A Role of YlBud8 in the Regulation of Cell Separation in the Yeast *Yarrowia lipolytica*

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The spatial landmark protein Bud8 plays a crucial role in bipolar budding in the budding yeast *Saccharomyces cerevisiae*. The unconventional yeast *Yarrowia lipolytica* can also bud in a bipolar pattern, but is evolutionarily distant from *S. cerevisiae*. It encodes the protein YALI0F12738p, which shares the highest amino acid sequence homology with *S. cerevisiae* Bud8, sharing a conserved transmembrane domain at the C-terminus. Therefore, we named it YlBud8. Deletion of YlBud8 in *Y. lipolytica* causes cellular separation defects, resulting in budded cells remaining linked with one another as cell chains or multiple buds from a single cell, which suggests that YlBud8 may play an important role in cell separation, which is distinct from the function of Bud8 in *S. cerevisiae*. We also show that the YlBud8-GFP fusion protein is located at the cell membrane and enriched in the bud cortex, which would be consistent with a role in the regulation of cell separation. The coiled-coil domain at the N-terminus of YlBud8 is important to the correct localization and function of YlBud8, as truncated proteins that do not contain the coiled-coil domain cannot rescue the defects observed in *YlBud8Δ*. This finding suggests that a new signaling pathway controlled by YlBud8 via regulation of cell separation may exist in *Y. lipolytica*.

Keywords: YlBud8, cell separation, cell polarity, coiled-coil

Introduction

Cell polarization is central to the development of most organisms as it plays important roles in cell differentiation, division, cell-cell signaling, and cell migration [1]. Many cellular physiological activities are directional, including nutrient transport, nerve signal transmission, as well as cell movement, and cell polarity is necessary for the normal function of these and other cellular activities. *Saccharomyces cerevisiae* is a classic model organism. Its cells display significant polarity when sensing and responding to external or internal signals, and polar growth is involved in many steps of the cell cycle, including budding site selection, bud growth, mating, mycelial growth and cell separation [2, 3]. Cell separation is important for both increased cell number and cell differentiation, and is considered to be a special form of polar growth [4]. In budding yeast, both budding and cell separation require actin polarization and exocytosis. During the process of

budding, polar growth is directed to the cortex of the bud to assemble a new bud, while at the later stage of the cell cycle, polar growth is directed to the bud neck to facilitate cytokinesis [5, 6]. Site selection in budding and cell division is nonrandom, but rather is controlled by spatial signals [7]. Thus, it is important to understand how spatial cues determine sites of polarized cell growth.

The molecular regulation of this process has been thoroughly studied in *S. cerevisiae*. A large number of spatial landmarks that are involved in polarized cell growth during bud site selection have been identified, which exist in and are expressed in different cell types. There are two sets of landmark cues that can mark new bud sites. In haploid cells, the polar sites are marked by the axial landmarks Axl1, Axl2, Bud3, and Bud4. In diploid cells, the polar sites are marked by the bipolar landmarks Rax1, Rax2, Bud8, and Bud9 [8, 9]. The bud-site GTPase Rsr1 and its regulators, the GTPase activating protein (GAP) Bud2 and the guanine nucleotide exchange factor

(GEF) Bud5 make up the core module, which can recognize the positional cues that mark the poles of the haploid and diploid cells [10]. Then, the Rsr1 GTPase module can regulate the GTPase Cdc42, which associates with effector proteins to establish cell polarity, initiate polarized growth, and assemble the new bud at specific sites [11–13].

