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Following nature's roadmap

Published in: Biotechnology Journal

DOI: 10.1002/biot.201600631

Published: 01/08/2017

Document Version Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

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Please cite the original version:

Koskela, E. V., de Ruijter, J. C., & Frey, A. D. (2017). Following nature's roadmap: folding factors from plasma cells led to improvements in antibody secretion in S. cerevisiae. *Biotechnology Journal*, *12*(8), Article 1600631. https://doi.org/10.1002/biot.201600631

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1	Research Article
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3	Following nature's roadmap: folding factors from plasma cells led to improvements in
4	antibody secretion in <i>S. cerevisiae</i>
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19	Keywords: Synthetic biology, yeast, antibodies, protein folding
20	
21	Abbreviations: PDI, protein disulfide isomerase; PPIase, peptidyl-prolyl cis-trans isomerase
22	

1 Abstract

2 Therapeutic protein production in yeast is a reality in industry with an untapped potential to expand 3 to more complex proteins, such as full-length antibodies. Despite multiple numerous engineering 4 approaches, cellular limitations are preventing the use of Saccharomyces cerevisiae as the titers of 5 recombinant antibodies are currently not competitive. Instead of a host specific approach, we 6 demonstrate the possibility of adopting the features from native producers of antibodies, plasma cells, to improve antibody production in yeast. We selected a subset of mammalian folding factors 7 8 upregulated in plasma cells for expression in yeast and screened for beneficial effects on antibody 9 secretion using a high-throughput ELISA platform. Co-expression of the mammalian chaperone 10 BiP, the co-chaperone GRP170, or the peptidyl-prolyl isomerase FKBP2, with the antibody 11 improved specific product yields up to two-fold. By comparing strains expressing FKBP2 or the 12 yeast PPIase Cpr5p, we demonstrate that speeding up peptidyl-prolyl isomerization by 13 upregulation of catalyzing enzymes is a key factor to improve antibody titers in yeast. Our findings 14 show that following the route of plasma cells can improve product titers and contribute to 15 developing an alternative yeast-based antibody factory.

1 **1 Introduction**

2 Antibodies are sophisticated agents of the mammalian immune system to fight against foreign 3 pathogens and neoplastic tissue. In addition to their established use in diagnostics and research, 4 antibodies, and especially IgG molecules, are used as advanced human therapeutics [1]. One of the 5 main limitations in their use as a treatment is their high cost of production, as therapeutic antibodies 6 are most often expressed in mammalian cell lines such as CHO cells. Due to ease of manipulation 7 and cultivation, also lower eukarvotes such as yeasts have been considered as an alternative for 8 production of recombinant antibodies [2–4]. However, obtaining the target glycoform of the 9 antibody is crucial for the therapeutic and clinical efficacy. Yeasts typically produce 10 hypermannosylated glycoproteins, which is a clear hindrance for therapeutic glycoprotein 11 production for human use. Although glycoengineering of yeasts has greatly advanced during recent 12 years [5–7], glycan structures are not the only hurdle for obtaining significant yields of full-length 13 IgG molecules for therapeutic applications. Especially in the case of Saccharomyces cerevisiae 14 product titers have remained modest, for example due to inefficient folding or proteolytic 15 processing [8-10]. Despite the fact that commercial production of therapeutic proteins such as 16 insulin in S. cerevisiae is practiced [11], transforming yeast cells into an antibody factory requires 17 extensive cellular engineering to improve target protein yields.

18

The roadmap for antibody production with high efficiency is already explicated by nature: studies of how the immune system's B cells differentiate into antibody-secreting plasma cells provide a comprehensive blueprint of the factors needed to equip the cell for complex protein production. The most striking feature of these professional secretors is their extensive endoplasmic reticulum (ER), which fills most of the cytoplasm. Accordingly, many of the biochemical activities of the ER are upregulated during differentiation, including the abundance of folding factors, such as chaperones, co-chaperones, protein disulfide isomerases (PDIs), and peptidyl-prolyl isomerases (PPIases) [12–14]. Overexpression of folding factors, most notably PDI and the chaperone BiP, have been studied in recombinant protein production for over two decades in different expression systems amongst others including yeasts, insect cells and mammalian cells [15–19]. In the case of antibody production in *S. cerevisiae*, overexpressing PDI (Pdi1p) and BiP (Kar2p), and ER engineering have been effective strategies in several occasions [8, 9, 20].

8

9 Previously, we demonstrated that following the example of plasma cells by increasing the size of 10 ER prior to protein expression, through deletion of the lipid regulator gene OPII, is beneficial for 11 antibody production in S. cerevisiae [9]. Mimicking steps in B cell differentiation has been 12 proposed as an engineering strategy for mammalian cell platforms and interestingly, in yeast the 13 strategy has been exploited in expanding antibody diversity [21, 22]. In this study, we further 14 explore the use of plasma cells as a blueprint for the development of a S. cerevisiae antibody 15 factory by co-expressing a comprehensive set of mammalian derived ER folding factors together 16 with a full-length IgG in yeast. We screened constructs for expression of ten folding factors shown 17 to interact with nascent antibodies in their endogenous environment and identified elements 18 beneficial for improving IgG titers in yeast. We show that the improvements of the engineered 19 strains were specific for recombinant mammalian proteins, as the effects were similar with human 20 erythropoietin but absent in expression of the endogenous yeast acid phosphatase. Although the 21 yeast PPIase Cpr5p expressing strain generated the highest antibody titers, we demonstrate that 22 mammalian folding factors can successfully be used in engineering of folding in yeast.

