Constitutively-stressed yeast strains are high-yielding for recombinant Fps1: implications for the translational regulation of an aquaporin

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Abstract

Background: We previously selected four strains of Saccharomyces cerevisiae for their ability to produce the aquaporin Fps1 in sufficient yield for further study. Yields from the yeast strains spt3Δ, srb5Δ, gcn5Δ and yTHCBMS1 (supplemented with 0.5 μg/mL doxycycline) that had been transformed with an expression plasmid containing 249 base pairs of 5' untranslated region (UTR) in addition to the primary FPS1 open reading frame (ORF) were 10–80 times higher than yields from wild-type cells expressing the same plasmid. One of the strains increased recombinant yields of the G protein-coupled receptor adenosine receptor 2a (A2aR) and soluble green fluorescent protein (GFP). The specific molecular mechanisms underpinning a high-yielding Fps1 phenotype remained incompletely described.

Results: Polysome profiling experiments were used to analyze the translational state of spt3Δ, srb5Δ, gcn5Δ and yTHCBMS1 (supplemented with 0.5 μg/mL doxycycline); all but gcn5Δ were found to exhibit a clear block in translation initiation. Four additional strains with known initiation blocks (rpl31aΔ, rpl22aΔ, ssf1Δ and nop1Δ) also improved the yield of recombinant Fps1 compared to wild-type. Expression of the eukaryotic transcriptional activator GCN4 was increased in spt3Δ, srb5Δ, gcn5Δ and yTHCBMS1 (supplemented with 0.5 μg/mL doxycycline); these four strains also exhibited constitutive phosphorylation of the eukaryotic initiation factor, eIF2α. Both responses are indicative of a constitutively-stressed phenotype. Investigation of the 5'UTR of FPS1 in the expression construct revealed two untranslated ORFs (uORF1 and uORF2) upstream of the primary ORF. Deletion of either uORF1 or uORF1 and uORF2 further improved recombinant yields in our four strains; the highest yields of the uORF deletions were obtained from wild-type cells. Frame-shifting the stop codon of the native uORF (uORF2) so that it extended into the FPS1 ORF did not substantially alter Fps1 yields in spt3Δ or wild-type cells, suggesting that high-yielding strains are able to bypass 5' uORFs in the FPS1 gene via leaky scanning, which is a known stress-response mechanism. Yields of recombinant A2aR, GFP and horseradish peroxidase could be improved in one or more of the yeast strains suggesting that a stressed phenotype may also be important in high-yielding cell factories.

Conclusions: Regulation of Fps1 levels in yeast by translational control may be functionally important; the presence of a native uORF (uORF2) may be required to maintain low levels of Fps1 under normal conditions, but higher levels as part of a stress response. Constitutively-stressed yeast strains may be useful high-yielding microbial cell factories for recombinant protein production.
Background

In recent years, our understanding of how to synthesize recombinant membrane proteins in microbes has benefitted from insights into the underlying molecular mechanisms [1] in a range of host cells including *Escherichia coli* [2, 3], *Lactococcus lactis* [4], *Saccharomyces cerevisiae* [5–8] and *Pichia pastoris* [9, 10]. Despite these advances, several membrane proteins still remain intractable to recombinant production [5]. The aquaporin Fps1, which has a central role in yeast mating [11] as well as the cellular response to stresses including osmotic, acetic acid and toxic metalloid stress [12, 13], is one such protein.

In order to obtain sufficient yields of Fps1 for further study, we previously used comparative transcriptome analysis to identify genes that were up- or down-regulated in *S. cerevisiae* when recombinant Fps1 was produced under a range of different culture conditions [8]. We noted that relatively high-yielding conditions were associated with the down-regulation of *SPT3*, *SRE5* or *GCN5* or the up-regulation of *BMS1* [8]. We used these findings to select yeast strains specifically for the production of recombinant Fps1: we chose three strains in which each of the down-regulated genes was singly deleted and one in which the upregulated gene was over-expressed. When cultured in stirred-tank bioreactors, Fps1 yields from strains *spt3Δ, srb5Δ, gcn5Δ* and *yTHCBMS1* (in the latter strain, the promoter is tuned by addition of 0.5 μg/mL doxycycline) were 10–80 times higher than yields from wild-type cells [6]. We also demonstrated more modest yield improvements when other target proteins were produced in *yTHCBMS1* (10 μg/mL doxycycline): yields of the G protein-coupled receptor, adenosine receptor 2a (A2aR) and soluble green fluorescent protein (GFP) [6] were doubled compared to controls in some cases.

We noted that all four strains had elevated levels of *BMS1* transcript compared to wild-type [6]; Bms1 is involved in ribosome biogenesis [14], suggesting that post-transcriptional mechanisms might be responsible for these high-yielding phenotypes. Notably, the expression plasmid used to produce Fps1 contained 249 base pairs (bp) of 5' untranslated region (UTR) in addition to the primary *FPS1* open reading frame (ORF) meaning that translational control might have a role in defining the final yield of Fps1 in our four strains. The aim of this study was therefore to investigate the translational mechanisms in our four high-yielding strains (*spt3Δ, srb5Δ, gcn5Δ* and *yTHCBMS1*) in order to understand the molecular determinants underpinning the expression of *FPS1*.

