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Comparative Proteome Analysis in *Schizosaccharomyces pombe* Identifies Metabolic Targets to Improve Protein Production and Secretion

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**Abbreviations**

CDW, cell dry weight;
FDR, false discovery rate;
GIS, global internal standard;
GO, gene ontology;
IP-RP, ion pairing reversed phase;
PPP, pentose phosphate pathway;
TCA, tricarboxylic acid cycle;
μ, growth rate;
2D-LC, two-dimensional liquid chromatography

**Authors contributions**

CWH, TK, SL, LC performed the experiments; CWH, TK, LC, DL, UA, MB, EH, KS, AT interpreted data; CWH, TK, LC, KS and AT wrote the manuscript.
Summary

Protein secretion in yeast is a complex process and its efficiency depends on a variety of parameters. We performed a comparative proteome analysis of a set of *Schizosaccharomyces pombe* strains producing the α-glucosidase maltase in increasing amounts to investigate the overall proteomic response of the cell to the burden of protein production along the various steps of protein production and secretion. Proteome analysis of these strains, utilizing an isobaric labeling / two dimensional LC-MALDI MS approach, revealed complex changes, from chaperones and secretory transport machinery to proteins controlling transcription and translation. We also found an unexpectedly high amount of changes in enzyme levels of the central carbon metabolism and a significant upregulation of several amino acid biosyntheses. These amino acids were partially underrepresented in the cellular protein compared to the composition of the model protein. Additional feeding of these amino acids resulted in a 1.5-fold increase in protein secretion. Membrane fluidity was identified as a second bottleneck for high-level protein secretion and addition of fluconazole to the culture caused a significant decrease in ergosterol levels, while protein secretion could be further increased by a factor of 2.1. In summary, we show that high level protein secretion causes global changes of protein expression levels in the cell and that precursor availability and membrane composition limit protein secretion in this yeast. In this respect, comparative proteome analysis is a powerful tool to identify targets for an efficient increase of protein production and secretion in *S. pombe*. Data are available via ProteomeXchange with identifiers PXD002693 and PXD003016.
**Introduction**

The field of recombinant protein production is constantly growing, providing both bulk scale enzymes for industrial processes as well as tailor-made proteins, such as antibody fragments, for medical usage. Yeasts have become a well utilized platform for recombinant protein production thanks to their ability to secrete proteins of interest (1-2). This greatly facilitates product purification and thus helps to make a process economically competitive (3). As unicellular microorganisms they are easy to cultivate and high cell densities can be reached for industrial production processes. As eukaryotes they possess both protein folding machinery and the ability to perform posttranslational modifications related to higher eukaryotes, which can be advantageous compared to prokaryotic microorganisms (1). Additionally, a large amount of research has been performed over the past decades into improving the secretion of recombinant proteins from yeast cells, engineering intracellular transport of secreted proteins as well as glycosylation patterns and protein folding. Recent studies also deal with the complex interactions between metabolism and recombinant protein production and systems biology tools allow the identification and overcoming of metabolic bottlenecks in recombinant protein production and secretion (4-5).

While *Saccharomyces cerevisiae* and *Pichia pastoris* are by far the most commonly utilized yeasts for industrial production processes (6-7), alternative host systems should also be considered. The fission yeast *Schizosaccharomyces pombe* is one such alternative cell factory. While this yeast is well known as a model organism for molecular and cell biology, its application for recombinant protein production has been demonstrated previously (8). In addition to having a well sequenced and annotated genome for which data is freely available (www.pombase.org), *S. pombe* is very well characterized in respect to cell cycle regulation, DNA replication, transcription and translation as well as in terms of protein folding and protein quality control (9). Posttranslational modification of secreted proteins, especially glycosylation, is closely related to mammalian cells (10), making *S. pombe* an attractive host for recombinant mammalian protein production. Secretion of recombinant human transferrin has been reported, and single-chain antibody fragments have been produced with
titers up to 5 mg/L (11-12). In recent times a significant amount of effort has been focused towards engineering *S. pombe* as a competitive host system for recombinant protein secretion (3, 11). Despite these efforts, *S. pombe* is still underdeveloped as an industrial cell factory and further research will be necessary to create a host system competitive to *S. cerevisiae* and *P. pastoris*.

In this study, we performed a quantitative (comparative) proteome analysis on three different strains of *S. pombe* secreting the model protein maltase in varying amounts: the wild type strain (NW8), a moderate maltase producing strain (NW9), and a strong maltase producing strain (NW10). In a recent study, we investigated the effects of increased maltase secretion on the central carbon metabolism of *S. pombe* using $^{13}$C-assisted metabolic flux analysis (13). In this study, we were interested in the system wide proteomic response of *S. pombe* cells to the increased level of protein secretion. Our model protein maltase, with an approximate size of 110 kDa, is a highly secreted $\alpha$-glucosidase of *S. pombe* (14). It is encoded by the *agl1* gene and expression of the wildtype gene is tightly regulated by extracellular glucose levels. While a strong secretion up to 10 mg (gCDW$^{-1}$) takes place under glucose starvation, high glucose concentrations in the media suppress gene expression (14). We expressed the *agl1* gene under the control of the strong *nmt1* promoter to achieve constitutive expression by plasmid as well as via additional chromosominal integration, which led to increased amounts of secreted protein even when high amounts of glucose are present in the media. Proteome analysis was performed using a two dimensional LC coupled offline to MALDI MS. To reduce measurements time, a pooling scheme for the multidimensional separation was established. Quantitative information was generated by isobaric labeling using the iTRAQ approach (15). A global internal standard approach was applied.

The quantitative proteomics results revealed changes in protein levels across numerous biological pathways within the cell. This finding provides a broad set of targets for further genetic engineering and media design with the aim of improved protein secretion. We selected two of these targets, amino acid biosynthesis and membrane fluidity, which could be influenced via supplementation of the media with specific compounds in order to validate the efficacy of the proteome data for increasing protein secretion in *S. pombe*.
Experimental procedures

Strain construction

Construction of *S. pombe* strains NW8 and NW9 has been described before (13). For the construction of strain NW10, the fission yeast gene *agl1* (16) was amplified from genomic fission yeast DNA by PCR with flanking primers that introduce restriction sites for *Nde*I (5’) and *Bam*HI (3’), respectively. After restriction digest the amplified *agl1* gene was cloned into the integrative expression vector pCAD1 (17) to yield the new plasmid pCAD1-agl1 and the episomal expression vector pREP1 to yield pREP1-agl1. The correctness of all expression constructs was confirmed by sequencing. The parental strain NCYC 2036 (18) was transformed first with the integrative vector pCAD1-agl1 and after successful integration transformed with plasmid pREP1-agl1 using cryocompetent cells (19). The resulting strain NW10 carries a genomic copy of the *agl1* gene integrated into the *leu1* locus and a second *agl1* gene on the episomal plasmid pREP1-agl1, both under control of the strong *nmt1* promoter. All strains are listed in Supplemental Table S1.

Media and cultivation conditions

Minimal media consisting of [g/L] glucose 20.0; NH₄SO₄ 15.0; KH₂PO₄ 11.0; MgCl₂ 1.0; NaCl 1.0; CaCl₂ 0.014 was used for cultivations. Vitamins and minerals were added to the following final concentrations [mg/L]: calcium pantothenate 1.0; nicotinic acid 10.0; myo-inositol 10.0; pyridoxine 0.5; biotin 0.01; FeSO₄ 0.7; ZnSO₄ 0.8; MnSO₄ 0.8; boric acid 1.0; CoCl₂ 1.0; NaMoO₄ 5.0; KI 2.0; CuSO₄ 0.08. The pH of the media was set to 5.5. For cultivation with addition of amino acids, 5 mM of the following amino acids were added: asparagine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, tryptophan and valine. Media components, amino acids and fluconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluconazole was applied in cultivations at concentrations ranging from 0.5 to 10 µg/mL.

Cell growth was observed via optical density measurement at 595 nm. Correlation between optical density at 595 nm (OD₅₉₅) and cell dry weight was taken from Klein et al. (20). For analysis of culture supernatants, 1 mL of cells was harvested in regular intervals during the growth phase on glucose and
supernatants were analyzed by HPLC as described before (20). Ethanol was determined enzymatically (Ethanol UV method, product code 10176290035, R-Biopharm, Germany).

For determination of the average amino acid composition of cellular protein, 1 mL of cells were harvested during the glucose growth phase, the pellet resuspended in 100 µL of 6 M HCl and the cellular protein hydrolyzed for 24 h at 100°C. The suspension was neutralized by addition of NaOH, the cell debris removed by filtration through a 0.22 µm filtration unit, and the remaining solution lyophilized. The lyophilisate was resuspended in 500 µL of 200 µM α-butyric acid, which served as internal standard for HPLC analysis, which was performed as described before (21). Ergosterol was extracted from dry cell mass as described for *Saccharomyces cerevisiae* (22). Quantification was performed by HPLC with 95 % methanol as eluent at a flow rate of 1 mL/min and 30°C oven temperature (22). Ergosterol standards were in the range of 0.1 to 2 mg/mL. All measurements were performed in biological and technical duplicates.

