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Analysis of antibody production in *Saccharomyces cerevisiae*: effects of ER protein quality control disruption

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Abstract

One of the main limitations for heterologous protein production in the yeast *Saccharomyces cerevisiae* is the protein folding capacity in the endoplasmic reticulum (ER). Accumulation of unfolded proteins triggers the unfolded protein response (UPR), which resolves the stress by increasing the capacity for protein folding and removal of unfolded proteins by the ER associated degradation (ERAD) system. In order to analyse the influence of ERAD on production of a human IgG, we disrupted ERAD at different stages by deletion of the *HTM1*, *YOS9*, *HRD1*, *HRD3* or *UBC7* gene, with or without a disruption of the UPR by deletion of the *IRE1* gene. All deletion strains were viable and did not exhibit a growth phenotype under normal growth conditions. Deletion of *HTM1* resulted in a small increase in antibody production, whereas a small decrease in antibody production was observed in the $\Delta hrd1$, $\Delta hrd3$ and $\Delta ubc7$ yeast strains, and a stronger decrease in the $\Delta yos9$ yeast strain. Deletion of the *IRE1* gene had contrasting effects in the ERAD mutants, with a strongly decreased production in wild type cells and partially reversed effects in combination with the $\Delta htm1$ or the $\Delta yos9$ deletions. In order to study IgG clearance from the ER, an assay was developed using the inhibitory effect of glucose on the *GAL1* promoter that is driving IgG expression. The $\Delta yos9\Delta ire1$ and $\Delta htm1\Delta ire1$ strains showed a delayed IgG clearance from the cells, showing that removal of components for the generation and recognition of the glycan signal needed for ERAD mediated protein degradation might increase the IgG ER residence time.

Introduction

The yeast *Saccharomyces cerevisiae* has been widely used as an expression host for the production of heterologous proteins. The advantages of this host as an expression system include a long history of scientific research leading to a vast amount of data from single component to cellular level. With the advent of glycoengineered yeasts, the production of therapeutic glycoproteins with yeast is now tangible (Li et al. 2006; Parsaie Nasab et al. 2013; Piirainen et al. 2014). However, it is known that bottlenecks in the secretion pathway lead to suboptimal and variable production levels.

Although possessing a eukaryotic protein folding and quality control system, expression of heterologous glycoproteins such as full-length antibodies remains a challenge and production titers remain low. It has been shown that by optimizing the production process, yields can be increased; however, without increasing the specific productivity of the cells (Gasser and Mattanovich 2007; Potgieter et al. 2009, 2010). Therefore, the cellular limitations still remain. One particular bottleneck might be found in the ER and it has been reported that residence times of heterologously expressed mammalian proteins in the yeast endoplasmic reticulum (ER) are up to 5 hours (Wittrup et al. 1994), putatively leading to the elimination of parts of the slow folding proteins.

Cell engineering approaches to increase specific productivity have proven to be complicated. Although many hurdles have been overcome by overexpression of molecular chaperones, other folding factors and transport factors to enhance vesicular transport or by deletions of sorting receptors to prevent miss-sorting and of proteins involved in proteolytic degradation, it has been shown that these results tend to be host and protein specific (Delic et al. 2014). In addition, often multiple steps are limiting protein production, therefore complicating the cell engineering process. However, systems biology might give more insight in the role of individual processes in the complex protein synthesis network and thus aid in finding the right approach for cell engineering (Hou et al. 2012).

One critical factor in protein folding is the maintenance of protein homeostasis in the ER. ER homeostasis can be perturbed when the folding capacity of the ER is saturated by expression of high amounts of heterologous proteins leading to upregulation of the yeast ER quality control (QC) machinery by the unfolded protein response (UPR). Aiming to enhance correct protein folding and to dispose of incorrectly folded proteins, the QC system includes amongst others folding enzymes, chaperones, and the ER associated protein degradation (ERAD) system. Although constitutively active, the QC machinery is vastly upregulated by the UPR (Friedlander et al. 2000). Key components of this ER process are shown in Fig. 1.

Nascent proteins are imported into the ER and need to reach their native state before they can be exported. In the case of glycoproteins, the folding state is mainly monitored via their glycoforms and the N-glycans control the fate of the proteins (Helenius and Aebi 2004). The nascent glycoprotein is initially N-glycosylated with a $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ glycan and during the folding process the three glucoses are trimmed off. Mns1p, a slowly acting mannosidase, removes the terminal mannose from the B-branch of the glycan tree, to signal removal of

