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Following nature's roadmap

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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 *S. cerevisiae***

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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

7   cells, to improve antibody production in yeast. We selected a subset of mammalian folding factors

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 Introduction

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

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

3

1   ER are upregulated during differentiation, including the abundance of folding factors, such as

2   chaperones, co-chaperones, protein disulfide isomerases (PDIs), and peptidyl-prolyl isomerases

3   (PPIases) [12–14]. Overexpression of folding factors, most notably PDI and the chaperone BiP,

4   have been studied in recombinant protein production for over two decades in different expression

5   systems amongst others including yeasts, insect cells and mammalian cells [15–19]. In the case of

6   antibody production in *S. cerevisiae*, overexpressing PDI (Pdi1p) and BiP (Kar2p), and ER

7   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 *OPI1*, 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.

23

1 **2 Materials and methods**

2 Enzymes used for DNA amplification and manipulation were purchased from Thermo Fisher

3 Scientific (Vantaa, Finland). Media components and reagents were obtained from Sigma-Aldrich

4 (Helsinki, Finland) unless stated otherwise.

5 **2.1 Yeast strains**

6 As described in detail in [9], two yeast strains served as the basic chassis in all screening

7 experiments: W303α and W303αΔopi1, from here on referred to as wt and Δopi1, respectively.

8 Both strains harbor a single copy of the light and heavy chain encoding genes of the monoclonal

9 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 cyc1 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 matα-

16 leader sequence (Matα) 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 Matα-

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**

2 cDNAs enconding human ERdj3, mouse GRP94, and human GRP170 were obtained from Dr.

3 Linda M. Hendershot, while human ERp57 cDNA was received from Dr. Kari Kivirikko. Dr.

4 Lloyd Ruddock provided coding regions for human FKBP2, cyclophilin B, P5 L20-L440 and

5 ERp72. Human PDI cDNA was from Dr. Neil Bulleid. cDNA for human BiP was obtained from

6 Source Bioscience (IMAGE clone 5020098). The used oligonucleotides are presented in

7 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 Eco31I the sticky ends became compatible with SpeI and XhoI sites,

18 which were used to cut the vectors.

19 The cloning of P5 and GRP94 to pRS41X-vectors is described in [25]. Other cDNAs were cloned

20 into pRS41X-vectors using ELIC [25]: the cDNAs were amplified in the first round of PCR with

21 gene specific oligonucleotides. An adaptor sequence of 16 bp was added to the 5' end. The yeast

22 specific ER retrieval signal (HDEL) was either mutated in the original gene sequence (GRP94,

23 PDI, GRP170, P5, BiP), or inserted at the C-terminal end of the genes (ERdj3, ERp57, ERp72,

1   FKBP2, CypB). 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 OD_{600} 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. OD_{600} 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 Na_2HPO_4, 1.75 mM

20 KH_2PO_4 with 0.05 % Tween-20) and stored at -20 ˚C until analysis. Each strain was cultivated in

21 at least three biological replicates.

22 Cultivations of EPO and acid phosphatase expressing strains were conducted as described for the

23 screening process but protein expression was performed only at 30 ˚C, 250 rpm. Samples were

1 prepared from three biological replicates, using two technical replicates for each. Acid phosphatase

2 activity assay was conducted immediately after the cultivations. Culture supernatants of EPO

3 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**

5 Antibody titers were determined in duplicate measurements with ELISA as specified in [28]. Data

6 was analyzed by using Gen5 (BioTek Winooski, USA) and R software. Statistical analyses were

7 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**

17 Strains were inoculated to 5 ml SD-URA-LEU with 2 % glucose and grown overnight at 30 ˚C,

18 250 rpm to OD_{600} 5-10. Ten OD_{600} of cells were collected by centrifugation at 3,000 g for 5

19 minutes and the cell pellet was resuspended in 10 ml of expression media (SD-URA-LEU with 2

20 % galactose) obtaining a starting OD_{600} of 1.0. Strains were cultivated in 250 ml shake flasks in

21 inducing conditions at 30 ˚C, 200 rpm for 24 hours and samples were collected at time points 0, 6,

22 12, and 24 hours. Supernatant samples were collected as follows: 1 or 2 ml was taken from culture,

23 cells were spun down and the supernatant was adjusted to 1xPBT and stored at -20 ˚C until

1 analysis. Resulting cell pellets were resuspended in the same volume of fresh, prewarmed media

2 and returned to the expression culture. Sample collection resulted in culture media replacement at

3 a rate of 1 ml per six hours. OD_{600} was measured with an eppendorf Biophotometer plus –

4 spectrophotometer (Eppendorf AG, Hamburg, Germany). IgG titers were measured in 2-3

5 technical replicates as described in [28]. All shake flask cultures were repeated with three

6 biological replicates. To account for the dilution due to addition of fresh media, averaged

7 volumetric productivity P_{avg} ($ngml^{-1}h^{-1}$) 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.

