FEBS Letters

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Mitotic cyclin Clb4 is required for the intracellular adaptation to glucose starvation in *Saccharomyces cerevisiae*

Midori Umekawa[1], Daiki Shiraishi[2], Marin Fuwa[2], Kazuna Sawaguchi[2], Yosuke Mashima[3], Takane Katayama[3] and Shuichi Karita[1]

1 Graduate School of Bioresources, Mie University, Tsu, Japan
2 Faculty of Bioresources, Mie University, Tsu, Japan
3 Graduate School of Biostudies, Kyoto University, Japan

Correspondence
M. Umekawa, Graduate School of Bioresources, Mie University, Kurimamachiya-cho 1577, Tsu 514-8507, Japan
Tel: +81-59-231-9559
E-mail: midoriumekawa@bio.mie-u.ac.jp

(Received 31 October 2019, revised 8 December 2019, accepted 9 December 2019, available online 29 December 2019)

doi:10.1002/1873-3468.13722

Edited by Ivan Sadowski

Cellular homeostasis in response to glucose availability is maintained through the tight coordination of various physiological processes, including cell proliferation, transcription, and metabolism. In this study, we use the budding yeast *Saccharomyces cerevisiae* to identify proteins implicated in carbon source-dependent modulation of physiological processes. We find that the mitotic cyclin Clb4 is required for optimal regulation of glucose-starvation-responsive pathways through the target of rapamycin complex 1. Cells lacking Clb4 are characterized by dysregulation of autophagy and impaired modulation of cell size. Notably, cell viability after prolonged glucose starvation is severely reduced by disruption of Clb4. We conclude that Clb4, in addition to its function in the cell cycle, plays a role in the intracellular adaptation to glucose starvation.

Keywords: autophagy; glucose starvation; nutrient response

Glucose is a fundamental source of energy for living cells. Cells have evolved the systems that respond to glucose availability to modulate cellular physiology, including cell proliferation and metabolism, which are critical for the maintenance of intracellular homeostasis. Perturbations in these systems have broad physiological implications for cells, including cell death. It is therefore important to identify molecular components of the machinery responsible for the intracellular response to glucose and/or glucose starvation. However, the mechanistic bases of these modulation systems, as well as their component molecules, are complicated and are yet to be fully understood. In this study, we use the budding yeast *Saccharomyces cerevisiae* as a eukaryotic model organism to search for the unknown components responsible for cellular adaptation to glucose starvation.

Three key and highly conserved components of eukaryotic nutrient-sensing systems exist in yeast: the target of rapamycin complex 1 (TORC1), the protein kinase A (PKA), and Snf1, each of which regulates cellular responses to nutrient availability through Ser/Thr kinase activities [1,2]. The central role of TORC1 as a key modulator of cell proliferation, translation, metabolic changes, and autophagy is well-established and is the subject of intense research [1,2]. Under nutrient-rich conditions, TORC1 is activated, resulting in the modulation of enzymes and expression of genes that promote anabolic processes and cell proliferation [1,2]. In contrast, TORC1 is inactivated upon starvation for nutrients including nitrogen, carbon, and

Abbreviations
Ams1, α-mannosidase; HA, influenza hemagglutinin; NTCB, 2-nitro-5-thiocyanatobenzoic acid; PKA, the protein kinase A; TCA, trichloroacetic acid; TORC1, the target of rapamycin complex 1.

phosphorus, which causes the suppression of cell proliferation and induction of catabolic pathways such as autophagy [1–4]. Meanwhile, PKA and Snf1 are known to play a particularly critical role in carbon source signaling [2]. Yeast Snf1, which is analogous to mammalian AMP-activated protein kinase, is thought to be activated and subsequently inactivate TORC1 upon glucose starvation [1,5]. On the other hand, PKA is activated in the presence of replete glucose and may be responsible for the inactivation of Snf1 when glucose is available [1,2]. Recent reports suggest intricate yet poorly characterized crosstalk between TORC1 and PKA/Snf1 during carbon depletion [1,5–9]. Taken together, these reports indicate that TORC1 forms a component of the cellular response to glucose availability, most likely through interplay with PKA/Snf1.

The vacuolar α-mannosidase (Ams1) is an important glycolytic enzyme that is involved in the degradation of intracellular free oligosaccharides in *S. cerevisiae* [10]. We have previously shown that the expression of Ams1 protein is tightly repressed under nutrient-rich conditions but is strongly induced in response to glucose starvation [11]. We further uncovered that Ams1 expression is regulated by the stress-responsive transcription factor Msn2/4, which is under the control of TORC1 [11]. We have also found that the enzymatic activity of Ams1 is enhanced strikingly upon glucose starvation by its autophagy-dependent transport to the vacuole, where it is processed by the vacuolar proteases to an active form [11,12]. Therefore, the intracellular Ams1 activity represents an ideal means of quantitatively monitoring the intracellular signaling pathways underlying the response to glucose starvation. In the present study, we set out to identify unknown molecules involved in the physiological response to glucose starvation by means of measuring the intracellular Ams1 activity in *S. cerevisiae*. We find that the mitotic cyclin Clb4, which is a component of the cell cycle progression pathway, also functions in the suppression and promotion of glucose-starvation-inducible pathways. We reveal for the first time a role for Clb4 in the cellular response and adaptation to glucose starvation.

