Journal of Bioscience and Bioengineering
VOL. 128 No. 6, 704–709, 2019

ELSEVIER

www.elsevier.com/locate/jbiosc

Heterologous expression and functional analysis of the F-box protein Ucc1 from other yeast species in *Saccharomyces cerevisiae*

Kunio Nakatsukasa,[1,*] Tomoyuki Kawarasaki,[2] and Akihiko Moriyama[2]

Graduate School of Natural Sciences, Nagoya City University, 1 Yamanohata, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8501, Japan[1] *and Graduate School of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai-shi, Aichi 487-8501, Japan*[2]

Received 26 December 2018; accepted 5 June 2019
Available online 25 June 2019

The ubiquitin-proteasome system plays an important role in metabolic regulation. In a previous study, we reported that, in *Saccharomyces cerevisiae*, when glucose is available, the SCF^{Ucc1} ubiquitin ligase complex targets citrate synthase 2 (Cit2) for proteasomal degradation, thereby suppressing the glyoxylate cycle, an anabolic pathway that replenishes the TCA cycle with succinate for the activation of gluconeogenesis. However, the roles of Ucc1 in other yeast species remain unclear. Here, we cloned orthologs of the F-box protein Ucc1 from *Zygosaccharomyces bailii*, an aggressive food spoilage microorganism that is the most acetic acid-tolerant yeast species, and *Candida glabrata*, an emerging fungal pathogen. These orthologs were expressed in *S. cerevisiae*, and their activities were tested genetically and biochemically. The results showed that *Z. bailii* Ucc1 rescued the *ucc1*Δ phenotype, suggesting the existence of a similar mechanism regulating the glyoxylate cycle in *Z. bailii*. By contrast, *C. glabrata* Ucc1 did not complement the *ucc1*Δ phenotype or exhibit a dominant negative effect on Ucc1. These results suggest the importance of analysing the regulatory mechanisms of glyoxylate cycle in a broad range of yeast species.

[**Key words:** Ubiquitin ligase; F-box protein; Glyoxylate cycle; *Saccharomyces cerevisiae*; *Zygosaccharomyces bailii*; *Candida glabrata*]

The ubiquitin-proteasome system has a critical function in many cellular processes including metabolic regulation. Ubiquitination is achieved through the action of three enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) (1,2). Among these enzymes, E3 ubiquitin ligases are the largest group of proteins and facilitate substrate-specific ubiquitination by binding to both E2s and substrates. Approximately 60–100 putative E3s are encoded in the genome of *Saccharomyces cerevisiae* (1). To elucidate the physiological functions of the ubiquitin-proteasome system, it is essential to identify the specific substrates of each ubiquitin ligase.

One type of cullin-RING E3 ligase (CRL), the SCF complex, consists of Skp1, Cdc53/Cul1, the F-box protein and the RING-finger domain-containing protein Rbx1/Roc1/Hrt1 (3,4). Cdc53/Cul1 acts as scaffold and its carboxy-terminus binds to Rbx1, which associates with the E2 enzyme (e.g., Cdc34). The amino-terminus of Cdc53/Cul1 binds to Skp1, which is an adaptor protein that mediates the interaction between Cdc53/Cul1 and F-box proteins that recruit substrates for ubiquitination and degradation (5). *S. cerevisiae* encodes approximately 20 F-box proteins (6,7). Although many substrates have been identified for several F-box proteins (e.g., Cdc4 and Grr1) in *S. cerevisiae*, only a few degradation substrates have been identified for most of the remaining F-box proteins. Therefore, it is essential to identify the substrates of each F-box protein to elucidate the physiological functions of SCF complexes (3,8).

In a previous study, we reported that the SCF^{Ucc1} ubiquitin ligase complex targets citrate synthase 2 (Cit2) for proteasomal degradation in glucose-grown cells, thereby suppressing the glyoxylate cycle, an anabolic pathway that replenishes the TCA cycle with succinate for the activation of gluconeogenesis (9). Although the important role of Ucc1 in the regulation of metabolic pathways in *S. cerevisiae* is known, its function in other yeast species remains unclear. In the present study, we cloned Ucc1 orthologs from *Zygosaccharomyces bailii*, a common spoilage yeast, and *Candida glabrata*, an emerging fungal pathogen, and analysed their activities in *S. cerevisiae*. *Z. bailii* Ucc1 could rescue the *ucc1*Δ phenotype, suggesting the existence of a similar mechanism regulating the glyoxylate cycle in *Z. bailii*. By contrast, *C. glabrata* Ucc1 did not complement the *ucc1*Δ phenotype or exhibit a dominant negative effect on Ucc1 in *S. cerevisiae*. These results suggest the importance of analysing the mechanism by which the glyoxylate cycle is regulated in a broad range of yeast species.

MATERIALS AND METHODS

Strains, plasmids and culturing conditions The yeast strains (*S. cerevisiae*) and plasmids used in the study are listed in Supplementary Tables S1 and S2, respectively. Standard genetic techniques were used to construct the strains and plasmids. Cells were grown in YPD-rich medium (1% yeast extract, 1% peptone and 2% glucose) or SD medium (0.67% yeast nitrogen base with 2% glucose and lacking the appropriate amino acids). Serial dilution analysis of yeasts was performed on SD minimal plates (0.67% yeast nitrogen base without amino acids,

* Corresponding author. Tel./fax: +81 52 872 5856.
E-mail address: nakatsukasa@nsc.nagoya-cu.ac.jp (K. Nakatsukasa).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.06.003

2% glucose, 0.01% adenine hydrochloride, 0.01% L-methionine, 0.01% L-tryptophan, 0.01% L-histidine, 0.01% L-leucine and 2% agar) and SAce minimal plates (0.67% yeast nitrogen base without amino acids, 2% sodium acetate, 0.01% adenine hydrochloride, 0.01% L-methionine, 0.01% L-tryptophan, 0.01% L-histidine, 0.01% L-leucine and 2% agar).