Results and discussion

High-yielding yeast strains exhibit blocks in translation initiation

On the basis that our four strains had elevated levels of *BMS1* transcript compared to wild-type [6] and that Bms1 is involved in ribosome biogenesis [14], polysome profiles were generated from shake-flask cultures. The initiation phase of translation is the rate-limiting step of protein synthesis [15, 16]. Using polysome profiling, it is possible to measure the numbers of ribosomes bound to a cell's mRNA pool under defined conditions. Differences in the ratio of bound polyribosomes (“polysomes”; two or more ribosomes) to bound single ribosomes (“monosomes”) are indicative of alterations in translation. A translation initiation block is typically associated with the majority of mRNAs being monosomal; in contrast, highly-translated mRNAs are polysomal. Figure 1a shows a typical polysome profile for the parental wild-type strain BY4741, which was not altered on supplementation with 0.5 μg/mL doxycycline (profile not shown; Table 1). The polysome profile of *yTHCBMS1* (with no doxycycline supplementation; Fig. 1b; Table 1) suggests a block in translation initiation, which is defined as an increase in the ratio of the 80S monosome peak area to the area of the polysome peaks, compared to the wild-type ratio (Table 1). Cells subjected to stresses such as heat shock or nutrient starvation typically exhibit initiation blocks; this is consistent with the down-regulation of translation in order to conserve energy, protect the proteome from damage and elicit alterations in gene expression to promote protection [15, 16]. Supplementation of *yTHCBMS1* with 0.5 μg/mL doxycycline increased the severity of the initiation block and increased Fps1 yield (Fig. 1c; Table 1). The decrease in the 40S ribosomal subunit peak and relative increase in the 60S peak is probably responsible for the initiation block in this case, since fewer functional ribosomes would be able to form. Strains *spt3Δ* and *srb5Δ* also exhibited a clear initiation block, but not on account of an altered ribosomal subunit ratio as seen for *yTHCBMS1* with 0.5 μg/mL doxycycline; for these two strains the subunit ratio is not perturbed (Fig. 1d, e; Table 1). The polysome profile of *gcn5Δ* resembled that of cells with a mild initiation block because of the relatively high 80S peak compared to the wild-type control (Fig. 1f; Table 1) [18]. The deletion strains have...
not previously been reported to have altered translation capacities; SPT3, GCN5 and SRB5 are global regulators of transcription [19, 20].

To test the hypothesis that high-yielding strains may exhibit a block in translation initiation, four additional strains with altered translational profiles were selected. The ribosomal biogenesis deletion mutants, ssf1Δ, nop12Δ, rpl31aΔ and rpl22aΔ have a decrease in the levels of 60S ribosomal subunit [21, 22]. Figure 2 shows a representative polysome profile containing halfmers after each monosome and polysome peak. Halfmers occur when the pre-initiation complex (PIC) binds to mRNA in the absence of the 60S subunit; the PIC then scans through the start codon in a mechanism known as leaky scanning [23]. When these four additional mutant strains were transformed with the vector pYX222-FPS1-HA3 and Fps1 production was analysed in shake flask cultures, Fps1 yields were higher than in wild-type cells (Fig. 2). These data suggest that yeast cells that are high-yielding for Fps1 may also exhibit an initiation block, but that the extent of the initiation block and the Fps1 yield are not correlated. We therefore continued to analyze the

Fig. 1 Some high-yielding Fps1 strains exhibit initiation blocks. Representative polysome profiles for the high-yielding strains and the wild-type control. Cells were cultured in YPD medium and harvested at A600 ~ 1. Values in square brackets are the monosome/polysome ratio with the standard error of the mean shown in parentheses, as follows: a BY4741 wild-type control [0.15 (n = 3, 0.01)], b yTHCBMS1 [0.24 (n = 3, 0.03)], c yTHCBMS1 supplemented with 0.5 μg/mL doxycycline [0.43 (n = 3, 0.14)], d spt3Δ [0.18 (n = 3, 0.03)], e srb5Δ [0.39 (n = 3, 0.07)], f gcn5Δ [0.16 (n = 2, 0.02)]
translational capacity of spt3Δ, srb5Δ, gcn5Δ and yTH-CBMS1 (supplemented with 0.5 µg/mL doxycycline).

**Phosphorylation of eIF2α is constitutive in high-yielding yeast strains**

Phosphorylation of the eukaryotic initiation factor subunit eIF2α was investigated. When eIF2α is phosphorylated, during times of stress, it prevents the exchange of eIF2α-GDP for eIF2α-GTP, decreasing the level of ternary complex (TC), which in turn reduces the pool of PIC and may cause an initiation block. Figure 3 shows that eIF2α is constitutively phosphorylated in all four high-yielding strains; the upper, control panel was detected with an anti-eIF2α antibody, whilst the lower panel was detected with an anti-phospho-eIF2α antibody. The data in the lower panel suggest that all four of our strains are constitutively stressed compared to wild-type cells.