Materials and methods

Yeast strains and plasmids used in this study

The *S. cerevisiae* strains used in this study are listed in Table S1. Yeast MATalpha Collection (YSC1054) was purchased from Funakoshi (Tokyo, Japan). Gene disruptions were performed by a standard PCR-based procedure [13]. The plasmids used in this study, pRS416-GFP-Atg8 and pRS315-Atg13-3HA, were kindly provided by D. Klionsky (University of Michigan). pRS416-Sch9-3HA was kindly provided by R. Loewith (University of Geneva).

Growth conditions

Yeast cells were cultured at 30 °C in YPD (1% yeast extract, 2% peptone, and 2% glucose) to early-log phase. Then, cells were washed twice and subjected to glucose starvation in YP (1% yeast extract and 2% peptone) or nitrogen starvation in synthetic medium without nitrogen (SD-N, 0.17% yeast nitrogen base without ammonium sulfate/amino acids and 2% glucose) for appropriate time periods. If required, 200 ng·mL^{-1} of rapamycin (Funakoshi) was added to the media.

Measurement of the intracellular α-mannosidase activity

The assay for the intracellular Ams1 activity was carried out as we established previously [9]. Briefly, 40 OD_{600} cells subjected to glucose starvation or nitrogen starvation were collected and disrupted with glass beads in 550 μL of lysis buffer [20 mM PIPES/NaOH (pH 6.8), 1 mM EDTA, 1 mM PMSF, 1% Triton X-100]. Cell-free extracts were used for the determination of Ams1 enzymatic activity using para-nitrophenyl-α-D-mannopyranoside as a substrate of Ams1. The amount of *p*-nitrophenol was measured at $A_{400,}$ and the protein concentrations were determined using protein assay CBB solution (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. One unit was defined as the amount of enzyme that catalyzes hydrolysis of 1 nmol of *p*-nitrophenol per minute.

Immunoblotting

The magnitude of autophagy was assessed by the GFP-Atg8 processing assay, using cells harboring pRS416-GFP-Atg8, according to the established protocols [14]. For assessment of phosphorylation states of Atg13, cells harboring pRS315-Atg13-3HA were used. For these assays, 4.0 OD_{600} cells were harvested at indicated time points before proteins were precipitated by adding 6.5% trichloroacetic acid (TCA) and washing with acetone. The precipitated proteins were dissolved in 80 μL of MURB buffer [50 mM sodium phosphate buffer (pH 7.0), 25 mM MES, 1% SDS, 3 M urea, 1 mM NaN_3, 1% β-mercaptoethanol, and 0.01% bromophenol blue]. After heating at 65 °C for 10 min, the samples were subjected to SDS/PAGE and immunoblotting.

The phosphorylation of Sch9 was assessed by monitoring the C-terminal fragment of Sch9-3HA truncated by 2-nitro-5-thiocyanatobenzoic acid (NTCB), according to the previously reported procedures [15]. Briefly, 5.0 OD_{600} cells harboring pRS416-Sch9-3HA were harvested at indicated

time points and proteins were precipitated by adding 6.0% TCA and washing with acetone. The precipitated proteins were lysed with glass beads in urea buffer [50 mM Tris buffer (pH 7.5), 5 mM EDTA, 6 M urea, 1% SDS, 10 mM sodium fluoride, 10 mM sodium azide, 10 mM *p*-nitrophenylphosphate, 10 mM $Na_2P_2O_4$, and 10 mM β-glycerophosphate, phosphatase inhibitor cocktail, protease inhibitor cocktail, and 1 mM PMSF]. After heating at 65 °C for 10 min, samples were treated with 1 mM NTCB in 0.1 M CHES buffer (pH 10.5) overnight. Protein extracts were then subjected to SDS/PAGE and immunoblotting.

Immunoblotting was carried out using anti-GFP mouse monoclonal antibodies (JL-8) (Clontech, Mountain View, CA, USA) at 1 : 5000 for the GFP-Atg8 processing assay. Anti-influenza hemagglutinin (HA) mouse monoclonal antibody (F-2) (Santa Cruz Biotechnology, Dallas, TX, USA) was used at 1 : 2000 to monitor the phosphorylation of Sch9-3HA or Atg13-3HA. Anti-Pgk1 mouse monoclonal antibody (22C5D8) (Abcam, Cambridge, UK) was used at 1 : 2500 to monitor endogenous Pgk1 as the protein-loading control. Mouse IgGκ light chain-binding protein conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used at 1 : 10 000 as the secondary antibody. Immunoreactive bands were visualized using a Chemi-Lumi One L Kit (Nacalai Tesque) and images captured using an IMAGEQUANT LAS500 (GE Healthcare, Piscataway, NJ, USA).

Microscopy

To monitor the nuclear localization of endogenous Sfp1, wild-type and *clb4*Δ cells expressing chromosomally GFP-tagged Sfp1 (Sfp1-GFP) under the control of its native promoter were grown in YPD to early-log phase before being subjected to glucose starvation in YP for 1-20 h. Live-cell microscopy was performed using an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a DP72 CCD camera. Captured images were deconvolved using cellSens standard software. For measurement of cell size under each condition, differential interference contrast (DIC) images were captured and the long diameters of 60 cells were measured using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA).

