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Investigation of the location and secretion features of *Candida albicans* enolase with monoclonal antibodies

Zhengxin He[1*], Jingzi Piao[2], Yanguang Qiu[3], Daxin Lei[4], Yanhui Yang[5], Lanchun Shi[3] and Fukun Wang[4*]

Abstract

Purpose: The glycolytic enzyme enolase plays important role in the pathogenesis of *Candida albicans* infection and has been also considered as a promising molecular marker for the diagnosis of invasive candidiasis. This study aimed to investigate the location and secretion features of *Candida albicans* enolase (CaEno) with a couple of specific monoclonal antibodies (mAbs).

Methods: Two mAbs named 9H8 and 10H8 against CaEno were generated by fusing SP2/0 myeloma cell with the spleen lymphocytes from CaEno immunized mice. The specificity of the mAbs was then validated by Western blot and liquid chromatography-mass spectrometry (LC–MS/MS). A diverse set of experiments were conducted based on the pair of mAbs which involved immunohistochemical staining analysis, whole cell enzyme-linked immunosorbent assay (ELISA), double antibody sandwich ELISA, and confocal microscopy to analyze the possible location and secretion features of CaEno.

Results: CaEno is abundantly expressed in the cytoplasm of *C. albicans* blastospores and is distributed in a ring-shaped pattern along the cell wall. CaEno appeared in the hyphal *C. albicans* as just a "mushroom" form. CaEno was found to be weakly expressed on the surface of blastospores but constantly expressed at various stages of growth. CaEno concentrations in *C. albicans* blastospores culture supernatant are considerably higher than in *C. albicans* hyphae culture supernatant. The dynamic changes of supernatant CaEno concentration in blastospores and hyphal *C. albicans* exhibit distinct features, although both appear to be associated with the *C. albicans* growth state. When cultivated under normal circumstances, however, no apparent CaEno degradation was seen in the cell-free supernatant.

Conclusion: Our results implied that CaEno was constantly expressed on the cell surface and its secretion features varied according to the growth stage of *C. albicans*. However, further experimental and theoretical studies are needed in future to identify the specific mechanisms by which this phenomenon can arise.

Introduction

Enolase (Eno), also known as phosphopyruvate hydratase, is a 48 kD enzyme responsible primarily for the dehydration of conversion of 2-phosphoglycerate to phosphoenolpyruvate. This protein was first identified by Lohman and Mayerho in 1934. A number of subsequent studies have indicated that Eno is widely spread from archae to mammals and highly conserved across the different species (Breitenbach et al. 1997; Tracy and Hedges 2000). For instance, in *Candida albicans*, Eno exists as an

*Correspondence: hezx1979@126.com; wangfk8@sina.com

[1] Basic Medical Laboratory, The 980Th Hospital of PLA Joint Logistical Support Force (Bethune International Peace Hospital), 398 Zhongshan Road, Shijiazhuang, Hebei 050082, People's Republic of China
[4] Clinical Laboratory, The 980Th Hospital of PLA Joint Logistical Support Force (Bethune International Peace Hospital), 398 Zhongshan Road, Shijiazhuang, Hebei 050082, People's Republic of China
Full list of author information is available at the end of the article

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abundant cytosolic protein that comprises 0.7% and 2.0% of the total protein isolated from yeast and hyphal forms (Sundstrom et al. 1994).

Despite intracellular metabolism activity, Eno is also known as a multifunctional protein termed as moonlighting protein. For example, Eno isolated from a number of different microbial pathogens including *C. albicans* has been identified to function as a plasminogen receptor, which can effectively promote the degradation of extracellular matrix (ECM), thus enhancing dissemination ability of the pathogens by stimulating the activation of plasmin activity (Jong et al. 2003; Funk et al. 2016; Rahi et al. 2017). The *Eno* null mutation in *C. albicans* can result in altered drug susceptibility, hyphal formation, and virulence (Ko et al. 2013). Eno has been also referred as an adhesion-related protein by virtue of its interaction with ECM proteins such as fibronectin and laminin (Kozik et al. 2015). Moreover, Elizabeth et al. identified *C. albicans* Eno1 as the cell wall protein responsible for transglutaminase (TGase) activity, which indicate that TGase/Eno1 is a putative target for designing new drugs to control *C. albicans* infection (Reyna-Beltrán et al. 2018). Additionally, in the field of lab diagnosis, the CaEno antigen and anti-CaEno antibody detection have been considered as potential strategies for diagnosing invasive candidiasis (IC). In 1991, TJ Walsh observed and reported the presence of CaEno antigenemia in patients with cancer and IC. Similarly, diverse in-house immunoassays including our previous work developed for detecting CaEno antibody in patient serum showed promising diagnostic value (Walsh et al. 1991; He et al. 2015; He et al. 2016).

A detailed understanding of the subcellular location of Eno can yield deeper insights into the role of Eno in the pathogen-host interaction and the development of IC. As expected from its metabolic function, Eno was first identified as predominantly a cytosolic protein. It was also found to be present at the cell surface of the different pathogenic organisms including *Streptococcus pneumoniae* (Mori et al. 2012), *C. albicans* (Eroles et al. 1997), and *Streptococcus suis* (Feng et al. 2009). The cell surface location can facilitate its potential interaction with the various factors present in the extracellular environment. As specifically for CaEno, there are numerous proteomics papers demonstrating that this protein could localize on the cell surface or secreted in the yeast surroundings (Pitarch et al. 2006; Chaffin 2008; Cabezón et al. 2009; Vialás et al. 2012; Gil-Bona et al. 2015a, b; Vargas et al. 2015; Luo et al. 2016). However, extensive investigations related to the CaEno location and secreted features are still necessary for further in-depth physiological study and to develop novel strategies for IC prevention. In the present study, using a couple of CaEno-specific mAbs,

we investigated the subcellular location and secretion features of CaEno in both *C. albicans* blastospores and hyphae.

Materials and methods

Microorganism and growth condition

C. albicans strain SC5314 was grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 37ºC, 5% CO_2 to obtain blastospores. Hyphae were prepared by allowing SC5314 cells to germinate in culture at 37 °C, in 5% CO_2 in RPMI medium supplemented with 10% fetal bovine serum (FBS). For standardization, 1×10^6 CFU of blastospores per mL of culture medium was fixedly seeded when performing experiments to measure the CaEno concentration in culture supernatants. *C. albicans* growth was monitored by turbidity measurement (McFarland units). The cultured blastospores/hyphae and its corresponding supernatants were then separated by centrifugation and prepared for analysis at the different time points if needed.

Generation of monoclonal anti-enolase antibodies

Recombinant CaEno (Walsh et al. 1991; He et al. 2015; He et al. 2016) was first injected into female BALB/c mice ($n = 4$) for mAb generation. The animals were obtained from the Experimental Animal Center of Hebei Medical University. Each mouse was administered 200 μg of recombinant CaEno protein emulsified in complete Freund's adjuvant (i.p.). for the first immunization or 100 μg of protein emulsified in incomplete Freund's adjuvant (i.p.) for the subsequent immunizations. Mice were immunized up to 4 times, with a 2-week interval between each immunization. Thereafter, antibody levels in the mice sera were determined by direct ELISA using recombinant CaEno and horseradish peroxidase-conjugated anti-mouse IgG (IgG–HRP) antibodies (Sangon Biotech, China). One mouse with a 1:100,000 serum titer of anti-CaEno was then sacrificed by cervical dislocation, and its spleen was removed for fusion with the SP2/0 myeloma cell line (Chinese Academy of Sciences, China). A total of 56 positive different hybridomas were screened out by ELISA and expanded. After verification the specificity by Western blot and preliminary antibody pairing experiment (data not shown), 2 were purified by passing the supernatants through protein G column, yielding specific antibodies of 9H8 and 10H8. The purity of the purified monoclonal antibodies was confirmed by SDS-PAGE.