Maltase activity assay was performed as described earlier (14). Glucose produced from maltase hydrolysis was determined enzymatically (D-Glucose UV method, product code 10716251035, R-Biopharm, Germany).

**Chemicals and reagents for proteome analysis**

Dithiothreitol (DTT), CHAPS, urea, glass beads, iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), triethylamine, acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid (FA), α-cyano-4-hydroxycinnamic acid (CHCA), and Glu-1-fibrinopeptide B (Glu-Fib) were from Sigma-Aldrich (Taufkirchen, Germany). Protease inhibitor cocktail was from Roche (Mannheim, Germany). Trypsin was obtained from Promega (Madison, WI, USA). 4-plex iTRAQ reagent kit was from Applied Biosystems (Darmstadt, Germany). Tris-HCl was from Roth (Karlsruhe, Germany). Water used for all experiments was purified by an arium® 611VF System (Sartorius, Göttingen, Germany).

**Preparation of S. pombe cell lysates**

Strains of *S. pombe* grown in 50 mL culture were pelleted by centrifugation, frozen immediately in liquid nitrogen and stored at -80 °C. For preparation of the total cell lysates of *S. pombe*, 20 mL cells
(ca. $2 \times 10^9$ cells) were re-suspended in 550 µL of freshly prepared ice-cold denaturing lysis buffer containing protease inhibitors (10 mM Tris-HCl pH 7.4, 0.55% CHAPS, 8 M urea, 200 mM DTT, 5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 1 mM phenylmethylsulfonylfluoride) (23) and ca. 200 mg of 0.25-0.5 mm glass beads were added. Cells were disrupted in an oscillating mill device (MM400, Retsch, Haan, Germany) at 30 Hz/s for 120 s, paused for another 120 s on ice, the procedure was repeated five times. Cell homogenate was centrifuged at 14,000 g for 15 min at 4 °C and the supernatant (clear cell lysate) were transferred to a 2 mL Eppendorf tube. The cell lysate extraction step was repeated twice and the supernatants were combined. The protein concentration was determined by Bradford assay (Bio-Rad Inc., Hercules, CA). 400 µg cell lysate proteins from each strain of the \textit{S. pombe} were alkylated with 20 µL of 375 mM IAA at RT for 30 min in the dark before transferring to the 0.5 mL Amicon ultra centrifugal filter unit (MWCO: 3 kDa, Millipore, Billerica, MA) for buffer exchange and concentration. The ultrafiltration microcentrifuge tube was centrifuged at 14,000 g for 25 min at 4 °C, re-buffered twice with 150 µL of H$_2$O, once with 150 µL of 200 mM TEAB, and the concentrate was recovered by reverse spinning at 1,000 g for 2 min at 4 °C. The protein concentration of the purified cell lysates was determined again by Bradford assay.

**Protein digestion and peptide 4-plex iTRAQ labeling (cell lysates)**

For trypsin digestion, 2 µg of trypsin was added to 100 µg of extracted cell lysate (adjusted with 0.2 mM TEAB to a final volume of 115 µL) and incubated at 37 °C overnight. Digestion was stopped by freezing samples at -20 °C.

Isobaric labeling of the resulting peptides was accomplished with 4-plex iTRAQ reagent. Prior to labeling, one quarter of the peptide solution from each of the three samples (i.e., NW8, NW9, and NW10) were combined to create a reference sample (i.e., the global internal standard, Mix), as shown in Fig. 1A. The peptide solutions were concentrated to less than 30 µL in a SpeedVac (Eppendorf Vacuum Concentrator Plus, Wesseling-Berzdorf, Germany) prior to labeling with 4-plex iTRAQ reagents according to the manufacturer’s protocol. Peptides derived from the Mix, NW8, NW9, and NW10 were labeled with iTRAQ tags 114, 115, 116 and 117, respectively, at room temperature for 2
h. The four iTRAQ-labeled peptide samples were pooled and stored at -80°C until MS analysis. Three independent biological replicates were performed.

**Two-dimensional LC-MALDI MS/MS analysis of cell lysates**

First dimension peptide separation was performed by reversed-phase RP-HPLC at high pH (24) on an Ultimate 3000 Binary Analytical HPLC system (Dionex, Dreieich, Germany) using a reversed phase Gemini 3 µm C18 110 Å column (2 mm i.d. × 150 mm, Phenomenex, Aschaffenburg, Germany). The pooled iTRAQ-labeled peptides from 100 µg protein digests were injected. Mobile phase A was 72 mM triethylamine in water (pH 10), and mobile phase B was 72 mM triethylamine in ACN (apparent pH 10). The LC gradient started isocratic with 10% solvent B for 5 min, followed by a linear ramp to 55% solvent B over 30 min; after a second linear ramp to 95% B (in 2 min), this solvent was kept isocratic for an additional 10 min before re-equilibration at 5% B. The flow rate was 200 µL/min; the column temperature was 30 °C; UV detection was performed at 214, 254, and 280 nm. Two-minute wide fractions were collected manually from 7 to 47 min. Every fourth fraction was pooled (i.e. fraction 1 plus 5 plus 10 etc.), yielding a total of four, ten-minute wide fractions. The four pooled fractions were concentrated in a SpeedVac, and re-buffered with 0.1% TFA for second dimension peptide separation.

Second dimension peptide separation was performed using ion-pairing-reversed-phase HPLC (IP-RP-HPLC) at pH 2. It was carried out with an integrated Ultimate 3000 RSLCnano system (Dionex) equipped with a µ-precolumn Acclaim PepMap 100 (300 µm i.d. × 5 mm, 100 Å pore and 5 µm particle size, Dionex) and an analytical reversed-phase Acclaim PepMap 100 C18 column (75 µm i.d. × 250 mm, 100 Å pore and 3 µm particle size, Dionex) that was coupled online to a Probot microfraction collector (LC Packings, Amsterdam, the Netherlands) for MALDI target spotting. Mobile phase A was 0.05% TFA (pH 2), mobile phase B was 0.04% TFA in 80% ACN (pH 2). The LC gradient was held at initial conditions of 15% B for 5 min followed by a linear ramp to 50% over 120 min; 95% B was reached via a linear gradient over the next 2 min and isocratically held for an additional 10 min before re-equilibration at 10% B. The flow rate was 300 nL/min; the column temperature was 30 °C; UV detection at 214 nm. Eluting peptides were directly mixed in the Probot
with matrix solution (3 mg/mL CHCA, 0.1 % TFA and 5 nM Glu-Fib in 70 % ACN) in a ratio of 1:3 (v/v) and spotted in 15 s intervals. Three technical (injection) replicates from each of the four fractions of first dimension separation were performed.

All MS and MS/MS measurements were performed on an AB Sciex TOF/TOF™ 5800 (Darmstadt, Germany) mass spectrometer operated in reflector positive ion mode. MS data were acquired with a total of 1,200 shots per spectrum (200 shots per sub-spectrum) with a laser pulse rate of 400 Hz. MS spectra in LC-MALDI experiments were internally calibrated on Glu-Fib and the matrix cluster signal at m/z of 877.034. MS/MS data were acquired at 1,000 Hz in 1 kV MS/MS mode with 4,080 shots/spectrum (255 shots per sub-spectrum). Non-redundant and fully automated precursor ions were selected by the TOF/TOF Series Explorer software (AB Sciex) for MS/MS fragmentation. Precursor ions were separated by timed ion selection with a resolution window of 200 (FHWM), for each spot the 20 most intense precursors with a minimum S/N ratio of 30 were selected for MS/MS, using ambient air as collision gas with gas pressure of \(4.0 \times 10^{-6}\) mbar.