1 2 Materials and methods

Enzymes used for DNA amplification and manipulation were purchased from Thermo Fisher
Scientific (Vantaa, Finland). Media components and reagents were obtained from Sigma-Aldrich
(Helsinki, Finland) unless stated otherwise.

5 2.1 Yeast strains

As described in detail in [9], two yeast strains served as the basic chassis in all screening
experiments: W303α and W303αΔ*opi1*, from here on referred to as wt and Δ*opi1*, respectively.
Both strains harbor a single copy of the light and heavy chain encoding genes of the monoclonal
antibody C2B8 under control of the GAL1-promoter integrated to the genomic *HIS3*-site.

10 The acid phosphatase gene (PHO5) and cDNA for human EPO (Source Bioscience, clone 11 OHu20340) were cloned under P_{GAL1} into the integrative pRS305K-plasmid [23]. P_{GPD}-PHO5-12 cycl cassette from plasmid pAX391 [24] was inserted between SmaI and BssHII sites of the 13 pRS305K-backbone creating plasmid pAX401. P_{GPD} was replaced with a PCR amplified P_{GAL1} 14 promoter (oligonucleotides 28 and 29, Supporting information, Table S1) using exonuclease and 15 ligation independent cloning (ELIC) after BamHI SpeI digestion of the vector [25]. The mata-16 leader sequence (Mata) EPO fusion was assembled in two consecutive steps in plasmid pSL1180. 17 PCR amplified Matα (oligonucleotides 32 and 33) and EPO (oligonucleotides 30 and 31) were 18 digested with BamHI NheI and NheI HindIII, respectively and inserted into pSL1180. The Mata-19 EPO fusion was excised and inserted into BamHI HindIII sites of pRS426-GAL1 [26]. The P_{GAL1}-20 EPO expression cassette was amplified with oligonucleotides 27 and 28 and inserted into BamHI 21 and XhoI digested pAX401 with ELIC, replacing the promoter and ORF before the CYC1-22 terminator. The resulting plasmids were linearized with either Eco31I or AatII and introduced into 23 the *LEU2* locus of both wt and Δopi .

1 2.2 Cloning the plasmid library

cDNAs enconding human ERdj3, mouse GRP94, and human GRP170 were obtained from Dr.
Linda M. Hendershot, while human ERp57 cDNA was received from Dr. Kari Kivirikko. Dr.
Lloyd Ruddock provided coding regions for human FKBP2, cyclophilin B, P5 L20-L440 and
ERp72. Human PDI cDNA was from Dr. Neil Bulleid. cDNA for human BiP was obtained from
Source Bioscience (IMAGE clone 5020098). The used oligonucleotides are presented in
Supporting information, Table S1.

8 The selected cDNAs were cloned into the low-copy number yeast shuttle vectors pRS415 and 9 pRS416 [26] under GAL1 (P_{GAL1}), GPD (P_{GPD}), and TEF (P_{TEF}) promoters and under the UPR-10 controlled promoters derived from the *PDI1* (P_{PDI1}) and *KAR2* (P_{KAR2}) genes, as described in [9]. 11 Native ER targeting signals were used for all genes except P5, which had the leader peptide from 12 yeast PDI [25]. Oligonucleotides 1 and 2 were used to amplify cDNA for human BiP replacing the 13 human ER retrieval sequence with the yeast sequence. Vectors and insert were both digested with 14 SpeI and XhoI and T4 ligase was used for fragment ligations generating plasmids with BiP under 15 control of P_{GAL1}, P_{GPD}, and P_{TEF}. The ERdj3 insert was amplified with oligonucleotides 3 and 4 16 so that the fragment ends contained Eco31I sites. The oligonucleotides were designed so that after 17 digestion of the insert with Eco311 the sticky ends became compatible with SpeI and XhoI sites, 18 which were used to cut the vectors.

The cloning of P5 and GRP94 to pRS41X-vectors is described in [25]. Other cDNAs were cloned into pRS41X-vectors using ELIC [25]: the cDNAs were amplified in the first round of PCR with gene specific oligonucleotides. An adaptor sequence of 16 bp was added to the 5' end. The yeast specific ER retrieval signal (HDEL) was either mutated in the original gene sequence (GRP94, PDI, GRP170, P5, BiP), or inserted at the C-terminal end of the genes (ERdj3, ERp57, ERp72,

1 FKBP2, CvpB). If HDEL resulted from mutation, the first round of PCR introduced a 17 bp 2 adaptor sequence to the 3' end. In case of HDEL insertion, an additional round of PCR was used 3 to add the 3' adaptor sequence by using gene specific 5' oligonucleotide and oligonucleotide 4 number 19 to add the adaptor sequence to 3' end. The last round of PCR introduced complementary 5 ends to the plasmids (adaptor specific oligonucleotides number 20 and 21-25). BiP cDNA was 6 introduced under P_{KAR2} and P_{PDI} by amplifying the gene with oligonucleotides 26 and 27 thus 7 adding the adaptor sequence only to the 5' end. All amplified coding sequences were cloned into 8 linearized vectors with ELIC [25]. The destination vectors were linearized with SpeI and XhoI as 9 above, except for plasmids containing P_{KAR2} where XbaI and XhoI were used. Plasmids were 10 transformed into the yeast strains using the lithium-acetate method [27]. Control strains contained 11 only the empty vectors to complement the auxotrophies.