the protein from further folding cycles (Herscovics 2001). The resulting Man₈GlcNAc₂ glycan, when present on a still unfolded protein, is recognized by the Pdi1p/Htm1p complex (Gauss et al. 2011). The Htm1p mannosidase of this complex further trims down the glycan to the Man₇GlcNAc₂ form by removing the terminal mannose at the C-branch of the glycan, in order to create the substrate for HRD ligase-mediated glycoprotein degradation (Clerc et al. 2009). The lectin Yos9p is part of this HRD ligase complex and recognizes glycans containing the now available terminal α 1,6 linked mannose residues (Quan et al. 2008). Next, the protein is targeted towards the HRD ligase complex for retrotranslocation, ubiquitination, and finally proteasomal degradation (Benitez et al. 2011). The HRD ligase core-complex consists of the proteins Hrd1p, Hrd3p, Usa1p and Der1p. The Hrd3 protein works concertedly with Yos9p for targeting of the unfolded protein to the complex. After this, the ERAD substrate is retrotranslocated across the ER membrane and ubiquitinated by Hrd1p together with the ubiquitin conjugating enzyme Ubc7p, which is located on the cytoplasmic site of the ER membrane (Hiller et al. 1996). The substrate is finally degraded by the proteasome, which is recruited by Hrd1p (Nakatsukasa et al. 2013).

In the present study, we studied the effects of the cellular glycoprotein QC system components on expression of a full-length antibody in *S. cerevisiae*. We analysed the effect of deleting various components of ERAD, alone or in combination with an inactivated UPR, on cellular growth, protein secretion and IgG clearance.

Materials and methods

Generation of strains and plasmids

All *S. cerevisiae* strains used in this study were derived from the parental strain SS328 (ATCC® MYA193™) and are listed in Table 1. Gene deletion strains were constructed using the method from Hegemann and Heick (2011). The target gene loci were deleted by replacement with a PCR product containing the *KanMX4* or the *NatMX* cassette and flanking target sequences. Plasmids pRS305K (Taxis and Knop 2006) and pUG74 (Hegemann and Heick 2011) were used as template for PCR amplification of the kanamycin (*KanMX4*) or the nourseothricin resistance marker cassettes (*NatMX*), respectively. The lithium acetate method was used for transformation of yeast cells. After transformation, cells were grown on medium supplemented with 200 µg/mL G418 (Sigma-Aldrich, Helsinki, Finland), 200 µg/mL LEXSY NTC (Jena Bioscience, Jena, Germany), or a combination of both antibiotics for selection of a second deletion.

The light and heavy chains of the human monoclonal anti-CD20 IgG (Li et al. 2006) were genetically fused to the secretion signal comprising amino acids 1-17 of the endogenous yeast acid phosphatase (*PHO5* gene). Heavy and light chains of the IgG were amplified from plasmid pAX88 using PCR and oligonucleotide pairs OAF015 (5'-

AAACTAGTATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTTCTTTGGCCAATGCACAGGTA
CAACTGCAGC) and OAF016 (5'-AACTCGAGTCATTTACCCGGAGACAGGG), and OAF017 (5'-
AATCTAGAATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTTCTTTGGCCAATGCACAAATT
GTTCTCTCCAGTC) and OAF018 (5'-AACTCGAGCTAACACTCTCCCCTGTTG) for heavy and light
chain, respectively. The forward primers contained the signal sequence. The PCR amplified fragments were
inserted into *XbaI XhoI* or *SpeI XhoI* sites of the CEN/ARS plasmid pRS416-Gal (Mumberg et al. 1995),
creating pEK1 and pEK3 respectively. The pEK1 *NaeI-NdeI* fragment, encompassing vector backbone,
promoter, light chain cDNA and terminator was ligated with pEK3 *EclI36II-NdeI* fragment, encompassing the
heavy chain cDNA with promoter and terminator, creating pEK5. All strains were transformed with the
antibody expression vector.

Cultivation and growth assays

Strains were grown in YPD (1% yeast extract, 2% peptone and 2% glucose), in minimal medium (0.67% yeast nitrogen base without amino acids (YNB) and 2% glucose or raffinose, with supplementation of 20 mg/l of adenine, 20 mg/l of histidine and 30 mg/l of lysine) or in synthetic drop out (SD) medium lacking uracil for plasmid selection. All media components and reagents were obtained from Sigma-Aldrich (Helsinki, Finland), unless stated otherwise. YNB was obtained from BD (Vantaa, Finland)

Spotting assays were performed on YPD, SD and SGal (supplemented with 2% galactose) agar plates (2% w/v). Exponentially growing cultures were harvested, washed with water, diluted to an OD₆₀₀ of 0.4 and

serially diluted into ddH₂O. 5 µL of each serial dilution were spotted. The plates were incubated for 48 and 72 hours at 25°C, 30°C and 37°C, after which pictures were taken.

For recording of growth curves, cultures of exponentially growing cells were diluted to OD₆₀₀ of 0.1 in YPD. 200 µL of these cultures was grown in a microtiter plate, with continuous shaking at 30°C using a Bioscreen C MBR system (Oy Growth Curve Ab Ltd, Finland). OD₆₀₀ measurements were taken every 30 minutes for 24 hours. Statistical analysis of $\mu_{\max}$ values was done using single factor ANOVA.