The integrating plasmids encoding *S. cerevisiae UCC1* were constructed as follows: the promoter region (~1000 bp) and the open reading frame of *UCC1* (P_{UCC1}-*UCC1*) were amplified by PCR using OKN1099 (GCG GAG CTC AAA TGG TTA TTG CTT ATG ATG CTC ACC GAA) and OKN870 (GCG CTC GAG TCA TCT TCG AAG ATA AGG GGT ATT CC) from pKN248, which is marked with *LEU2* and encodes P_{UCC1}-*UCC1*-T_{UCC1}. T_{UCC1} represents the 3′ untranslated region (terminator) of the *UCC1* gene. The open reading frame of *UCC1* was amplified by PCR using OKN869 (GCG TCT AGA ATG AAT CAG AGC GAT AGC AGC TT) and OKN870 from pKN248. The resultant P_{UCC1}-*UCC1* fragment and *UCC1* fragment were digested with *Sac*I/*Xho*I and *Xba*I/*Xho*I, respectively, and inserted into the same sites of p416ADH (10) to generate pKN330 and pKN331. To construct the integrating plasmids, pKN330 and pKN331 were digested with *Sac*I/*Kpn*I. The resultant P_{UCC1}-*UCC1*-T_{CYC1} fragment and P_{ADH1}-*UCC1*-T_{CYC1} fragment were inserted into the same site of pRS306 to generate pKN333 and pKN334, respectively.

The integrating plasmids encoding *C. glabrata UCC1* were constructed as follows: the *C. glabrata UCC1* gene (CAGL0C01155g) was amplified by PCR using OKN1366 (GCG ACT AGT ATG AAT CTC CCA ATA GAA ATT ACA CTG CG) and OKN1367 (GCG CTC GAG CTA ATC CAA ATC TTT TGT AGG TCT CCG T) from *C. glabrata* genomic DNA (a gift from Chibana H., Chiba University). The amplicon was digested with *Spe*I/*Xho*I and cloned into the same sites of p416ADH to generate pKN323. The promoter region of *S. cerevisiae UCC1* was amplified by PCR using OKN1099 and OKN1379 (ATG CAC TAG TCT CTT GTT TGC ACA CTC TGT CTT C) from pKN248. The resultant fragment (P_{UCC1}) was digested with *Sac*I/*Spe*I and inserted into the same sites of pKN323 to generate pKN342. pKN323 was digested with *Sac*I/*Kpn*I, and the resultant fragment (P_{ADH1}-*C. glabrata UCC1*-T_{CYC1}) was inserted into the same sites of pRS306 to generate pKN343.

The integrating plasmids encoding *Z. bailii UCC1* were constructed as follows: *Z. bailii* (NBRC 1098) was purchased from National Institute of Technology and Evaluation Biological Resource Centre, Japan. *Z. bailii UCC1* (locus tag:

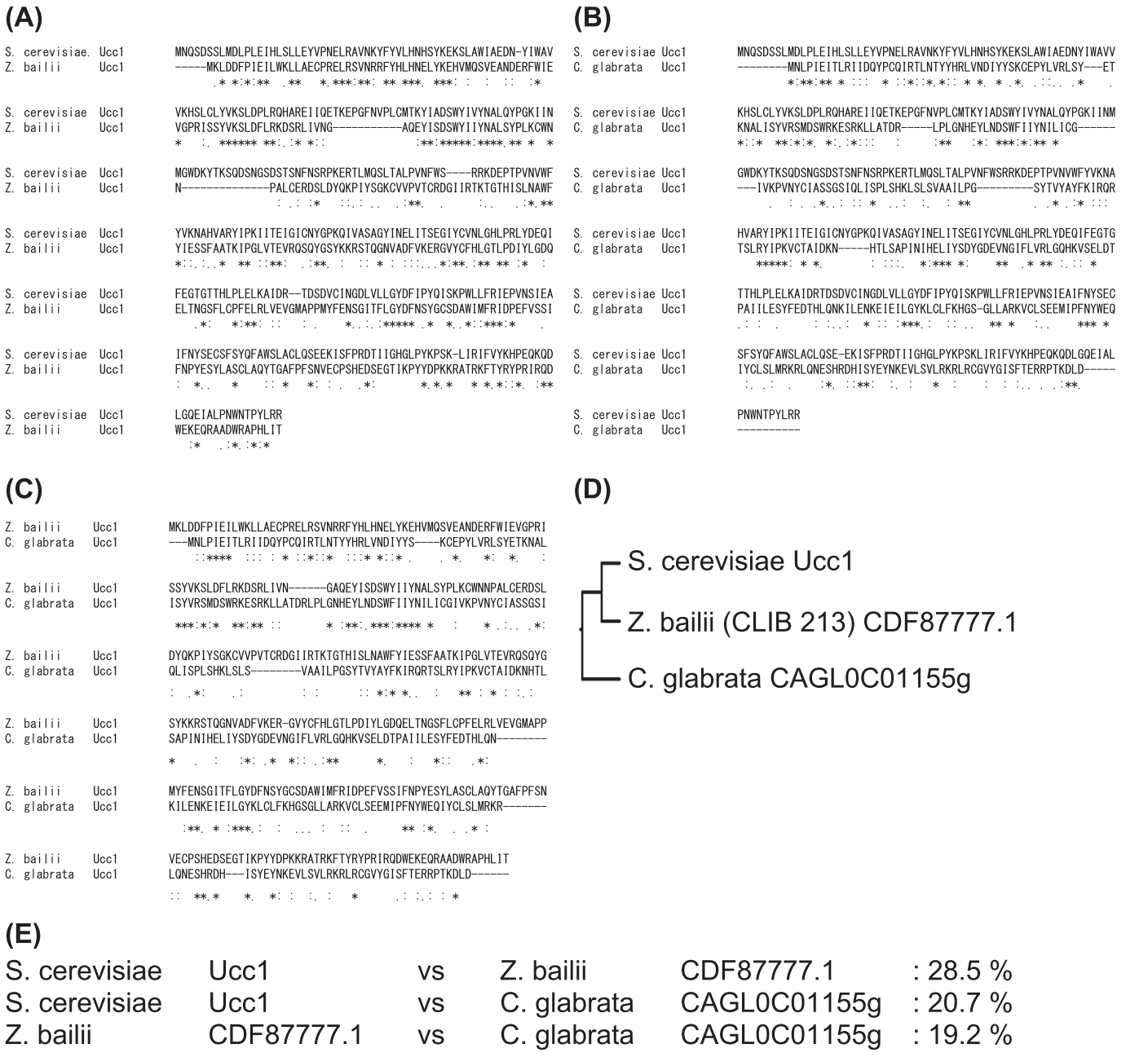


FIG. 1. Protein sequence analysis of *S. cerevisiae* Ucc1, *Z. bailii* Ucc1 and *C. glabrata* Ucc1. (A–E) The amino acid sequences of *S. cerevisiae* Ucc1 (Ylr224w), *Z. bailii* Ucc1 (CDF87777.1) and *C. glabrata* Ucc1 (CAGL0C01155g) were aligned with each other, and the rooted phylogenetic tree was generated by ClustalW. The sequence identity values were calculated with Clustal Omega.