**Database search for protein identification and quantification of cell lysates**

Protein identification was performed with the ProteinPilot v4.0 software (AB Sciex) using the Paragon algorithms (25). MS/MS data from 36 HPLC runs (i.e. 3 biological replicates × 4 pooled fractions from first dimension separation × 3 technical replicates) were searched separately against the PomBase database (released on 09th of December 2010, 5,038 entries) downloaded from EMBL-EBI (ftp://ftp.sanger.ac.uk/pub/yeast/pombe/Protein_data/OLD/Pompep/). The following search parameters were selected: iTRAQ 4-plex peptide label, cysteine carbamidomethylation, trypsin specificity, one missed cleavage site allowed ID focused on biological modification and amino acid substitution, and processing including quantification and “Thorough ID”, in which the precursor ion mass accuracy and fragment ion mass accuracy are MALDI 5800 built-in function of ProteinPilot software. All reported proteins were identified with 99% confidence as determined by ProteinPilot Unused ProtScores (2.0) with the corresponding local false positive discovery rate (FDR) (26) below 5% (Supplemental Table S2). The identified proteins were grouped by the ProGroup algorithm (ABSciex) to minimize redundancy.
Protein quantification was performed following extraction of the Peptide Summary data that was obtained from each LC run (a total of 36 spot sets). Only peptides that were confidently identified via the Paragon algorithm and classified as “Used” were exported to Microsoft Excel for further data merging and protein ratio calculation using the in-house VBA (visual basic for applications) scripts (see Supplemental Information). These scripts performed steps identical to those of ProteinPilot for the calculation of protein ratios within the three biological replicates; however, they were necessary as with our computational setup ProteinPilot was not able to perform the merging and statistical calculations of all datasets. Only proteins with two or more unique peptides quantified in all three LC-MS experiments were considered for relative quantification, excluding those common to other isoforms or proteins of the same family. A scheme illustrating the iTRAQ ratio comparison strategy used in this study is shown in Fig. 1B. Proteins with significantly different abundances between the conditions were identified by multiple hypotheses testing (Students t-test, two tailed assuming equal variance) that was controlled by Benjamini Hochberg false discovery rate (FDR) analysis (27), with FDR of < 5% used as threshold to define biologically changed proteins (Supplemental Table S3).

**GO annotation of differentially abundant proteins**

To characterize the various differentially abundant proteins for biological interpretation, a gene ontology (GO) analysis at AmiGO website (http://amigo.geneontology.org/cgi-bin/amigo) was undertaken using both the GO Slimmer and GO Enrichment tools (28-29). For GO slim analysis the systematic IDs of all the regulated proteins were uploaded as input file, the PomBase and the fission yeast GO slim (30) (updated on 2014-06-03, including regulates links) was selected as filter and GO slim set, respectively. For GO enrichment analysis the systemic IDs of all regulated proteins were uploaded as input, PomBase was selected as the database filter, and a maximum $p$-value of 0.01 with a minimum of 2 gene products was selected for the changeable thresholds. The results of the analysis are summarized in Supplemental Tables S4 (GO slim analysis) and S5 (GO term enrichment).

**Protein profiles of cultivation supernatants**

Supernatants from the cultivation of NW8, NW9 and NW10 (as used for the full cellular proteome analysis) were collected following centrifugation (as described above). A protease inhibitor cocktail
(cOmplete mini, Roche) was included and the samples were frozen at -80°C. Three biological replicates of the supernatants of each strains cultivations were analyzed. Five mL of growth media supernatant was lyophilized before being re-suspended in 500 µL of 50 mM HEPES buffer pH 7.4. The re-suspended supernatant was subjected to an on-filter wash (10 kDa membrane filter) with 500 µL of HEPES buffer before being re-suspended in 250 µL of fresh HEPES buffer and transferred to a clean micro-centrifuge tube. 30 µL of each sample and 10 µL of 4× Laemmli loading buffer were heated at 98°C for 5 minutes before being loaded onto a 12 % T gel for SDS-PAGE stacking gel clean up. The gel was run for 45 minutes (40 V [constant] × 15 minutes × 2 (double loading into each well), then 100 V [constant] × 15 minutes). Once the proteins had migrated to the boarder of the stacking gel, the resolving gel was removed from the cassette, fixed, washed two times, stained with Coomassie brilliant blue, and destained overnight. The stacking gel area for each band was excised (approximately 1 cm² for each sample), destained and subjected to reduction (10mM DTT, 56°C 1 h, alkylation (IAA, RT, 30 min) and in-gel digestion with trypsin (133 ng, 37°C, overnight). Peptides from each sample were extracted in increasing concentrations of acetonitrile, dried under vacuum and re-suspended in 40 µL of sample loading buffer (3% ACN, 0.1% TFA).

Nano-HPLC separations were performed on a Dionex UltiMate™ 3000 RSLCnano system (Dionex) equipped with a µ-precolumn Acclaim PepMap 100 (300 µm i.d. × 5 mm, 100 Å pore, 5 µm particle size, Dionex) and an analytical reversed-phase Acclaim PepMap 100 C18 column (75 µm i.d. × 500 mm, 100 Å pore, 2 µm particle size, Dionex) coupled online to a mass spectrometer. Eluent A: 0.05% FA in water, eluent B: 0.04% FA in 80% ACN; gradient: 0 – 2 min isocratic at 4% B, 2 – 95 min linear gradient from 4 to 40% B, 95 – 101 min linear gradient from 40 to 90% B, 101 – 104 min isocratic at 90% B, 104 – 105 min, 90 – 4% B, 105 – 125 min at 4% B. The flow rate was 300 nL/min; column temperature was 40°C. MS analysis was performed on a QExactive™ Plus mass spectrometer (Thermo Scientific, Germany) operated in positive ion mode, Full scan 300 – 2000 m/z at a resolution of 70,000; MS² top 10, at a resolution of 17,500; isolation window of 3.0 m/z; NCE 27.5; dynamic exclusion 20s; lock mass (m/z = 445.12003) enabled.
Raw data files were searched against the *S. pombe* FASTA database as described above. A combined search algorithm approach was performed in Proteome Discoverer (Version 1.4.0.288) using Mascot (Version 2.2.7) and SequestHT using the following search parameters: enzyme specificity = trypsin; fixed modification = cysteine (carbamidomethyl); variable modification = methionine (oxidation); min peptide length = 6; peptide FDR 0.01; missed cleavages = 2; precursor mass tolerance = 7 ppm; fragment mass tolerance = 0.02 Da. A minimum of two high confidence peptides, of which at least one was unique peptide, were required for a protein to be considered as identified. Percolator (Version 2.04) was used for calculation of q-values and posterior error probabilities.

The same analytical setup was used for a second series of supernatant analysis for the comparison of NW10 with / without addition of fluconazole and amino acids.

**Raw data repository**

All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (31) via the PRIDE partner repository with the dataset identifiers PXD002693 (2D-LC-MALDI MS analysis of supernatant analysis for comparison of NW8, NW9 and NW10); and PXD003016 (assessment of NW10 supernatant with / without fluconazole and amino acid supplementation).
Results

Physiological characterization of maltase secreting strains

Cultivation of the strains NW8, NW9 and NW10 in minimal media containing 20 g/L of glucose results in repression of the wildtype maltase gene; however, copies of the maltase gene under control of the nmt1 promoter are constitutively expressed provided no thiamine is present. As expected, NW8 produced very low amounts of maltase, with a yield ($Y_{\text{maltase/X}}$) of 15.7 ± 4.2 U (g CDW)$^{-1}$ at the end of the glucose growth phase. Under the same growth conditions, strain NW9 produced 439 ± 23 U (g CDW)$^{-1}$ and the strain NW10 a maximum of 592 ± 17 U (g CDW)$^{-1}$. This corresponds to yields of 0.45, 12.5 and 16.9 mg (g CDW)$^{-1}$, respectively (Supplemental Table S6 and Supplemental Fig. S1).

Strains NW8 and NW9 exhibited a specific growth rate ($\mu$) of 0.22 h$^{-1}$ while growth of strain NW10 was slower with a specific growth rate of 0.17 h$^{-1}$ (Supplemental Table S6). However, yield coefficients for glucose uptake ($Y_{\text{Glc/X}}$) and byproduct formation for the different strains did not change significantly (Supplemental Table S6). Ethanol was the main fermentative product with yields ($Y_{\text{EtOH/X}}$) of 45.59 ± 2.32, 47.13 ± 2.78 and 45.12 ± 3.88 mmol (g CDW)$^{-1}$ for NW8, NW9 and NW10, respectively. Glycerol was identified as second major byproduct besides ethanol with yields ($Y_{\text{Gly/X}}$) in the range of 3.7 to 4.1 mmol (g CDW)$^{-1}$ (Supplemental Table S6). During all cultivations we detected small amounts of the organic acids pyruvate and acetate; (less than 0.015 mmol per mmol of glucose consumed). Consistent with the product yields, biomass yields ($Y_{\text{xb}}$) of the strains NW8, NW9 and NW10 were in a comparable range.

