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# Ste20 and Cla4 modulate the expression of the glycerol biosynthesis enzyme Gpd1 by a novel MAPK-independent pathway

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ABSTRACT

p21-activated kinases (PAKs) are important signalling molecules with a wide range of functions. In budding yeast, the main PAKs Ste20 and Cla4 regulate the response to hyperosmotic stress, which is an excellent model for the adaptation to changing environmental conditions. In this pathway, the only known function of Ste20 and Cla4 is the activation of a mitogen-activated protein kinase (MAPK) cascade through Ste11. This eventually leads to increased transcription of glycerol biosynthesis genes, the most important response to hyperosmotic shock. Here, we show that Ste20 and Cla4 not only stimulate transcription, they also bind to the glycerol biosynthesis enzymes Gpd1, Gpp1 and Gpp2. Protein levels of Gpd1, the enzyme that catalyzes the rate limiting step in glycerol synthesis, positively correlate with glucose availability. Using a chemical genetics approach, we find that simultaneous inactivation of *STE20* and *CLA4* reduces the glucose-induced increase of Gpd1 levels, whereas the deletion of either *STE20* or *CLA4* alone has no effect. This is also observed for the hyperosmotic stress-induced increase of Gpd1 levels. Importantly, under both conditions the deletion of *STE11* has no effect on Gpd1 induction. These observations suggest that Ste20 and Cla4 not only have a role in the transcriptional regulation of *GPD1* through Ste11. They also seem to modulate *GPD1* expression at another level such as translation or protein degradation.



## 1. Introduction

The p21-activated kinases (PAKs) are highly conserved effectors of the Rho GTPases Cdc42 and Rac. Three members of this family of important signalling molecules can be found in the budding yeast *Saccharomyces cerevisiae*: Ste20, Cla4 and Skm1 [1]. Very little is known about Skm1. In contrast, Ste20 and Cla4 have a wide range of functions, some of them overlapping [1–3]. Ste20 activates three different mitogen-activated protein kinase (MAPK) cascades regulating the hyperosmotic stress response, filamentation and mating [1,4–7]. Importantly, these pathways are also excellent models for the adaptation to changing environmental conditions. Triggering these MAPK cascades eventually leads to changes in gene expression. In all three MAPK pathways, Ste20 phosphorylates Ste11, the most upstream component of these signalling cascades [8,9]. In the hyperosmotic stress response MAPK cascade, Ste11 is also activated by Cla4 [3]. Stimulation of this pathway results in the translocation of the MAPK Hog1 into the nucleus where it induces expression of osmo-responsive genes through several transcription factors [10,11]. One of the most important adaptive mechanism to hyperosmolarity is the biosynthesis of the osmolyte glycerol [10–12].

Glycerol is synthesized from the glycolytic intermediate dihydroxyacetone phosphate in two steps (Fig. 1A). The two homologous NADH-dependent glycerol 3-phosphate dehydrogenases Gpd1 and Gpd2 convert dihydroxyacetone phosphate to glycerol 3-phosphate [13–15], and the two homologous glycerol 3-phosphatases Gpp1 and Gpp2 metabolize glycerol 3-phosphate to glycerol [16]. Despite their similarities the homologous enzymes only have partially overlapping functions. Under conditions of high osmolarity, glycerol is predominantly synthesized by Gpd1 and Gpp2 [13,14,16,17], whereas in the absence of oxygen glycerol synthesis is catalysed by Gpd2 and Gpp1 [18–20].

Hyperosmotic stress leads to the Hog1-dependent upregulation of expression of *GPD1*, *GPP2* and hundreds of other genes [10,11,14,16]. However, out of these only *GPD1* is essential for osmoadaptation, and the upregulation of *GPD1* and *GPP2* is sufficient for efficient adaptation to hyperosmotic stress, highlighting the central role that glycerol biosynthesis plays in osmoadaptation [12].