**GCN4 expression is increased in high-yielding yeast strains**

It is well known that in yeast, cellular stress is associated with increased expression of the eukaryotic transcriptional activator gene, GCN4. Translation of GCN4 is regulated by four upstream open reading frames (uORF; Fig. 4a). At low levels of eIF2α phosphorylation, such as those found under normal conditions, the TC is abundant. It has been demonstrated experimentally that ribosomes that initiate at uORF1 can also continue scanning; the AT-rich sequences around the stop codon of uORF1 promote ribosome retention, enabling re-initiation of translation at uORF2, uORF3 or uORF4. However, ribosomes that terminate at uORFs 2–4 do not resume scanning because GC-rich sequences promote ribosome disassociation, decreasing the probability of the GCN4 ORF being translated [24]. During stress conditions, increased levels of eIF2α phosphorylation (e.g. Fig. 3) reduce the abundance of the TC so that re-initiation at uORFs 2-4 becomes even less frequent, allowing scanning ribosomes to reach the GCN4 ORF and translate it.

**GCN4 expression (assayed in our four strains using a β-galactosidase reporter), was shown to be increased in all strains relative to wild-type cells (Table 1)**. Increased Gcn4 levels in srb5Δ, gcn5Δ, spt3Δ and yTH-CBMS1 (supplemented with 0.5 µg/mL doxycycline) may be the result of initiation blocks due to the eIF2α phosphorylation observed in these strains. For the ribosomal biogenesis and ribosomal protein mutant strains, a decrease in the level of the 60S subunit enables the PIC to scan through the uORFs in the GCN4 transcript; it has already been shown that ribosomal mutants have increased levels of Gcn4 [21] on account of leaky scanning by 40S subunits [17] through uORFs 1-4.

**High-yielding yeast strains can bypass uORFs in the FPS1 transcript**

On account of a possible association between increased Fps1 yields and constitutive stress phenotypes, the sequence of pYZ222-FPS1-HA3 was analysed for the presence of start codons upstream of the FPS1 ORF and downstream of the TP1 promoter. To define the 5'UTR region, transcriptional start site information for TP1 was obtained from the literature [25]. Additional file 1: Figure S2; Fig. 4b and Table 2 illustrate the 2 uORFs in the 5'UTR of the FPS1 expression construct. The first FPS1

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**Table 1 Characteristics of high-yielding yeast strains**

| Yeast strain                      | Fps1 yield (µg/L) FPS1-HA3 | S'Δ1-43-FPS1-HA3 | S'Δ1-215-FPS1-HA3 | M:P ratio | GCN4 expression | Constitutive phosphorylation of eIF2α |
|----------------------------------|-----------------------------|------------------|-------------------|-----------|-----------------|--------------------------------------|
| Wild-type (BY4741)               | 0.02 (0.01)                 | 0.21 (0.06)      | 12.14 (1.68)      | 0.15 (0.01) | 1.0 (0.4)       | N                                    |
| Wild-type + 0.5 µg/mL doxycycline| 0.02 (0.01)                 | –                | –                 | 0.15 (0.01) | –               | –                                    |
| yTHCBMS1                         | 0.12 (0.02)                 | –                | 0.24 (0.03)       | 2.7 (0.1)  | Y               |                                      |
| yTHCBMS1 + 0.5 µg/mL doxycycline | 0.73 (0.15)                 | 2.39 (0.54)      | 8.00 (2.50)       | 0.43 (0.14) | 2.2 (0.3)      | Y                                    |
| srb5Δ                           | 0.13 (0.03)                 | –                | –                 | 0.39 (0.07) | 1.5 (0.1)      | Y                                    |
| gcn5Δ                           | 0.23 (0.04)                 | –                | –                 | 0.16 (0.02) | 6.0 (0.5)      | Y                                    |
| spt3Δ                           | 0.91 (0.07)                 | 1.36 (0.28)      | 4.70 (1.11)       | 0.18 (0.03) | 4.1 (0.4)      | Y                                    |

**Yeasts were transformed with a plasmid expressing FPS1-HA3, S'Δ1-43-FPS1-HA3 or S'Δ1-215-FPS1-HA3 as indicated. Single transformants were cultured in shake flasks in 2 x CBS lacking histidine to maintain the plasmid. Cells were harvested just before the diauxic shift by monitoring residual glucose concentration (cultures had a typical biomass yield of 0.9 g/L; A600 ~ 4). Fps1 yields (µg/L) were determined with reference to a BSA standard curve [38]; a yield of 12.14 µg/L may also be expressed as 13.49 µg/g dry cell weight. The ratio of monosome to polysome peaks (M:P) was calculated from polysome profiles obtained by fractionation on a 10–50% sucrose gradient. To determine GCN4 expression, yeast cells were transformed with plasmid B1805 [31] or for yTHCBMS1, B1805-HS. Yeast cells expressing GCN4-LacZ were cultured in shake flasks, harvested at A600 ~ 1 and β-galactosidase levels were determined using ONPG. β-Galactosidase levels are expressed relative to wild-type control cells. For analysis of eIF2α phosphorylation, cells were cultured in the absence (amino-acid-starved cells) or presence (control cells) of amino acids and cells lysates were analysed by immunoblot using anti-eIF2α and anti-phospho-eIF2α antibodies as previously described [39]. All data shown are the mean of biologically-independent, triplicate determinations; where relevant, the standard error of the mean (SEM) is shown in parentheses. Y indicates yes, N indicates no and – indicates that the experiment was not performed.
uORF (uORF1) is 34 codons in length and 154 nucleotides upstream of the FPS1 ORF. The second uORF (uORF2) is 3 codons in length, similar to those found in GCN4, but 5 nucleotides from the FPS1 ORF; uORF2 occurs natively in the yeast genome in the 5'UTR of FPS1 [25].