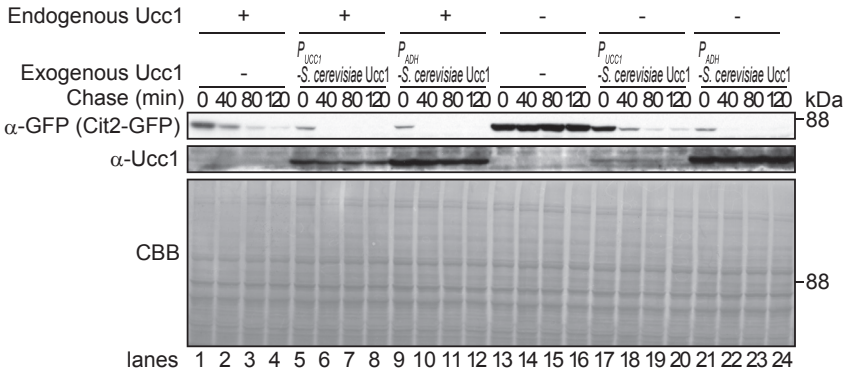


FIG. 2. *S. cerevisiae* Ucc1 induces the degradation of Cit2 in a dose-dependent manner. Typically, the indicated cells were grown in SD medium lacking appropriate amino acids overnight. Next morning, the cell culture was diluted and grown to log-phase. After addition of cycloheximide at a final concentration of 100 μg mL^{-1}, cells were collected at the indicated time points. Lysates were prepared and subjected to immunoblotting with anti-GFP antibody and anti-Ucc1 antibodies. Proteins on the immunoblot membrane were stained with Coomassie Brilliant Blue (CBB) and used as a loading control. Strains used in this assay were KNY415, KNY416, KNY417, KNY418, KNY419 and KNY420.

BN860_14246g; Protein ID: CDF87777.1; other names: ZYBA0S01-14246g, S6EZA6) was amplified by PCR using OKN1356 (GCG TCT AGA ATG AAA TTA GAT GAC TTC CCC ATT GAG A) and OKN1357 (GCG GTC GAC TCA GGT TAT TAG GTG CGG TGC C) from *Z. bailii* genomic DNA. The amplicon was digested with *Xba*I/*Sal*I and cloned into the same sites of p416ADH. The resultant plasmid was digested with *Xba*I/*Kpn*I and cloned into the same sites of pKN334 to generate pKN412.

The integrating plasmids were linearised with *Nco*I and integrated at the *ura3-1* locus of KNY346 or KNY347. Correct integration was confirmed by PCR using OKN1426 (GGC AAC GGT TCA TCA TCT CAT GG) and OKN1428 (ATG GTT CAC GTA GTG GGC CAT C).

Sequence analysis Protein sequences were analysed by ClustalW (https://www.genome.jp/tools-bin/clustalw), Clustal Omega (https://www.uniprot.org/align/) and PhylomeDB (http://phylomedb.org/) (11,12). The probability of a mitochondrial pre-sequence in each citrate synthase was calculated with MitoFates (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi) (13).

Antibodies and immunoblotting Mouse monoclonal antibody against GFP was purchased from Nacalai Tesque (Kyoto, Japan). The rabbit polyclonal anti-Cdc48 antibodies were described in our previous study (14). The polyclonal anti-Ucc1 antibodies were generated in rabbits against recombinant Ucc1. I note that the resultant anti-Ucc1 antibodies weakly detected endogenous Ucc1, whereas they detected overexpressed Ucc1. Anti-mouse IgG-HRP (Sigma–Aldrich Japan, Tokyo, Japan) and anti-rabbit IgG-HRP (Sigma–Aldrich Japan) were used as secondary antibodies. Proteins were dissolved in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (80 mM Tris–Cl pH 7.5, 8 mM EDTA, 0.2 M DTT, 4% SDS, 8 M Urea, 15% glycerol, 0.08% Tris-base, 0.01% BPB), separated by SDS–PAGE, transferred to Immobilon-P membranes (Merck, Tokyo,