12 2.3 Screening process and cultivation of yeast strains

13 Screening the yeast strains was conducted in deep-well plate format as outlined in [9]. Briefly, the 14 saturated pre-cultures of OD₆₀₀ between 5 and 10 were diluted 1:4 in fresh SD-URA-LEU media, 15 which contained 2 % raffinose, and grown at 30 °C, 250 rpm for 5.5 hours before protein 16 expression was induced by adding galactose to a final concentration of 0.5 or 2 %. Protein 17 expression was continued for 24 hours at 20, 25, or 30 °C, 250 rpm. OD₆₀₀ was measured with an 18 Eon Microplate Spectrophotometer (BioTek, Winooski, USA). Samples for ELISA were clarified 19 by centrifugation, adjusted to 1xPBT (135 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.75 mM 20 KH₂PO₄ with 0.05 % Tween-20) and stored at -20 °C until analysis. Each strain was cultivated in 21 at least three biological replicates.

Cultivations of EPO and acid phosphatase expressing strains were conducted as described for the
 screening process but protein expression was performed only at 30 °C, 250 rpm. Samples were

prepared from three biological replicates, using two technical replicates for each. Acid phosphatase
 activity assay was conducted immediately after the cultivations. Culture supernatants of EPO
 cultures were clarified by centrifugation and stored at -20 °C until analysis.

4 2.4 Quantification of IgG and EPO titers and acid phosphatase activity

Antibody titers were determined in duplicate measurements with ELISA as specified in [28]. Data
was analyzed by using Gen5 (BioTek Winooski, USA) and R software. Statistical analyses were
conducted as described in [9].

8 The acid phosphatase assay was conducted as described in [24], with the exception that the assay 9 was done at 22 °C. The substrate, 20 mM para-nitrophenyl- phosphate in sodium acetate buffer pH 10 4.2, was added to start the reaction. Reaction was terminated after 0, 2, 10, and 20 minutes and 11 absorbance was read at 405 nm with BioTek Synergy 2 spectrophotometer. Relative acid 12 phosphatase activity was determined from the colorimetric product formation as the slope of 13 absorbance versus time. Concentration of EPO was determined with a commercial ELISA-kit 14 (RAB0654, Sigma Aldrich) following the manufacturer's instructions. Absorbance at 450 nm was 15 read with BioTek Synergy 2 spectrophotometer.

16 **2.5 Shake flask cultivations**

Strains were inoculated to 5 ml SD-URA-LEU with 2 % glucose and grown overnight at 30 °C,
Strains were inoculated to 5 ml SD-URA-LEU with 2 % glucose and grown overnight at 30 °C,
The OD₆₀₀ 5-10. Ten OD₆₀₀ of cells were collected by centrifugation at 3,000 g for 5
minutes and the cell pellet was resuspended in 10 ml of expression media (SD-URA-LEU with 2
% galactose) obtaining a starting OD₆₀₀ of 1.0. Strains were cultivated in 250 ml shake flasks in
inducing conditions at 30 °C, 200 rpm for 24 hours and samples were collected at time points 0, 6,
12, and 24 hours. Supernatant samples were collected as follows: 1 or 2 ml was taken from culture,
cells were spun down and the supernatant was adjusted to 1xPBT and stored at -20 °C until

analysis. Resulting cell pellets were resuspended in the same volume of fresh, prewarmed media and returned to the expression culture. Sample collection resulted in culture media replacement at a rate of 1 ml per six hours. OD_{600} was measured with an eppendorf Biophotometer plus – spectrophotometer (Eppendorf AG, Hamburg, Germany). IgG titers were measured in 2-3 technical replicates as described in [28]. All shake flask cultures were repeated with three biological replicates. To account for the dilution due to addition of fresh media, averaged volumetric productivity P_{avg} (ngml⁻¹h⁻¹) was defined as

8

$$P_{avg} = \frac{[AB]_2 V - [AB]_1 (V - V_s)}{V(t_2 - t_1)}$$

9

10 Where $[AB]_2$ is the measured antibody titer, $[AB]_1$ is the antibody titer of the previous time point, 11 *V* is the culture volume, V_s is the sample volume taken in the previous time point and t_2 and t_1 are 12 the time points in hours.

1 **3 Results**

3.1 Selected mammalian folding factors were overexpressed in two chassis strains and showed an increase in specific product yields with increasing temperature

4 Guided by studies on plasma cell differentiation and IgG folding, we selected a subset of ER 5 resident folding factors known to be involved in IgG synthesis and folding [29]. We selected two 6 PPIases, FKBP2 and cyclophilin B (CypB), four PDIs (P5, ERp57, ERp72, and PDI), the 7 chaperones BiP and GRP94, and the co-chaperones of BiP, ERdj3 and GRP170. GRP170 has also 8 been reported to have hold as activity, as it binds unfolded proteins directly [30]. All the selected 9 genes are significantly upregulated both at protein and transcript level after four days of B cell 10 differentiation (Supporting information, Table S3), as we concluded from our own analysis of 11 microarray data published in [31] and proteomics data reported in [14]. To be able to adjust 12 expression levels of the folding factors, the selected mammalian cDNAs were expressed under 13 control of five different promoters. In order to allow co-expression, the expression constructs were 14 cloned into plasmids with LEU2 and URA3 selection markers resulting in 65 different plasmids 15 (Supporting information, Table S2).