Cell viability assay

The cell viability of wild-type and *clb4*Δ cells was evaluated as follows. The 1.0 OD_{600} cells, grown in YPD to early-log phase or subjected to glucose starvation in YP for the indicated days, were collected and diluted with the same medium. The serial dilution of each cell was spotted (5 μL) on YPD agar plates and incubated at 30 °C until the colonies were grown up. For calculating the colony-forming unit per 1 mL of each cell culture, cells were diluted appropriate times to spread (100 μL) on YPD agar plates and incubated until the colonies were grown up.

Results

Glucose-starvation-induced Ams1 activity was significantly decreased by deletion of *CLB4*

In order to identify unknown factors involved in regulation of glucose-starvation-induced pathways, we first conducted a screen for diminished Ams1 activity under glucose starvation in knockout strains. As pathways responsible for the response to glucose starvation are often controlled by kinases or kinase-related molecules, we selected 150 genes encoding putative kinases or kinase-related proteins according to *Saccharomyces* Genome Database (https://www.yeastgenome.org) [16] and obtained knockout strains of each gene from the yeast knockout collection [17]. We observed that several of these knockout strains showed reduced Ams1 activity under glucose starvation, in comparison with that of wild-type cells (Fig. S1A). In order to verify these results, we generated our own strains knocked out for these genes and reassessed the Ams1 activity under glucose starvation. Among these strains, cells lacking Clb4 showed significantly reduced Ams1 activity under glucose-starvation conditions (Figs 1A and S1B,C). In contrast, increased activity under nutrient-rich conditions was observed for *clb4*Δ cells, more than three times of that of the wild-type cells (Fig. 1D). On the other hand, Ams1 activity was increased clearly upon nitrogen starvation in *clb4*Δ cells, similar to that of the wild-type cells (Fig. 1B). These results suggest that Clb4 is required for the upregulation of Ams1 upon glucose starvation as well as the suppression of Ams1 under nutrient-rich conditions by a pathway independent of nitrogen signaling.

Clb4 is one of the six B-type cyclins that are involved in G_2/M cell cycle progression of proliferating cells through the activation of cyclin-dependent kinase1/Cdc28 [18]. Therefore, we assessed whether the deficiency of *CLB* genes or Cdc28 affects Ams1 activity. The deletion of *CLB4* had a dramatically pronounced effect among all of the *CLB* genes under both glucose-depleted and glucose-replete conditions (Fig. 1C,D). A temperature-sensitive mutant of the essential gene *CDC28*, *cdc28-1*, also showed enhanced Ams1 activity in nutrient-rich conditions, similar to that of *clb4*Δ cells, although this mutant showed only a partial decrease in Ams1 activity under glucose-depleted conditions (Fig. 1E). Taken together, these findings indicate that one of the B-type cyclins, Clb4, plays a particularly striking role in the efficient enhancement of the intracellular Ams1 activity in response to changes in glucose availability.

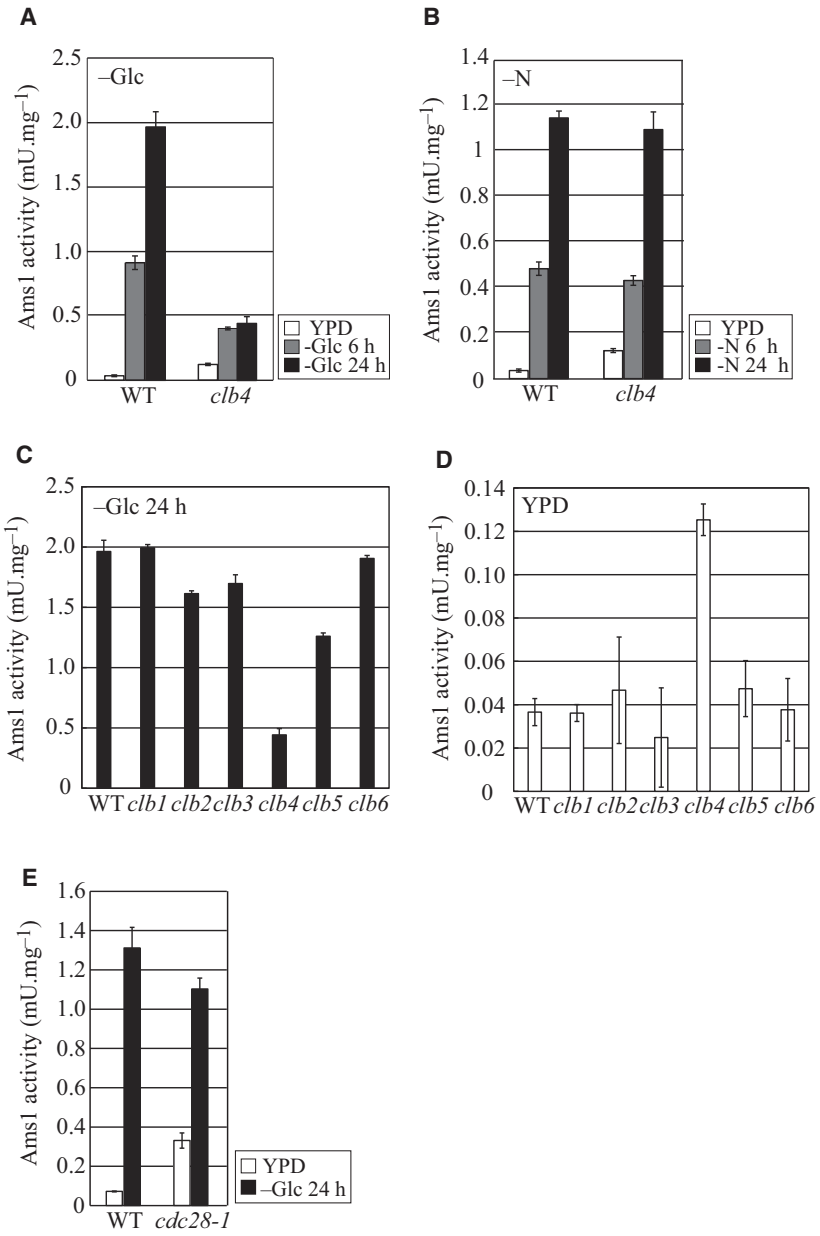
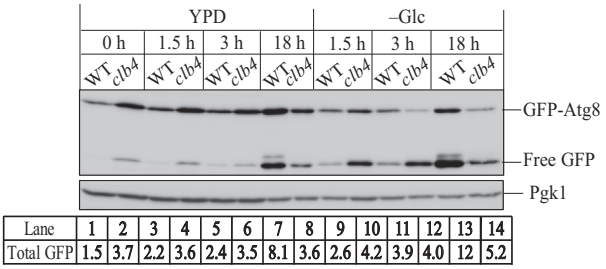


