cleaved during entry into the ER and the pro-peptide cleaved by the Kex2 pro-peptidase in the Golgi. The three N-glycosylation sites on the pro-peptide are depicted by the branched, blue-coloured structures.
of the light chain in the P_{AOX1}-Fab samples seem to be glycosylated, on account of the observable band shift upon PNGase treatment (marked by [>] in P_{AOX1}-Fab Fig. 3a). By comparing the major Fab light chain populations in the P_{GAP}- and P_{AOX1}-Fab cell lysate fractions, we can observe that the P_{AOX1}-Fab producer contains markedly more Fab light-chain populations with significantly higher molecular sizes. The major fraction of these higher molecular size variants also seems to be sensitive to PNGase treatment. This indicates the presence of N-glycans and therefore a current localization within the secretory pathway (either ER or Golgi). The higher molecular size variants even after N-glycan removal (P_{AOX1}-Fab in Fig. 3a) indicate the presence of at least the pro-peptide of the M{\textalpha} secretion leader (Fig. 3e), which is no longer present after successful secretion to the supernatant (Fig. 2d).

The P_{GAP}-Fab strain does not seem to have any detectable amounts of obviously un- or semi-processed signal peptide light-chain fusions. The major population of Fab light chain in P_{GAP}-Fab has similar retention times to the fully processed and secreted light chain (Fig. 2d). On the other hand, it also seems to be ubiquitinated although the estimated size does not match an additional ubiquitin (~8.5 kDa), even less polyubiquitination. The exact route of these detected Fab light chains in P_{GAP}-Fab is therefore ambiguous. However, we think that we are detecting partly degraded light chains. This supposition is supported by strongly visible ubiquitin signals in the insoluble (membrane-associated) fraction detected previously (Fig. 2c).

The other peptide chain required for Fab assembly in the ER is the heavy-chain fragment. Here again, we are interested in the molecular size variants around the ER membrane. As regards the P_{GAP}-Fab samples, the most prominent heavy chain bands are found at around 33 and 52 kDa (both marked by [>] in Fig. 3d). The detected variant at around 33 kDa most likely still possessed the pre-pro-peptide of the M{\textalpha} secretion leader, whereas the variant at around 52 kDa seems to be additionally ubiquitinated. In addition, there was a fainter band at around 31 kDa, which seems to be the heavy chain fused to the pro-peptide and missing the pre-peptide of the M{\textalpha} secretion leader. In any case, both variants are larger than the fully processed heavy chain detected in the supernatant (Fig. 2d). The intracellular heavy-chain bands in P_{GAP}-Fab were not PNGase sensitive, indicating the absence of N-glycans. This therefore indicates that the majority of the heavy chain, in contrast to the light chain, never reaches the interior of the ER and is targeted for proteasomal degradation before translocation. The P_{AOX1}-Fab samples did not show a heavy-chain pattern (Fig. 3d) differing from that of the light chain (Fig. 3a). More heavy-chain fragments seem to translocate successfully into the ER during methanol cultivation (P_{AOX1}-Fab).

**DISCUSSION**

In order to successfully improve *P. pastoris* for Fab production, one needs to be able to specifically pinpoint the bottlenecks of the intracellular process. That process begins with product gene transcription and ends with exocytosis of the recombinant protein. In this study, the hypothesis at the outset was an excessive intracellular degradation of recombinant Fab [8], possibly due to an overshooting ERAD [9]. The approach taken to reduce ERAD through gene disruptions did not yield significant increases in Fab secretion. This is in agreement with similar studies in *S. cerevisiae* [33], even though we targeted different components of the ERAD pathway. Instead of improving secretion, we were able to shed light on the processes that lead to intracellular retention of the product and ensuing differences in specific product yield (Fab titre in supernatant per biomass) between P_{GAP}-Fab (grown on glucose) and P_{AOX1}-Fab (grown on methanol).