Comparative proteome analysis of maltase secreting strains

Analytical strategy applied for quantitative proteome analysis

An isobaric labeling strategy encompassing four-plex iTRAQ was utilized to compare the protein abundance ratios between the three different S. pombe strains. In addition, a global internal standard (GIS) approach was applied, in which an equal amount (1/4) from each of three tryptic peptide samples was pooled to create a reference sample (Mix) (Fig. 1A). The use of a two-dimensional LC
A separation scheme encompassing high-pH (pH 10) reversed-phase HPLC in the first and low-pH (pH 2) ion-pairing reversed-phase HPLC in the second dimension (RP × IP-RP-HPLC) (24, 32) resulted in the comprehensive proteomic profiling of *S. pombe*. A complete list of protein identifications and quantitative values of the triplicate iTRAQ experiments is summarized in Supplemental Tables S2 and S3, respectively. In these three replicates, a total of 41,018, 33,399, and 33,254 MS/MS spectra were acquired; 38,068, 30,784, and 30,511 distinct peptides were identified with a 5% local FDR that led to the identification of 1,610, 1,538 and 1,466 proteins with 99% confidence, respectively. After redundancies and single peptide identifications were removed, a total of 1,700 unique proteins were identified (Supplemental Fig. S2). Of these, 1,315 (78.8%) proteins were shared across all three iTRAQ experiments while 213 (12.3%) were shared by two experiments. Thus, more than 85% of the identified proteins (1,528 of 1,700 proteins) were detected in at least two of the three replicates (Supplemental Fig. S2A).

Among the 1,700 identified proteins a total of 1,580 (92.9%) could be quantified by at least two unique peptides in at least one LC-MS experiment. Of these, 1,041 (65.8%) were shared by all three LC-MS experiments and incorporated in this study for the analysis of differential abundance. In addition, at key positions we also manually inspected values quantified in only two out the three LC-MS experiments when the biological data (e.g. the change in abundance of other proteins in the same pathway) hinted for potential importance of the particular proteins; these values are clearly assigned as such in the following parts of the manuscript.

**Relative quantification of protein levels**

In order to identify statistically significant differential protein abundances, a multiple hypothesis testing analysis with a controlled false discovery rate (FDR) correction was carried out. For this purpose, volcano plots of the 1,041 quantifiable proteins in datasets R[NW8 v 9] and R[NW8 v 10], respectively were generated by plotting the averaged relative abundance ratio against its *p*-value on a
logarithmic scale (Fig. 2A and 2B). Protein ratios for both datasets \( R[\text{NW8 v 9}] \) and \( R[\text{NW8 v 10}] \) were ranked by increasing \( p \)-values; the cumulative number of protein ratios (detections), as well as the percentage of detections (%) at specified \( p \)-values (\( \leq p \)-value), were counted, and two global FDR curves were calculated for \( R[\text{NW8 v 9}] \) and \( R[\text{NW8 v 10}] \) (Fig. 2C, D). As a result, specific \( p \)-values can be defined as the thresholds to differentiate statistically significant (true positive, \( \leq p \)-value) protein ratios from non-significant (false positive, \( > p \)-value) ones.

In this study significant protein abundance changes were based on global FDR’s of \( \leq 5\% \), which resulted in thresholds of \( p \leq 0.0155 \) (\( \log_{10}[p \text{-value}] = -1.81 \)) for \( R[\text{NW8 v 9}] \), and \( p \leq 0.0165 \) (\( \log_{10}[p \text{-value}] = -1.78 \)) for \( R[\text{NW8 v 10}] \). In addition to the significance threshold, we applied a protein–fold change filter to underline proteins that are relevant biologically. A protein fold-change cutoff value of 0.704 (\( \log_2(0.704) = -0.5 \)) and 1.420 (\( \log_2(1.420) = 0.5 \)) was chosen to define proteins with higher or lower abundance, respectively. This resulted in, for NW9, 22 proteins with higher and 9 proteins with lower abundance compared to the NW8 (Fig. 2E), while for NW10, 31 proteins were of higher and 16 proteins of lower abundance in comparison to the wildtype (Fig. 2E).

In case of applying a least stringent \( p \)-value of \( p < 0.05 \), and fold changes of \( < 0.8 \) and \( > 1.25 \) a further 75 proteins were identified as differentially abundant for NW8 vs. NW9 and 106 for NW8 vs. NW10 (Fig. 2E). We also took these proteins into account in the following discussion but distinguished between both significance criteria throughout the further discussion.

Among the proteins identified as differentially abundant, the overexpressed target protein Agl1p (maltase) exhibited the most drastic changes showing a 2.6-fold increase in strain NW9 and a 3.3-fold increase in strain NW10 compared to NW8 (intracellular values). In the remainder of the text, higher or lower protein abundances or amounts are stated in relation to the wild type NW8 strain unless otherwise stated.
Proteomic response of the central metabolism to elevated levels of maltase secretion

Of the differentially abundant proteins identified in strain NW9 and NW10 compared to the wildtype, a large number were found to be related to central energy metabolism and anabolic pathways. This trend was supported via gene ontology analysis which identified these metabolic processes (or related terms) as both containing a high number of gene products, as well as being significantly enriched (Supplemental Tables S4 and S5).

Central carbon metabolism

The majority of enzymes involved in the central carbon metabolism, i.e. from glycolysis, the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA), anaplerotic and fermentative pathways (Fig. 3) were identified in this study.

Compared to the wildtype, the NW9 strain showed abundance changes in two enzymes from glycolysis (fbp1p, eno102p), while the NW10 strain showed altered abundances for six enzymes (ght5p, pgi1p, fbp1p, tp1p, tdh1p, eno102p). In yeast, the glycolytic flux is primarily controlled by glucose uptake, the reaction of hexokinase and pyruvate kinase (33). *S. pombe*’s major glucose transporter Ght5p and three further enzymes in the glycolytic pathway (Fbp1p, Tdh1p, Eno102p) were significantly decreased in the NW10 strain. NW9 also showed a decrease for both enolase (Eno102p) and fructose-1,6-bisphosphate (Fbp1p). On the other hand, two glycolytic enzymes were increased, glucose-6-phosphate isomerase (Pgi1p) and triosephosphate-isomerase (Tpi1p), both in the NW10 strain. The oxidative branch of the pentose phosphate pathway (PPP) exhibited lower expression levels in NW9 and NW10 compared to the reference strain (Fig. 3). In the non-oxidative part of the PPP only transaldolase was decreased in NW9 whereas the remaining enzyme abundances were not altered among the strains.

Levels of anaplerotic enzymes remained unchanged in both strains. However, abundances of pyruvate decarboxylase and acetaldehyde dehydrogenase were significantly decreased (Fig. 3). Also, abundance levels of several TCA cycle enzymes, namely oxoglutarate dehydrogenase (Kdg1p) and
malate dehydrogenase (Mdh1p), were decreased in both NW9 and NW10, while citrate synthase (Cit1p) and isocitrate dehydrogenase (Idh1p) were reduced only in NW9.

In addition, a major effect was apparent for proteins involved in electron transport and energy metabolism. Mitochondrial alcohol dehydrogenase (Adh4p), Cox6p and Etp1p, which are involved in the electron transport chain, as well as the ATPase related proteins, Vma5p, Vma7p and Vma10p were increased in both maltase producing strains. In contrast, we found reduced protein levels for Qcr2p, which is involved in ubiquinol-mediated electron transport across the inner mitochondrial membrane.

**Amino acid metabolism**

In contrast to the general decrease in abundances observed across the majority of enzymes involved in central carbon metabolism in both maltase producing strains, enzymes involved in amino acid biosynthesis were typically only found to be altered in the NW10 strain, and of those proteins all but one (Leu1p) were increased (Fig. 3 and Supplemental Figure S3).

Two enzymes involved in asparagine biosynthesis, aspartate aminotransferase (Aat2p) and asparagine synthase (Asn1p), and the majority of enzymes involved in the synthesis of branched chain amino acids (aspartate kinase (SPBC19F5.04), acetalactate synthase (SPBC14C8.04), branched chain amino acid aminotransferase (Eca39p) and 3-isopropylmalate dehydratase (Leu2p)), were significantly higher in abundance (Supplemental Fig. S3A and B).

The enzymes essential for lysine biosynthesis, homocitrate synthase (Lys4p), amino adipate-semialdehyde dehydrogenase (Lys1p) and saccharopine dehydrogenase (Lys3p), were all significantly increased in the NW10 strain (Supplemental Fig. S3C). In addition, the aminotransferase (SPAC56E4.03), which is involved in lysine biosynthesis, aromatic amino acid biosynthesis and methionine scavenging, was more abundant (Supplemental Fig. S3C, G and H).
A key enzyme within the histidine biosynthetic pathway, imidazole-glycerol-phosphate synthase (His4p), was increased in abundance in the NW10 strain. This finding is of special interest as the catalyzed reaction also produces 5-aminimidazole-4-carboxamide ribonucleotide (AICAR). AICAR is a precursor in purine biosynthesis and has been shown to be involved in cross-regulation of histidine and purine biosynthesis by transcriptional regulation in *S. cerevisiae* (34-35).