Fig. 1. Clb4 is required for the upregulation of Ams1 activity in response to glucose availability. Cells grown in nutrient-rich (YPD) media to early-log phase were shifted to glucose-starvation (-Glc) or nitrogen-starvation (-N) media for indicated times. Cell-free extracts were used to determine Ams1 enzyme activity. Comparison of Ams1 activities of wild-type and the *clb4*Δ cells after glucose starvation (A) or nitrogen starvation (B). Comparison of Ams1 activities of wild-type and other *clb1-6*Δ cells during glucose-starvation (C) or nutrient-rich conditions (D). Comparison of Ams1 activities of wild-type and *cdc28-1* cells at nonpermissive temperature (37 °C) under nutrient-rich and glucose-starved conditions (E). Error bars indicate the SEM of at least three independent experiments.

Clb4 is required for optimal control of autophagy induction

Next, we asked whether disruption of Clb4 causes dysregulation of autophagy. As well as Ams1 activity, autophagy is suppressed under nutrient-rich conditions but induced by glucose starvation through the activity of the TORC1 and PKA/Snf1 pathways [1,8,9]. To quantify the magnitude of autophagy, we utilized the GFP-Atg8 processing assay, according to reported procedures [14]. Since an ubiquitin-like protein Atg8 is a key component of the autophagosomal membrane, the N-terminal fusion of GFP (GFP-Atg8) can be used as a marker of autophagosome formation [19]. Upon the fusion of an autophagosome with the vacuole, the GFP-Atg8 present on the inner vesicle is incorporated into the vacuole inside the autophagic body. Atg8 is degraded after lysis of the autophagic body in the vacuole, whereas the GFP moiety of a GFP-Atg8 chimera is relatively resistant to proteolysis. Accordingly, the increase in GFP-Atg8 with the accumulation of free GFP, the processed form of GFP-Atg8, reflects the magnitude of autophagy [14,19]. In wild-type cells, free GFP was not observed under nutrient-rich conditions



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Total GFP	1.5	3.7	2.2	3.6	2.4	3.5	8.1	3.6	2.6	4.2	3.9	4.0	12	5.2

Fig. 2. Regulation of autophagy response to glucose availability is impaired by deletion of *CLB4*. Wild-type (BY4742) and *clb4*Δ cells harboring pRS416-GFP-Atg8 were grown in nutrient-rich (YPD) media to early-log phase and shifted to glucose starvation (-Glc) for the indicated times. Cells were collected at the indicated time points, and total protein extracts were subjected to immunoblotting using anti-GFP and anti-Pgk1 antibodies to detect GFP-Atg8, free GFP and endogenous Pgk1, respectively. The ratio of the signal of total GFP (GFP-Atg8 and free GFP)/Pgk1 was quantified using Image J software, and the values are indicated below the panel.

(Fig. 2, lane 1). After a prolonged period of glucose starvation or cultivation in YPD for 18 h, autophagy was clearly evident in wild-type cells in which free GFP was accumulated prominently (Fig. 2, lanes 7 and 13). On the other hand, the accumulation of free GFP was severely attenuated in *clb4*Δ cells (Fig. 2, lanes 8 and 14), in comparison with that of the wild-type cells. The quantification data show that the amount of total GFP, including both free GFP and GFP-Atg8 per Pgk1, in *clb4*Δ cells after glucose starvation for 18 h was less than half of that of wild-type cells, suggesting that autophagy induction is severely subdued by deletion of *CLB4*. In contrast, free GFP as well as GFP-Atg8 was clearly accumulated in *clb4*Δ cells even under nutrient-rich conditions (Fig. 2, lane 2), whereas autophagy is stringently suppressed in wild-type cells. In addition, after the onset of 1.5–3 h of glucose starvation, the amount of total GFP (free GFP and GFP-Atg8/Pgk1) was increased gradually in the wild-type, whereas it remained almost constant in *clb4*Δ cells (Fig. 2, lanes 1, 9, and 11 vs. lanes 2, 10, and 12). These results suggest that the disruption of *CLB4* results in the broad dysregulation of autophagy induction in cells under both nutrient-rich and nutrient-starvation conditions, with an intermediate degree of induction.