16 The plasmids were transformed individually and in combinations into two different IgG producing 17 strains, the nonengineered wt and the $\Delta opil$ strain, containing the deletion of the OPII-gene that 18 results in an enlarged ER and significantly higher IgG secretion [9, 32]. To determine the specific 19 product yields of our strains, we used a previously established screening platform described in [9]. 20 We cultivated all strains at 20, 25, and 30 °C using 0.5% and 2.0% galactose for induction. Specific 21 product yields (IgG titer normalized by cell density) increased with temperature while the wt and 22 *Aopi1* strains remained as two distinct populations (Supporting information, Fig. S1). Generally, 23 IgG titers and specific product yields were higher with 0.5 % than with 2 % induction, although the difference in specific product yields was smaller than in titers between the two induction levels
 (Supporting information, Table S4, S5).

3.2 PPIases and the chaperone BiP with its co-chaperone GRP170 were the most successful folding factors to improve specific product yields

5 In order to identify constructs capable of improving specific product yields, we took the overall 6 performance of the strains with both induction levels into account. By first comparing the strains 7 to the respective background strain within a condition (Supporting information, Table S4), we 8 determined the mean fold-change in specific product yield for each construct, as shown in Fig. 9 1A. The corresponding IgG titer distributions are shown in Fig. 1B for wt and in Fig. 1C for 10 $\Delta opil$ strains. In most cases, expression of folding factors resulted in relatively minor effects, 11 and did not change the specific product yields considerably (Fig. 1A). Neither the different 12 molecular functions of proteins nor the promoters allowed separating the tested strains into 13 different groups, indicating that the effect of overexpression of different folding factors remained 14 largely unpredictable. Based on the results from the screening approach, we identified the 15 PPIases FKBP2 and CypB, BiP and its co-chaperone GRP170 as the most interesting candidates, 16 which improved specific product yields up to 1.6-fold. Interestingly, the strains with the co-17 chaperone performed slightly better than those with BiP (Fig. 1A). In the *Aopil* strains, the UPR-18 controlled promoters P_{KAR2} and P_{PDII} seemed to produce the best level of chaperone expression 19 (Fig. 1A, right panel). This is in line with our previous observation that these promoters are 20 useful modules in adjusting overexpression of chaperones [9]. In contrast, stronger promoters 21 were responsible for the highest fold-changes in wt strains (Fig. 1A, left panel), demonstrating 22 that a different level and pattern of expression was required in these two strain backgrounds.

Although the highest fold changes were observed at 25 °C, the highest IgG titers were observed
 at 30 °C (Supporting information, Table S4).

The results in Figure 1 show one clear deviation: the wt strain expressing P5 under control of P_{GAL1} performed extraordinarily well in antibody secretion. All the measured specific product yields of this strain were above 1.5 –fold, in lower temperatures up to 3.6 –fold (Fig. 1A, Supporting information, Table S4). This strain also had the highest mean IgG titer from all wt samples (Supporting information, Table S4).

8

9 3.3 Co-overexpression of two folding factors revealed limited benefits of several 10 modifications to ER and folding

11 Based on the reported interactions of the different folding factors [33] depicted in Fig. 2A, we 12 tested all the shown pairs of genes and determined their effect on antibody titers (Fig. 2B). We 13 selected one or two promoters for each folding factor based on the screening results obtained with 14 the single construct cultivations. In total, we tested 35 different combinations (Supporting 15 information, Table S5). For the wt strains, we observed a small shift towards higher antibody titers 16 upon co-expression of two folding factors (Fig. 2B lower panel compared to Fig. 1B). In contrast, 17 $\Delta opil$ strains co-expressing two folding factors hardly exceeded the improvement obtained by the 18 OPI1-deletion alone (Fig. 2B). Generally, the overexpression of a second folding factor in a strain 19 that had already displayed high specific product yield did not result in an additional benefit.

The best strains of the two screening sets are summarized in Table 1, which shows the highest overall specific product yields from both strain backgrounds. In the best producing $\Delta opil$ strains, all folding factors were expressed under control of P_{KAR2} and P_{PDI1}. Combined with the *OPI1*gene deletion, GRP170 overexpression led up to 6-fold higher specific product yield compared to

1 the wt (Table 1). Although the improvement in specific product yield was smaller, the $\Delta opil$ strains 2 expressing FKBP2 or BiP under P_{PDI} reached comparable titers to GRP170 overexpression strain, 3 being slightly over 100 μ g/l (Table 1). None of the strains with simultaneous overexpression of 4 two folding factors was among the ten best strains in the $\Delta opil$ background. In contrast, four of 5 such pairs in the wt background are listed in Table 1. In addition, the folding factors improving 6 specific product yields in $\Delta opil$ background were noted to be more effective when expressed under 7 stronger promoters in wt (Table 1). It is possible that deletion of OPII gene already enables the 8 cell to circumvent major portion of the folding bottleneck, whereas the demand for improvement 9 in wt strains is larger and requires a greater amount of folding factors.

10

3.4 Folding factor overexpression affected only mammalian protein secretion

Our main assumption was that the folding factors would improve secreted antibody titers through increasing the folding rate of IgG in the yeast ER, but other cellular effects of folding factor overexpression cannot be excluded. In the case of BiP, we investigated the interaction of this protein with intracellular antibody by co-immunoprecipitation, and confirmed that the antibody interacts with human BiP in the yeast cell (Supporting information, Fig. S2). For most other folding factors, we verified their co-expression in yeast and interestingly, their expression levels varied considerably even when expressed under the same promoter (Supporting information, Fig. S3).

