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RESEARCH ARTICLE

Protein markers for *Candida albicans* EVs include claudin-like Sur7 family proteins

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ABSTRACT

Background: Fungal extracellular vesicles (EVs) have been implicated in host-pathogen and pathogen-pathogen communication in some fungal diseases. In depth research into fungal EVs has been hindered by the lack of specific protein markers such as those found in mammalian EVs that have enabled sophisticated isolation and analysis techniques. Despite their role in fungal EV biogenesis, ESCRT proteins such as Vps23 (Tsg101) and Bro1 (ALIX) are not present as fungal EV cargo. Furthermore, tetraspanin homologs are yet to be identified in many fungi including the model yeast S. cerevisiae.

Objective: We performed de novo identification of EV protein markers for the major human fungal pathogen Candida albicans with adherence to MISEV2018 guidelines.

Materials and methods: EVs were isolated by differential ultracentrifugation from DAY286, ATCC90028 and ATCC10231 yeast cells, as well as DAY286 biofilms. Whole cell lysates (WCL) were also obtained from the EV-releasing cells. Label-free quantitative proteomics was performed to determine the set of proteins consistently enriched in EVs compared to WCL.

Results: 47 proteins were consistently enriched in C. albicans EVs. We refined these to 22 putative C. albicans EV protein markers including the claudin-like Sur7 family (Pfam: PF06687) proteins Sur7 and Evp1 (orf19.6741). A complementary set of 62 EV depleted proteins was selected as potential negative markers.

Conclusions: The marker proteins for C. albicans EVs identified in this study will be useful tools for studies on EV biogenesis and cargo loading in C. albicans and potentially other fungal species and will also assist in elucidating the role of EVs in C. albicans pathogenesis. Many of the proteins identified as putative markers are fungal specific proteins indicating that the pathways of EV biogenesis and cargo loading may be specific to fungi, and that assumptions made based on studies in mammalian cells could be misleading.

Abbreviations: A1 – ATCC10231; A9 – ATCC90028; DAY B – DAY286 biofilm; DAY Y – DAY286 yeast; EV – extracellular vesicle; Evp1 – extracellular vesicle protein 1 (orf19.6741); GO – gene ontology; Log2 (FC) – log2(fold change); MCC – membrane compartment of Can1; MDS – multidimensional scaling; MISEV – minimal information for studies of EVs; sEVs – small EVs; SP – signal peptide; TEMs – tetraspanin enriched microdomains; TM – transmembrane; VDM – vesicle-depleted medium; WCL – whole cell lysate

Introduction

Fungal infections are ubiquitous and are estimated to affect over 20% of the world's population [1]. These infections range from non-life-threatening mycoses of the skin and nails, to deadly systemic infections. They represent a significant burden on healthcare systems, with the direct medical cost of fungal disease in the USA in 2017 estimated to be 7.2 billion USD [2]. The fungi responsible for most fungal infections worldwide are the various species of Candida [3]. Candida is primarily associated with relatively harmless superficial infections [4], but infections of the bloodstream or internal organs are associated with mortality rates as high as 75% [4,5]. Over 12,000 people were hospitalised for invasive candidiasis in the US in 2017 alone, at a cost of almost 1.2 billion USD [2]. The species responsible for the majority of Candida infections is Candida albicans [6]. C. albicans infections are most often treated with two classes of antifungals, the azoles

which target biosynthesis of the fungal-specific sterol ergosterol, and the echinocandins, which target biosynthesis of the major cell wall polysaccharide 1,3-β-glucan [7]. Resistance to these and other antifungals has begun to emerge [8–11] making the development of new antifungals with novel mechanisms of action paramount. Understanding the interface between the host and pathogen will help to identify new targets for controlling *C. albicans* infections.

Extracellular vesicles (EVs) are key factors in the progression of many diseases ranging from cancer [12] to neurodegenerative diseases [13] and bacterial infections [14]. EVs are defined as "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate" [15]. Packaging of cargo into EVs is a method for the coordinated release of biological molecules via a non-classic method of secretion. This facilitates intercellular communication both within an organism [16] and between organisms [17].

Recently, EVs have been proposed to function in the pathogenesis of fungal disease, particularly in yeast infections including those from the genera *Cryptococcus* [18–20], *Paracoccidioides* [21,22] and *Candida* [23–25]. The cargo of fungal EVs comprises proteins [26], nucleic acids [27], lipids [23,24,28,29] and carbohydrates [22,28]. Fungal EVs activate innate immune cells in vitro [18,23,24,30] and are responsible for the hypervirulence of a *Cryptococcus gattii* strain that caused disease in immunocompetent individuals [20]. Given the potential roles for EVs in host-pathogen interactions, there is significant motivation to better understand the biology of fungal EVs.

C. albicans EVs are typical of fungal EVs. They contain proteins, lipids, nucleic acids and carbohydrates [23,24,27,31,32]. Activation of murine macrophages and dendritic cells by EVs from *C. albicans* indicates a potential role for EVs in modulating the innate immune response to the fungus [23,24]. Formation of biofilms is a critical component of *C. albicans* pathogenesis [33] and the biofilm matrix provides a protective layer for the fungus against antifungal drugs [34]. The cargo of EVs produced by *C. albicans* biofilms includes key proteins and carbohydrates for biofilm biogenesis, making EVs crucial contributors to the establishment of biofilms [32]. Furthermore, "add-back" of EVs from wildtype biofilms restored the loss of azole resistance in biofilms formed by strains with deletions in genes encoding important enzymes for matrix generation [32]. That is, EVs also function in antifungal drug resistance.

The tools and techniques for the study of fungal EVs have been adapted from those established for mammalian systems, where EVs are most intensively studied. Many of the cutting-edge experimental techniques for isolation,

tracking and analysis of mammalian EVs are dependent on protein markers [35]. They include identifying fractions containing EVs after density gradient centrifugation [36] or size exclusion chromatography [37], purification of EVs via immunoaffinity capture [38], and imaging of EV release and uptake [39]. Unfortunately, proteomic analyses of fungal EVs have revealed that the markers that have been so useful for the isolation and study of mammalian EVs are not present in fungal EVs [26]. Thus, there are differences in the protein cargo and possibly the biogenesis of EVs from fungi and mammalian cells, and *de novo* identification of fungal EV markers is essential. Inconsistencies between published data sets on the protein content of fungal EVs [26] means that a robust proteomic analysis of EVs from a particular species is required to provide an adequate data set for EV marker identification. In this study, we have addressed this problem by performing in-depth proteomic analyses on multiple *C. albicans* strains to define a set of broadly applicable *C. albicans* EV markers.

Label-free quantitative proteomics was employed to generate an overview of the differences in EV and whole cell lysate (WCL) proteomes for a selection of different *C. albicans* strains and morphologies; DAY286 yeast, DAY286 biofilm, ATCC90028 yeast and ATCC10231 yeast. By comparing the EV versus WCL data across all strains and morphologies we identified 47 putative EV markers which are enriched in *C. albicans* EVs regardless of source, cell strain, or morphology. These candidate protein markers include GTPases, enzymes crucial for cell wall synthesis, and plasma membrane proteins resembling mammalian tetraspanins or claudins. The "minimal information for studies of EVs" (MISEV) 2018 position statement indicates that, in addition to demonstrating the enrichment of EV marker proteins in EV preparations, a deficiency of non-EV proteins should also be shown [15]. With this in mind, we identified 62 proteins that were depleted in *C. albicans* EVs compared to the WCL from the four data sets. Validation of these putative EV markers *in vitro* will allow future *C. albicans* EV research to align better with the recommendations set out in MISEV2018. Furthermore, robust *C. albicans* EV markers will allow adaptation of mammalian EV analysis techniques for use in the study of EVs in *C. albicans* pathogenesis and will provide potential new targets for the development of novel antifungals.

Materials and methods

C. albicans strains and maintenance

C. albicans strains used in this study were DAY286 [40], ATCC90028 and ATCC10231. All strains were

maintained on YPD Agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). Liquid starter cultures were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C and 300 rpm.

Yeast culture conditions

For isolation of EVs from yeast form *C. albicans* (DAY286, ATCC90028, ATCC10231), YPD starter cultures were used to inoculate 150 or 300 mL YPD at an $OD_{600} = 0.2$ in a baffled flask. Cultures were incubated at 30°C overnight (approximately 20 h). The culture OD_{600} was measured immediately prior to EV isolation.

Biofilm culture conditions

Biofilms (DAY286) were generated based on the method described in [41] with modifications. Briefly, 500 mL pre-warmed ½ strength RPMI (250 mL RPMI 1640 (Sigma), 0.165 M MOPS (Sigma) and 250 mL 3.6% dextrose) was inoculated using a liquid starter culture to 1.0×10^6 cells/mL. Five 25×25 cm non-treated bioassay dishes (Thermo Fisher Scientific) were each filled with 100 mL of inoculated ½ RPMI and incubated statically for 4 h at 37°C to allow for adhesion of cells to the dish. Following cell adhesion, the culture medium (and any non-adherent cells) was decanted and replaced with 200 mL pre-warmed full strength RPMI. Biofilms were formed by incubating the dishes for 54 h at 37°C with agitation at 28 rpm in an Incu-Shaker Mini (Benchmark Scientific).

EV isolation

EVs were isolated according to the method described in [28] with minor modifications. In brief, yeast cells or biofilm fragments were separated from the culture supernatants by centrifugation at 4000 x g for 15 min in an Heraeus Multifuge X3 R (75,003,607 rotor, k_{adj} = 11,778.7, Thermo Scientific), then 15,000 x g for 30 min in an Avanti J-E centrifuge (JLA 16.250 rotor, k_{adj} = 2790.4, Beckman Coulter). Supernatants were 0.45 μm filtered (Millipore) then ultracentrifuged at 100,000 x g for 1 h in a Beckman Coulter Optima L-100XP using 70 mL polycarbonate bottle assemblies (45Ti rotor, k_{adj} = 312.6, Beckman Coulter) to pellet EVs, which were resuspended in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) (PBS) and transferred to 1.5 mL polypropylene tubes (Beckman Coulter). EVs were ultracentrifuged again at 120,000 x g for 1 h using a Beckman TL-100 ultracentrifuge (TLA55 rotor, k_{adj} = 102.3, Beckman Coulter) then resuspended in 100 μL

PBS. An aliquot of yeast cells (250 μL) was reserved for preparation of yeast whole cell lysate. Similarly, 250 μL of biofilm scrapings were resuspended in PBS for preparation of biofilm lysate.