Western blot analysis

Western blotting analysis was performed according to the standard protocols. The total lysates of *C. albicans* (Wiśniewski et al. 2009) and recombinant CaEno were transferred to polyvinylidene difluoride (PVDF)

membrane (Merck millipore, Germany) after 12% SDS-PAGE electrophoresis. After blocking with tris buffer (pH 7.5) containing 3% bovine serum albumin (BSA), the membrane was incubated with primary 1:500 diluted 9H8 or 10H8 and then with HRP-conjugated goat anti-mouse IgG. Western blot was developed using diaminobenzidine (DAB) substrate (Solarbio, China).

Immunocapture assay

The specificities of 9H8 and 10H8 antibodies were evaluated by an immunocapture assay and subsequently by using mass spectrometry analysis. The immunocapture assay was performed using anti-CaEno mAbs coupled Berpharose FF beads and *C. albicans* total lysates. Briefly, 15 mg of purified 9H8 or 10H8 solution was concentrated to 1.5 mL and dialyzed against the coupling buffer (0.2 M NaHCO$_3$, 0.5 M NaCI, pH 8.3). Thereafter, the antibodies were coupled to N-hydroxysuccinimide (NHS) activated Berpharose FF beads (Bersee, China) at 4 °C overnight. After washing and blocking, the antibody-coupled beads were then packed into empty columns with 0.02 M phosphate-buffered saline (PBS). Affinity columns were equilibrated by passing 10 column volumes (CV) of 0.02 M PBS, and the total lysates of *C. albicans* were thereafter loaded onto the columns. The columns were washed with 40 volumes of PBS till the read value of the UV detector (Shimadzu, Japan) decreased to zero. Finally, the various bound fractions were eluted with 0.1 M glycine-HCI (pH 3.4) and neutralized with 1.5 M Tris-HCI (pH 9.5). Eluted proteins were collected and analyzed by SDS-PAGE and mass spectrometry.

Mass spectrometry

The mass spectrometry identification of the immunocapture fraction of 9H8 mAb was performed by Sangon Biotech (Shanghai, China). The protein in the eluted solution was first precipitated by trichloroacetic acid (TCA) treatment and then treated with 8 M urea/100 mM Tris–HCl solution (pH 8.0) for causing denaturation and exposed to 10-mM dithiothreitol (DTT) to open the disulfide bond. Subsequently, the protein sample was digested by trypsin and desalinated by Sep-Pak C18. MS was performed using a Triple TOF 5600 System (AB SCIEX, USA). The mass spectrum data generated were thereafter retrieved by Protein Pilot (V4.5), and the database retrieval algorithm was Paragon. The database used for the retrieval purpose was the proteome reference database of *Candida albicans* in Uniprot.

The identification of the elute fraction generated by 10H8 mAb was performed by Shanghai Applied Protein Technology Co., Ltd. (China). The bands were excised from the silver-stained gels and digested with trypsin, and then, the peptides were separated by chromatography using an Easy nLC system (Thermo Scientific, Germany) and then used for mass spectrometry with a Q-Exactive (Thermo Scientific, Germany) mass spectrometer. The original raw files were imported into Max Quant software for the database retrieval. The database used for retrieval was "uniprot_candida_albicans_6040_20210421.fasta".

Immunohistochemical staining

Immunohistochemical staining analysis was done to acquire an overall image of CaEno distribution. After a 48-h culture, the collected blastospores/hyphae were first fixed with 10% phosphate-buffered formalin at 4ºC for 24 h. After paraffin embedding and sectioning, the slices were blocked by 5% BSA at room temperature for 20 min, followed by incubation with 1:25 diluted 10H8 at 37ºC for 1 h. After washing in PBS for three times, the sections were then incubated with 1:400 diluted anti-mouse IgG-HRP antibody (Solarbio, China) for 90 min at room temperature. Thereafter, the sections were reacted with 0.05% 3,3′-diaminobenzidine (DAB) and 0.003% H$_2$O$_2$ in 0.05 M tris buffer (pH 7.2–7.4). The stained sections were then evaluated under a BX43 microscope (Olympus, Japan). Digital images of the stained slides were captured at about 7 200 × magnification with an Olympus BX43 microscope equipped with a digital amplification system (DP26, Olympus).

Whole cell ELISA

The 24-h cultured *C. albicans* SC5314 cells were harvested and then washed three times with ice-cold 0.1 M PBS. After centrifugation, the cells were adjusted to a density of OD600 = 0.5 or 0.1 in pH 9.6 carbonate buffer solution (CBS). The prepared suspensions were thereafter used to coat ELISA plates (100 μL/well) at 4ºC overnight. CBS suspension of *Escherichia coli* (OD600 = 0.5) was used as a negative control. After blocking and washing, the plates were treated with serial dilutions of 10H8 (from 1:1000 to 1:128,000) at 37ºC for 1 h and then with 1:5000 diluted HRP goat anti-mouse IgG (Solarbio, China). One hundred microliters of the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added into each well and incubated at 37ºC for 10 min after the washing. The reaction was terminated with 2 N H$_2$SO$_4$, and the optical density values at 450 nm (OD450) were finally measured using the Versa Max plate reader (Molecular Devices Co., USA).

Flow cytometry (FCM)

Flow cytometry was applied to determine the surface location of CaEno on live *C. albicans* cells. In brief, 24 h-cultured *C. albicans* cells were adjusted to a density of 1×10^7 CFU/mL and treated for 1 h with 1:100 diluted 10H8 mAb. Following that, cells were washed and treated

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for 1 h with 1:100 FITC goat anti mouse IgG, followed by 30 min of paraformaldehyde fixation. After washing, the immunofluorescence stained *C. albicans* cells were analyzed by an EPICS XL4 flow cytometry (Beckman coulter, USA).

Confocal microscopy
The confocal microscopy analysis was completed by the Wuhan Servicebio Technology CO., LT., Wuhan, China. *C. albicans* cells were first centrifuged, washed, and fixed in 4% paraformaldehyde-PBS for 20 min. After washing with PBS, a 10 μl of cell suspension was dripped onto the glass slides to dry at room temperature overnight. The slides containing yeast were then blocked with 3% BSA and incubated with 1:200 diluted 10H8 overnight at 4 °C. The slides were subsequently incubated with Cy3-conjugated goat anti-mouse IgG (Servicebio, China) diluted 1:300 for 1 h in the dark after washing. Nikon Eclipse T1 confocal microscope (Nikon Inc., Japan) at 400 × magnification was used for image acquisition and a Nikon C2 confocal system (Nikon Inc., Japan) was employed to record images.

Double antibody sandwich ELISA (DAS-ELISA)
DAS-ELISA was developed to determine the levels of CaEno released in the culture medium. 9H8 mAb was diluted to 3 μg/ml in CBS and then used to coat ELISA plates (100 μL/well) at 4°C overnight. After blocking and washing, culture supernatant collected from *C. albicans* blastospores or hyphea at different time points (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 72, 96, and 120 h) were added (100 μL/well) and thereafter incubated at 37°C for 1 h. Afterwards, the plate was washed and 100 μL of 1:5000 diluted HRP-10H8 was added to each well and incubated at 37°C for 1 h. After incubation and washing, 100 μL of TMB substrate was added into each well and incubated at 37°C for 10 min. The reaction was then terminated and the OD450 were determined with a plate reader. The diluted recombinant CaEno were used as the standard substances to establish a standard curve, and the results were analyzed by the software of ELISACalc V0.2.