To determine if the folding factors had a general influence on protein production, we tested for their possible effects on the secretion of two additional model proteins. We created strains with and without the *OPII*-gene deletion that had an overexpression cassette of the endogenous yeast protein, Pho5p, a secreted acid phosphatase, or of human erythropoietin (EPO), a heavily glycosylated cytokine. Acid phosphatase activities and EPO concentrations were recorded after

cultivation in the same conditions as for the IgG titer measurements at 30 °C (Table 1). Although 1 2 the OPII-gene deletion increased IgG titers significantly, it halved the amount of secreted acid 3 phosphatase activity in the supernatant (Fig. 3A). Some of the most interesting folding factor 4 constructs were tested together with Pho5p overexpression (P5, the GRP94/ERp72 pair, BiP, 5 GRP170, and FKPB2, constructs from strains 87, 306, 151, 142 and 124, see Table 1), but they 6 had little effect on Pho5p production (Fig. 3A). Moreover, in contrast to IgG, the differences in 7 induction strength had a clear impact on Pho5p activity; 2 % galactose at least doubled the activity 8 of Pho5p in the supernatant (Fig. 3A). Overall, the secretion patterns of acid phosphatase and IgG 9 were completely different in the strains tested. When testing expression of EPO, the improvements 10 in the production of this cytokine followed the same trends as observed for IgG secretion (Figure 11 3B). In addition to the titers shown in Figure 3B, also patterns in specific product yields were 12 comparable with IgG and EPO (data not shown). Overall, the effects of the beneficial 13 modifications identified here seemed to be specific only for the two mammalian glycoproteins 14 tested (Figure 3). Interestingly, increasing induction hampered EPO secretion more than measured 15 for IgG (Figure 3B). As secretion of Pho5p could simply be increased with stronger induction, this 16 demonstrates that the secretory pathway is more adapted for the expression of the endogenous 17 protein Pho5p than for IgG and EPO.

18 **3.5 Mammalian PPIase FKBP2 displayed similar improvements to the yeast PPIase CPR5**

We cultivated a selection of strains in shake flasks and followed the production over time to gain more insight into the secretion behavior of these strains. We chose strains expressing BiP, FKBP2, and GRP170 from the *Δopi1* strains (numbers 142, 124 and 151), and the strains expressing P5, and the GRP94/ERp72 pair (numbers 87, and 306) from the wt background, as these resulted in the highest improvements in specific product yield in the two backgrounds (Table 1). Secretion

1 phenotypes of these strains were confirmed in shake flask cultivations, as the IgG titers were 2 comparable to the screening cultures (Fig. 4A, 4B). We previously reported a yeast PPIase 3 construct, P_{GPD}-CPR5, which strongly increased specific antibody yields [9]. As we wanted to 4 compare the two approaches of having a native versus a heterologous folding enhancer, we 5 included this *Aopil* -based strain expressing the native ER localized PPIase in this experiment. Although the Cpr5p-overexpression strain reached the highest titer at 24 hours, the mammalian 6 7 PPIase FKBP2 expressing strain produced similar titers in a shorter time (Fig. 4B). When we 8 plotted averaged volumetric productivity against OD₆₀₀, we noticed that the increase in 9 productivity was accompanied with a shift to lower cell densities, especially in the $\Delta opil$ strains, 10 indicating that high titers were achieved at the expense of culture density (Fig. 4C, 4D). Thus, 11 although the increase in specific product yield was much greater with overexpression of Cpr5p 12 ([9]) than for FKBP2 (Table 1), the difference in IgG titer at 24 hours was relatively small.

13 Overexpression of folding factors seemed to improve volumetric productivity especially around 14 the midpoint of the cultivations (Fig. 4C, 4D). In the wt background, the folding factor expressions 15 actually decreased productivity during the first six hours (Fig. 4C). Afterwards, the cells seem to 16 adapt to the production phase, as the productivity strongly increases (Fig. 4C). Interestingly, in the 17 shake flasks, most of the strains reach their maximum IgG titer already at 12 hours after induction 18 (Fig. 4A, 4B). In accordance to other studies [10], protein degradation might be a prevalent 19 problem in our yeast system, as the productivity turned to negative values at the last time point 20 (Fig. 4C, 4D).

1 4 Discussion

2 Improving folding and secretion of recombinant proteins is a complicated task as the effects of 3 overexpressing folding factors are not straightforward to anticipate and dependent on the target 4 recombinant protein [18, 34]. In contrast to upregulating endogenous folding factors, in this study 5 we explored an alternative approach for folding engineering: we looked at the native environment 6 of the target protein and transferred the antibody-specific folding factors to the selected host. The 7 folding helpers we selected represent each of the different classes of folding factors that interact 8 with the folding heavy and light chain polypeptides [35]. Judging by the reported abundance of 9 interactions with the IgG molecule, human BiP would be the first choice to transfer as a folding 10 enhancer [29]. Although not as successful in shake flasks, co-expression of human BiP was 11 improving the specific product yields of IgG up to 1.5-fold (Table 1), more than overexpression 12 of the yeast equivalent Kar2p [9, 36]. The PPIase FKBP2 and the chaperone GRP170 were also 13 effective in improving titers, resulting in an up to 6-fold increase in specific product yields when 14 combined with the deletion of OPI1-gene (Table 1, Fig. 4B). GRP170 is a homolog of yeast co-15 chaperone Lhs1p, but the beneficial effects of GRP170 are most likely to rise from its activity to 16 bind unfolded proteins directly [30]. Although improvements were achieved, the antibody- and 17 plasma cell inspired engineering approach yielded similar results to the host-specific approach. In 18 both cases, the most striking observation was the criticality of the expression levels of the folding 19 factors (Figure 1A, [9]). Fine-tuning the expression levels with the selection of promoters did not 20 display much predictability, and the same promoter did not guarantee similar protein levels (Fig. 21 1A, Supporting information, Figure S3). In addition, the combination of folding factors were 22 generally not improving IgG titers to the same extent (Fig. 2B), probably due to the overload from

multiple protein overexpression. For future approaches of ER engineering, the selection of
 promoters should be broadened, mainly to weaker promoters.