Spatial control of polar growth is not a phenomenon unique to *S. cerevisiae*, but exists ubiquitously in other yeasts. For example, the non-model yeast *Yarrowia lipolytica* buds in a bipolar pattern: mother cells can choose the bud site at either the proximal or distal poles to the preceding site of cytokinesis, whereas the daughter cells usually bud at the distal pole to the birth site, this process is thought to be controlled by spatial cues [14]. With *Y. lipolytica*, the bipolar bud takes on a very steady budding pattern that exists without the influence of cell type change, cell morphology change, or changes in the external environment; this characteristic is different from those of *Candida albicans* and *S. cerevisiae* [15, 16]. We are interested in the spatial landmarks that are involved in regulating the stable budding pattern of *Y. lipolytica*, a hemiascomycetous yeast species distantly related to the model organism *S. cerevisiae*. This species exhibits several special characteristics at the physiological, biochemical and metabolic levels and has recently become a model organism as unconventional yeast [17].

Research on the spatial landmark for bipolar budding in *Y. lipolytica* appears to be thus far absent. In *S. cerevisiae*, Bud8 and Bud9 are thought to act as bipolar spatial landmarks that provide spatial signals in diploid cells [18, 19]. Bud8 is a part of the distal landmark, which is required for distal bud site selection; Bud9 is localized to the proximal pole and is required for proximal pole selection

[20]. These two proteins can interact with Bud5 to deliver the landmark signals to the Rsr1 GTPase module [9]. We had previously discovered that YIRsr1 is involved in the regulation of the bipolar budding pattern and cell separation in *Y. lipolytica*. Cells without YIRsr1 cannot separate from one another and form cell chains or multi-buds [21]. To understand whether spatial landmarks similar to Bud8 and Bud9 exist to provide spatial signals to the YIRsr1 GTPase module in *Y. lipolytica*, the amino acid sequences of Bud8 and Bud9 from *S. cerevisiae* were used in a BLAST search for homologue proteins in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) where the optional organism is *Y. lipolytica*.

Here, we show that the Bud8 homologue YIBud8 plays a crucial role in cell separation, but is not involved in regulating bipolar budding. A YIBud8-GFP fusion protein can be located at the cell membrane and the small bud cortex, which is different from where *S. cerevisiae* Bud8 is located. We further show that the coiled-coil domain in YIBud8 is required for its normal function and localization.

Materials and Methods

Strains, Media and Growth Conditions

The *Y. lipolytica* strains used in this study are listed in Table 1. The construction of strains and strain source are described below or in Table 1. *Escherichia coli* strain DH5α was used for plasmids amplification. *Y. lipolytica* strains were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or in synthetic YNBD medium (0.67% yeast nitrogen base without amino acid, 2% glucose) at 30°C. YNBD media were supplemented with 20 mg/l uracil, 80 mg/l leucine, or both when required. Agar was added to 2% concentration for solid media, and glucose was used in place of glycerol to keep cells in their oval form in synthetic media.

Table 1. *Y. lipolytica* strains used in this study.

Strain	Genotype	Source
PO1a	<i>MATA leu2-270 ura3-302</i>	22
YLY403	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P</i>	This study
YLYJ3	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [Integrative URA3 YIBUD8]</i>	This study
YLYJ4	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [Integrative URA3 Ylbud8-1]</i>	This study
YLYJ5	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [Integrative URA3 Ylbud8-2]</i>	This study
YLYJ8	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [CEN LEU2 EGFP-YIBUD8]</i>	This study
YLYJ9	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [CEN LEU2 EGFP-Ylbud8-1]</i>	This study
YLYJ10	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [CEN LEU2 EGFP-Ylbud8-2]</i>	This study
YLYJ14	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [Integrative URA3 Ylbud8-3]</i>	This study
YLYJ15	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [Integrative URA3 Ylbud8-4]</i>	This study
YLYJ16	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [CEN LEU2 EGFP-Ylbud8-3]</i>	This study
YLYJ17	<i>MATA leu2-270 ura3-302 Ylbud8Δ::loxR/P [CEN LEU2 EGFP-Ylbud8-4]</i>	This study

Table 2. Plasmids used in this study.