Preparation of whole cell lysate and protein quantification

C. albicans cells were washed three times with PBS prior to the addition of approximately 400 μL of acid washed glass beads (Sigma). Cells were lysed using a TissueLyser (Qiagen) at 30 bps for 3×1 min bursts with 1 min incubations on ice between bursts. Debris and beads were pelleted by centrifugation for 5 min at 23,000 x g at 4°C. The supernatant was retained as the respective lysate sample. Protein concentration of EVs and whole cell lysates (WCL) was determined using a Qubit™ 4 fluorimeter (Life Technologies) and the Qubit™ protein assay kit according to the manufacturer's instructions.

Nanoparticle tracking analysis

The particle size and concentration of EV samples was determined using a NanoSight NS300 equipped with a 405 nm (blue) laser (Malvern Instruments). Immediately prior to injection, EVs were diluted between 1:250 and 1:6000 in 0.22 μm (Millipore) filtered PBS to bring the particle concentration within the manufacturer's recommended range (10^8–10^9 particles/mL). NTA software version 3.2 was used to record data in 60 s reads in triplicate. Instrument settings included: a syringe infusion rate of 70, temperature held at 25°C, a camera level of 11, and a detection threshold of 6. The particle concentrations and particle size parameters were recorded. Dilution factor correction and averaging of data across biological replicates was performed using the R package *tidyNano* [42].

Transmission electron microscopy

Carbon-coated 400-mesh copper grids (ProSciTech) were glow discharged for 1 min in a K950X turbo evaporator coupled to a K350 glow discharge unit (Quorum Technologies Ltd). EVs (5 μL) were deposited onto the grid and incubated for 1 min. Grids were then washed once with ultrapure water and negatively stained three times with 2% (v/v) uranyl acetate (Agar Scientific). Excess solution was blotted off and the grids were dried overnight. AJEM 2100 electron microscope (JEOL Ltd) operated at 200 kV was used for imaging.

Sample preparation for mass spectrometry

A total of 14 EV and WCL samples from four different *C. albicans* strains were used for proteomics and NTA analyses; DAY286 yeast (n = 3), ATCC90028 yeast (n = 3), ATCC10231 yeast (n = 3), and DAY286 biofilm (n = 5). Details of each independent culture from which the EV and WCL samples were isolated are provided in Supplementary Table 1. Preparation of EV and WCL proteomics samples and MS data acquisition was performed in three separate batches; DAY286 yeast, DAY286 biofilm, and then both ATCC strains.

EVs or WCLs (15 µg protein) were boiled in LDS sample buffer (Life Technologies) and TCEP (tris(2-carboxyethyl)phosphine) (Thermo Fisher Scientific), then separated by short-range SDS-PAGE [43]. The gel was fixed in 50% (v/v) methanol, 7% (v/v) acetic acid for 30 min, and the samples were excised. The proteins were reduced (2 mM TCEP, 1 h) and alkylated (40 mM iodoacetamide, 30 min in the dark) before digestion with 1 µg trypsin (Promega) for 18 h at 37°C. Peptides were then extracted from the gel pieces with 85% (v/v) ACN, 0.5% (v/v) TFA, lyophilised and resuspended in 20 µL of 5% (v/v) ACN, 0.5% (v/v) TFA.

Mass spectrometry (ESI-LC-MS/MS) of EVs and WCL proteins

Two injections of 6 µL were used for each biological replicate and the samples were randomised prior to injection. Peptides were analysed as described previously [44] with modifications. Using a nanoflow UPLC instrument (UltiMate^{TM} 3000 RSLCnano, Thermo Fisher Scientific), the reconstituted peptides were loaded onto a precolumn (C18 PepMap 300 µm ID x 2 cm trapping column, Thermo Fisher Scientific) and washed (water with 0.1% formic acid, 2% ACN) prior to separation using a 90 min linear ACN gradient on an analytical column (BEH C18, 1.7 µm, 130 Å and 75 µm ID x 25 cm, Waters).

The nanoflow UPLC was coupled on-line to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Nanospray Flex, Thermo Fisher Scientific). The separation of peptides was performed at 45°C, 250 nL/min using a linear ACN gradient of buffer A (water with 0.1% formic acid, 2% ACN) and buffer B (water with 0.1% formic acid, 80% ACN). The gradient started from 2% buffer B to 13% in 6 min, to 33% buffer B over 70 min, followed by 50% at 80 min. The gradient was then increased from 50% buffer B to 95% for 5 min and stayed 95% for 1 min. The column was then equilibrated for 4 min with buffer A.

Data were collected in data-dependent acquisition mode using an MS scan range of m/z 350–1500 and resolution of 60,000. HCD MS/MS spectra were collected for the 15 most intense ions per MS scan at 30,000 resolution with a normalised collision energy of 28% and an isolation window of 1.4 m/z. Dynamic exclusion parameters were set as follows: exclude isotope on, duration 30 s and peptide match preferred. Other Orbitrap instrument parameters included an MS maximum injection time of 30 ms with AGC target 3 x 10^6, for a maximum injection time of 110 ms with an AGT target of 1×10^5.

Database search and protein identification

Tandem MS data collected for EVs and WCLs were processed in MaxQuant version 1.6.0.16 [45]. Andromeda was used to search the data against the UniProt *C. albicans* reference proteome (UP000000559; downloaded 16/06/2018; 6035 entries) and the common contaminants and decoys list [46]. The raw data from both injections of each biological replicate was combined into one sample using the "set experiment" option in MaxQuant. Trypsin was selected as the digestion enzyme and up to 2 missed cleavages were allowed. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine and N-terminal acetylation were set as variable modifications. Peptide mass tolerances were 20 ppm (first search) and 4.5 ppm (main search), and false discovery rate (FDR) cut offs were 0.01 for both proteins and peptides. Minimum and maximum peptide lengths were set to 8 and 25 respectively and match between runs was not selected.

Label-free quantification (LFQ) of protein abundances was performed using the MaxLFQ algorithm [47]. Fast LFQ was selected and normalisation was skipped. The proteinGroups.txt MaxQuant output was used for further analyses. Metadata for identified proteins (localisation, topology, function) were obtained from the UniProt *C. albicans* reference proteome as well as the *C. albicans* SC5314 Assembly 22 chromosomal feature file available from the Candida Genome Database (candidagenome.org/download/chromosomal_feature_files) [48,49].

Protein filtering and proteomic analysis

All proteomics statistical and data analyses were performed using R version 3.6.0 [50], Statistical tests, multiple comparison corrections and important individual R and Bioconductor packages are referenced where they have been used. The DAY286 yeast, DAY286 biofilm and ATCC proteinGroups.txt MaxQuant output files were initially processed and analysed separately. The analysis

Table 1. Candidate positive protein markers for *C. albicans* EVs. This list of proteins consists of those that were found to be exclusive to EVs or significantly enriched in EVs across the four *C. albicans* strains examined in this study. Proteins are grouped according to their subcellular localisation as annotated in the Candida Genome Database (candidagenome.org) [48,49] unless otherwise indicated. The log2 ratio of the abundance (mean MaxQuant LFQ intensity) of each protein in EVs compared to whole cell lysate (WCL) for each strain is listed. "ex" indicates where a protein was only quantified in the EV fraction and not the WCL for that strain. The "TM" column indicates the number of transmembrane domains for each protein as annotated in UniProt. "SP" indicates whether a protein is annotated as having a signal peptide according to UniProt. "VDM" shows whether a protein has been detected previously in vesicle-depleted culture media (i.e. the proteins may also be in the soluble secretome) [31]. Underlined proteins are those identified as the best candidates for positive EV markers according to the criteria depicted in Supplementary Figure S1.