Clb4 is involved in the regulation of TORC1

Previous studies have shown that TORC1, which is at least partly synchronized with PKA/Snf1, regulates autophagy as well as the Ams1 activity [8,9,11]. We next thought to determine whether deletion of *CLB4* affects TORC1 activity. To this end, we first assessed the phosphorylation status of Sch9, a well-established direct target of TORC1 kinase activity [15]. In wild-type cells, the phosphorylated form of Sch9 was observed clearly under nutrient-rich conditions, reflecting TORC1 activity as expected (Fig. 3A, lane 1). As previously reported, Sch9 phosphorylation decreased rapidly upon glucose starvation or rapamycin treatment (Fig. 3A, lanes 3, 5, and 9) [5]. However, the phosphorylated form of Sch9 was detected only slightly in *clb4*Δ cells under nutrient-rich conditions (Fig. 3A, lane 2). We therefore conclude that Sch9 phosphorylation, which reflects TORC1 activity, is severely reduced in *clb4*Δ cells irrespective of nutrient conditions.

Atg13 is phosphorylated directly by TORC1, and this phosphorylation is known to suppress autophagy under nutrient-rich conditions [20]. Notably, it has been shown that the expression of mutants of Atg13 lacking key phosphorylation sites results in the partial induction of autophagy under nutrient-rich conditions [20]. Consistent with the data for Sch9, we found that while Atg13 is hyperphosphorylated in wild-type cells grown on nutrient-rich media, *clb4*Δ cells under the same conditions were characterized by a reduction in Atg13 phosphorylation (Fig. 3B, lanes 1–2 and Fig. 3C). Partial dephosphorylation of Atg13 observed within 30 min in both wild-type and *clb4*Δ cells, with dephosphorylation particularly marked in *clb4*Δ cells (Fig. 3B, lanes 3–10 and Fig. 3C). These results suggest that Clb4 is required for optimal tuning of TORC1 pathway response to glucose availability in the cells.

Clb4 is required for the optimal control of cell size through Sfp1

Next, we further analyzed whether deletion of *CLB4* affects the localization of the nutrient-responsive transcription factor Sfp1. Previous reports suggest that nuclear localization of Sfp1 is regulated by TORC1 and the TORC1-controlled nuclear localization of Sfp1 is required for carbon source-dependent modulation of cell size [21]. We adopted a similar approach to monitor the localization of chromosomally tagged Sfp1-GFP by fluorescence microscopy [21,22]. As reported previously, while Sfp1-GFP was clearly localized to the interior of the nucleus under nutrient-rich conditions, dispersal of Sfp1-GFP throughout the cytosol was observed after rapamycin treatment or glucose starvation in the wild-type cells (Fig. 4A). In contrast, in *clb4*Δ cells under nutrient-rich conditions, Sfp1-GFP was localized only partially to the nucleus and was mostly dispersed throughout the cytosol (Fig. 4B).