The NAD$^+$-dependent glutamate dehydrogenase (isoform Gdh2p) involved in glutamate biosynthesis exhibited elevated levels in NW10 (Supplemental Fig. S3E). However, the alternative isoform (Gdh1p) and the glutamate synthase (Glt1p) were unaffected in both strains. Several enzymes involved in arginine biosynthesis were also affected (Supplemental Fig. S3F). The enzymes ornithine carbamoyltransferase (Arg3p), carbamoylphosphate synthase (Arg4p, Arg5p), argininosuccinate synthase (Arg12p) and arginase (Car1p) were observed with higher abundances.

A significant alteration of protein levels in strain NW10 was also observed in the biosynthesis of the aromatic amino acids tyrosine and phenylalanine (Supplemental Fig. S3H). The aromatic aminotransferase (SPAC56E4.03) catalyzing the conversion of phenylpyruvate and 4-hydroxyphenylpyruvate to phenylalanine and tyrosine, respectively, was found in higher amounts (Supplemental Fig. S3H).

One of the most influenced pathways observed was the methionine salvage pathway. Here, in strain NW10, the enzymes *S*-adenosylmethionine synthase (Sam1p), and aromatic aminotransferase (SPAC56E4.03) were increased in strain NW10, and in addition the enzyme acireductone dioxygenase (Adi1p) was significantly increased in strain NW9 (Supplemental Fig. S3G).

**Ergosterol and lipid metabolism**

Lipid and ergosterol biosyntheses were also affected by increasing maltase secretion, in particular in the strain NW10. We observed an increase in the isopentenyl-diphosphate delta-isomerase Idi1p, a key enzyme of the ergosterol biosynthetic route. Regarding lipid metabolism, we observed a reduction
of protein abundance of both glycerol-3-phosphate dehydrogenases (Gld1p and Gut2p). It has previously been shown that deletion of these proteins in the yeast *Yarrowia lipolytica* leads to a 3-fold increase in lipid production and accumulation (36), and that high-level protein secretion in *S. pombe* correlates with increased cellular lipid content (13). In addition, the protein Lsd90, which plays a role in the metabolism of very long chain fatty acids (37), and a predicted 3-hydroxyacyl-CoA dehydrogenase (SPAC4H3.08), involved in lipid degradation, were significantly decreased in abundance in the NW10 strain.

*Other metabolic pathways*

Nucleotide metabolism was also affected in the maltase producing strains. However, changes in enzyme levels were not as strong as found for enzymes in central carbon metabolism and amino acid biosynthetic pathways. Increased abundance of enzymes of pyrimidine metabolism was observed for orotate phosphoribosyltransferase (Ura5p), uridylic kinase (SPCC1795.05c), deoxyuridine 5’-triphosphate nucleotidohydrolase (SPAC644.05c) and guanine deaminase (SPCC1672.03c). In contrast, Ura4p was less abundant in NW10. However, changes related to the pyrimidine metabolism, in particular, the enzyme Ura4p must be treated with caution, as Ura4p was used as selection marker in the construction of the NW8 and NW9 strains.

Vitamin metabolism, in particular enzymes for the biosyntheses for thiamine (Thi2p, Pho4p, Nmt1p), and pyridoxine (Plr1p, Snz1p, SPAC222.08c), showed increased abundance in the maltase producing strain NW10 (Fig. 4). In the case of Nmt1p this finding is in particular interesting, as it might point to an increased activity of the *nmt1* promoter in the maltase producing strains, although all three strains were cultivated under the same conditions. Besides the increased gene copy number of *agl1* this might further enhance maltase production compared to the NW8 wild type strain.
Impact of elevated maltase secretion on the cellular protein synthesis and processing machinery

We primarily focused on those parts of the proteome involved in protein biosynthesis, protein folding, modification and transport as well as the associated degradation pathways. However, this reflects only a fraction of the total response of the proteome due to an elevated maltase secretion by NW9 and NW10. A complete overview on all quantifiable proteins is given in the Supplemental Table S3. GO-slim terms for these pathways were well represented; however, fewer terms were specifically identified via the enrichment analysis (Supplemental Tables S4 and S5).

Protein biosynthesis

We were able to quantify 88 ribosomal proteins (small 40S and large 60S ribosomal proteins). 12 of these ribosomal proteins exhibited altered levels in the maltase secreting strains: 3 were lower abundant in the high maltase secreting strain NW10 and 3 proteins showed increased abundance. In strain NW9 only 7 proteins were found in higher amounts (Fig. 5).

Related to protein biosynthesis, we found significantly changed protein abundances concerning translation initiation and elongation factors in both maltase secreting strains. In this context, the transcription initiation factors eIF6 (Tif6p) and eIF5A (Tif51ap and Tif51bp) as well as the translation elongation factor eEF1B (Tef5p) and Mnp1p, involved in translation, were higher abundant in both maltase secreting strains NW9 and NW10. In contrast, eIF4A (Tif412p) exhibited reduced protein levels in NW10 (Fig. 5). Further, several proteins involved in RNA polymerase formation showed distinct changes upon increased maltase secretion. Rpb4p (RNA polymerase III), and Rpc19p (RNA polymerase I and II) were present in higher amounts in both maltase producing strains. Rpc11p exhibited elevated protein levels in NW9.

The general transcription factor TFIIA (Toa1p) showed elevated abundance in strain NW9 and Sus1p involved in the regulation of transcription was higher abundant in NW10. In addition, we could quantify aminoacyl–tRNA synthetases for all 20 proteinogenic amino acids (Supplemental Table S2),
but did not observe any further statistically significant changes between the maltase secreting strains NW9 and NW10 and the parental strain NW8.

Protein folding, processing and transport

Several enzymes involved in the processing and transportation of proteins were identified as differentially abundant in this study. Of these, a number of protein disulfide isomerases, which play a pivotal role in the formation and breakage of disulfide bonds between cysteine residues to allow rapid and correct folding, were significantly altered in abundance. The cyclophilin family peptidyl-prolyl cis-trans isomerases (Cyp4) were increased in the NW9 strain as well as the FKBP-type peptidyl-prolyl cis-trans isomerase Fkh1.

The protein Spo20p/Sec14p which is required for the transport of secretory protein from the Golgi complex (38) had, with the exception of Agl1p, the highest increase in abundance of all proteins in the NW10 strain. In addition to this, clatharin light chain protein (Clc1p), a structural protein involved in vesicles formation for intracellular trafficking was also increased in abundance in both strains.

Degradation pathways and RNA metabolism

A number of proteins involved in both degradation pathways and RNA metabolism were identified (Fig. 5). There was an up-regulation of proteins Pip1p and Skp1p, both involved in the formation of the SCF E3 ubiquitine ligase complex, in both maltase producing strains. Further, several proteasomal proteins exhibited altered amounts in these strains. The β subunit, Pre3p, of the 20S particle was increased in NW10. Increased protein levels of the proteasome maturation factor Ump1p were observed in NW9. Proteins involved in the ubiquitine dependent proteasomal protein degradation, the deubiquinating enzyme Ubx3p, which is involved in substrate delivery to the 26S proteasome, showed increased abundance in NW10. Further, vacuolar serine protease Isp6p was present in lower amounts in NW9 strain.

Proteins involved in the formation of the lsm1-7/pat1 complex, which is involved in mRNA decapping followed by its subsequent degradation, were more abundant in both maltase producing
strains with the protein Lsm1p significantly higher in the NW10 strain. Two additional proteins (Ini1p and Srp1p) participating in mRNA splicing were increased in both the NW9 and NW10 strains (Fig.5). Additionally, Trm112p (higher in NW9), which is involved in RNA methylation, and SPCC74.02c (lower in NW10), that is associated with the cleavage and polyadenylation specificity factor complex, were identified.

**Supernatant Analysis**

Qualitative analysis of the supernatants of NW8, NW9, and NW10 identified in total 432 protein matches meeting our search criteria; however, the signals of the majority of these proteins were weak. The top 50 proteins (according to the number of peptide spectrum matches (PSM)) for each of the strains were compared. This resulted in a list of 78 proteins of which more than 60% were shared between at least two of the strains. In addition 37 (74%) of the proteins identified in the NW8 strain were identified as shared with at least one of the higher maltase producing strains (Supplemental Table S7).

While there was a high degree of similarity observed between the top 50 secreted protein profiles for each of the three strains, of those proteins observed in either only NW8 or NW9 and NW10, there were also a number of clear differences (Supplemental Table S7). In the secretome of strain NW8, a number of proteins involved in general energy production, stress protection and nucleotide production were present (e.g. ATP synthase alpha and beta subunits, HSP 60 and 70, and pyridoxine biosynthesis PDX-1 like protein). In contrast to this, of those proteins only identified in the NW9 or NW10 strains, 50% of the proteins (14 of 28) were either members of glycoside hydrolase families (e.g. glucan 1,3-beta-glucosidase, 1,3-beta-glucanosyltransferase, and glucan endo-1,3-alpha-glucosidase) or involved in lipid metabolism (e.g. lysophospholipase 1). It can be speculated that these changes are a direct response to the NW9 and NW10 mutations, as many of the observed proteins are involved in lipid and cell wall formation and an increased secretion of maltase could influence the composition of both plasma membrane and cell wall (39). However, as observed, the changes to the protein secretion
profile (with the exception of maltase) are not extensive, and do not result in the production and secretion of an excessive number of unwanted proteins into the media.