Name	Function	log$_2$(fold change) EV vs WCL				TM	SP	VDM
		DAY Y	A9	A1	DAY B			
Plasma membrane								
ARF3	Arf family GTPase[a]	ex	1.69	ex	3.45			
CDC42	Rho family GTPase	ex	ex	ex	ex			
CDR1;CDR2	Multidrug transporter of ABC superfamily	ex	ex	ex	11.19	12;12		
CHS3	Major chitin synthase of yeast and hyphae	ex	4.12	ex	ex	5		
ENA21	Predicted P-type ATPase sodium pump[a]	ex	ex	ex	ex	9		
FAA4	Long-chain fatty acid-CoA ligase[a]	2.89	2.24	2.28	1.45			
FET34	Multicopper feroxidase	ex	ex	ex	6.18	1	Y	
GAP4	High-affinity S-adenosylmethionine permease	ex	ex	ex	4.28	12		
GSC1	1,3-beta-glucan synthase	2.57	3.45	5.97	8.01	15		
HGT1	High-affinity MFS glucose transporter	ex	ex	ex	ex	12		
HGT6	Putative high-affinity MFS glucose transporter	4.04	4.78	6.53	5.68	11		
HGT7	Putative MFS glucose transporter	ex	5.95	ex	ex	11		
MTS1	Sphingolipid C9-methyltransferase	2.20	1.86	1.41	2.13	2		
NCE102	Non-classical protein export protein	3.34	2.20	ex	5.32	4		
EVP1[d]	*S. cerevisiae* ortholog is Pun1, plasma membrane protein[a]	ex	ex	ex	ex	3		Y
PHM7	Putative ion transporter	ex	ex	ex	ex	11		
PMA1	Plasma membrane ATPase	5.65	4.38	5.60	6.46	8		
RAC1	G-protein of RAC subfamily	ex	ex	ex	3.92			
RHO1	Rho family GTPase	2.81	3.68	3.74	4.03			
RHO3	Rho family GTPase	ex	ex	ex	ex			
SSO2	Plasma membrane t-SNARE[a]	ex	1.88	3.57	ex	1		
SUR7	Protein required for normal cell wall, plasma membrane[c]	ex	ex	ex	7.04	4		
YCK2	*S. cerevisiae* ortholog is Yck2, casein kinase	ex	4.42	ex	ex			
Cell wall, cell surface								
BGL2	1,3-beta-glucanosyltransferase	7.26	9.44	11.08	ex		Y	Y
CRH11	GPI-anchored cell wall transglycosylase	ex	ex	ex	ex		Y	Y
ECM33	GPI-anchored cell wall protein	4.39	5.28	8.31	7.03		Y	Y
GPD2	Glycerol-3-phosphate dehydrogenase	ex	3.48	2.39	1.30			
MP65	Cell surface mannoprotein	ex	ex	ex	ex		Y	Y
MSB2	Mucin family adhesin-like protein	ex	ex	ex	ex	1	Y	Y
PGA4	1,3-beta-glucanosyltransferase	ex	ex	ex	ex		Y	Y
PGA52	GPI-anchored cell surface protein of unknown function	ex	ex	ex	ex		Y	Y
PHR1	Cell surface glycosidase	ex	ex	ex	5.65		Y	
PHR2	Glycosidase	ex	3.51	6.44	ex		Y	Y
PLB4.5	Phospholipase B	ex	ex	ex	ex		Y	Y
SAP9	Secreted aspartyl protease	ex	ex	ex	ex	1	Y	Y
YWP1	Secreted yeast cell wall protein	2.37	3.51	6.17	ex		Y	Y
Endoplasmic reticulum								
ERO1	ER oxidoreductin	ex	ex	2.32	1.68		Y	Y
orf19.1054	*S. cerevisiae* ortholog is Pom33, transmembrane nucleoporin[b]	5.66	ex	ex	2.51	4		
orf19.2168.3	*S. cerevisiae* ortholog is Yop1, reticulon-interacting protein[a]	4.77	1.98	2.29	1.81	4		
orf19.3799	*S. cerevisiae* ortholog is Rtn1, reticulon protein[b]	2.24	1.40	3.00	1.29	2		
SEC61	ER protein-translocation complex subunit	2.53	2.16	2.10	1.46	8		
Endosome, Golgi, transport vesicle								
SEC4	Rab family GTPase[a]	1.88	1.20	1.86	2.90			
YKT6	Palmitoyltransferase, putative vacuolar SNARE complex protein[a]	ex	4.01	5.46	3.20			
YPT31	Rab family GTPase[a]	ex	1.57	1.33	3.33			
Vacuole								
VAC8	Protein involved in vacuolar inheritance[a]	2.79	ex	ex	3.83			
Mitochondrion								
MIR1	Putative mitochondrial phosphate transporter[a]	3.68	4.38	4.24	1.60			
POR1	Mitochondrial outer membrane porin[a]	3.44	2.51	3.69	1.38			

[a]Protein localisation was inferred from sequence similarity with *S. cerevisiae* homolog as annotated in the Candida Genome Database [48,49].
[b]Protein localisation was obtained from the GO Cellular Component annotation in the *C. albicans* UniProt reference proteome UP000000559 [58].
[c]Presence of transmembrane domains and absence of a signal peptide was predicted using TOPCONS2 [59].
[d]The name Evp1 for the protein encoded by orf19.6741 was proposed in the present study.

methodology and some modified functions were derived from the Bioconductor package *DEP* [51].

First, reversed and contaminant proteins as well as proteins identified by less than 2 unique peptides were

removed. The resulting proteins were filtered for those quantified (LFQ intensity > 0) in a minimum of 2/3 yeast or 4/5 biofilm EV or WCL biological replicates (Supplementary Data S1). LFQ intensities were $\log_2$ transformed, then normalised using the cyclic loess normalisation method available in the Bioconductor package *limma* [52]. Proteins were then separated into two groups: those exclusive to EVs or WCL, and those quantified in both samples. Missing LFQ values in the latter group were imputed using the bpca method from the Bioconductor package *pcaMethods* [53], while exclusive proteins were left untouched. Finally, differential abundance analyses of proteins in EV versus WCL or EV vs EV were performed using the empirical Bayes method available in *limma* [52,54]. The resulting p-values were adjusted using the Benjamini-Hochberg approach [55] and the significance threshold was set at an adjusted p-value of 0.01 and a $\log_2$(fold change) of 1. Results of the differential abundance analyses are available in Supplementary Data S2 and Supplementary Data S6.

Functional enrichment analysis and clustering

The online tool FungiFun2 version 2.2.8 (sbi.hki-jena.de/fungifun) was used for all GO term enrichment analyses [56]. The hypergeometric distribution significance test was used and resulting p-values were adjusted using the Benjamini-Hochberg procedure. Enriched GO terms were those with an adjusted p-value less than 0.01. Full results from the functional enrichment analyses are presented in Supplementary Data S3 and S7. Multidimensional scaling (MDS) plots were constructed using the plotMDS function from *limma* [52]. To construct the heatmap in (Figure 5), unsupervised clustering of $\log_2$(FC) for the proteins common to EVs from all strains was performed using Gower's formula via the daisy function from the R package *cluster* [57].

Protein localisation and attribute prediction

Location information of proteins was obtained from the Candida Genome Database (candidagenome.org) or the *C. albicans* UniProt reference proteome (UP000000559; downloaded 16/06/2018; 6035 entries) [48,49,58]. When this information was not available, the subcellular location of a *C. albicans* protein was inferred from the *S. cerevisiae* homolog as annotated in the Candida Genome Database [48,49]. Signal peptide and transmembrane domain annotations for each protein were obtained from UniProt or were predicted using TOPCONS2 (topcons.cbr.su.se) [58,59]. Prediction of protein palmitoylation sites was

performed using CSS-PALM 4.0 (csspalm.biocuckoo.org) [60].

C. albicans EV marker definition

Lists of proteins significantly enriched and exclusive to EVs from each strain were compared using the R package *VennDiagram* [61]. Proteins common to all the three strains and both morphologies were selected as potential EV positive protein markers. This was repeated for significantly enriched and exclusive proteins in WCL to define potential EV negative protein markers. Initial candidate EV marker proteins were refined using the following criteria to obtain a shortlist of the best potential markers. Ideal positive EV markers were proteins that satisfied MISEV2018 marker categories 1 and 2 [15] and had less than five transmembrane domains, no recognisable signal peptide, and were not detected in vesicle-depleted media [31]. Ideal negative EV marker proteins were selected based on prior tagging with fluorescent proteins in literature, prior detection via Western blot, and adherence to MISEV2018 marker categories 3 or 4 [15].

Data availability

The proteomics data have been deposited in the ProteomeXchange Consortium database via the PRIDE partner repository [62,63] with the data set identifiers PXD014367 (DAY286 yeast), PXD014388 (ATCC90028 and ATCC10231) and PXD014389 (DAY286 biofilm). These data include "RAW" files, peak list files, MaxQuant search parameters, MaxQuant search engine txt output and the UniProt *C. albicans* reference proteome FASTA file. The proteinGroups.txt files and source code used in this study is freely available in an online research compendium available at github.com/csdaw/candidaev. It was constructed using the R package *rrtools* [64] and is structured as an R package which can be used to reproduce the results as submitted. The compendium has also been permanently archived at zenodo (https://www.doi.org/10.5281/zenodo.3747549).

Results

EV size distribution is dependent on the C. albicans source strain

EVs were isolated from different strains using a modified version of the differential ultracentrifugation method defined by [28] (Supplementary Figure S1). The strains included the clinical isolate strains ATCC90028 (isolated from blood) and ATCC10231 (isolated from a patient with bronchomycosis). A reference strain commonly used in experiments with *C. albicans* mutants, DAY286,

was also assessed in both yeast and biofilm morphology. Characterisation was performed in accordance with the MISEV2018 recommendations wherein the EV preparation and the EV source are imaged and described quantitatively [15]. Information on each EV preparation used for proteomics experiments and the EV source culture is provided in Supplementary Table S1.

The final protein yield of the EV samples ranged from 18.0 to 71.6 µg with concentrations of 0.192 to 0.716 µg/µL (Supplementary Table S1). EVs from DAY286 yeast and ATCC90028 had a higher protein content than those from ATCC10231 cultures and DAY286 biofilms.

TEM visualisation revealed cup-shaped particles typically 200 nm or less in diameter as well as some non-vesicular co-isolated components (Figure 1(A)). Nanoparticle tracking analysis (NTA) of the EV preparations agreed with the TEM imaging, revealing that the mode size of *C. albicans* EVs isolated at 100,000 x g was less than 200 nm for all samples (Supplementary Table S1). The EVs examined in this study are primarily "small EVs" (sEVs) as defined in MISEV2018 [15], hence the proteomics results are relevant for sEVs and not necessarily larger EVs.

The distribution of EV particle size differed slightly for each strain (Figure 1(B), Supplementary Figure S2). The yeast form strains had a broader range of EV sizes, with particles up to 500 nm being detected. Conversely, the biofilm EVs showed a tight, symmetrical

distribution centred around 100 nm. Interestingly, EVs from both ATCC strains had a significantly larger mode diameter than EVs from either morphology of DAY286 (Supplementary Figure S3A) which was reflected by a lack of detected particles for ATCC strain EVs below 100 nm (Figure 1(B)). The total particle concentration of EVs from ATCC90028, DAY286 yeast and DAY286 biofilm was not significantly different (Supplementary Figure S3B), and typically ranged from 1 to 3×10^{12} particles/mL; although one exceptional biofilm EV preparation had 8×10^{12} particles/mL that skewed the biofilm average (Supplementary Table S1). Conversely, the particle concentration of ATCC10231 EVs was lower than all the other EV samples (2 to 5×10^{11} particles/mL), but this difference was only significant compared to the biofilm EV preparations (Supplementary Figure S3B). Comparing the ratio of particles per µg of protein for each EV sample revealed that DAY286 biofilm EVs had a significantly higher ratio of particles per µg of protein than the other *C. albicans* EVs.