Statistical analysis
The statistical analysis was performed using the Graph-Pad Prism software version 7.00. The continuous measures have been presented as the mean and standard deviation at each time point. The statistical significance of the differences between the groups at different times was determined by the one-way analysis of variance test, followed by the LSD *t* test. A *P* value of less than 0.05 ($P < 0.05$) was considered as statistically significant.

Results

Specificity of the monoclonal antibodies
It was found that both 9H8 and 10H8 mAbs exhibited excellent specificity towards CaEno. The results of western blot analysis demonstrated that mAbs of 9H8 and 10H8 could significantly react with the 55 kD recombinant CaEno protein and could specifically recognize a protein band with a molecular weight of approximately 48 kD in whole *C. albicans* lysates (Figs. 1A and 2A). The results of SDS-PAGE analysis indicated that both the elutes of immunocapture method with 9H8 and 10H8 mAbs exhibited high-purity proteins with a molecular weight of about 48 kD (Figs. 1B and 2B). Thereafter, the eluted samples were subjected to further determination by different LC MS/MS system. The mass spectrometry method for the identification of 9H8 elute used the collected spectra numbers to approximately determine the abundance of the target protein. The results indicated that a total of 602 CaEno spectra were obtained, which was markedly higher than the other non-specific proteins in the sample (Fig. 1C). The method for the identification of 10H8 elutes used the intensity-based absolute protein quantification (iBAQ) to determine the relative abundance of the target proteins in the samples. Similarly, the main protein component of the 10H8 immunocapture elute was found to be CaEno with an iBAQ value of 6,883,900, which was much substantially higher than the other components present in the sample (Fig. 2C).

Immunohistochemical staining characteristics of CaEno
To find morphological evidence for the subcellular localization of CaEno, *C. albicans* blastospores and hyphae were examined by immunohistochemical staining with the 10H8 as the probe antibody. For *C. albicans* blastospores, a specific immune-reaction was then detected in the cytoplasm and the cell wall. It was demonstrated that the CaEno was abundantly present in the cell plasma and formed a ring-shaped pattern along with the cell wall. The unstained center areas of the fungi cells implied very low abundance or lack of distribution of CaEno in this area (Fig. 3A). The staining on *C. albicans* hyphae is faint, with high staining at the terminal, showing a "mushroom" form under the microscope. The cell wall of the hyphae appeared to be stained more intensely than the cytoplasmic portion, highlighting the hyphae's distinct outline (Fig. 3B).
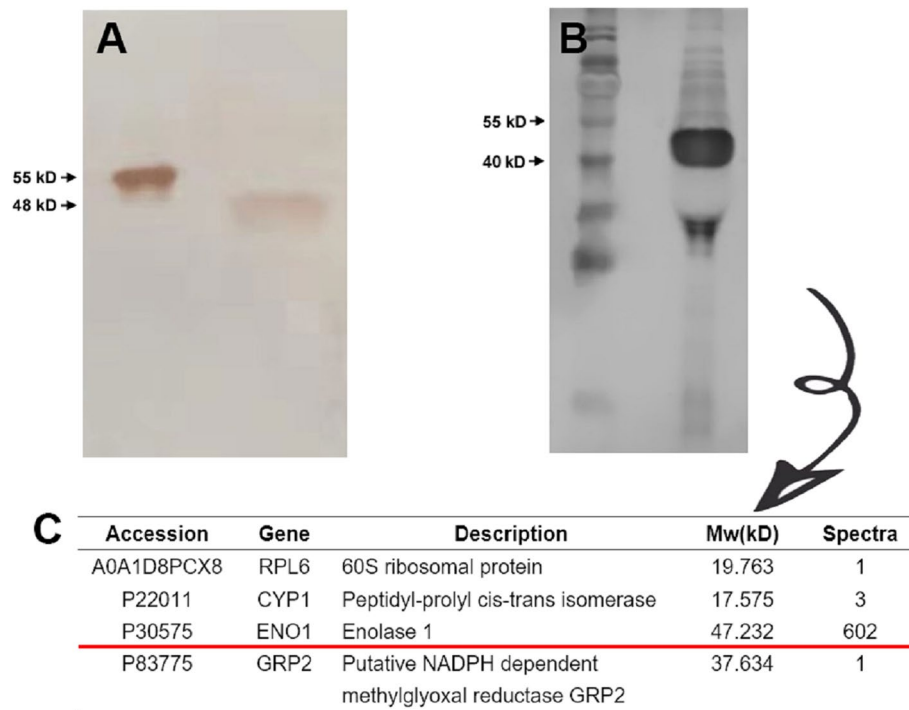
Fig. 1 Validation of the specificity of 9H8 mAb. **A** Western blot analysis showed the 9H8 mAb specifically reacted with the 55 kD recombinant CaEno and recognized a 48-kD protein in the *Candida albicans* total lysates. **B** SDS-PAGE (sliver stained) analysis of the 9H8 immunocapture elute. **C** LC MS/MS analysis of the 9H8 immunocapture elute
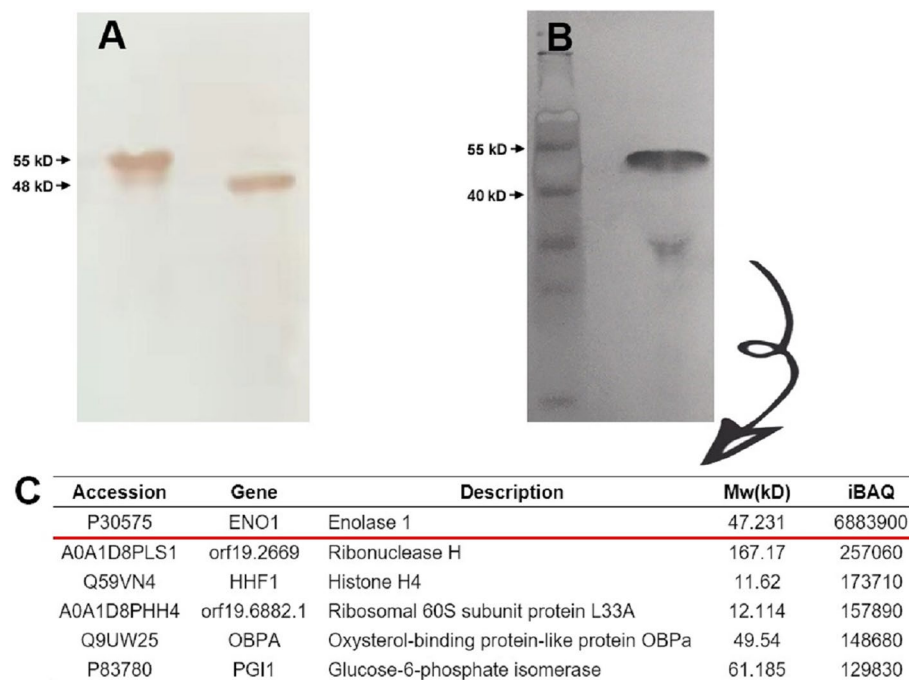
Accession	Gene	Description	Mw(kD)	Spectra
A0A1D8PCX8	RPL6	60S ribosomal protein	19.763	1
P22011	CYP1	Peptidyl-prolyl cis-trans isomerase	17.575	3
P30575	ENO1	Enolase 1	47.232	602
P83775	GRP2	Putative NADPH dependent methylglyoxal reductase GRP2	37.634	1