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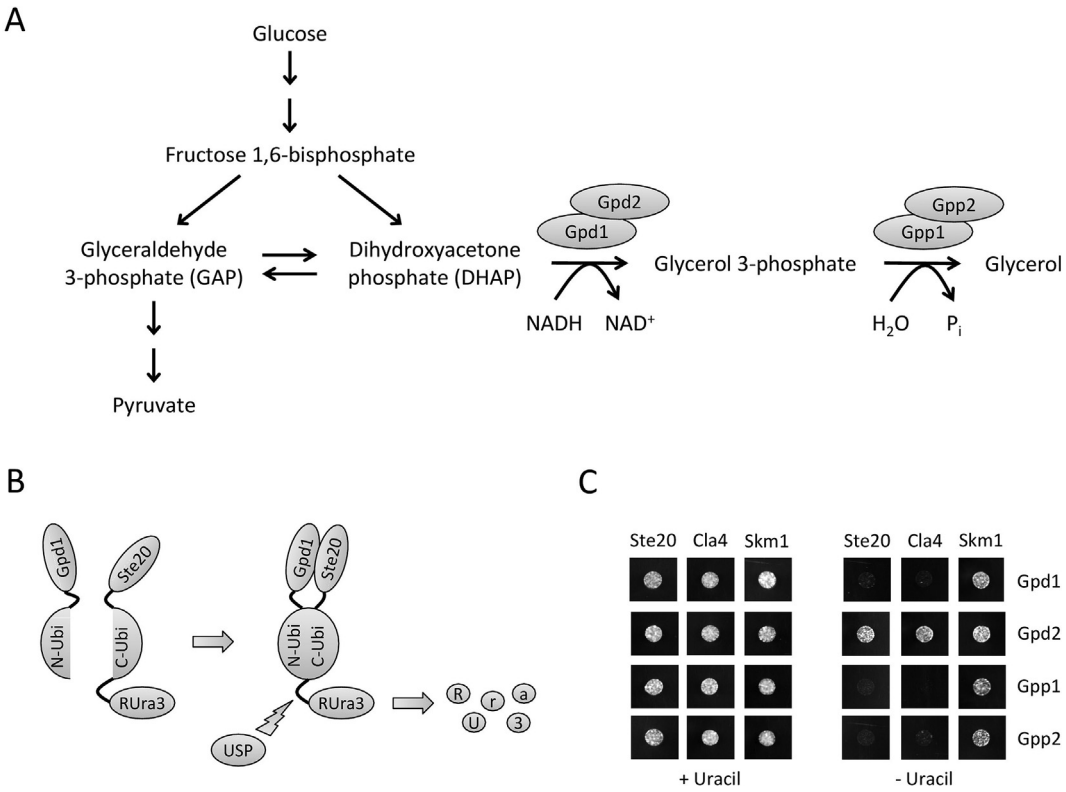


**Fig. 1.** Ste20 and Cla4 bind to glycerol biosynthesis enzymes. (A) Glycerol biosynthesis in budding yeast. (B) The split-ubiquitin system. See text for details. USP, ubiquitin-specific proteases. (C) Ste20 and Cla4 both bind to Gpd1, Gpp1 and Gpp2. Cells of the indicated plasmid combinations were either spotted onto plates lacking uracil to monitor protein-protein interactions, or onto plates supplemented with uracil as control.

In this study, we examined further links between glycerol biosynthetic enzymes and the PAKs Ste20 and Cla4.

## 2. Materials and methods

### 2.1. Yeast strains and plasmids

All yeast strains and plasmids used in this study are listed in Table 1. Yeast strains were constructed using PCR-amplified cassettes [21,22]. For the filamentation experiment, cells of the Σ1278b background were used [23]. For all other experiments, cells of the YPH499 background were used [24]. The split-ubiquitin constructs were derived from the plasmid pADNX [25]. The YCplac22-*cla4-as3* construct was kindly provided by Eric Weiss [26].

### 2.2. Growth conditions and inhibitor treatment

Yeast strains were either grown in 1% yeast extract, 2% peptone, 2% glucose (YPD) or synthetic complete (SC) medium.