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

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

The plasmids were transformed individually and in combinations into two different IgG producing strains, the nonengineered wt and the Δ*opi1* strain, containing the deletion of the *OPI1*-gene that results in an enlarged ER and significantly higher IgG secretion [9, 32]. To determine the specific product yields of our strains, we used a previously established screening platform described in [9]. We cultivated all strains at 20, 25, and 30 ˚C using 0.5% and 2.0% galactose for induction. Specific product yields (IgG titer normalized by cell density) increased with temperature while the wt and *Δopi1* strains remained as two distinct populations (Supporting information, Fig. S1). Generally, IgG titers and specific product yields were higher with 0.5 % than with 2 % induction, although

1   the difference in specific product yields was smaller than in titers between the two induction levels

2   (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   $\Delta opi1$ 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 $\Delta opi1$ strains, the UPR-

18   controlled promoters P_{KAR2} and P_{PDI1} 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.

1 Although the highest fold changes were observed at 25 ˚C, the highest IgG titers were observed

2 at 30 ˚C (Supporting information, Table S4).

3 The results in Figure 1 show one clear deviation: the wt strain expressing P5 under control of P$_{GAL1}$

4 performed extraordinarily well in antibody secretion. All the measured specific product yields of

5 this strain were above 1.5 –fold, in lower temperatures up to 3.6 –fold (Fig. 1A, Supporting

6 information, Table S4). This strain also had the highest mean IgG titer from all wt samples

7 (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 *Δopi1* 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.

20 The best strains of the two screening sets are summarized in Table 1, which shows the highest

21 overall specific product yields from both strain backgrounds. In the best producing *Δopi1* strains,

22 all folding factors were expressed under control of P$_{KAR2}$ and P$_{PDI1}$. Combined with the *OPI1*-

23 gene deletion, GRP170 overexpression led up to 6-fold higher specific product yield compared to

the wt (Table 1). Although the improvement in specific product yield was smaller, the *Δopi1* strains

expressing FKBP2 or BiP under P_{PDI} reached comparable titers to GRP170 overexpression strain,

being slightly over 100 µg/l (Table 1). None of the strains with simultaneous overexpression of

two folding factors was among the ten best strains in the *Δopi1* background. In contrast, four of

such pairs in the wt background are listed in Table 1. In addition, the folding factors improving

specific product yields in *Δopi1* background were noted to be more effective when expressed under

stronger promoters in wt (Table 1). It is possible that deletion of *OPI1* gene already enables the

cell to circumvent major portion of the folding bottleneck, whereas the demand for improvement

in wt strains is larger and requires a greater amount of folding factors.

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 *OPI1*-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

1   cultivation in the same conditions as for the IgG titer measurements at 30 ˚C (Table 1). Although

2   the *OPI1*-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**

19   We cultivated a selection of strains in shake flasks and followed the production over time to gain

20   more insight into the secretion behavior of these strains. We chose strains expressing BiP, FKBP2,

21   and GRP170 from the *Δopi1* strains (numbers 142, 124 and 151), and the strains expressing P5,

22   and the GRP94/ERp72 pair (numbers 87, and 306) from the wt background, as these resulted in

23   the highest improvements in specific product yield in the two backgrounds (Table 1). Secretion

14

phenotypes of these strains were confirmed in shake flask cultivations, as the IgG titers were comparable to the screening cultures (Fig. 4A, 4B). We previously reported a yeast PPIase construct, P_{GPD}-*CPR5*, which strongly increased specific antibody yields [9]. As we wanted to compare the two approaches of having a native versus a heterologous folding enhancer, we included this *Δopi1* -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 PPIase FKBP2 expressing strain produced similar titers in a shorter time (Fig. 4B). When we plotted averaged volumetric productivity against OD_{600}, we noticed that the increase in productivity was accompanied with a shift to lower cell densities, especially in the *Δopi1* strains, indicating that high titers were achieved at the expense of culture density (Fig. 4C, 4D). Thus, although the increase in specific product yield was much greater with overexpression of Cpr5p ([9]) than for FKBP2 (Table 1), the difference in IgG titer at 24 hours was relatively small.