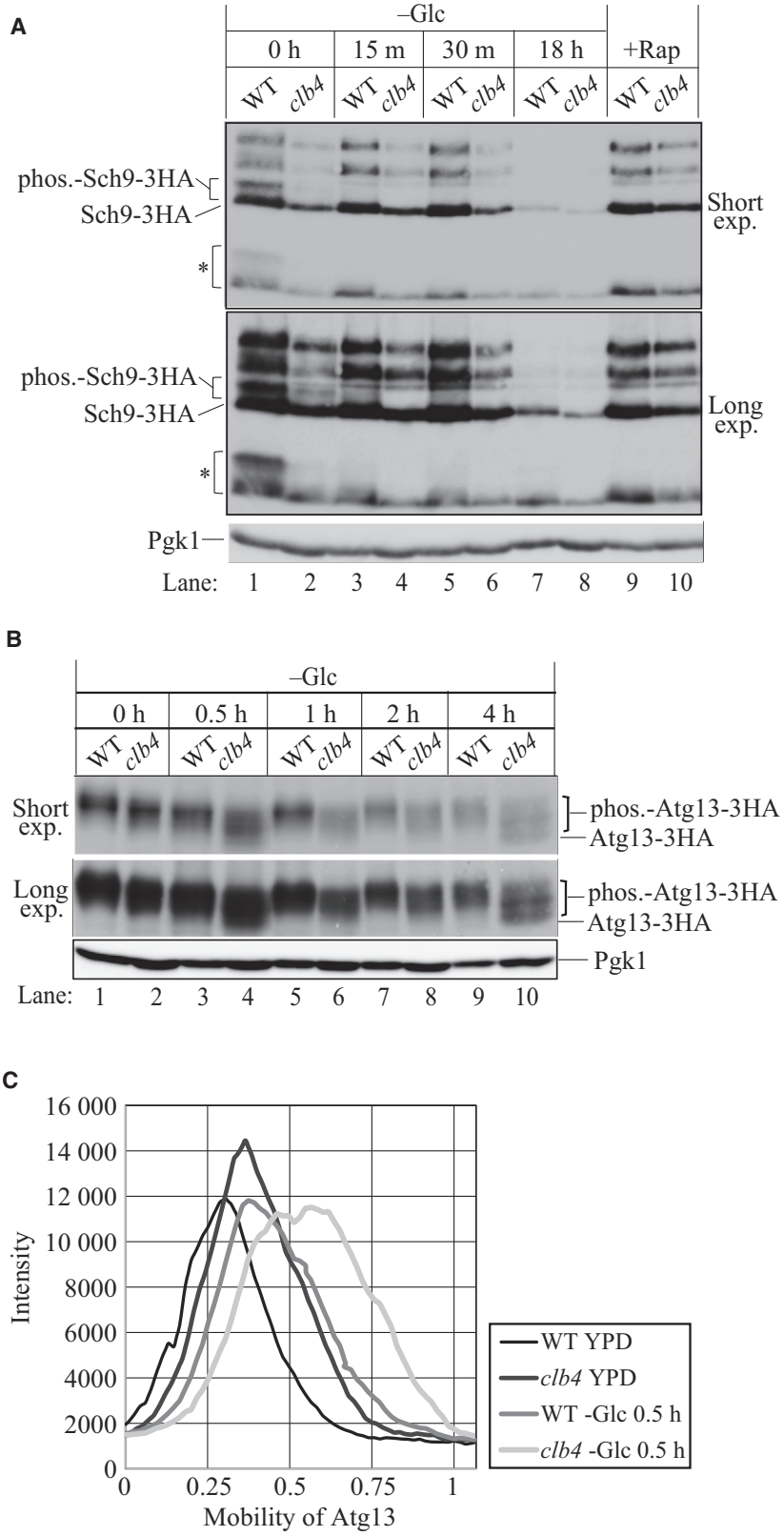


Fig. 3. Phosphorylation of Sch9 and Atg13 is markedly reduced in *clb4*Δ cells, irrespective of glucose availability. Wild-type (BY4742) and *clb4*Δ cells harboring pRS416-Sch9-3HA (A) or pRS315-Atg13-3HA (B) were grown in nutrient-rich (YPD) media to early-log phase and transferred to glucose-starvation (-Glc) media. Cells were collected at the indicated time points, and the total protein extracts were prepared for immunoblotting with anti-HA and anti-Pgk1 antibodies. The phosphorylation of the C-terminal fragment of NTCB-treated Sch9-3HA (about 40–60 kDa) was analyzed by immunoblotting using an anti-HA antibody (A), as reported previously [15]. Pgk1, a loading control. Asterisks (*), degradation products of the C-terminal fragment of NTCB-treated Sch9-3HA. +Rap, cells were treated with rapamycin (200 ng·mL^{-1}) for 30 min. The phosphorylation of Atg13-3HA was analyzed by immunoblotting using an anti-HA antibody (B). The signal intensity and mobility shift of Atg13 were quantified using Image J software to compare the phosphorylation states of Atg13 between the wild-type and the *clb4*Δ cells grown on nutrient-rich media (YPD) or subjected to glucose starvation (-Glc) for 0.5 h (C).

After glucose starvation, the intermediate nuclear localization of Sfp1-GFP was lost and dispersal of Sfp1-GFP throughout the cytosol was observed in *clb4*Δ cells, as for wild-type cells. Since the nuclear localization of Sfp1 is known to be critical for the cell size control, we also compared the cell sizes of wild-type and *clb4*Δ cells by light microscopy [21]. This experiment revealed that the size of *clb4*Δ cells is significantly smaller than that of wild-type cells in both nutrient-rich and glucose-starvation conditions (Fig. 4C). These results suggest that Clb4 also plays a role in the maintenance of cell size mediated by the glucose-starvation response of Sfp1, which is dependent on TORC1 control of nuclear localization.

Clb4 is required for cell survival under prolonged glucose restriction

These above results suggested that Clb4 plays a role in the physiological response mounted by cells upon glucose starvation. Accordingly, we evaluated whether *CLB4* deletion affects cell viability under glucose-starved conditions. Under nutrient-rich conditions, a significant difference in cell viability was not observed between wild-type and *clb4*Δ cells (Fig. 5A,B, day 0). However, after a prolonged glucose starvation of more than 6 days, survival of *clb4*Δ cells was markedly reduced in contrast to that of wild-type cells, which experienced little change in survival, even after 20 days of glucose starvation. These results suggested that Clb4 plays an indispensable role in cell survival under glucose-starvation conditions, likely through its role in regulating the TORC1 pathway and thereby stress response pathways such as autophagy.

Discussion

The intracellular mechanisms facilitating adaptation to glucose starvation, as well as the molecules implicated, are not well-clarified. The results presented in this study suggest that Clb4 is required to effectively mount the cellular response to glucose starvation that supports cell survival in budding yeast. We found that deletion of Clb4 causes dysregulation of glucose-starvation-induced autophagy and Ams1 activation at least partly by dysregulation of the TORC1 pathway (Figs 1–3 and S1). In addition, optimal modulation of cell size was impaired by *CLB4* deletion, which perturbed the nuclear localization of Sfp1, a transcription factor that is regulated by TORC1 activity in response to glucose availability (Fig. 4). Notably, the data suggest that Clb4 is required for the maintenance of cell viability under extended glucose starvation (Fig. 5).