C. albicans strains show similar numbers of differentially abundant EV proteins

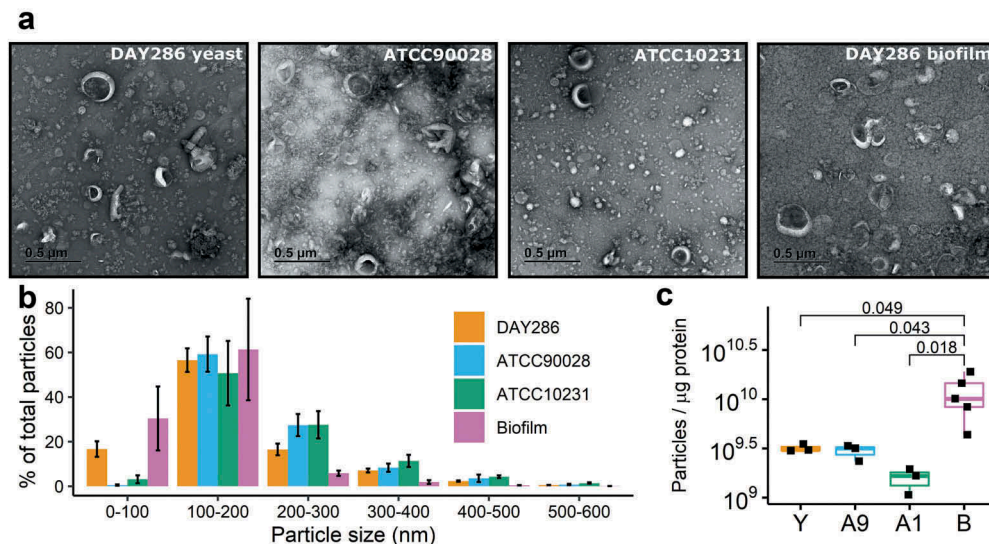MaxQuant and the Andromeda search engine were used to identify proteins in EVs and WCL for each

Figure 1. Characterisation and quantification of EVs from different *C. albicans* strains. *(a)* Representative TEM images of EVs isolated from different *C. albicans* strains. Scale bar indicates 0.5 µm. *(B)* Size distribution of DAY286 yeast (n = 3), ATCC90028 yeast (n = 3), ATCC10231 yeast (n = 3), and DAY286 biofilm (n = 5) EVs as measured by nanoparticle tracking analysis. Bar plots show the percentage of the total number of EVs that can be assigned to each size range. The size of the bars indicates the mean percentage across each biological replicate and the error bars indicate the standard error of the mean. NTA traces for the individual biological replicates are presented in Supplementary Figure S2. *(C)* Comparison of the ratio of particle concentration to protein concentration of the EV fraction, (particles/mL) ÷ (µg protein/mL), across the four EV sources. Each dot represents one biological replicate. Sample means were compared using One-way ANOVA followed by Tukey's HSD *post-hoc* test. Adjusted p-values indicating significant differences are shown. The particle and protein concentrations for each biological replicate are provided in Supplementary Table S1.

strain (Figure 2(A)). Raw MS/MS spectra were used to query the *C. albicans* (SC5314) reference proteome in the UniProt database. Initially, proteins identified by less than two unique peptides were removed. Proteins with two or more unique peptides were filtered to ensure that they were detected in at least 2/3 replicates of EV or WCL for the yeast strains and 4/5 replicates of EV or WCL for the biofilm strain. EV proteins remaining post-filtering ranged from 690 in DAY286 yeast EVs to 1202 proteins in ATCC90028 yeast EVs. Similarly, WCL proteins numbered from 760 for DAY286 yeast WCL to 1229 for ATCC90028. There was at least a 50% overlap between the proteins quantified in EVs and those in WCL for each strain. The biofilm samples had the highest degree of overlap at 77.1% whilst for ATCC10231 54.8% of proteins were common to EVs and WCL (Figure 2(A)). For all strains, the number of proteins exclusive to EVs was less than WCL exclusive proteins. Of the proteins

quantified in EVs from DAY286 biofilms or yeast cells, 44% were common (Supplementary Figure S4A).

Differential abundance analysis was performed on the common EV and WCL proteins from each strain to identify enriched proteins (Figure 2(B)). The full results including $\log_2$(FC) values and p-values are available in Supplementary Data S2. Following imputation of missing values, the mean normalised $\log_2$ LFQ intensities for each protein were compared using the empirical Bayes method implemented in *limma* [52,54]. The resulting t-statistics were adjusted using the Benjamini-Hochberg method [55] and an adjusted p-value cut-off of 0.01, along with a $\log_2$(FC) cut-off of 1, was used to identify significantly enriched EV and WCL proteins for each strain (Figure 2(B)). a similar number of proteins were significantly enriched in EVs compared to WCL across all datasets. The number of EV-enriched proteins ranged from 101 to 121 whereas the number of WCL-enriched proteins ranged from
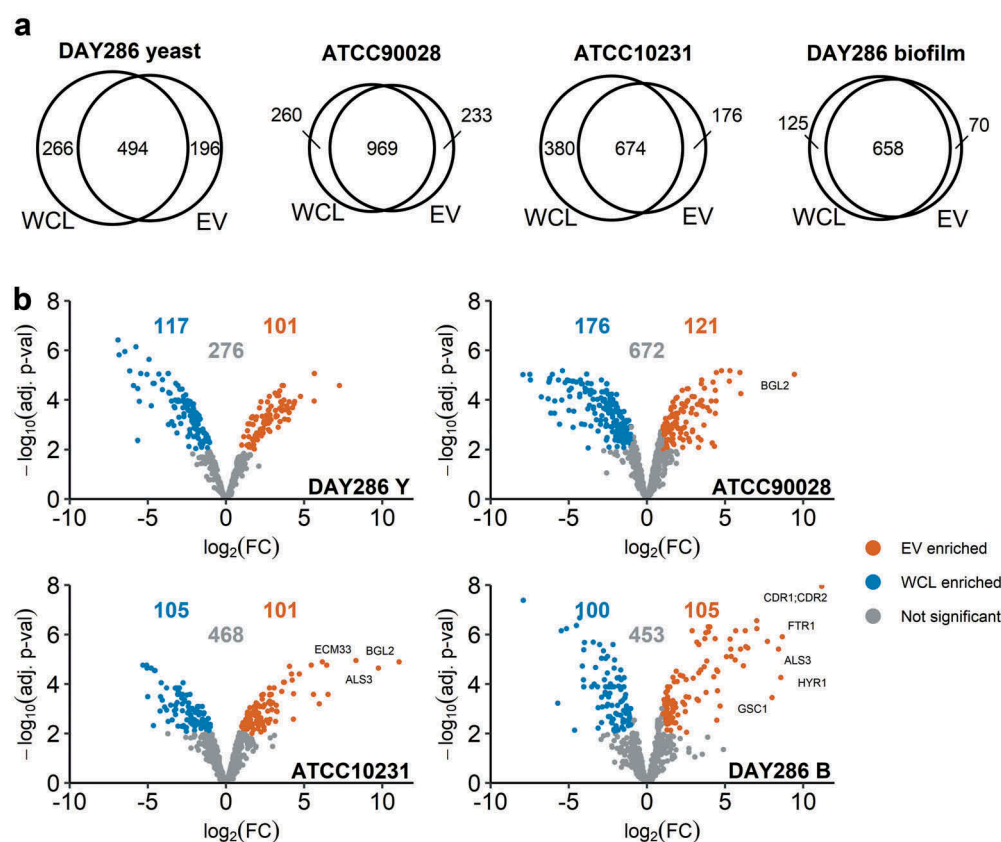

Figure 2. **Differential abundance analysis of proteins identified in *C. albicans* EVs and whole cell lysates (WCL)**. *(a)* Venn diagrams comparing the EV and WCL proteomes from four *C. albicans* strains; DAY286 yeast (n = 3), ATCC90028 yeast (n = 3), ATCC10231 yeast (n = 3), and DAY286 biofilm (n = 5). Whole cell lysates were prepared from the EV source cells. Proteins were quantified (MaxQuant LFQ intensity) in a minimum of 2/3 (yeast) and 4/5 (biofilm) biological replicates of EV or WCL. *(B)* Volcano plots depicting significantly enriched EV or WCL proteins. Differential abundance analysis was performed by comparing the mean normalised LFQ intensities of proteins identified in both EV and WCL (i.e. proteins in the Venn overlap) using the package *limma* [47,52]. Significantly enriched proteins were identified using a Benjamini-Hochberg adjusted p-value cut-off of 0.01 and $\log_2$(FC) cut-off of 1. Counts of significant and non-significant proteins are indicated on each graph. Proteins with a $\log_2$(FC) greater than 8 are labelled. Data tables underlying the Venn diagrams and volcano plots are provided in Supplementary Data S2.

100 to 176. The differential abundance analysis was repeated to compare the enrichment of proteins common to DAY286 yeast and biofilm EVs (Supplementary Figure S4B, Supplementary Data S6). From the 433 shared proteins, 129 were significantly enriched in biofilm EVs versus 136 proteins which were significantly more abundant in DAY286 yeast EVs.

Cell wall and pathogenesis proteins are overrepresented in EVs

We performed functional enrichment analyses on proteins significantly enriched or exclusive to *C. albicans* EVs from each strain (Figure 2(A,B)) to explore whether there were common biological roles for the cargo, and to provide insight on potential functions of *C. albicans* EVs (Figure 3). This was implemented using the online resource FungiFun2 which is a webtool specific for analysis of fungal genes and proteins [56]. In agreement with previous reports [24,31], fungal-type or yeast-type cell wall organisation were significantly enriched biological processes that were identified for all four types of EVs. Furthermore, *C. albicans* EV proteins from all isolations were commonly associated with the *C. albicans* cell wall, the plasma membrane, or generally to the cell surface. Proteins involved in ER localisation and protein glycosylation were overrepresented in the three yeast EV data sets (Supplementary Data S3) and may relate to the production of glycoproteins that are directed to the fungal cell wall. Enrichment of ER, cell wall and plasma membrane proteins suggest that the EVs contain a mix of EV subtypes derived from potentially different vesicle biogenesis pathways.