Since translation can be controlled at ORFs by uORFs [24, 26], we reasoned that the presence of FPS1 uORFs might limit recombinant Fps1 yields. Two new vectors were therefore created (Table 2), one to delete uORF1 (pYX222-5'D1-43-FPS1-HA3) and the second to delete both uORFs (pYX222-5'D1-215-FPS1-HA3). To investigate the impact of deleting these uORFs, Fps1 yields were analysed in wild-type cells, the yTHCBMS1 strain (supplemented with 0.5 μg/mL doxycycline) and the spt3Δ strain, each transformed with pYX222-FPS1-HA3, pYX222-5'D1-43-FPS1-HA3 or pYX222-5'D1-215-FPS1-HA3. Table 1 shows Fps1 yields compared with wild-type cells transformed with pYX222-FPS1-HA3 (the control condition). When uORF1 was deleted from the FPS1 transcript, the yield of Fps1 increased 11 times in wild-type yeast relative to the control; when both uORFs were removed, the yield increased 607 times relative to the control. When two uORFs were present in the FPS1 transcript, yTHCBMS1 supplemented with 0.5 μg/mL doxycycline increased Fps1 yield 37 times relative to the control. Deleting uORF1 increased Fps1 yields 120 times; deleting both increased yields 400 times. When both uORFs are present, yield improvement was 46 times higher than control for spt3Δ cells, 68 times higher when the first uORF was deleted and 235 times higher when both uORFs were deleted. Notably, when both uORFs were deleted, wild-type cells produced the highest yields of Fps1 compared to any other combination of strain and vector. Overall, these data suggest that (a) in wild-type cells, the expression of FPS1 is less than 2% of its maximum value in the presence of native uORF2 (12.14 µg/L or 13.49 µg/g dry cell weight, Table 1) and (b) that our four high-yielding, bespoke strains can circumvent both uORFs (Table 1; Figs. 1, 3).

High-yielding yeast strains bypass uORFs by leaky scanning to produce Fps1

Translational control by uORFs is known to occur through two main mechanisms: re-initiation or leaky scanning [27]. As exemplified by the case of GCN4, re-initiation occurs when, after completing translation of
Table 2 Sequences upstream of the FPS1 ORF in the four expression constructs used in this study

| Vector name | Sequence (sense strand; 5' to 3') | Reference |
|-------------|----------------------------------|-----------|
| pXY222-FPS1-HA3 | ...TAACATACAAAAAACACTAACAGAAATTCAGATCGATCTATAATGAGTAATCCTCAAAAAGCTCTAAACGACTT ... | [8]; Additional file 1: Figure S2 |
| pXY222-5’Δ1-43-FPS1-HA3 | ...TAACATACAAAAAACACTAACAGAAATTCAGATCGATCTATAATGGCTCTAAACGACTT ... | This study; Additional file 1: Figure S3 |
| pXY222-5’Δ1-43-uORF2-stop-removed-FPS1-HA3 | ...TAACATACAAAAAACACTAACAGAAATTCAGATCGATCTATAATGGCTCTAAACGACTT ... | This study; Additional file 1: Figure S4 |
| pXY222-5’Δ1-215-FPS1-HA3 | ...TAACATACAAAAAACACTAACAGAAATTCAGATCGATCTATAATGGCTCTAAACGACTT ... | This study; Additional file 1: Figure S5 |

Four vectors were used in this study; their construction is described in the "Methods" section and their sequences are given as Additional file 1, as indicated. The sequences directly upstream of the FPS1 ORF (in bold), are shown. The uORFs are underlined; uORF1 is 34 codons long; uORF2, which is found natively in the genomic FPS1 sequence, is 3 codons long. The two additional cytosines in pXY222-5’Δ1-43-uORF2-stop-removed-FPS1-HA3 are italicised and the consequent extension of uORF2 is highlighted in grey.
more consistent with a leaky scanning mechanism than re-initiation. Furthermore, only 5 nucleotides separate uORF2 from the \( FPS1 \) ORF; Kozack previously demonstrated that re-initiation is inefficient if fewer than 79 nucleotides separate uORF and ORF (in that study the ORF encoded preproinsulin) [30]. In our strains it is possible that leaky scanning may be a consequence of defects such as subunit joining [23] or start codon recognition [31]. Others have also demonstrated that leaky scanning of uORFs occurs during times of stress, mediated by eIF1 phosphorylation and resulting in the translation of a subset of mRNAs important for survival or apoptosis [28, 29]. Notably yields of recombinant GFP, horseradish peroxidase (HRP) and \( A_{2a} \)R can be improved in the high-yielding yeast strains suggesting more generic benefits (Table 3).