Recombinant proteins that fail to translocate into the ER decrease productivity and are ultimately degraded. The light chain of the Fab in glucose-grown cells (P_{GAP}-Fab) does not seem to have this problem. The major size variants of the light chain we detected seem to lack the pro-peptide of the M{\textalpha} secretion leader. This means that these light chains must have passed through the ER (pre-peptide removal) and the Golgi (pro-peptide removal). The M{\textalpha} pro-peptide is cleaved off by Kex2, which is located in the late Golgi. After the Fab leaves the ER to the Golgi, the only possible route of degradation is to the vacuole. If the detected light chain (~27 kDa; Fig. 3a) passed the ER/Golgi and ended up in the vacuole [18], then no ubiquitin would be attached. Another possibility might be the rather unlikely event of Kex2 (pro-peptide) cleavage in the ER followed by ERAD [34]. This event would generate a transient ubiquitinated light-chain variant. Most likely, the prevailing portion of light chain is simply secreted. Indeed, it was shown previously that LCs are dimerized and readily secreted in the absence of Fab heavy chain [35].

The major fraction of the light chain in methanol-grown cells seems to be located in the ER. This is indicated by the presence of glycans on the uncleaved pro-peptide and the absence of ubiquitin. This marked contrast to the glucose-grown cells (P_{GAP}-Fab) especially as regards quantity, shows that much more light chain is in a state ready for pairing or has already paired with the heavy chain in the methanol-grown cells (P_{AOX1}-Fab). This means that a much larger fraction of the Fab is *en route* to successful secretion in the P_{AOX1}-Fab, which matches the higher secretion rates obtained per biomass with this system. Additionally, ER-localized Fab chains in the P_{AOX1}-Fab strain seem to be protected from ubiquitination and subsequent degradation by the cytosolic proteasome. The heavy chain in methanol grown cells did not show an obviously different pattern in the cell lysate compared to the light chain.

The heavy-chain size variants detected in the glucose-grown cells (P_{GAP}-Fab) do, however, differ from those of the light chain. The sizes of ~33 kDa and greater indicate that at least the pro-peptide is still present. The ubiquitin signal further indicates targeting for proteasomal degradation, and the...
lack of glycans calls into question whether those heavy-chain species ever reached the inside of the ER. Glycan removal during ERAD in the cytosol, however, may render it impossible to detect glycosylated ERAD substrates undergoing processing [36]. Nonetheless, the absence of any effect upon disruption of ERAD-L components on secretion or on accumulation of intracellular membrane-associated heavy chain further substantiates the notion that the majority of heavy chain is degraded prior to translocation. A possible cause of this problem may be the intrinsic aggregation prone property of the heavy chain’s C_{H1} domain, which is described to be intrinsically disordered and likely requires dedicated chaperones to be kept soluble in the cytosol [37, 38]. Premature cytosolic folding (which might eventually lead to aggregation) of recombinant proteins targeted for secretion was addressed recently as a bottleneck [30], as only soluble substrates are translocation-competent. One possible reason for the differences between the two Fab chains might be that the LC could have an advantage for translocation due to being better soluble and thus out-compete the HC.

We propose that the majority of Fab heavy chain is degraded before ever reaching the inside of the ER, at least in the case of glucose-grown cells (Fig. 4). This issue of intracellular heavy chain retention has been reported previously [35] but, instead of attributing it to an insufficient folding capacity in the ER, we interpret the problem to be degradation prior to the secretory pathway. Bottlenecks in secretion, which are independent of folding, have been reported previously [39], as well as the very detailed pinpointing of a translocation bottleneck [30]. Interestingly, the methanol-grown cells (P\textsubscript{AOX1}-Fab) show a greater secretory capacity and no apparent degradation prior to the secretory pathway, which is underlined by around 2-fold higher product yields. So far, neither transcriptomics, proteomics or fluxomics have been able to exactly pinpoint the major reason for generally increased productivity of the methanol-based \textit{P. pastoris} expression system [40]. Besides higher expression levels, a possible explanation may be a beneficial effect of the pulsed Fab expression under P\textsubscript{AOX1}, due to repeated methanol feeding in this study, in contrast to the constitutive expression profile with P\textsubscript{GAP} which continuously adds newly synthesized Fab and thus maintains.