Precultures for IgG expression experiments were grown in 5 mL of minimal medium for 16 hours at 30°C, 220 rpm, and used to inoculate 1 mL of fresh media to a starting OD₆₀₀ of 0.1 in 96-deepwell plates. Cells were grown for 6 hours at 30°C, 280 rpm, after which protein expression was induced with addition of 4% galactose at 25°C. After 24 hours of production, OD₆₀₀ values were measured in 96-well plates using a BioTek Eon spectrophotometer (BioTek, Winooski, USA). For ELISA analysis, the cultures were clarified by centrifugation for 5 minutes at relative centrifugal force (rcf) of 3,214. The clarified culture supernatants were adjusted to 1x PBT (PBS (135 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.75 mM KH₂PO₄) + 0.5% Tween-20) and stored at -20°C until analysis.

Antibody titer determination

An enzyme linked immunoassay (ELISA) was used to determine antibody titers. The ELISA assay was automated on a HAMILTON Star line liquid handling station (Hamilton, Bonaduz, Switzerland). All antibodies were obtained from Sigma-Aldrich (Helsinki, Finland); human IgG standard antibody was obtained from Calbiochem (Merck Millipore, Billerica, USA). 96-well plates were coated with 100 µL/well of 4.2 µg/mL of goat anti Human IgG (Fc specific) antibody in PBS shaking overnight at 4°C. Plates were washed four times with 200 µl PBT and blocked for 45 minutes in PBT at 22°C with occasional shaking. Serial dilutions of standard antibody were performed in PBT. 200 µl of samples in triplicates or standard antibody in duplicates were added to the wells and the plates were incubated for 90 minutes while shaking at room temperature. After incubation wells were washed three times with 200 µl of PBT. 100 µL/well of 1:4,000 dilution of goat anti-Human IgG (Fc specific)-peroxidase labelled antibody was added and allowed to bind for 1 hour at room temperature while shaking. After incubation wells were washed three times with 200 µL of PBT. For detection, 80 µL/well substrate solution (0.2 mg/mL *o*-phenylenediamine, 3 µL of 30% H₂O₂ per 10 mL solution in 0.05 M phosphate citrate buffer) was added. After 8 minutes the reaction was quenched by addition of 80 µL/well of 3 M H₂SO₄. Absorbance was read at 405 nm using a BioTek Synergy 2 spectrophotometer. Data evaluation was done with Gen5 software (BioTek, Winooski, USA).

ER clearance assay

Precultures were grown in 5 mL of minimal medium at 30°C, 220 rpm for 15 hours. Exponentially growing cells were induced with 2% galactose when they reached an OD₆₀₀ of 0.8, and transferred to 25°C. After four hours of expression, either 200 µg/ml of cycloheximide, 2% glucose, or 200 µg/ml of cycloheximide + 2%

glucose, was added to repress the IgG production, or the same volume of water for control samples. The cells were collected 0, 2, 4 and 6 hours after addition of inhibitors or water.

10 OD₆₀₀ units of cells were collected by centrifugation and the cell pellets were resuspended in 500 µL of lysis buffer (100 mM Tris HCl buffer, pH 7.4 containing 1x cOmplete®, EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The same volume of acid washed glass beads was added, and samples were vortexed for 10 minutes at 4°C using a Disruptor genie (Scientific industries, Bohemia, USA). After settling of the beads, the supernatant was transferred to a new tube. The glass beads were washed with 500 µL of lysis buffer by vortexing the tubes twice for 30 seconds, while keeping them for 30 seconds on ice in between. The combined supernatants were pelleted and the pellets were resuspended in 100 µL of SDS-Page sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.005% bromophenolblue). Samples were heated for 5 minutes at 65°C, after which they were centrifuged for 5 min at 14,000 rcf and the supernatants were used for analysis.

10 µL of sample was loaded onto a 12.5% SDS-polyacrylamide gel and the electrophoresis was run in SDS-tris-glycine buffer. For each sample, replicate gels and blots for detection of IgG and tubulin were prepared. After the SDS-PAGE proteins were transferred to a nitrocellulose membrane using western blotting. A 1:10,000 dilution of anti-human IgG (Fc specific)-peroxidase labelled antibody produced in goat was used for staining. A 1:10,000 dilution of anti-tubulin [EPR13799] produced in rabbit (Abcam, Cambridge, UK) in combination with a 1:10,000 dilution of goat anti-rabbit IgG (Fc specific)-peroxidase labelled was used. Signal detection was done with the ECL western Blotting Detection kit (GE Healthcare, Helsinki, Finland) following the manufacturer's instructions. Analysis and quantification of band intensities were performed using the Bio-Rad Image Lab Software (Bio-Rad, Hercules, USA). Anti-tubulin blots were used as loading controls.

Results

The influence of deletions on growth rates

The ERAD pathway participates in ER protein homeostasis by targeting and removing misfolded proteins from the ER and channeling them towards the ubiquitin-proteasomal degradation machinery. We have selected five genes from different stages of the ERAD pathway to analyze the effects of ERAD disruptions on production of a model antibody. The genes *HRD1*, *HRD3*, *UBC7*, *HTM1*, and *YOS9* were deleted in the wild type *S. cerevisiae* strain SS328. Additionally, *IRE1* was deleted in all 5 deletion strains and SS328 to prevent induction of UPR and mitigation of putative ERAD phenotypes. Finally, the *ALG3* gene was deleted in the SS328 and *Aire1* strains to create a glycoform that bona fide escapes the quality control system.