3 PPIases present an intriguing class of factors to improve antibody folding, as specific peptidyl 4 prolyl-isomerizations have been reported to constitute the rate-limiting steps in immunoglobulin 5 fold formation and IgG tetramer assembly [37–39]. In fact, all antibody domains require at least 6 one cis configured proline residue [29]. Lilie et al. [37] reported that the isomerization of Pro159 7 might have been responsible for slow folding of a Fab fragment, which would correspond to 8 Pro157 in our heavy chain. This proline is located in the $C_{\rm H}$ 1 domain that is key in the control of 9 IgG assembly [39]. Under normal conditions, ER-specific PPIase activity in *S. cerevisiae* is barely 10 needed [40], so upregulating PPIase activity seems to be crucial for improving IgG titers in yeast. 11 The two mammalian PPIases CypB and FKBP2 were both successful in improving specific 12 product yields and IgG titers (Table 1, Figure 4), although not to the same extent as the yeast 13 PPIase Cpr5p (Figure 4B). It is possible that Cpr5p acts on several of the proline residues, while 14 it has been shown for CypB and FKBP2 that they have differential substrate specificity limited to 15 certain peptidyl-prolyl isomerizations of antibodies [37].

16 Although we demonstrated that at least BiP interacts with IgG in yeast (Supporting information, 17 Figure S2), the effects of the constructs might partially results from pleiotropic effects. For 18 example, the wt strain Y87 with P_{GAL}-P5 expression cassette was an outlier in our screening experiments (Figure 1A). P5 was the only folding factor that was expressed with a yeast-derived 19 20 leader peptide, and interestingly, the folding factor showed significant effects only when expressed 21 under P_{GAL1} . A possible scenario for a pleiotropic effect would be the overload of the translocation 22 machinery. Perhaps the yeast-derived leader peptide in combination with a strongly induced 23 promoter resulted in P5, light and heavy chains competing with each other for availability of 1 translocation machinery. Overloading the translocation step might explain the initial decrease in 2 productivity in the wt-based strains expressing P5, or the GPR94/ERp72 pair (Fig. 4A, 4C). If 3 translocation is initially slowed down, the passing heavy and light chains might have more space 4 available in the ER to assemble and exit while overloading of ER is circumvented. Transit through 5 the ER has been shown to determine the limits of secretion capacity [41], so effectively reducing 6 the translocation rates of target proteins might prevent protein accumulation and lead to increase 7 in secretion. Clearly, events related to the ER seem to be most crucial in determining the speed of 8 IgG secretion and are likely rate limiting also for secretion of the other mammalian recombinant 9 glycoprotein tested, EPO (Figure 3B).

10 Co-overexpressing mammalian folding factors together with human antibodies proved to be an 11 interesting alternative to engineering the yeast host for improved IgG secretion, although the native 12 folding factor Cpr5p remained superior to heterologous folding factors. Nevertheless, several of 13 the mammalian proteins were successful in improving titers and it would be interesting to 14 determine how these improvements transfer to the fermentation scale. In any case, our antibody 15 titers of up to 100 μ g/l are very modest compared to the commercially employed mammalian cell 16 lines, working with titers up to g/l scale [42]. Use of the IgG's native folding helpers could display 17 better transferability between production systems, so our approach could be adopted for 18 engineering other expression hosts as well. For example, glycoengineered Pichia pastoris strain 19 have already demonstrated commercial potential in producing therapeutic antibodies [43]; this 20 yeast might benefit from co-expression of mammalian folding factors. Creating a superior antibody 21 producer requires optimization at the whole cell level and benefits from both IgG and host specific 22 engineering approaches.

1 Acknowledgement

The study was supported by Aalto University Bioeconomy facilities and Aalto University School
of Chemical Engineering. EV Koskela is a recipient of a doctoral study grant from the School of
Chemical Engineering, Aalto University. We thank Dr. Linda M. Hendershot, Dr. Kari Kivirikko,
Dr. Lloyd Ruddock, and Dr. Neil Bulleid for kindly providing requested cDNAs.
EK carried out the molecular cloning work, performed the screening experiments, carried out the

7 interaction study, carried out the statistical analyses, and drafted the manuscript. JR contributed to

8 the screening experiments and the cloning work, and helped to draft the manuscript. AF conceived

9 the study, participated in its design, and helped to draft the manuscript. All authors read and

