UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Microbiología II

TESIS DOCTORAL

Estudio proteómico de las vesículas extracelulares y del secretoma de *Candida albicans* y análisis funcional de proteínas de superficie celular

Proteomic profiling of the extracellular vesicles and the secretome of *Candida albicans* and functional analysis of cell surface proteins

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Ana Gil de Bona

Directores

Concha Gil García
Lucía Monteoliva Díaz

Madrid, 2016

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Memoria presentada para optar al Título de Doctor por la Universidad Complutense de Madrid

Dña. Ana Gil de Bona

Directoras:

Dra. Concha Gil García

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Madrid, 2015

Dª. GLORIA MOLERO MARTÍN-PORTUGUÉS, DIRECTORA DEL DEPARTAMENTO DE MICROBIOLOGÍA II DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA: Que Doña ANA GIL DE BONA ha realizado en el Departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid bajo la dirección de las Doctoras Concha Gil García y Lucía Monteoliva Díaz, el trabajo que presenta para optar al grado de Doctor con el título:

Estudio proteómico de las vesículas extracelulares y del secretoma de *Candida albicans* y análisis funcional de proteínas de superficie celular

Y para que así conste, firmo la presente certificación en Madrid, 2015

Fdo. Prof. Dra. Dª. Gloria Molero Martín-Portugués

ESTA TESIS DOCTORAL HA SIDO POSIBLE GRACIAS A LA CONCESIÓN DE DIFERENTES AYUDAS:

- Beca del Programa Nacional de Formación de Profesorado Universitario (FPI) del Ministerio de Educación y Ciencia durante el periodo Septiembre 2010-2014.
- Ayuda para estancias científicas breves asociada a la Beca FPI en el laboratorio del Dr. Scott G. Filler en Infectious Disease Clinical Outcome Research Unit, Los Angeles Biomedical Research Institute at Harrbor-UCLA Medical Center, Torrance, California, USA, durante el periodo Julio a Diciembre de 2013.
- Ayuda concedida por la "Cátedra de Genómica y Proteómica" por el Banco Santander, de la cual es director el Dr. César Nombela Cano, durante el periodo Septiembre a Diciembre 2014.

EL TRABAJO DESCRITO HA ESTADO INCLUIDO EN LOS SIGUIENTES PROYECTOS:

- "Interacción *Candida albicans*-hospedador. Aproximaciones proteómicas globales para el diagnóstico, tratamiento e inmunoprevención de las candidiasis invasivas". BIO2009-07654 del Ministerio de ciencia e innovación.

- "Proteómica dirigida aplicada al estudio de la interacción *Candida*-hospedador y a nuevas estrategias de diagnóstico y pronóstico de la candidiasis invasiva". BIO2012-31767 del Ministerio de economía y competitividad.

- Red Española de Investigación en Patología Infecciosa (REIPI), del Instituto de salud Carlos III (RD06/008).

- Plataforma en Red de Recursos Biomoleculares y Bioinformáticos (PT13/0001/0038), del Instituto de salud Carlos III (PRB2-ISCIII) y la Federación Española de Enfermedades Raras (FEDER).

- "Interacción microorganismo-hospedador. Proyecto proteoma humano" (920685), financiado por el programa Universidad Complutense de Madrid-Banco Santander (GR3/2014).

- Programación de circuitos microbianos en medicina protectiva y terapeútica (PROMTP) de la Comunidad de Autónoma de Madrid. Grupo APOPROT (S2010/BMD-2414).

A mis padres.

A mi hermana.

Por fin llegó el momento de escribir estas palabras. Creo que es la parte más fácil de este proceso. Y eso es porque me he rodeado de gente increíble que me han ayudado a estar donde estoy ahora mismo. Esta etapa ha sido maravillosa y a veces tediosa. Un camino con curvas y rectas que finalmente ha llegado a su fin. Una experiencia en la que he aprendido mucho y que me ha traído alegrías, tristezas, nuevas y fantásticas amistades e infinidad de experiencias.

Me gustaría comenzar agradeciéndole esta tesis a mis jefas, Concha y Lucia, sin vosotras no hubiese podido llegar este momento. Gracias por confiar en mi trabajo y apoyarme en todo momento. También a la tercera jefa, Gloria. Aunque no eres directamente mi jefa y has estado unos añitos al margen, sin tu apoyo y consejos no habría sido lo mismo. Muchas gracias a las tres.

Gracias de todo corazón a la Unidad 1. A los que están y a los que ya se fueron, incluyendo a los estudiantes y a los nómadas que han pasado por aquí. Sois geniales, todos y cada uno de vosotros. Las horas de trabajo y las de ocio que hemos compartido han sido, y son maravillosas. Gracias por apoyarme en los momentos duros. Me llevo grandes amistades y sabéis que siempre ocuparéis una parte de mi corazón.

Por supuesto agradecerle a todo el departamento de Micro II, a los técnicos, a los estudiantes de doctorado, a los postdoc, a los profesores, a las chicas de secretaría y a Merce. Y por supuesto a los miembros de la unidad de Proteómica. Gracias a todos por formar parte de esta experiencia, porque de alguna manera todos habéis participado en esta etapa.

Una mención especial a Kaiki, Raúl y Ahmad, parte del "núcleo duro". Curiosamente miembros de distintas unidades que a lo largo de estos años hemos forjado una amistad que durará a lo largo de los años aunque cada uno estemos en una punta del mundo siguiendo nuestra vida profesional y personal. Muchas gracias por todo chicos.

No puedo dejar de dedicarle unas palabras a parte de mis pilares de esta etapa. Jose, mi hermano mayor. Gracias por todo. Por las alegrías, los enfados, los viajes, los éxitos, los no tan exitosos logros, por la convivencia y, sobretodo, por estar ahí siempre. Eres una de las personas más especiales de mi vida y no sé que habría hecho estos años sin ti. Y a mis gallegas, Andrea y Noe. No tengo palabras suficientes para agradeceros todo, sobretodo mis continuas subidas y bajadas de ánimo. Por aguantarme y apoyarme en los buenos y malos momentos. Gracias a los tres. Os quiero mucho.

I would like to thank Scott G. Filler for the opportunity of going to his lab at LA-BioMed at Harbor-UCLA Medical Center. Thanks for your dedication and thanks for the opportunity to learn with you. Thanks to my lab mates, Norma, Hong, Trang, Yaoping and all the members of the department, for your dedication and make me feel like a part of your group. Y por supuesto a mis primos angelinos, Yair y Silvia, gracias por acogerme en esa etapa del viaje.

Agradecimientos

Agradecer a mis biotecnólogas las cañas a la salida del labo y todo lo que conlleva la amistad. Blanca, llevamos mucho camino recorrido juntas. No sé qué va a ser de mí sin ti al lado. Nos esperan grandes cambios y sé que todo nos va a ir genial aunque no estemos ya juntas en esta nueva etapa. Al resto de las niñas, cada vez más lejos unas de las otras, siempre encontraremos un punto de reunión para planear nuevos viajes, bodas, bautizos y todo lo que está por venir. Gracias a Pac, mi chico biotecnólogo, ya sabes que necesito que me pongas las pilas de vez en cuando.

Muy especialmente les dedico esta tesis a mi familia y amigos. Sin saber mucho del mundo de la investigación, os habéis interesado y me habéis apoyado en estos años. Sin vosotros no hubiese podido llegar a donde estoy ahora.

Este huequito se lo dedico a Vital. Gracias por estar a mi lado durante estos años. Gracias por tener infinita paciencia y saber calmarme cuando lo necesito. Por darme esa visión de la vida que para mí era totalmente desconocida. Eres muy importante en mi vida y espero que esta nueva etapa que empezamos prácticamente a la vez sea maravillosa, al igual que la que dejamos atrás.

Y por último, esta tesis va dedicada a mis padres. Para mí, los mejores padres del mundo. Sin vuestro esfuerzo, ayuda, dedicación y amor no estaría aquí y no sería quien soy. No tendré años suficientes para agradeceros todo lo que os debo. A mi hermana, Sarah. ¿Qué puedo decir? Eres especial en ti misma y espero que nunca cambies aunque eso nos lleve al caos más absoluto. Os quiero a los tres.

Gracias a todos por vuestro apoyo. Ha sido un placer compartir con todos esta etapa.

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2-DE Two-Dimensional Gel Electrophoresis

° C Celsius degrees

ACN Acetonitrile

BSA Bovin seroalbumin

CFU Colony forming unit

CGD *Candida albicans* database

CHAPS 8-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate)

CID Collision induced dissociation

CLS Chronological life span

CR Calorie restriction

CV Crystal violet

CWI Cell wall integrity

CWP Cell wall protein

DAPI 4',6-diamidino-2-phenylindole

DHR Dihydrorhodamine

DIGE Difference gel electrophoresis

DNA Deoxyribonucleic acid

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

ER Endoplasmic reticulum

ESI Electrospray ionization

EV Extracellular vesicle

FBS Fetal bovine serum

FDA Food and Drug Administration

FDR False discovery rate

FGSC Fungal Genetics Stock Center

FITC Fluorescein isothiocyanate

FT-MS Fourier transform ion cyclotron

GO Gene Ontology

GPI Glycosylphosphatidylinositol

h Hour

HBSS Hanks' Balanced Salt Solution

HIV Human immunodeficiency virus

HPLC High-performance liquid chromatography

IEF Isoelectric focusing

IT Ion trap

kDa kiloDaltons

LC Liquid chromatography

LC-MS/MS Liquid chromatography-tandem mass spectrometry

LDH Lactate dehydrogenase

LRR Leucine rich repeat

LTQ Linear trap quadrupol

m/z Mass-to charge ratio

MALDI Matrix assisted laser desorption ionization

MAPK Mitogen-activated protein (MAP) kinase

min Minutes

MOI Multiplicity of infection

MS Mass spectrometry

MS/MS Tandem mass spectrometry

MVB Multivesicular body

MW Molecular weight

NADH Nicotinamide adenine dinucleotide

NSAF Normalized spectral abundance factor

OD Optical density

OMV Outer membrane vesicle

OPC Oropharyngeal candidiasis

ORF Open reading frame

PAGE Polyacrylamide gel electrophoresis

PAMP Pathogen-associated molecular pattern

PASS PeptideAtlas Submission System

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PI Propidium iodide

PMF Peptide mass fingerprinting

PMSF Phenylmethanesulfonyl fluoride

PRR Pattern recognition receptor

Q Quadrupole

QS Quorum sensing

QSM Quorum sensing molecule

RNA Ribonucleic acid

ROS Reactive oxygen species

SD Synthetic defined

SDS Sodium dodecyl sulphate

SEM Scanning electron microscopy

SP Signal peptide

TEAB Triethylammonium bicarbonate

TEM Transmission electron microscopy

TFA Trifluoroacetic acid

TLCK N-tosyl-L-lysine chloromethyl ketone hydrochloride

TOF Time-of-flight

TOR Target of Rapamycin

TPCK N-p-tosyl-L-phenylalanine chloromethyl ketone

UV Ultraviolet

YNB Yeast nitrogen base

YPD Yeast extract peptone dextrose

Summary

Introduction:

Candida albicans is a commensal fungus in humans which causes different infections ranging from superficial to systemic. Invasive candidiasis is an important cause of disease and mortality in immunocompromised patients. The ability to switch from yeast to hypha growth is essential for virulence in *C. albicans* which express distinct cell surface proteins in these stages. The cell surface is the most external structure and the initial point of contact between the fungus and the host. Surface proteins play an important role in the structural integrity of the cell, adherence and invasion of host cells. One of these proteins is Ecm33, a glycosylphosphatidylinositol (GPI)-linked cell wall protein. The absence of this protein affects both yeast and hypha morphology and results in an aberrant wall structure and reduced virulence *in vitro* and *in vivo*.

The *C. albicans* secreted proteins are also relevant in host-pathogen interaction. *C. albicans* secretes many important proteins involved in different processes, including biofilm formation, cell nutrient acquisition and cell wall integrity maintenance. Some secreted proteins, such as secreted aspartyl proteinases (Sap) and phospholipase B (Plb) families have been detected in the cell wall since they must pass through it to be secreted. These proteins have an amino-terminal signal peptide that is responsible for directing them into the classical secretory pathway. Furthermore, close to one-third of extracellular proteins identified in the *C. albicans* secretome do not possess a secretion signal. These proteins lacking the N-linked signal peptide should use alternative routes of exportation. Extracellular vesicles (EVs) have been described as a mechanism of molecular traffic across the cell wall to the extracellular space in fungi. All fungal species studied to date were apparently able to use EVs as a general mechanism of molecular traffic to transport intracellular proteins across the cell wall.

Objectives:

The present study is focused on 4 different aims:

1. The analysis of the function and importance in virulence of 17 proteins of unknown function identified at the cell surface of the *C. albicans* by the phenotypic analysis of the corresponding deletion mutants.

2. The proteomic study of the *C. albicans* SC5314 secretome including extracellular vesicles and extracellular vesicle-free secretome.

3. The proteomic analysis of *C. albicans ecm33* mutant (RML2U) secretome including extracellular vesicles and extracellular vesicle-free secretome and the comparative analysis with the SC5314 data.

4. The analysis of the function of Ecm33 cell wall protein in *C. albicans* by an extensive phenotypic analysis of the RML2U mutant.

Results:

In the first chapter, the study of 17 proteins of unknown function identified by *C. albicans* cell shaving was tackled. The different phenotypic analysis of the corresponding deletion mutants allowed the identification of four proteins involved in oxidative, osmotic and cell wall stress resistance, in yeast to hypha transition and in the ability to damage and invade oral epithelial cells. These are the putative NADH-ubiquinone-related proteins, Ali1, Mci4, Orf19.287 and Orf19.7590. Other four proteins, Pst3, Orf19.3060, Orf19.5352 and Tos1, were found to be involved in cell wall integrity and in *C. albicans* engulfment by murine macrophages. However, none of these last four proteins were involved in virulence during experimental murine oropharyngeal candidiasis.

The second chapter describes the proteomic study of the extracellular media of *C. albicans* or secretome. The cell-free culture supernatant was separated into EVs and EV-free supernatant and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This approach allowed the identification of 96 proteins, including 75 and 61 proteins detected in EVs and EV-free supernatant, respectively. All the identified proteins involved in cell metabolism or in the exocytosis and endocytosis process were exclusively detected in EVs. In addition, cell wall-related proteins and membrane proteins, including GPI-anchored proteins, are transported by the EVs. Most of the proteins detected in the EV-free supernatant were classical secretory proteins with predicted N-terminal signal peptide (more than 90% of the proteins identified), including cell wall and secreted pathogenesis related proteins.

The third chapter includes the proteomic analysis of the EVs and EV-free supernatant of the *ecm33* mutant RML2U. A total of 170 proteins were identified, corresponding 114 and 154 proteins to the EV-free supernatant and EVs, respectively. In this case, the proteins more represented in both samples were cell-wall related and metabolic proteins. RML2U showed altered the formation of EVs. Also, the pattern of proteins secreted by the classical secretion pathway was affected. Specifically, the secretion of the secreted aspartyl proteinase 2 (Sap2) was compromised but not its intracellular expression. The secretion of other members of the Sap family was not affected. The RML2U growth was compromised in bovine serum albumin (BSA) medium that induces the Sap2 expression and secretion. Because of the relation of Sap2 expression to TOR pathway, the sensitivity of RML2U to rapamycin (the inhibitor of Target Of Rapamycin kinase) was tested and found to be enhanced, connecting Ecm33 with the TOR pathway.

The last chapter of this thesis was the characterization of the Ecm33 protein by an extensive phenotypic study of the RML2U mutant strain. This study revealed that RML2U displays defects in cell wall regeneration, hypersensitivity to high temperatures, oxidative and osmotic stress-inducing agents. Morphological defects were observed in RML2U, such as abnormal cell morphologies, irregular septa distribution, nuclear disorganization and actin patches with unusual distribution in the cell. The abnormal actin and DNA distribution points to a possible arrest in cell cycle. In addition, RML2U presents short chronological lifespan, which connects again with TOR pathway. Furthermore, a new "veil growth" never described in *C. albicans* was observed in RML2U static stationary cultures after long periods. A film of yeast cells covers the surface of the liquid culture. The cells observed in this film were giant round cells with translucent cytoplasm, with large cell wall and large vacuoles. The cornmeal medium mimics the type of cells observed in the veil. In addition, a host-pathogen interaction was performed with murine macrophages. RML2U caused less damage to macrophages and its death response to macrophage was different than the wild type.

Conclusions:

Four new proteins detected in yeast and hypha cell surface, corresponding to putative NADH-ubiquinone proteins, are involved in cell wall integrity, yeast to hypha transition, stress response and host-pathogen interaction: Ali1, Mci4, Orf19.287 and Orf19.7590. Other four proteins, Pst3, Tos1, Orf19.3060 and Orf19.5352 are involved

in the maintenance of cell wall integrity and in the *C. albicans* engulfment by macrophages.

The proteomic analysis of EVs and EV-free supernatant of *C. albicans* allowed the proposal of a model for protein secretion in the studied conditions based on the concept that proteins in the EV-free medium are secreted by the classical secretory pathway, and the EVs that might be formed from the plasma membrane carry cytoplasmic proteins and cell wall-related proteins. Thus, the EVs are the most important mechanism used by *C. albicans* to secrete proteins without predicted N-terminal signal peptide.

The deletion of *ECM33* affects the EVs morphology and their protein content. The classical secretion pathway is also altered, increasing the number of proteins secreted. However, the secretion of Sap2 is specifically compromised in RML2U, suggesting a different mechanism of Sap2 secretion. This result, together to the hypersensitivity to rapamycin and the reduced chronological life span showed by RML2U mutant, indicates a relation between Ecm33 and the TOR pathway.

RML2U presents morphological defects and it is sensitive to high temperatures and to oxidative and osmotic stress-inducing agents. This mutant is able to growth as a "veil growth" which is a new growth stage never described for *C. albicans* with giant round cells with translucent cytoplasm. This form of growth is probably the RML2U adaptation to survive in extreme environmental conditions.

Resumen

Introducción:

Candida albicans es un importante patógeno oportunista en humanos, que puede causar distintos tipos de infecciones, desde micosis superficiales hasta sistémicas. La candidiasis invasiva es una enfermedad que puede causar mortalidad en pacientes inmunocomprometidos. Para causar daño en el hospedador, *C. albicans* cuenta con una serie de factores de virulencia. Entre ellos destaca la capacidad de cambiar su forma de crecimiento de levadura a hifa. La superficie celular es la estructura más externa de la célula y el punto de contacto entre el hongo y el hospedador. Las proteínas de superficie tienen un papel importante en la integridad estructural de la célula y en la adherencia e invasión de células del hospedador. Una de las proteínas localizadas en la superficie celular es Ecm33, una proteína de pared celular con anclaje glicosilfosfatidilinositol (GPI). La deleción de esta proteína afecta a la morfología tanto de levaduras como de hifas, dando como resultado células con la pared celular alterada y virulencia reducida tanto en condiciones *in vitro* como *in vivo*.

El secretoma o las proteínas secretadas por *C. albicans* son también relevantes en la interacción patógeno-hospedador. *C. albicans* secreta muchas proteínas importantes relacionadas con diferentes procesos, entre los que se incluyen la formación de biofilms, la adquisición de nutrientes y el mantenimiento de la integridad de la pared celular. Muchas de estas proteínas secretadas, como las pertenecientes a las familias de aspartil proteasas (Sap) y la familia de fosfolipasas B (Plb), también han sido detectadas en la pared celular, ya que deben pasar a través de ella en su tránsito hacia el medio extracelular. Estas proteínas tienen un péptido señal en el extremo N-terminal que es el responsable de dirigirlas a la ruta clásica de secreción. Sin embargo, cerca de un tercio de las proteínas identificadas en el medio extracelular de *C. albicans* no poseen dicho péptido señal en su secuencia. Estas proteínas deben utilizar una ruta alternativa de secreción. Las vesículas extracelulares han sido descritas como un mecanismo para el tráfico de moléculas al espacio extracelular a través de la pared celular en hongos. Todas las especies fúngicas en las que se han estudiado dichas vesículas son capaces de usarlas como mecanismo general de transporte de proteínas intracelulares a través de la pared celular.

Objetivos:

Los objetivos concretos de este trabajo son los siguientes:

1. Análisis de la función e implicación en virulencia de 17 proteínas identificadas en la superficie de *C. albicans* con función desconocida mediante el análisis fenotípico de los mutantes delecionados en los genes correspondientes.

2. Estudio proteómico del secretoma de la cepa SC5314 de *C. albicans* incluyendo las vesículas extracelulares y el secretoma libre de vesículas.

3. Estudio proteómico del secretoma del mutante *ecm33* de *C. albicans* (RML2U) incluyendo las vesículas extracelulares y el secretoma libre de vesículas y su análisis comparativo con los datos de la cepa SC5314.

4. Análisis de la función de la proteína de pared celular Ecm33 de *C. albicans* mediante un análisis fenotípico exhaustivo del mutante RML2U.

Resultados:

En el primer capítulo se ha llevado a cabo el estudio de 17 proteínas de función desconocida identificadas en la superficie celular de *C. albicans*. Se realizaron diferentes análisis fenotípicos con los mutantes carentes de las proteínas seleccionadas que permitieron la identificación de cuatro proteínas relacionadas con la resistencia a estrés osmótico, oxidativo y a daño en la pared celular, con la transición levadura-hifa y con la habilidad para dañar e invadir células del epitelio oral. Estas cuatro proteínas, Ali1, Mci4, Orf19.287 y Orf19.7590, están descritas como posibles NADH-ubiquinona oxidorreductasas o deshidrogenasas. Los mutantes carentes de las proteínas Pst3, Orf19.3060, Orf19.5352 y Tos1, mostraron fenotipos relacionados con la integridad de la pared celular y la fagocitosis de *C. albicans* por los macrófagos murinos. Sin embargo, ninguna de estas cuatro últimas proteínas está implicada en virulencia en el modelo murino de candidiasis orofaríngea.

El segundo capítulo describe el estudio proteómico del medio extracelular de *C. albicans*. El sobrenadante del cultivo libre de células fue separado en vesículas extracelulares y sobrenadante libre de vesículas, y fue analizado por cromatografía

líquida seguida de espectrometría de masas en tándem (LC-MS/MS). Esta aproximación permitió la identificación de 96 proteínas, incluyendo 75 proteínas detectadas en vesículas extracelulares y 61 proteínas identificadas en el sobrenadante libre de vesículas. Todas las proteínas identificadas relacionadas con el metabolismo celular o con los procesos de exocitosis y endocitosis fueron exclusivamente detectadas en las vesículas extracelulares. Además, algunas proteínas relacionadas con la pared celular y las proteínas de membrana, incluyendo las proteínas de anclaje GPI, también son transportadas por las vesículas extracelulares. Respecto a las proteínas detectadas en el sobrenadante libre de vesículas, más del 90% presentan péptido señal para su entrada en la ruta de secreción de proteínas, entre las que se incluyen proteínas de pared celular y proteínas secretadas relacionadas con patogénesis.