First, in order to study the effect of these different mutations on yeast, growth curves were recorded. In short, exponentially growing cultures were diluted in YPD to OD₆₀₀ of 0.1 and 200 µl of the diluted culture were added to the wells of microtiter plates. The cultures were grown for 24 hours at 30°C with constant shaking and OD₆₀₀ measurements were automatically recorded every 30 minutes. The resulting growth curves are shown in Fig. 2. All strains displayed a similar growth behavior, reaching the stationary phase after ten hours of cultivation. The $\mu_{\max}$ was reached for all strains after 2.5 to 4 hours of cultivation (Table 2). The $\mu_{\max}$ values of the different strains ranged from 0.505 ± 0.007 to 0.581 ± 0.015 in the wild type strain background and 0.497 ± 0.012 to 0.562 ± 0.019 in the *Aire1* genetic background. However, statistical analysis of $\mu_{\max}$ values using single factor ANOVA did not find any significant differences between the means of the different strains ($P>0.05$).

Effects of deletions of QC and ERAD components on antibody production and intracellular accumulation.

To examine the effects of deletions in the ERAD and UPR pathways on heterologous protein production, all deletion and wild type strains were transformed with the antibody producing plasmid pEK5. The yeast strains carrying the plasmid were diluted from precultures into 96-deepwell plates. Cultures were grown to exponential growth phase at 30°C and antibody expression was induced by the addition of 4% galactose. After 24 hours of growth at 25°C, the cultures were harvested and the OD₆₀₀ values and the IgG levels in the culture supernatants were determined. The variation between OD₆₀₀ values of the different strains was 13%. IgG levels were normalized to cell densities of the cultures (Fig. 3).

Inactivating the ERAD pathway by deleting different genes led to several production phenotypes. Deletion of the genes encoding for three components of the retrotranslocation complex (*HRD1*, *HRD3* and *UBC7*) gave similar results, and the secreted antibody titers reached 63%, 69% and 68%, respectively, of the wild type production level. In contrast, deletion of the *HTM1* gene was the only beneficial deletion for protein production yield, with an increase in antibody production of 15%. However, when disrupting a later step in the ERAD, the recognition of the trimmed N-glycan mediated by Yos9p, production levels dropped to 18%.

In a next set of experiments, we tested these different gene disruptions in a yeast background incapable to mount the UPR. Therefore, each gene disruption was combined with the *IRE1* deletion. Blocking the UPR by deletion of the *IRE1* gene was detrimental for protein production as IgG levels dropped to 7% relative to the wild type levels. Interestingly, combining the *IRE1* deletion with deletions in the ERAD pathway partially rescued the production phenotype of the *IRE1* deletion strain. In the $\Delta hrd1\Delta ire1$, $\Delta hrd3\Delta ire1$, and $\Delta ubc7\Delta ire1$ strains production levels were comparable to their respective single mutants reaching 74%, 62% and 81% of the wild type levels. Remarkably, combining $\Delta htm1$ with $\Delta ire1$ led to an almost 2/3 decrease compared to the single $\Delta htm1$ strain. However, production was still almost 7 times higher than the $\Delta ire1$ strain. In contrast, introducing the *IRE1* deletion in the $\Delta yos9$ deletion strain increased production levels 4-fold reaching 76% of wild type levels. The $\Delta alg3$ deletion strain showed improved production and seemed not to be affected by an abolished UPR, with production levels of 120% and 131% compared to the wild type and the $\Delta ire1$ strain, respectively.

Due to the observed differences in the amount of secreted antibodies in the culture supernatant, we analyzed whether these differences would be reflected in the intracellular concentrations of IgG. Therefore, we determined relative concentrations of the intracellular IgG amounts by measuring signal intensities of IgG on western blots and normalizing them with the signal intensities of tubulin of corresponding cell extracts. The results indicated that 24 hours after induction intracellular IgG levels varied between the different strains. For the strains with deletions of ERAD components the normalized IgG levels ranged from 1.1 to 1.8 times the amount of IgG compared with wild type cells. For the strains in the $\Delta ire1$ background, the normalized IgG levels ranged from 0.8 to 1.6 times the amount of IgG compared with the $\Delta ire1$ deletion strain or 0.7 to 1.5 times the amount of IgG compared with wild type cells. No correlation between the total secreted amount of IgG detected in the culture supernatant and the intracellular amounts of IgG was found.