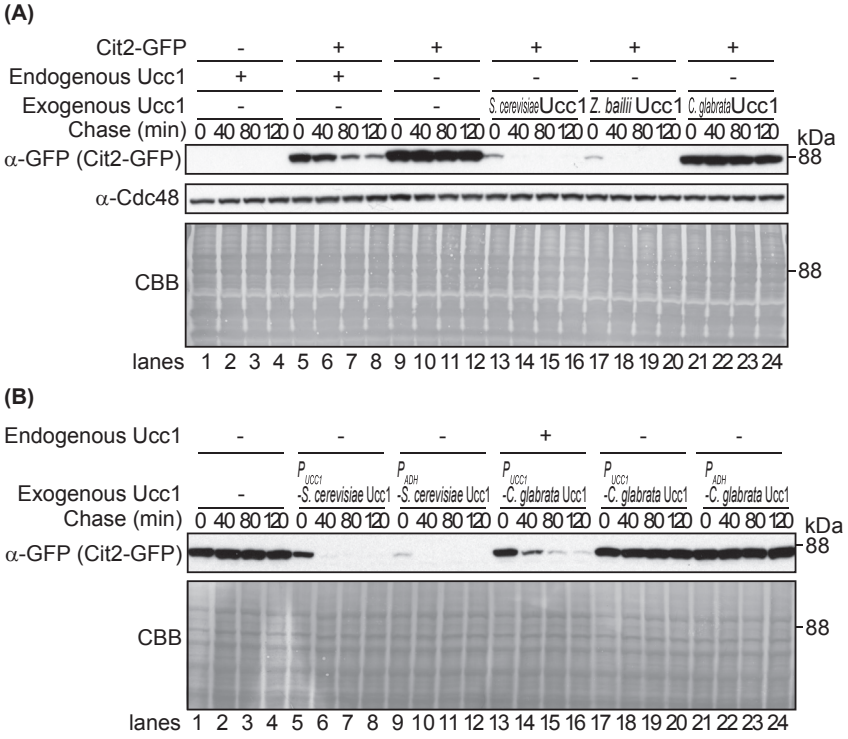


FIG. 3. The defect of Cit2 degradation in *ucc1*Δ *S. cerevisiae* can be complemented by *Z. bailii* Ucc1 but not by *C. glabrata* Ucc1. (A) Typically, the indicated cells were grown in SD medium lacking appropriate amino acids overnight. Next morning, the cell culture was diluted and grown to log-phase. After addition of cycloheximide at a final concentration of 100 μg mL^{-1}, cells were collected at the indicated time points. Lysates were prepared and subjected to immunoblotting with anti-GFP antibody. Cdc48 served as a loading control. Proteins on the immunoblot membrane were stained with CBB, which was used as a loading control. Strains used in this assay were TKY2586 transformed with p416ADH, KNY415, KNY418, KNY420, KNY457 and KNY424. Lanes 1–4 demonstrate the specificity of the anti-GFP antibody used in this study. (B) The cycloheximide chase experiment was performed as in panel A. Proteins on the immunoblot membrane were stained with CBB, which was used as a loading control. Strains used in this assay were KNY418, KNY419, KNY420, KNY421, KNY423 and KNY424.

Japan), and subjected to western blotting. Signals were detected using Chemi-Lumi One (Nacalai Tesque).

Protein degradation assay The cycloheximide chase protein degradation assay was performed as described previously (9,15). Typically, yeast cells were grown to log-phase (30–40 mL, OD_{600} = 0.4–0.8) and cycloheximide was added to the medium at the final concentration of 100 μg mL^{-1}. At the indicated time points, 5 mL of cultured cells were transferred to a conical tube and mixed with 30 mM azide on ice. Cells were collected by centrifugation at 3000 rpm for 2 min at 4 °C, and the resultant pellet was stored at −80 °C until use. Cells were then disrupted by vortexing in presence of 300 μL of 20% trichloroacetic acid (TCA) and acid-washed glass beads (Yasui Kikai, Osaka, Japan) for 30 s, followed by incubation on ice for another 30 s. This procedure was repeated 8 times. Next, 600 μL of 5% TCA was added, and 650 μL of suspension was transferred to a new eppendorf tube. Glass beads were added to 400 μL of 5% TCA, and 450 μL of the suspension was pooled with the preserved cell suspension. Proteins were precipitated by centrifugation at 20,000 ×*g* for 10 min at 4 °C, rinsed with 900 μL of ice-cold acetone, and dissolved in SDS–PAGE sample buffer.

RESULTS

Cloning of *S. cerevisiae UCC1* orthologs from *Z. bailii* and *C. glabrata* Although many orthologs of well-characterized F-box proteins such as Cdc4 and Met30 have been identified in other fungal species (approximately 45 and 65 orthologs for Cdc4 and Met30, respectively), the number of Ucc1 orthologs is limited (approximately 11 orthologs) (PhylomeDB) (11,12). To determine the extent to which the function of Ucc1 is conserved among the limited species, Ucc1 orthologs were cloned from *Z. bailii* (CDF87777.1) and *C. glabrata* (CAGL0C01155g) (Fig. 1A–C). *Z. bailii* was selected for this study since it is an important yeast species from the aspect of food preservation; it is an aggressive food spoilage microorganism and is the most acetic acid-tolerant yeast species. *C. glabrata* was also selected considering it an emerging fungal pathogen. The corresponding proteins have been referred to as *Z. bailii* Ucc1 and *C. glabrata* Ucc1 in the present study. The N-terminal F-box domain was relatively conserved between the species, whereas others were less conserved. Overall, *S. cerevisiae* Ucc1 found to share 28.5% and 20.7% identity with *Z. bailii* Ucc1 and *C. glabrata* Ucc1, respectively, whereas *Z. bailii* Ucc1 and *C. glabrata* Ucc1 share 19.2% identity (Fig. 1D and E).

***Z. bailii* Ucc1 but not *C. glabrata* Ucc1 can restore Cit2 degradation in *ucc1*Δ cells** In a previous study, we demonstrated that, when *S. cerevisiae* is grown in glucose-containing medium, Cit2 is degraded by the proteasome in a SCF^{Ucc1} ubiquitin ligase-dependent manner (9). In addition, we showed that the Cit2-GFP-SKL fusion protein, in which EGFP was inserted just upstream of the C-terminal peroxisome targeting signal (serine-lysine-leucine), was fully functional and degraded in a manner similar to the original Cit2 (9). To determine whether the exogenously expressed *S. cerevisiae* Ucc1 could complement the *ucc1*Δ phenotype, a cycloheximide chase experiment was performed, and the half-life of the Cit2-GFP-SKL fusion protein (hereafter described as Cit2-GFP) expressed from the *CIT2* locus was analysed. Consistent with the results of the previous study, Cit2-GFP was degraded with a half-life of approximately 60 min (Fig. 2, lanes 1–4), whereas it was almost completely stabilised in the absence of Ucc1 (Fig. 2, lanes 13–16). Expression of *S. cerevisiae* Ucc1 under the control of its endogenous promoter or the relatively strong *ADH1* promoter (10) from the *URA3* locus restored Cit2-GFP degradation (Fig. 2, lanes 17–20 and 21–24). An increase in the amount of Ucc1 (compare the level of Ucc1 in Fig. 2, lanes 17–20 and 21–24) correlated with a decrease in Cit2-GFP, suggesting that Cit2-GFP degradation was facilitated by Ucc1 in a dose-dependent manner. Endogenous Ucc1 was almost undetectable using the anti-Ucc1 antibody (Fig. 2, lanes 1–4), whereas Ucc1 expressed under the control of its own promoter from the *URA3* locus was easily detected (Fig. 2, lanes 17–20), suggesting that the level of exogenously expressed Ucc1 was slightly higher than the endogenous level.