**Fig. 4.** Schematic overview of the fate of the recombinant Fab fragments, especially in glucose-grown cells (P\textsubscript{GAP}-Fab). The majority of Fab heavy chain is degraded before being translocated into the ER. The light chain, on the other hand, is largely translocated into the ER and subsequently secreted or degraded. The same complexes are very likely involved in methanol-grown cells too (P\textsubscript{AOX1}-Fab), but with the difference that more recombinant protein is secreted and less is degraded in the cytosol. Successful gene disruptions are marked with an asterisk (\textdagger) and unsuccessful disruptions with a dagger (\textdaggerdbl). Protein complexes are not depicted completely and only show relevant proteins.
stressful conditions. Alternatively, the previously described pre-induction of the UPR upon methanol feeding might cause a preconditioning for recombinant protein secretion [41, 42]. Our findings add a further piece to the puzzle, but the detailed underlying physiological differences, causing higher secretion and lower pre-insertional degradation rates in methanol-grown P. pastoris, are yet to be discovered by further investigations.

All things considered, pre-insertional degradation of the Fab HC would also better explain the fact that inhibition of proteasomal activity increased secretion of intact Fab [9], meaning that actually immature, instead of terminally misfolded, product was targeted for degradation. Pre-insertional ERAD (prERAD) has been described when substrate builds up at the cytosolic leaflet of the ER [43]. This may also be the case if recombinant protein accumulates prior to translocation, especially when folding has already progressed in a post-translational manner [30]. Pre-insertional ERAD is facilitated by the Doa10 (Ssm4) complex, which is not the Hrd1 complex we targeted in this study. One possible explanation is that components of the Hrd1 complex (ERAD-L) are more relevant when the recombinant protein is more unstable. Transcriptional up-regulation of ERAD components was reported to be stronger with decreasingly stable variants of human lysozyme in P. pastoris [44]. Similarly, ERAD-L was required in S. cerevisiae for degradation of the prion protein PrP [28]. Both represent folding-impaired substrates, but we did not observe ERAD induction in any of our P. pastoris strains. Nevertheless, for generally stable recombinant proteins, like the Fab in this study, ERAD-L does not seem to be significant. Therefore, Doa10 (Ssm4) represents an interesting target to study, either its role in degrading recombinant proteins or to completely rule out a significant involvement of any of the ERAD complexes for generally stable recombinant proteins. The substrate range of the Doa10-complex also includes a variety of soluble proteins [45] and has recently been extended to the transmembrane protein Sbh2, which is part of the translocation pore in S. cerevisiae [46]. It remains to be investigated in future whether the presumably pre-insertional degradation of recombinant Fab chains might be prevented by disruption of Doa10, and if this effect is direct or due to stabilization of the translocon component Sbh2. In the former case, it would also be interesting to see whether Doa10 indeed recognizes the intrinsically disordered C_31_1 domain of the Fab heavy chain or if other parts of the recombinant protein are acting as degron.

In conclusion, we were able to narrow down the reported issue of intracellular Fab degradation in P. pastoris [8, 35]. We propose that most intracellular degradation is a symptom rather than the primary problem. A major problem in yeast seems to be transport of the recombinant proteins into the ER, which has been observed for different model proteins in P. pastoris and S. cerevisiae [30, 47]. Furthermore, we can exclude overshooting ER quality control (ERQC) as having a significant role to play in decreasing secretion, similar to what has also been reported for S. cerevisiae with disrupted ERAD or ERQC components [33].

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The manuscript contains neither experiments using animals nor human studies.

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