The protein of interest for this study, maltase (Agl1p), based on PSM, was the most highly abundant protein in both the NW9 (average PSM of three replicates ± standard error: 681 ± 139) and NW10 (1179 ± 41) strains while it ranked as only the seventh most abundant (based on PSM counts) in the control NW8 strain (145 ± 7); these numbers strongly indicate also for the increase protein production and specific secretion of maltase in NW9 and NW10. In addition, the sequence coverage of maltase was correspondingly higher in the strains with 29.10%, 36.12% and 39.93% in the strains NW8, NW9 and NW10 respectively (Supplemental Figure S4).

**Application of proteomic data to improve maltase secretion**

Quantitative proteomics data showed, that amongst other pathways and protein classes, both amino acid biosyntheses and membrane fluidity (ergosterol biosynthesis) were significantly altered in the maltase production strains, in particular in the strongest producing strain NW10. We therefore chose to test improvement of maltase secretion in *S. pombe* via targeted supplementation of the media with specific metabolites/compounds.

*Feeding of selected amino acids to improve maltase secretion*

The proteome analysis revealed higher abundances of a number of enzymes involved in amino acid biosynthetic pathways in the high maltase secretion strain NW10 (Fig. 4, Supplemental Figure S3). An analysis of the average amino acid composition of the cellular proteins in *S. pombe* was performed and compared against the amino acid composition of maltase (Supplemental Table S8). This analysis revealed that the amino acids aspartate/asparagine, leucine and phenylalanine were moderately overrepresented in maltase, whereas the amino acids proline, methionine, tyrosine, tryptophan, valine and isoleucine were strongly overrepresented in maltase compared to the cellular protein. Many enzymes involved in the biosynthetic pathways for these amino acids were also found to exhibit significantly elevated levels in NW10 (Supplemental Figure S3). Additionally, His4p catalyzing four
steps in histidine biosynthesis exhibited significantly increased levels in NW10, although histidine abundance (1.9%) was similar between the cellular proteins and the homologous expressed maltase. The second exception was lysine, which was actually more abundant in the cellular proteins (8.1%) than in maltase (1.8%) but whose biosynthesis was strongly upregulated.

Combining these results, we supplemented the glucose minimal media with 5 mM of the amino acids asparagine, valine, isoleucine, histidine, methionine, proline, lysine, phenylalanine and tryptophan (GlcAA). Cultivation of the NW10 strain in GlcAA media produced 26.0 ± 1.3 mg (g CDW)^{-1} of maltase, which was a 1.5-fold increase, compared to the cultivation on the glucose minimal media (Supplemental Table S6 and Supplemental Fig. S1). Further, we studied if this increase in protein secretion was due to a generally increased availability of carbon and energy to the cell or actually dependent on the type of amino acid fed. Control experiments were performed using glucose minimal media supplemented with either 5mM of alanine, glycine, cysteine, threonine, serine, glutamate and arginine (C1), or with all 20 amino acids except leucine (C2) (due to its use as auxotrophic selection marker). Maltase secretion in C1 media produced 19.2 ± 0.5 mg (g CDW)^{-1}, a 1.2-fold increase compared to glucose as the sole carbon source. Cultivation in C2 media resulted in a maltase secretion of 27.5 ± 1.6 mg (g CDW)^{-1}, which is not significantly different to the GlcAA media. Addition of amino acids caused a slight increase of the growth rate of NW10 but did not significantly affect the biomass yield compared to cultivations using glucose as sole carbon source (Supplemental Table S6).

**Membrane fluidity as target for improved maltase secretion**

In the maltase producing strains, we observed a clear impact on several proteins involved in lipid and sterol biosynthesis. We concluded that membrane composition, and thus fluidity, changed with increasing protein secretion due to abundance changes in Lsd90p and 3-hydroxyacyl-CoA dehydrogenase. At the same time, down-regulation of Gld1p and Gut2p strongly indicated that the overall lipid content of the cell increased with elevated protein secretion. We also observed an increased amount of intracellular retained maltase with increasing gene copy number. Together with the increased levels of proteins involved in folding but also degradation of misfolded proteins, these data suggested a limitation of maltase secretion along the secretory pathway.
Deletion of genes of the lipid and sterol metabolism often ends in inviable phenotypes or has severe effects on cell growth and morphology. Therefore, we decided against genetic engineering of the lipid metabolism based on the above mentioned targets. Instead, we applied the antifungal drug fluconazole, which is a general inhibitor of cytochrome P450 (CYP) enzymes (40), and affects the ergosterol biosynthesis by inhibition of the two fission yeast CYP enzymes Erg5p (CYP61) and Erg11p (CYP51). This alters the membrane composition, but should not affect the overall lipid content of the cell and was therefore considered as an acceptable strategy to enhance the effects deduced from the comparative proteome analysis. We examined the effect of fluconazole on ergosterol biosynthesis of strain NW10 and its impact on maltase secretion; in addition, an assessment of the toxicity of fluconazole was performed that showed no negative impact on growth up to a concentration of 5 µg/mL (data not shown).

As expected, ergosterol content within the cells was found to decrease with increasing concentrations of fluconazole in the media (data not shown). At the highest applied concentration of 5 µg/mL, a reduction to 66.8 ± 1.3% of the control ergosterol level was achieved while the maltase yield increased to 22.0 ± 0.9 mg (g CDW)^{-1}, an increase by a factor of 1.3 in comparison to the untreated cells.

Next, we investigated if a combination of both approaches could further increase maltase secretion. Cultivation of the NW10 strain in GlcAA media supplemented with fluconazole at a concentration of 5 µg/mL resulted in the secretion of 37.0 ± 0.3 mg (g CDW)^{-1} of maltase with no observable negative effect on cell growth (Supplemental Table S6). This yield corresponded to a 1.4-fold increase compared to GlcAA without fluconazole, and a 2.2-fold increase compared to NW10 grown in simple glucose minimal media (Supplemental Fig. S1). However, according to the MS-based supernatant analysis, there was no significant difference in the number of proteins released into the supernatant in cultivations of NW10 in media with or without fluconazole and amino acids. A total of 641 unique proteins were identified in the NW10 samples (561 ± 22 proteins) cultivated in non-supplemented medium, whereas 651 unique proteins were identified in the NW10 in medium plus fluconazole plus amino acids (477 ± 88 proteins) (triplicate replicates), rather pointing towards an actual increase in the
secretion capacity of the cells than to an unspecific leakage of proteins upon fluconazole treatment of the culture (data not shown, data accessible via ProteomeXchange PXD003016).
Discussion

Maltase secretion

Under the selected cultivation conditions, a repression of the *agl1* wild type locus was observed as described by Jansen and coworkers (14). Consequently, only small amounts (0.36 mg (g CDW)$^{-1}$) of maltase were detected in culture supernatants of *S. pombe* strain NW8. Using the strong *nmt1* promoter for expression of *agl1*, protein secretion increased to 12.5 and 16.9 mg (g CDW)$^{-1}$ for NW8 and NW9, respectively. While a linear correlation between gene copy number and cytoplasmic protein production has been demonstrated in yeast, this was not true for protein secretion (41). Recombinant protein secretion is often hampered along the secretory pathway or due to limitations in the central carbon metabolism (5). This was also true for high-level maltase secretion, where we could overcome these limitations by influencing membrane composition and targeted amino acid feeding.

Physiology of maltase secreting strains

Physiological characterization of the strains showed that the maximum specific growth rate was lower for NW10 with 0.17 h$^{-1}$ than for strains NW8 and NW9 with a specific growth rate of 0.22 h$^{-1}$. This resulted in decreased rates for glucose uptake and byproduct formation. However, the decrease of $\mu_{\text{max}}$ was not caused by an increased metabolic burden to the cell due to the increased maltase secretion of this strain. The influence of different auxotrophic markers on growth rates of yeast strains has been reported before (42) and most likely, the use of different auxotrophic markers between strains NW8 and NW9 (*ura4+* marker) and the NW10 (*LEU2* marker) caused the difference in $\mu_{\text{max}}$. Finally, biomass and byproduct yields of all strains were comparable, indicating that the strains share comparable physiologies. Also the additional feeding of amino acids as well as fluconazole treatment did not influence the cellular physiology with respect to biomass yields and yields for the main metabolites glucose, ethanol and glycerol (Supplementary Table S6). Thus, the metabolic burden caused by the increased maltase secretion clearly observable from the proteome data was not reflected in the extracellular metabolites. These findings are apparently in contrast to a previous study of maltase overexpression (13). However, in the previous study, respiratory growth conditions were
applied, which results in highly efficient substrate utilization. Consequently, already small changes in biomass composition and increased precursor demand for maltase production can cause significant changes in substrate uptake rates. In the present study, we applied fermentative growth conditions, resulting in excessive substrate uptake at high rates. This might somewhat buffer the direct effects of increased maltase secretion on biomass and metabolite yields as also observed for fermentative growth of recombinant protein secreting *S. cerevisiae* (43).