Effects of deletions on growth under IgG production conditions

To analyze if any of the IgG producing deletion strains displayed a growth phenotype, serial dilutions of exponentially growing cells were spotted on solid media. The plates used were rich media (YPD) and selective media (SD-URA and SGal-URA) and were incubated at 25°C, 30°C and 37°C. Pictures were taken after 48 hours (YPD and SD-URA) or 72 hours (SGal-URA). All strains grew indistinguishably from each other on rich media (data not shown). In the selective medium set of experiments the strains harboring the IgG expression plasmids were grown under inducing and non-inducing conditions. The results showed that under inducing conditions, the $\Delta yos9\Delta ire1$ and $\Delta htm1\Delta ire1$ strains displayed strongly reduced growth compared to the parental strains. In particular at elevated temperatures of 30°C and 37°C, respectively, these two strains were not able to grow. In contrast, the $\Delta hrd3\Delta ire1$ and $\Delta ubc7\Delta ire1$ strains displayed improved growth properties under inducing conditions at elevated temperatures (Fig. 4). The $\Delta alg3$ deletion strain in SS328 and in the $\Delta ire1$ strain background displayed a growth phenotype when grown on the SD-URA and SGal-URA

plates at 37°C. This effect is most likely unrelated to ERAD or UPR effects, and has been described as being caused by a changed cell wall composition (Bailey and Schulz 2013).

Deletions of ERAD components and UPR abolishment lead to differences in IgG clearance

In order to follow the fate of the folding IgG in the ER we developed an assay to study ER depletion. Exponentially growing cultures of wild type cells were induced with galactose and allowed to express IgG for 4 hours. After 4 hours, protein production was inhibited by the addition of 2% glucose, 200 µg/mL cycloheximide, or a combination of both to the cultures. An equal amount of ddH₂O was added to control samples. 0, 2, 4 and 6 hours after inhibition, 10 OD₆₀₀ units of cells were collected for preparations of cell extracts. The cell extracts were separated using SDS-PAGE and transferred to nitrocellulose membranes. The presence of IgG was visualized using an anti-IgG western blot and equal loading of the gels was verified using an anti-tubulin western blot, thus for each sample two replicates were analyzed. The signal intensities were quantified and the IgG signal intensity was normalized to the corresponding intensity of the tubulin signal. The IgG clearance was fastest in the cultures where glucose alone or glucose in combination with cycloheximide was added, with an IgG signal half-life of 1.36 hours and 1.18 hours respectively. Upon cycloheximide treatment of the cells a delayed cellular IgG clearance with an IgG signal half-life of 1.88 hours was observed (Fig. 5A), indicating that the general inhibition of protein synthesis by cycloheximide negatively influences normal protein processing. Therefore, in contrast to assays relying on the inhibition of protein synthesis using cycloheximide, we decided to take advantage of the repressible effect of glucose on the galactose promoter to switch off expression, without affecting the general protein synthesis and ensuring conservation of normal processing conditions.

Next, the experimental setup was extended to include all strains used in the earlier experiments, and performed as described above, using 2% glucose as inhibitor of the IgG production. Equal loading of the blots was confirmed using an anti-tubulin antibody. Selected western blots are shown in Fig. 5B, with a representative anti-tubulin blot as a loading control. As expected, the non-repressed cultures kept producing the IgG protein during the course of the experiment. In samples prepared from the wild type yeast expressing IgG, the IgG levels decreased fastest and no IgG was detected anymore after 6 hours. In contrast, the IgG levels decreased more slowly in samples prepared from the *Δubc7*, *ΔhmlΔire1* and *Δyos9Δire1* strains, indicating that the IgG processing was reduced in these strains. In the other strains the IgG clearance was similar to the clearance observed in the wild type yeast. In general, a higher variability in IgG clearance was observed in single deletion strains compared to the double deletion strains.

In order to get quantitative data of the IgG clearance, intensities of IgG and tubulin bands were measured using Bio-Rad Image Lab software. Band intensity of time point 0 was set to 100% and relative IgG quantities were calculated for the different time points. Quantifications of corresponding anti-tubulin blots were used to correct the anti-IgG intensities for loading differences and the resulting clearance curves are shown in Fig. 6.