El tercer capítulo incluye el análisis proteómico de las vesículas extracelulares y del sobrenadante libre de vesículas del mutante en *ecm33,* RML2U. Se identificaron 170 proteínas, correspondientes a 114 proteínas en el sobrenadante libre de vesículas y 154 proteínas en las vesículas extracelulares. En este caso, las proteínas más representadas en ambas muestras fueron proteínas relacionadas con la pared celular y proteínas metabólicas. Se comprobó que RML2U tiene alterada la formación de vesículas extracelulares. Además, el patrón de proteínas secretadas por la ruta clásica de secreción también está afectado. Específicamente, RML2U es incapaz de secretar la aspartil proteasa 2 (Sap2), mientras que la secreción del resto de los miembros de la familia Sap no se ve afectada. El crecimiento de RML2U está comprometido en medio con albúmina de suero bovino, el cual induce la expresión y secreción de Sap2. Debido a la relación que existe entre la expresión de Sap2 y la ruta TOR (diana de la rapamicina), se analizó la sensibilidad de RML2U a rapamicina y se observó que RML2U es sensible a rapamicina, lo cual conecta a Ecm33 con la ruta TOR.

El último capítulo de esta tesis fue la caracterización de la proteína Ecm33 mediante un estudio fenotípico del mutante RML2U. Este estudio reveló que RML2U es sensible a altas temperaturas y a agentes que producen estrés osmótico y oxidativo, y tiene problemas para regenerar la pared celular. RML2U tiene defectos morfológicos, como morfologías aberrantes, distribución irregular de los septos, desorganización nuclear y parches de actina con distribución inusual en la célula. La distribución atípica de actina y del DNA señala a un posible fallo en el ciclo celular. Además, RML2U presenta una

longevidad menor que la de su cepa parental, lo cual apoya la relación de Ecm33 con la ruta TOR. Además, el "crecimiento en velo", nunca descrito hasta la fecha en *C. albicans*, fue observado en cultivos de RML2U en fase estacionaria y estáticos tras largos periodos de tiempo. Este velo está compuesto por una película de células que cubre la superficie del cultivo líquido. Las células observadas en esta película son gigantes, redondas, con citoplasma transparente, gruesa pared celular y grandes vacuolas. El medio de cultivo de maíz genera células similares a las observadas en el velo. Por otra parte, se estudió la interacción del mutante RML2U con macrófagos murinos. RML2U causó menos daño a los macrófagos que la cepa silvestre y presentó un patrón de muerte diferente.

Conclusiones:

Cuatro nuevas proteínas detectadas en la superficie celular de *C. albicans* y descritas como posibles NADH-ubiquinona oxidorreductasas o deshidrogenasas, están implicadas en la resistencia a estrés osmótico, oxidativo y de pared celular, en la transición levadura-hifa y en la interacción con células de epitelio orofaríngeo: Ali1, Mci4, Orf19.287 y Orf19.7590. Otras cuatro proteínas, Pst3, Tos1, Orf19.3060 y Orf19.5352, están relacionadas con el mantenimiento de la integridad de la pared celular y con la fagocitosis por los macrófagos.

El análisis proteómico del secretoma de *C. albicans* ha permitido proponer un modelo de mecanismos de secreción de proteínas basado en el concepto de que existen al menos dos rutas principales. Las proteínas identificadas en el medio libre de vesículas son secretadas mayoritariamente por la ruta clásica de secreción, y las vesículas extracelulares, posiblemente formadas a partir de la membrana plasmática, transportan las proteínas citoplasmáticas y de membrana, algunas de ellas relacionadas con la reorganización de la pared celular. Por lo tanto, las vesículas extracelulares constituyen el mecanismo más importante utilizado por *C. albicans* para secretar proteínas que no presentan péptido señal.

La deleción de *ECM33* afecta a la morfología y al contenido proteico de las vesículas extracelulares. El mutante RML2U también tiene alterada la ruta clásica de secreción, ya que secreta un número mayor de proteínas. Sin embargo, no es capaz de secretar específicamente Sap2, lo que sugiere un posible mecanismo de secreción distinto para

esta proteína. Estos resultados unidos a la hipersensibilidad de RML2U a rapamicina y su reducida longevidad indican que existe una relación entre Ecm33 y la ruta TOR.

RML2U presenta defectos en su morfología y es sensible a las altas temperaturas, al estrés osmótico y al estrés oxidativo. El "crecimiento en velo" observado en cultivos estacionarios y estáticos de RML2U está formado por células gigantes, redondas y con citoplasmas transparente y representa una forma de crecimiento no descrita anteriormente en *C. albicans*, que puede estar relacionada con la adaptación y supervivencia en condiciones ambientales extremas.

General introduction

1. *Candida albicans* and candidiasis

Fungi are important organisms present everywhere, from the land to sea water or human mucosal. The number of fungi species estimated on the Earth is approximately 600.000, a 7% of the total number of eukaryotic species, but only 600 are known to cause disease, and some of them are human pathogens (Mora et al. 2011). Also, yeasts are part of the commensal fungi of the healthy population which are normally avirulent in healthy people but could cause important infections in diseased individuals. The most common opportunistic yeast infection in human is candidiasis, which involves the *Candida* species. More than 150 species of *Candida* were discovered, but only five of them are responsible of 90% of cases of candidemia: *C. albicans, C. parapsilopsis*, *C. glabrata*, *C. tropicalis,* and *C. krusei*. The world distribution in clinical isolates from invasive *Candida* infections shows geographic differences (Figure 1) (Calderone 2011, Guinea 2014, Klingspor et al. 2015). Out of them, *C. albicans* is still the most prevalent cause of candidaemia and invasive candidiasis, accounting for 50-70% of all cases (Quindos 2014, Yapar 2014).

C. albicans is a polymorphic fungus capable of switching between different morphologies and adapting to nutritional and environmental situations. It is able to grow in distinct morphological states, such as yeast, pseudohypha, chlamydospore and true hypha, critical for its virulence and corresponding to an adaptative response to environmental changes (Figure 2). Hyphal cells are long, with parallel sides and no obvious constriction at the neck of the mother cell. The yeast form is characterized by its oval form and its role for dissemination through the blood stream. It is stimulated by lower temperatures and more acidic pH, absence of serum and high concentration of glucose. Pseudohyphae are elongated cells that fail to separate from the mother cell, producing filaments with retained constrictions at the septal junctions, which are considered to represent an intermediate form between yeast and true hyphal growth forms (Sudbery et al. 2004). Other morphologies comprise chlamydospores, large, thick-walled, spherical cells produced on specific nutrient-poor media without biological without biological function or role in life cycle identified until date (Staib and Morschhauser 2007). The biological function proposed is to allow survival in severe environmental conditions, but it remains to be demonstrated.

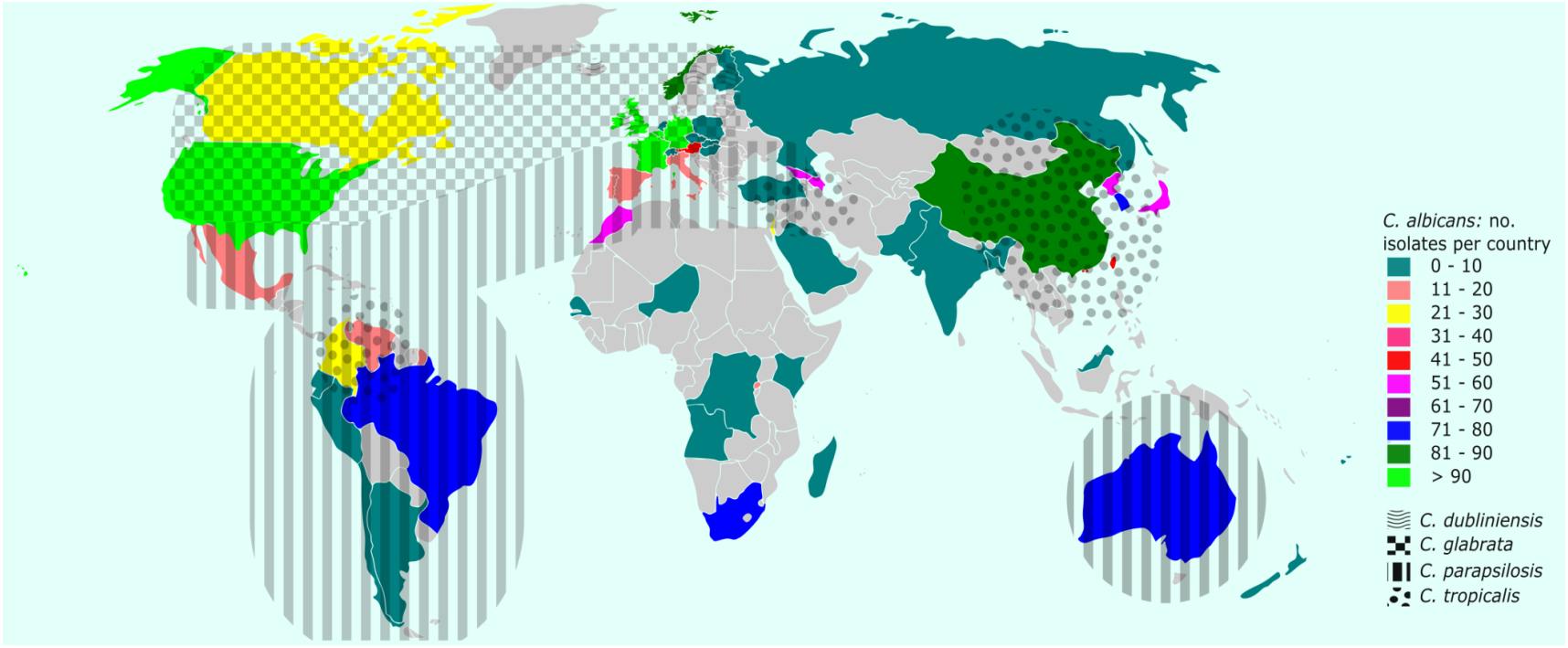


Figure 1. Distribution of most frequent species of *Candida*. View of the geographical locations of isolates of *C. albicans* in the database and details regarding Sequence Types per country. *C. albicans* data were obtained from Multi Locus Sequence Typing (MLST) database. Other *Candida* spp data were obtained from Quindos *et al*. (Quindos 2014). In the case of *Candida dubliniensis* the areas represent those places reporting more than 2% of blood isolates corresponding to this species.

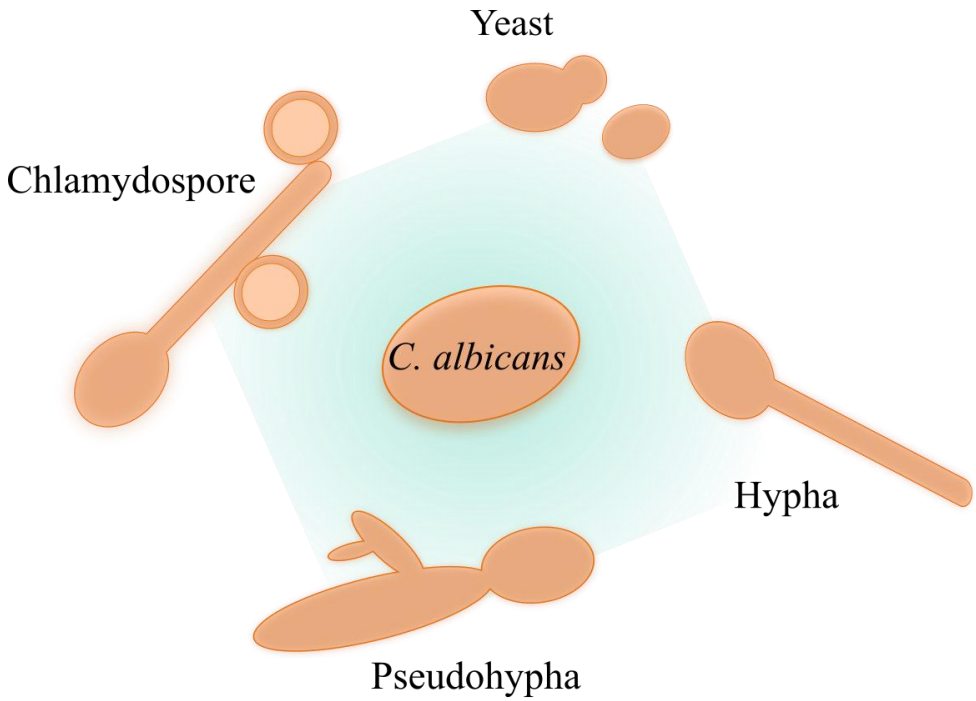


Figure 2. *C. albicans* morphological states. Yeast, true hypha, pseudohypha and chlamydospore.

C. albicans is a common species in genitourinary and gastrointestinal tracts of healthy people, but it is also able to cause problems, mainly vaginal infections in woman. Also, it is responsible for mouth and mucocutaneous infections (Odds 1988, Calderone 2011). *C. albicans* acts as an opportunistic pathogen under certain conditions, such as in immunocompromised people who receive chemotherapy sessions, in HIV-infected people or diabetics, among others. Under these conditions it is able to cause a range of infections, from superficial to systemic candidiasis, making it the most prevalent fungal pathogen in humans.

In the last years, an increase in the frequency of *Candida* infections has been observed, not only in immunocompromised patients, but also in the healthy population. This increase could be associated to the raise in the number of organ transplantations, the use of invasive devices, the use of broad-spectrum antibiotic therapies, the increase of immunocompromised persons, as well as other predisposing factors (Eggimann et al. 2003, Martins et al. 2014). However, the mortality rate of candidaemia has decreased in both HIV infected and non-infected patients, which could be due to an early diagnosis, increased awareness and enhanced therapy (Quindos 2014).

2. *C. albicans* virulence factors

Determinants of pathogenicity are called virulence factors and are described as the components of a microorganism that cause damage to the host (Casadevall and Pirofski 1999). A virulence factor becomes evident when loss of this factor leads to the loss of virulence, and when restoring the factor brings back the virulence. When considering the case of *C. albicans*, it is able to regulate the expression of certain genes and their products as virulence factors to produce disease. One important aspect, is its ability to survive as a commensal in various anatomical sites, each with its own environmental stresses (Calderone and Fonzi 2001). The combination of host factors and the different expression of virulence factors described below, results in a successful *C. albicans* infection (Figure 3).

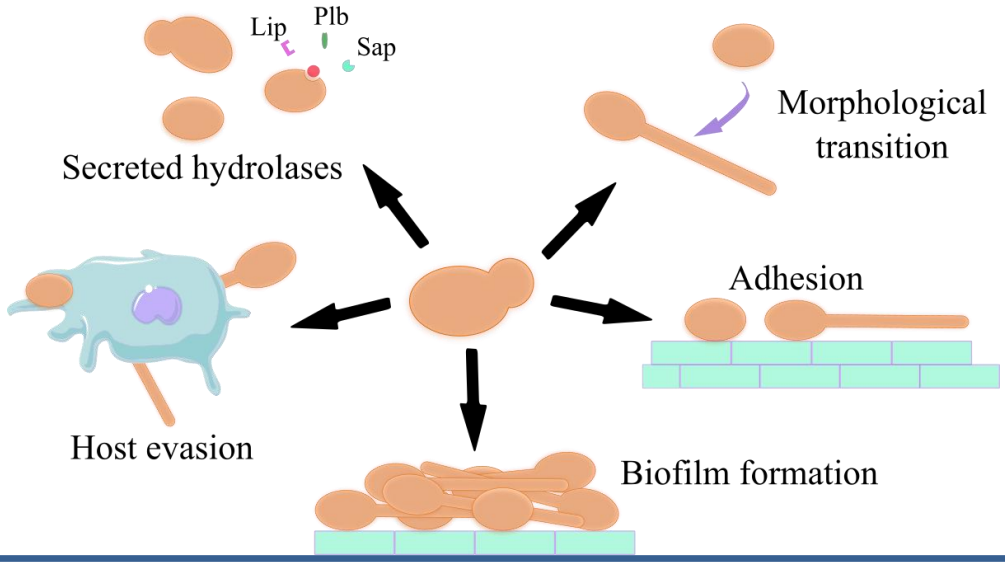


Figure 3. Overview of *C. albicans* pathogenicity mechanisms. Yeast-to -hypha transition, yeast adhesion to host cell surfaces, the attachment of yeast cells to abiotic or biotic surfaces rising to the formation of biofilms with yeast and hyphal cells, host evasion and secretion of fungal hydrolases to facilitate the mechanism of invasion.

2.1. Morphological transition

As mentioned above, *C. albicans* is a polymorphic fungus, which is able to growth as yeast, as elongated cells with constricted septum (pseudohypha) or as true hypha. The ability to change between its yeast, hyphal and pseudohyphal morphologies is

considered to be important for virulence. The hyphal growth is induced under several environmental signs, like exposure to physiological temperature of 37 ºC, addition of serum, neutral to higher pH values, N-acetyl-D-glucosamine, 5% CO_2, hypoxia and nutrient starvation (Eckert et al. 2007).

The yeast to hypha transition is termed dimorphism. The ability to switch between the yeast to filamentous forms is considered to be crucial for its virulence. Little is known about the role of pseudophyphal form in virulence, but it is known that the mutants that are impaired to form hyphae under *in vitro* conditions are generally avirulent (Lo et al. 1997). Yeast and hyphal morphologies have distinct and important roles during infection, being accepted that the yeast form is primarily involved in the dissemination into the bloodstream and the hyphae form is implicated in the invasion, damage and evasion of phagocytic cells (Saville et al. 2003).

Cell cycle progression is different between yeast and hypha. The progression of the yeast and pseudophyphal cell cycle is not considerably different, except for the bud elongation and the failure of cells to completely separate after septum formation. The hyphal cell cycle is completely different to the others (Figure 4).

The yeast to hypha transition involves various regulatory pathways that contribute to a different expression of cell surface proteins in both stages, that will be explained in the corresponding section. Many triggers for this morphological switch have been described and cited before. Quorum sensing (QS) is a cell-to-cell communication system regulated by cell density which also regulates morphogenesis. In *C. albicans*, QS was described after the observation that cell densities lower than 10^6 cells/ml favor hyphal formation mediated by a QS molecule (QSM), Farnesol (Hornby et al. 2001). In addition to farnesol, other molecules like tyrosol, have been found to be *C. albicans* QSM that induced the yeast-to-hyphal shift (Kruppa 2009, Albuquerque and Casadevall 2012).

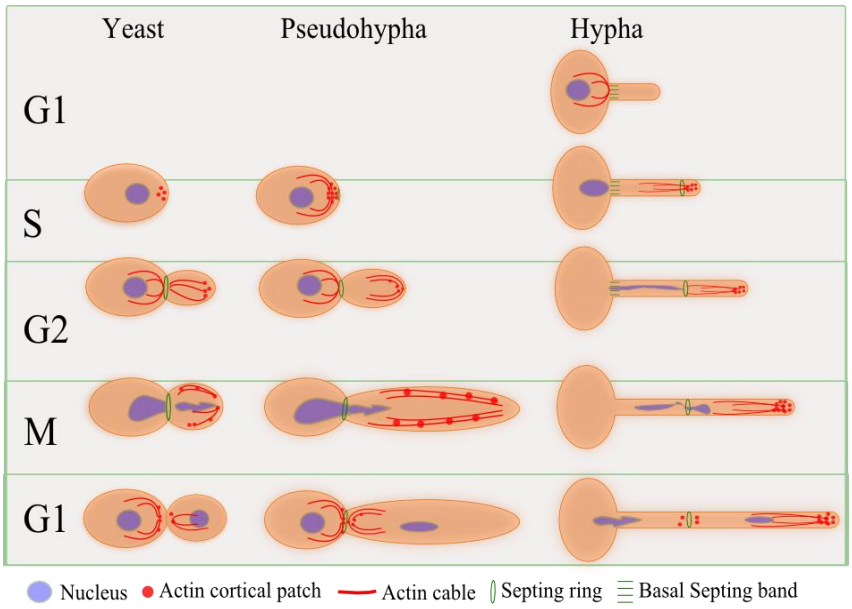


Figure 4. Cell cycles progression in yeast, hypha and pseudohypha. Modified from Sudbery *et al.* (Sudbery et al. 2004, Martinez-Lopez et al. 2006).

2.2. Adhesion

The ability of *C. albicans* to adhere to the host tissue is considered essential in the early stages of colonization and tissue invasion. It is a complex multifactorial event that comprises a series of specific and non-specific mechanisms, which allow the yeast to attach to abiotic surfaces and to host cells. To achieve this it exposes surface proteins such as adhesins and many other pathogenic factors (Chaffin 2008). Adhesins are a specialized set of proteins expressed in *C. albicans* that mediate adherence. The best studied *C. albicans* adhesins are the agglutinin-like sequences (Als) proteins (Hoyer 2001). The *ALS* genes encode a family of glycosylphosphatidylinositol (GPI)-linked cell surface proteins composed by eight members (Als1 to Als7 and Als9). The hypha-associated protein Als3 is very important for adhesion and it is involved in the attachment to host cells, extracellular matrix proteins and biofilm formation on biomedical surfaces (Liu and Filler 2011). Another protein involved in cell-surface adhesion is the hyphal wall protein, Hwp1. A study with *C. albicans hwp1* null mutant has shown reduced adherence and mortality in a murine model (Staab et al. 1999). Both

proteins, Als3 and Hwp1, contribute to biofilm formation by acting as complementary adhesins (Nobile et al. 2008).

Other interesting proteins have been related to adhesion, such as the integrin-like protein Int1, the GPI-linked proteins Eap1, Ecm33 and Iff4, the cell surface mannoprotein Mp65, the secreted aspartyl proteinases Sap9 and Sap10, and the α-1,2-mannosyltransferase Mnt1 (Buurman et al. 1998, Calderone and Fonzi 2001, Sundstrom 2002, Li and Palecek 2003, Albrecht et al. 2006, Martinez-Lopez et al. 2006, Kempf et al. 2007, Sandini et al. 2007).

2.3. Biofilm formation

C. albicans is able to growth in the form of free-living (planktonic) cells or biofilms. Biofilms are defined as structured microbial communities that are attached to a surface and surrounded by a selfproduced extracellular matrix (Costerton et al. 1995). In the last few decades there have been increased *Candida*-biofilm-related infections associated to medical implant devices because of their high resistance to antifungal treatment and their host defense mechanisms (Ramage et al. 2006). Once *C. albicans* has adhered to the medical devices and evaded the immune system of the patient, it can form biofilms that colonize the internal organs and medical implants, such as central venous or urinary catheters, artificial heart valves, prosthetic joints or dentures. *C. albicans* biofilms are formed in a sequential process including 4 steps (Figure 5) (Blankenship and Mitchell 2006, Polke et al. 2015):

1. Settlement and adhesion of yeast cells to the surface.
2. Proliferation of the attached yeast cells.
3. Maturation of the biofilm through development of hyphae and pseudohyphae and accumulation of extracellular matrix material.
4. Dispersion and dissemination of yeast cells from the biofilm complex.

The first phase of biofilm formation is the adherence of *Candida* to medical devices mediated by cell wall proteins. During this step, the adhesins seem to be important in the establishment of the first contact with the host cell and/or the surface of the inert material during the formation and development of the biofilm, e.g. Hwp1 and Als3. A real-time PCR expression profiling of genes encoding potential virulence factors in

C. albicans biofilms showed that *HWP1* and the genes member of the *ALS*, *SAP* and *LIP* gene families are upregulated in various biofilm model systems (Nailis et al. 2010).