Plasmid	Description	Source
pRRQ2	<i>CEN YlLEU2 hp4d-CRE</i>	Jean-Marc Nicaud
pWU24	<i>YlURA3</i> (178 bp promoter, ORF and 772 bp 3' UTR) in pBlueScript KS(+)	23
pWU24-YlBUD8	<i>YlBUD8</i> in pWU24	This study
pWU25	<i>P_{YlBUD8}</i> (680 bp promoter) in pWU24	This study
pWU25-Ylbud8-1	<i>Ylbud8-1</i> in pWU25	This study
pWU25-Ylbud8-2	<i>Ylbud8-2</i> in pWU25	This study
pWU25-Ylbud8-3	<i>Ylbud8-3</i> in pWU25	This study
pWU25-Ylbud8-4	<i>Ylbud8-4</i> in pWU25	This study
pYL8	<i>loxR-YlURA3-loxP YlLEU2</i> in pBlueScript KS(+)	23
pYL8-YlBUD8-PT	<i>P_{YlBUD8}-loxR-YlURA3-loxP-T_{YlBUD8}</i> in pYL8	This study
pYL15	<i>P_{YlTEF1}-EGFP</i> in pINA445	24
pYL15-YlBUD8	<i>P_{YlTEF1}-EGFP-YlBUD8</i> in pYL15	This study
pYL15-Ylbud8-1	<i>P_{YlTEF1}-EGFP-Ylbud8-1</i> in pYL15	This study
pYL15-Ylbud8-2	<i>P_{YlTEF1}-EGFP-Ylbud8-2</i> in pYL15	This study
pYL15-Ylbud8-3	<i>P_{YlTEF1}-EGFP-Ylbud8-3</i> in pYL15	This study
pYL15-Ylbud8-4	<i>P_{YlTEF1}-EGFP-Ylbud8-4</i> in pYL15	This study

Construction of Plasmids and Yeast Strains

Plasmids and primers used in this study are respectively listed in Tables 2 and 3. *YlBUD8* was deleted in the wild-type strain PO1a using homologous recombination, which was used in the deletion of *YlTEC1* [23]. PCR was used to identify the mutants that carried the correct replacement of YlBud8. Plasmid pRRQ2 was transformed into the *YlBUD8* deletion mutant to remove the selection marker *YlURA3*. We used PCR to confirm the deletion strains with the *YlBUD8*-5CK & *YlBUD8*-1R primers after the *YlURA3* marker was removed.

The plasmid pWU24-YlBUD8 was constructed to complement the *Ylbud8Δ* mutant. The 4,135-bp *YlBUD8* gene containing promoter (845 bp) and a 282-bp 3'-untranslated region (UTR) was digested by *Cla*I and *Bam*HI and then ligated into pWU24 (integrative, *YlURA3*) [24]. The plasmids were linearized by *Pml*I to introduce them into yeast cells for the complementation test.

To generate a version of pWU25 that tests the activity of truncated fragments of YlBud8, the 668-bp *Kpn*I-*Cla*I *YlBUD8* promoter was amplified by PCR and was inserted into pWU24. YlBud8 segments were inserted into the plasmid pWU25 digested by *Cla*I and *Bam*HI, resulting in pWU25-Ylbud8-1, pWU25-Ylbud8-2, pWU25-Ylbud8-3, and pWU25-Ylbud8-4. The plasmids were also linearized, leading to *Ylbud8Δ*, which was used to identify the function of the four fragments.

The plasmid pYL15-YlBUD8 was constructed to examine the subcellular localization of YlBud8. To do this, the 3,280-bp *YlBUD8* gene containing the full ORF and a 282-bp 3'-UTR sequence was amplified by PCR from genomic DNA and ligated into *Xba*I- and *Bam*HI-digested pYL15 (*CEN*, *YlLEU2*, *P_{YlTEF1}-EGFP*) [27], the N-terminus of YlBud8 was fused with EGFP. The truncated fragments were also amplified from genomic DNA and inserted into the *Xba*I- and *Bam*HI-digested pYL15. The five plasmids were

all introduced into *Ylbud8Δ* cells to examine the differences in localization of the different truncated fragments.