**Implications for the regulation of Fps1**

Fps1 facilitates glycerol efflux from yeast cells under conditions of hypo-osmotic stress; the \( fps1\Delta \) strain is sensitive to osmotic down-shifts [32, 33]. The regulation of Fps1 by gating is known to be mediated by its amino- and carboxy-terminal regions [34, 35], but it is not known whether other mechanisms are also involved. In order to investigate a physiological role for uORF2, we aligned the 5′UTR of \( FPS1 \), using the alignment facility of the Saccharomyces Genome Database (SGD; www.yeastgenome.org/cgi-bin/FUNGI/ShowAlign), which revealed that the yeasts \( S.\ mikatae \) and \( S.\ paradoxus \) also have a similar uORF in their \( FPS1 \) gene. We next confirmed that the Fps1 produced from our expression plasmids (Table 2) was functional. Figure 5 shows that Fps1 produced from all three vectors (with 0, 1 or 2 uORFs; Table 2) rescues the well-established osmosensitive phenotype of \( fps1\Delta \) cells [32, 33], and that sufficient Fps1 is produced in the presence of the uORF(s) for this purpose. The specific growth rates of the transformants were calculated from corresponding liquid cultures and were found to be consistent with the qualitative serial spot assays (Fig. 5). Notably, the transformants (WT and \( fps1\Delta \)) with no uORF grew marginally more slowly than those with one or two uORFs suggesting that unregulated translation of \( FPS1 \) may be detrimental to yeast cells (Fig. 5). This observation was also seen in cultures of yTHCBMS1 and \( spt3\Delta \). The growth rate decreased by 12.5 and 6.9% in yTHCBMS1 and \( spt3\Delta \) respectively when there were no uORFs in the \( FPS1 \) transcript compared to the presence of two uORFs (Fig. 5).

The translational state of yeast cells during hyper-osmotic shock was analysed because Fps1 is involved in glycerol efflux under these conditions. The yields of Fps1 produced in wild-type cells from the vector pYX222-5′/Δ1-43-\( FPS1\)-HA3 were analysed after 15, 30, 60 and 90 min of hypo-osmotic shock. Notably, the Fps1 yield and the translational state of the cells (as determined by polysome analysis) were indistinguishable from control (unshocked) cells (data not shown), suggesting that the primary functional role of Fps1 as a glycerol efflux channel following osmotic down-shift is not translationally controlled by uORF2.

Translation initiation blocks are observed in yeast following hyper-osmotic shock [36], which we confirmed for wild-type strain BY4741 (Table 4); the block was immediate and alleviated after 90 min, as previously described [36]. In the presence of native uORF2, Fps1 yields increased ~threefold following hyperosmotic shock compared to control, unshocked cells (Table 4). The yield decreased as the initiation block was alleviated (Table 4). When both uORFs were removed (using pYX222-5′/Δ1-215-\( FPS1\)-HA3) there was no increase in

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**Table 3** Yields of recombinant GFP, HRP and \( A_{2a} \)R can be improved in the high-yielding yeast strains

| Yeast strain                | GFP yield (AU) | HRP yield (U/mL) | \( A_{2a} \)R yield (pmol/mg) |
|----------------------------|----------------|-----------------|-------------------------------|
| Wild-type (BY4741)         | 1.0 (0.2)      | 5.9 (0.7)       | 7.4 (0.7)                     |
| yTHCBMS1                   | 0.9 (0.2)      | 28.1 (4.0)      | –                             |
| yTHCBMS1 +0.5 \( \mu \)g/mL doxycycline | 1.3 (0.1) | –              | 9.6 (1.3)                     |
| yTHCBMS1 +10 \( \mu \)g/mL doxycycline | 4.7 (0.8) | –              | 16.1 (1.7)                    |
| srb5Δ                      | 1.6 (0.3)      | –               | –                             |
| gcn5Δ                      | 1.5 (0.4)      | –               | –                             |
| spt3Δ                      | 0.8 (0.1)      | 16.0 (2.0)      | –                             |

Yeast cells were transformed with a plasmid expressing green fluorescent protein (GFP; Additional file 1: Figure S7), horseradish peroxidase (HRP; Additional file 1: Figure S8) or the adenosine \( A_{2a} \) receptor (\( A_{2a} \)R; Additional file 1: Figure S9). Single transformants were cultured in shake flasks in 2× CBS lacking histidine (to maintain the plasmid). GFP yields were determined by fluorometry of the culture supernatant as previously described [6], but with cells harvested from 50 mL cultures at 25° C. Data for \( A_{2a} \)R were published previously [6] and are reported as B\( _{max} \) values (pmol/mg membrane). All data shown are the mean of biologically-independent, triplicate determinations; the standard error of the mean (SEM) is shown in parentheses.
Fps1 production following hyperosmotic shock (data not shown). A physiological role for uORF2 may therefore be to control the levels of Fps1 (to 2% of its maximum possible value) by keeping it low during normal conditions (Table 1), but increasing it as part of a stress response (Table 4). Notably, high levels of Fps1 may be detrimental to the cell under normal conditions because Fps1 has been shown to be involved in the uptake of cytotoxic compounds such as arsenite and acetic acid that lead to enzyme inhibition or apoptosis, respectively [12, 37].