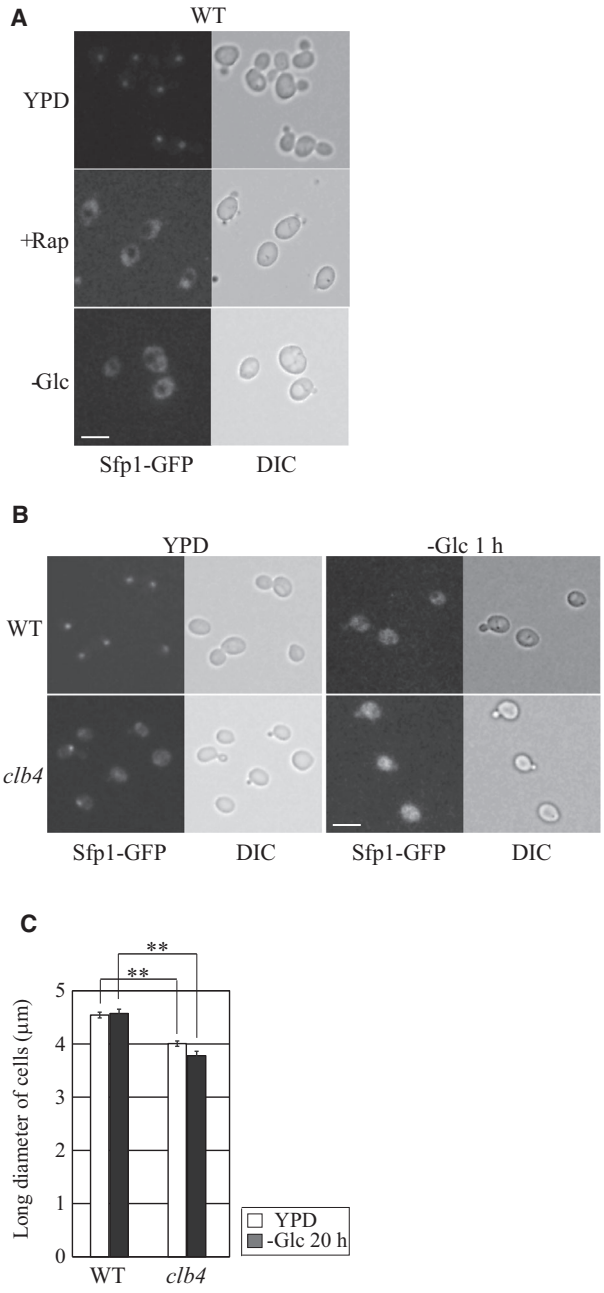


Fig. 4. Clb4 is required for modulation of cell size through the glucose-responsive nuclear localization of Sfp1. Wild-type (BY4741) cells expressing Sfp1-GFP were grown in nutrient-rich (YPD) media to early-log phase and transferred to glucose-starvation (-Glc) media for 1 h or treated with rapamycin (100 ng·mL^{-1}) for 1 h (+Rap) (A). The localization of endogenous Sfp1-GFP was monitored by fluorescence microscopy in wild-type and *clb4*Δ cells on nutrient-rich (YPD) or glucose-starvation (-Glc) media (B). Scale bar, 10 μm. DIC, differential interference contrast. The long diameter (cell size) of 60 cells was determined by DIC microscopy (C). The size of cells grown under the indicated conditions was measured using IMAGEJ software. Error bars indicate the SEM of at least 60 cells. **, $P < 0.001$.