Yeast Transformation

The lithium acetate method was used to introduce plasmids to *Y. lipolytica* strains. This method has been used in the transformation of *S. cerevisiae*, but with *Y. lipolytica*, the cells were heat shocked at a lower temperature for a shorter period of time before plating on selective medium: 37°C for 15 min.

Western-Blot Analysis of Proteins

Cells of strain *Ylbud8Δ* carrying pYL15 or pYL15-YlBud8 segments were grown in YNBL+Ura medium at 30°C for 16 h. To extract total cellular proteins the Yeast Protein Extraction Reagent (Takara, Japan) was used. Proteins were separated by 8.0% SDS-PAGE. Mouse monoclonal antibody against GFP (Abcam, England) was used as primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG was used as secondary antibody.

Microscopy

Cell morphology was observed using an Olympus BX51 microscope (Japan) and a DP80 charge-coupled-device (CCD) camera. The images were acquired using CellSens Standard. For the count of the percentage of cells with abnormal morphology, a minimum of 200 cells were counted.

Results

Identification of YlBud8 in *Y. lipolytica*

In order to find the potential bipolar landmark in *Y. lipolytica*, the amino acid sequences of Bud8 and Bud9 from

Table 3. Primers used in this study.

Name	Sequence (5' → 3')	Use
YIBUD8-PF	CGCGGATCCGAGATATAGCGTATGGTCAGC	Amplification of P _{YIBUD8} and YIBUD8
YIBUD8-PR	CCGGAATTCAGAAGTTGTAGTGAAGTAGCG	Amplification of P _{YIBUD8}
YIBUD8-TF	ACCCAAGCTTTGGTTGCTGGCGTTTTAACGA	Amplification of T _{YIBUD8}
YIBUD8-TR	CCCAGCGTCGACTAGGAGTCTAGCCAAACGACC	Amplification of T _{YIBUD8}
YIBUD8-5CK	ACAACAGATGAAGAAGAG	Checking Ylbud8Δ deletion
YIBUD8-1PF	CGGGGTACCGACATGAAATCTCATGGCTTCAG	Amplification of P _{YIBUD8}
YIBUD8-1PR	CCCATCGATAGAAGAAGTTGTAGTGAAGTAG	Amplification of P _{YIBUD8}
YIBUD8-1R	ACATGCATGCGCTTTGTCAGACTGTCTACC	Checking Ylbud8Δ deletion
YIBUD8-1F	CCCATCGATTGCACGAACTCCACATTCCACC	Amplification of YIBUD8
YIBUD8-2R	CGCGGATCCAAATGCTCAGTAATGACTGATGG	Amplification of YIBUD8, Ylbud8-1, Ylbud8-2
YIBUD8-2F	CCCATCGATATGCTCACCACATTAACAACGA	Amplification of Ylbud8-1
YIBUD8-3F	CCCATCGATATGGTGGATAATATTGTCACAG	Amplification of Ylbud8-2
YIBUD8-7F	CCCATCGATATGAATGTCTCTGTTGACTCCTT	Amplification of Ylbud8-3
YIBUD8-8F	CCCATCGATATGGGCGAGGGTGAGGATGAG	Amplification of Ylbud8-4
YIBUD8-4F	TGCTCTAGAAATGGACACGAAACGAAGCCTTTC	Amplification of YIBUD8
YIBUD8-5F	TGCTCTAGACTACCGACATTAACAACGAG	Amplification of Ylbud8-1
YIBUD8-6F	TGCTCTAGAGTGGATAATATTGTCACAGTC	Amplification of Ylbud8-2
YIBUD8-9F	TGCTCTAGAAATGTCTCTGTTGACTCCTT	Amplification of Ylbud8-3
YIBUD8-10F	TGCTCTAGAGGCGAGGGTGAGGATGAG	Amplification of Ylbud8-4