**Conclusions**

In common with many membrane proteins, the recombinant production of Fps1 (a facilitator for glycerol efflux from yeast cells [32, 33]) has proved challenging. We previously identified four strains of *S. cerevisiae* (*spt3Δ, srb5Δ, gcn5Δ* and *yTHCBMS1* supplemented with 0.5 μg/mL doxycycline) that specifically produced Fps1 in sufficient yield for further study [6]. However, the mechanism underpinning their high-yielding phenotype was unknown. A significant finding emerging from this work is that strains that are high-yielding for Fps1 may have translation initiation blocks (Fig. 1), exhibit constitutive phosphorylation of eIF2α (Fig. 3) and have increased expression of *GCN4* (Table 1). Our data suggest that the strains can bypass, via a leaky scanning mechanism, a native uORF that we identified in the *FPS1* gene (see http://doi.org/10.17036/researchdata.aston.ac.uk.00000176; Additional file 1: Figures S2, S4). While the strains were selected for improved expression of Fps1 and so give the best yields for that target, a second significant finding is that these strains can also be used to increase the yields of other heterologous proteins (Table 3).

A third significant finding of this work is the identification of a previously-unknown regulatory element in the *FPS1* gene. The discovery of a native uORF suggests that Fps1 may be regulated translationally. It is known that regulation of Fps1 function occurs through gating by its amino- and carboxy-terminal regions and that unregulated Fps1 renders yeast cells sensitive to osmotic [34, 35], arsenite [37] and acetic acid [12] stresses. A physiological role for uORF2 may therefore be to control the levels of Fps1 (to a little as 2% of its maximum possible value) during normal conditions (Table 1), but increase it as part of a stress response (Table 3).

**Methods**

**Strains and vectors**

The haploid *S. cerevisiae* BY4741 strain (MATα, *ura3Δ0, leu2Δ0, met15Δ0, his3Δ1*) is the parental strain of the
Wild-type yeast cells were transformed with a plasmid expressing 5′Δ1-43-FPS1-HA3; uORF2 was therefore present (Table 1). Single transformants were cultured in shake flasks in 2× CBS lacking histidine (to maintain the plasmid). At A600 = 4, cultures were divided into two, harvested by centrifugation and the pellets suspended in either 2× CBS (no shock) or 2× CBS supplemented with 0.8 M NaCl (hyperosmotic shock). Fps1 yields were determined by immunoblot and analyzed using ImageJ [6] after 15, 30, 60 and 90 min; values are reported relative to control conditions (wild-type cells after 15 min in 2× CBS). All data shown are the mean of biologically-independent, triplicate determinations; where relevant, the standard error of the mean (SEM) is shown in parentheses. A two-tailed paired t test was used to compare Fps1 yields or polysome ratios of shocked cells with the corresponding values for control cells at the equivalent time point.

**Table 4** Fps1 yields following hyperosmotic shock

| Osmotic shock | Time post-shock (min) | Fps1 yield relative to control | M:P ratio |
|---------------|-----------------------|-------------------------------|-----------|
| No shock (2× CBS) | 15 | 1.00 (0.61) | 0.24 (0.00) |
| | 30 | 0.59 (0.17) | 0.22 (0.03) |
| | 60 | 0.90 (0.48) | 0.14 (0.01) |
| | 90 | 1.21 (0.63) | 0.10 (0.00) |
| Hyperosmotic shock (2× CBS, 0.8 M NaCl) | 15 | 3.03 (0.21)* | 1.08 (0.14)** |
| | 30 | 2.62 (0.83) | 1.24 (0.16)*** |
| | 60 | 1.73 (0.29) | 0.29 (0.03) |
| | 90 | 2.08 (0.58) | 0.21 (0.02)* |

Deletion mutants, gen5Δ, spt3Δ, srb5Δ, rpl22aΔ, rpl31aΔ, ssf1Δ and nop12Δ (from the EUROSCARF collection) and the yTHCBMS1 strain (Open Biosystems) used in this study, and as such provided the wild-type control. Expression of BMS1 in the yTHCBMS1 strain is under the control of a doxycycline-repressible promoter; we have previously shown that supplementing the strain with 0.5 μg/mL doxycycline gives maximum Fps1 yields [6]. pYX222-FPS1-HA3 (found to contain two uORFs; uORF1 and uORF2) was described previously [8]. pYX222-5′Δ1-43-FPS1-HA3 (containing only the native uORF, uORF2; Table 1) was made by digesting pYX222 [8] with EcoRI and HindIII to remove the Kozak sequence and inserting a FPS1 fragment with a truncated 5′UTR starting 216 bp upstream of the FPS1 ORF. pYX222-5′Δ1-215-FPS1-HA3 (containing no uORFs; Table 1) contained a FPS1 fragment with 1 bp upstream of the FPS1 ORF. pYX222-5′Δ1-43-uORF-stop-removed-FPS1-HA3 (Table 1) was made by inserting two cytosines before the stop codon of uORF2 to cause a frame shift (forward primer: CCCCCCGAATTTCATGCACCTTTAAAAGA-CATG). The vector B1805 (the kind gift to Mark P. Ashe of Alan Hinnebusch) contains the GCN4 promoter and complete 5′UTR; LacZ is inserted into the BamHI site of the GCN4 ORF [31]. For experiments requiring nutrient selection on histidine-deficient medium, B1805-HIS was used in which the URA3 gene was replaced with the HIS3 gene by restriction digest with Xmal and KasI. Yeast cells were transformed using the lithium acetate method [8].