The decrease of the IgG levels in cell extracts was fastest in the wild type cells and a highly similar IgG depletion was observed in the *Aire1* strain. In general, the strains with deletions in ERAD showed a delayed clearance, but the levels of IgG depletion were comparable to the wild type strain and the *Aire1* strain at the end of the assay. Similar clearance kinetics as in the wild type cells were observed in the *Ahtml*, *Aalg3Aire1*, *Ahrd1Aire1*, *Ahrd3Aire1* and *Aire1* strains. For these strains only 15% to 25% of the IgG was left after two hours, after 4 hours this was further decreased to approximately 5%, and at the latest time point on average less than 2% of the original IgG amount was observed in these strains. For rest of the strains with only deletions in ERAD components, the *Ahrd1* and *Aubc7* strains showed a delayed clearance. In the *Ahrd1* strain the normalized IgG amount after 2 hours was similar to other ERAD mutants, but after 6 hours, more than 12% of the initial amount was left. This is on average more than double the amount than in the other strains, leaving only the *Ayos9Aire1* strain with a higher IgG load. A notable difference in IgG depletion was observed for the *Aubc7* strain, 68% of the IgG was left two hours after addition of glucose, but the cellular IgG load became similar to other strains in this ERAD deletion strain at later time points. The *Aubc7Aire1* strain also showed a slightly slower IgG depletion than the other strains in the *Aire1* background. After 2 hours only 25% of the initial IgG levels remained in the cell, but at later time points the strain showed comparable signals to the other strains. In the *Aire1* strain background, the IgG clearance was slowest in the *AhtmlAire1* and the *Ayos9Aire1* strains. In particular the *Ayos9Aire1* strain failed to deplete the total amount of IgG during the 6 hours, whereas the *AhtmlAire1* strain displayed a strongly delayed clearance, but IgG levels were depleted to similar levels as observed in the other strains after 6 hours. After two hours still 42% and 51%, respectively, of the initial amount of IgG were detected, which was approximately twice the amount when compared to the other strains. For the *AhtmlAire1* strain the decrease was slightly higher, with 14% and 6% of the initial IgG concentration left after 4 and 6 hours, respectively, whereas in the *Ayos9Aire1* strain 30% and 21%, respectively, of the initial IgG concentration was left.

Discussion

Many studies have been performed to understand the underlying molecular mechanisms of the protein quality control systems in *S. cerevisiae*. Mechanistic insight of the functioning of components of the cellular protein QC system has been mainly gained from studies using model substrates which are prone to misfolding. Examples of these model ERAD substrates include CPY*, a truncated version of the vacuolar hydrolase carboxypeptidase Y, and PrA*, a mutated version of the glycoprotein proteinase A. These substrates have been used to gain information on the roles of, among others, Yos9p (Szathmary et al. 2005; Benitez et al. 2011), Htm1p (Clerc et al. 2009) and Ubc7p (Hiller et al. 1996). From a biotechnologists' perspective, it is paramount to study these processes under more natural conditions and using real protein substrates. Unfortunately, so far very few articles report on the influence of ERAD in combination with heterologous protein production.

Producing heterologous proteins at high levels is prone to activate UPR and ERAD through crowding of the ER. For example, a study by Pfeffer et al. (2012) found that expression of the antibody fragment Fab3H6 in *Pichia pastoris* increased proteasomal activities by 20% decreasing the product yield significantly. This indicates that ER quality control systems can also overshoot their requirements when expressing recombinant proteins, by targeting more than misfolded proteins for degradation. Manipulation of UPR has been used as well to improve heterologous protein production. Disruption and overexpression of *HAC1* has been shown to lead to a decrease and increase, respectively, in the production of α -amylase in *S. cerevisiae* (Valkonen et al. 2003). However, activation of the UPR as a whole has a great impact on various cellular processes and therefore does not improve our understanding of the contribution of individual components of UPR regulated genes in resolving the ER stress. Here, we studied the influence of selected proteins of the cellular QC system on the expression of a human IgG as a model protein and analysed productivity, intracellular accumulation of IgG and IgG clearance.

The data presented here shows that all strains used bearing a single deletion of an ERAD component, a deletion of *IRE1* leading to an inactivated UPR, or a combination of both, are viable and do not exhibit a growth phenotype under normal growth conditions. Similar results under normal conditions have been previously reported for the strains $\Delta ubc7$, $\Delta ire1$, $\Delta hrd1$ (Friedlander et al. 2000; Apodaca et al. 2006; Li et al. 2012), and for the disruption of putative ERAD components in the filamentous fungi *Aspergillus niger* (Carvalho et al. 2011). We observed a negative effect on growth only for $\Delta htm1\Delta ire1$ and $\Delta yos9\Delta ire1$ strains when grown at 30°C and 37°C, and combining the elevated temperature with protein overexpression stress. However, growth of these strains was not affected under the normal conditions used for growth and expression of IgG in our experiments.

Deletion of single components of the ERAD system in the wild type strain background allowed distinguishing between three distinct effects. A small increase in production was observed upon deletion of the *HTM1* gene, a small decrease in IgG production of strains lacking the *HRD1*, *HRD3*, and *UBC7* gene, and a more strong decrease was associated with the deletion of the *YOS9* gene. In addition, IgG secretion was increased in the

Δalg3 strains, a genotype which is considered not to produce the glycan signal for ERAD. It has been shown that removal of a mannose by Htm1p is required for efficient binding of ERAD targeted proteins to Yos9p (Szathmary et al. 2005; Clerc et al. 2009). Therefore, this could imply that the slowly folding IgG protein escape from degradation through ERAD in the *Δhtm1* background, giving it more time to fold and be secreted. In contrast, in all strains where the IgG is already marked for degradation, a reduced secretion was observed. In order to exclude any possibility for a rescue of IgG accumulation in the ER by the induction of UPR, we combined the single deletions with the *IRE1* deletion. Such strains should be incapable to resolve ER stress by increased expression of folding chaperones. Interestingly, deletion of *IRE1* had a strong effect on productivity in wild type and partially reversed effects in *Δhtm1* and *Δyos9* background.