Fig. 2 Validation of the specificity of 10H8 mAb. **A** Western blot analysis showed the 10H8 mAb specifically reacted with the 55 kD recombinant CaEno and recognized a 48-kD protein in the total *Candida albicans* lysates. **B** SDS-PAGE (sliver stained) analysis of the 10H8 immunocapture elute. **C** LC MS/MS analysis of the 10H8 immunocapture elute
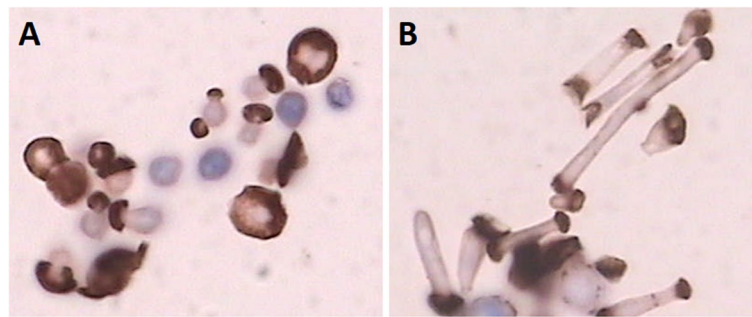
Accession	Gene	Description	Mw(kD)	iBAQ
P30575	ENO1	Enolase 1	47.231	6883900
A0A1D8PLS1	orf19.2669	Ribonuclease H	167.17	257060
Q59VN4	HHF1	Histone H4	11.62	173710
A0A1D8PHH4	orf19.6882.1	Ribosomal 60S subunit protein L33A	12.114	157890
Q9UW25	OBPA	Oxysterol-binding protein-like protein OBPa	49.54	148680
P83780	PGI1	Glucose-6-phosphate isomerase	61.185	129830

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Fig. 3 Immunohistochemical analysis of *Candida albicans* SC5314 blastospores (**A**) and hyphae (**B**) by 10H8 (× 7 200)
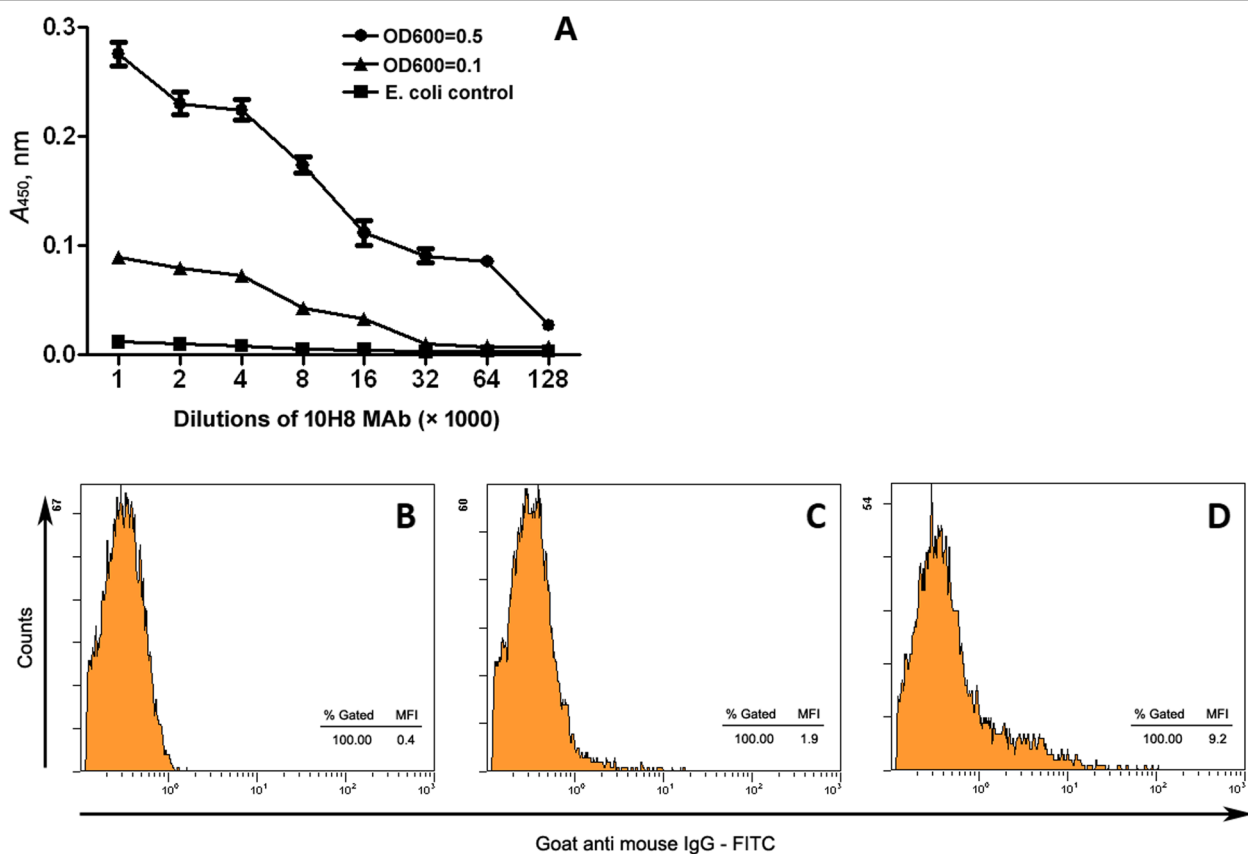

Fig. 4 Surface display of *Candida albicans* enolase. **A** Whole cell ELISA showing different dilutions of 10H8 reacted with two *C. albicans* density (OD600 = 0.5, OD600 = 0.1); **B**–**D** Extracellular detection of *C. albicans* enolase by flow cytometry (FCM) analysis. **B** Mean fluorescence intensity (MFI) of the unlabeled *C. albicans*. **C** MFI of *C. albicans* treated with FITC-goat anti mouse IgG. **D** MFI of *C. albicans* treated with 10H8 and FITC-goat anti mouse IgG

CaEno was constantly present on the cell surface

It was found that *C. albicans* cells prepared at the different densities (OD600 = 0.5 or OD600 = 0.1) can specifically reacted with 10H8 by whole cell ELISA (Fig. 4A). For the plate coated at a *C. albicans* density of OD600 = 0.5, the OD450 value was found to decrease markedly from 0.285 to 0.009 as 10H8 diluted from 1:1 000 to 1:128 000. Likewise, the OD450 value reduced from 0.098 to 0.006 when 10H8 was diluted from 1:1 000 to 1:128 000 for the plate with a coated density of OD600 = 0.1. The flow cytometry analysis revealed that the mean fluorescence intensity (MFI) of *C. albicans*

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cells treated with 10H8 was significantly higher (about 4 times) than the negative control (Fig. 4D, E). These results indicated the presence of the CaEno antigen on the *C. albicans* cell surface, which was identified by the 10H8 mAb.

Thereafter, confocal microscopy analysis was applied to further understand the kinetic change characteristics of cell-surface located CaEno during the culture (Fig. 5). Cy3 signals were detected and its areal density (AD) values were calculated to assess the possible expression intensity of cell surface CaEno. As can be seen in Fig. 6, weak red fluorescence signals were observed on the *C. albicans* cells at each time point. The AD values were basically maintained in a stable state during the culture period, and there was no statistical significant differences found between the AD values at the different culture time points.