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

4 Discussion

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

1   multiple protein overexpression. For future approaches of ER engineering, the selection of

2   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_H1 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

19   experiments (Figure 1A). P5 was the only folding factor that was expressed with a yeast-derived

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 µg/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**

11

12 **Conflict of interest**

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

1 **5 References**

2 [1] Buss, N.A.P.S., Henderson, S.J., McFarlane, M., Shenton, J.M., et al., Monoclonal

3 antibody therapeutics: History and future. *Curr. Opin. Pharmacol.* 2012, *12*, 615–622.

4 [2] Gasser, B., Mattanovich, D., Antibody production with yeasts and filamentous fungi: on

5 the road to large scale? *Biotechnol. Lett.* 2007, *29*, 201–212.

6 [3] Durocher, Y., Butler, M., Expression systems for therapeutic glycoprotein production.

7 *Curr. Opin. Biotechnol.* 2009, *20*, 700–707.

8 [4] Frenzel, A., Hust, M., Schirrmann, T., Expression of recombinant antibodies. *Front.*

9 *Immunol.* 2013, *4*, 217.

10 [5] Nasab, F.P., Aebi, M., Bernhard, G., Frey, A.D., A combined system for engineering

11 glycosylation efficiency and glycan structure in *Saccharomyces cerevisiae*. *Appl. Environ.*

12 *Microbiol.* 2013, *79*, 997–1007.

13 [6] Piirainen, M.A., Boer, H., De Ruijter, J.C., Frey, A.D., A dual approach for improving

14 homogeneity of a human-type N-glycan structure in *Saccharomyces cerevisiae*.

15 *Glycoconj. J.* 2016, *33*, 189–199.

16 [7] Laukens, B., De Visscher, C., Callewaert, N., Engineering yeast for producing human

17 glycoproteins: where are we now? *Future Microbiol.* 2015, *10*, 21–34.

18 [8] Rakestraw, J.A., Sazinsky, S.L., Piatesi, A., Antipov, E., et al., Directed evolution of a

19 secretory leader for the improved expression of heterologous proteins and full-length

20 antibodies in *Saccharomyces cerevisiae. Biotechnol. Bioeng.* 2009, *103*, 1192–201.

21 [9] de Ruijter, J.C., Koskela, E. V., Frey, A.D., Enhancing antibody folding and secretion by

22 tailoring the *Saccharomyces cerevisiae* endoplasmic reticulum. *Microb. Cell Fact.* 2016,

23 *15*, 1–18.

1   [10]   Tyo, K.E.J., Liu, Z., Magnusson, Y., Petranovic, D., et al., Impact of protein uptake and

2   degradation on recombinant protein secretion in yeast. *Appl. Microbiol. Biotechnol.* 2014,

3   *98*, 7149–7159.

4   [11]   Ferrer-Miralles, N., Domingo-Espín, J., Corchero, J.L., Vázquez, E., et al., Microbial

5   factories for recombinant pharmaceuticals. *Microb. Cell Fact.* 2009, *8*, 17.

6   [12]   Cenci, S., Sitia, R., Managing and exploiting stress in the antibody factory. *FEBS Lett.*

7   2007, *581*, 3652–3657.

8   [13]   van Anken, E., Romijn, E.P., Maggioni, C., Mezghrani, A., et al., Sequential waves of

9   functionally related proteins are expressed when B cells prepare for antibody secretion.

10   *Immunity* 2003, *18*, 243–253.

11   [14]   Romijn, E.P., Christis, C., Wieffer, M., Gouw, J.W., et al., Expression clustering reveals

12   detailed co-expression patterns of functionally related proteins during B cell

13   differentiation. *Mol. Cell. Proteomics* 2005, *4*, 1297–1310.

14   [15]   Robinson, a S., Hines, V., Wittrup, K.D., Protein disulfide isomerase overexpression

15   increases secretion of foreign proteins in *Saccharomyces cerevisiae. Biotechnology. (N.*

16   *Y).* 1994, *12*, 381–384.

17   [16]   Hsu, T.A., Eiden, J.J., Bourgarel, P., Meo, T., et al., Effects of Co-Expressing Chaperone

18   BiP on Functional Antibody Production in the Baculovirus System. *Protein Expr. Purif.*

19   1994, *5*, 595–603.