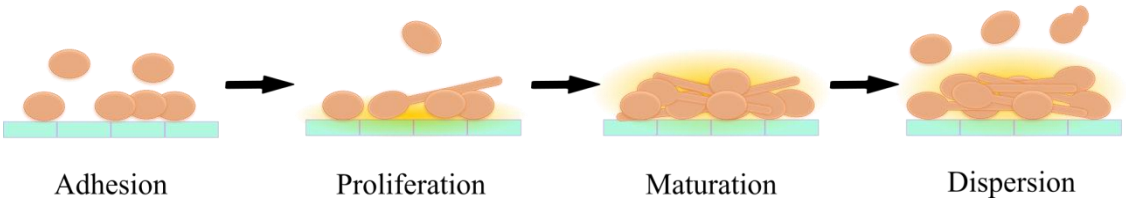


Figure 5. Stages in the *C. albicans* biofilm development. Sequence of steps in biofilm formation representing the categories listed at the text.

The formation of the exopolymeric matrix, composed of polysaccharides, carbohydrates, proteins and other components, enables *C. albicans* to protect itself from the phagocytic cells, maintaining nutrients and serving as a barrier to the diffusion of drugs and substances toxic to the yeast. In fact, biofilms are more resistant to drugs and the host immune system because of its matrix, architecture, increased expression of drug efflux pumps and metabolic plasticity (Fanning and Mitchell 2012). In line with this, *C. albicans* has other drug resistance mechanisms, such as differential regulation of drug targets that block binding of the drug to the target, and reduced growth rate that decrease the antifungal efficacy (Mathe and Van Dijck 2013). The major heat shock protein Hsp90, required also for biofilm antifungal drug resistance, is involved in the dispersion in *C. albicans* biofilms (Robbins et al. 2011).

2.4. Host evasion

C. albicans employs multiple avenues to avoid the antimicrobial activity of the immune system by inhibiting recognition, trafficking and effectors release, as well as by overcoming important stresses (Cheng et al. 2012, Jimenez-Lopez and Lorenz 2013). The innate immune system recognizes *C. albicans* through pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs), being the most important the cell wall components (see section 3.1. *C. albicans* cell wall organization). *C. albicans* is able to evade the host innate system through different evasion strategies:

a. Yeast-to-hypha transition.
b. Passive epithelium invasion through Als3 recognition by oral epithelial cells.

c. Shielding of PAMPs from PRRs.
d. Inhibition or degradation of complement system.
e. Inhibition of phagolysosome formation.
f. Modulation of cytokine production by soluble factors.

The binding of negative regulators of the complement cascade to the cell surface inhibit complement activation. All of these activities aim to reduce immune recognition of *C. albicans* (Meri et al. 2002, Meri et al. 2004, Gantner et al. 2005, Phan et al. 2007, Gropp et al. 2009, Cheng et al. 2012).

2.5. Secreted hydrolases

C. albicans expresses important families of secreted proteins, including proteases (Saps), lipases (Lips) and phospholipases (Plbs), which contribute to facilitate active penetration into the host cells and to enhance the efficiency of extracellular nutrient acquisition. The family of Saps comprises ten members, Sap1 through Sap10. Sap9 and Sap10 are retained at the cell surface via a GPI anchor while Sap1 to Sap8 are secreted into the extracellular media (Albrecht et al. 2006). Saps are involved in multiple processes, like degradation of proteins and nutrient uptake, but the relative contribution of Saps to *C. albicans* pathogenicity is controversial. Previous studies have shown that Sap1-3 are required for virulence in a mouse model of systemic infection and for damage of human epithelium *in vitro* (Hube et al. 1997, Schaller et al. 1999); but other results indicate that Saps are dispensable for these purposes (Lermann and Morschhauser 2008, Correia et al. 2010). However, recent study showed that *C. albicans* Saps interfere and inactivate host innate effector components, suggesting a role for these proteases in virulence (Gropp et al., 2009).

The family of phospholipases includes four different classes (A, B, C and D). Their activity consists in tissue disruption and evasion contributing to pathogenecity (Niewerth and Korting 2001). The five members of class B (Plb1-5) contain a signal sequence for secretion to extracellular environment, and 3 out of them, Plb3, Plb4.5 and Plb5, have a putative GPI signal (Richard and Plaine 2007). Their role in virulence implies the ability to destroy the components of the host membranes. Of the Plb proteins, Plb1 appears to be the most important contributor to the virulence. The *plb1* null mutant is viable without an obvious phenotype or an effect on adherence to human

endothelial or epithelial cells. However, *PLB1* gene is required for virulence in a murine model for hematogenously disseminated candidiasis and its reintroduction restores virulence *in vivo* (Leidich et al. 1998, Mukherjee et al. 2001).

The third important family of secreted hydrolases is the lipase family. This family consists of 10 members (Lip1-10) containing an N-terminal signal for secretion (Fu et al. 1997, Hube et al. 2000). All of them have a role in *C. albicans* pathogenecity. The differential expression of the *C. albicans LIP* genes in human clinical specimens confirmed that the transcription of these genes depends on the stage of infection (Stehr et al. 2004).

3. The external face of *C. albicans*: the cell wall and secreted proteins

3.1. *C. albicans* cell wall organization

The cell wall of *C. albicans* is one of the most important organelles because it is the first site of contact between the cell and the environment. As the external structure of yeast, it plays a significant role in cell shape, physical strength, colonization and invasion of human tissues, in the adhesion to medical materials and to biofilm formation. In addition, the cell wall provides a protective barrier against a wide range of environmental conditions such as temperature, osmotic stress and oxidative stress. The cell wall is a layered structure composed of polysaccharides, chitin, β-1,3- and β-1,6-glucan, manann, and proteins (Figure 6). The cell wall integrity must be balanced and the loss of this integrity could result in sensitivity to environmental factors and it could affect the growth, morphology and viability of the yeast.

The chitin and β-glucans are the main components of the skeletal inner layer of the cell wall, closer to the plasma membrane. The **β-1,3-glucan** is part of an ordered structure to which β-1,6-glucan and chitin are attached through their reducing ends. The function of the β-1,3-glucan is to confer a degree of elasticity and tensile strength to the cell wall, functioning as the major cell wall building block. It is synthesized by the plasma membrane-associated enzyme β-1,3-glucan synthase, Fks1 (Mio et al. 1997). The β-1,3-

glucan contributes to 40% of the yeast cell wall dry weight increasing twice in hypha, being one of the most abundant cell wall components.

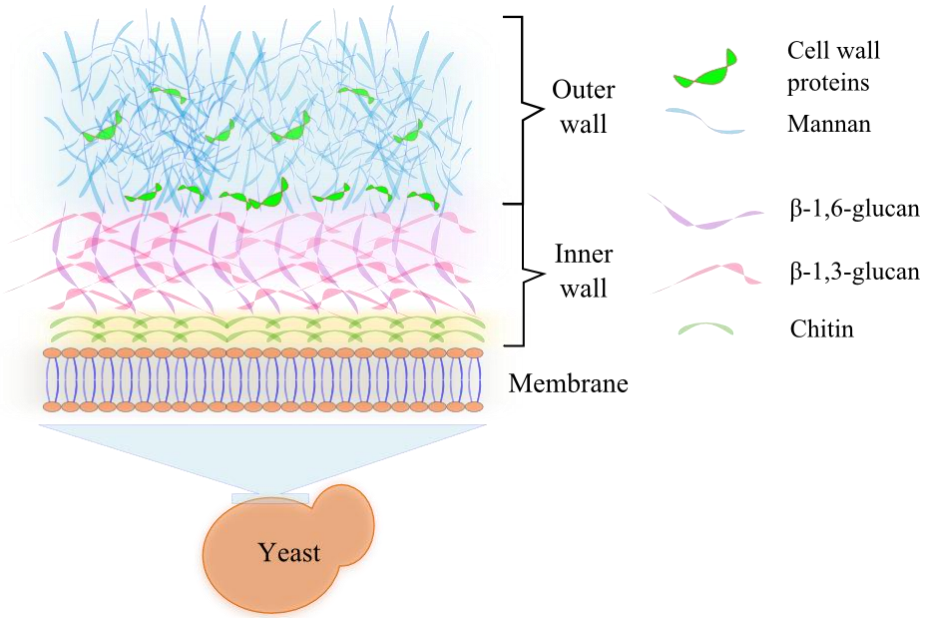


Figure 6. *C. albicans* cell wall structure. General representation of the cell wall of *C. albicans*. The inner wall contains chitin and glucan matrix, including β-1,3-glucan and β-1,6-glucan. GPI-anchored proteins and non-anchored proteins are attached by mannans and represent the outer wall.

The **β-1,6-glucan** has been shown to form cross-links with the β-1,3-glucan, chitin and the GPI anchor and it is an important component of the cell wall, representing around 20% of its dry weight. The biosynthesis of this polysaccharide is carried out by Kre family and Skn1, the latter is induced upon induction of hyphal formation (Boone et al. 1991, Mio et al. 1997, Lussier et al. 1998, Gilbert et al. 2010).

The last component of the cell wall polysaccharides is the **chitin**, which represents about 1-2% of the yeast and 4-6% of the hyphae dry weight. It is a β-1,4-linked polymer essential for cell viability and covalently attached to β-1,3-glucan (Lenardon et al. 2010). The major classes of cell wall proteins are attached through the remnant of a GPI residue to β-1,3-glucan or via a branched β-1,6-glucan linker to chitin. Chitin is synthesized by a large family of chitin synthase (Chs). Of them, Chs1 is an essential chitin synthase required for the synthesis of primary septa in yeast and hyphae cells and

cell wall integrity (Munro et al. 2001). Chs2 is a non-essential hypha-specific enzyme responsible of the elaboration of about 40% of chitin found in this morphology (Gow et al. 1994). Chs3 and Chs8 synthesize short- and long-chitin fibrils, respectively, being Chs3 the major chitin synthase (Lenardon et al. 2007).

The outermost layer of the cell wall is highly enriched with **mannans** that are covalently associated with proteins and represent 40% of total cell wall polysacchararide content. Mannose sugar is incorporated into three structures: linear O-linked mannan, highly branched N-linked mannan and phospholipomannan (Poulain and Jouault 2004). N-linked mannan is linked to a protein component via asparagine. It is a comb-like structure comprised of a core glycan and an extensive outer branched structure, which has an α-1,6-linked mannose backbone and a variety of α-1,2-, α-1,3-linked, and sometimes β-1,2-linked side chains and an acid-labile β-1,2-linked phosphomannan side chain joined to the backbone by a phosphodiester bond. Several enzymes are involved in the synthesis of N-linked mannan. Och1 is responsible for the addition of the first α-1,6-linked mannose to the core glycan (Bates et al. 2006). Bmt1-9 are a novel family of *C. albicans* β-1,2-mannosyltransferases that synthesise the β-1,2-linked oligossacharide side chains (Mille et al. 2008). Mnn2 is responsible for the addition of the first α-1,2-linked mannose to the α-1,6-linked mannose backbone. The synthesis of the O-linked mannans begins with a mannose residue being added to a serine or threonine residue in the amino acid sequence of the protein. The enzymes responsible for this addition are the protein mannosyltransferase (PMT), a family of six proteins (Pmt1 to Pmt6). The mannosyltransferases (Mnt1, Mnt2) are responsible for the addition of the second and third mannose residue during O-linked mannosylation to create a short α-1,2-mannose chain (Munro et al. 2005). As a final point, a α-1,3-mannosyltransferase (Mnn1, Mnt2, Mnt3) added α-1,3-mannose to end the O-linked oligossacharides. Almost all cell wall proteins contain substantial amounts of O-linked oligosaccharides and a substantial amount of N-linked glycosylation. Initial N-linked glycosylation take place in the endoplasmic reticulum.

As commented before, associated with these carbohydrates are **cell wall proteins** (CWPs) **covalently** attached to this framework of carbohydrates. The **GPI**-CWP are the most abundant class of CWP linked to β-1,6-glucan by a truncated GPI-anchor. Most GPI-CWPs are organized in distinct domains (Figure 7):

- N-terminus contains a signal peptide, which is cleaved off when exiting the secretory pathway. The remaining N-terminal domain constitutes the active part of the protein.
- The middle part contains the serine/threonine-rich domain. It has a structural function and contains the majority of glycosylation sites.
- The ω-site is located between the C-terminus and the S/T domain. During GPI anchor addition, the ω-site is cleaved and the protein is attached to an ethanolamine residue in the GPI anchor.
- The C-terminal hydrophobic domain is for transient attachment to the ER membrane.

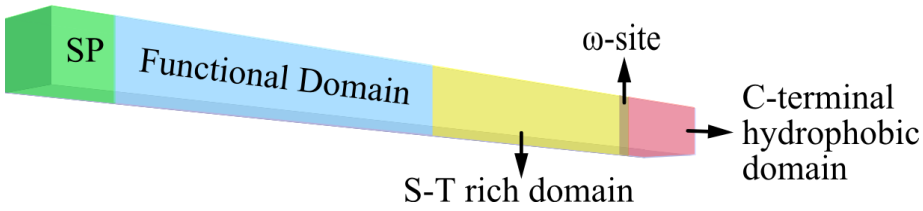


Figure 7. Domain structure of a GPI-CWP. From left to right the domains mentioned in the text: N-terminal signal peptide (SP), functional domain, serine/threonine domain, ω-site and C-terminal hydrophobic domain. (Thomas et al. 1990, de Groot et al. 2003, Nather and Munro 2008).

While all GPI-proteins are initially inserted into the plasma membrane, the GPI-proteins that are to become resident proteins of the wall get cleaved off and the GPI-remnant is attached to the β-1,6-glucan (Pittet and Conzelmann 2007). Some GPI-proteins can be found at both locations.

Richard and Plaine (2007) published a putative GPI-proteins list corresponding to the *C. albicans* genome and based on previous studies. In that list, the number of putative GPI-proteins identified in *C. albicans* was 115. Out of them, a vast majority of the proteins are of unknown function. These proteins, in particularly whose function is unknown, are important targets of *C. albicans* pathogenicity because their putative localization at the cell surface permits interactions with the environment. One of these proteins is Ecm33, a GPI-linked cell wall protein whose absence affects the wall structure (More details in section "3.1.1. Ecm33"). Another GPI-linked cell wall protein

is the secreted yeast wall protein Ywp1. This protein is linked covalently to glucans of the wall matrix and has the highest expression during yeast exponential growth. The *ywp1Δ/Δ* mutant has increased adhesiveness and biofilm formation but no obvious change in growth, morphology or virulence, suggesting that Ywp1p promotes dispersal of yeast form cells in *C. albicans* (Granger et al. 2005).

Several CWPs make up families with the same function but slightly different specificities like pH or morphological state. Furthermore, as commented before, these proteins are involved in adhesion, biofilm formation, acquisition of nutrients and interaction with the host. Several single proteins and families included in pathogenicity were commented in previous section "2. Pathogenesis and virulence factors of *C. albicans*", for example Als, Plb and Sap families, and Hwp1. Another family is the pH response (*PHR*) family composed by the homologous *PHR1* and *PHR2* genes. The expression of *PHR1* and *PHR2* depends to the pH conditions, with *PHR1* being expressed at neutral or basic pH and *PHR2* being expressed at low pH, both required for normal morphology and virulence (Fonzi 1999).

The other class of proteins, named **Pir** (Proteins with integral repeats) are linked directly to the β-1,3-glucan. They have a role in cell wall architecture and rigidity. *PIR1* expression increased during the protoplast regeneration and *PIR32* is upregulated in response to macrophage interaction (Martinez et al. 2004, Fernández-Arenas et al. 2007). Furthermore, the homozygous null mutant of *PIR32* showed increased virulence in a mouse model of disseminated candidiasis, stress response and cell wall chitin deposition (Bahnan et al. 2012).

Among the **noncovalently** attached **CWP**, Bgl2 is the major β-1,3-glycosyltransferase and it is involved in cell wall biogenesis (Sarthy et al. 1997). Mp65 is a cell surface mannoprotein that is induced during cell wall regeneration and it is required for hyphal morphogenesis and experimental pathogenicity (Gomez et al. 1996, Castillo et al. 2006, Sandini et al. 2007). Also, Sap and Plb families, have been detected in the cell wall. These proteins have the capacity to hydrolyze substrates for their nutrition and they also function as virulence factors (Chaffin 2008, Mayer et al. 2013, Pericolini et al. 2015). All of these noncovalently attached CWP have been shown to function as structural and nutrient acquisition proteins (Chaffin 2008, Free 2013). In addition, these proteins share the property of having an N-terminal signal peptide and pass through the classical

secretory pathway before being delivered to the cell wall space. However, many proteins detected on the surface of *C. albicans* lack this signal peptide and are often referred to "nonconventional" cell wall proteins. The set of "cytosolic" proteins identified in cell wall preparations include enolase (Eno1), glyceraldehyde-3-phosphate dehydrogenase (Tdh3), and heat shock proteins such as Hsp70 (Angiolella et al. 1996, Gil-Navarro et al. 1997, de Groot et al. 2004, Ebanks et al. 2006, Castillo et al. 2008, Chaffin 2008).

3.1.1. Ecm33

Different proteomic approaches have been used in order to obtain a comprehensive and integrated view of the *Saccharomyces cerevisiae* and *C. albicans* cell wall. The analysis of proteins secreted during *S. cerevisiae* protoplast regeneration allowed the identification of a protoplast-secreted protein (Pst1) that leads to the identification of *C. albicans* homolog (Pardo et al. 1999, Pardo et al. 2000, Monteoliva et al. 2002). Other three *S. cerevisiae* proteins showed similarity to Pst1 (*ECM33*, *SPS2* and *YCL048W* gene products), but only *ECM33* deletion affects the cell wall integrity under the growth conditions analyzed, resulting in hypersensitivity to cell wall perturbing agents and increased amount of β-1,6-glucan-linked proteins secreted to the extracellular medium. However, the deletion of its homolog *PST1* did not show these effects, although the effects in the double deletion *ECM33/PST1* strain were increased (Pardo et al. 2004). Both deletions resulted in a higher phosphorylation of the Slt2 protein, the MAP kinase involved in regulating maintenance of cell wall integrity.

In *C. albicans*, several studies of Ecm33 protein have tried to elucidate the function of the protein in the cell. In *C. albicans*, Ecm33 is a GPI-CWP member of a three-gene family that includes *ECM33*, *ECM331* and *C1_13290W*. Its role in cell wall remodeling, cell wall maintenance and cell biogenesis was observed in the *ecm33* deleted mutant, RML2U (Martínez-Lopez et al. 2004). Electron microscopy imaging of the RML2U strain showed an abnormal electron-dense outer mannoprotein layer, evidencing an abnormal wall structure that was supported by the aberrant surface localization of the adhesin Als1 (Martinez-Lopez et al. 2006). In addition, it showed defects to produce hyphae and media invasiveness on solid medium and it was delayed in liquid medium (Martínez-Lopez et al. 2004). The adherence and invasion capacity to endothelial and FaDu oral epithelial cell lines was also reduced in the mutant strain and it is less virulent

in the murine systemic infection model, indicating a role of Ecm33 in virulence. In addition the vaccination of mice with the RML2U strain protected them from a lethal infection with virulent strain SC5314 in a systemic candidiasis model (Martínez-Lopez et al. 2008). In the same work, the analysis of cell surface proteins (surfome) showed that RML2U exposed a larger number of proteins to the environment than the wild type, and proteins exclusively identified in this strain were also detected as immunogenic, supporting the idea that their surface localization enhances their immunoprotective capacity.

These observations support the importance of Ecm33 for normal adherence and for the interactions with host cells. However, there are no studies that determine the function of the Ecm33 cell wall protein in the human pathogen *C. albicans*.

3.2. Extracellular secretion

The secretion of proteins are important for the commensal to pathogenic change because they are necessary for their adaptation to the environment and host, and they help to invade and evade host defenses by the secretion of hydrolytic enzymes. These secreted proteins are involved in different vital processes such as nutrient acquisition or cell wall integrity, and other virulence-related processes, such as tissue invasion, immune evasion or biofilm formation. Protein secretion in yeast follows the classical secretory pathway which involves the conventional endoplasmic reticulum-*trans*-Golgi network-plasma membrane route, in which a coordinated network of intracellular vesicles transport promote vesicular fusion with the plasma membrane and release of cargo to the extracellular space (Figure 8) (Schekman 2010).

Apart from hydrolytic enzymes, mentioned in section "2.5. Secreted hydrolases", other proteins involved in cell wall protein, such as the exoglucanase Xog1 and the endoglucanase Eng1, are also secreted to the medium. The core set of seven secreted proteins detected in different studies under different growth conditions encloses Cht3, Mp65, Scw11, Sim1, Sun41, Tos1 and Xog1, that are responsible for maintaining cell wall integrity and wall remodeling (Sorgo et al. 2013). These proteins are secreted by the classical protein secretory pathway because they have an amino-terminal signal peptide responsible for directing them to the endoplasmic reticulum, and then redirection to the Golgi apparatus and transportation to the plasma membrane or the

extracellular region through a complex system of internal vesicles. Xog1 is a glycosidase responsible for the major exoglucanase activity in *C. albicans.* Besides Cht3, other two chitinases have been detected in the extracellular media, Cht1 and Cht2. Cht2 is a GPI-anchored protein, whereas Cht1 and Cht3 are non-GPI-proteins. The cell surface mannoprotein Mp65 and Tos1, are both abundant secreted proteins under all conditions examined. Besides these proteins, *C. albicans* also secretes proteins to sequester metal ions, such as zinc (Pra1 and Zrt1) or iron (Csa1, Csa2, Pga7, Pga10 and Rbt5).

However, proteins without a signal peptide have been detected in the extracellular environment of *C. albicans* and other yeast species (Chaffin et al. 1998, Nombela et al. 2006, Nickel 2010). Most of these unconventionally secreted proteins are determined as intracellular proteins and some of them have been described as "moonlighting" or multifunctional proteins, meaning that they are capable of performing dual or multiple functions, in some cases depending on their location in the cell (Jeffery 1999, Nombela et al. 2006, Chaffin 2008). These proteins lacking the N-linked signal peptide should use alternative routes of exporting, including vesicular pathways (Figure 8).

One of the unconventional secretory mechanisms is mediated by the extracellular vesicles (EVs), recently characterized in fungi and extensively studied in mammalian cells (Raposo and Stoorvogel 2013, Rodrigues et al. 2013). However, the presence of a cell wall in fungi makes it different from other eukaryotic cells. All the fungal species examined are able to use vesicular mechanisms to transport proteins across the cell wall, which lack secretion signals and have cytoplasmic origin. The mechanism of vesicles biogenesis remained unknown as well as the pathway used by them to traverse plasma membrane and cell wall. The use of transmission electron microscopy (TEM) to observe the fungal EVs unravels different vesicles sizes and types in the culture medium of several fungi including *C. albicans* (Albuquerque et al. 2008). Moreover, suggestive EVs formation was observed by this methodology in the membrane of *S. cerevisiae* cells (Rodrigues et al. 2013). Based on these observations, the fungi plasma membrane invagination englobes intracellular components forming an EV. This is completely compatible with the proteomic analysis of the fungi EVs where cytoplasmic proteins lacking secretory tags are a higher proportion of the proteins detected (Albuquerque et al. 2008, Rodrigues et al. 2008, Oliveira et al. 2010, Vallejo et al. 2012, Rodrigues et al.