Secretion features of CaEno differed in blastospores and hyphal C. albicans

The cultured *C. albicans* supernatants which were collected at different time points (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 72, 96, 120 h) were applied to the

Fig. 6 The areal density (AD) values of 10H8-stained *Candida albicans* blastospores at different culture time points in YPD medium
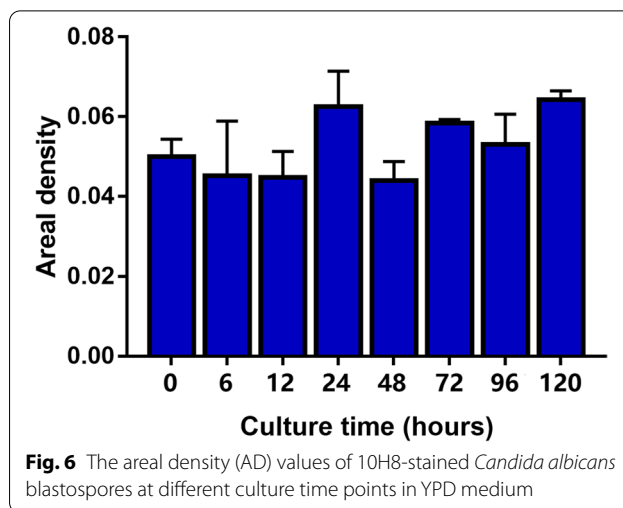
DAS ELISA to observe the dynamic changes of secreted CaEno in both blastospores and hyphae morphology. The CaEno concentration was determined using a standard curve established by serially diluted recombinant CaEno, as shown in Fig. 7A.
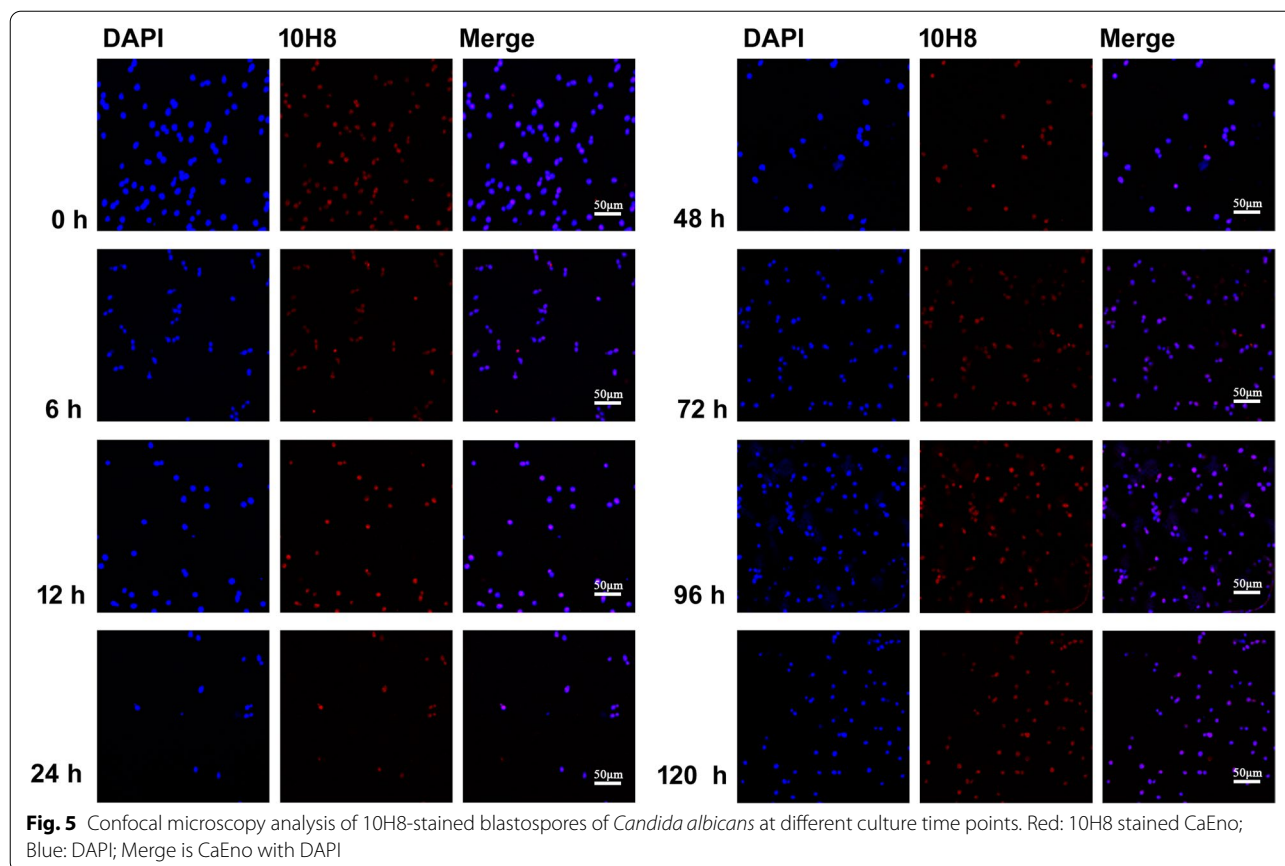
Fig. 5 Confocal microscopy analysis of 10H8-stained blastospores of *Candida albicans* at different culture time points. Red: 10H8 stained CaEno; Blue: DAPI; Merge is CaEno with DAPI
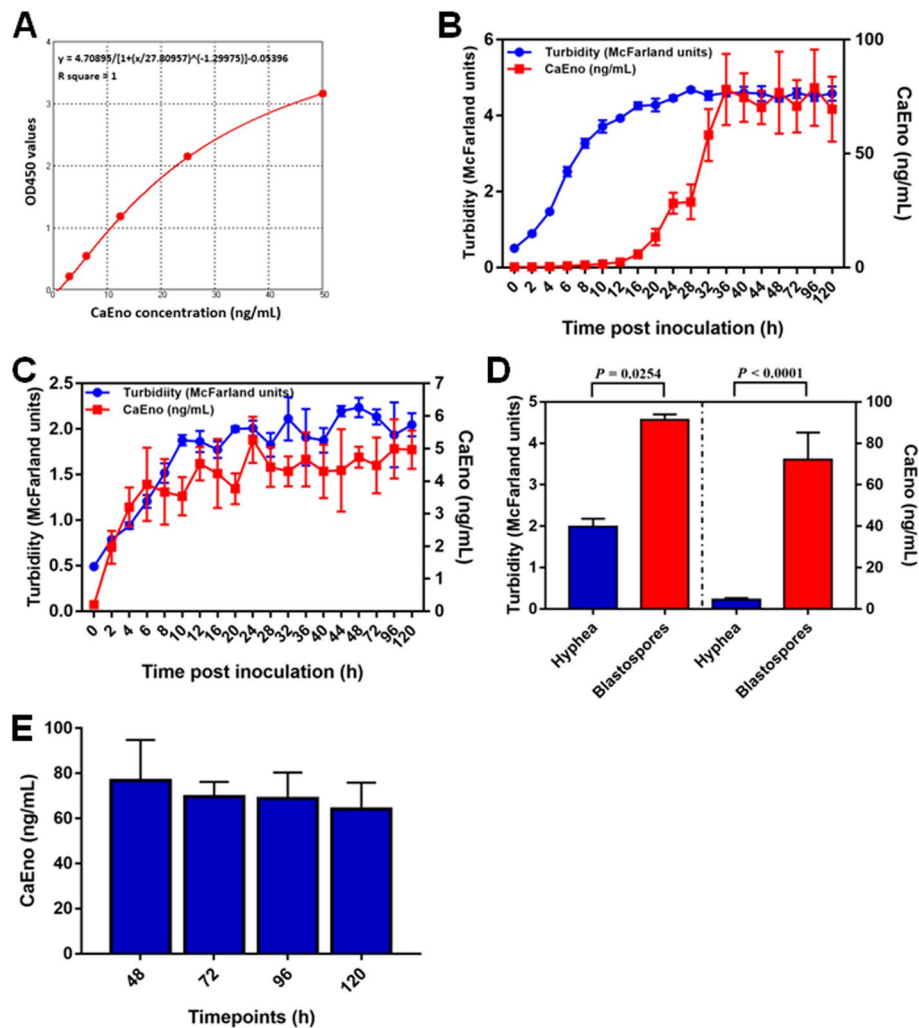
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Fig. 7 Analysis of CaEno concentrations in the culture supernatants. **A** Calibration curve for determining CaEno by double antibody sandwich ELISA. **B** Growth curve of *C. albicans* SC5314 in YPD medium and supernatant CaEno concentration dynamic curve. **C** Growth curve of *C. albicans* SC5314 in RMPI with 10% FBS and supernatant CaEno concentration dynamic curve. **D** Comparison of average turbidity (left) and supernatant CaEno concentrations (right) between *C. albicans* blastospores and hyphea in stationary phase. **E** Dynamic changes of CaEno concentration in cell-free *C. albicans* blastospore culture supernatant when maintained under 37 ℃