- 10 approved the final manuscript.
- 11

12 **Conflict of interest**

13 The authors declare no financial or commercial conflict of interest.

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						Specific			Fold-change
	Strain			IgG titer	OD .600. ^b	productivity	Relative	Р-	relative to
Rank ^{a)}	number	Added elements	Strain	(µg/ml) ^{b)})	(µg/ml*OD.600) ^{b)}	frequency ^{c)}	value ^{d)}	wild-type ^{b)}
1	142	GRP170-Ppdi	∆opil	0.1050	0.7227	0.1482	0.1071	0.0127	6.37
2	143	GRP170-P _{KAR2}	∆opil	0.0966	0.7371	0.1373	0.0714	0.0042	5.89
3	151	BiP-P _{PDI}	∆opi1	0.1031	0.8027	0.1306	0.0714	0.0005	5.58
4	124	FKBP2-P _{PDI}	∆opil	0.1028	0.8069	0.1295	0.0357	0.0008	5.56
5	127	CypB-P _{KAR2}	∆opi1	0.0958	0.7880	0.1209	0.0179	0.0004	5.17
6	152	BiP-P _{KAR2}	∆opi1	0.0917	0.7873	0.1203	0.0357	0.0056	5.14
7	126	CypB-P _{PDI}	∆opi1	0.0993	0.8571	0.1197	0.0357	0.0396	5.15
8	136	P5-P _{PDI}	∆opil	0.0897	0.7807	0.1168	0.0179	0.0051	5.05
9	138	ERp72-P _{PDI}	∆opi1	0.0937	0.8453	0.1153	0.0536	0.0144	5.01
10	137	P5-P _{KAR2}	∆opil	0.0904	0.8013	0.1152	0.0357	0.0274	4.95
35	83	none ^{e)}	∆opi1	0.0792	0.9123	0.0918	0.1071		3.94
		GRP94-P _{PDI} &							
1	306	ERp72-P _{TEF}	wt	0.0462	0.9433	0.0503	0.1579	< 0.0001	2.14
2	87	P5-PGAL1	wt	0.0573	1.3133	0.0440	0.1053	< 0.0001	1.86
3	69	FKBP2-P _{TEF}	wt	0.0472	1.2333	0.0389	0.0526	0.0001	1.66
		GRP170-P _{TEF} &							
4	340	FKBP2-P _{TEF}	wt	0.0467	1.3007	0.0342	0.0702	0.1074	1.46
		FKBP2-P _{KAR2} &							
5	303	ERp57-P _{PDI}	wt	0.0396	1.1693	0.0339	0.0351	0.0004	1.46
6	68	FKBP2-P _{GPD}	wt	0.0409	1.2633	0.0328	0.0175	0.0017	1.41
7	96	GRP170-PGAL1	wt	0.0385	1.2400	0.0318	0.0000	0.0051	1.36
8	141	GRP170-P _{KAR2}	wt	0.0347	1.0827	0.0316	0.0000	0.1204	1.35
9	129	PDI-P _{KAR2}	wt	0.0349	1.1147	0.0315	0.0000	0.0027	1.36
		PDI-P _{KAR2} &							
10	356	FKBP2-PTEF	wt	0.0403	1.2727	0.0315	0.0702	0.1074	1.36
47	73	none ^{e)}	wt	0.0280	1.2080	0.0235	0.0351		1.00

Table 1. *The strains with highest specific product yields in each strain background.*

- 1 ^{a)} Ranking based on descending specific product yield within parental strain background.
- 2 b) The mean from 0.5% and 2% induction at 30 °C. Averages were calculated from fold-changes that were first compared to the
- 3 respective wild-type samples. OD₆₀₀-values were measured with a microtiter plate and are not path length corrected: the values are
- 4 proportional in approximately 1:5.5 ratio to measurements with regular path length of 1 cm.
- 5 ^{c)} Relative frequency in the 95th percentile of measurements within each parental strain at 30 °C.
- 6 d) Calculated with the nonparametric equivalent of t-test, Wilcoxon signed rank test using the parental strain without added elements as
- 7 the reference.
- 8 ^{e)} Values from the parental strains are added for comparison.

1 Figure legends

2 Figure 1: Summary of the effects of the folding factor constructs on specific product 3 yields. (A) The fold-changes in specific product yields are shown as a heatmap for each 4 construct in the two strain backgrounds and at each cultivation temperature. Fold-changes 5 were normalized to the control in the respective condition and the means of the two 6 induction conditions were used. The corresponding IgG titers are depicted as distribution 7 plots at the three temperatures in (B) for all wt strains and (C) for all *Aopil* strains. The 8 y-axis is proportional to relative frequency and the range of the measured IgG titers is 9 given in the x-axis. The area under each curve is equal to 1.

10 Figure 2: Co-expression of folding factor pairs. The selected chaperones are shown to 11 interact with each other and their reported primary associations [33] are summarized in 12 (A). All folding factor pairs connected by lines in (A) were tested and the mean fold-13 changes and distribution of IgG titers of each strain in each condition are shown in (B). 14 Fold-changes were calculated from specific product yields (titer normalized by OD₆₀₀). 15 The corresponding mean antibody titers are shown in the x-axis. Dots indicate wt samples 16 and crosses $\Delta opil$ samples, and the symbols are colored according to temperature as 17 indicated. The horizontal lines describe the baseline of the fold-changes, one for wt and approximately 4.5 for *dopi1* alone. The lower panel depicts the range of IgG titers in each 18 19 temperature, shown as distribution plots, solid lines for wt and dashed lines for $\Delta opil$ 20 samples. The y-axis is proportional to relative frequency and the range of the measured 21 IgG titers is given in the x-axis. The area under each curve is equal to 1. Each data point 22 represents the mean of at least three biological replicates, each with two technical 23 replicates.

1 Figure 3: Modifications leading to high IgG titers improved EPO secretion but had no 2 effect on acid phosphatase titers. IgG titers were contrasted to (A) secreted acid 3 phosphatase and (B) human EPO secretion in wt, *Aopi1*, and several folding factor 4 overexpression strains. BiP, GRP170, and FKBP2 were expressed under P_{PDI} in the *Aopi1* 5 background, while constructs in the wt strains were P5-P_{GAL1} and the GRP94-P_{PDI}/ 6 ERp72-P_{TEF} pair. Protein yields were measured from identical culture conditions, both 7 with 0.5 % and 2 % galactose induction. Acid phosphatase activity is described with 8 arbitrary units. Means of three biological replicates, each with two technical replicates, 9 are shown.