2014). Proteomic analysis of the content of EVs from several fungal species reveals similar protein composition that includes cytoplasmic, mitochondrial, vacuolar, cell wall

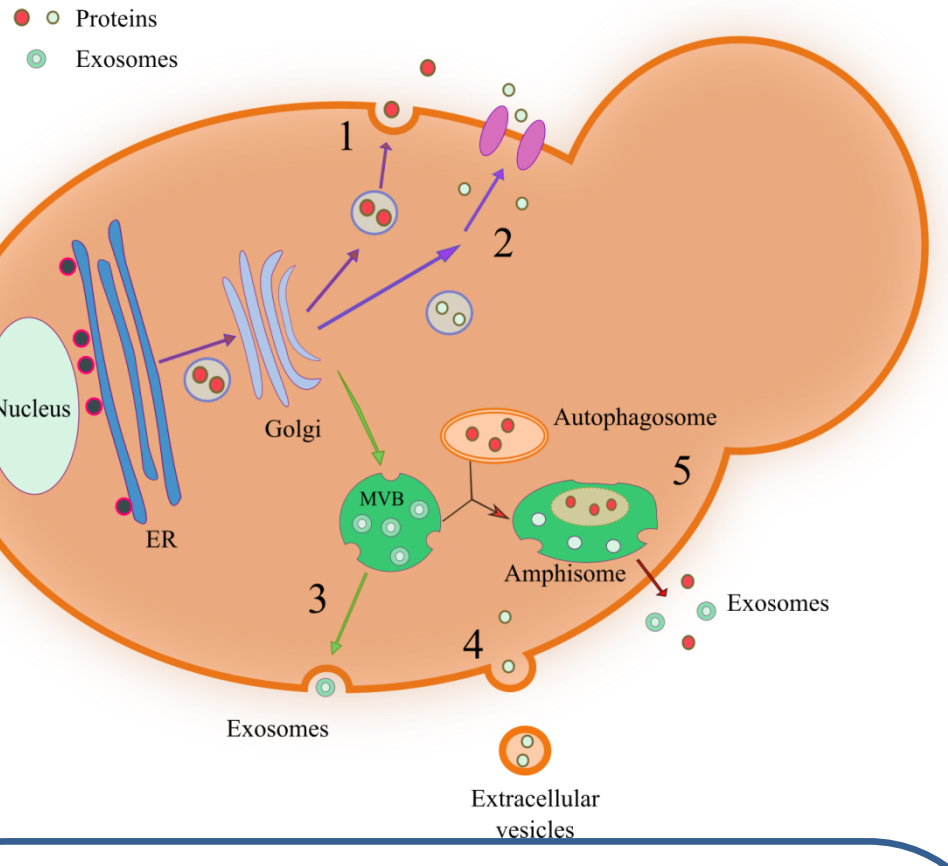


Figure 8. Conventional and unconventional protein secretion pathways proposed in yeast. The numbers denote the names of the protein transport pathways. **1**: Classical secretory pathway from proteins with signal peptide. **2**: Unconventional transport of the α-pheromone peptide specifically driven by the transporter Ste6. **3**: Other Golgi-independent pathway involves the formation of internal vesicles in the lumen of endosomes that generates the multivesicular bodies (MVB), which can fuse with the plasma membrane, resulting in the release of internal vesicles to the extracellular space as exosomes. **4**: Soluble cytoplasmic proteins are captured during microvesicle shedding from the cell surface. **5**: Pathway that connects the unconventional protein secretion and autophagy. Autophagosomes fuse with multivesicular bodies to form so-called amphisomes, which fuse with the plasma membrane, releasing exosomes and other proteins. Data obtained from (Nombela et al. 2006, Nickel and Rabouille 2009, Oliveira et al. 2010, Ding et al. 2012, Miura and Ueda 2015).

architecture, plasma membrane, signaling and virulence related proteins. After the initial description and further confirmation of the existence of EVs in all of these fungal species including *C. albicans*, and together with secretome proteomic data, in which about one third of the extracellular proteins detected in its growth culture does not possess a secretion signal, this alternative mechanism of secretion could be considered a logical explanation for cytoplasmic proteins in the extracellular media.

4. Proteomic technology applied to the advance in the knowledge of *C. albicans*

The term "proteome" was coined by Mark Wilkins in 1995 and it refers to the entire set of proteins, produced or modified by an organism or a cellular system which vary with time and different cell states. The large-scale comprehensive study of a specific proteome is named "proteomics" which goal is the qualitative and quantitative description of protein expression and its changes under different biological conditions in order to understand cellular processes. This term usually is used to enclose all of the technology currently available to analyze global patterns of protein expression. Proteomics is an interdisciplinary field emerging from the overall level of intracellular protein composition.

Genomics is the study of the entire DNA sequence of organisms and fine-scale genetic mapping. There is only one definitive genome of an organism. This genome codes for multiple proteomes, since the accumulation of protein changes in response to the environment is the result of a combination of transcription, translation and post-translation modifications. Proteomics gives biological information that cannot be obtained through DNA analysis, such as the subcellular localization of the protein, if it is post-translationally modified, its relative abundance or its possible interaction with other proteins (Patterson and Aebersold 2003). The combined application of advanced techniques to resolve, identify, quantify and characterized proteins, combined with bioinformatics tools to store and interlink protein information is the goal of proteomics to study complex biological phenomena.

The field of proteomics has grown in the last few years, mainly due to the improvements in the accuracy, sensitivity, speed and throughput of mass spectrometry

(MS), and the development of analytical software. Proteome identification can be divided in several steps that include sample preparation, sample fractionation/separation and finally MS analysis. The accuracy and care in the sample preparation is vital to avoid sample contamination, which often leads to false positives, reduced quantification fidelity and unreliable identification results. Proteomics is based in different types of procedures for protein separation and identification. Within protein separation techniques, proteomics approaches can be classified in two important methods (Monteoliva and Albar 2004, Abdallah et al. 2012):

1. Gel-based methods: Two-Dimensional Gel Electrophoresis (2-DE). Introduced in 1975 (O'Farrell 1975), it became the most used technique of protein separation. Protein first undergo isoelectric focusing (IEF) based on their net charge at different pH values and in the second dimension further separation is performed based on the molecular weight (MW). It is possible to visualize over 10,000 spots corresponding to over 1,000 proteins. Its limitations include limited reproducibility, poor representation of low abundant proteins or difficulties in automation of the technique (Gygi et al. 2000, Tonge et al. 2001, Lilley et al. 2002). Separated proteins have to be enzymatic digested (usually with trypsin) in order to be identified in the next step.
2. Gel-free methods. Liquid chromatography (LC) connected with the mass analysis capabilities of MS: LC-MS. LC is able to separate proteins or peptides according to their differences in physical and chemical properties, first by an ion exchange column and then the eluted fractions are separated in reverse phase microcolums (Gao et al. 2010).

Protein identification of digested proteins is performed by MS, the most common method for large-scale protein identification (Patterson 1998). The MS allows for protein identification, rapid posttranslational modification analysis, identification of components in complex mixtures, and direct mass analysis of gel-separated proteins. It is able to produce and separate ions according to their mass-to-charge ratio (m/z). The MS consist of three elements: an ion source, a mass analyzer and a detector. In the first element, the sample is ionized, causing charged fragments (ions). Among the diverse ionization sources only two produce peptide and protein ions efficiently: electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI). ESI ionizes the analytes

out of a solution and it is therefore readily coupled to liquid-based separation tools. MALDI sublimates and ionizes the samples out of a dry, crystalline matrix via laser pulses (Aebersold and Mann 2003). Then, ions are then separated according to their spatial trajectories, velocity and/or direction by electric or magnetic fields generated inside the mass analyzer. There are four different types of mass analyzer that can be used: quadrupole (Q), Fourier transform ion cyclotron (FT-MS), time-of-flight (TOF) and ion trap (IT) (Kicman et al. 2007). These analyzers can be stand alone or put together in tandem to take advantage of the strengths of each. MALDI is usually coupled to TOF analyzers, whereas ESI has mostly been coupled to ion traps and triple quadrupole instruments. MS produces a mass spectrum that consists in a plot of ion abundance versus its mass-to-charge ratio from the sample. Mass spectrometry data analysis is specific to the type of experiment producing the data. Two strategies have been developed to identify the proteins: the Peptide Mass Fingerprinting (PMF) and the analysis of the resulting masses of the fragmented peptide. In PMF the peptide masses of digested proteins are compared to a database containing known protein sequences. The computer programs (search engine) translate the known genome of the organism into proteins, then theoretically cut the proteins into peptides and calculate the absolute masses of the peptides from each protein. Then the masses of the peptides of the unknown protein are compared to the theoretical peptide masses of each protein encoded in the genome. The results are statistically analyzed to find the best match (Pappin et al. 1993). In the analysis of the resulting masses of fragmented peptides, the interpretation of fragmentation spectra allow the identification of protein fragments by comparing them with theoretical fragmentation spectra obtained by the search engines from protein databases, or by "*de novo*" peptide sequencing which is the analytical process that derives a peptide´s amino acid sequence from its MS/MS spectrum without the assistance of a sequence database. This last strategy is the only possibility if the sequence of the organism to study is unknown or there is no homologue organism (Patterson and Aebersold 1995). Following the acquisition of the data, bioinformatic analyses are necessary. Figure 9 summarizes the proteomics workflow from the sample to the results.

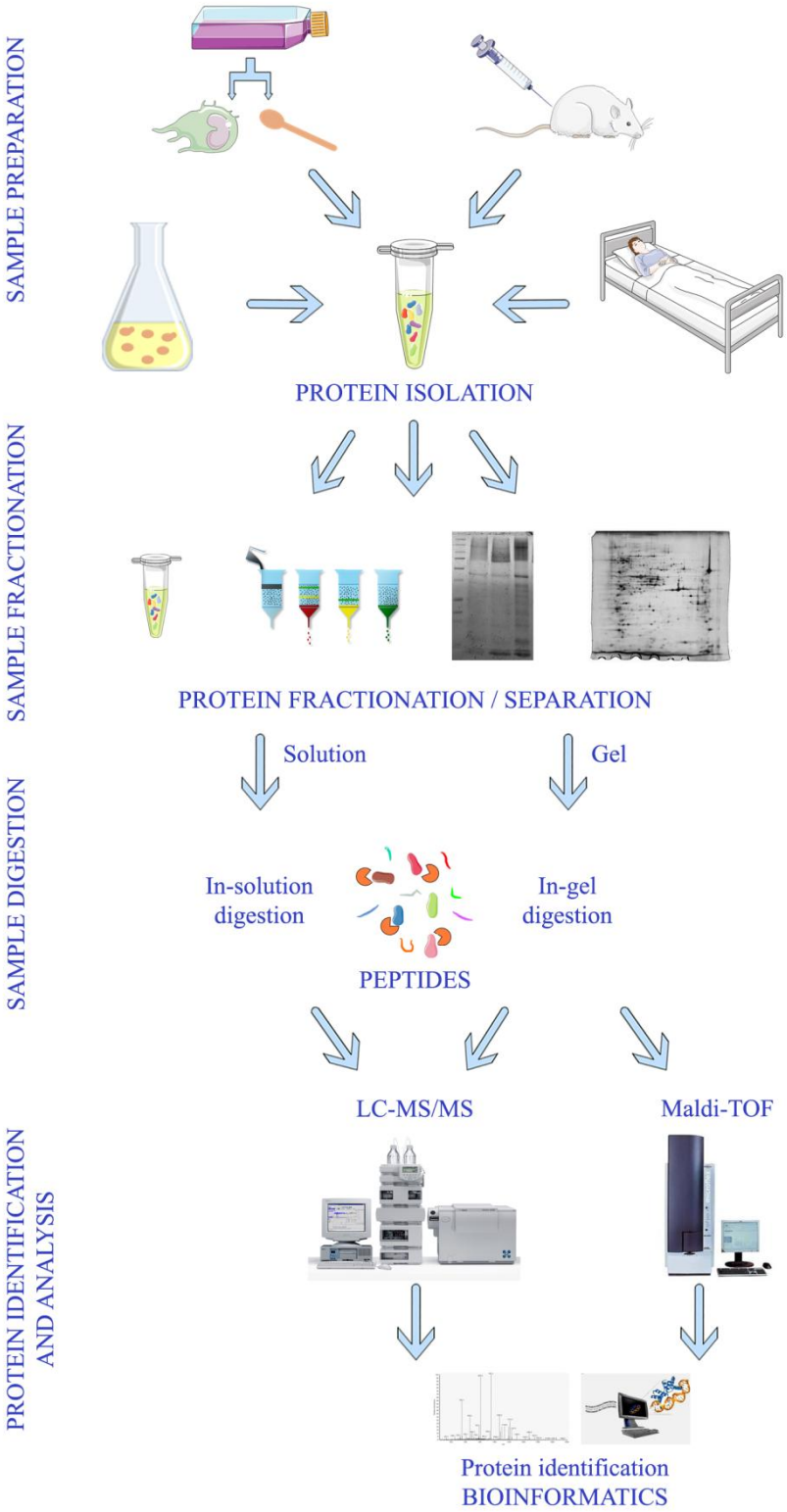


Figure 9. Proteomics workflow. Schematic diagram of proteomic analysis.

Through the application of proteomics to the study of *C. albicans*, new insights into the biology and pathogenicity of this opportunistic fungus are gradually coming out. The availability of the complete diploid genome sequence of *C. albicans* has significantly enhanced the application of proteomics for the study of the behavior of the yeast and the host-fungus interaction. To date, proteome analysis of *C. albicans* has focused on the understanding of different aspects of its biology and:

- **Virulence factors:** the pathogenicity of *C. albicans* is attributed to several virulence factors, as commented in the corresponding section. There are several works analyzing *C. albicans* proteins involved in biofilm formation (Martinez-Gomariz et al. 2009) and protein secretion (Thomas et al. 2009, Sorgo et al. 2010, Ene et al. 2012).
- **The structure and composition of the fungal cell wall and cell surface**. The fungal cell wall mediates the interactions between the fungus and its environment linked to different aspects of its pathogenicity. Diverse proteomic studies have focused on the analysis of *C. albicans* cell wall proteins, in view of the fact that these constitute the major antigens and host recognition molecules (Pitarch et al. 2002, de Groot et al. 2004, Castillo et al. 2008, Hernáez et al. 2010). Even more, proteomic analyses of *C. albicans* proteins secreted from protoplast in active cell wall regeneration provided insights into the protein framework related to cell wall biogenesis (Pitarch et al. 1999, Pitarch et al. 2006).
- **Dimorphism**. *C. albicans* can exist in different forms, especially as yeast cells, pseudoyphae and true hyphae, depending on the environment conditions. Proteomic techniques have been used in several studies to identify differences in the protein profiles associated with these forms and to create reference maps for their protein components (Hernandez et al. 2004, Monteoliva et al. 2011, Vialas et al. 2012).
- **Drug response**: a reduced number of antifungal drugs are used for candidiasis therapy. These different compounds have different modes of action and some proteomic studies have analyzed the changes in *C. albicans* proteome after the treatment (Bruneau et al. 2003, Sorgo et al. 2011).
- **Host response:** the study of the host response to *Candida* infections can be a very useful tool to discover new therapeutic strategies. This is the goal of several recent works published in which the protein profile of murine-derived and

human macrophages after interaction with *C. albicans* was analized by several proteomic strategies, such as quantitative proteomics, phosphoproteomics, 2-DE and SILAC (Martínez-Solano et al. 2006, Martínez-Solano et al. 2009, Reales-Calderon et al. 2012, Reales-Calderon et al. 2013, Reales-Calderon et al. 2014). In addition, the research using proteomic strategies with serum of patients or in a murine model systemic candidiasis, detected a high number of immunogenic proteins (Pitarch et al. 2001, Pitarch et al. 2004, Pitarch et al. 2006).

All of these proteomic results contribute to the dissection of the molecular mechanisms by which *C. albicans* interacts with the environment and how the host copes with it. It can pave the way for the advancement in novel diagnostic strategies and even for the development of antifungal drug or vaccine design.

Aims of the thesis

The overall goal of this thesis was to deepen the knowledge concerning the surface and secreted proteins of *C. albicans* that play important roles in virulence and pathogenesis, because they are the first to come into contact with the host.

The specific aims of the present study are:

1. The analysis of the function and importance in virulence of 17 proteins of unknown function identified at the cell surface of the *C. albicans* by the phenotypic analysis of the corresponding deletion mutants.

2. The proteomic study of the *C. albicans* SC5314 secretome including extracellular vesicles and extracellular vesicle-free secretome.

3. The proteomic analysis of *C. albicans ecm33* mutant (RML2U) secretome including extracellular vesicles and extracellular vesicle-free secretome and the comparative analysis with the SC5314 data.

4. The analysis of the function of Ecm33 cell wall protein in *C. albicans* by an extensive phenotypic analysis of the RML2U mutant.

Objetivos

El objetivo general de este trabajo es profundizar en el conocimiento de las proteínas localizadas en la superficie celular, así como de las proteínas secretadas por *C. albicans*, ya que ambas tienen un papel importante en virulencia y patogénesis, debido a que son el primer punto de contacto con el hospedador.

Los objetivos concretos de este trabajo son los siguientes:

1. Análisis de la función e implicación en virulencia de 17 proteínas identificadas en la superficie de *C. albicans* con función desconocida mediante el análisis fenotípico de los mutantes delecionados en los genes correspondientes.

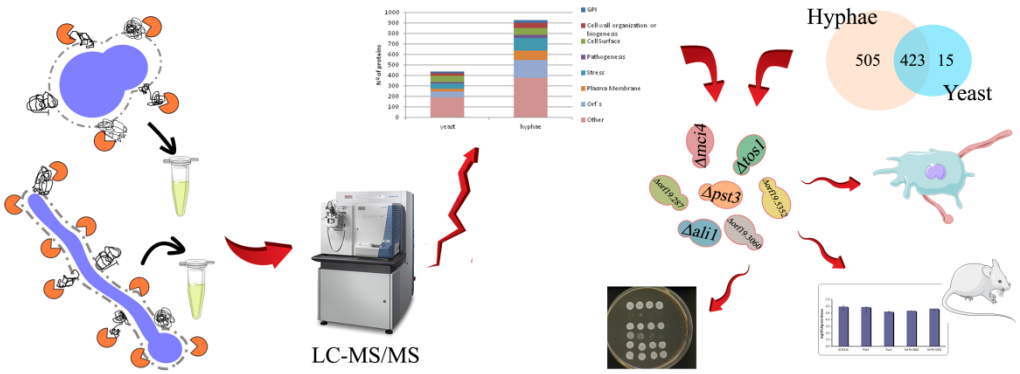
2. Estudio proteómico del secretoma de la cepa SC5314 de *C. albicans* incluyendo las vesículas extracelulares y el secretoma libre de vesículas.

3. Estudio proteómico del secretoma del mutante *ecm33* de *C. albicans* (RML2U) incluyendo las vesículas extracelulares y el secretoma libre de vesículas y su análisis comparativo con los datos de la cepa SC5314.

4. Análisis de la función de la proteína de pared celular Ecm33 de *C. albicans* mediante un análisis fenotípico exhaustivo del mutante RML2U.

Chapter 1:

Candida albicans cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host-pathogen interaction



*In this chapter, the participation of Gil-Bona includes part of the proteomic data analysis to select the proteins of study and the phenotypic and virulence analysis of the mutants of the interesting identified proteins

Journal of Proteomics 127 (2015) 340–351

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Candida albicans cell shaving uncovers new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host–pathogen interaction☆

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 25 May 2015
Accepted 9 June 2015
Available online 15 June 2015

Keywords:
Candida albicans
Trypsin digestion
Surface proteins
Cell wall integrity
Osmotic stress
Oxidative stress

ABSTRACT

The ability to switch from yeast to hyphal growth is essential for virulence in *Candida albicans*. The cell surface is the initial point of contact between the fungus and the host. In this work, a free-gel proteomic strategy based on tryptic digestion of live yeast and hyphae cells and protein identification using LC–MS/MS methodology was used to identify cell surface proteins. Using this strategy, a total of 943 proteins were identified, of which 438 were in yeast and 928 were in hyphae. Of these proteins, 79 were closely related to the organization and biogenesis of the cell wall, including 28 GPI-anchored proteins, such as Hyr1 and Sod5 which were detected exclusively in hyphae, and Als2 and Sap10which were detected only in yeast. A group of 17 proteins of unknown function were subsequently studied by analysis of the corresponding deletion mutants. We found that four new proteins, Pst3, Tos1, Orf19.3060 and Orf19.5352 are involved in cell wall integrity and in *C. albicans*' engulfment by macrophages. Moreover, the putative NADH-ubiquinone-related proteins, Ali1, Mci4, Orf19.287 and Orf19.7590, are also involved in osmotic and oxidative resistance, yeast to hypha transition and the ability to damage and invade oral epithelial cells. This article is part of a Special Issue entitled: HUPO 2014.

1. Introduction

The cell wall is the most external structure in fungi, confers organization and form to the cell and protects the organism from physical and osmotic damage. Surface proteins play an important role in the pathogenic process as they are the initial point of contact between the cell and the environment. *Candida albicans* is an important opportunistic fungus that causes a wide variety of diseases in patients, ranging from the superficial mucocutaneous candidiasis (affecting the nails, skin, and oral and genital mucosae) to life-threatening disseminated infections [1,2]. In patient in critical care, candidemia is the most important fungi disease with a 30% mortality rate [3]. The *C. albicans*' cell wall maintains structural integrity and acts as intermediate between the cell and the environment. As the initial point of contact with host cells, the cell wall is an obvious target for development of antifungals and vaccines. It is composed of β-1,3-glucan, β-1,6-glucan, a small percentage of chitin and different wall proteins, most of them covalently attached to β-1,6-glucan linkage through a remnant of glycosylphosphatidylinositol (GPI) anchors [4–6]. The non-glucan-linked proteins traffic to the cell surface by either the classical or alternative secretory pathway [7,8]. These cell wall proteins maintain structural integrity, mediate adherence and/or invasion of host cells, or function as enzymes [5,8]. One of these proteins is Ecm33p, a GPI-linked cell wall protein whose absence affects both yeast cells and hyphal morphology and results in an aberrant wall structure and reduced virulence *in vitro* and *in vivo* [9,10]. Another GPI-linked cell wall protein is the secreted yeast wall protein Ywp1p, which is covalently linked to glucans of the wall matrix and has the highest expression during yeast exponential growth. The *ywp1*Δ/Δ mutant has increased adhesiveness and biofilm formation but no obvious change in growth, morphology or virulence, suggesting that Ywp1p promotes dispersal of yeast form cells in *C. albicans* [11]. Pir proteins (proteins with internal repeats) are an additional group of *C. albicans*' cell wall proteins and are linked directly to β-1,3-glucan [12,13]. *PIR1* is an essential gene and its abundance changes in response to environmental conditions [13–15]. Among no covalent attachment proteins, Bgl2p is involved in cell wall biogenesis [16]. It is the major β-1,3-glycosyltransferase and *bgl2*Δ/Δ mutants have attenuated virulence in mice. Furthermore, Bgl2 is recognized by IgG antibodies from patients with invasive candidiasis, which has diagnostic and prognostic usefulness [17]. Some

☆ This article is part of a Special Issue entitled: HUPO 2014.

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http://dx.doi.org/10.1016/j.jprot.2015.06.006
1874-3919/

secreted proteins, such as secreted aspartyl proteinase (SAP) and phospholipase B (PLB) families, must pass through the cell wall and have been detected there [18]. These proteins are hydrolytic enzymes which enable the organism to break down proteins for nutrition, but their relative contribution to *C. albicans*' pathogenicity is controversial [19–23]. In addition, many proteins identified on the surface of *C. albicans* lack classical secretion signal peptides and are dual function proteins, that function as enzymes or chaperones in the cytoplasm and as adhesins, invasins, or immunogens when expressed on the cell surface. These proteins include glyceraldehyde-3-phosphate dehydrogenase (Tdh3), enolase (Eno1) and heat shock proteins such as Hsp70 [24–26].