For the analysis of protein levels during filamentation, cells were

**Table 1**
Strains and plasmids used in this study.

| Strain | Genotype | Source |
|---|---|---|
| IJY1 | YPH499 *GPD1-3HA-His3MX6* | This study |
| IJY3 | YPH499 *GPD1-3HA-His3MX6 ste20Δ::KanMX6* | This study |
| IJY5 | YPH499 *GPD1-3HA-His3MX6 cla4Δ::hphNT1* | This study |
| IJY20 | YPH499 *GPD1-3HA-His3MX6 ste20Δ::KanMX6 cla4Δ::hphNT1* YCplac22-*cla4-as3* | This study |
| IJY21 | YPH499 *GPD1-3HA-His3MX6 ste11Δ::KanMX6* | This study |
| PPY966 | *MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52* | [23] |
| YPH499 | *MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1* | [24] |
| **Plasmid** | **Genotype** | **Source** |
| pADNX | 2 μm, *LEU2*, *pADH1-NUbiquitin-HA* | [25] |
| pIJ6 | pADNX carrying *GPP1* | This study |
| pML68 | pRS313 carrying *pMET25-SKM1-CUbiquitin-RURA3* | This study |
| pML70 | pRS313 carrying *pMET25-CLA4-CUbiquitin-RURA3* | This study |
| pRS313 | *CEN*, *HIS3* | [24] |
| pTH197 | pRS313 carrying *pMET25-STE20-CUbiquitin-RURA3* | [23] |
| pTH442 | pADNX carrying *GPP2* | This study |
| pTH445 | pADNX carrying *GPD1* | This study |
| pTH468 | pADNX carrying *GPD2* | This study |
| YCplac22-*cla4-as3* | *CEN*, *TRP1*, carrying *cla4-as3* | [26] |

grown to exponential phase in SC medium. Cells were washed with water and $10^5$ cells were plated onto SC medium lacking glucose and incubated for 14 h at 30 °C. Cells were then scraped from a plate for protein analysis.

To induce hyperosmotic stress, NaCl was added to the medium to a final concentration of 0.8 M. For the inhibition of Cla4 activity, cells were incubated for 2 h with 1-tert-butyl-3-(naphthalen-1-ylmethyl)-1H-pyrazolo [3,4-d]pyrimidin-4-amine (1NM-PP1) to a final concentration of 10 μM. To determine the half-life of Gpd1 protein, cycloheximide was added to exponentially growing cells in YPD to a final concentration of 100 μg/ml.

### 2.3. Split-ubiquitin technique

$10^4$ cells carrying the split-ubiquitin plasmids were spotted on SC plates lacking histidine and leucine to select for the plasmids or onto SC plates lacking histidine, leucine and uracil to monitor protein-protein interactions. The plates also lacked methionine and cysteine to induce expression of the *STE20*, *CLA4* and *SKM1* fusion genes under control of the *MET25* promoter.

### 2.4. Cell extracts and immunoblotting

Generation of cell extracts and immunoblotting was performed as described previously [27].

## 3. Results and discussion

### 3.1. Ste20 and Cla4 bind to glycerol biosynthesis enzymes

Using the split-ubiquitin technique [28,29], we have previously shown that Ste20 interacts with many enzymes of glycolysis, the pentose phosphate pathway and gluconeogenesis (I. M. Joshua, M. Lin, A. Mardjuki, A. Mazzola and T. Höfken, manuscript in preparation). Here, we examined whether Ste20 also binds to Gpd1, Gpd2, Gpp1 and Gpp2 which catalyze glycerol biosynthesis from a glycolytic metabolite (Fig. 1A) employing the split-ubiquitin method. For this analysis, we also included the other two PAKs, Cla4 and Skm1. The split-ubiquitin technique detects protein-protein interactions *in vivo* using artificially separated N-terminal and C-terminal halves of ubiquitin (Fig. 1B) [28]. If two proteins, which are attached to the N-terminal and C-terminal halves, interact, a native-like ubiquitin may assemble. Ubiquitin-specific proteases recognize the reconstituted ubiquitin and cleave off the reporter protein RUra3, which is linked to the C-terminal domain of ubiquitin [29]. RUra3 is a modified version of the enzyme Ura3 which is essential for uracil biosynthesis. The freed RUra3 is rapidly degraded by proteases of the N-end rule pathway. Interaction between two proteins fused to the N-terminal and C-terminal halves of ubiquitin, therefore, results in non-growth on medium lacking uracil. Using this technique, we identified Ste20 and Cla4 as binding partners of Gpd1, Gpp1 and Gpp2 (Fig. 1C). These interactions were quite specific since Gpd1, Gpp1 and Gpp2 did not bind to Skm1, and Ste20 and Cla4 did not associate with Gpd2 (Fig. 1C). This specificity is consistent with other observations. Ste20 and Cla4 have overlapping functions and in some cases even bind to the same proteins [1–3]. In contrast, very little is known about Skm1. However, there seems to be rather little functional overlap with Ste20 and Cla4 [1]. It is, therefore, not surprising that Ste20 and Cla4 but not Skm1 bound to Gpd1, Gpp1 and Gpp2.