S. cerevisiae were used in a BLAST search and compared against the NCBI database (<https://www.ncbi.nlm.nih.gov/>) to find proteins that share the highest degree of similarity in amino acid sequences. The protein encoded by the ORF YALI0F12738 satisfied the above condition, sharing 11% identity with Bud8 and 10% identity with Bud9 from *S. cerevisiae*. Although these are not considered to be high degrees of homology, YALI0F12738 was the sequence sharing the highest degree of homology among *Y. lipolytica* proteins. We then analyzed the conserved domains of these three proteins use SMART (<http://smart.embl-heidelberg.de/>) and discovered that YALI0F12738p shares the same conserved transmembrane (TM) domain at its C-terminus and that this domain shares 23% and 22% amino acid sequence identity with the TM domains of Bud8 and Bud9, respectively (Fig. 1). In addition, we found that YALI0F12738 is 995 amino acids in length with a long N-terminus containing a coiled-coil domain. In comparison, Bud8 and Bud9 encode a 603-amino acid protein and a 547-amino acid protein, respectively, with a shorter N-terminus and without a coiled-coil domain, which may be the reason that the degree of homology between these proteins is so low. We named the *Y. lipolytica* homolog protein YIBud8 and analyzed its function.

YIBud8 Plays an Important Role in Cell Separation

S. cerevisiae cells lacking either Bud8 or Bud9 are normal in cell morphology and growth, with changes observed only in bipolar budding. To determine whether YIBud8 is required for bipolar budding, homologous recombination

Fig. 1. Homology domains in Bud8 and Bud9.

(A) Map of *S. cerevisiae* Bud8, Bud9 and *Y. lipolytica* YIBud8 domains, TM domain is shown in black and the coiled-coil domain is shown in gray. (B) Sequence alignment of the TM domains in *S. cerevisiae* Bud8, Bud9 and YIBud8. Black shading: identical residues; gray shading: similar residues.

Fig. 2. Verification of *Y. lipolytica* gene deletion mutant.

(A) Schematic representation of *YIBUD8* deletion procedure. The *YIBUD8* ORF was first replaced by the *loxR-YIURA3-loxP* marker via homologous recombination. Then, the *YIURA3* marker was removed. The location of the primers YIBud8-5CK & YIBud8-1R used for PCR is shown. (B) Verification of *YIBUD8* deletion in three independent clones of the strain *Ylbud8Δ::loxR/P*. The primer pair YIBUD8-5CK/YIBUD8-1R was used to amplify the *YIBUD8* locus in strains PO1a (WT) and *Ylbud8Δ::loxR/P*. Expected amplified DNA fragments are 5.1 kb (WT) and 2.1 kb (*Ylbud8Δ::loxR/P*).

was used to delete *YIBUD8* from the *Y. lipolytica* wild-type strain PO1a. We successfully obtained three mutants where *YIBUD8* was correctly deleted, and the deletion was verified by PCR. When the wild-type genome was used as a template, we were able to obtain a PCR product of 5.1 kb, while a PCR product of 2.1 kb was obtained when the mutant genomes were used as the PCR template (Fig. 2), which indicated that *YIBUD8* was correctly deleted. We then named the mutant YLX403 and observed its phenotype to help identify the function of YIBud8.

In YPD liquid medium, cells that are *Ylbud8Δ* exhibit bipolar budding, which is the same as observed in the wild-type PO1a (data not shown). This result suggests that YIBud8 is not involved in the regulation of bipolar budding