EVs from the two clinical isolates ATCC90028 and ATCC10231 as well as the DAY286 biofilms were enriched for proteins that function in pathogenesis, consistent with the notion that EVs are crucial to the interaction between the fungus and the host. For example, proteins with aspartic protease activity and adhesion functions which were enriched in ATCC10213 and biofilm EVs are linked to *C. albicans* virulence [65,66]. All EV data sets were enriched with proteins with 1,3 beta-glucanosyltransferase activity. However, the biofilm EVs were also enriched with proteins with exo-1,3-beta-glucosidase activity indicating that the role for EVs in cell wall dynamics may change between morphologies.

Consequently, we performed functional analyses on the differentially abundant proteins found in DAY286 biofilm and yeast EVs to explore how EV function may change depending on source cell morphology (Supplementary Figure S5). Compared to biofilm EVs, DAY286 yeast EVs were enriched in ribosomal and mitochondrial proteins and proteins that function in post-translational modification. EVs from *C. albicans* biofilms have been shown to function in extracellular matrix biogenesis [32], which was reflected here by an enrichment of cell surface and cell wall proteins that function in carbohydrate metabolism. Furthermore, biofilm EVs were enriched in oxidative and heat stress response proteins, which is reflective of the different growth conditions compared to DAY286 yeast EVs.

WCL proteomes have a higher degree of similarity than the EV proteomes from four *C. albicans* strains

EV proteins from each isolation were compared to identify proteins that are packaged into *C. albicans* EVs, regardless of cell morphology or strain. Of the 1487 proteins quantified in EVs across the four data sets, 396 (26.7%) were common to all (Figure 4(A)). Conversely, more WCL proteins; 556 out of 1389 proteins (40.0%) were common to the four proteomes (Figure 4(B)).

The relationship between the *C. albicans* proteomes presented in this study was explored further via multidimensional scaling plots (Figure 4(C,D)). Dimension 1, which represents the leading factor explaining the $\log_2(FC)$ between the four data sets, separates the samples based on source strain; DAY286 versus both ATCC strains. The second dimension shows a batch effect associated with each LC-MS/MS experiment. The two ATCC strains cluster closely together, away from both DAY286 yeast and biofilm. Likewise, the two ATCC sample sets were run on the MS and analysed together, whereas the other two strains were run separately and analysed separately. Finally, dimension 3 separates the samples according to the primary condition of interest; EVs versus WCL (Figure 4(D)).

Immunogenic cell surface and cell wall organisation proteins are universally EV enriched

The 396 proteins common to EVs from all sources (Figure 4(A)) were assessed by unsupervised hierarchical clustering to group proteins based on their $\log_2$(FC) across the four data sets, with the results plotted in a heatmap (Figure 5). The proteins were grouped into eight clusters, C1 to C8. C1, C2 and C3 contained proteins that were highly enriched or exclusive to EVs with an abundance of proteins that were exclusive to one or more EV isolations. C4 also contained EV enriched proteins which were largely not EV exclusive. C5 and C6 contained proteins with varied relative abundance in EVs versus WCL, with C7 containing highly WCL enriched proteins. Finally, C8 included
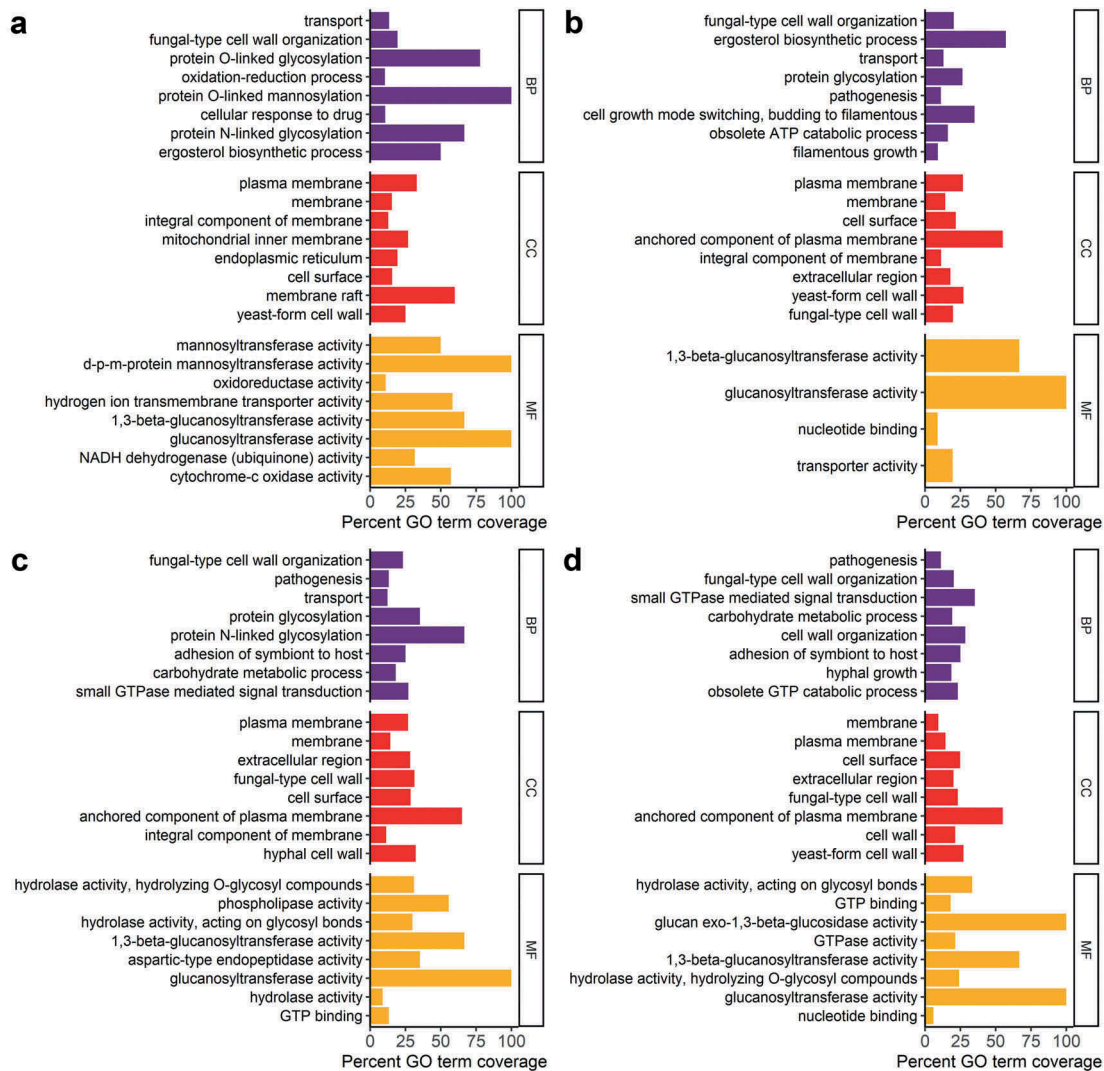
Figure 3. Functional enrichment analyses of the significantly enriched and exclusive *C. albicans* EV proteins. The online tool FungiFun2 (elbe.hki-jena.de/fungifun) was used to identify enriched biological process (BP), cellular component (CC), and molecular function (MF) GO terms [56] based on EV enriched or exclusive proteins from *(a)* DAY286 yeast, *(B)* ATCC90028, *(C)* ATCC10231, and *(D)* DAY286 biofilm. The top 8 (where present) significantly enriched terms for each GO domain are shown (Hypergeometric distribution, Benjamini-Hochberg adjusted p-value < 0.01). They are presented top to bottom in order of increasing Benjamini-Hochberg adjusted p-value. Full lists of enriched GO terms can be found in Supplementary Data S3.

proteins that were exclusive to EVs across all four data sets. These could not be included in the cluster analysis because they did not have any valid $\log_2$(FC) values and were appended to the heat map manually. Supplementary Data S5 contains the names and functions of all the proteins represented in the heatmap in order of appearance from top to bottom.

GO enrichment analyses of the proteins in each of these clusters were performed to provide further insight into the functional roles of *C. albicans* EV proteins with similar $\log_2$(FC) patterns. The 13 proteins in C8 are cell surface/wall (Crh11, Mp65, Msb2, Pga4, Pga52, Plb4.5, Sap9) or plasma membrane-

localised proteins (Cdc42, Ena21, Hgt1, orf19.6741, Phm7, Rho3) involved in cell wall organisation, establishment of cell polarity and pathogenesis. Many of the cell surface/wall proteins, such as Mp65 and Bgl2, are immunogenic and are released extracellularly by soluble secretion as well as in vesicles *in vitro* [31,67,68]. C1, C2 and C3 also contain primarily plasma membrane-associated proteins, many of which function in cell wall biosynthesis and organisation, morphogenesis and pathogenesis. Of note is the enrichment of proteins from the eisosome; a fungal plasma membrane domain also known as the membrane compartment containing Can1 (MCC). Several
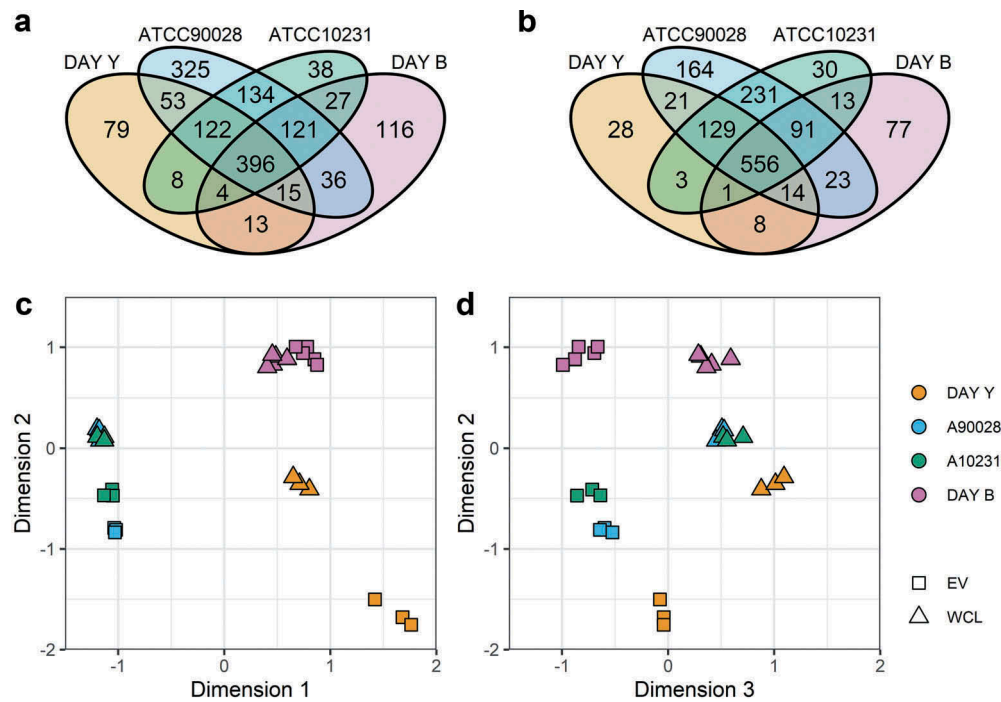
Figure 4. Combined comparison of *C. albicans* proteomes. *(a)* Venn diagram comparing four *C. albicans* EV proteomes. Lists of EV proteins present in the Venn diagrams in Figure 2A were compared to identify proteins common to all four EV datasets. *(B)* Venn diagram comparing four *C. albicans* WCL proteomes. Lists of WCL proteins present in the Venn diagrams in Figure 2A were compared to identify proteins common to all four WCL datasets. The data tables underlying Figuress 4A and 4B are provided in Supplementary Data S4. *(C)* Multidimensional scaling (MDS) plot of each LC-MS/MS biological replicate sample with the first 2 dimensions shown. Pairwise distance between samples approximately indicates the log2(FC) between samples. The plotMDS function from *limma* was used to generate the MDS plots [52]. *(D)* MDS plot of each LC-MS/MS biological replicate sample with the second and third dimensions shown.