We next tested whether *Z. bailii* Ucc1 and *C. glabrata* Ucc1 could complement the *ucc1*Δ phenotype when expressed in *S. cerevisiae*. To this end, Cit2-GFP degradation was assessed using the cycloheximide chase experiment in cells expressing *Z. bailii* Ucc1 and *C. glabrata* Ucc1 under the control of the *ADH1* promoter from the *URA3* locus. As shown in Fig. 3A, *Z. bailii* Ucc1 could restore Cit2-GFP degradation to the same extent as *S. cerevisiae* Ucc1 (compare Fig. 3A, lanes 17–20 and 13–16), whereas *C. glabrata* Ucc1 could not restore the degradation (Fig. 3A, lanes 21–24).

Overexpression of a ubiquitin ligase does not always facilitate degradation, whereas it sometimes stabilises the substrate. This is probably because the ligase tightly captures the substrate, which cannot be released for a subsequent step (our unpublished observation). We therefore hypothesised that ligase overexpression may have caused the unsuccessful complementation of the *ucc1*Δ phenotype by *C. glabrata* Ucc1. To test this possibility, *C. glabrata* Ucc1 was expressed under the control of the *S. cerevisiae UCC1* promoter from the *URA3* locus. As shown in Fig. 3B, *C. glabrata* Ucc1 was unable to complement the depletion of Ucc1 (Fig. 3B, lanes 17–20). The expression of *C. glabrata* Ucc1 under the control of the *ADH1* promoter did not suppress Cit2 degradation in wild-type cells (Fig. 3B, lanes 13–16), suggesting that *C. glabrata* Ucc1 does not cause a dominant negative effect on the endogenous *S. cerevisiae* Ucc1.

Overexpression of *Z. bailii* Ucc1 but not *C. glabrata* Ucc1 causes a growth defect of *S. cerevisiae* on acetate medium Cells lacking Cit2 show a significant growth defect on medium containing acetate as the sole carbon source (Fig. 4, compare lines 1 and 2) (9). This is likely because Cit2 depletion causes a defect in the glyoxylate cycle, which is essential for growth on acetate medium. In the previous study, we demonstrated that overexpression of *S. cerevisiae* Ucc1 results in severe growth defect on acetate medium because it decreases the level of Cit2 (Fig. 4, line 3) (9). Here, it should be noted that the endogenously expressed Cit2-GFP, in which GFP is tagged at the C-terminus of Cit2, was functional (Fig. 4, line 7). In addition, cells lacking Ucc1 grew normally on acetate medium, suggesting that the accumulation of Cit2-GFP does not affect the yeast growth on acetate medium (Fig. 4, line 6). To further examine the function of *Z. bailii* Ucc1 and *C. glabrata* Ucc1, the growth of *S. cerevisiae* overexpressing *Z. bailii* Ucc1 and *C. glabrata* Ucc1 on acetate medium was tested. Overexpression of *Z. bailii* Ucc1 suppressed growth, whereas *C. glabrata* Ucc1 had no significant effect (Fig. 4, lines 4 and 5). This was consistent with the above observation

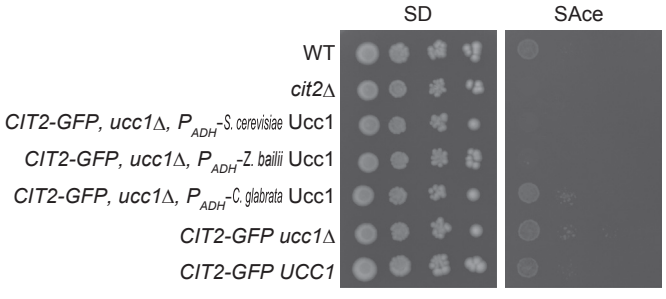


FIG. 4. Growth analysis of *S. cerevisiae* strains overexpressing *S. cerevisiae* Ucc1, *Z. bailii* Ucc1 or *C. glabrata* Ucc1. The indicated cells were grown to log-phase and spotted onto synthetic minimal medium plates containing glucose (SD) or sodium acetate (SAce). *S. cerevisiae* Ucc1, *Z. bailii* Ucc1 or *C. glabrata* Ucc1 was overexpressed under the control of the *ADH* promoter from the *URA3* locus in the genome. Plates were incubated at 30 °C for 6 days. Strains used in this assay were TKY2586 transformed with p416ADH, TKY1751 transformed with p416ADH, KNY420, KNY457, KNY424, KNY418 and KNY415.