C. albicans is able to grow in different morphological forms. The ability to switch between yeast and hypha is necessary for virulence [23,27,28]. Both morphological forms are important during infection. The yeast form probably disseminates *via* the bloodstream, spreading the organism to different host niches, while the hyphal form is invasive and enables the organism to evade phagocytic cells [29,30]. Consequently, *C. albicans* expresses distinct cell surface proteins in these stages.

The study of cell surface protein composition of yeast and hypha morphologies and their differences will help to find novel therapeutic targets. In recent years, the response of the cell wall proteome to changes in ambient pH and with respect to yeast to hyphal transition has been investigated [31–35]. These classical proteomic approaches involve several steps based on subcellular fractionation which are time-consuming and laborious. Hernáez et al. [36] and Vialás et al. [35] used a proteomic strategy based on cell shaving of extracellular peptides to identify surface proteins in *C. albicans* yeast and hyphae forms, using Nano-LC followed by off-line MS/MS for peptide separation and identification. By this method, many novel surface proteins were identified that had not previously been reported as being on the cell surface. These proteins included some with unknown functions and aerobic respiration-related and ribosomal proteins, such as Rpl15A, Rps16A and Rps4A. In the present work, growing yeast and hypha cells were analyzed using the same strategy and more sensitive separation and identification equipment, enabling the identification of a larger number of proteins in each sample. In addition, a phenotypic analysis *in vitro* and/or *in vivo* of mutants with undescribed function of 17 identified proteins was performed to investigate their role in cell wall biogenesis, stress and virulence.

2. Materials and methods

2.1. Strains and growth conditions

C. albicans SC5314 [37] was used as wild type in this work. *C. albicans* mutant strains used in the *in vitro* and *in vivo* phenotypic studies were acquired from Noble collection [38] stored in the Fungal Genetics Stock Center (Kansas City, Missouri USA) [39]. *C. albicans* strains were maintained on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar plates at 30 °C. Yeast cells were pre-cultured in liquid YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) in a rotary shaker at 200 rpm and 30 °C overnight. For the cell surface study, yeast cells were collected at an OD of 0.6 ± 0.2, which corresponds to logarithmic growth phase, and washed. A total of 5×10^6 cells/mL were resuspended in Lee's medium [40] (0.2 g/l magnesium sulfate, 2.5 g/l dipotassium phosphate, 5 g/l sodium chloride, 5 g/l ammonium sulfate, 0.5 g/l L-alanine, 1.3 g/l Leucine, 1 g/l L-lysine, 0.1 g/l L-methionine, 0.07 g/l L-ornithine, 0.5 g/l L-phenylalanine, 0.5 g/l L-proline, 0.5 g/l L-threonine, 25 ml/l 50% glucose and 1 ml/l 0.1% biotin) at two different pHs, pH 4.2 for yeast growth and pH 6.7 for hyhae growth, and incubated at 37 °C for 6 h. At the end of the incubation period, cells were analyzed by light microscopy showing yeast shape at pH 4.2 and *C. albicans* hyphae at pH 6.7. To determine the generation time, strains were cultured in liquid YPD at 30 °C with OD_{600} 0.1. Absorbance of the cultures was measured at 600 nm every hour. Linear regression was used to calculate specific growth rate and generation time. For phagocytosis and cytotoxicity assays, RAW 264.7 murine macrophages were cultured in RPMI 1640 medium supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml), L-Glutamine (2 mM) and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. The FaDu oral epithelial cell line was obtained from the American Type Culture Collection and maintained in Eagle's minimum essential medium with Earle's balanced salt solution (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM pyruvic acid 2 mM L-glutamine and 0.1 mM non essential amino acids.

2.2. Surface shaving

For surface protein identification, Hernáez et al. [36] method was used. Briefly, after 6 h of culture under different conditions, cells were collected and washed several times. Subsequently, cells were resuspended in 800 μl of 25 mM ammonium bicarbonate buffer (pH 8.0). A total amount of 5 μg of trypsin (Roche) in the presence of 0.1 mM DTT was added to the cell suspensions. After incubation at 37 °C for 5 min in a rotary shaker at 600 rpm, proteolytic reactions were stopped by adding 0.1% trifluoroacetic acid (TFA) v/v. Samples were centrifuged and supernatants were collected. The peptide supernatants were filtered through 0.22 μm pore-size filters (Millipore). Cell lysis was controlled by propidium iodide (PI) staining using flow cytometry in case of yeast and by fluorescence microscopy in case of hyphae. Before mass spectrometry analysis, the peptide supernatants were cleaned up with a microcolumn filled with Poros 50 R2 packing (PerSeptive Biosystems). Peptides were eluted with 80% acetonitrile (ACN) in 0.1% TFA, dried in a Speed-vac and resuspended in 0.1% formic acid. The samples were stored at −20 °C before nano-LC–MS/MS analysis.

2.3. LTQ-Orbitrap Velos analysis and protein identification

Prior to nano-LC–MS/MS analysis, all peptide samples were purified and desalted using C18-A1 ASY-column 2 cm pre-column (Thermo Scientific) and then eluted onto a Biosphere C18 column (Nano-Separations). Peptides were separated with a 140 min gradient (110 min from 0 to 40% Buffer B; Buffer A: 0.1% formic acid/2% acetonitrile; Buffer B: 0.1% formic acid in acetonitrile) at a flow-rate of 250 nl/min on a nano-Easy HPLC (Proxeon) coupled to a nano-electrospray ion source (Proxeon). Mass spectra were acquired on the LTQ-Orbitrap Velos (Thermo Scientific) in the positive ion mode. Full-scan MS spectra (m/z 400/1400) were acquired in the Orbitrap with a target value of 1,000,000 at a resolution of 60,000 at m/z 400 and the 15 most intense ions were selected for collision induced dissociation (CID) fragmentation in the LTQ with a target value of 10,000 and normalized collision energy of 38%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 s.

2.4. Protein identification and analysis

Protein identification from raw data was carried out using a licensed version of search engine MASCOT 2.3.0 with Proteome Discoverer software version 1.4.1.14 (Thermo Scientific). A database search was performed against the CGD21 database (6221 sequences). Search parameters were oxidized methionine as variable modification, peptide mass tolerance 10 ppm, 1 missed trypsin cleavage site and MS/MS fragment mass tolerance of 0.8 Da. In all protein identification, the FDR was <1%, using a Mascot Percolator [41], with a q-value of 0.01. As an estimation of the relative protein abundances the normalized spectral abundance factor (NSAF) was used [42], and the average of the normalized values was calculated. The MS output files have been submitted to PeptideAtlas through the PeptideAtlas Submission System (PASS) with identifier PASS00446.

2.5. Low Temperature Scanning Electron Microscopy (LTSEM)

Untreated *C. albicans* cells were mechanically fixed onto the specimen holder of a cryotransfer system (OxfordCT-1500), plunged into subcooled liquid nitrogen, and then transferred to the microscope's preparation unit *via* an air-lock transfer device. The frozen samples were cryofractured and etched for 2 min at −90 °C. After ice sublimation, etched surfaces were gold sputter coated and the specimens were then placed on the cold stage of the SEM chamber. Fractured surfaces were observed under a DSM960 Zeiss SEM microscope at −135 °C.

2.6. Assays to test susceptibility to stressors

C. albicans wild type and mutant strains were grown overnight in liquid YPD medium at 200 rpm and 30 °C. Yeast suspensions were adjusted at OD_{600}0.8 and serial 5 μl of 10-fold dilutions were spotted on YPD agar plates. For heat-shock stress assay the plates were incubated at 30 °C, 37 °C, 42 °C and 45 °C. For treatment with cell wall-, osmotic- and oxidative-perturbing agents, the plates were supplemented with Calcofluor white (28 μg/ml), Congo red (175 μg/ml), sorbitol (1.5 M), KCl (1.2 M) and menadione (0.2 mM). Identical plates of Cell wall-perturbing agents were prepared supplemented with 1 M Sorbitol. The wild-type and mutant strains were assayed for ability to withstand lethal dose of hydrogen peroxide. Strains were grown at 0.8 OD at 600 nm on 5 ml YPD liquid media containing hydrogen peroxide to a concentration of 100 mM incubated at 30 °C for 90 min. Five μl of each culture was spotted on YPD plates at different periods of time: 0, 2, 5, 10, 15, 30, 60 and 90 min. To assay the utilization of the polyol mannitol as a carbon source, strains were spotted onto mannitol agar plates (20 g/l mannitol, 5 g/l ammonium sulfate, 1.7 g/l nitrogen base and 2.2 g/l amino acids mix). After incubation at 30 °C for 24–48 h, the plates were imaged.

2.7. Filamentation assays

For invasive and filamentous growth on solid media, strains and wild-type were plated onto Spider agar (2% nutrient broth, 2% mannitol, 0.8% K_2HPO_4, 5.4% Bacto Agar) and YPD plates supplemented with 10% fetal bovine serum at a density of 30 CFUs/plate. Spider plates were incubated at 30 °C and 37 °C, and 10% fetal bovine serum plates at 30 °C for 7 days.

2.8. C. albicans' phagocytosis assay and cytotoxicity measurement

For phagocytosis assay, macrophages were plated onto 18-mm glass sterile coverslips placed in 24-well plates. *C. albicans* strains were pre-labeled with 1 μM Oregon Green 488 (Molecular Probes) in the dark with gentle shaking (30 °C) for 1 h. Macrophages were confronted with the yeast at a MOI (multiplicity of infection; macrophage/yeast ratio) of 1 at 37 °C and 5% CO_2. Interaction was stopped after 45 min, 1.5 and 3 h and cells were then washed with ice-cold PBS and fixed in 4% paraformaldehyde for 30 min. To distinguish between internalized and attached/non-ingested yeasts, *C. albicans* cells were counterstained with 2.5 M Calcofluor white (Sigma) for 15 min in the dark. The number of ingested cells (green fluorescence) and/or adhered/non-ingested (Calcofluor white blue fluorescence) was quantified by fluorescence microscopy with FITC and UV [43]. Three different replicates with two different slides were prepared for each MOI and time point. At least 400 *C. albicans* cells were scored per slide, and results were expressed as the percentage of yeasts internalized by macrophages. For cytotoxicity measurement, macrophages were co-incubated (in a new complete media without phenol red (pH indicator) to avoid the background in the LDH test) with *C. albicans* strains at a MOI (multiplicity of infection; macrophage/bacteria ratio) of 1 during 3 and 8 h. Staurosporine 5 mM was used as a positive control. After the incubation, LDH was measured with the Cytotoxicity Detection Kit^{PLUS} (Roche) according to the manufacturer's protocol.

2.9. Endocytosis and damage assays

The number of organisms that were endocytosed by FaDu cells was determined using a differential fluorescence assay as described previously [44]. Briefly, 2.5×10^5FaDu epithelial cells were grown on fibronectin-coated glass coverslips placed in a 24-well tissue culture plate and were infected with 10^5 cells of either *C. albicans* in RPMI 1640 medium the next day. After a 90 min incubation, the cells were rinsed once with PBS to remove the organisms that were not cell-associated and then fixed in 3% paraformaldehyde. The non-endocytosed organisms were stained for 1 h with an anti-*C. albicans* rabbit antiserum conjugated with Alexa 568 (Meridian Life Sciences, Inc.). After rinsing with PBS, the FaDu cells were permeabilized in 0.5% Triton X-100 for 10 min, and then the cell-associated organisms (defined as the endocytosed plus non endocytosed organisms) were stained with the anti-*C. alb*icans rabbit serum conjugated with Alexa 488 (Molecular Probes). The coverslips were observed with epifluorescence microscope. The number of organisms' endocyted by the FaDu cells was determined by subtracting the number of cell-associated organisms (labeled with Alexa 568, which fluoresced red) from the total number of organisms (labeled with Alexa 488, which fluoresced green). Organisms that were partially internalized were counted as being endocytosed. At least 100 organisms were counted on each coverslip, and all experiments were performed in triplicate.

The extent of damage caused by the various *C. albicans* strains to the FaDu cell line was measured using a ^{51}Cr release assay as described previously [45]. The host cells were grown in a 96-well tissue culture plate containing detachable wells and incubated overnight with $Na_2^{51}CrO_4$ (MP Biomedicals, Inc., Irvine, CA) per well. The following day, the unincorporated tracer was aspirated and the cells were washed with HBSS and then infected with 10^5 organisms in RPMI 1640. To measure the spontaneous release of ^{51}Cr, uninfected FaDu cells were exposed to medium alone. After 3 h incubation at 37 °C, the upper 50% of medium was removed from each well and then the wells were manually detached from one another. The amount of ^{51}Cr in the aspirates and the well was determined by gamma counting. After correcting for the amount of ^{51}Cr incorporated in each well, the specific release of ^{51}Cr was calculated by the formula: (experimental release − spontaneous release)/(total incorporation − spontaneous release). Experimental release was the amount of ^{51}Cr released into the medium by cells infected with *C. albicans*. Spontaneous release was the amount of ^{51}Cr released into the medium by uninfected host cells. Total incorporation was the sum of the amount of ^{51}Cr released into the medium and remaining in the host cells. Each experiment was performed in triplicate at least three different times.

2.10. Mouse model of oropharyngeal candidiasis

The virulence of the different *C. albicans* strains was assessed using the mouse model of oropharyngeal candidiasis as described previously [46]. Briefly, five male BALB/c mice per strain of *C. albicans* were immunosuppressed by subcutaneous injection with 225 mg/kg of cortisone acetate (Sigma-Aldrich) on days −1, 1 and 3 relative to the day of infection. On the day of infection, each mouse was anesthetized and inoculated sublingually for 75 min with a swabs saturated with 10^6 cells per ml. After 5 days of infection, each mouse was sacrificed, the tongue was excised, weighted, and homogenized, and the number of CFUs was determined. The animal experiments were approved by the Animal Care and Use Committee at the Los Angeles Biomedical Research Institute.

3. Results

3.1. Global analysis of C. albicans yeast and hypha-surface proteins by cell shaving

In this work, a modified and optimized proteomic strategy to identify surface proteins in *C. albicans* cells was followed [35,36]. Live cells were treated with trypsin to digest the cell surfaces. Cell lysis was monitored by flow cytometry and fluorescence microscopy in yeasts and hyphae, respectively, to control for cell lysis after trypsin digestion and avoid intracellular protein contamination. Aliquots of cells were taken from each sample before and after trypsin digestion and tested in each treatment. There was no significant increase in the percentage of PI population after incubation with the trypsin, being always under 0.7%. In addition, the cell surface was imaged by scanning electron microscopy after cryofracture before and after trypsinization. Before digestion, the cell surface was smooth, and it became wrinkled after digestion (Supplemental Fig. S1).

Proteomic analysis of yeast and hyphae was performed with proteins identified in at least two replicates with more than two peptides in each and proteins identified with one peptide in at least three replicates. A total of 438 and 928 proteins were identified in yeast and hypha samples, respectively (Supplemental Tables S1 and S2). The data corresponded to three biological replicates for yeast samples and four biological replicates for hyphal samples. They are deposited in the PeptideAtlas database [47], and information on each protein can be accessed through the CGD.

The identified proteins were classified in eight different categories based on their extracellular localization and their function related to cell wall maintenance, stress resistance or pathogenesis. These categories include GPI-anchored proteins, cell wall organization or biogenesis related proteins, cell surface proteins, proteins involved in pathogenesis or stress response, plasma membrane proteins, open reading frames (ORFs) and other identified proteins (Table 1). The Orfs are proteins that are unnamed in CGD and include proteins with verified feature type, meaning that there is experimental evidence for the existence of a gene product, or uncharacterized, without experimental evidence. The 'other identified proteins' category includes proteins with no relationship to the other categories above (Table 1). In both samples, this category included almost 40% of the proteins identified. Table 1 also includes the number of proteins in each type of sample and the corresponding percentage based on the total number of proteins in the sample. Proteins were classified according to Gene Ontology (GO) terms and the groups were ordered by importance in this work. This categorization is hierarchical and mutually exclusive, meaning that proteins included in one group are not classified again in any of the other groups.

Table 1
Number and percentage distribution of different proteins identified by shaving *C. albicans* SC5314 yeast and hyphae cells. Proteins were classified in the 8 designated protein categories according to Candida Genome Database.

Categories	Yeast		Hypha	
	No. of proteins	%	No. of proteins	%
GPI-anchored	19	4.34	26	2.8
Cell wall organization or biogenesis	23	5.25	50	5.39
Cell surface	60	13.7	64	6.9
Pathogenesis	13	2.97	34	3.66
Stress	53	12.1	119	12.82
Plasma membrane	24	5.48	87	9.38
Orf's	53	12.1	172	18.53
Other identified proteins	193	44.06	376	40.52
Total	438	100	928	100

3.2. Comparative analysis of C. albicans yeast and hyphae-surface proteins

Of the proteins identified, hyphae had slightly more GPI proteins than did yeast (Table 1). The number of cell surface proteins is similar in the two different samples, however looking at the percentage based on the total proteins identified per sample this group is more highly enriched in yeast (13.7%) than in hyphae (6.9%). In the cell wall, pathogenesis and stress response-related identified proteins twice as many of these proteins were identified in hyphae as compared to yeast, while the percentage of proteins in these categories was similar for both morphologies. Moreover, three-fold more proteins in the plasma membrane and Orfs categories were identified in hypha. In addition, the averaged normalized spectral abundance factor (NSAF) [42] of each sample was performed to analyze the relative abundance of the proteins. It was noticeable that in the top 20 proteins of both samples, 14 were present in both yeast and hyphae, with six cell surface proteins (Tdh3, Adh1, Eno1, Pgk1, Pdc11 and Tal1), three stress response-related (Ahp1, Hsp12 and Orf19.4216), one related to pathogenesis (Wh11) and four categorized as other (Rpl38, Rps14b, Rps20 and Tef2) (Table 2). In the case of yeast, cell surface proteins were more abundant (seven) followed by other surface proteins (six), stress-related (four), Orfs (two) and pathogenesis-related (one). The distribution of hyphal was slightly different, with other surface proteins being the most representative with nine proteins, followed by cell surface (seven), stress-related (three) and pathogenesis-related proteins (one).

Venny analysis was carried out to find proteins common to both morphologies and proteins identified exclusively in a cellular morphology. A total of 943 unique proteins were detected. Of these, 423 proteins were present in both yeast and hyphae. The other 520 remaining proteins were found exclusively in yeast (15 proteins) and hyphae (505 proteins). All of these proteins were re-ordered in the eight groups

Table 2
Ranking of the top twenty proteins according to the averaged normalized spectral abundance factor (NSAF) in yeast and hyphal *C. albicans* cells. In gray, proteins that appear in both hits.

Yeast		Hyphae	
Protein	NSAF	Protein	NSAF
Tdh3	0.0230	Tdh3	0.0238
Wh11	0.0213	Adh1	0.0089
Hsp12	0.0171	Eno1	0.0087
Orf19.4216	0.0170	Pgk1	0.0084
Pgk1	0.0127	Wh11	0.0083
Eno1	0.0113	Pdc11	0.0082
Rps14b	0.0102	Rpl38	0.0078
Crd2	0.0098	Rps14b	0.0075
Mp65	0.0094	Tef2	0.0073
Cyp1	0.0089	Orf19.4216	0.0070
Orf19.3690.2	0.0087	Hsp12	0.0070
Pdc11	0.0087	Tal1	0.0066
Tal1	0.0083	Ahp1	0.0065
Glx3	0.0082	Rps1	0.0064
Tef2	0.0074	Rpl14	0.0063
Orf19.6415.1	0.0073	Rps20	0.0062
Rpl38	0.0073	Rps3	0.0061
Rps20	0.0072	Ece1	0.0061
Ahp1	0.0071	Rpl23a	0.0060
Adh1	0.0070	Rps13	0.0059

Table 3
Categorization of proteins identified in *C. albicans* SC5314 surfome of yeast and hyphae. Proteins were classified in the 8 designated categories according to Candida Genome Database (CGD) Gene Ontology (GO) terms (in parentheses the GO identifier): Cell wall organization or biogenesis (71554), cell surface (9986), pathogenesis (9405), response to stress (6950) and plasma membrane (5886). GPI-anchored category is based on Richard et al. [2]. Orf's category refers to proteins without standard name in CGD. Other identified proteins category is composed by proteins not classified in previous sections.