MCC/eisosome proteins are EV enriched or exclusive across all *C. albicans* strains including the transmembrane proteins Sur7, Nce102, orf19.6741. The cytosolic eisosome proteins Lsp1 and Pil1 were also detected in EVs but were not enriched. C1 also contains the multidrug transporter Cdr1 (and Cdr2, which cannot be distinguished from Cdr1 based on the peptides identified in this study) that is associated with azole resistance [69]. The locations and functions of C4 proteins are more varied but importantly include GTPases and secretory pathway proteins. Small GTPases are associated with intracellular vesicular trafficking and regulate a variety of membrane fusion events [70,71]. C5, C6 and C7 proteins are mostly cytosolic or ribosomal proteins with functions in glycolysis and translation.

Putative *C. albicans* *EV positive markers include trans-plasma membrane domain Sur7 family proteins*

Potential protein markers for *C. albicans* EVs were selected firstly on the criteria that they were EV exclusive or significantly enriched in EVs. That is, the proteins had a $\log_2$(FC) greater than 1 and adjusted p-value of less than 0.01 across all four datasets (Figure 6(A)). The 47 candidate positive EV markers that were identified are listed in Table 1. These proteins are mostly located on the plasma membrane and cell surface, with ER, endosome and vacuole proteins also represented. Of note were two mitochondrial proteins, which were unexpectedly enriched across all strains.

The 47 candidate proteins were filtered according to specific criteria (Supplementary Figure S1) to obtain a list of the best potential markers for future use. Proteins with signal peptides that had previously been detected in a proteomic analysis of *C. albicans* vesicle-depleted culture medium (VDM) [31], and proteins with more than 5 predicted transmembrane domains were not considered the best marker candidates. This was because proteins with signal peptides that have been detected in VDM are likely secreted canonically as well as in EVs, hence they would not be EV-specific markers. Furthermore, though marker candidates with numerous transmembrane domains such as GSC1 are likely to be secreted specifically in EVs, they may prove difficult to work with in subsequent applications. Following this refinement, 22 proteins were identified as ideal *C. albicans* EV positive markers (Figure 7). These
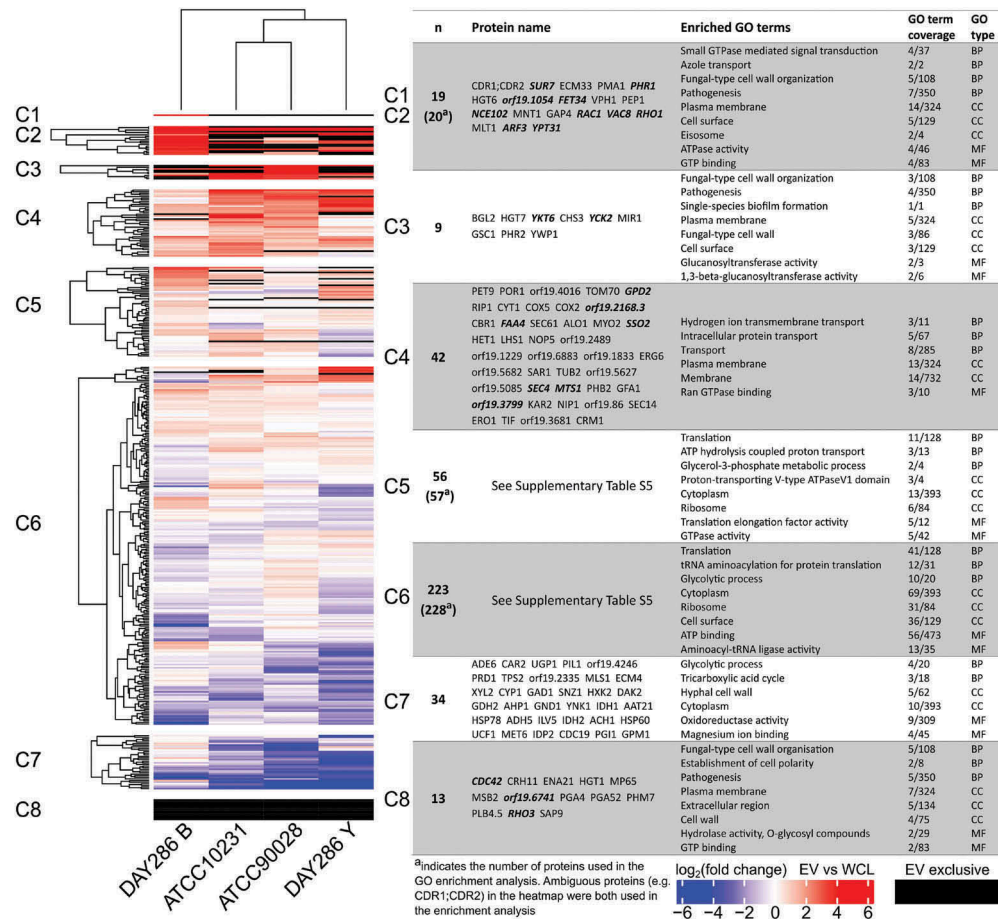
Figure 5. Identification of commonly enriched EV proteins across different *C. albicans* strains. Heatmap of proteins identified in all four EV samples (n = 396, Venn overlap in Figure 4A). Each column represents a *C. albicans* strain and each row represents a protein. The colours indicate the log$_2$(FC) of the protein for that particular strain; red is EV enriched, blue is EV depleted, and black is EV exclusive. Unsupervised clustering of rows and columns was performed using Gower's formula via the daisy function from the R package *cluster* [57]. Proteins which were exclusive to EVs in all four data sets and therefore had no valid log$_2$(FC) values were separated prior to clustering, in the group designated "Cluster 8". Functional enrichment analyses were performed on the heatmap protein clusters using the online tool FungiFun2 [56]. a selection of significantly enriched GO terms, biological process (BP), cellular component (CC), and molecular function (MF), are presented in the table inset. Full lists of enriched GO terms and proteins in each cluster are available in Supplementary Data S3 and S5 respectively. Proteins in bold italics are the 22 putative *C. albicans* EV marker proteins presented in Figure 7.

included trans-plasma membrane proteins such as the Sur7 family proteins orf19.6741 and Sur7 (Pfam: PF06687) and the MARVEL family protein Nce102 (Pfam: PF01284). Anumber of GTPases, which associate with the plasma membrane (Arf3, Cdc42, Rac1, Rho1, Rho3) and secretory pathway (Sec4, Ypt31), respectively, were also determined to be ideal marker candidates. The 22 proteins were only present in clusters C1-C4 and C8 of the heatmap in Figure 5 which included only proteins that were consistently enriched across all four proteomics experiments. Metadata regarding these proteins including their localisation and associated GO terms are provided in Supplementary Data S8.

The process for defining candidate EV positive markers was repeated to identify potential EV negative markers.

Specifically, the lists of exclusive and significantly enriched WCL proteins from each strain were compared (Figure 6 (B)). Sixty-two proteins were selected as potential negative EV markers (Supplementary Table S2). These candidates were refined to a short-list of seven proteins which have been fluorescently tagged or detected by Western blot in previous literature. The 7 proteins are located in the mitochondria (Lpd1, Sod2), vacuole (Apr1, Cpy1, Lap41), cell wall (Gpm1) and actin cortical patches (Abp1).