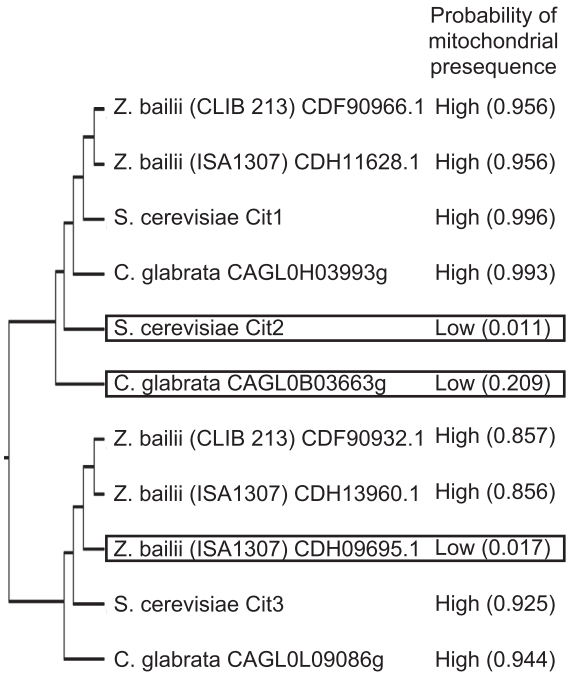
(A)

```
S. cerevisiae.  Skp1    --MVTSNVVLVSGEGERFTVDKKIAERSLLLKNYLNDMHDSNLQNNSDSESDSDSETNHK
Z. bailii       Skp1    --MPESQVVLVSGEGERFTVERRIAERSLLLKNYLNDMHDSVLE----------------
C. glabrata     Skp1    MDKKGKYVVLVSGEGEKFTVEKKIAQRSLLLKNYLNDMHESSLDDEDEEQ----------
                        . *********:***:::**:************:* *:

S. cerevisiae   Skp1    SKDNNNGDDDDEDDDEIVMPVPNVRSSVLQKVIEWAEHHRDSNFPDEDDDDSRKSAPVDS
Z. bailii       Skp1    ---------DSDEEEDVVMPIPNVRSSVLQKVIEWAEHHRDSTFPDEDDDDSRKSAPMDS
C. glabrata     Skp1    -------DEDEDEDDEIVMPVPNVRSSVLQKVIEWAEHHRDSNFPDEDDDDSRKSAPVDA
                          *.::::::***:**********************.***************:*:

S. cerevisiae   Skp1    WDREFLKVDQEMLYEIILAANYLNIKPLLDAGCKVVAEMIRGRSPEEIRRTFNIVNDFTP
Z. bailii       Skp1    WDREFLKVDQEMLYEIIQAANYLNIKPLLDAGCKVVAEMIRGRSAEEIRRTFNIVNDFTP
C. glabrata     Skp1    WDREFLKVDQEMLYEIILAANYLNIKPLLDAGCKVVAEMIRGRSPEEIRRTFNIVNDFTP
                        ***************** *************************.***************

S. cerevisiae   Skp1    EEEAAIRRENEWAEDR
Z. bailii       Skp1    EEEAAIRRENEWAEDR
C. glabrata     Skp1    EEEAAIRRENEWAEDR
                        ****************
```

(B)

S. cerevisiae	Skp1	vs	Z. bailii	CDF88787.1	: 75.9 %
S. cerevisiae	Skp1	vs	C. glabrata	CAGL0M01364g	: 79.2 %
Z. bailii	CDF88787.1	vs	C. glabrata	CAGL0M01364g	: 81.6 %

(C)



(D)

```
S. cerevisiae   Cit2        MTVPYLNSNRNVASYLQSNSSQEKTLKERFSEIYPIHAQDVRQFVKEHGKTKISDVLLEQ
C. glabrata     CAGL0B03663g ----------MPNLSHQWESVSLKEAYARNLPAQAERLKRFKSEFGSTVIGEVTVEQ
Z. bailii       CDH09695    --------MRRAFHCTKSASSSSVGLKQVLQDLIPERKHDILELKKNYGNVKIGDITIGS
                                    *. ..  **:     * : . : : :*. *.:: : .

S. cerevisiae   Cit2        VYGGMRGIPGSVWEGSVLDPEDGIRFRGRTIADIQKDLPK----AKGSSQPLPEALFWLL
C. glabrata     CAGL0B03663g ALGGMRGVTGLVWEGSVLDPEEGIKFRGLTIDEVRVSLG-----VAGEREPQPEALFWLL
Z. bailii       CDH09695    VIGGMRGNASMFWQGTSLDPYEGIKFQGKTISDCQRLLPKSPKDSNGKGNFLPESMLWLL
                . ***** .. .*:*: *** :**:*:* ** : :  *       *. :  **:::***

S. cerevisiae   Cit2        LTGEVPTQAQVENLSADLMSRS-ELPSHVVQLLDNLPKDLHPMAQFSIAVTALESESKFA
C. glabrata     CAGL0B03663g LTGQIPNEDQVAQLSRELAARG-ELPGHVETLLDTLPRDLHPMAQLSIAVTALEGESHFA
Z. bailii       CDH09695    MTGEVPSAEQALHLSAELAARGRQLPDYTDKLMSNLPSGMHPMTQLAIGLASMNKGSEFA
                :**::*.  *. :** :* :*. :**.:.  *:.,** .:***:*::*.::::::  *.**

S. cerevisiae   Cit2        KAYAQG-ISKQDYWSYTFEDSLDLLGKLPVIAAKIYRNVFKDGKMG---------EVDPN
C. glabrata     CAGL0B03663g RAYLAG-VPRGELWEHTYEDALNLIAKLPAIAGRIYHNVFGGAQGAQGQGTQGAFVLDPQ
Z. bailii       CDH09695    RKYEKGDIGKNEYWEYVFDDAINLIAALPQLTGKIYSNIVAGGKPLG--------QFRED
                : *  * : : : *.:.::*::*:. ** ::.:** *:. ..:            .  :

S. cerevisiae   Cit2        ADYAKNLVNLIGSKD--------------EDFVDLMRLYLTIHSDHEGGNVSAHTSHLVG
C. glabrata     CAGL0B03663g ADYSENLCRALGVQPDG----------ETEDFHNLMRLYLTIHADHEGGNVSAHTTHLVG
Z. bailii       CDH09695    RDWSYNICSLLGMTSGDSCNRQNLGGKQSEDFVNLMRLYTGIHVDHEGGNVSAHTTHLVG
                 *:: *:   :*                 *** :***** ** ***********:*****

S. cerevisiae   Cit2        SALSSPYLSLASGLNGLAGPLHGRANQEVLEWLFALKEEVN----DDYSKDTIEKYLWDT
C. glabrata     CAGL0B03663g SALSSPYLSFAAGLDGLAGPLHGRANQEVLEWLLVLKGELVREGKTVTDREAIRQYLWRT
Z. bailii       CDH09695    SALSDPYLSYSSGIMGLAGPLHGLAAQEVVRFLTEMNALIS----SPQSGREIEQYLWKL
                ****.**** ::*: ******** * ***:.:*  ::  :         .  *.:***

S. cerevisiae   Cit2        LNSGRVIPGYGHAVLRKTDPRYMAQRKFAMDH---FPDYELFKLVSSIYEVAPGVLTEHG
C. glabrata     CAGL0B03663g LNSGKVVPGYGHAVLRKTDPRFLAQREFGRTR---FPGDELYQLVSTVYEVAPQVLKEHG
Z. bailii       CDH09695    LSCKRVIPGYGHAVLRKADPRFIAMLDFAKARPEEFAEDNNVILMEKLSEIAPKVLKEHG
                *. .:*:**********:***::*  .*.  :   *.  :   *:.: *:** **.***

S. cerevisiae   Cit2        KTKNPWPNVDAHSGVLLQYYGLKESSFYTVLFGVSRAFGILAQLITDRAIGASIERPKSY
C. glabrata     CAGL0B03663g KTRNPYPNVDAHSGVLLTHYGLDQPSFYTVLFGISRCFGVMAQLITDRALGAPIERPKSL
Z. bailii       CDH09695    KTKNPYPNVDSASGILFYHYGVTELLFFTVIFGCSRSMGPLTQLVWDRALGLPIERPKSL
                **:**:****: **:*: :**: :  *:**:** **.:* ::**: ***:* .******

S. cerevisiae   Cit2        STEKYKELVKNIESKL
C. glabrata     CAGL0B03663g STDRYIELATRHHNKL
Z. bailii       CDH09695    DLKSLGALCR------
                . .    *
```