### Comparative proteome analysis

**Assessment of analytical strategy**

An isobaric tag labeling strategy combined with a 2D-LC-MALDI-TOF/TOF platform was applied in this study. One of the unique features of this analytical approach is the independent operation of the three key steps chromatographic separation, MS and MS/MS acquisition. Another advantage is the possibility to archive the sample plate, and to reanalyze the samples using data-driven, optimized MS/MS parameters on the basis of prior experiments. As a result, identification of minor species can be significantly improved without further sample consumption. However, compared to LC-ESI MS, LC-MALDI MS based proteomics studies are characterized by significantly longer measurement times. Therefore, an efficient reduction of LC-MS runs in two dimensional separation schemes becomes mandatory. Together with a prescreening procedure (44) the reduction of fractions to be measured in second dimension LC bears significant potential for improvement. We used a 2D-LC separation encompassing micro-RP-LC at pH 10 in the first followed by nano-IP-RP-LC at pH 2 in the second dimension. Two-minute wide fractions from first dimension were pooled by combination of every fourth fraction, finally yielding only four pools to be measured in second dimension LC-MALDI MS. This pooling scheme not only reduces the number of samples but also combines 1st dimension fractions containing peptides with different polarities; therefore, the chromatographic space in second dimension separation can be efficiently used.
A “global internal standard” was generated by combining equal amounts of the three samples to generate a master channel which was labeled with the fourth iTRAQ reagent. This GIS channel served as an internal technical replicate for each of the multiplexed samples within an iTRAQ labeling experiment. This approach is particular advantageous when a limited number of biological samples are available and determination of biological variations or estimation of experimental errors via an internal standard in a single experiment is desired.

In this study we employed the strict requirement of the presence of quantification data in all three experiments for consideration as a statistically valid quantification result. However, this criterion results in the exclusion of proteins yielding high quality quantitative information in only two out of three experiments. In this respect, assessment of the data, together with a careful visual inspection of the raw spectra, delivered of a small number (NW9: 18, NW10: 17) of additional proteins. However, of these proteins we only took into consideration Erg25p, a key enzyme in ergosterol metabolism, as other significantly quantified proteins (e.g. Lsd90p and SAP4H3.08) revealed potential perturbation of the lipid metabolism in the different strains. Erg25p presented with more than one quantifiable peptide in two out of the three experiments, however, was excluded due to the presence of only a single quantifiable peptide in the third experiment. If included, this protein is found to be significantly increased in abundance in both the NW9 and NW10 strains.

Changes in protein abundance as reaction on increased maltase production

In the maltase producing strain NW10, a total of 47 proteins were identified as significantly altered in abundance compared to the wildtype NW8 strain (31 increased and 16 decreased), while in the maltase producing strain NW9 fewer changes were observed (22 increased, 9 decreased) with thresholds of 1.42- and 0.704-fold, respectively. Furthermore, the abundances of more than 181 other proteins were differentially affected in the maltase producing strains at lesser, but still statistically significant (p<0.05) threshold changes of 1.25- and 0.8-fold.
A large number of differentially abundant proteins were found to be associated with pathways involved in (i) the central carbon metabolism, with relevance for energy production, the production of biosynthetic reduction equivalents (NADPH), and precursors for biosynthetic pathways, (ii) the biosynthesis of precursors for protein synthesis, in particular amino acid and transfer-RNA biosynthesis and components of the cell membrane, and (iii) the processing and secretion of proteins.

(a) Central carbon metabolism and biosynthesis of precursors and amino acids

Even though there was no significant difference observable regarding the extracellular strain physiology (Supplementary Table S6) increased protein production caused a reduction of protein levels for several key enzymes of glycolysis, PPP and TCA cycle. This phenomenon was unexpected, since increased protein production will demand more ATP and reduction power in the form of NADPH. While proteome data and actual metabolic activities were well in agreement in a study using recombinant \textit{P. pastoris} (45), this is not generally true for \textit{S. cerevisiae} (46-47). However, a reduction of TCA cycle fluxes has been described for a human superoxide dismutase producing \textit{S. cerevisiae} strain (48). For \textit{S. pombe} strains NW8 and NW9 a reduction of glycolytic and TCA cycle fluxes has been recently demonstrated and was attributed to an increased precursor demand and increasing efficiency of substrate utilization due to high level protein production (13). One major difference was observed between the previous study and the present findings: the abundance decrease of glucose-6-phosphate dehydrogenase and fructose-1,6-bisphosphatase as well as the increase in glucose-6-phosphate isomerase here suggest a switch in the split ratio between PPP and glycolysis towards the latter, while elevated protein secretion resulted in an increase of PPP fluxes in the former study (13). This difference might however be also due the domination of regulation effects on the metabolite level, e.g. concerning the oxidative branch of the PPP and to the different cultivation conditions applied in both studies (fermentative vs. respiratory growth conditions): while \textit{S. pombe} shows extensive carbon cycling through the PPP under respiratory growth conditions, fermentative growth is in general associated with minimal PPP fluxes in Crabtree-positive yeasts (49). Thus, it is reasonable
to assume that expression levels of enzymes involved in the central carbon metabolism and metabolic flux distributions in general correlate well in S. pombe.

In summary, the decreased expression levels of key enzymes of main catabolic pathways together with the observed reduction of expression levels for proteins involved in energy formation strongly suggests a limitation of maltase formation due to precursor and energy availability. Targeted amino acid feeding was one strategy to overcome this limitation by reducing the metabolic burden of precursor supply.

Increased abundance of proteins involved in the biosynthesis of the amino acids proline asparagine, lysine and histidine as well as branched chain amino acids (Val, Ile) and aromatic amino acids (Phe, Trp), in addition to scavenging pathways for methionine were observed. Feeding of the above mentioned amino acids, increased the protein secretion by a factor of 1.5. In contrast, feeding of 8 non-increased amino acids did only slightly improve maltase secretion. Addition of all 20 proteinogenic amino acids to minimal media has been shown in earlier studies to improve protein production and secretion in P. pastoris (50) and S. cerevisiae (51) and improved secretion of human transferrin produced in S. pombe (11). As expected, feeding of all 20 amino acids resulted in a comparable increase in maltase as targeted feeding. However, feeding of only the selected amino acids was sufficient to increase protein production to a comparable level, thus decreasing fermentation costs by decreasing the amount of supplemented amino acids.

We could show that proteome analysis is a powerful tool to identify those amino acids which are actually limiting protein production. Determination of the average amino acid composition of the cellular protein showed that, most of these amino acids appeared with higher abundance in the heterologously expressed maltase than in the cellular protein. Exceptions were histidine and lysine, which were not significantly overrepresented in maltase. Thus, proteome analysis is also useful to determine the limitations of precursors, which are not necessarily obvious from straightforward biomass analyses.
(b) Influence of membrane composition

Membrane composition, and in particular its fluidity, are known as critical parameters for the secretion of proteins. Our quantitative proteome study revealed significantly changed abundances of enzymes involved in sterol biosynthesis and lipid metabolism, which led us to the conclusion that membrane composition is a worthwhile target for manipulating protein secretion of *S. pombe*. Besides genetic engineering of the cell, membrane composition can be altered by addition of suitable detergents that directly affect the membrane or addition of compounds that influence the production of membrane compounds, e.g., ergosterol biosynthesis. Manipulation of cellular membrane by addition of surfactants has been shown to improve protein secretion in *S. pombe* (11). The decrease of ergosterol levels by addition of the fungicide fluconazole has been shown to enhance protein secretion in *P. pastoris* (45). Addition of fluconazole to *S. pombe* NW10 decreased ergosterol levels by more than 30% while at the same time improving maltase secretion 1.3-fold. We hypothesize that increased membrane fluidity might allow for easier vesicle formation and fusion, which might improve intracellular transport of maltase through the secretory pathway. For *S. cerevisiae*, improving vesicle formation resulted in improved secretion of α-amylase (52).

Conclusively, the highest yields of secreted maltase were obtained using media containing a combination of the amino acid and supplemented with fluconazole. Maltase secretion was increased by a factor of 2.2, showing the synergistic effect of a combination of the targets identified by quantitative proteome data.