C. albicans grew as blastosportes when cultured with YPD. The dynamic curve of CaEno concentration in the YPD supernatant was remarkably comparable to the growth curve. Both curves show a typical logarithmic increase before reaching a plateau, where the rise of CaEno delayed by more than 16 h (Fig. 7B). *C. albicans* grew as hyphae in RPMI with 10% FBS culture. Interestingly, the growth curve and CaEno dynamics in the RPMI supernatant exhibited entirely different features from *C. albicans* cultivated with YPD. The two curves show no significant hysteresis and essentially overlap. A minor quantity of CaEno was identified in the cultured supernatant after 2 h and rises continually with the turbidity of

the culture, reaching a plateau at about 12 h after inoculation (Fig. 7C).

CaEno concentrations in the hyphae supernatant were much lower than in the blastospore supernatant. The CaEno concentration throughout the secretion plateau phase differed significantly ($P < 0.0001$), with an average concentration of 4.54 ± 0.74 ng/ml in the hyphae supernatant (12–120 h) and 74.16 ± 12.22 ng/ml in the blastospore supernatant (36–120 h). Similarly, turbidity differed significantly between hyphal and blastospores *C. albicans* ($P = 0.0254$). During the stationary phase, the average turbidity of hyphal *C. albicans* (10–120 h) was

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1.98 ± 0.20, which was 4.57 ± 0.13 for the blastospores *C. albicans* (28–120 h) (Fig. 7D).

Since the CaEno was reported as a protein susceptible to the degradation (Silva et al. 2014), we investigated whether the CaEno could decay in the cell-free supernatant. One batch of 48-h cultured *C. albicans* supernatant was separated by centrifugation and maintained at 37 ℃. As indicated in Fig. 7E, we observed that although CaEno in the separated culture supernatant showed progressive degradation after isolation, most of the CaEno could be effectively retained until the time point of 120 h.

Discussion

CaEno was initially identified as a cytosolic protein for its catalytic function in the second step of the glycolytic pathway. However, accumulated experimental evidences clearly indicate that this glycolytic enzyme exists not only in the cytosol but is also present in the surface of the yeast cell (Eroles et al. 1997) and even in the culture medium (Sundstrom et al. 1994). The multi-location distribution of Eno has also been reported in many other species (Jung et al. 2014; Wang and Jeffery 2016). For instance, in the 1990s, a series of research papers concurred that CaEno could be found in the cell wall as a result of cell lysis or unknown mechanism related to the leakage. This assumption was primarily based on the following indirect facts: (i) formation of blocks of β-glucan skeleton resulted in the release of CaEno into the supernatant (Font de Mora et al. 1993); (ii) the CaEno sequence lacks typical features of intrinsic cell wall proteins, such as presence of signal peptide and glycosylation sites (Eroles et al. 1995; Angiolella et al. 1996); (iii) incubation with SDS solution can release the CaEno from the cell wall, thus clearly indicating that the protein is retained by the non-covalent bonds (Eroles et al. 1997).

In the present study, highly specific mAbs were found to facilitate the development of visible techniques and quantitative detection methods for the sub-cellular investigation of CaEno which included both the protein expressed on the fungal cell surface and secreted into the medium. Immunohistochemical results showed that the CaEno was distributed with a ring-shaped pattern in blastospores and a mushroom form in hyphea. It is worth noting that the staining of the cells was not consistent, some were clear, while the others were light. Since the immunohistochemical was performed multiple times, and the specificity of 10H8 mAb has been verified, we speculate that the reason for this phenomenon were the differences between the slice position (e.g., only cut through the cell surface) and the growth state for each cell. Future single-cell proteomics research may provide more accurate results (Zhu et al. 2016). Immunofluorescence staining revealed weak fluorescence on the cell surface of *C. albicans* blastospores, and the MPI obtained by FCM after live cell staining was correspondingly very low. The findings imply that the quantity of CaEno on the blastospores surface of *C. albicans* is limited, which is consistent with the observations of Karkowska-kuleta et al. (2021). However, the impact of mAb affinity cannot be completely excluded. We did not assess the quantity of CaEno expression on the *C. albicans* hyphea due to technical limitations. Given the importance of hyphea morphology in *C. albicans* pathogenesis, future quantitative investigations could be interesting.

The DAS-ELISA assay established by using two mAbs ensured the test sensitivity and specificity when detecting the CaEno in the supernatant samples (Himananto et al. 2020). Our findings suggested that the main source of extracellular CaEno might not be simply derived from only fungal lysis. First, as the concentration in the supernatant was increased with the culture time, we did not accordingly observe a significant change of the cell wall located CaEno, which implicated that CaEno might only be a major functional component of the cell wall (Gil-Bona et al. 2015a, b; Reyna-Beltrán et al. 2018); Secondly, the secretion of CaEno reached a stationary phase after a period of culture, showing direct correlation with the growth but not the death of *C. albicans*.

The intracellular/secreted moonlighting proteins that are displayed on the cell surface have been found in various pathogenic bacteria and fungus. The atypical cell wall proteins of *C. albicans* include enzymes involved in evolutionarily conserved central metabolic pathways such as CaEno and fructose-bisphosphate aldolase (Fba1), factors associated with protein synthesis such as elongation factor 2 (Eft2), and chaperones such as Ssa1 and enzymes involved in redox homeostasis such as superoxide dismutase (Sod3) (Satala et al. 2020). These proteins have been found to play active roles in interaction with the host, stress protection, candidal virulence, and atypical enzymatic activity. The surface location and functionary secretion can cause the CaEno to come in direct contact with the host immune system and epithelial cells. It has been established that high titers of anti-enolase can be detected in the sera of patients diagnosed with infections cause by different *Candida* species (Li et al. 2013; He et al. 2015). However, for a long time, the molecular mechanism through which this unconventional secretory protein could be potentially transported to the cell surface and extracellular medium remained a matter of speculation and debate. Interestingly, recent proteomic studies have revealed that extracellular vesicles (EVs) of *C. albicans* can act as carriers of atypical secretary proteins (Gil-Bona et al. 2015a, b; Wolf et al. 2015). Among the various unconventionally secreted proteins known,

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the CaEno was one of the only six proteins identified in *C. albicans* EVs commonly across all variations of the culture condition (Karkowska-Kuleta et al. 2021). We found that CaEno was secreted extra-cellularly but did not show significant degradation in the cell-free supernatant under 37 °C culture condition, which varies from the degradable cytosol CaEno reported in the previous studies (Silva et al. 2014; Karkowska-Kuleta et al. 2021). Since the lipid bilayer of EVs was reported to be able to protect the encapsulated proteins from degradation (Ohno et al. 2016), it is plausible that the CaEno inside or on the surface of EVs could remain stable in the medium through this mechanism.