(E)

S. cerevisiae	Cit2	vs	Z. bailii	CDH09695.1	: 44.0 %
S. cerevisiae	Cit2	vs	C. glabrata	CAGL0B03663g	: 57.4 %
Z. bailii	CDH09695.1	vs	C. glabrata	CAGL0B03663g	: 43.4 %

FIG. 5. Protein sequence analysis of *Skp1* and citrate synthase in *S. cerevisiae*, *Z. bailii*, and *C. glabrata*. (A, B) The amino acid sequences of *S. cerevisiae* Skp1 (YDR328c), *Z. bailii* Skp1 (CDF88787.1) and *C. glabrata* Skp1 (CAGL0M01364g) were aligned with each other by ClustalW. The sequence identity values were calculated with Clustal Omega. (C) The rooted phylogenetic tree was generated by ClustalW for the protein sequences of three citrate synthases from *S. cerevisiae* (Cit1, Cit2 and Cit3), two citrate synthases from *Z. bailii* (CLIB 213) (CDF90966.1 and CDF90932.1), three citrate synthases from *Z. bailii* (ISA 1307) (CDH11628.1, CDH13960.1 and CDH09695.1) and three citrate synthases from *C. glabrata* (CAGL0H03993g, CAGL0B03663g and CAGL0L09086g). The probability of a mitochondrial pre-sequence in each citrate synthase was calculated with MitoFates. (D, E) The amino acid sequences of *S. cerevisiae* Cit2, *Z. bailii* CDH09695.1 and *C. glabrata* CAGL0B03663g were aligned with each other by ClustalW. The sequence identity values were calculated with Clustal Omega.

that *Z. bailii* Ucc1, but not *C. glabrata* Ucc1, supports Cit2 degradation in *S. cerevisiae*.

DISCUSSION

In the present study, we demonstrated that *Z. bailii* Ucc1 rescues the *ucc1*Δ phenotype in *S. cerevisiae*, thereby suggesting that the proteolytic regulation of glyoxylate cycle is not limited to *S. cerevisiae*, rather is basically conserved across the yeast species. Interestingly, however, *C. glabrata* Ucc1 could not complement the *ucc1*Δ phenotype in *S. cerevisiae* or exhibit a dominant negative effect on Ucc1.

Since *C. glabrata* Ucc1 could not rescue the *ucc1*Δ phenotype in *S. cerevisiae*, we analysed the sequence of Skp1, an adaptor that connects F-box proteins to Cul1 (Cdc53 in *S. cerevisiae*). In contrast to Ucc1, which shares only 20–30% identity with *S. cerevisiae* Ucc1, *Z. bailii* Ucc1 and *C. glabrata* Ucc1 (Fig. 1), Skp1 is highly conserved in the three yeast species, sharing 75–81% identity (Fig. 5A and B). In addition, we extracted the sequences of citrate synthases from these organisms (Fig. 5C–E). *S. cerevisiae* had three citrate synthases, and Cit1 and Cit3 were mitochondrial isoforms. Both Cit1 and Cit3 had a high probability of mitochondrial pre-sequence. A citrate synthase with a lower probability of a mitochondrial pre-sequence was not identified in the *Z. bailii* (CLIB213) strain, whereas one (CDH09695.1) was identified in the *Z. bailii* (ISA1307) strain and one (CAGL0B03663g) in *C. glabrata*. The underlying reason for the intra-species difference between *Z. bailii* (CLIB213) strain and *Z. bailii* (ISA1307) strain remains unknown. Although further study is recommended to fully elucidate the roles of these citrate synthase orthologs, the lack of a clear mitochondrial targeting sequence suggests these citrate synthases (ISA1307 and CAGL0B03663g) to be able to function in a manner similar to *S. cerevisiae* Cit2 in the glyoxylate cycle.

To answer, why *C. glabrata* Ucc1 could not complement the *ucc1*Δ phenotype in *S. cerevisiae* or exhibit a dominant negative effect on Ucc1, a simple explanation would be that *C. glabrata* Ucc1 interacts with *S. cerevisiae* Skp1 and/or *S. cerevisiae* Cit2 in *S. cerevisiae*, since Ucc1 is less conserved in *C. glabrata* than in *Z. bailii*. Another possible explanation could be that other proteolytic machineries regulate the glyoxylate cycle in *C. glabrata*. In this regard, it would be important to analyse the role of the Gid complex, which is known to regulate the glyoxylate cycle and gluconeogenic pathway in *S. cerevisiae* (16) in *C. glabrata*. Finally, it is also possible that the glyoxylate cycle in *C. glabrata* is transcriptionally regulated, and proteolytic regulation was not be the main regulatory mechanism. Similar differences can be found for ergosterol synthesis pathway in *S. cerevisiae* and *Schizosaccharomyces pombe*; ergosterol synthesis pathway may be regulated by endoplasmic reticulum-associated degradation (ERAD) of several enzymes in *S. cerevisiae*, while the same pathway may be regulated by their synthesis rate in *S. pombe* (17).