Interestingly, Gpd2 did not bind to Ste20 and Cla4. Gpd2 shares only 69% sequence identity with Gpd1 [15], and both proteins have distinct functions. Gpd1 has functions under aerobic conditions including hyperosmotic stress response, whereas Gpd2 is required in the absence of oxygen [13–15,17,18]. In contrast, Gpp1 and Gpp2 sequences are 95% identical and they have several overlapping functions including osmoadaptation [16,20]. Our data, therefore, suggest that Ste20 and Cla4 might have a function associated with Gpd1 such as aerobic glycerol biosynthesis, in particular in response to hyperosmotic stress, rather than a process associated with Gpd2 such as anaerobic glycerol synthesis.

The reaction catalysed by Gpd1 is not only required for glycerol synthesis. The oxidation of NADH to $NAD^+$ in this reaction plays an important role in the maintenance of a cytoplasmic redox balance and peroxisomal lysine biosynthesis [30,31]. Furthermore, glycerol 3-phosphate formed here is also used for the biosynthesis of glycerolipids (triacylglycerols and glycerophospholipids) [32]. However, since Ste20 and Cla4 also bind to Gpp1 and Gpp2 which catalyse glycerol formation it seems likely that Ste20 and Cla4 are rather involved in glycerol synthesis and not other metabolic pathways.

Since Ste20, Cla4 and also Gpd1, Gpp1 and Gpp2 all predominantly localize to the cytoplasm it seems likely that Ste20 and Cla4 interact with glycerol biosynthesis enzymes in the cytoplasm [13,33–37].

Taken together our data suggest that Ste20 and Cla4 might have a function that involves the enzymes Gpd1, Gpp1 and Gpp2, most likely glycerol biosynthesis under aerobic conditions.

### 3.2. Gpd1 expression and glucose availability

Since we did not observe any interactions for Skm1 we decided to focus on Ste20 and Cla4 for this study. It was tested whether there are any links between glycerol biosynthetic enzymes and processes that are regulated by Ste20 and Cla4. Ste20 is essential for filamentation, and Cla4 also seems to play an important role in this process [4,5,38]. Cells switch from the yeast form to a filamentous form when grown on agar plates lacking a fermentable carbon source such as glucose [39]. For this study, we focused on Gpd1 because unlike Gpp1 and Gpp2, Gpd1 is the primary regulator of glycerol synthesis [20,40,41]. When grown overnight on agar plates without glucose, conditions that trigger filamentous growth, Gpd1 protein levels were about three times lower compared to cells in liquid glucose medium which keeps cells in the yeast form (Fig. 2A and B). However, our controls demonstrated that this reduction of Gpd1 protein levels was not specific for filamentation. Gpd1 protein expression was also decreased about three times in cells grown in liquid medium lacking glucose compared to cells growing on glucose plates and liquid glucose medium, all conditions that favour the yeast form (Fig. 2A and B). Thus, Gpd1 protein levels rather seem to correlate with glucose availability. A positive correlation between glucose concentration and Gpd1 protein levels would make physiological sense since glycerol is synthesized from a glycolytic intermediate [10,11]. This potential link was further investigated. First, we looked into changes of Gpd1 protein levels after cells were initially grown in medium with standard glucose concentration (2%) and then switched to glucose-free medium. After 4 h we detected no significant decrease of protein levels (Fig. 2C and D). Thus, Gpd1 protein levels only seem to be reduced after long exposure to glucose-free medium. For a protein with a relatively high turnover rate such a decrease could be achieved by just reducing transcription or translation. The half-life of Gpd1 has been determined in a proteome-wide study to be 25 h [42]. In our hands, Gpd1 has a much shorter half-life of about 2 h (Fig. 2E and F). The long-term decrease of Gpd1 protein levels when shifted to glucose-free medium could therefore simply be explained by a reduction of *GPD1* transcription.