Discussion

EVs are increasingly being identified as crucial components of cell-cell communication. Most EV studies have
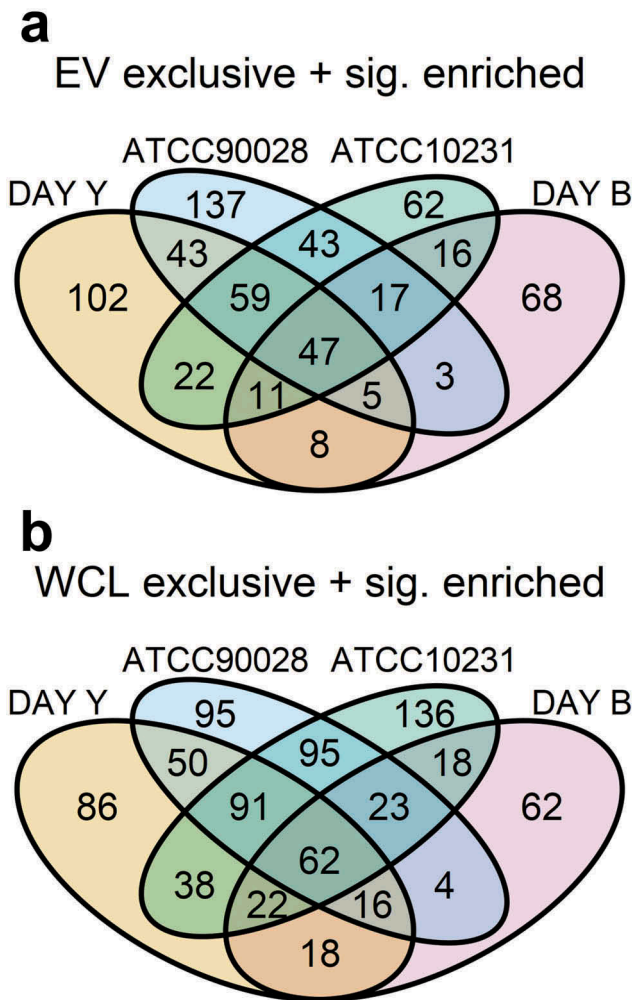
a
EV exclusive + sig. enriched

b
WCL exclusive + sig. enriched

Figure 6. **Identification of common significantly enriched or exclusive *C. albicans* EV and WCL proteins**. Proteins identified as significantly enriched or exclusive to EVs in Figure 2 were compared to select those which were common to all four datasets. (A) 47 proteins were identified as potential positive EV protein markers and (B) 62 proteins as potential negative EV protein markers. The data tables underlying Figure 6 are provided in Supplementary Data S4. Details of the 47 positive EV protein marker candidates and 62 negative candidates can be found in Table 1 and Supplementary Table S2, respectively.

Figure 7. **Twenty-two best potential *C. albicans* positive EV protein markers**. The most promising EV marker proteins are underlined in Table 1. They are enriched or exclusive to EVs isolated from all the *C. albicans* strains investigated in this study. The proteins fit within the EV biomarker categories 1 and 2 from MISEV2018 which includes transmembrane, GPI-anchored, and cytosolic proteins that are EV enriched [15].

the investigation of mammalian EVs because of the absence of suitable marker proteins to assist in purification and tracking in biological systems. Many of the mammalian EV marker proteins either have no homologs in fungi, or the homologs are not found in fungal EVs [26]. This has led to the need for *de novo* identification of fungal EV protein markers based on the recommendations outlined in MISEV2018 [15]. These markers will enable researchers to adapt cutting edge techniques from mammalian EV research to the study of fungal EVs.

We have defined a suite of 22 proteins that are putative markers of *C. albicans* EVs (Figure 7). These positive markers are complemented by seven negative markers that are depleted or absent from EVs versus cell lysates. Both sets of markers were identified from analysis of robust proteomic data sets generated from multiple, independent isolations of EVs from three *C. albicans* strains grown in the yeast form and one strain grown as a biofilm. This represents a step change in the quality of proteomic data published for *C. albicans* EVs as three out of four previous studies analysed EVs from a single isolation [26]. Thus, we are highly confident that the proteins we have identified as putative EV markers are present in *C. albicans* EVs. These markers will need to be validated in future studies using techniques including Western blotting, immunogold labelling on TEM and/or Nano-FACS. The validation studies will depend on generation of

been performed in mammalian systems and a reliable set of EV-specific marker proteins has been identified [15]. These markers have been invaluable for tracking EVs during isolation and have also been exploited for immunoaffinity purification and monitoring of EV release and distribution *in vivo* with fluorescently tagged markers [72–74]. More recently EVs have been identified in a range of yeast and filamentous fungi and their importance in fungal biology has been acknowledged particularly in the interaction between the fungal pathogens and their hosts [75]. However, research on fungal EVs has been limited to the basic techniques developed early in
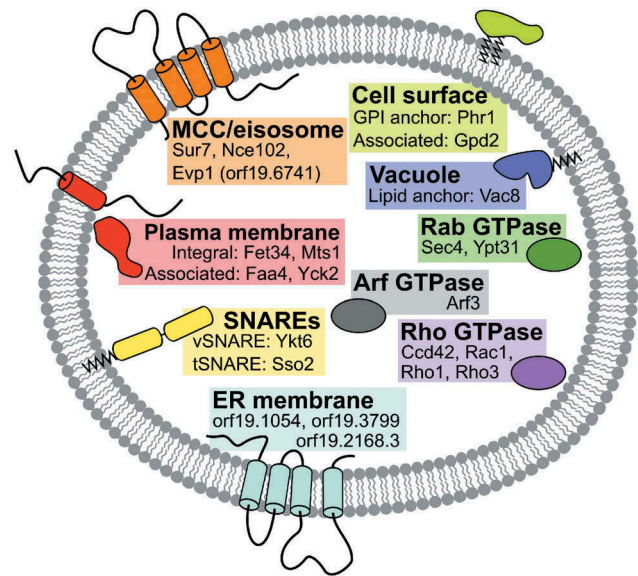
antibodies to EV marker proteins, particularly the extracellular loops of membrane proteins, as no commercial antibodies to specific *C. albicans* proteins are available. One putative marker, Hgt1, has already been independently confirmed to be packaged into *C. albicans* EVs using immunogold labelling and electron microscopy [76]. The immunogold TEM images from Kenno et al. [76] show the plasma membrane protein Hgt1 present in the membrane of small EVs released from the *C. albicans* strain SN152. This result not only confirms Hgt1 as *C. albicans* EV cargo, but also demonstrates that the markers presented here may be applicable to EVs isolated from *C. albicans* strains other than those assessed in this study.

Generally, the EVs isolated in this study had morphologies and size distributions consistent with published data [23,24,31,32]. The main difference was in the number of proteins identified in EVs. Initial investigations into the proteome of *C. albicans* yeast EVs repeatedly found fewer than 100 proteins [23,24,31]. We identified 690–1202 proteins in EVs, which is consistent with the most recent *C. albicans* EV report which identified 862 and 729 proteins in yeast and biofilm EVs, respectively [32]. In addition to the improved quality of our data due to analysis of multiple biological replicates for EVs from each strain, this work also represents the first comparative label-free quantitative analyses of proteins in *C. albicans* EVs and their parent planktonic cells. This is particularly important because our meta-analysis of previously published EV proteomes revealed only 12 proteins that were common to all available data sets [26]. Ten of these were also detected in all four EV proteomes in this study (Supplementary Data S1) but there was no consistent significant difference in abundance between EVs and WCL for seven of the proteins (Eft2, Eno1, Hsp70, Pdc11, Pgk1, Tal1 and Tdh3) (Supplementary Data S2). Furthermore, one protein (Gpm1) was consistently EV depleted compared to WCL. Two out of ten proteins were confirmed as enriched in all EVs tested; the cell surface glycosidase Phr2 and the aspartyl protease Sap9.

Phr2 and Sap9 were among 47 proteins that were either unique or significantly enriched in EVs across the four EV proteomes analysed in this study. To identify putative markers for *C. albicans* EVs, we refined these 47 proteins to eliminate those with unfavourable characteristics for downstream applications or where there was published data indicating that they are not secreted exclusively within EVs [31]. This left us with 22 potential markers that could be grouped into two broad categories, transmembrane/GPI-anchored proteins and lumenal/cytosolic proteins, which align with MISEV2018 biomarker categories 1 and 2.

The cytosolic/lumenal group of marker proteins consists mainly of GTPases, which are associated with different internal membranes. Rho-type GTPases (Rho1, Rho3, Rac1 and Cdc42) are of particular interest because they are from the same protein family as the mammalian EV marker RhoA, which is involved in microvesicle biogenesis alongside Rac1 [77,78]. The primary role of *C. albicans* Rho GTPases is in polarised growth and morphogenesis [79–81], but we show they may also have a role in vesicle release from the fungal plasma membrane. This is supported by the enrichment of Arf3 in all four EV data sets (Table 1). Arf3 is the *C. albicans* homolog of human ARF6, a key regulator of selective cargo recruitment and microvesicle shedding [82].

Potential functional roles of *C. albicans* EVs may also be related to other activities of Rho GTPases. EVs from *S. cerevisiae* are enriched for the cell wall 1,3-β-glucan synthase Fks1 and can provide a protective effect against the 1,3-β-glucan synthase inhibitor caspofungin [83]. Rho1 is the essential regulatory GTPase that forms a complex with the 1,3-β-glucan synthase in *C. albicans*, Gsc1 [84]. This 1,3- β-glucan synthase in *C. albicans* is also enriched in EVs, supporting a conserved role for EVs in cell wall maintenance between *S. cerevisiae* and *C. albicans*.

The four non-plasma membrane associated cytosolic/luminal markers are predicted to associate with secretory compartments (Ykt6, Sec4, Ypt31), and the vacuole (Vac8). These proteins all have roles in intracellular vesicle transport in yeast [85–87], particularly the v-SNARE Ykt6 which is involved in vesicle fusion to the ER, Golgi and vacuole [88,89]. In *Drosophila* and human cells, Ykt6 is required for sorting of Wnt proteins into exosomes for secretion [90,91]. Interestingly, another SNARE protein similar to human syntaxin-2 was enriched in all the EV proteomes (Supplementary Data S2). This t-SNARE, Sso2, is an integral membrane protein involved in the fusion of secretory vesicles with the plasma membrane [92]. Enrichment of these proteins may reflect an endocytic origin of some *C. albicans* EVs, similar to exosomes produced via fusion of multivesicular bodies with the plasma membrane in mammalian cells [73]. This is supported by the abundance of the Rab GTPases Sec4 and Ypt31 which function in the vesicular exocytic pathway of *C. albicans* and *S. cerevisiae* [87,93].

The transmembrane/GPI-anchored proteins could be grouped into four subcellular locations; the membrane compartment of Can1 (MCC)/eisosome, the cell surface, the plasma membrane and the ER membrane. Of these the MCC/eisosome proteins, Sur7 and orf19.6741 were the most intriguing.