**Culture conditions**

Yeast strains were cultured in shake flasks in either YPD or 2× CBS. YPD contains 1% yeast extract, 2% bacto peptone and 2% glucose. 1 L of 2× CBS is composed of 10 g/L ammonium sulfate, 6 g/L potassium dihydrogen phosphate, 1 g/L magnesium sulfate heptahydrate supplemented with 2% glucose, 2× DO solution, 100 mM MES, 2 mL/L each of trace element solution and vitamin stock solution. 10× DO solution (/L) was composed of 200 mg adenine hemisulfate, 200 mg L-arginine hydrochloride, 300 mg L-isoleucine, 1000 mg L-leucine, 300 mg L-lysine hydrochloride, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 200 mg L-tryptophan, 300 mg L-tyrosine, 200 mg uracil, 1500 mg L-valine. 250 mL trace element solution was composed of 3.75 g EDTA, 1.125 g zinc sulfate heptahydrate, 0.25 g magnesium chloride tetrahydrate, 0.075 g cobalt (II) chloride hexahydrate, 0.075 g copper (II) sulfate pentahydrate, 0.1 g sodium molybdenum dehydrate, 1.125 g calcium chloride dehydrate, 0.75 g iron (II) sulfate heptahydrate, 0.25 g boric acid and 0.025 g potassium iodide. 250 mL vitamin stock solution was composed of 0.0125 g biotin, 0.25 g calcium-D-pantothenate, 0.25 g nicotinic acid, 6.25 g myo-inositol, 0.25 g thiamine hydrochloride, 0.25 g pyridoxine hydrochloride and 0.05 g D-amino benzoic acid; pH was maintained at 6.5.

**Cell membrane preparation and Fps1 yield analysis**

Yeast strains were cultured in shake flasks in 2× CBS. Cells were harvested prior to the diauxic shift as determined by residual glucose concentration readings using a Roche Accu-Chek Active diabetes monitor. Cells were pelleted at 5000×g, 4 °C for 3 min, the supernatant discarded and the pellet frozen at −20 °C until required. The pellet was washed once, suspended in 1 mL breaking buffer (50 mM Na2HPO4, 50 mM NaH2PO4, 2 mM EDTA pH 7.4, 100 mM NaCl, 5% glycerol) and transferred to a breaking tube containing 1 mL glass beads. Protease inhibitor cocktail IV (Calbiochem) was added at a dilution factor of 1:500 (typically 2 μL). Cells were broken...
in a cold TissueLyser (Qiagen) at 50 Hz for 10 min. The cell lysate was cleared by centrifugation at 25,000 × g for 15 min. Membranes were harvested by centrifugation at 190,000 × g for 1 h. The membrane pellet was suspended in 100 μL Buffer A (20 mM HEPES, 50 mM NaCl, 10% glycerol, pH 7) and stored at −20°C until required. Total protein concentration was determined by BCA assay. Fps1 yield was determined by a densitometric analysis of silver-stained SDS-PAGE gels as previously described [38]. The Fps1 band in the membrane fraction was compared with a series of known amounts of bovine serum albumin (0.75, 0.5, 0.25, 0.125, 0.05, 0.025 and 0.01 μg) on the same gel. The presence and location of the Fps1 protein band was confirmed by immunoblot using a mouse anti-HA antibody (Sigma); samples were diluted serially to allow accurate comparisons within the linear range of the standard curve. All determinations were done in at least triplicate (n = 3). The intensity of protein bands was determined by quantitative densitometry using ImageJ (http://rsb.info.nih.gov/ij/).

**GFP and HRP yield measurements**

GFP yields from recombinant yeast cultures were assayed as previously described [6], except 25 mL cultures were harvested at A<sub>600</sub> = 4 (just before the diauxic shift) rather than 50 mL cultures at A<sub>600</sub> = 1. Briefly, samples were withdrawn and the cells pelletted at 5000 × g, 4°C for 5 min and the supernatant collected. 200 μL supernatant were loaded in triplicate in a black Nunc MaxiSorp 96-well plate and the fluorescence recorded on a SpectraMax Gemini XS plate reader (Molecular Devices, Wokingham, UK) with excitation and emission wavelengths of 390 and 510 nm respectively, and a cut-off of 495 nm. Doxycycline fluorescence accounted for less than 5% of the signal up to concentrations of 10 μg/mL.

HRP yields from recombinant yeast cultures, grown as described for GFP [6], using a commercial kit according to the manufacturer's instructions (EY Laboratories, Inc). One unit of HRP activity decomposes 1 μmol of peroxide/min at 25°C.