Categories	Common in yeast and hypha	Only identified in yeast	Only identified in hypha	Total
GPI-anchored	17 Als1, Cht2, Crh11, Ecm33, Ihd1, Pga4, Phr1, Phr2, Pir1, Plb4.5, Rbt1, Rbt5, Rhd3, Sap9, Ssr1, Utr2, Ywp1.	2 Als2, Sap10.	9 Als3, Eap1, Hyr1, Pga10, Pga45, Pga52, Pga53, Plb3, Sod5.	28
Cell wall organization or biogenesis	22 Act1, Agm1, Bgl2, Bmh1, Cmk2, Eng1, Gda1, Gfa1, Mnt1, Msb2, Pmi1, Ras1, Rho1, Sim1, Slk19, Sod1, Srb1, Sun41, Sur7, Tsa1, Yps7, Ypt31.	1 Xog1.	28 Arp2, Bud7, Cdc10, Chs5, Clc1, Cwh43, Dck1, End3, Flc2, Gsc1, Hog1, Kex2, Kre9, Lmo1, Mnn26, Pbs2, Pho85, Pmr1, Pmt1, Pmt2, Pmt4. Pop2, Rac1, Rvs161, Rvs167, Smi1, Snf1, Tps2.	51
Cell surface	58 Adh1, Atp1, Atp2, Cdc19, Cdc48, Cef3, Cht3, Coi1, Csp37, Ddr48, Efb1, Eft2, Egd2, Eno1, Fba1, Gpd2, Gph1, Gpm1, Hem13, Hsp104, Hsp21, Hsp70, Hsp90, Ino1, Kar2, Met6, Mp65, Orf19.2478.1, Pdc11, Pdi1, Pgk1, Pma1, Rbe1, Rpl10, Rpl13, Rpl14, Rpl17b, Rpl19a, Rpl20b, Rpl3, Rpl4b, Rpl6, Rps10, Rps6a, Rps7a, Rsp8a, Sam2, Scw11, Ssa2, Ssb1, Ssc1, Ssz1, Tal1, Tdh3, Tkl1, Tos1, Tpi1, Ugp1.	2 Fet99, Pho113.	6 Aip2, Cdr1, Fre10, Rax2, Rbt4, Sdh2.	66
Pathogenesis	12 Ade5.7, Age3, Asc1, Cdc42, Fas2, Msi3, Tfp1, Ttr1, Tup1, Vma2, Wh11, Yhb1.	1 Hex1.	22 Alo1, Cat1, Cdc11, Cla4, Crk1, Csh3, Gna1, Gpa2, Het1, Mlt1, Mts1, Nmt1, Not5, Obpa, Ole1, Rsr1, Slr1, Srv2, Ssd1, Ssn6, Vps27, Ypt72.	35
Stress response	51 Ade1, Ade12, Ade13, Aha1, Ahp1, Atc1, Bcy1, Ccp1, Cdc54, Ded1, Dot5, Erf1, Erg13, Gis2, Glc7, Gln1, Glx3, Gnd1, Gre3, Hsp12, Imh3, Lsp1, Orf19.1340, Orf19.4216, Orf19.4246, Orf19.4622, Orf19.5281, Orf19.5620, Orf19.5943.1, Orf19.6358, Pet9, Pil1, Pol30, Prx1, Rdi1, Rhr2, Rpg1a, Rpl16a, Rpt4, Sbp1, Sgt2, Skp1, Smt3, Sub2, Sui2, Tma19, Trr1, Trx1, Ynk1, Ypt1, Zpr1.	2 Orf19.4150, Orf19.7196.	68 Apm1, Arf2, Bdf1, Bre1, Cct8, Cdc28, Cdc37, Cip1, Crm1, Cta3, Dhh1, Doa1, Erg1, Gea2, Gpd1, Grx3, Hat2, Hbr1, Hrr25, Hsp60, Hta1, Hta2, Lag1, Mcr1, Mxr1, Myo2, Ncb2, Orf19.2265, Orf19.2304, Orf19.239, Orf19.512, Orf19.5833, Orf19.5917.3, Orf19.6250, Orf19.6424, Orf19.7160, Orf19.86, Orf19.933, Osm2, Pob3, Ptc2, Pyc2, Rad23, Rad6, Rfa2, Rfc1, Rgd1, Rli1, Rpo21, Rp6, Sec18, Sec7, She3, Shp1, Sin3, Sis1, Spt5, Spt6, Svf1, Taf14, Tfg1, Tif34, Ura7, Vac8, Yck2, Yim1, Ypt52, Zrt2.	121
Plasma membrane	23 Ade17, Adh2, Cof1, Erg6, Erv25, Faa4, Glk1, Hgt6, Hgt7, Ist2, Mdg1, Met15, Orf19.1564, Orf19.6160, Orf19.6553, Pfy1, Pgi1, Pho88, Sac6, Tif, Tom22, Tom70, Ycp4.	1 Fet3.	64 Ali1, Apm4, Atp17, Atp3, Atp4, Atp7, Cbr1, Chc1, Cox13, Cox4, Cox5, Cox6, Cox9, Ctr1, Cyt1, Ddi1, Dpm1, Elf1, Emp24, Erg11, Frp3, Gap4, Gca1, Gut2, Hmg1, Hom2, Kin2, Lem3, Mir1, Nde1, Orf19.1840, Orf19.1970, Orf19.3003, Orf19.3235, Orf19.3290, Orf19.3335, Orf19.3430, Orf19.409, Orf19.4396, Orf19.4864, Orf19.5006.1, Orf19.5095, Orf19.5340, Orf19.5669, Orf19.6082, Orf19.7310, Pan1, Phm7, Por1, Pst3, Qcr2, Rho3, Rip1, Sec26, Sec4, Sec61, Sec62, Shm2, Stt3, Tcp1, Tim23, Tim50, Tom20, Vma5.	88
Orf's	50	3	122	175
Other identified proteins	190	3	186	379
Total	423	15	505	943

detailed above and according to the morphology in which they were identified (common or exclusive to one of the morphologies) (Table 3). Again, the results showed that a large proportion of the proteins identified were present in both morphologies. The largest differences were found in cell wall-, pathogenesis-, stress response- and plasma membrane-related proteins, and clearly in the Orf's group, where more than twice as many proteins were identified in hyphae. In the GPI-anchor protein group, the hypha-specific protein Hyr1 was identified exclusively in this morphology. Sap10 and Als2 were yeast exclusive GPI proteins. In the case of cell wall-related proteins, the difference was higher, Xog1 was detected only in yeast, 28 proteins were detected only in hyphae and 22 were present in both. Clearly, more proteins are expressed on the surface of hyphae than on yeast. It is interesting to note that a high number of Orf's were detected in both samples, with 172 proteins identified in hyphae (Table 1), and with 53 in yeast (Table 3).

3.3. Phenotypic and virulence analysis of interesting identified proteins

Among the identified proteins, 17 were selected to investigate their possible involvement in the formation of the cell wall as the outermost structure of the cell, due to their being proteins of unknown function, their implication in the interaction with the host and the fact that they have been characterized only in large-scale studies. The availability of the mutants and the morphologic form of the organism in which the protein had been identified were also used as criteria to select the proteins. These 17 proteins are listed in Table 4 and they are classified in the following categories: GPI (two), cell surface (one), stress-related (one), plasma membrane (six) and Orf's (five). Two proteins classified in the 'Other identified proteins' category were selected to be studied, Mci4 and Ptp3. The first was selected because its feature type at CGD is uncharacterized and the second because it is more abundant in hypha.

Table 4
List of *C. albicans* selected proteins for the phenotypic and virulence analysis of the corresponding mutants.

CGD accesion	Protein name	Category annotated (CGD)	Description[a]	Replicates with protein (peptides for each replicate)	
				Yeast	Hyphae
orf19.1690	Tos1	Cell surface	Protein similar to alpha agglutinin anchor subunit	3 (11, 9, 11)	4 (10, 10, 10, 8)
orf19.1710	Ali1	Plasma membrane	Putative NADH-ubiquinone oxidoreductase	–	2 (2, 2)
orf19.2451	Pga45	GPI	Putative GPI-anchored cell wall protein	–	4 (4, 5, 5, 2)
orf19.2570	Mci4	Other	Putative NADH-ubiquinone dehydrogenase	–	3 (1, 1, 1)
orf19.287	–	Orf's	Putative NADH-ubiquinone oxidoreductase subunit	–	3 (1, 1, 1)
orf19.3060	–	Orf's	OPutativedolichyl-diphoshooligosaccharide–proteinglycotransferase	–	3 (3, 3, 2)
orf19.3290	–	Plasma membrane	Plasma membrane-localized protein	–	3 (2, 2, 1)
orf19.3335	–	Plasma membrane	Plasma membrane protein of unknown function	–	4 (1, 1, 4, 4)
orf19.5285	Pst3	Plasma membrane	Putative flavodoxin	–	4 (2, 3, 3, 8)
orf19.5286	Ycp4	Plasma membrane	Putative flavodoxin	3 (1,1,2)	4 (2, 2, 3, 2)
orf19.5352	–	Orf's	Protein with a predicted magnesium transporter domain	–	3 (1, 1, 1)
orf19.5760	Ihd1	GPI	GPI-anchored protein	3 (2,2,1)	4 (2, 2, 2, 2)
orf19.6553	–	Plasma membrane	Membrane-localized protein of unknown function	2 (3,2)	4 (1, 1, 1, 1)
orf19.7196	–	Stress	Putative vacuolar protease	2 (2,2)	–
orf19.7328	–	Orf's	Protein with a staphylococcal nuclease domain	–	4 (14, 8, 8, 7)
orf19.7590	–	Orf's	Putative NADH-ubiquinone oxidoreductase	–	3 (2, 5, 4)
orf19.7610	Ptp3	Other	Putative protein tyrosine phosphatase	–	4 (3, 3, 6, 7)

[a] Description according to *Candida* Genome Database (CGD).

With this purpose, the homozygotic mutants were acquired from the Fungal Genetics Stock Center (FGSC) and different phenotypic assays were carried out. Strain SC5314 was used for the study of surfome. Although the wild type of the mutant collection is the SN250, we validated that both strains have the same behavior under all conditions tested, thus SC5314 can be used as a reference strain.

The assay conditions were separated into the broad categories of stress (including temperature, cell wall perturbing agents, osmotic and oxidative stress), morphology and nutrition. The sensitivity of the mutants to 175 μg/ml Congo red and 28 μg/ml Calcofluor white, compounds that interfere in the cross-linking of cell wall components [9,48], was tested. Several mutants were shown to be sensitive to those compounds, with *ali1*Δ, *orf19.287*Δ, *orf19.3060*Δ, *orf19.5352*Δ and *pst3*Δ sensitive to both Calcofluor white and Congo red, and *orf19.7590*Δ and *tos1*Δ slightly sensitive to Calcofluor white (Fig. 1 and Table 5). Sensitivity to these compounds was suppressed on osmotic medium stabilized with sorbitol 1 M, suggesting that the sensitivity observed was due to a defect in the cell wall [49]. Heat shock stress was tested growing the strains at different temperatures. All strains grew well at 37 °C, while at 45 °C *ali1*Δ, *orf19.287*Δ, *orf19.3060*Δ, *orf19.3290*Δ, *orf19.7590*Δ and *pst3*Δ had an altered growth (Table 5).

Oxidative stress was tested using 0.2 mM menadione, which can generate reactive oxygen species (ROS) by redox cycling which leads to stress, and 100 mM H_2O_2, a ROS that damages cellular components, as described in Section 2. Three mutants were sensitive to both H_2O_2 and menadione, *ali1*Δ, *orf19.287*Δ and the *orf19.7590*Δ. The *pst3*Δ mutant was sensitive just to menadione and *mci4*Δ and *ycp4*Δ just to H_2O_2 (Fig. 2 and Table 5). Strains were also exposed to osmotic stress by adding 1.5 M sorbitol and 1.2 M KCl to the medium. The a*li1*Δ, *orf19.3290*Δ and *mci4*Δ mutants were sensitive to both compounds while the *orf19.287*Δ mutant was sensitive only to KCl (Fig. 2 and Table 5).

In addition, the capacity of the mutants to form filaments was studied. When growing on YPD agar supplemented with 10% serum the *ali1*Δ, *orf19.7590*Δ, *orf19.287*Δ and *mci4*Δ mutants had a completely smooth colony morphology showing a severe defect in the yeast to hypha transition (Fig. 3A and Table 5). On Spider agar, the same mutants grew very poorly and very small, barely visible colonies appeared (Fig. 3B). This phenotype could be due to inability to metabolize the mannitol carbon source in Spider medium. For this reason, growth in mannitol agar plates was assayed. As expected, the mutant strains exhibit impaired growth on this medium, showing that they had an

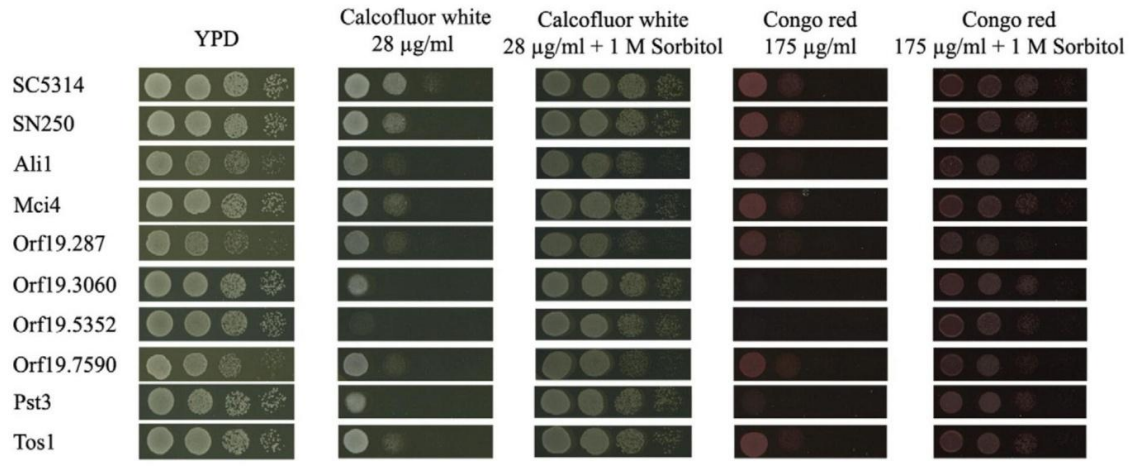

Fig. 1. Phenotypic analysis of *C. albicans* mutants to cell wall perturbing agents. Sensitivity to Calcofluor white and Congo red of the wild-type strains SC5314 and SN250 and *C. albicans* mutants.

Table 5
Phenotypes of *C. albicans* mutants. Sensitivity to the compounds was indicated with "+" and means reduction of growth relative to the controls SC5314 and SN250. Blank box stand for the same growth as the controls. "n.d." or Not Determined. Stress media growth, colony morphology and nutrient media growth phenotypes were indicated in the table. The data shown are from at least three replicates. 8 out 17 mutants studied (*idh1*, *orf19.3335*, *orf19.6553*, *orf19.7196*, *orf19.7238*, *pga45*, *ptp3* and *ycp4*) did not exhibit any phenotype.

C. albicans strain	Generation time 30 °C (min)	Stress									Morphology			Nutrition
		Cell Wall				Osmotic		Oxidative		T°				
		Sensitivity to calcofluor white 28 μg/ml	Calcofluor white 28 μg/ml + 1 M sorbitol	Sensitivity to Congo red 175 μg/ml	Congo red 175 μg/ml + 1 M sorbitol	Sensitivity to sorbitol 1.5 M	Sensitivity to KCl 1.2 M	Sensitivity to menadione 0.2 mM	Sensitivity to H_2O_2 100 mM	45 °C	Serum 10% 37 °C	Spider 30 °C	Spider 37 °C	C-source: mannitol
SC5314	72	Control strain (wild-type)												
SN250	n.d.	Collection control strain												
ali1	100	+	√	+	√	+	+	+	+	+	+	+	+	+
mci4	88					+	+		+		+	+	+	+
orf19.287	96	+	√	+	√		+	+	+	+	+	+	+	+
orf19.3060	n.d.	+	√	+	√					+				
orf19.3290	n.d.					+	+			+				
orf19.5352	71	+	√	+	√									
orf19.7590	99	+	√					+	+	+	+	+	+	+
pst3	n.d.	+	√	+	√			+		+				
tos1	73	+	√											

impaired ability to use mannitol as a carbon source (Fig. 3C and Table 5). In addition to investigating the growth of these mutants under different stress conditions, we also tested their growth at 30 °C to determine if any had a global growth defect. All strains had approximately the same growth rate to the parental strain, with the exception of *ali1*Δ, *orf19.7590*Δ, *orf19.287*Δ and *mci4*Δ mutants, which grew more slowly than the wild-type strain (Table 5).

Based on these results, the subsequent analyses were focused on the following eight mutants: *ali1*Δ, *mci4*Δ, *orf19.287*Δ, *orf19.7590*Δ, *pst3*Δ, *tos1*Δ, *orf19.3060*Δ and *orf19.5352*Δ. These mutants were sensitive to cell wall disturbing agents, oxidative stress and osmotic stress, and in the case of *ali1*Δ, *mci4*Δ, *orf19.287* and *orf19.7590*Δ had filamentation defects and were unable to use mannitol as a carbon-source (Table 5).

The interaction of the eight *C. albicans* mutants and the parental *C. albicans* SC5314 with RAW 264.7 macrophages was studied. Phagocytosis by RAW 264.7 macrophages were evaluated at an MOI of 1:1 and at different interaction times. As shown in Fig. 4A, at earlier time points, the *ali1*Δ, *mci4*Δ, *orf19.287*Δ, *pst3*Δ, *tos1*Δ and *orf19.3060*Δ mutants were phagocytosed statistically significant more than the wild-type strain. This difference was no longer evident at the 3 h time point for all strains tested.

To determine the capacity of the different *C. albicans* mutants to damage RAW 264.7 macrophages, the lactate dehydrogenase (LDH) cytotoxicity detection kit was used to measure the amount of LDH released from the damaged cells into the medium. As shown in Fig. 4B, *C. albicans mci4*Δ, *orf19.287*Δ, *pst3*Δ and *orf19.3060*Δ caused significantly more damage to the macrophages as compared to the wild-type strain at one or both time points.

Fungal invasion of the superficial epithelial lining of the oral mucosa is an important feature of oropharyngeal candidiasis [50]. For this reason, the interactions of these mutants with the FaDu oral epithelial cell line *in vitro* were tested. After 90 min of infection, the number of cell-associated (adherent) organisms was similar to that of the wild-type strain for all the mutants (Fig. 5A). At this time point, the endocytosis of the *ali1*Δ, *mci4*Δ, *orf19.287*Δ, *orf19.3060*Δ and *orf19.7590*Δ mutants was significantly less than that of the wild-type strain (Fig. 5A). Of note, the *ali1*Δ, *mci4*Δ and *orf19.287*Δ mutants germinated poorly on the FaDu cells and grew mainly as yeast, which likely explains their impaired endocytosis.

Next, the extent of damage caused by the different *C. albicans* mutants to FaDu oral epithelial cells was investigated. The *ali1*Δ, *mci4*Δ, *orf19.287*Δ and *orf19.7590*Δ mutants caused significantly less

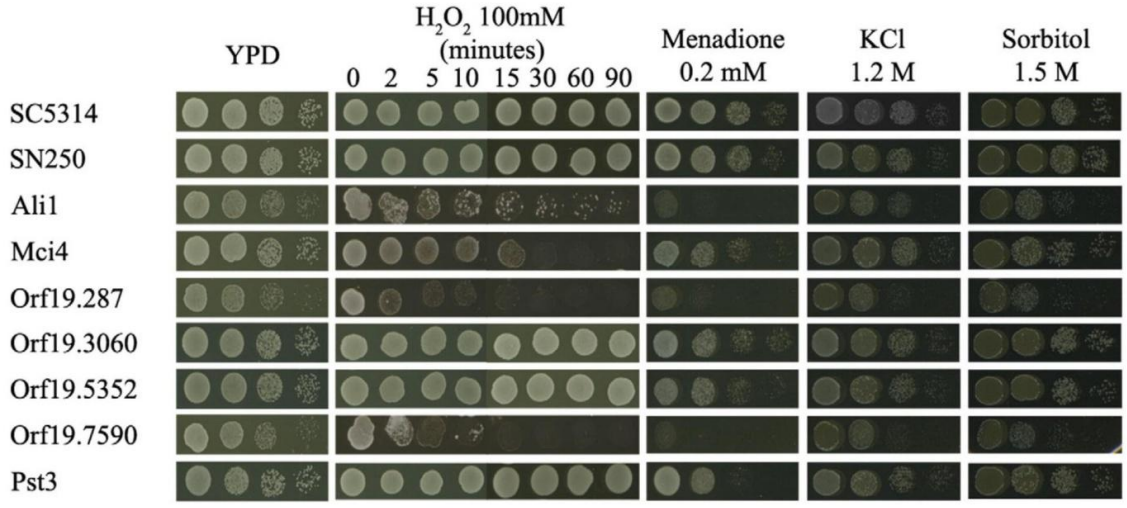

Fig. 2. Oxidative and osmotic-perturbing agents assays in *C. albicans* mutants. Mutants were exposed to oxidative-perturbing agents (H_2O_2 and menadione) and osmotic-perturbing agents (KCl and sorbitol) onto YPD plates containing the indicated amounts of the compound to be tested.

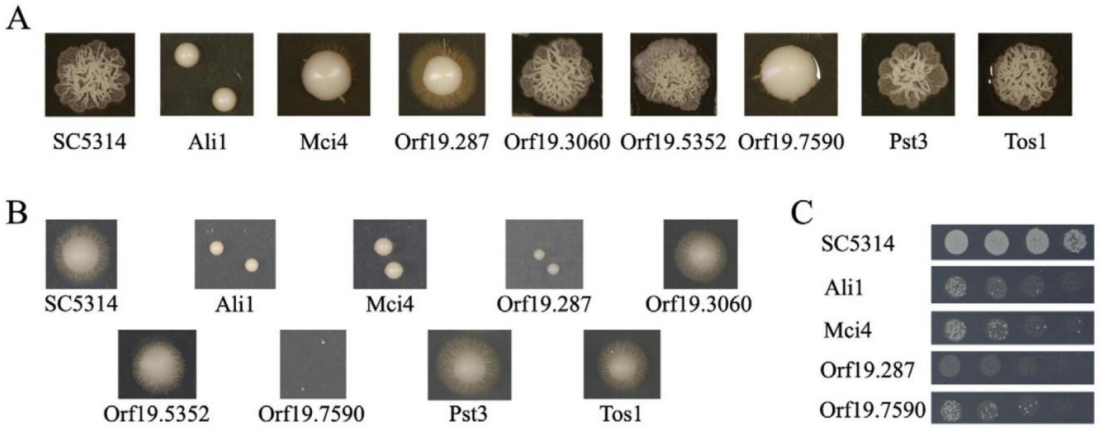

Fig. 3. Yeast-to-hypha transition assays in *C. albicans* mutants. (A) Yeast-to-hypha transition in YPD plates supplemented with 10% serum incubated at 37 °C for 7 days. (B) Yeast-to-hypha transition in Spider plates incubated at 37 °C for 7 days. (C) *C. albicans* mutants with growth defect on Spider plates exhibited a reduced growth rate on the C-source: mannitol medium.

damage to the cells compared with the wild-type strain (Fig. 5B). Interestingly, the *orf19.3060*Δ mutant caused slightly increased epithelial cell damage as compared with the wild-type strain.

The diverse phenotypes shown in the mutant strains' *in vitro* test suggest that these mutants might have increased virulence during oropharyngeal infection. This hypothesis was tested using a mouse model of OPC. The oral fungal burden of mice infected with either *pst3*Δ, *tos1*Δ, *orf19.3060*Δ or *orf19.5352*Δ strains was similar to that of mice infected with the wild-type strain (Supplemental Fig. S2). Therefore, these proteins do not appear to be involved in virulence during oropharyngeal candidiasis.

4. Discussion

4.1. Cell shaving for the identification of C. albicans yeast and hyphae surface protein profile

This work is mainly based on the comprehensive study of the surface proteome of *C. albicans* and the selection of some identified and unknown-function proteins to analyze their relevance in the cell surface, stress and virulence. For the identification of surface proteins of both yeast and hyphae, a proteomic approach based on cell surface shaving through live cell trypsin digestion and identification by MS was used. A total of 438 and 928 proteins were identified in yeast and hyphae morphology, respectively (Supplemental Tables S1 and S2). Other previously published proteomic analysis of *C. albicans* surface in yeast and/or hyphal morphology reported a smaller number of detected proteins [31–36]. Even using the same methodology optimized to identify surface proteins in *C. albicans* yeast cells by Hernaez et al. [36] and by Vialás et al. [35], the number of proteins identified in this work is much higher. Thus the proteomics approximation used has been validated and improved in this work by using LTQ-Orbitrap Velos, an ultra-high resolution mass analyzer with increased sensitivity, *versus* the MALDI TOF/TOF used in previous works. Moreover, also the proportion of proteins identified in each of the more interesting categories is more enriched. Among the new identified proteins Als2, Cht2, Crh11, Ihd1, Pir1 and Sap9-10 were found. All of these were described as GPI-anchored proteins by Richard and Plaine [4].