Since Gpd1 protein levels decreased in response to lower glucose concentrations we also examined whether Gpd1 expression changes in response to higher glucose levels. When cells were

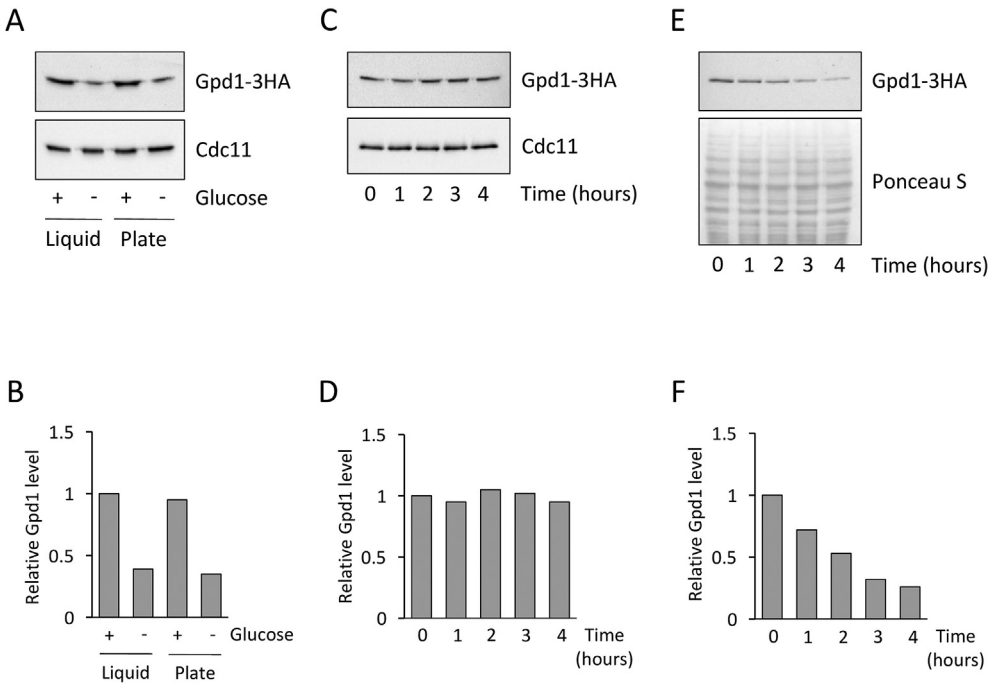


**Fig. 2.** Gpd1 protein levels decrease in response to glucose starvation. (A) Cells were first grown in minimal medium with 2% glucose and then shifted overnight to liquid medium or plates with (2%) or without glucose as indicated. Gpd1 expression was analysed by immunoblotting using antibodies against the HA epitope. Cdc11 was used as loading control. (B) Quantification of (A). Data were normalised to the loading control. (C) Cells were grown to exponential phase in medium containing 2% glucose, washed in medium lacking glucose and then resuspended in medium without glucose. (D) Quantification of (C) normalised to the loading control. (E) Gpd1 half-life was determined by treating cells with cycloheximide. The blot stained with Ponceau S confirms that equal amounts of protein were loaded. (F) Quantification of (E).

first grown to stationary phase, and were, therefore, glucose-starved, and then transferred to fresh medium containing 2% glucose, Gpd1 protein levels increased (data not shown). This effect was even more pronounced when cells were starved overnight, then shifted to fresh medium (2% glucose) for 2 h and then grown in medium that contains another 2% glucose (Fig. 3A and B). Deletion of either *STE20* or *CLA4* had no effect on the increase of Gpd1 protein concentration (Fig. 3A and B). Ste20 and Cla4 have some overlapping functions [1–3]. This might also include the regulation of *GPD1* expression. Since a *STE20 CLA4* double deletion strain is not viable [3] we used a chemical genetic approach in which *STE20* was deleted and *CLA4* was replaced by the analogue-sensitive allele *cla4-as3* [26]. The inhibitor 1NM-PP1 rapidly and specifically blocks kinase activity of the mutated Cla4 protein but not the wild type Cla4 or any other kinase. The *ste20*Δ *cla4-as3* double mutant displayed a slight increase of Gpd1 protein levels in response to higher glucose concentrations but overall Gpd1 expression was much lower compared to the wild type (Fig. 3C and D). Ste20 and Cla4 are, therefore, both needed for the increased Gpd1 expression. It is well established that Ste20 and Cla4 induce the transcription of hyperosmotic stress response genes such as *GPD1* through activation of Ste11, the most upstream component of the Hog1 MAPK cascade [3,6–9]. In order to test whether Ste20 and Cla4 have a role in the regulation of *GPD1* expression beyond MAPK cascade activation, we analysed Gpd1 protein levels in cells lacking *STE11*. In the *ste11*Δ strain, the increase of Gpd1 protein levels in response to higher glucose concentrations was comparable to the wild type (Fig. 3C and D). This suggests that the reduction of Gpd1 levels observed in the *ste20*Δ *cla4-as3* double mutant is due to a novel function of Ste20 and Cla4 which is independent of Ste11. This could for example be transcriptional activation independent of a MAPK cascade. Ste20 and Cla4 not only control transcription indirectly through cytoplasmic activation of MAPK cascade, they can also translocate to the nucleus where they regulate the transcription factor Sut1 [43]. Other aspects of gene expression such as mRNA export from the nucleus, transcript stability, translation efficiency and protein stability could also be regulated by Ste20 and Cla4. Notably, Ste20 modulates transcript-specific mRNA degradation via the decapping enzyme Dcp2 [44]. Furthermore, it has been shown that *GPD1* expression is not only regulated transcriptionally but also at other levels. This includes the modulation of *GPD1* translation and Gpd1 protein degradation [45,46]. It is, therefore, conceivable that Ste20 and Cla4 either regulate *GPD1* transcription in an MAPK-independent pathway or modulate other levels of *GPD1* expression. However, since Ste20 and Cla4 also bind to the Gpd1 protein it seems likely that they also regulate Gpd1 protein stability.