20   [17]   Hou, J., Tyo, K.E.J., Liu, Z., Petranovic, D., et al., Metabolic engineering of recombinant

21   protein secretion by *Saccharomyces cerevisiae. FEMS Yeast Res.* 2012, *12*, 491–510.

22   [18]   Delic, M., Göngrich, R., Mattanovich, D., Gasser, B., Engineering of protein folding and

23   secretion-strategies to overcome bottlenecks for efficient production of recombinant

proteins. *Antioxid. Redox Signal.* 2014, *21*, 414–437.

[19] Dorner, A.J., Wasley, L.C., Kaufman, R.J., Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *EMBO J.* 1992, *11*, 1563–1571.

[20] Shusta, E. V., Raines, R.T., Plückthun, A., Wittrup, K.D., Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nat. Biotechnol.* 1998, *16*, 773–777.

[21] Dinnis, D.M., James, D.C., Engineering mammalian cell factories for improved recombinant monoclonal antibody production: lessons from nature? *Biotechnol. Bioeng.* 2005, *91*, 180–189.

[22] Gorlani, a, Hulsik, D.L., Adams, H., Vriend, G., et al., Antibody engineering reveals the important role of J segments in the production efficiency of llama single-domain antibodies in *Saccharomyces cerevisiae*. *Protein Eng. Des. Sel.* 2012, *25*, 39–46.

[23] Taxis, C., Knop, M., System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*. *Biotechniques* 2006, *40*, 73–78.

[24] Frey, A.D., Aebi, M., An enzyme-based screening system for the rapid assessment of protein N-glycosylation efficiency in yeast. *Glycobiology* 2015, *25*, 252–257.

[25] Koskela, E. V., Frey, A.D., Homologous Recombinatorial Cloning Without the Creation of Single-Stranded Ends: Exonuclease and Ligation-Independent Cloning (ELIC). *Mol. Biotechnol.* 2015, *57*, 233–240.

[26] Mumberg, D., Müller, R., Funk, M., Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 1995, *156*, 119–122.

[27] Gietz, D., St Jean, A., Woods, R. a, Schiestl, R.H., Improved method for high efficiency

transformation of intact yeast cells. *Nucl Acid Res* 1992, *20*, 1425.

[28] de Ruijter, J.C., Frey, A.D., Analysis of antibody production in *Saccharomyces cerevisiae*:

effects of ER protein quality control disruption. *Appl. Microbiol. Biotechnol.* 2015, *99*,

9061–9071.

[29] Feige, M.J., Hendershot, L.M., Buchner, J., How antibodies fold. *Trends Biochem. Sci.*

2010, *35*, 189–198.

[30] Behnke, J., Hendershot, L.M., The large hsp70 grp170 binds to unfolded protein

substrates in vivo with a regulation distinct from conventional hsp70s. *J. Biol. Chem.*

2014, *289*, 2899–2907.

[31] Le Gallou, S., Caron, G., Delaloy, C., Rossille, D., et al., IL-2 requirement for human

plasma cell generation: coupling differentiation and proliferation by enhancing MAPK-

ERK signaling. *J. Immunol.* 2012, *189*, 161–173.

[32] Schuck, S., Prinz, W. a, Thorn, K.S., Voss, C., et al., Membrane expansion alleviates

endoplasmic reticulum stress independently of the unfolded protein response. *J. Cell Biol.*

2009, *187*, 525–536.

[33] Jansen, G., Määttänen, P., Denisov, A.Y., Scarffe, L., et al., An interaction map of

endoplasmic reticulum chaperones and foldases. *Mol. Cell. Proteomics* 2012, *11*, 710–

723.

[34] Idiris, A., Tohda, H., Kumagai, H., Takegawa, K., Engineering of protein secretion in

yeast: strategies and impact on protein production. *Appl. Microbiol. Biotechnol.* 2010, *86*,

403–417.

[35] Meunier, L., Usherwood, Y., Chung, K.T., Hendershot, L.M., A subset of chaperones and

folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent

proteins. *Mol. Biol. Cell* 2002, *13*, 4456–4469.

[36] Xu, P., Raden, D., Doyle, F.J., Robinson, A.S., Analysis of unfolded protein response during single-chain antibody expression in *Saccaromyces cerevisiae* reveals different roles for BiP and PDI in folding. *Metab. Eng.* 2005, *7*, 269–279.