in *Y. lipolytica*. In order to find the function of YIBud8, we examined *Ylbud8Δ* cells in yeast form and during filamentous growth. We did not observe a detectable growth defect in *Ylbud8Δ* cells at 30°C (data not shown), but cells showed morphological defects during yeast form and filamentous growth. In liquid YPD medium (yeast form), most *Ylbud8Δ* (80%, $n = 200$) cells remain linked to one another and form cell chains or multiple buds, while the wild-type PO1a forms single and oval form cells, and only 10% ($n = 200$) of the cells form cell chains (Figs. 3A and 3B). We also examined the cell morphology in YNBD and YNDC7 media that can promote mycelial growth to detect their ability to form pseudohyphae and hyphae. The PO1a strain formed an elongated morphology in YNBD medium and a few hyphae in YNDC7 medium. In YNBD medium, more pseudohyphae (83%, $n = 200$) could be observed in *Ylbud8Δ* cells than in wild-type cells (7%, $n = 201$) (Fig. 3C). The same phenomenon was observed when cells were grown in YNDC7 medium, which can induce hyphae growth, as the majority of cells (85%, $n = 200$) formed pseudohyphae where the length of a single cell is the same as that of the wild-type cell (Fig. 3D). When the intact plasmid pWU24-*YIBUD8* was used for the complementation of the *Ylbud8Δ* strain, the cell morphology recovered well. This result proves that the defects we observed are caused by the deletion of *YIBUD8* and that *YIBUD8* was correctly deleted.

Together, we conclude that YIBud8 is not involved in the regulation of bipolar budding but plays an important role in cell separation of *Y. lipolytica* during cell growth.

The Coiled-Coil Domain in YIBud8 is Important for Regulating Cell Separation

YIBud8 has a long N-terminus with a coiled-coil domain which is absent in Bud8 and Bud9 from *S. cerevisiae*. The coiled-coil domain is a common structural pattern that mediates protein-protein interactions [25]. Therefore, we wanted to find out whether the long N-terminus or the coiled-coil domain plays an important role in the normal function of YIBud8. We constructed four truncated constructs of YIBud8, named *Ylbud8-1*, *Ylbud8-2*, *Ylbud8-3* and *Ylbud8-4*. The long N-terminus of YIBud8 was removed but the coiled-coil domain was retained in *Ylbud8-1* and *Ylbud8-3* while in *Ylbud8-2* and *Ylbud8-4* the long N-terminus and the coiled-coil domain were both removed (Fig. 4A). The truncated *Ylbud8* segments were expressed in *Ylbud8Δ* cells under the control of the YIBud8 promoter to determine whether they can rescue the defects in cell morphology found in *Ylbud8Δ*. We examined the cell morphology of *Ylbud8Δ* cells expressing YIBud8 segments in YPD liquid.

Fig. 3. Phenotypes of *Ylbud8Δ* cells. (A) Cells of PO1a strain with plasmid pWU24 (WT/Vec) and YLX403 strain with plasmid pWU24 (*Ylbud8Δ*/Vec) or pWU24-YIBUD8 (*Ylbud8Δ*/YIBUD8) were grown in liquid YPD medium at 30°C for 12 h, then were stained with Calcofluor white. (B) The percentage of single cells and cell chains were counted. (C and D) The same cells shown in A were grown in liquid YNBD and YNDC7 media at 30°C for 16 h. Scale bar, 5 μm.

We found that YlBud8, *Ylbud8-1*, and *Ylbud8-3* can rescue the cell morphology defect of *Ylbud8Δ* cells during yeast form growth (Fig. 4C). In YPD medium, 83% ($n = 200$) of *Ylbud8Δ/Ylbud8-1* and 87% ($n = 200$) of *Ylbud8Δ/Ylbud8-3* cells show normal cell shape and size. Only 17% or 13% of cells are connected with one another in short cell chains or multiple buds, which is close to the 8% ($n = 221$) short cell chains or multiple buds found in *Ylbud8Δ/YlBud8* cells (Fig. 4B). These results suggest that the long N-terminus deletion in YlBud8 is largely functional in cell separation and further investigation will be needed to elucidate the function of the coiled-coil domain.