There are a substantial number of reports that have demonstrated that human alpha-Eno was responsible for the proliferation and metastasis of a variety of tumors (Fu et al. 2015; Sun et al. 2019; Xu et al. 2020). For example, a study by Gil-Bona et al. hypothesized that fungal EVs might also have a possible role in the regulation of intracellular communication as it has been reported for EVs of mammalian cells (Gil-Bona et al. 2018). Our results reveal that the dynamic secretion curve of CaEno has a strong similarity or correlation with the growth curve of *C. albicans* in either hyphea or blastospores morphology, indicating that extra-cellular CaEno may be connected with *C. albicans* growth. The CaEno concentration in blastospores *C. albicans* supernatant is 16 times greater than in hyphal supernatant, according to our results. *C. albicans* blastospores have a turbidity that is more than twice that of hyphal *C. albicans.* As hyphal cells are substantially larger than blastospores cells, the quantity of cells in blastospores *C. albicans* solution should be far more than twice that of hyphal cells in the stationary phase. As a result, it will be an interesting issue to analyze if the peak concentration of CaEno in the stationary phase is associated to cell number. The specific molecular mechanisms for these phenomena will be investigated further in future studies.

Conclusion

Using a pair of specific mAbs, our work clearly indicated novel subcellular localization features of CaEno, which was constantly expressed on the cell surface and was secreted during the growth stage of *C. albicans*. However, further experimental and theoretical studies are needed in the future to reveal the specific mechanisms by which this phenomenon might occur.

Authors' contributions
He ZX: Conceptualization, methodology, writing—original draft, and funding acquisition; Piao JZ, Qiu YG, Lei DX, Yang YH: Data curation, methodology, and formal analysis; Shi LC: Data curation and writing—review and editing; Wang FK: Funding acquisition, project administration, supervision, and writing—review and editing. The authors read and approved the final manuscript.

Availability of data and materials
The data that support the findings of this study are openly available in 4TU. ResearchData at http://doi.org/10.4121/15040701.

Declarations

Ethics approval and consent to participate
The animal experiments were performed complied with the ARRIVE (animal research of reporting in vivo experiments) guidelines and approved by the ethics committee board of the 980th Hospital of PLA Joint Logistical Support Force.

Consent for publication
N/A

Competing interests
Author Yanhui Yang is employed by the Heya Biotechnology Co., Ltd. The other authors declare that they have no competing interests.

Author details
[1]Basic Medical Laboratory, The 980Th Hospital of PLA Joint Logistical Support Force (Bethune International Peace Hospital), 398 Zhongshan Road, Shijiazhuang, Hebei 050082, People's Republic of China. [2]College of Plant Protection, Shenyang Agricultural University, 120 Dongling Road, Shenyang, Liaoning 110866, People's Republic of China. [3]Department of Basic Courses, NCO School, Army Medical University, 450 Zhongshan Road, Shijiazhuang, Hebei 050081, People's Republic of China. [4]Clinical Laboratory, The 980Th Hospital of PLA Joint Logistical Support Force (Bethune International Peace Hospital), 398 Zhongshan Road, Shijiazhuang, Hebei 050082, People's Republic of China. [5]Heya Biotechnology Co., Ltd, 368 Xinshi North Road, Shijiazhuang, Hebei 050091, People's Republic of China.

References

Angiolella LMF, StringaroB. MarasN. SimonettiA. Cassone, (1996) Identification of a glucan-associated enolase as a main cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. J Infect Dis 173(3):684–690. https://doi.org/10.1093/infdis/173.3.684

Breitenbach MBS, ProbstH. OberkoflerF. FerreiraP. BrizaG. AchatzA. UngerC. EbnerD. KraftR. Hirschwehr, (1997) Enolases are highly conserved fungal allergens. Int Arch Allergy Immunol 113(1–3):114–117. https://doi.org/10.1159/000237521

Cabezón V-P, NombelaL. MonteolivaC. Gil, (2009) Analysis of *Candida albicans* plasma membrane proteome. Proteomics 9(20):4770–4786. https://doi.org/10.1002/pmic.200800988

Chaffin WL (2008) *Candida albicans* cell wall proteins. Microbiol Mol Biol Rev 72(3):495–544. https://doi.org/10.1128/mmbr.00032-07

Eroles PMS, ElorzaRSentandreu V (1995) Cloning of a DNA fragment encoding part of a 70-kDa heat shock protein of *Candida albicans*. FEMS Microbiol Lett 128(1):95–100. https://doi.org/10.1111/j.1574-6968.1995.tb07506.x

Eroles PMS, ElorzaRSentandreu V (1997) The highly immunogenic enolase and Hsp70p are adventitious *Candida albicans* cell wall proteins. Microbiology (reading) 143(Pt 2):313–320. https://doi.org/10.1099/00221287-143-2-313

Feng Y, Pan X, Sun W, Wang C, Zhang H, Li X, Ma Y, Shao Z, Ge J, Zheng F, Gao GF, Tang J (2009) *Streptococcus suis* enolase functions as a protective

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antigen displayed on the bacterial cell surface. J Infect Dis 200(10):1583–1592. https://doi.org/10.1086/644602

Font de Mora JE, Sentandreu Herrero R (1993) A kinetic study on the regeneration of *Candida albicans* protoplasts in the presence of cell wall synthesis inhibitors. FEMS Microbiol Lett 111(1):43–47. https://doi.org/10.1111/j.1574-6968.1993.tb06359.x

Fu QF, Liu Y, Fan Y, Hua SN, Qu HY, Dong SW, Li RL, Zhao MY, Zhen Y, Yu XL, Chen YY, Luo RC, Li R, Li LB, Deng XJ, Fang WY, Liu Z, Song X (2015) Alpha-enolase promotes cell glycolysis, growth, migration, and invasion in non-small cell lung cancer through FAK-mediated PI3K/AKT pathway. J Hematol Oncol 8:22. https://doi.org/10.1186/s13045-015-0117-5

Funk J, Schaarschmidt B, Slesiona S, Hallström T, Horn U, M. Brock (2016) The glycolytic enzyme enolase represents a plasminogen-binding protein on the surface of a wide variety of medically important fungal species. Int J Med Microbiol 306(1):59–68. https://doi.org/10.1016/j.ijmm.2015.11.005

Gil-Bona A, Llama-Palacios A, ParraVivanco CMF, Nombela C, Monteoliva L, Gil C (2015a) Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in *Candida albicans*. J Proteome Res 14(1):142–153. https://doi.org/10.1021/pr5007944

Gil-Bona A, Parra-Giraldo CM, Hernáez ML, Reales-Calderon JA, Solis NV, Filler S G, Monteoliva L, Gil C (2015b) *Candida albicans* cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host-pathogen interaction. J Proteomics 127(Pt B):340–351. https://doi.org/10.1016/j.jprot.2015.06.006

Gil-Bona A, Amador-García A, Gil C, Monteoliva L (2018) The external face of *Candida albicans*: a proteomic view of the cell surface and the extracellular environment. J Proteomics 180:70–79. https://doi.org/10.1016/j.jprot.2017.12.002

He ZX, Chen J, Li W, Cheng Y, Zhang HP, Zhang LN, Hou TW (2015) Serological response and diagnostic value of recombinant *candida* cell wall protein enolase, phosphoglycerate kinase, and β-glucosidase. Front Microbiol 6:920. https://doi.org/10.3389/fmicb.2015.00920

He ZX, Shi LC, Ran XY, Li W, Wang XL, Wang FK (2016) Development of a lateral flow immunoassay for the rapid diagnosis of invasive candidiasis. Front Microbiol 7:1451. https://doi.org/10.3389/fmicb.2016.01451