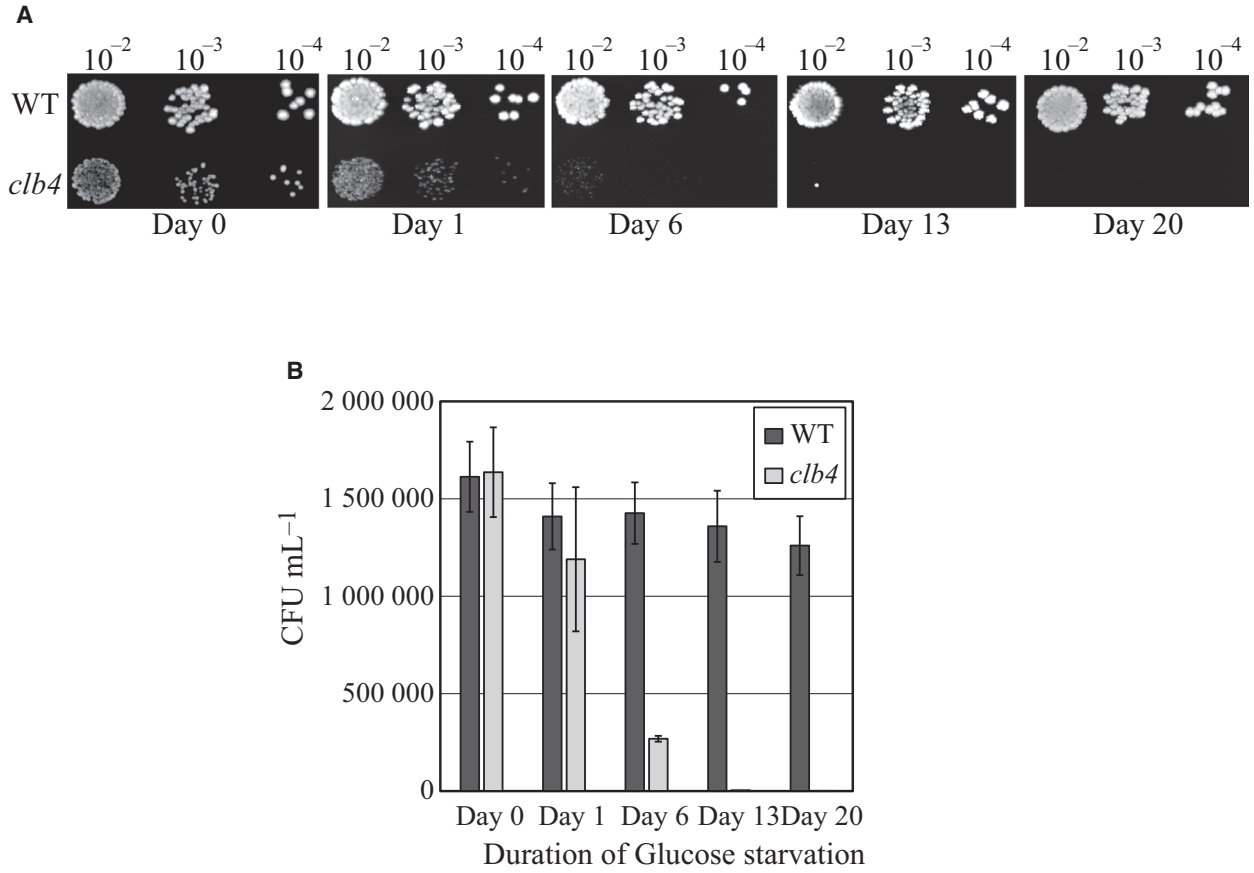


Fig. 5. *clb4*Δ cell survival is impaired following glucose starvation. Wild-type (BY4742) and the *clb4*Δ cells were grown in nutrient-rich (YPD) media to an early-log phase and transferred to glucose-starvation (-Glc) media. At the indicated time points, 5 μL aliquots of serially diluted culture solutions were spotted on YPD agar plates and incubated at 30 °C (A). 100 μL aliquots of dilution cultures were spread on YPD agar plates (B). After the colonies were grown at 30 °C, the number of colonies appearing on each plate was counted. Error bars indicate the SEM of at least three independent experiments.

Clb4 is considered to be a redundant mitotic cyclin that plays a role in the progression from S to M phase of the cell cycle [18]. However, our finding that Clb4 also contributes to glucose-starvation adaptation indicates a new role for Clb4 in addition to cell proliferation. Recently, a separate report has found that another mitotic cyclin, Clb2, may contribute to the DNA damage response as well as cell survival through the G_2/M arrest [23]. Further, glucose restriction appears to induce G_2/M arrest, thereby extending chronological lifespan as reported in fission yeast [24]. The modulation of TORC1 pathway is also thought to play a role in both G_2/M transition and the G_2/M arrest response to nutrient signals [25]. Our microscopic analyses suggest that Clb4 is required for the nuclear localization of the carbon source-responsive transcription factor Sfp1. It has been reported that Sfp1 may act as a repressor of the G_2/M transition, which is required for G_2/M checkpoint arrest as well as the modulation of cell size [26]. Taken together, Clb4, unlike other mitotic cyclins, may contribute to the execution of the glucose-starvation response as well as intracellular reorganization during the G_2/M arrest, partly by fine-tuning of the TORC1 pathway.

In *clb4*Δ cells, autophagy was partially induced under glucose-replete conditions, corresponding to elevated dephosphorylation of Atg13 (Figs 2 and 3). On the other hand, autophagy induction remained subdued in *clb4*Δ cells, even after prolonged glucose starvation and despite the relative dephosphorylation of Atg13 in contrast to wild-type cells. The regulatory mechanisms governing induction and physiological role of glucose-starvation-induced autophagy remain unclear. However, a recent study has suggested that glucose-starvation-induced autophagy requires the metabolic shift from fermentation to respiration, most likely because the maintenance of intracellular pools of

ATP is enhanced following the onset of respiration [27]. Given our findings that *clb4*Δ cells are characterized by the perturbation of glucose-starvation-induced pathways, including autophagy and Ams1 activation, we suggest that Clb4 may be required for metabolic shift and/or the promotion of catabolic processes, for example, utilization of storage carbohydrates to maintain intracellular ATP under glucose-restricted conditions, in part through the proper control of Sch9 and Sfp1. In conclusion, we propose that Clb4 plays a hitherto uncharacterized role in the cellular adaptation to glucose starvation, at least partly through the TORC1 signaling pathway.

Acknowledgements

We thank Daniel Klionsky (University of Michigan, USA), Robbie Loewith (University of Geneva, Switzerland), Hiromu Takematsu (Fujita Health University, Japan), and the National BioResource Project (Japan) for generous gifts of materials. Alexander I May (Tokyo Institute of Technology, Japan) contributed helpful discussions and revisions of the manuscript. We also thank Masaya Nagao (Kyoto University, Japan) for helpful discussions. This work was financially supported by JSPS KAKENHI No. 19K15806 and in part by the Sugiyama Chemical & Industrial Laboratory (Japan).

Author contributions

MU designed the experiments and wrote the manuscript. MU, DS, KS, MF, and YM performed the experiments. TK and SK revised the manuscript and provided technical support.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Ams1 activity is reduced in clb4Δ cells during glucose starvation.

Table S1. Yeast strains used in this study.