### 3.3. Gpd1 expression and hyperosmotic stress response

Both, Ste20 and Cla4 play a crucial role in the transcription of hyperosmotic stress response genes such as *GPD1* through the activation of Ste11 [3,6–9]. Since we have shown that Ste20 and Cla4 modulate Gpd1 protein levels in response to changing glucose concentrations by a Ste11-independent mechanism, we wanted to know whether this is also the case during osmoadaptation. In wild type cells, Gpd1 protein levels increase within minutes of exposure to high salinity (0.8 M NaCl) which is in line with published observations (Fig. 4A and B) [14,17]. This increase is less pronounced in the *ste20*Δ *cla4-as3* double mutant but the deletion of either *STE20* or *CLA4* alone has no effect (Fig. 4A–D). Gpd1 induction in the *ste11*Δ strain is comparable to the wild type (Fig. 4C and D). It has previously been shown that *STE11* deletion does not affect the expression of other hyperosmotic stress genes [3]. This is not surprising since the Hog1 MAPK cascade is not only activated by Ste11 but also by a second redundant branch [10,11]. The observation that Gpd1 protein levels were reduced in the *ste20*Δ *cla4-as3* strain suggests that Ste20 and Cla4 have an additional function in the

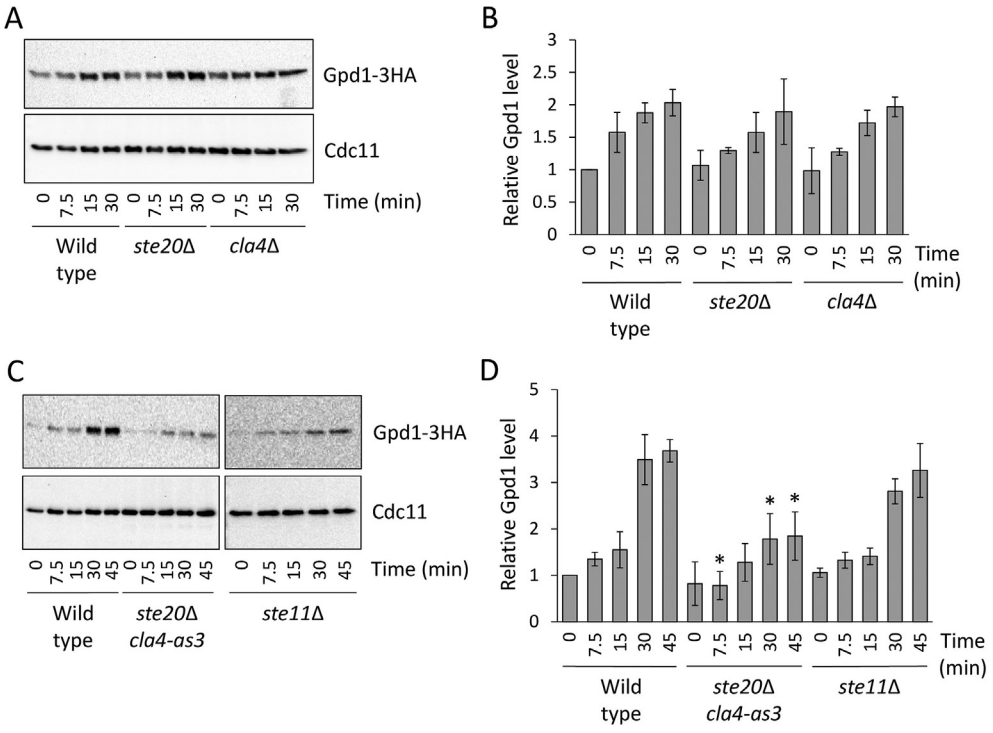