Himananto O, Yoohat K, Danwisetkanjana K, Kumpoosiri M, Rukpratanporn S, Theppawong Y, Phuengwas S, Makornwattana M, Charlermroj R, Karoonuthaisiri N, Thummabenjapone P, Kositcharoenkul N, Gajanandana O (2020) Double antibody pairs sandwich-ELISA (DAPS-ELISA) detects *Acidovorax citrulli* serotypes with broad coverage. PLoS ONE 15(8):e0237940. https://doi.org/10.1371/journal.pone.0237940

Jong AYSHM, ChenM F, StinsK S, KimT L, TuanS H, Huang, (2003) Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J Med Microbiol 52(Pt 8):615–622. https://doi.org/10.1099/jmm.0.05060-0

Jung DW, Kim WH, Williams DR (2014) Chemical genetics and its application to moonlighting in glycolytic enzymes. Biochem Soc Trans 42(6):1756–1761. https://doi.org/10.1042/bst20140201

Karkowska-Kuleta J, Wronowska E, Satala D, Zawrotniak M, Bras G, Kozik A, Nobbs AH, Rapala-Kozik M (2021) Als3-mediated attachment of enolase on the surface of *Candida albicans* cells regulates their interactions with host proteins. Cell Microbiol 23(4):e13297. https://doi.org/10.1111/cmi.13297

Ko HC, Hsiao TY, Chen CT, Yang YL (2013) *Candida albicans* ENO1 null mutants exhibit altered drug susceptibility, hyphal formation, and virulence. J Microbiol 51(3):345–351. https://doi.org/10.1007/s12275-013-2577-z

Kozik A, Karkowska-Kuleta J, Zajac D, Bochenska O, Kedracka-Krok S, Jankowska U, Rapala-Kozik M (2015) Fibronectin-, vitronectin- and laminin-binding proteins at the cell walls of *Candida parapsilosis* and *Candida tropicalis* pathogenic yeasts. BMC Microbiol 15:197. https://doi.org/10.1186/s12866-015-0531-4

Li FQ, Ma CF, Shi LN, Lu JF, Wang Y, Huang M, Kong QQ (2013) Diagnostic value of immunoglobulin G antibodies against *Candida* enolase and fructose-bisphosphate aldolase for candidemia. BMC Infect Dis 13:253. https://doi.org/10.1186/1471-2334-13-253

Luo T, Krüger T, Knüpfer U, Kasper L, Wielsch N, Hube B, Kortgen A, Bauer M, Giamarellos-Bourboulis EJ, Dimopoulos G, Brakhage AA, Kniemeyer O (2016) Immunoproteomic analysis of antibody responses to extracellular proteins of *Candida albicans* revealing the importance of glycosylation for antigen recognition. J Proteome Res 15(8):2394–2406. https://doi.org/10.1021/acs.jproteome.5b01065

Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S (2012) α-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. J Biol Chem 287(13):10472–10481. https://doi.org/10.1074/jbc.M111.280321

Ohno S, Drummen GP, Kuroda M (2016) Focus on extracellular vesicles: development of extracellular vesicle-based therapeutic systems. Int J Mol Sci 17(2):172. https://doi.org/10.3390/ijms17020172

Pitarch A, Jiménez A, Nombela C, Gil C (2006) Decoding serological response to *Candida* cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatic analyses. Mol Cell Proteomics 5(1):79–96. https://doi.org/10.1074/mcp.M500243-MCP200

Rahi A, Matta SK, Dhiman A, Garhyan J, Gopalani M, Chandra S, Bhatnagar R (2017) Enolase of *Mycobacterium tuberculosis* is a surface exposed plasminogen binding protein. Biochim Biophys Acta Gen Subj 1861(1 Pt A):3355–3364. https://doi.org/10.1016/j.bbagen.2016.08.018

Reyna-Beltrán E, Iranzo M, Calderón-González KG, Mondragón-Flores R, Labra-Barrios ML, Mormeneo S, Luna-Arias JP (2018) The *Candida albicans* *ENO1* gene encodes a transglutaminase involved in growth, cell division, morphogenesis, and osmotic protection. J Biol Chem 293(12):4304–4323. https://doi.org/10.1074/jbc.M117

Satala D, Karkowska-Kuleta J, Zelazna A, Rapala-Kozik M, Kozik A (2020) Moonlighting proteins at the candidal cell surface. Microorganisms 8(7):1046. https://doi.org/10.3390/microorganisms8071046

Silva RCAC, PadovanD C, PimentaR C, da FerreiraC V, SilvaMBriones R (2014) Extracellular enolase of *Candida albicans* is involved in colonization of mammalian intestinal epithelium. Front Cell Infect Microbiol 4:66. https://doi.org/10.3389/fcimb.2014.00066

Sun L, Lu T, Tian K, Zhou D, Yuan J, Wang X, Zhu Z, Wan D, Yao Y, Zhu X, He S (2019) Alpha-enolase promotes gastric cancer cell proliferation and metastasis via regulating AKT signaling pathway. Eur J Pharmacol 845:8–15. https://doi.org/10.1016/j.ejphar.2018.12.035

Sundstrom P, Jensen J, Balish E (1994) Humoral and cellular immune responses to enolase after alimentary tract colonization or intravenous immunization with *Candida albicans*. J Infect Dis 170(2):390–395. https://doi.org/10.1093/infdis/170.2.390

Tracy MR, Hedges SB (2000) Evolutionary history of the enolase gene family. Gene 259(1–2):129–138. https://doi.org/10.1016/s0378-1119(00)00439-x

Vargas G, Rocha JD, Oliveira DL, Albuquerque PC, Frases S, Santos SS, Nosanchuk JD, Gomes A M, Medeiros LC, Miranda K, Sobreira T J, Nakayasu ES, Arigi EA, Casadevall A, Guimaraes AJ, Rodrigues ML, Freire-de-Limal CG, Almeida C, Nimrichter L (2015) Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. Cell Microbiol 17(3):389–407. https://doi.org/10.1111/cmi.12374

Vialás V, Perumal P, Gutierrez D, Ximénez-Embún P, Nombela C, Gil C, Chaffin WL (2012) Cell surface shaving of *Candida albicans* biofilms, hyphae, and yeast form cells. Proteomics 12(14):2331–2339. https://doi.org/10.1002/pmic.201100588

Walsh TJ, Hathorn JW, Sobel JD, Merz WG, Sanchez V, Maret SM, Buckley HR, Pfaller MA, Schaufele R, Slivaet C et al (1991) Detection of circulating candida enolase by immunoassay in patients with cancer and invasive candidiasis. N Engl J Med 324(15):1026–1031. https://doi.org/10.1056/nejm199104113241504

Wang W, Jeffery CJ (2016) An analysis of surface proteomics results reveals novel candidates for intracellular/surface moonlighting proteins in bacteria. Mol Biosyst 12(5):1420–1431. https://doi.org/10.1039/c5mb00550g

Wiśniewski J R, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6(5):359–362. https://doi.org/10.1038/nmeth.1322

Wolf J M, Espadas J, Luque-Garcia J, Reynolds T, Casadevall A (2015) Lipid biosynthetic genes affect *Candida albicans* extracellular vesicle morphology, cargo, and immunostimulatory properties. Eukaryot Cell 14(8):745–754. https://doi.org/10.1128/ec.00054-15

Xu W, Yang W, Wu C, Ma X, Li H, Zheng J (2020) Enolase 1 correlated with cancer progression and immune-infiltrating in multiple cancer types: a pan-cancer analysis. Front Oncol 10:593706. https://doi.org/10.3389/fonc.2020.593706

Zhu Y, Li H, Bhatti S, Zhou S, Yang Y, Fish T, Thannhauser TW (2016) Development of a laser capture microscope based single cellN type proteomics tool for studying proteomes of individual cell layers of plant roots. Horticulture Res 3:16026. https://doi.org/10.1038/hortres.2016.26

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