In a broader sense, since the glyoxylate cycle and gluconeogenic pathways are crucial for the virulence of pathogenic yeast (18), it is important to further analyse their regulatory mechanisms in pathogenic yeast species. In addition, from the engineering point of view, overexpression of Ucc1 has been shown to confer *n*-butanol tolerance in *S. cerevisiae* (9,19). Genetic engineering of alcohol-tolerant yeast strain is therefore crucial for overcoming the toxicity that offers the upper limit to product titers. However, genetic analysis of minor yeast species is sometimes difficult due to the limitation of molecular biology tools. Complementation assay, using a mutant of *S. cerevisiae*, would be the starting point to analyse gene function in minor yeast species.

Supplementary data to this article can be found online at doi: https://doi.org/10.1016/j.jbiosc.2019.06.003.

ACKNOWLEDGMENTS

We would like to thank Ayumi Aoyama for helpful discussions. We are especially grateful to Shuang Li and Yasushi Yukawa (Nagoya City University) for technical assistance, Takumi Kamura and Fumihiko Okumura (Nagoya University) for yeast strains, Hiroji Chibana (Chiba University) for the genomic DNA of *Candida glabrata* and Kazuya Nishio and Tsunehiro Mizushima (Hyogo University) for insightful discussions. This work was supported by the Naito Foundation, the Daiko Foundation and the Takeda Science Foundation (to K.N.). The authors declare no conflict of interests.

References

1. **Finley, D., Ulrich, H. D., Sommer, T., and Kaiser, P.:** The ubiquitin-proteasome system of *Saccharomyces cerevisiae*, Genetics, **192**, 319–360 (2012).
2. **Komander, D. and Rape, M.:** The ubiquitin code, Annu. Rev. Biochem., **81**, 203–229 (2012).
3. **Deshaies, R. J. and Joazeiro, C. A.:** RING domain E3 ubiquitin ligases, Annu. Rev. Biochem., **78**, 399–434 (2009).
4. **Harper, J. W. and Tan, M. K.:** Understanding cullin-RING E3 biology through proteomics-based substrate identification, Mol. Cell Proteomics, **11**, 1541–1550 (2012).
5. **Mark, K. G., Simonetta, M., Maiolica, A., Seller, C. A., and Toczyski, D. P.:** Ubiquitin ligase trapping identifies an SCFSaf1 pathway targeting unprocessed vacuolar/lysosomal proteins, Mol. Cell, **53**, 148–161 (2014).
6. **Willems, A. R., Schwab, M., and Tyers, M.:** A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin, Biochim. Biophys. Acta, **1695**, 133–170 (2004).
7. **Jonkers, W. and Rep, M.:** Lessons from fungal F-box proteins, Eukaryot. Cell, **8**, 677–695 (2009).
8. **Skaar, J. R., Pagan, J. K., and Pagano, M.:** Mechanisms and function of substrate recruitment by F-box proteins, Nat. Rev. Mol. Cell Biol., **14**, 369–381 (2013).
9. **Nakatsukasa, K., Nishimura, T., Byrne, S. D., Okamoto, M., Takahashi-Nakaguchi, A., Chibana, H., Okumura, F., and Kamura, T.:** The ubiquitin ligase SCFUcc1 acts as a metabolic switch for the glyoxylate cycle, Mol. Cell, **59**, 22–34 (2015).
10. **Mumberg, D., Muller, R., and Funk, M.:** Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds, Gene, **156**, 119–122 (1995).
11. **Huerta-Cepas, J., Capella-Gutierrez, S., Pryszcz, L. P., Denisov, I., Kormes, D., Marcet-Houben, M., and Gabaldon, T.:** PhylomeDB v3.0: an expanding repository of genome-wide collections of trees, alignments and phylogeny-based orthology and paralogy predictions, Nucleic Acids Res., **39**, D556–D560 (2011).
12. **Huerta-Cepas, J., Capella-Gutierrez, S., Pryszcz, L. P., Marcet-Houben, M., and Gabaldon, T.:** PhylomeDB v4: zooming into the plurality of evolutionary histories of a genome, Nucleic Acids Res., **42**, D897–D902 (2014).
13. **Fukasawa, Y., Tsuji, J., Fu, S. C., Tomii, K., Horton, P., and Imai, K.:** MitoFates: improved prediction of mitochondrial targeting sequences and their cleavage sites, Mol. Cell. Proteomics, **14**, 1113–1126 (2015).
14. **Nakatsukasa, K., Brodsky, J. L., and Kamura, T.:** A stalled retrotranslocation complex reveals physical linkage between substrate recognition and proteasomal degradation during ER-associated degradation, Mol. Biol. Cell, **24**, 1765–1775 (2013).
15. **Nakatsukasa, K., Huyer, G., Michaelis, S., and Brodsky, J. L.:** Dissecting the ER-associated degradation of a misfolded polytopic membrane protein, Cell, **132**, 101–112 (2008).
16. **Menssen, R., Schweiggert, J., Schreiner, J., Kusevic, D., Reuther, J., Braun, B., and Wolf, D. H.:** Exploring the topology of the Gid complex, the E3 ubiquitin ligase involved in catabolite-induced degradation of gluconeogenic enzymes, J. Biol. Chem., **287**, 25602–25614 (2012).
17. **Christiano, R., Nagaraj, N., Frohlich, F., and Walther, T. C.:** Global proteome turnover analyses of the yeasts *S. cerevisiae* and *S. pombe*, Cell Rep., **9**, 1959–1965 (2014).
18. **Lorenz, M. C. and Fink, G. R.:** The glyoxylate cycle is required for fungal virulence, Nature, **412**, 83–86 (2001).
19. **Gonzalez-Ramos, D., van den Broek, M., van Maris, A. J., Pronk, J. T., and Daran, J. M.:** Genome-scale analyses of butanol tolerance in *Saccharomyces cerevisiae* reveal an essential role of protein degradation, Biotechnol. Biofuels, **6**, 48 (2013).