Several of the identified proteins are classical metabolic cytoplasmic proteins, such as Eno1 or Tdh3. They have been described at the *C. albicans* and *Saccharomyces cerevisiae* cell wall using different proteomic approaches [35,36,51–54]. In all cell surface protein studies of different microorganisms using the shaving approach, this type of proteins was detected (reviewed in [55]). The mechanisms used for this type of proteins to reach the cell wall are under study and different possibilities have been proposed: non classical exporting/secretory mechanisms, membrane-vesicle structures in which proteins are trapped and residual cell lysis [55]. A recent work describes that *C. albicans* extracellular vesicles contains several classical cytoplasmic and membrane proteins, such as Eno1, Gpm1, Met6, Pdc11, Pgk1, Ssa2 or Tdh3 [18] suggesting that they could pass through the cell wall inside these vesicles. In the present work, more classical cytoplasmic proteins

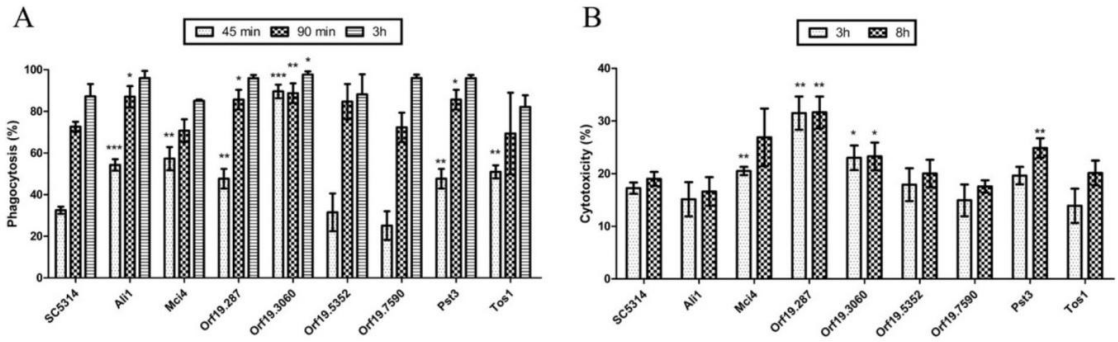

Fig. 4. Murine macrophage response to *C. albicans* mutants. (A) Quantification of phagocytosis of *C. albicans* yeasts at 45 min, 1.5 and 3 h of coincubation. (B) Cytotoxicity of the different mutants of *C. albicans* in RAW 264.7 macrophages at 3 and 8 h of coincubation. Data are represented as mean ± SD (n = 3), and statistical significance relative to wild-type is indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

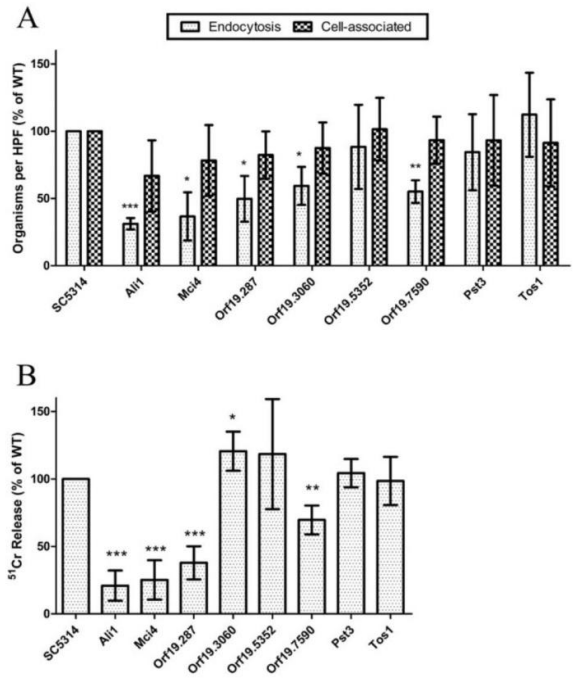

Fig. 5. Role of the proteins for the adherence, invasion and induce damage to FaDu oral epithelial cells. (A) The indicated strains of *C. albicans* were incubated with the FaDu oral epithelial cell line for 90 min, after which the numbers of endocytosed and cell-associated organisms were determined by a differential fluorescence assay. (B) The indicated host cells were loaded with ^{51}Cr and then incubated with the indicated strains of *C. albicans* for 3 h. The extent of host cell damage was measured by the release of ^{51}Cr into the medium. Results are the mean standard deviation of three experiments, each performed in triplicate. Data are represented as mean ± SD (n = 3), and statistical significance relative to wild-type is indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

or proteins described as located at intracellular membranes were detected. This might be related with the more sensitivity of this study, but also, although cell lysis controls were carried out, a very low contamination of intracellular proteins cannot be absolutely discarded.

Regarding the different profiles of *C. albicans* cell surface proteins of yeast or hyphae, Heilmann et al. [33] proposed Rhd3, Sod4 and Ywp1 as indicative of yeast cells while in this work Rdh3 and Ywp1 were identified in both morphologies as in Sosinska et al. [34]. However, Als2 and Sap10 were detected only in yeast. For proteins exclusively detected in hyphae, some are adhesins and lytic enzymes associated with virulence and hyphal growth, such as Als3, Hyr1 or Sod5 [56–60] (Table 3). The detection of some of these proteins only in one of the morphological forms might suggest that they have a role in this morphology, but other factors can also regulate their expression; for example the pH could influence the composition of these proteomes. It is known that environmental pH has effects on *C. albicans* morphology and its ability to respond to stress [61]. In addition, proteins have a pH optimum for activity, thus their functionality depends on the media conditions, including secreted and surface exposed proteins. For example, the homologous *PHR1* and *PHR2* genes encode pH regulated cell surface glycosidases. Although Phr2 mRNA and protein levels are higher preferentially at acidic pH and Phr1 at neutral [34,62,63], here both proteins were identified in the two morphologies obtained at different pHs (Table 3). Consistent with our results, recent works, in which an enhanced mass analyzer with increased sensitivity was used, have described the increase in abundance of both proteins under wall stress and different pHs, strongly at pH 7.4, in the cell wall [64,65]. Furthermore, the expression of these proteins together with others such as Als1or Ihd1, is enhanced, but not exclusive, at determined pHs [66,67].

It is important to highlight the large number of Orf's identified (annotated as verified or uncharacterized at CGD), which totalled 175; the vast majority identified just in hyphae. Due to their implication in cell is unknown and based on published data and mutant availability, 17 mutants were selected to examine their involvement in different cellular roles and virulence.

4.2. New identified C. albicans proteins relevant for dimorphic transition, cell wall maintenance, oxidative or osmotic stress responses

The response of the selected mutants to different stresses, including temperature, oxidative, osmotic or cell wall stress, was analyzed. Half of the mutants did not show any sensitivity to the compounds or temperatures studied (eight out of 17, *ihd1*Δ, *orf19.3335*Δ, *orf19.6553*Δ, *orf19.7196*Δ, *orf19.7238*Δ, *pga45*Δ, *ptp3*Δ and *ycp4*Δ), meaning that these proteins do not have a relevant cell role in responding to these stresses. The other nine mutant strains, *ali1*Δ, *mci4*Δ, *orf19.287*Δ, *orf19.3060*Δ, *orf19.3290*Δ, *orf19.5352*Δ, *orf19.7590*Δ, *pst3*Δ and *tos1*Δ, presented sensitivity to some of the stresses tested.

In addition, *ali1*Δ, *mci4*Δ, *orf19.287*Δ and *orf19.7590*Δ showed filamentation defects in 10% serum agar plates and growing defects in the Spider plates containing mannitol. Indeed, these mutants were unable to grow using this C-source. These four proteins are described in CGD as putative NADH-ubiquinone oxidoreductases (Ali1, Orf19.287 and Orf19.7590) and a putative NADH-ubiquinone dehydrogenase (Mci4), and no *S. cerevisiae* orthologs are described. Previous work showed that *C. albicans* uses mannitol only after the exhaustion of glucose, increasing the activity of NAD-linked mannitol dehydrogenase [68]. A possible relation between this activity and the four NADH-ubiquinone-related proteins remains to be investigated. In fact, little is known about the mannitol catabolic process: just one protein has been directly related to it, Zcf7, a predicted Zn(II)2Cys6 transcription factor, to date [69]. Thus, it can only be suggested that these four proteins appear to be linked to the use of mannitol and to link specific cues in environment to colony phenotype. In addition, these strains showed sensitivities to the vast majority of stresses studied.

Out of the 17 initial mutants, eight had a marked increase in susceptibility to a diverse panel of stressors, correspond to eight proteins identified in hyphae, including the four NADH-ubiquinone related proteins noted before (Ali1, Mci4, Orf19.287 and Orf19.7590), Orf19.3060, Orf19.5352 and Pst3, and Tos1 that were detected in both morphologies. The Tos1 mutant, *tos1*Δ, together with *ali1*Δ, *orf19.287*Δ, *orf19.3060*Δ, *orf19.5352*Δ, *orf19.7590*Δ and *pst3*Δ, presented sensitive to cell wall-disturbing agents and this sensitivity was suppressed in osmotic medium stabilized with sorbitol 1 M, suggesting that the sensitivity observed was due to a defect in the cell wall [49]. Pst3 protein has an ortholog in *S. cerevisiae*, Pst2, described in the plasma membrane by electronic annotation in CGD, and curiously it was also identified in the DIGE proteomic analysis of *C. albicans* yeast-to-hypha transition, that analyzed mainly soluble proteins, but in this case Pst3 was detected as less abundant in hypha than in yeast cells [70]. *S. cerevisiae* Pst2 was also identified in a proteomic approach for the study of cell wall biogenesis, in accordance with the sensitivity shown for *pst3*Δ to the cell wall-disturbing agents [71] and is similar to a family of flavodoxin-like proteins which is in agreement with the increased oxidative stress sensitivity of *pst3*Δ mutant strain. It is important to note that *orf19.3060*Δ, *orf19.5352*Δ, and *tos1*Δ mutant strains only presented defects when they grew in the presence of the cell wall-disturbing agents; thus, they seem to have a cell wall-specific defect. The Orf19.5352 is an uncharacterized protein with a predicted magnesium transporter domain located in the membrane and no *S. cerevisiae* ortholog is described [72]; and therefore, it is difficult to correlate this predicted function with the cell wall damage. On the contrary, the *orf19.3060*Δ is defective in a protein with a possible role in protein N-linked glycosylation according to the function of its ortholog in *S. cerevisiae*, Swp1, a 30 kDa type I transmembrane [73];

which would lead to a weaker cell wall according to our results. Tos1 is a protein similar to an alpha-agglutinin anchor subunit. It was previously detected in extracellular vesicles and proteins secreted by *C. albicans* in different studies, but its function is not known [18,74–76] and according to our results would be involved in cell wall maintenance.

Of the other 10 proteins, where the mutant strains did not present any sensitivity to the stressors tested, or only to osmotic stress such as *orf19.3290*Δ, little is known about their function to date. The *IHD1* gene was defined as part of the core filamentation network although Ihd1 is not essential for hyphal development according to current work [77].

4.3. C. albicans mutant strains produce different degrees of cell damage in host–pathogen interaction assays

Phagocytosis analysis reveals that six of the eight mutants used in this study showed an increase in engulfment by this type of immune cell. The mutation of these proteins could affect the composition of specific components of the *C. albicans* cell wall, leading to an increase in recognition by macrophages, as occurs with different O-linked and N-linked mannan-deficient strains in which the recognition by macrophages is increased [73]. Of these more phagocytized mutants, unexpectedly, the *mci4*Δ, *orf19.287*Δ, *pst3*Δ and *orf19.3060*Δ mutants, showed an increase in their ability to cause damage in the host cells. The increase in the amount of phagocytized yeast is crucial for macrophages in order to contain and kill *Candida*. The clearance of the internalized cells seems to be less efficient at increasing cytotoxicity to the macrophages, as occurs in macrophages coincubated with *C. albicans* at different MOIs where the higher MOIs are more cytotoxic to the macrophages [78]. The *orf19.3060*Δ mutant showed the most important differences in phagocytosis. As commented above, this mutant strain is defective in a protein with a role in protein N-linked glycosylation. McKenzie et al. [73] assessed the contribution of *C. albicans* cell wall glycosylation in macrophage response and related the lack of *MSN1* (N-glycosylation defect) with an increase in macrophage recognition and phagocytosis according to our data. The higher rate of *orf19.3060*Δ cells phagocyted and their normal hyphal formation can explain the increase in macrophage damage. The most cytotoxic strain was *orf19.287*Δ, which was more than 50% more cytotoxic than the wild type. Although this mutant strain is deficient in yeast-hypha transition, the large number of phagocyted cells could cause the increased damage by another mechanism, such as high secretion of proteins or enzymes involved in virulence. It is known that *C. albicans* is able to induce an apoptotic signal in macrophages during the interaction in the ratio of 1:1 [79]; thus, another possibility is that under the circumstances in which the number of phagocyted cells is higher than usual, the apoptotic damage caused to the immune cells could be greater. In the case of *ali1*Δ and *orf19.7590*Δ, macrophages had a higher ability to phagocytize and kill them. As these proteins are membrane oxidoreductases, and the mutants are more sensitive to oxidative stress, this result suggests a role for these proteins in avoiding the damage produced by reactive oxidative species inside the macrophages that would be impaired in the mutant cells.

Five out of the eight mutants examined were shown to be of important significance in regulating *C. albicans* interactions with oral epithelial cells *in vitro* (*ali1*Δ, *mci4*Δ, *orf19.287*Δ, *orf19.3060*Δ and *orf19.7590*Δ). However, while almost all of them showed decreased endocytosis and reduced capacity to damage the oral epithelial cell line, *orf19.3060*Δ showed decreased endocytosis but a significant increase in cell damage compared with the wild type. As mentioned above, the glycosylation status of the *C. albicans* cell wall is critical in host–pathogen interaction. Moreover, besides being involved in phagocytosis, it is also critical for the stimulus and regulation of epithelial responses [80]. Moreover, *tos1*Δ showed high variability in both assays with FaDu cells, showing slightly increased endocytosis and acting similarly to the wild-type strain in the damage assays, suggesting that Tos1 is not essential for adhesion and endocytosis by FaDu cells. As happens with *tos1*Δ, the *orf19.5352*Δ mutant showed a high variability in the results. As predicted by their *in vitro* results in host–cell interactions, no difference was observed in virulence in the mouse model of OPC (Supplemental Fig. S2). Therefore, these proteins de not appear to be necessary for virulence during oropharyngeal candidiasis.

5. Conclusions

The combination of proteomics studies with functional analysis of selected mutant strains allowed the identification of proteins involved in relevant cellular processes such as cell wall maintenance, osmotic and oxidative stress resistance and host–pathogen interplay. More studies are needed to understand the complexity of the *C. albicans* surface to find new drug targets and biomarkers for *Candida* infections.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

This work was supported by BIO2012-31767 from the Ministerio de Economía y Competitividad, PROMPT (S2010/BMD-2414) from the Comunidad Autónoma de Madrid, REIPI, Spanish Network for the Research in Infectious Diseases (RD12/0015/0004), and PRB2-ISCIII (PT13/0001/0038) funded by ISCIII and FEDER. It was also supported by grant R01DE017088 from the National Institutes of Health, USA. A. Gil-Bona was the recipient of a fellowship from Ministerio de Ciencia e Innovación. CM. Parra was the recipient of a fellowship from the program Continuous training for teachers of the Pontificia Javeriana University (Colombia) in conjunction with COLCIENCIAS-LASPAU Harvard University. The proteomic analyses were carried out in the Proteomics Unit UCM-Parque Científico de Madrid, a member of ProteoRed network. Strains and plasmids were obtained from the Fungal Genetics Stock Center (Kansas City, Missouri, USA). The authors would like to thank J. Aguilera and I. García-Barbazán for their helpful technical support in phenotypic assays and SG. Filler lab personnel for their support and help in the works performed in their laboratory. These results are lined up with the Spanish Initiative on the Human Proteome Project (B/DHPP).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.06.006.

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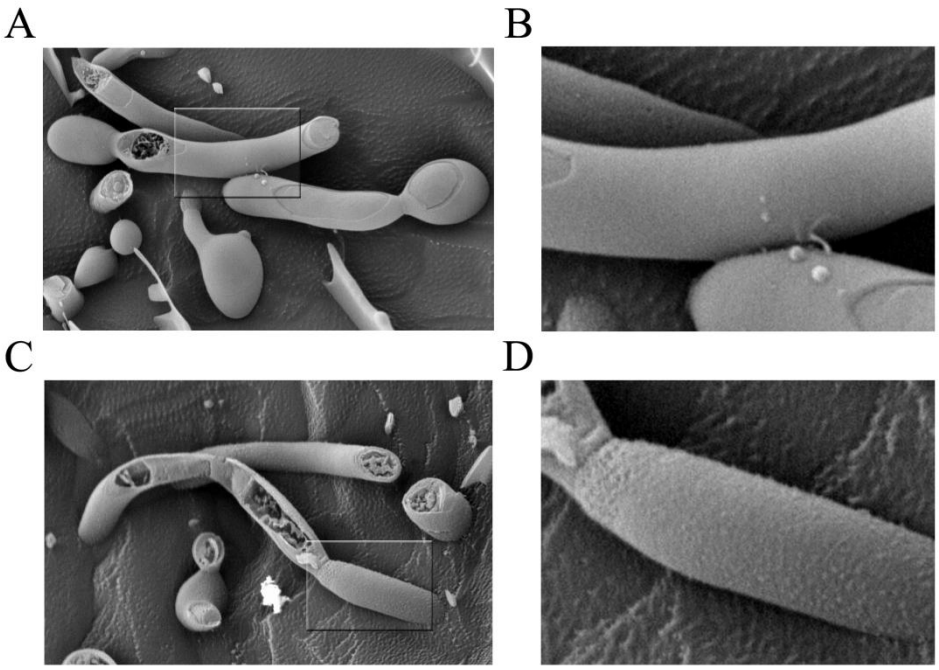
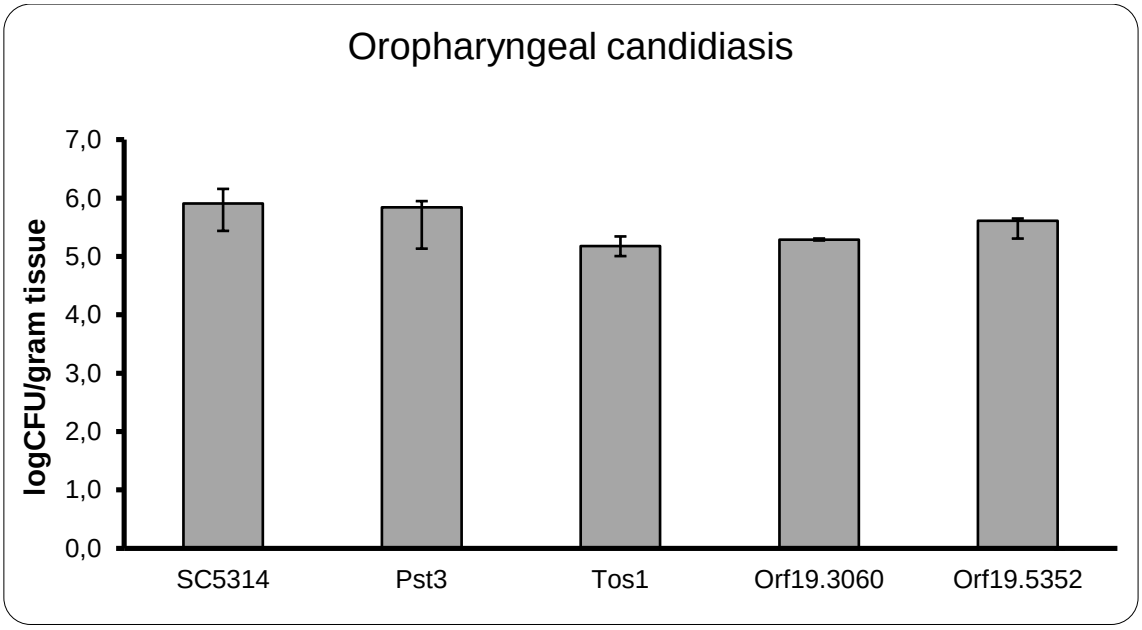
Appendix Chapter 1



Supplemental Figure 1. Low Temperature Scanning Electron Microscopy (LTSEM) of the cell surface of *C. albicans* hyphae cells before (A, B) and after (C, D) trypsinization. The untreated cell surface was intact (A, B) whereas irregular and wrinkled cell surface was observed after treatment (C, D). B and D are enlargement of the section marked in A and C images, respectively.



Supplemental Figure 2. Oral fungal burden of mice with oropharyngeal candidiasis.

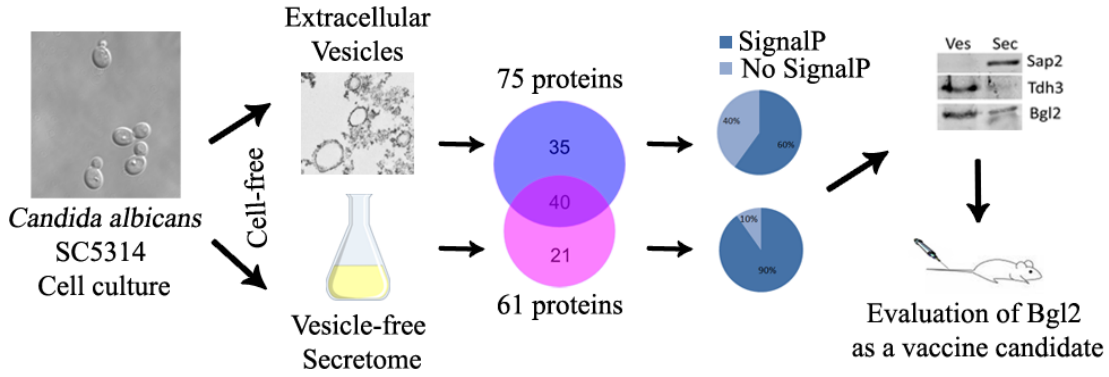
Remaining Supplemental material is available in the provided CD.

Supplemental Table 1. List of 438 proteins identified in *C. albicans* SC5314 yeast culture identified in at least two replicates with more than two peptides in both and proteins identified with one peptide in at least three replicates. Blue boxes correspond to proteins selected for phenotypic and virulence assays.

Supplemental Table 2. List of 928 proteins identified in *C. albicans* SC5314 hypha culture identified in at least two replicates with more than two peptides in both and proteins identified with one peptide in at least three replicates. Blue boxes correspond to proteins selected for phenotypic and virulence assays.

Chapter 2:

Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in *Candida albicans*



*In this chapter, the participation of Gil-Bona includes most of the work presented with the exception of the Bgl2 expression and vaccination assays in mice

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Proteomics Unravels Extracellular Vesicles as Carriers of Classical Cytoplasmic Proteins in *Candida albicans*

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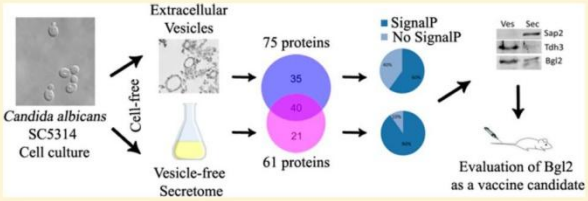
Supporting Information

ABSTRACT: The commensal fungus *Candida albicans* secretes a considerable number of proteins and, as in different fungal pathogens, extracellular vesicles (EVs) have also been observed. Our report contains the first proteomic analysis of EVs in *C. albicans* and a comparative proteomic study of the soluble secreted proteins. With this purpose, cell-free culture supernatants from *C. albicans* were separated into EVs and EV-free supernatant and analyzed by LC−MS/MS. A total of 96 proteins were identified including 75 and 61 proteins in EVs and EV-free supernatant, respectively. Out of these, 40 proteins were found in secretome by proteomic analysis for the first time. The soluble proteins were enriched in cell wall and secreted pathogenesis related proteins. Interestingly, more than 90% of these EV-free supernatant proteins were classical secretory proteins with predicted N-terminal signal peptide, whereas all the leaderless proteins involved in metabolism, including some moonlighting proteins, or in the exocytosis and endocytosis process were exclusively cargo of the EVs. We propose a model of the different mechanisms used by *C. albicans* secreted proteins to reach the extracellular medium. Furthermore, we tested the potential of the Bgl2 protein, identified in vesicles and EV-free supernatant, to protect against a systemic candidiasis in a murine model.