10 Figure 4: Adding folding factors improved productivity differentially during the time 11 *course*. Antibody titers were measured for strains in wt (A) and *Aopil* (B) background in 12 four time points. Averaged volumetric productivity was calculated from the previous time 13 point taking into account the dilution due to sample collection. Volumetric productivity 14 is shown as a function of OD₆₀₀ for wt (C) and $\Delta opil$ (D) strains. Time of the sampling 15 points is indicated. Error bars are omitted for clarity, but the standard deviations are 16 approximately 10-30% for samples with high IgG concentrations, and maximum 0.003 17 µg/ml for samples near zero. G+E; GRP94-P_{PDI} and ERp72-P_{TEF} pair expression, GRP; 18 GRP170-P_{PDI}. FKBP is referring to FKBP2-P_{PDI} and CPR5 to the yeast PPIase Cpr5p 19 expressed under P_{GPD}. Constructs BiP-P_{PDI} and P5-P_{GAL1} were used for expression of BiP and P5. 20

1 Figures

Color Key А <u> Дорі1</u> wt 0 0.5 1 2 3 P KAR1 P KAR1 P GAL1 < P_{GAL1} PDI1 PGPD PDDI Р Раро PTEF 0.54 0.85 1.02 1.02 0.82 BiP 0.91 0.B 1.31 1.42 0.76 BiP 0.96 1.1 1.06 1.32 1.1 CVDB 0.76 1.09 1.32 1.31 0.81 CypB 1.07 ERdj3 1.11 0.92 1.04 1.05 ERdj3 1.14 0.72 1.16 1.09 0.97 1.06 0.98 0.9 1.01 ERp57 0.69 1.13 1.18 0.96 0.95 ERp57 30 °C ERp72 1.12 11 ERp72 1.08 1.15 1.06 1.04 1.08 1.09 1.26 1.12 1.41 1.15 1.02 1.66 FKBP2 0.96 0.93 1.22 1.41 0.77 FKBP2 1.36 1.2 1.35 1,15 1.32 GRP170 0.94 1.14 1.49 1.61 1.03 **GRP170** GRP94 1.24 0.88 0.85 1.17 GRP94 0.99 0.98 1.22 0.74 0.6 0.97 1.21 1.86 0.96 1.18 1.18 P5 1.22 1.22 1.26 1.27 P5 1.11 0.83 1,36 1.12 1.09 PDI 0.89 1,1 0.98 0.8 PDI D 68 0.62 0.84 1.03 1.08 0.77 BiP 0.65 0.74 0.71 BiP 1.31 1.43 1.09 0.76 0.7 0.86 1.22 1.19 0.87 СурВ 0.65 1.5 СурВ 1.6 0.9 0.97 1.06 1.06 1.02 ERdj3 0.97 1.16 0.89 0.94 1.04 ERdj3 ERp57 0.85 0.91 1.06 1.13 0.92 ERp57 0.7 0.76 1.26 0.92 0.72 1.05 1.03 1.13 1.12 1.08 ERp72 0.9 0.95 ERp72 25 °C 0.98 1:36 1.36 0.99 1.18 1.12 1.26 FKBP2 0.69 0.81 1.11 1.42 0.73 FKBP2 1 1.14 1.15 1.14 **GRP170** 1.06 1.11 1.73 GRP170 1,4 1.09 1.63 0.99 1.03 1.05 GRP94 0.71 GRP94 0.99 0.95 0.93 0.76 0.92 0.99 0.68 3.5 0.92 1.1 1.26 0.95 P5 89.0 1.16 1.41 1.32 1.05 P5 0.97 1.28 1.13 PDI 0.7 PDI 0.97 0.67 1.4 1.2 0.69 0.78 0.87 0.7 0.86 0.91 0.77 BIP D.77 0.74 0.98 0.94 0.85 BiP 0.55 0.79 1.01 1.02 0.75 СурВ 0.85 0.7 0.99 1 0.84 СурВ ERdj3 0.89 1.08 1.07 1.12 1.04 ERdj3 0.87 0.96 0.77 0.89 0.93 ERp57 0.86 1.02 1.11 1.13 0.85 ERp57 0.86 0.72 0.9 0.85 0.76 20 °C 1.06 1.08 0.84 1.12 1.12 ERp72 0.84 0.87 0.98 0.77 0.93 ERp72 FKBP2 0.84 0.99 1.03 0.73 1.32 FKBP2 0.89 1 1.22 1.19 1.04 **GRP170** 1.12 1.12 1.03 0.92 1.12 GRP170 0.91 1.07 1.06 **1**2 0.97 0.79 0.89 0.84 0.82 GRP94 0.86 0.96 1.12 1.18 GRP94 0.69 0.98 0.87 0.99 0.97 0.94 0.93 P5 2.78 0.93 0.86 0.87 1 P5 PDI 0.84 1.22 0.82 0.93 PDI 0.89 0.81 0.91 0.93 0.86 0.87 В С 250 100 30 °C 25 °C 20 °C 30 °C 25 °C 20 °C 100 150 200 --Relative density 20 40 60 80 --Relative density 50 0 0 0.00 0.05 0.10 0.15 0.00 0.05 0.10 0.15 IgG titer (µg/ml) IgG titer (µg/ml)

2 Figure 1



2 Figure 2



2 Figure 3



3 Figure 4