**Fig. 3.** Ste20 and Cla4 have overlapping functions in the glucose-induced increase of Gpd1 levels. (A) Deletion of either *STE20* or *CLA4* alone does not affect Gpd1 concentrations. (B) Quantification of (A) normalised to the loading control. Shown is the average of 3 independent experiments with SD. (C) Inactivation of Cla4 in the absence of *STE20* results in decreased Gpd1 levels. (D) Quantification of (C). Data are expressed as average with SD of 3 experiments. Student's t-test $^{*}p < 0.05$.

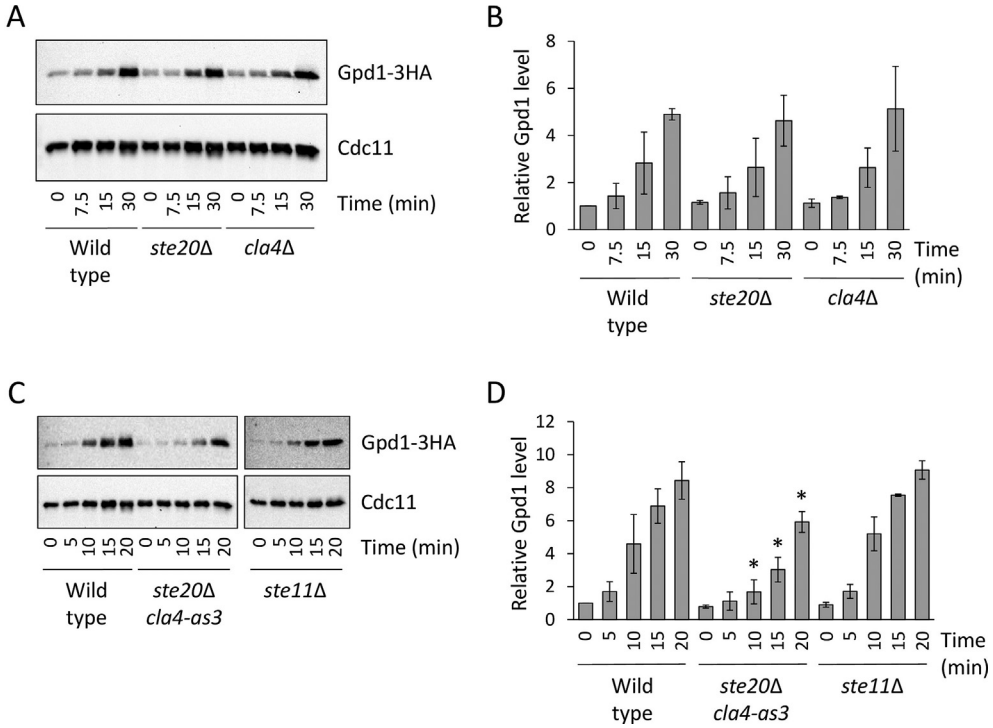


**Fig. 4.** Ste20 and Cla4 are jointly required for hyperosmotic stress-induced increase of Gpd1 levels. (A) The lack of either *STE20* or *CLA4* has no effect on Gpd1 protein levels. (B) Quantification of (A) normalised to the loading control. Shown is the average of 3 independent experiments with SD. (C) Simultaneous *STE20* deletion and Cla4 inactivation reduces Gpd1 level in a *STE11*-independent manner. (D) Quantification of (C). Data are expressed as average with SD of 3 experiments. Student's t-test $^{*}p < 0.05$.

regulation of *GPD1* expression as discussed above for the glucose-induced increase of Gpd1 protein. Such a regulation of gene expression at multiple stages seems to be an important feature for the adaptation to a changing environment because it ensures that the cell response is efficient and highly specific.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.07.072.

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