The truncated *Ylbud8-2* and *Ylbud8-4* segments were expressed in *Ylbud8Δ* cells under the control of the YIBUD8 promoter to study whether they can rescue the defect of *Ylbud8Δ* in cell morphology. Our results show that neither *Ylbud8-2* nor *Ylbud8-4* can rescue the cell morphology defect of *Ylbud8Δ* during yeast form growth. When grown in YPD medium, 75% ($n = 200$) of *Ylbud8Δ/Ylbud8-2* and 79% ($n = 200$) of *Ylbud8Δ/Ylbud8-4* cells are linked together

with one another and form cell chains or multiple buds, which is similar to the phenotype of *Ylbud8Δ* mutants (Fig. 4B and 4C). Therefore, we conclude that the coiled-coil domain is essential for the function of YlBud8 to regulate cell separation, but the long N-terminus is not required for this purpose.

The Coiled-Coil Domain Plays an Important Role in the Localization of YlBud8

Protein localization is often linked to function. To obtain more information about the function of YlBud8, we fused EGFP to the N-terminus of YlBud8 to observe the location of EGFP-YlBud8. The fusion construct EGFP-YIBUD8 was expressed using the YITEF1 promoter and was fully functional, as it rescued the cell morphology defect of *Ylbud8Δ*. The fusion protein can locate to the plasma membrane uniformly in unbudded cells, and is enriched in the bud cortex in both small-budded and large-budded cells (Fig. 5A). This result is consistent with the finding that YlBud8 may play an important role in cell separation.

Fig. 4. The coiled-coil domain in YlBud8 plays an important role in regulating cell separation.

(A) Schematic representation of the domains of YlBud8 and the position of YlBud8 truncated segments Ylbud8-1, 2, 3, 4. (B) Cells of strain Ylbud8 Δ integrated with PmlI-linearized plasmids pWU24-YlBud8 (YlBud8) and pWU25-YlBud8 fragments were grown in liquid YPD medium for 12 h, and the percentage of single cells and cell chains was determined. (C) Same cells in B were stained with calcofluor white. Scale bar, 5 μ m.

We also fused the truncated fragments of YlBud8 to EGFP at their N-terminus to study their localization. YlBud8 and the truncated fragments were all expressed correctly and stably as determined by immunoblotting with a monoclonal antibody against GFP (Fig. 5B). The functions of the fusion proteins are similar to the proteins that were expressed at the basal level in the pWU25 plasmid in the previous section. Fragments EGFP-Ylbud8-1 and EGFP-Ylbud8-3 localize to the same locations as EGFP-YlBud8, as they can be observed in the plasma membrane and the bud cortex. However, the fragments EGFP-Ylbud8-2 and EGFP-Ylbud8-4, which cannot rescue the cell separation defect of Ylbud8 Δ , cannot locate to the cell membrane and bud cortex, but rather remain dispersed in the cytoplasm (Fig. 5A). The above results indicate that the localization of YlBud8 to the cell membrane and the bud cortex are essential to its function in cell separation, and that the mutants Ylbud8-2 and Ylbud8-4, which do not contain the coiled-coil domain, are incorrectly localized. This finding indicates that the coiled-coil domain is important to the localization of YlBud8, and that correct localization of YlBud8 may be a functional determinant.

Discussion

YlBud8 Plays Important Roles in Cell Separation

In *S. cerevisiae*, Bud8 and Bud9 are the essential components of the bipolar landmarks in diploid strains. The deletion of either Bud8 or Bud9 can change the bipolar budding pattern of the diploid strain, but has no influence on cell morphology and cell separation [26, 27]. We have identified YlBud8 in *Y. lipolytica* as a homolog of Bud8 and Bud9 and studied its function. The data we present above show that YlBud8 plays an important role in cell separation, as YlBud8 deletion mutants cannot separate from one another and ultimately form cell chains or multi-bud cells. The program TMHMM server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) predicts that there are two short TM domains at the C-terminus of YlBud8 and we speculate that the N-terminus of YlBud8 may be located to the extracytoplasmic space, which can provide a spatial basis for YlBud8 to interact with other proteins that can regulate cell separation. In *Y. lipolytica*, deletion of YlRsr1 can also result in the formation of cell chains or multi-bud cells, which is similar to the phenotypes observed with YlBud8

Fig. 5. The coiled-coil domain plays an important role in the localization of YlBud8.