Sur7 is a four TM domain protein present in the plasma membrane in stable, sphingolipid and ergosterol enriched microdomains known as the MCC [94]. orf19.6741 is a non-essential, chlamydospore induced gene which encodes a predicted 3 TM domain protein homologous to the *S. cerevisiae* MCC/eisosome protein Pun1 [95,96]. Based on the detection of the orf19.6741 protein exclusively in all EV samples with particularly high abundance, we designate this protein as 'EV associated Protein 1′ or Evp1. Sur7 and Evp1 are both part of the fungal-specific Sur7 family (Pfam: PF06687). This family describes a group of fungal specific transmembrane proteins which localise to the plasma membrane and have a conserved cysteine motif similar to mammalian claudins [97,98]. Alongside their similarities to claudins, Sur7 and Evp1 have predicted topologies reminiscent of mammalian tetraspanins, which are key markers for mammalian EVs (Figure 8) [15]. Tetraspanin family (Pfam: PF00335) proteins have four transmembrane alpha helices and two extracellular

loops, one short and one long which contains four or more conserved cysteine residues. Similarly, Sur7 and Evp1 have 4 or 3 predicted transmembrane domains, with two extracellular loops of different lengths. The long extracellular loops of Sur7 and Evp1 have at least two cysteine residues in a conserved motif.

We hypothesize that Sur7 and Evp1 will be ideal *C. albicans* EV markers after validation because of their potential topological similarity to tetraspanin EV markers, specifically CD9, CD81 and CD63. The utility of tetraspanins as EV markers is characterised by their abundant and broad expression in different cell types, their stability as part of tetraspanin-enriched microdomains (TEMs) in EV membranes, and the plethora of monoclonal antibodies that are available for them. Antibodies raised against tetraspanins typically bind to epitopes in the large extracellular loop [99–102], which protrudes from the EV surface. This interaction has been exploited to isolate EVs from complex biological fluids such as plasma, serum and urine
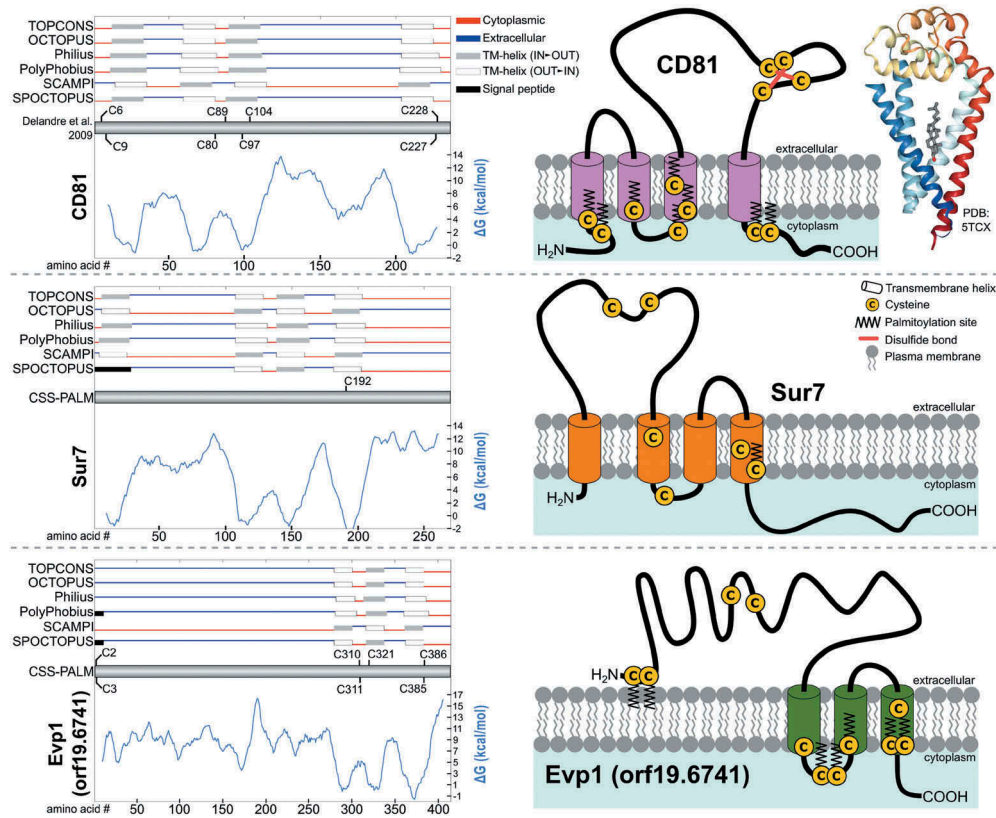

Figure 8. **Predicted topology and palmitoylation sites of *C. albicans* MCC/eisosome proteins Sur7 and Evp1 (orf19.6741) compared to the human tetraspanin CD81**. TOPCONS2 (topcons.cbr.su.se) [59] was used to analyse the amino acid sequences of human CD81 and *C. albicans* Sur7 and Evp1 to predict their topology and the location of transmembrane (TM) domains. The hydrophobicity plots (light blue chart) and topology diagrams (red and blue lines) are shown on the left side of the figure. Additionally, cysteine residues of Sur7 and Evp1 predicted to be palmitoylated using CSS-PALM 4.0 (csspalm.biocuckoo.org) have been annotated [60]. For CD81, palmitoylation sites experimentally determined in previous literature are shown [119]. The right side of the figure shows a cartoon representation of the TOPCONS2 consensus topology prediction for the three proteins as well as the crystal structure of CD81 (PDB: 5TCX) [120].

[72,103,104]. Unfortunately, commercial antibodies for Sur7 are not available and Evp1 has largely been investigated at the transcript level [96,105]. However, if these Sur7 and Evp1 are validated as *C. albicans* EV membrane proteins, then raising antibodies against their extracellular loops would be vital for future *in vivo* research. Alternatively, the genetic manipulability of *C. albicans* means that the expression of fusion proteins is relatively simple and rapid [106], as demonstrated for Sur7 [98]. Isolation of EVs from *C. albicans* strains with GFP-tagged Sur7 or Evp1 will enable monitoring of EVs via fluorescence or the use anti-GFP antibodies for EV marker detection via Western blot. Furthermore, if the predicted topology of Evp1 is correct, expression of Evp1 with a small N-terminal tag may permit immunocapture of *C. albicans* EVs.

Of the 22 putative *C. albicans* EV markers, the MCC/eisosome proteins Sur7 and Evp1 are certainly the most promising candidates. Interestingly, other MCC/eisosome proteins including Nce102, Pil1 and Lsp1 were detected in all four EV proteomes and Fmp45 was quantified in both DAY286 data sets. MCC/eisosomes are important for cell wall synthesis and organisation as well as the recruitment of proteins to the plasma membrane [107]. We speculate that these fungal-specific microdomains also have a role in EV biogenesis from *C. albicans*, and potentially other fungi, based on the role of TEMs in sorting specific cargo into mammalian EVs [108].

To supplement the positive EV markers, we selected 62 proteins that were significantly less abundant in, or were absent from *C. albicans* EVs as potential negative markers. They included ribosome associated proteins such as Tif11 and Tma19, which are potential MISEV2018 category 3 markers. Proteins of this category are part of non-EV co-isolated structures (such as ribosomes) and their depletion in EV isolations is a measure of purity [15]. The 62 candidates were refined to seven proteins that had been interrogated using antibodies or fluorescent constructs as reported in previous literature [109–114]; making them prime targets for initial validation. All seven were from intracellular compartments such as the mitochondria (Lpd1, Sod2), the vacuole (Apr1, Cpy1, Lap41), the cell wall (Gpm1) and actin cortical patches (Apb1) and can be roughly assigned to MISEV2018 marker category 4.

We have focused on the identification of EV marker proteins; however, the biological activities of these proteins provide insight into the function of *C. albicans* EVs. Functional enrichment analysis of the EV proteins indicated a likely role in pathogenesis (Figure 3). Several of the most enriched proteins in all four data sets are antigenic virulence factors involved in morphogenesis such as Bgl2, Mp65 and Phr1 [68,115,116]. Furthermore, hyphal-specific virulence proteins including Xog1, Sap5, Hyr1 and Ece1 are highly abundant in biofilm EVs but absent from the biofilm cell lysates or EVs from yeast-form cells of the same strain (Supplementary Data S2). Also, of note was the enrichment of Cdr1/Cdr2 in EVs from all strains (Table 1). These drug efflux pump proteins are important in the development of resistance to azole antifungals [69]. Their presence in *C. albicans* EVs indicates that EVs may function in azole resistance through transportation of efflux pumps from resistant to non-resistant *C. albicans* cells. This differs from previous studies where *C. albicans* EVs contribute to azole resistance through delivery of matrix components and glucan modification enzymes to promote biofilm growth [32].

Conclusion

In summary, the most promising putative markers for *C. albicans* EVs are the plasma membrane proteins Sur7 and Evp1, which have potential to be the fungal equivalents of the tetraspanin markers used for mammalian EV research. Complementary negative EV markers include the cell surface protein Gpm1, the actin binding protein Apb1, and the intracellular proteins Apr1, Cpy1, Lap41, Sod2 and Lpd1. We are confident that these positive and negative EV marker proteins will be valuable in future investigations on *C. albicans* EVs but antibody-based validation is required. Following *in vitro* validation, these markers will enable the use of a plethora of EV analysis methods, which have not yet been applied to fungal EVs due to the absence of protein markers. Examples include EV immunocapture, *in vitro* tracking of vesicle release and fusion, and *in vivo* monitoring of *C. albicans* EVs over the course of an infection, for example, in mice [73,117,118]. Gaining access to these techniques will allow researchers to answer important questions regarding *C. albicans* EV biology. Specifically, are EVs involved in *C. albicans* pathogenesis *in vivo*, what is their role during infection, and what are the EV biogenesis pathways in *C. albicans*? At this point, it is unclear whether the markers identified in this study will be applicable to other fungal species. Our previous analysis determined that the likelihood of cross-species fungal EV markers is low [26]. Therefore, the process of defining EV enriched markers should be repeated for other pathogenic fungi including *C. neoformans, C. gattii* and *P. brasiliensis*, all of which are known to release EVs.

Disclosure statement

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