According to the current model, a glycoprotein in the ER of yeast can either reach its native state quickly and continue to the Golgi apparatus, remain in a folding intermediate state, or be degraded by the ERAD. It has also been proposed that slowly folding proteins can be removed from the ER. Therefore, we wanted to study whether the differences in productivity are connected to differences in the protein folding.

The strains most different from the wild type and other strains tested in the clearance assay were the *Δhrd1* and the *Δubc7* strains and in the *Δire1* genetic background the *Δhtm1Δire1* and *Δyos9Δire1* strains. In these strains we observed a delayed clearance of IgG from the cells. Hrd1p and Ubc7p are part of the HRD-ligase complex. Changes in the IgG load over time can be attributed to two effects, either reaching the native folding state and leaving the ER to be secreted into the media, or degradation of the protein by the ERAD system. Based on the assumptions that once the IgG is properly folded, no differences, which would affect the secretion of the IgG, exist between different strains, it is fair to assume that all the observed changes are due to changes in the rate of protein degradation. However, it cannot explain the observed differences in the IgG load after 6 hours, unless after a prolonged incubation similar levels as for other strains would be reached. An explanation could be that as the IgG fraction of the total amount of protein in the ER decreases, the relative IgG depletion also decreases. This will then be reflected in the decrease of clearance speed over time that are seen in the clearance assay. It has been shown that deletion of *UBC7* leads to a decreased degradation of ERAD substrates (Hiller et al. 1996; Friedlander et al. 2000). Also for the *HRD1* deletion a delayed degradation of ERAD substrates has been found (Benitez et al. 2011). In addition, misfolded substrates have shown to have an increased ER retention when *HRD1* is deleted (Izawa et al. 2012), which might contribute to the increased IgG load at the end of the clearance assay. Htm1p and Yos9p are responsible for generating and recognizing the glycan degradation signal in ERAD, respectively. Deleting these two proteins will abrogate formation of the recognition signal for targeting towards the translocation and ligase complex, leading to a longer residence time of the protein in the cells. The effect of *Δhtm1Δire1* deletion on IgG clearance is less severe than in *Δyos9Δire1*, as Yos9p is known to also slowly recognize unfolded proteins based on hydrophobic patches and target them to the HRD-complex (Benitez et al. 2011). Supporting this, in the *Δhtm1Δire1* strain a decreased amount of secreted IgG compared with *Δhtm1* was found. However, removal of Yos9p from ERAD removes the targeting step for retrotranslocation, which increases their residence time in the ER. This is also reflected

by an increase in the specific production titers compared to the $\Delta yos9$ deletion strain. A reduced clearance had already been reported for CPY* and other artificial ERAD substrates (Szathmary et al. 2005; Clerc et al. 2009). In addition, a study in *A. niger* found indications for a delay in protein degradation when parts of the ERAD pathway were impaired, as high expression levels of heterologous proteins led to higher intracellular protein levels in these strains (Carvalho et al. 2011). This also supports our finding that strains with a defect in ERAD had higher intracellular IgG level than the wild type strain. However, almost all strains in the $\Delta ire1$ background had lower intracellular IgG levels, indicating that in the wild type strain background, the active UPR response pathway might obscure some of the observed effects.

In our study we included also a $\Delta alg3$ strain, which produced slightly more IgG compared with wild type yeast cells. Principally, this strain does not produce the typical $\text{Man}_7\text{GlcNAc}_2$ glycan which is considered to be the canonical Yos9p recognition signal, having an exposed terminal $\alpha 1,6$ -mannose at the C-branch. However, contrasting data has been published. It has been shown that the *ALG3* mutation led to an increased accumulation of CPY* in the ER (Jakob et al. 1998). In contrast, other reports indicate that the glycan structure produced in the $\Delta alg3$ background, bearing as well a terminal $\alpha 1,6$ -mannose, is also recognized by Yos9p. In our study we observe a similar IgG clearance in $\Delta alg3$ and wild type strains, indicating that a substantial amount of IgG carrying the $\text{Man}_5\text{GlcNAc}_2$ structure is cleared from the ER. Evidence for this hypothesis comes from a report of Clerc *et al.* (2009) who showed that in the $\Delta alg3$ strain, degradation of CPY* is similar in a $\Delta yos9$ deletion and $\Delta alg3\Delta yos9$ deletion strain.

In conclusion, deletion of various parts of the ERAD system, with or without an abolished UPR shows an interplay between these two quality control systems on production of antibodies and on cellular clearance of the produced antibody. However, the exact mechanism that causes these phenotypes in the different strains has yet to be elucidated

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Conflict of interest

The authors declare that they have no competing interests.

