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

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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 archa 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
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 immunosassays 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 37°C, 5% CO₂ to obtain blastospores. Hyphae were prepared by allowing SC5314 cells to germinate in culture at 37°C, in 5% CO₂ 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 NaHCO₃, 0.5 M NaCl, 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-HCl (pH 3.4) and neutralized with 1.5 M Tris-HCl (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 spectrometry 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 eluate 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 4 °C 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 37 °C 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% H2O2 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 7200 × 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 4°C 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 37 °C 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 37 °C for 10 min after the washing. The reaction was terminated with 2 N H2SO4, 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 × 10⁷ CFU/mL and treated for 1 h with 1:100 diluted 10H8 mAb. Following that, cells were washed and treated
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 ºC. 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 4ºC overnight. After blocking and washing, culture supernatant collected from *C. albicans* blastospores or hyphae 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 37ºC 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 37ºC for 1 h. After incubation and washing, 100 μL of TMB substrate was added into each well and incubated at 37ºC 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.

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 (silver 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 | 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 (silver 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 | orf19.0662.1 | 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  |
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*...
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 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.
C. albicans grew as blastospores 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 $4.54\pm0.74$ ng/ml in the hyphae supernatant (12–120 h) and $74.16\pm12.22$ 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...
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 °C. 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 hyphae. 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 hypha due to technical limitations. Given the importance of hypha 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 evolutionally 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 homoeostasis such as superoxide dismutase (Sod3) (Satalla 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 secretory proteins (Gil-Bona et al. 2015a, b; Wolf et al. 2015). Among the various unconventionally secreted proteins known,
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 hyphae 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.

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None.

**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.

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

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