(A) Cells of strain YLX403 (*YlBud8Δ*) carrying plasmids pYL15-YlBud8 fragments were grown in liquid YNBL medium supplemented with uracil at 30°C to observe GFP fluorescence. Scale bar, 5 μm. (B) Yeast strains in A were grown in liquid YNBL+Ura medium for 12 h and total proteins were extracted, which were separately subjected to 8.0% SDS-PAGE and immunoblotted with an anti-GFP antibody.

deletion cells [21]. We speculate that YlBud8 may act as the spatial landmark for cell separation and transmit spatial signals to YlRsr1, so that the function of YlBud8 in cell separation may be mediated by the Bud8 → Rsr1 → Cdc24 → Cdc42 signaling pathway.

We propose two reasons for the differences between the functions of the two homologous proteins Bud8 and YlBud8. Firstly, the structure of YlBud8 differs significantly from that of Bud8 as it has a long N-terminus and a coiled-coil domain at the N-terminus which is non-existent in *S. cerevisiae* Bud8. Coiled-coils are known as domains that can mediate protein-protein interactions [25]. We speculate that the coiled-coil domain endows YlBud8 with novel functions that may influence the roles it plays in *Y. lipolytica*. YlBud8 may interact with other proteins through the coiled-coil domain to take part in cell separation, but not in bipolar budding. Secondly, the location of YlBud8 differs from that of Bud8 in *S. cerevisiae*. In *S. cerevisiae*, Bud8 can localize to the bud tips and presumptive bud

sites, which provides the spatial basis for the role played by Bud8 in the regulation of bipolar budding [26]. However, in *Y. lipolytica*, YlBud8 localizes to the cell membrane and the small bud cortex, but cannot be observed at the bud neck or the presumptive bud sites. This localization is consistent with the data indicating that YlBud8 is not involved in the regulation of bipolar budding. Besides, Bud8 homolog proteins from other species also have different functions in different biological processes. For example, in *Cryptococcus neoformans*, *BUD8* was significantly up-regulated in the H99 strain, which demonstrates higher virulence than wild-type strains, but its function in cell morphology requires further investigation [28].

The Coiled-Coil Domain Is Essential for the Function of YlBud8

The coiled-coil domain is an abundant structural motif that is present in approximately 10% of all proteins of any given species [29]. The amino acids in this domain can

interact among themselves to form amphiphilic α -helices, which then form a super-helix, *i.e.* the coiled-coil [30]. Coiled-coil domains have been visualized as rod-like spacers separating functional domains and they always act as protein scaffolds to mediate interactions between proteins [31, 32]. A coiled-coil domain exists at the N-terminus of YlBud8 and we hypothesize that this domain can mediate the interaction between YlBud8 and other proteins to enable YlBud8 to perform its normal functions. To test this, truncated fragments of YlBud8 were constructed. Our results show that the fragments Yl*bud8*-1 and Yl*bud8*-3, which contain the coiled-coil domain but not the long N-terminus of YlBud8, can localize to the correct cellular sites, such as the cell membrane and the small bud cortex and perform their normal functions, as they are able to complement the cellular defects observed in the Yl*bud8* Δ mutant. However, when the coiled-coil domain was removed, as with Yl*bud8*-2 and Yl*bud8*-4 fragments, the fragments were neither able to perform normal functions nor localize to normal sites. These results indicate that it is the coiled-coil domain, but not the long N-terminus that plays important roles in the normal function of YlBud8. However, whether downstream and upstream signaling proteins of YlBud8 can act on YlBud8 through the coiled-coil domain has not been confirmed and further investigations will be required.

To date, no other homologs of Bud8 from organisms other than *S. cerevisiae* and *C. neoformans* have been reported, and our study will enrich the known functional diversity of Bud8 homologs. There are still many questions at present on the function of these proteins and further tests are required to determine if YlBud8 can act on YlRsr1 to play effector roles in cell separation. The functions of other domains of YlBud8 also require further study.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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