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Figures

Fig. 1 Overview of key players in UPR and ERAD. Nascent proteins are imported into the ER, where glycoproteins are glycosylated by the oligosaccharyltransferase. The folding process is monitored through their glycoforms, and when successfully folded, proteins are exported from the ER. However, glycans from misfolded proteins are trimmed down and recognized by components of the ERAD system, which targets them for retrotranslocation into the cytosol and subsequent degradation by the proteasome. Additionally, unfolded proteins can activate the UPR, which leads to upregulated transcription of various genes, including genes encoding various ERAD components and ER chaperones

Fig. 2 Growth of deletion strains in liquid rich media. Exponentially growing cells were diluted to OD₆₀₀ of 0.1 in YPD and grown in microtiter plates for 24 hours at 30°C with continuous shaking, while OD₆₀₀ was measured every 30 minutes. The growth curves represent average OD₆₀₀ of 4 replicates. The average % standard error ranges from 1.60 to 4.15%. WT = wild type

Fig. 3 Effects of deletion of ERAD components on antibody production. Strains were grown in 96-well plate format into the exponential phase and protein production was induced with galactose for 24 hours. IgG concentration in medium was quantified using ELISA and normalized to cell concentration using OD₆₀₀ measurements. Error bars indicate standard error from 9 independent cultures, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (versus wild type control, by Student's two-tailed t test). The strains used were wild type (WT) and deletions strains. Deletion of *IRE1* is marked with + and -

Fig 4 Growth analysis of deletion strains at different temperatures and solid media. Serial fivefold dilutions of exponentially growing cells of the indicated strains were spotted on selective synthetic medium plates with either glucose (SD-URA) or galactose (SGal-URA) as the carbon source. Plates were incubated at 25°C, 30°C or 37°C for 2 days (SD-URA) or 3 days (SGal-URA)

Fig. 5 Analysis of IgG clearance using western blot. Antibody expression was induced in exponentially growing cultures with galactose for 4 hours, after which protein expression was inhibited and IgG clearance was followed for 0, 2, 4 and 6 hours. After incubation periods cell extracts were prepared from the cultures and analysed using SDS-PAGE and western blot. (a) Cultures of the wild type strains expressing IgG were treated with glucose (gluc), cycloheximide (cyc), glucose and cycloheximide or water (control). (b) Cultures of the wild type (WT), and all strains containing a deletion were treated with glucose or water and IgG clearance was monitored over a period of 6 hours. A representative blot for tubulin, used as loading control, is shown for both sets of experiments.

Fig. 6 Quantitative analysis of IgG clearance. Cultures of (a) strains in the wild type (WT) background, and (b) strains containing an *IRE1* deletion were treated with glucose or water (control) and cellular IgG concentration was monitored over a period of 6 hours. Total protein was separated on SDS-PAGE and transferred onto membranes. Blots were stained with an anti-IgG and an anti-tubulin antibody, respectively.

Imagelab software was used for quantification of IgG and tubulin signal levels. Data represent the average of two blots normalized for loading differences using an anti-tubulin blot.

Tables

Table 1 Yeast strains used in this study

Strain	Genotype
SS328	MAT α <i>ade2-101 his3Δ200 lys2-801 ura3-52</i>
YJR043	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, hrd1Δ::kanMX6</i> with pEK5(Ura3)
YJR044	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, yos9Δ::kanMX6</i> with pEK5(Ura3)
YJR045	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, hrd3Δ::kanMX6</i> with pEK5(Ura3)
YJR046	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, ubc7Δ::kanMX6</i> with pEK5(Ura3)
YJR049	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, htm1Δ::kanMX6</i> with pEK5(Ura3)
YJR050	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52</i> with pEK5(Ura3)
YJR051	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, alg3Δ::kanMX6</i> with pEK5(Ura3)
YJR102	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR103	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, hrd1Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR104	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, hrd3Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR105	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, htm1Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR106	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, ubc7Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR109	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, yos9Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)
YJR110	SS328 MAT α <i>ade2-101, his3Δ200, lys2-801, ura3-52, alg3Δ::kanMX6, ire1Δ::natMX6</i> with pEK5(Ura3)

Table 2 Maximum growth rates of the indicated strains calculated from the growth data.

Strain	$\mu_{\max}^a$	Strain	$\mu_{\max}$
Wild type	0.581 ± 0.015	$\Delta ire1$	0.507 ± 0.009
$\Delta htm1$	0.552 ± 0.009	$\Delta htm1 \Delta ire1$	0.530 ± 0.015
$\Delta yos9$	0.518 ± 0.027	$\Delta yos9 \Delta ire1$	0.562 ± 0.019
$\Delta hrd1$	0.525 ± 0.017	$\Delta hrd1 \Delta ire1$	0.516 ± 0.024
$\Delta hrd3$	0.505 ± 0.007	$\Delta hrd3 \Delta ire1$	0.497 ± 0.012
$\Delta ubc7$	0.551 ± 0.015	$\Delta ubc7 \Delta ire1$	0.534 ± 0.009
$\Delta alg3$	0.517 ± 0.016	$\Delta alg3 \Delta ire1$	0.506 ± 0.008

^a Values represent average $\mu_{\max}$ with standard error from 4 cultures.

A **B**