**Polysome analysis**

Polysome analysis was done using an improved methodology compared to that used in our previous study [6]; this resulted in higher-quality profiles. Yeast strains were cultured in shake flasks in YPD to A<sub>600</sub> = 0.5–1, 10 mg cycloheximide were added per 100 mL culture and the cells were incubated for a further 15 min at 30°C. Cultures were instantly cooled by pouring over ice and the cells were harvested at 5300 × g for 5 min. All subsequent stages were done at 4°C in RNAase-free medium. Cells were washed in freshly-prepared lysis buffer (10 mM Tris–HCl pH7.5, 0.1 M NaCl, 30 mM MgCl<sub>2</sub>, 50 μg/mL cycloheximide, 200 μg/mL heparin, 0.2% diethyl-pyrocarbonate) and harvested at 5300 × g for 5 min. The pellet was suspended in 250 μL lysis buffer and transferred to a 2 mL breaking tube containing 1 mL glass beads. Cells were broken in a cold TissueLyser (Qiagen) at 50 Hz for 3 min, the cell debris removed by centrifugation (17,000 × g for 15 min) and the supernatant stored at −80°C until required. Supernatant containing 150 μg total nucleic acid was loaded onto a 10–50% sucrose gradient made in gradient buffer (50 mM NH₄Cl, 50 mM Tris-OAc pH7, 12 mM MgCl₂) and centrifuged at 25,000 × g for 2 h in a SW41 rotor (Beckman Instruments). Gradients were collected continuously from the top using a Biocomp Gradient profiler at A<sub>254</sub>. Areas under the curves were analysed using ImageJ.

**GCN4 expression analysis**

Yeast cells were transformed with B1805 (or B1805-HIS for yTHCBMSI) and grown in 2× CBS (with corresponding nutrient selection) to A<sub>600</sub> ~ 1. Cells were harvested and washed in sterile water at 5300 × g for 3 min. The pellet was suspended in 500 μL breaking buffer (100 mM Tris–HCl pH 8, 1 mM dithiothreitol, 20% glycerol), transferred to a 2 mL breaking tube containing 1 mL glass beads and 1:500 dilution of protease inhibitor cocktail IV (Calbiochem). Cells were lyzed in a cold TissueLyser (Qiagen) at 50 Hz for 3 min. Cell debris was removed by centrifugation at 17,000 × g for 15 min; 100 μL cell lysate was added to 900 μL Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>·7H₂O, 40 mM NaH<sub>2</sub>PO<sub>4</sub>·H₂O, 10 mM KCl, 1 mM MgSO<sub>4</sub>·7H₂O pH 7), incubated at 28°C for 5 min and the reaction started by adding 200μL 4 mg/mL o-nitrophenyl-β-d-galactopyranoside (ONPG) in Z buffer. The reaction was incubated at 28°C until a yellow colouration appeared (typically after 15–30 min) and was stopped by adding 500 μL Na<sub>2</sub>CO<sub>3</sub>. β-Galactosidase levels are expressed relative to wild-type.

**eIF2α phosphorylation analysis**

For analysis of eIF2α phosphorylation, cells were cultured in the absence (amino-acid-starved cells) or presence (control cells) of amino acids and cells lysates were analysed by immunoblot using anti-eIF2α and anti-phospho-eIF2α antibodies as described previously [39].

**Phenotypic analysis**

Fps1 facilitates glycerol efflux from yeast cells under conditions of hypo-osmotic stress; the fps1Δ strain is sensitive to osmotic down-shifts [32, 33]. To assess the phenotypic consequences of expressing the different FPS1-containing constructs shown in Table 1, yeast cells were exposed to osmotic shock. The consequences of hyperosmotic shock were analyzed by culturing transformed cells in 400 mL
2× CBS without histidine to A_{600} ~ 4. Cultures were then divided into two, harvested at 5300×g for 5 min at 4 °C and suspended in either 2× CBS (no shock) or 2× CBS supplemented with 0.8 M NaCl (hypersomotic shock) that has been pre-warmed to 30 °C. The A_{600} was adjusted to ~4 and 50 mL aliquots were sampled after 15, 30, 60 and 90 min. Cells were spotted (10 μL spots) in 10-fold serial dilutions (made using the same medium in which the cells were originally suspended) onto YNB agar plates with or without 0.8 M NaCl. For polysome analysis of hypersomotically-shocked cells, cultures were grown to A_{600} ~ 1, the culture was divided into two and the harvested cells were suspended in either 2× CBS or 2× CBS supplemented with 0.8 M NaCl. 50 mL samples were harvested after 15, 30, 60 and 90 min and processed as described under “Polysome analysis” section. The consequences of hypo-osmotic shock were analyzed by cultivating transformed cells to A_{600} ~ 2 in 2× CBS without histidine and supplemented with 1 M sorbitol. Cells were spotted (10 μL spots) in 10-fold serial dilutions (made using the growth medium) onto YNB plates either with or without 1 M sorbitol. Plates containing 1 M sorbitol were control plates whilst those without sorbitol caused hypo-osmotic shock.

Additional file

**Additional file 1.** Additional figures, also see http://doi.org/10.17036/researchdata.aston.ac.uk.00000176.

**Abbreviations**

ONPG: o-nitrophenyl-β-D-galactopyranoside; ORF: open reading frame; PIC: pre-initiation complex; uORF: upstream open reading frame; TC: ternary complex; UTR: untranslated region.

**Authors’ contributions**

SPC, RAJD, NB, SRG, MPA and RMB conceived and designed the experiments. SPC, RAJD, DS and NB performed the experiments. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

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