[37] Lilie, H., Rudolph, R., Buchner, J., Association of antibody chains at different stages of folding: prolyl isomerization occurs after formation of quaternary structure. *J. Mol. Biol.* 1995, *248*, 190–201.

[38] Thies, M.J., Mayer, J., Augustine, J.G., Frederick, C.A., et al., Folding and association of the antibody domain CH3: prolyl isomerization preceeds dimerization. *J Mol Biol* 1999, *293*, 67–79.

[39] Feige, M.J., Groscurth, S., Marcinowski, M., Shimizu, Y., et al., An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. *Mol. Cell* 2009, *34*, 569–579.

[40] Delic, M., Valli, M., Graf, A.B., Pfeffer, M., et al., The secretory pathway: Exploring yeast diversity. *FEMS Microbiol. Rev.* 2013, *37*, 872–914.

[41] Love, K.R., Politano, T.J., Panagiotou, V., Jiang, B., et al., Systematic single-cell analysis of pichia pastoris reveals secretory capacity limits productivity. *PLoS One* 2012, *7*, 1–11.

[42] Kunert, R., Reinhart, D., Advances in recombinant antibody manufacturing. *Appl. Microbiol. Biotechnol.* 2016, *100*, 3451–3461.

[43] Ye, J., Ly, J., Watts, K., Hsu, A., et al., Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. *Biotechnol. Prog.* 2011, *27*, 1744–1750.

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

Rank[a]	Strain number	Added elements	Strain	IgG titer (µg/ml)[b]	OD_{600}[b]	Specific productivity (µg/ml*OD_{600})[b]	Relative frequency[c]	P-value[d]	Fold-change relative to wild-type[b]
1	142	GRP170-P_{PDI}	$\Delta opi1$	0.1050	0.7227	0.1482	0.1071	0.0127	6.37
2	143	GRP170-P_{KAR2}	$\Delta opi1$	0.0966	0.7371	0.1373	0.0714	0.0042	5.89
3	151	BiP-P_{PDI}	$\Delta opi1$	0.1031	0.8027	0.1306	0.0714	0.0005	5.58
4	124	FKBP2-P_{PDI}	$\Delta opi1$	0.1028	0.8069	0.1295	0.0357	0.0008	5.56
5	127	CypB-P_{KAR2}	$\Delta opi1$	0.0958	0.7880	0.1209	0.0179	0.0004	5.17
6	152	BiP-P_{KAR2}	$\Delta opi1$	0.0917	0.7873	0.1203	0.0357	0.0056	5.14
7	126	CypB-P_{PDI}	$\Delta opi1$	0.0993	0.8571	0.1197	0.0357	0.0396	5.15
8	136	P5-P_{PDI}	$\Delta opi1$	0.0897	0.7807	0.1168	0.0179	0.0051	5.05
9	138	ERp72-P_{PDI}	$\Delta opi1$	0.0937	0.8453	0.1153	0.0536	0.0144	5.01
10	137	P5-P_{KAR2}	$\Delta opi1$	0.0904	0.8013	0.1152	0.0357	0.0274	4.95
35	83	none[e]	$\Delta opi1$	0.0792	0.9123	0.0918	0.1071		3.94
1	306	GRP94-P_{PDI} & ERp72-P_{TEF}	wt	0.0462	0.9433	0.0503	0.1579	<0.0001	2.14
2	87	P5-P_{GAL1}	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
4	340	GRP170-P_{TEF} & FKBP2-P_{TEF}	wt	0.0467	1.3007	0.0342	0.0702	0.1074	1.46
5	303	FKBP2-P_{KAR2} & 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-P_{GAL1}	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
10	356	PDI-P_{KAR2} & FKBP2-P_{TEF}	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

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. OD_{600}-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 *Δopi1* 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 OD_{600}).

15 The corresponding mean antibody titers are shown in the x-axis. Dots indicate wt samples

16 and crosses *Δopi1* 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

18 approximately 4.5 for *Δopi1* alone. The lower panel depicts the range of IgG titers in each

19 temperature, shown as distribution plots, solid lines for wt and dashed lines for *Δopi1*

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.