(c) Protein biosynthesis, processing and secretion.

The increasing expression and secretion of maltase in the new strains NW9 and NW10 was reflected in the observation of higher amounts of proteins involved in translation. Here, in particular proteins involved in translation initiation and in formation of ribosomes were significantly increased in the NW10 strain. Two of the most increased proteins belonged to the 40S ribosomal subunit. A perturbation of the ratio of ribosomal subunits of 60S to 40S from 1:1 to 2:1 has been reported for recombinant membrane protein expressing strains of *S. cerevisiae* (53). Thus the increased expression of the *agl1* gene in NW10 might cause a comparable perturbation of this ratio in *S. pombe*. The
observed increase in expression of ribosomal 40S subunit proteins may be an attempt of the cell to counteract this perturbation. A further observed response of the cell to control maltase translation levels in the presence of high amounts of mRNA transcripts was an increase in proteins involved in mRNA degradation in both maltase-overexpressing strains. For filamentous fungi, mechanisms for mRNA degradation upon secretory stress have been described (54), but to our knowledge this is the first time that such a mechanism has been proposed for yeast.

Up-regulated genes further included proteins involved in protein folding and transport of secretory vesicles, e.g. of Ssa2p, a protein of the Hsp70-family. These proteins are chaperones located in the endoplasmic reticulum and increased levels of these have been shown to improve secretion of several recombinant proteins in *S. cerevisiae* (55). Also proteins related to vesicular transport significantly increased, e.g. Sec14p, a protein involved in phospholipid transfer. Sec14p proteins have been shown to stimulate the secretory function of the Golgi apparatus (56). Tyo et al. could show that engineering the vesicular transport machinery can efficiently increase recombinant protein secretion in *S. cerevisiae* (43). These examples show that the changes in protein levels we observed are a response to the increased burden of protein secretion posed on the cells and that engineering approaches targeting these protein families seem generally applicable since it has already been shown to improve protein secretion in yeast and other eukaryotic organisms (52, 57).

However, the proteomic study also yielded a number of novel targets which have not been described yet. These included a number of proteins involved in vesicular transport, e.g. Cle1p, a clathrin-light chain protein. Tyo *et al.* showed an up-regulation of the vesicular transport by increased amounts of Sly1p and Sec1p, two proteins involved in membrane fusion between transport vesicles and the respective organelle (43). However, clathrins are directly part of these transport vesicles and recycled in the process of vesicle formation and breakdown. Due to this, they have not yet been considered as interesting targets for improving protein secretion. In this context we observed also significant increase in abundance of proteins involved in cytoskeleton formation, especially of the actin cytoskeleton as well as of proteins involved in transmembrane transport. It appears that, at least in *S. pombe*, not only proper folding of secreted proteins but especially intracellular transport and
exocytosis can have a limiting influence on protein secretion and the identified proteins may represent interesting targets for genetic engineering of this yeast in the future. We observed alterations in proteins involved in vitamin biology. The underlying reasons are difficult to determine, since these vitamins are involved in such diverse reactions as central carbon metabolism, amino acid biosyntheses and lipid metabolism. However, since these metabolic pathways were strongly influenced by elevated maltase expression, one can assume that also the corresponding expression levels of important cofactors can also be affected.

The analysis of the supernatants of the cultivations of the three strains in non-supplemented media revealed the presence of several hundred other proteins; however, the majority of these proteins were present in low amount according to the weak signal intensities and peptide spectrum matches observed. The by far most abundant protein secreted in both NW9 and in particular NW10 was the target protein maltase. The addition of fluconazole and amino acids to the cultivation medium for NW10 did not significantly increase the release of proteins compared to the additive free medium.

Conclusively, we could show that the use of quantitative proteomics can provide useful information about potential targets for strain manipulation and in particular the composition of reaction media, aiming for increased protein production and secretion. However, it has to be noted, that the protein abundance alone represents only one level of regulation in cellular networks. Other levels need to be taken into account in future investigations, potentially opening the path for further improvements in protein production are the combination of proteomics data with metabolome and fluxome data as well as taking into account the regulation of protein activities, e.g. by posttranslational modifications.
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References


Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J Biol Chem* 278, 45011-45020


Figures

Figure 1. Experimental design. (A) Workflow of iTRAQ labeling for quantitative comparison of cell lysate proteins extracted from three different strains of *S. pombe*. Proteins extracted from NW8, NW9 and NW10 were reduced, alkylated, and digested with trypsin. A reference channel (Mix) was generated by combing ¼ of tryptic peptides from NW8, NW9, and NW10. The four samples were labeled with 4-plex iTRAQ reagents separately. The pooled peptide mixture was first separated on an RP-microHPLC column at pH 10, followed by a second dimension IP-RP-nanoHPLC separation at pH 2. The separated peptides were analyzed by MALDI-MS/MS. (B) Strategy for performing iTRAQ ratio comparison among three strains of *S. pombe*. The iTRAQ ratio R(115/114), R(116/114), and R(117/114) represents the normalized protein ratio of NW8, NW9, and NW10, respectively. An averaged ratio R_{avg}(NW8), R_{avg}(NW9), and R_{avg}(NW10) calculated from three biological replicates was applied to compare the relative fold-change between NW8/NW9 (R[NW8 v 9]) and NW8/NW10 (R[NW8 v 10]). SEM denotes standard error mean calculated from three iTRAQ experiments.
Figure 2. Statistical versus biological relevance. (A) Volcano plot of all 1,041 quantified proteins from the NW8 versus NW9 dataset. The averaged protein ratio ($\log_{2}(R[\text{NW8 v 9}])$, x-axis) was plotted against the $p$-value ($-\log_{10}[p\text{-value, } t\text{-test}], y\text{-axis}$). (B) The same plot was employed to display the dataset of NW8 versus NW10. (C) The analysis of detected changes (detections, y-axis, right) and the calculated global FDR (y-axis, left) over the full range of NW8 v 9 $\log_{10}(p\text{-values})$. (D) The same plot was employed to display the NW8 v 10 $\log_{10}(p\text{-values})$. (E) From the 1,041 proteins quantified, 52 protein ratios from both NW8 v 9, and NW8 v 10, showed significant differential abundances (FDR $\leq 5\%$, $p$-value $\leq 0.0155$ and $\leq 0.0165$, respectively). Among them, 22 and 31 proteins showed a $\geq 1.42$-fold change (red dots in A and B), and 9 and 16 showed a $\leq 0.704$-fold change (blue dots) for NW8 v 9 and NW8 v 10, respectively. Under the less stringent criteria ($p$-value < 0.05) a further 56 and 66 proteins with a fold change $> 1.25$ (pink dots), and 19 and 40 proteins with a fold change $< 0.8$ (cyan dots) were observed while the remaining 935 and 888 proteins did not show any significant difference in expression levels (gray dots) in the NW8 v 9 and NW8 v 10 analyses, respectively.
Figure 3. Simplified central carbon and amino acid metabolic network. Proteins from pathways involved in glycolysis, pentose phosphate pathway, TCA cycle and amino acid metabolism identified as differentially abundant. Enzymes found to be significantly higher abundant (R ≥ 1.42, NW8 v 9 p ≤ 0.0155; NW8 v 10 p ≤ 0.0165) are labeled in red; higher abundant proteins (1.25 < R < 1.42, NW8 v 9 p ≤ 0.0155; NW8 v 10 p ≤ 0.0165 < p < 0.05) are labeled in pink; significantly lower abundant proteins (R ≤ 0.704, NW8 v 9 p ≤ 0.0155; NW8 v 10 p ≤ 0.0165) are labeled in blue, and the lower abundant proteins (0.704 < R < 0.8, NW8 v 9 p ≤ 0.0155; NW8 v 10 p ≤ 0.0165 < p < 0.05) are labeled in cyan. Proteins unchanged are labeled in gray (=). Left arrows/=: low maltase expression strain NW9 versus parental strain NW8 (NW8 v 9); right arrows/=: high maltase expression strain NW10 versus parental strain NW8 (NW8 v 10). n.d. denotes not identified proteins.
Figure 4. Correlation of proteins fold change identified as differentially abundant in NW9 and NW10 strains. Scatter plot depicting the protein fold change correlations between NW9 and NW10 strain related to central carbon metabolism (upper panel) and other metabolic pathways (lower panel). Significantly up- or down-regulated proteins are marked with asterisks (*).
Figure 5. Correlation of proteins fold change identified as differentially abundant in NW9 and NW10 strains. Scatter plot depicting the protein fold change correlations between NW9 and NW10 strain in related to protein synthesis (upper panel) and to protein degradations (lower panel). Significantly up- or down-regulated proteins were marked with asterisks (*).