Figure 3: *Modifications leading to high IgG titers improved EPO secretion but had no effect on acid phosphatase titers.* IgG titers were contrasted to (A) secreted acid phosphatase and (B) human EPO secretion in wt, *Δopi1*, and several folding factor overexpression strains. BiP, GRP170, and FKBP2 were expressed under P_{PDI} in the *Δopi1* background, while constructs in the wt strains were P5-P_{GAL1} and the GRP94-P_{PDI}/ERp72-P_{TEF} pair. Protein yields were measured from identical culture conditions, both with 0.5 % and 2 % galactose induction. Acid phosphatase activity is described with arbitrary units. Means of three biological replicates, each with two technical replicates, are shown.

Figure 4: *Adding folding factors improved productivity differentially during the time course.* Antibody titers were measured for strains in wt (A) and *Δopi1* (B) background in four time points. Averaged volumetric productivity was calculated from the previous time point taking into account the dilution due to sample collection. Volumetric productivity is shown as a function of OD_{600} for wt (C) and *Δopi1* (D) strains. Time of the sampling points is indicated. Error bars are omitted for clarity, but the standard deviations are approximately 10-30% for samples with high IgG concentrations, and maximum 0.003 µg/ml for samples near zero. G+E; GRP94-P_{PDI} and ERp72-P_{TEF} pair expression, GRP; GRP170-P_{PDI}. FKBP is referring to FKBP2-P_{PDI} and CPR5 to the yeast PPIase Cpr5p expressed under P_{GPD}. Constructs BiP-P_{PDI} and P5-P_{GAL1} were used for expression of BiP and P5.
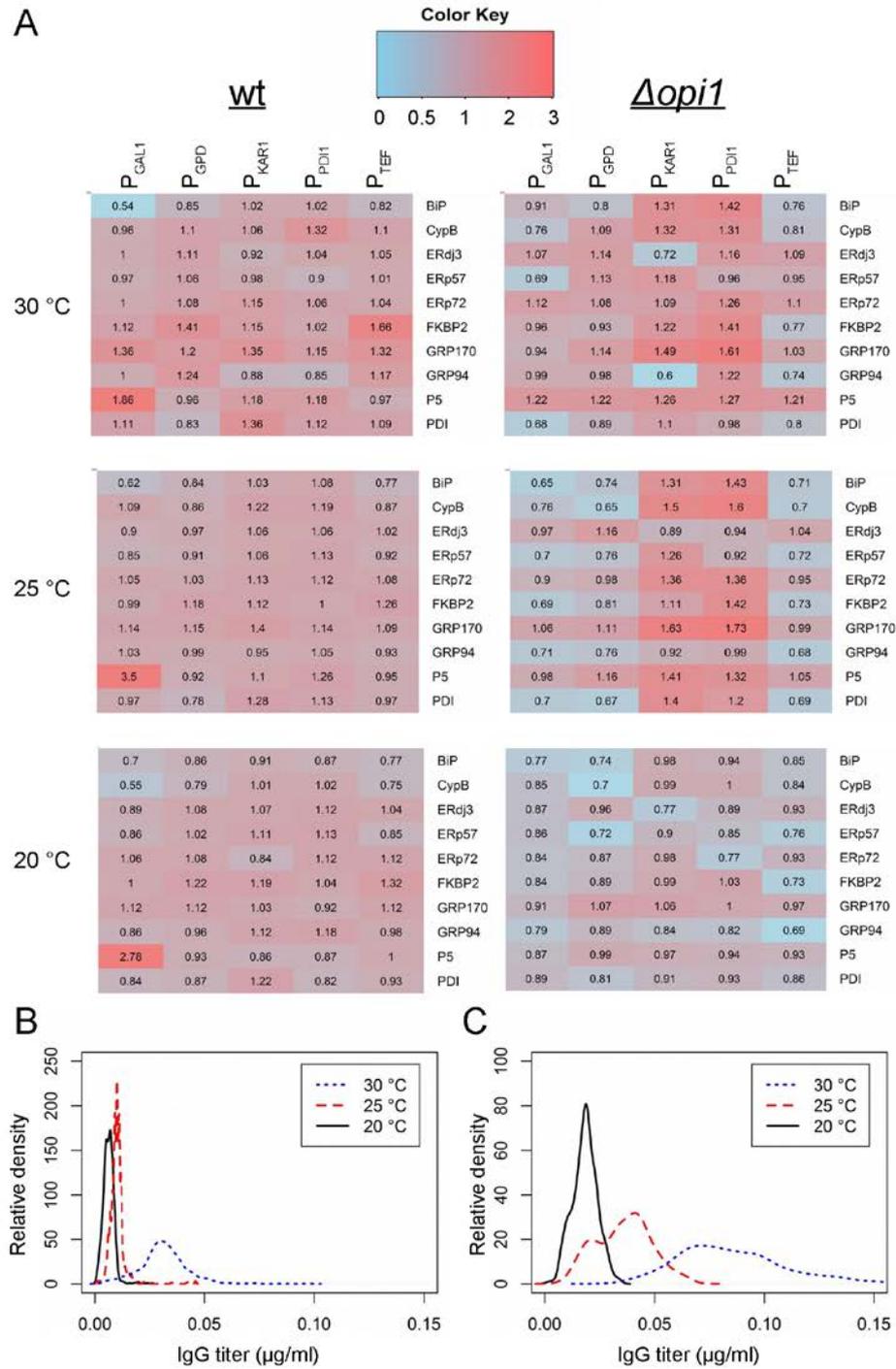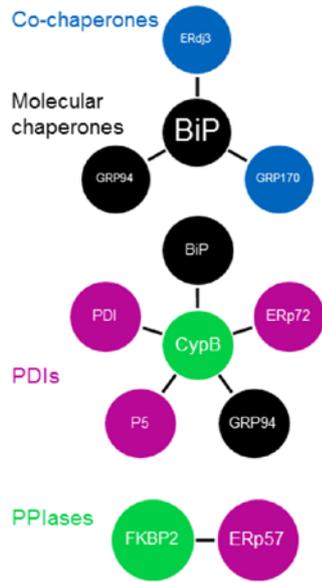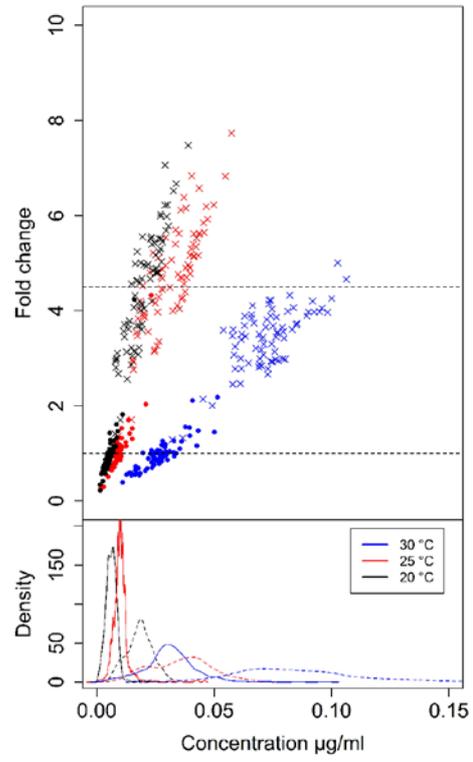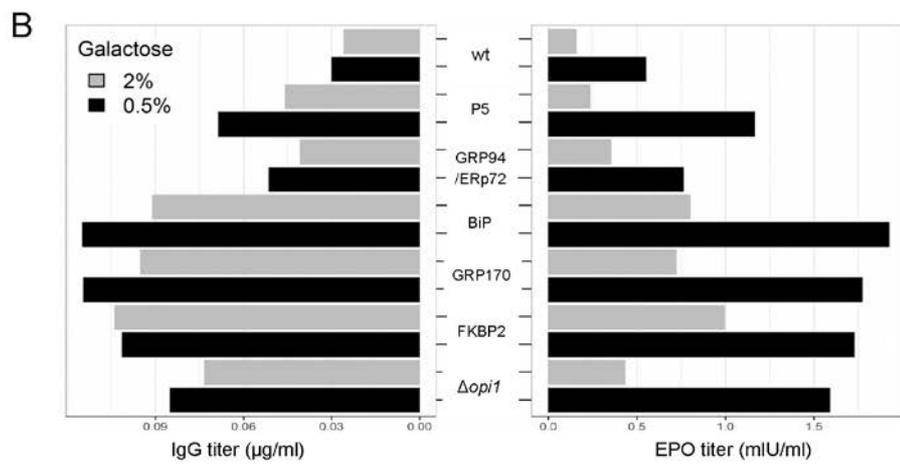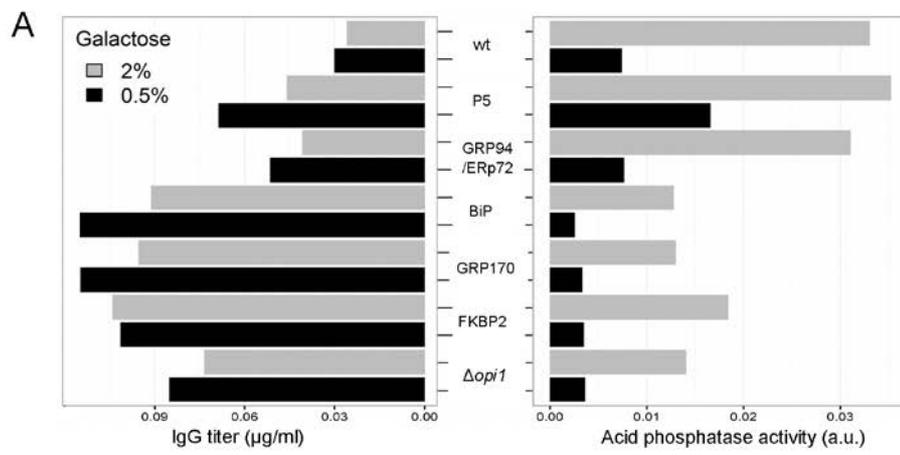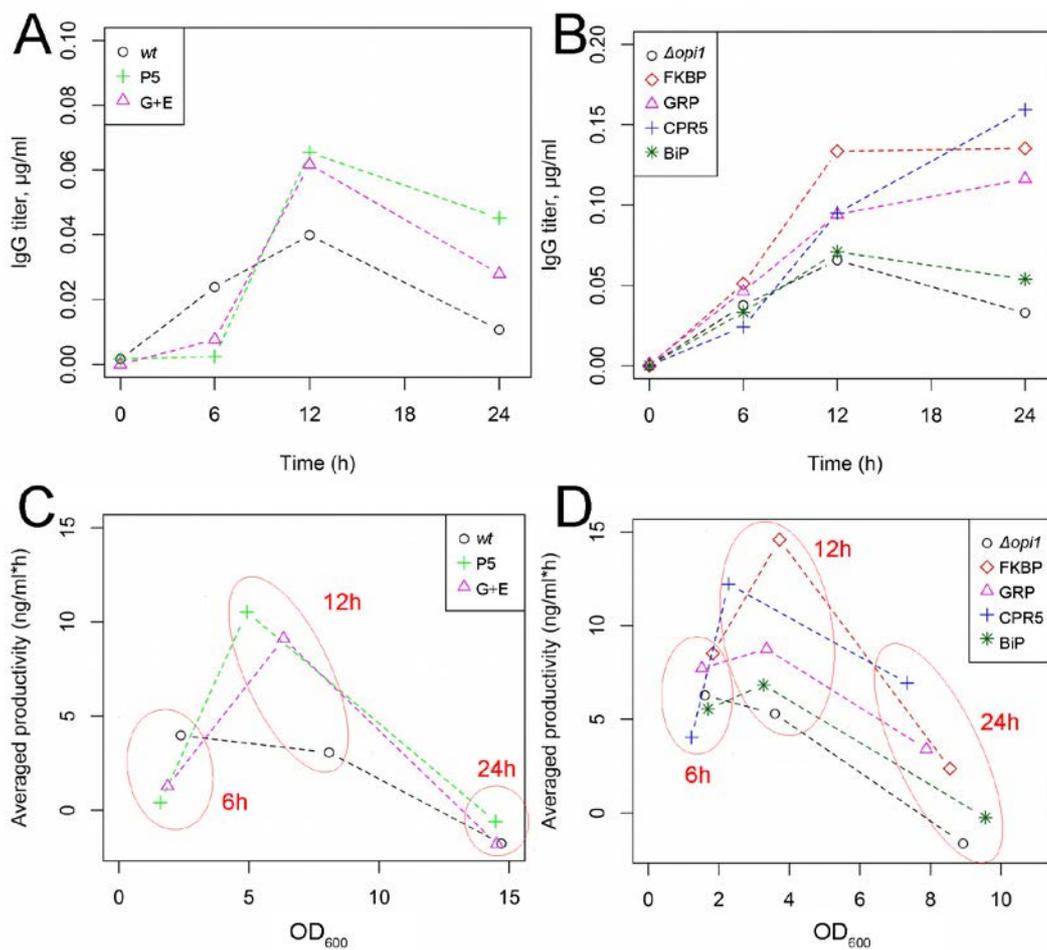
1   Figures

2   Figure 1

2   Figure 2

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2   **Figure 3**

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3　**Figure 4**