KEYWORDS: *Candida albicans, extracellular vesicles, LC−MS/MS analysis, secreted proteins, moonlighting proteins*

INTRODUCTION

Candida albicans is a commensal fungus in healthy humans and may cause different types of infections mainly in immunocompromised patients. The pathogenicity of *C. albicans* is attributed to several virulence factors, such as the ability to evade host defenses, adherence, biofilm formation and the secretion of hydrolytic enzymes such as proteases and phospholipases.[1−3] Secreted proteins are important for the commensal to pathogenic change, not only because some of them are hydrolytic enzymes, but also because they are necessary for their adaptation to the environment, in this particular case, the host. In all living organisms, and in particular in yeast, the secreted proteins are involved in different vital processes including biofilm formation, tissue invasion, immune evasion, cell wall integrity maintenance and nutrient acquisition.[4,5] Therefore, studies of the "secretome", as the part of the cell proteome secreted into the medium, are of outstanding interest.

All eukaryotic cells have a classical secretory pathway that includes the endoplasmic reticulum (ER), the Golgi apparatus and a complex system of vesicles, to transport proteins to the plasma membrane or to the extracellular region, including the periplasmic space, the cell wall or the extracellular medium.[6] Although few works analyzing *C. albicans* proteins involved in these pathways have been reported,[4,7] the *C. albicans* genome contains all the orthologues to *Saccharomyces cerevisiae* genes involved in the classical secretory pathway, which is highly conserved. Proteins secreted through this pathway have an amino-terminal signal peptide that is responsible for directing them to the inside of the ER. This signal sequence has been used in genetic and bioinformatic studies to define the *C. albicans* secretome.[8,9]

Several *C. albicans* virulence studies highlighted the importance of some families of proteins secreted through the secretory pathway as virulence factors, including secreted aspartyl proteases (Saps) and phospholipases (Plbs).[2] Proteins involved in cell wall synthesis, such as the exo-1,3-β-glucanase (Xog1) or the endoglucanase (Eng1), are also secreted by the classical protein secretory pathway.[10−12] In addition, an important group of plasma membrane and cell wall proteins are the glycosylphosphatidylinoditol (GPI)-anchored proteins such as Ecm33.[13,14] They are also secretory proteins with a signal peptide, serine- and threonine-rich region and a potential C-terminal domain for GPI anchor attachment.[15,16] However, proteins without a signal peptide have been detected in the extracellular medium of *C. albicans* and other yeast species as published in the last years.[17−23] Recent proteomic analysis of

Special Issue: Environmental Impact on Health

Received: July 30, 2014
Published: November 3, 2014

C. albicans secreted proteins identified a high number of hydrolases and other secreted proteins regulated by fungus under different growth conditions.[24]

It is remarkable that close to one-third of extracellular proteins identified in the *C. albicans* secretome do not possess a secretion signal, and several predicted cytosolic proteins are detected as secreted.[25] These proteins lacking the N-linked signal peptide should use alternative routes of exportation, including vesicular pathways.[26] Many of these cytoplasmic proteins have been identified as secreted or located at the cell surface of several organisms.[26,27] In addition, some of them have been described as "moonlighting" or multifunctional proteins that are capable of performing dual or multiple functions, in some cases depending on their location in the cell.[28−31] In this context, three different protein groups are notable: heat-shock proteins including Hsp90 or Hsp70; proteins involved in glycolysis such as the enolase (Eno1), the glyceraldehyde-3-phosphate dehydrogenase Tdh1 or the piruvate decarboxylase Pdc11; and the third group, corresponding to translation elongation factors including proteins such as the ribosomal protein Rps6 involved in mRNA metabolism.[30]

In the last years, extracellular vesicles (EVs) have been involved in protein export from mammalian, fungal and bacterial cells. Several outer membrane vesicle proteomes from bacteria have been evaluated recently, and the results showed that they provide a survival advantage and carry virulence-associated bacterial proteins that affect the host.[32,33] Also, the studied fungal species were apparently able to use vesicles as a general mechanism of molecular traffic to transport intracellular proteins across the cell wall. In this way, Albuquerque et al.[34] have described extracellular vesicles in the culture medium of several fungi including *C. albicans* by transmission electron microscopy (TEM). Proteomic analyses of the EVs from *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *S. cerevisiae* revealed the presence of proteins involved in diverse processes including metabolism, cell wall architecture, signaling and virulence.[22,34−39] Vallejo et al.[22] compared the EV proteomes of four fungal species (*P. brasiliensis*, *C. neoformans*, *H. capsulatum* and *S. cerevisiae*) revealing an effective overlap in protein composition, including cytoplasmic, mitochondrial, vacuolar and plasma membrane proteins. In fact, EV analysis of human pathogenic fungi discovered the presence of proteins with both immunological and pathogenic activities, suggesting that EVs might be actively involved in fungal pathogenesis.[39] In bacteria, some of the outer membrane vesicles (OMV) studied are enriched in toxins and other virulence-associated bacterial proteins, capable of activating the host innate and acquired immune response pathways to modulate the host response.[40]

The fact that *C. albicans* secreted proteins can potentially interact with the host immune system underlines their importance not only for the pathogenic process but also as putative biomarkers of *C. albicans* infections or as antigens for the development of new acellular vaccines. The 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase Met6, Hsp90, the phosphoglycerate kinase Pgk1, Tdh3, Eno1 and the cell wall 1,3-β-glucosyltransferase Bgl2, among others, have been detected by proteomic analysis as diagnostic and prognostic biomarkers for systemic candidiasis.[20,41−43] In addition, other *C. albicans* proteins implicated in virulence are being studied as possible vaccines to prevent disseminated candidiasis. Among them, immunization with *C. albicans* Sap2, the agglutinin-like sequences 1 and 3 (Als1 and Als3) and the hyphally regulated protein Hyr1 showed protective effects in mice against lethal doses of *C. albicans*.[44−48]

In this work, we describe the first proteomic study of *C. albicans* EVs and a comparative analysis with the *C. albicans* soluble secreted proteins. Data analysis rendered interesting results regarding the controversial mechanism of secretion of the classical cytoplasmic proteins, including moonlighting proteins. Furthermore, the previously detected as immunogenic protein Bgl2, that has been identified in secretome and in extracellular vesicles, was tested as a new antigen to be used as a *C. albicans* acellular vaccine.

■ MATERIALS AND METHODS

Growth Conditions

C. albicans SC5314[49] was used in this study. Yeast cells were precultured in liquid synthetic defined (SD) medium (20 g/L glucose, 5 g/L ammonium sulfate, 1.7 g/L nitrogen base and 2.2 g/L amino acids mix) with rotary shaking (200 rpm) at 30 °C, during 7 h. The preculture was used to inoculate flasks containing 1 l SD medium adjusting OD_{600} to recover the culture at a final OD_{600} of 4, 16 h later. At this point, cell viability was determined by propidium iodide staining.

Isolation of Extracellular Vesicles and Vesicles-Free Secretome

Vesicles were isolated according to Rodrigues et al.[50] All the steps were carried out at 4 °C to avoid proteinase activity and vesicle rupture. A tablet of protease inhibitor (complete mini, EDTA-free, Roche) was added per liter of culture. Three independent biological samples were performed. Briefly, yeast cells were separated from culture supernatants by centrifugation at 5524*g* for 15 min. The resulting supernatants were collected and centrifuged again at 15344*g* for 30 min to remove smaller debris. The supernatant was collected and concentrated using a Centricon Plus-70 centrifugal filter (cutoff filter 100 kDa, Millipore). The concentrated culture was centrifuged again at 4000*g* for 15 min and 15000*g* for 30 min to remove smaller debris. The resulting supernatant was separated into two tubes and ultracentrifuged at 100000*g* for 1 h. The supernatant was recovered for secretome analysis, and the pellet containing the EVs was washed in phosphate-buffered saline solution (PBS) and centrifuged again at 100000*g* for 1 h (Supporting Information Figure S1A). The supernatant was again removed from the pellet and one of the samples was embedded in a fixative solution (as described below) for electron microscopy analysis while the other was used for the proteomic analysis.

The flow through of the 100-kDa filter and the supernatant recovered from the first ultracentrifugation of the vesicles purification were concentrated approximately 20-fold using a 10-kDa cutoff filter (Millipore) to obtain *C. albicans* secreted proteins that were soluble in the culture medium. The methanol/chloroform precipitation procedure based on the Wessel et al.[51] protocol was used to precipitate and clean the sample.

Transmission Electron Microscopy (TEM)

TEM was used to visualize intact extracellular vesicles from *C. albicans* cells. The pellet obtained by ultracentrifugation was incubated in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 2 h and then fixed in 4% paraformaldehyde, 1% glutaraldehyde and 0.1% PBS overnight at 4 °C. Samples were incubated for 90 min in 2% osmium tetroxide (TAAB Laboratories, UK), serially dehydrated in

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142−153

ethanol, and embedded in EMBed-812 resin (Electron Microscopy Sciences). Thin sections (50−70 nm) were obtained by ultracut and observed in a JEOL JEM 1010 transmission electron microscope operating at 100 kV and pictures were taken with a Megaview II camera. TEM images were analyzed with Soft Imaging Viewer Software.

Protein Digestion

Two preparations were processed for proteomic analysis: (a) EVs and (b) EV-free supernatant proteome. The concentrated proteins of each sample were resuspended in 0.5 M Triethylammonium bicarbonate (TEAB) and quantified using the Bradford protein assay.

Aliquots of 13 μg of vesicles and EV-free supernatant samples were adjusted to the same volume of ammonium bicarbonate (NH_4HCO_3). All samples were reduced by adding 100 mM DTT for 30 min at 37 °C and alkylated with 55 mM iodacetamide for 20 min in the dark. Then, digestion was performed by adding recombinant sequencing grade trypsin (Roche) 1:20 (w/w) overnight at 37 °C. Subsequently, the produced peptides were cleaned up with a POROS R2 column. The peptides were eluted with 80% acetonitrile (ACN) in 0.1% TFA, dried in a Speed-Vac and resuspended in 0.1% formic acid. The samples were stored at −20 °C before the nano LC−MS/MS analysis.

LTQ-Orbitrap Velos Analysis

Desalted peptides were analyzed by RP-LC−MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos mass spectrometer (Thermo Scientific). The peptides were concentrated (online) on a C18-A1 ASY 0.1 × 20 mm C18 RP precolumn (Thermo Scientific) and then separated on a Biosphere C18 RP-column [C18, inner diameter 75 μm, 15 cm long, 3 μm particle size (NanoSeparations)] and were eluted using a 150 min gradient (0−140 min from 2 to 35% Buffer B, 140−150 min 35−95% Buffer B. Buffer A: 0.1% formic acid/2% ACN; Buffer B: 0.1% formic acid in ACN) at a flow-rate of 250 nL/min on a nanoEasy HPLC (Proxeon) coupled to a nanoelectrospay ion source (Proxeon). Mass spectra were acquired on the LTQ-Orbitrap Velos in the positive ion mode. Full-scan MS spectra (m/z 400−1800) were acquired in the Orbitrap with a target value of 1 000 000 at a resolution of 30 000 at m/z 400 and the 15 most intense ions were selected for collision induced dissociation (CID) fragmentation in the LTQ with a target value of 10 000 and normalized collision energy of 38%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 s.

Protein Identification and Analysis

Protein identification from raw data was carried out using a licensed version of search engine MASCOT 2.3.0 with Proteome Discoverer software version 1.4.1.14 (Thermo Scientific). A database search was performed against the CGD21 database (6221 sequences). Search parameters were oxidized methionine as variable modification, carbamidomethyl cysteine as fixed modification, peptide mass tolerance 10 ppm, 1 missed trypsin cleavage site and MS/MS fragment mass tolerance of 0.8 Da. In all protein identification, the FDR was <1%, using a Mascot Percolator, with a q-value of 0.01. As an estimation of the relative protein abundances the normalized spectral abundance factor (NSAF) was used,[52] and the average of the normalized values was calculated.

Mass spectrometry proteomics data (Supporting Information Table S4) has been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via PRIDE partner repository[53] with the data set identifier PXD000525. The identified proteins have been included into the Proteopathogen Database (http://proteopathogen.dacya.ucm.es)[54] and in the new version of *Candida albicans* PeptideAtlas[55] with the data set identifier PASS00408.

Bioinformatic Analysis

CGD (www.candidagenome.org) was the database used for the analyses. Proteins identified in at least two replicates with more than two peptides in one of them were used for the analysis. Venn diagrams were prepared using the Venn tool available http://bioinfogp.cnb.csic.es/tools/venny. Signal peptide was predicted using SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP). This program predicts the presence and location of signal peptide cleavage sites in amino acid sequences based on a combination of several artificial neural networks. Selected protein sequences were submitted to Gene Ontology (Go) annotation, using the CGD application (http://www.candidagenome.org/cgi-bin/GO/goTermFinder).

SDS-PAGE and Western Blotting

Vesicles and EV-free supernatant samples (10 μg of each) were denatured by heating in SDS buffer containing 100 mM Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue and 20% DTT for 5 min at 99 °C. Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis using the Miniprotean II electrophoresis system (Bio-Rad). The gel was stained with Coomassie blue. For silver staining, the gel was destained, fixed (40% MeOH, 10% acetic acid v/v) and stained using the Silver Stain Kit (Bio-Rad). For Western blotting, 15 μg of vesicles and EV-free supernatant proteins were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blocked in 5% milk. Also, 15 μg of *C. albicans* cytoplasmic extract obtained as previously described[56] were included as a positive control. Western blots were probed with anti-Tdh3 (a gift from M.L. Gil and J.P. Martínez) at 1:500, anti-Bgl2 at 1:250 and anti-Sap2 (a gift from M. Monod, Centre Hospitalier Universitaire Vaudois, Switzerland) at 1:3000 to identify the protein-enriched fraction. Human sera from patients with candidiasis were also tested at 1:3000. After incubation with primary antibodies, membranes were washed several times in PBS with 0.1% Tween-20, and then were incubated with fluorescently labeled secondary antibodies: 1/2000 IRDye 800 goat antirabbit IgG or 1/1000 IRDye 800 goat antihuman IgG depending on the first antibody (LI-COR Biosciences). The Western blotting was performed with the Odyssey system (LI-COR Biosciences, Nebraska, USA).

Expression of Bgl2

The expression of Bgl2 was performed as previously described by Díaz-Perales et al.[57] All DNA manipulations were carried out using standard protocols.[58] A fragment of *C. albicans* genomic DNA containing the *BGL2* gene was amplified by PCR using the following primers: Bgl2up 5′-GCTACTGCGGAGGAAGGACC-3′ and Bgl2lw 5′-CTCGTTCACTTTGACTTTGCC-3′. The PCR products were purified and cloned into the pGEM vector (Promega, Madison, WI, USA), and the construct was used to transform DH5αF′ *Escherichia coli* cells.

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142−153

Sequencing reactions were performed at the Genomic Unit at the University Complutense, Madrid (UCM) – Parque Científico (Madrid, Spain). The DNA sequence was double-checked by sequencing both strands and used as a template for the following PCR. The *BGL2* coding region was PCR amplified with the sense primer 5′-CTCGAGAAAAGA-GAAATGGGTGATTTGGCTTTC-3′ (the *Xho*I restriction site is underlined) and the antisense primer, 5′-GTAGTA-GCGGCCGCTTAGTTGAATTTACAGTC-3′ (the *Not*I restriction site is underlined). The hybridizing sequences were extended in the synthetic primers with in-frame sequences coding for the pre-prosequence of the α-mating factor of *S. cerevisiae* present in plasmid pPIC9 (Invitrogen Corporation, De Schelp, The Netherlands). The sense primer also included a codon for glutamic acid which enabled the processing of the signal peptide by the yeast. The PCR product was purified from an agarose gel and used for the ligation with the pGEM T vector. This construct was used to transform DH5αF′ *E. coli* cells. Plasmid DNA from one clone representative of the Bgl2-pGEM T-*Xho*I-*Not*I was isolated and digested with *XhoI/NotI* restriction enzymes. The DNA fragment was subcloned into the pPIC9 vector, rendering pPIC9-Bgl2, which was sequenced to confirm the in-frame arrangement of the leader sequence and the inset.

The pPIC9-Bgl2 construct was linearized with the *Bgl*II restriction enzyme and used to transform GS115 *Pichia pastoris* cells by electroporation following the manufacturer's recommendations (Invitrogen). Transformed cells were grown on regeneration dextrose medium plates [1 M sorbitol, 2% dextrose, 1.34% YNB (Yeast Nitrogen Base), 4×10^{-5}% biotin, 0.005% amino acids] at 30 °C for 3–4 days, and the screening for positive clones was performed by patching the colonies in replica-plating on minimal glucose plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) vs minimal methanol plates (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol). The *Pichia* integrants were analyzed by PCR to determine if the gene of interest was integrated into the *Pichia* genome (5′AOX1 5′-GACTGGTTCCAATTGACAAGC-3′; 3′AOX1 5′-GCAAATGGCATTCTGACATCC-3′ supplied with the *Pichia* Expression Kit (Invitrogen).

The selected transformants were cultured in buffered minimal glycerol medium (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) for 24 h at 30 °C. Cells were collected by centrifugation, and expression of the recombinant protein was induced by resuspension in buffered minimal methanol medium (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol). The highest expression level of recombinant Bgl2 protein was reached 4 days after induction as determined by SDS-PAGE.

One liter of the culture medium obtained after induction was dialyzed against water and freeze-dried. The lyophilized sample was resuspended in 20 mM ethanolamine (pH 9.0), and the amount of protein was calculated using the Bradford method. This preparation was fractioned by anion-exchange chromatography on an Acell Plus CM Waters SepPak (solid-phase extraction) cartridge (Waters Corpor, Milford, MA, USA), eluting with different concentrations of NaCl (1 mL/min). The fraction enriched in Bgl2 was dialyzed, freeze-dried, and then separated by FPLC on a MonoQ 5/50 GL Tricorn column (GE Healthcare, Uppsala, Sweden). Elution was performed with a linear gradient of NaCl in 20 mM ethanolamine pH 9.0 (0–50% for 50 min and 50–100% for 30 min; 1 mL/min). Peaks containing recombinant Bgl2 in each chromatographic separation were identified by SDS-PAGE and immunodetection. The SDS-PAGE bands corresponding to Bgl2 protein were identified by mass spectrometry at the Proteomic Unit at the University Complutense, Madrid (UCM) – Parque Científico (Madrid, Spain). The corresponding bands were excised from the colloidal Coomassie blue-stained gel and in-gel digested. The MALDI-TOF identification was performed as previously described.[56]

Vaccination Assays in Mice

Female BALB/c mice (aged from 13 to 16 weeks and with a weight of about 20 g) were obtained from Harlan France (Sarl, France) and housed at the Animal Facilities of the School of Medicine at the Complutense University of Madrid, Spain.

Immunization assays were carried out according the Spellberg protocol.[46] Five groups of eight mice were injected intraperitoneally twice with 2-week intervals with five different doses of the protein (0.003, 0.03, 0.3, 3, and 30 μg). As a negative control, mice were vaccinated with adjuvant; and the candidemia control was also performed. After 30 days of the first immunization mice were infected intravenously through the tail vein with 5×10^5 cells of the parental *C. albicans* strain SC5314. The mice were observed for 28 days and survival was determined for each vaccination group. These animals were treated according to the European Union Ethical Norm. Mantel-Cox test was done to determine the mice survival curves significance using adjuvant as control.

■ RESULTS

C. albicans Secretome Fractionation: Extracellular Vesicles (EVs) and EV-Free Supernatant Purification

In this work, the EVs produced by *C. albicans* yeast cells growing in SD medium were recovered from culture supernatants and separated from the rest of the secreted material according to the scheme shown in Supporting Information Figure S1A. Subsequently, the proteome composition of the EVs and EV-free supernatant was analyzed in parallel. To discard secretome contamination by cytoplasmic proteins or by the presence of vesicles arising from dead cells, cell viability was analyzed by fluorescence microscopy. The lack of propidium iodide (PI) fluorescence suggested that cells were viable and without compromised permeability.

Purified vesicles were observed by TEM, revealing spherical and bilayered structures with heterogeneous sizes: the majority of the observed vesicles presented sizes between 50 and 100 nm but a smaller percentage had larger sizes (more than 200 nm). Figure 1A and 1B show a representative view of the different vesicles sizes obtained and the bilayered membrane is clearly shown in Figure 1C. Furthermore, TEM analysis of SC5314 cells growing in exponential phase revealed interesting membrane invaginations and the formation of a membrane structure resembling a putative vesicle (Figure 1D).

Before the LC–MS/MS analysis, protein samples were separated by SDS-PAGE in order to compare the protein profile. As shown in Supporting Information Figure S1B, more intense bands were detected at high and low molecular weights in the vesicles sample; nevertheless, a very intense band between 25 and 37 kDa appeared only in the secretome fraction.

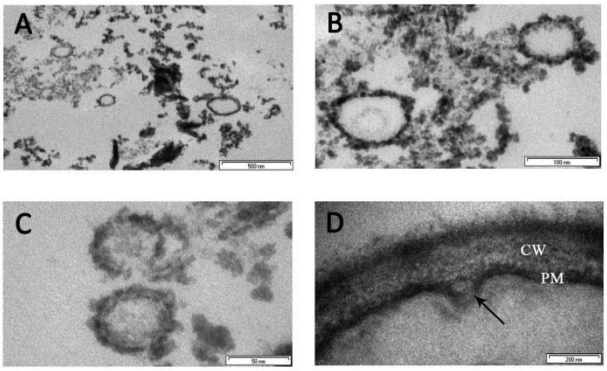


Figure 1. TEM images of purified EVs obtained by ultracentrifugation of culture supernatants from *C. albicans* showing heterogeneous size (A and B), bilayered membranes (C) and vesicular structures associated with the cell wall (D). Bars: 500 nm (A), 100 nm (B), 50 nm (C) and 200 nm (D).

Proteomic Analysis of *C. albicans* Extracellular Vesicles

The purified vesicles were digested with trypsin and analyzed by LC–MS/MS (Orbitrap). After the analysis of the three biological replicates, proteins detected in two or more replicates with at least two peptides in one of them were selected and listed in Supporting Information Table S1. A total of 75 proteins were identified in the vesicle fraction. Supporting Information Table S1 summarizes all the identified proteins with their biological characteristics and the number of identified peptides in each biological replicate. The protein composition of the vesicles content was very diverse. The vesicular proteins were categorized by the biological process in which they are involved (Figure 2A) with nine principal groups, including 31 cell wall-related proteins (41%), nine transmembrane transporters (12%), eight metabolic proteins (10%), seven proteins involved in protein folding (9%), five secreted hydrolases and adhesins (7%), five proteins related to exocytosis and endocytosis (7%) and three related to protein synthesis and degradation (4%). Of the remaining seven proteins, five (7%) were related to different processes and two (3%) are uncharacterized.

The prediction of signal peptides using SignalP 4.1 detected that 60% (45 proteins) are classical secretory proteins as they carry an N-terminal signal peptide. Of these proteins, 14 (18.7%) are GPI-anchored proteins (Figure 2B). The other 40% of proteins (30 out of 75) were not predicted as secreted.

Proteomic Analysis of the EV-Free Supernatant

For the global proteomic analysis of the soluble secreted proteins, the supernatant of the first ultracentrifugation including proteins >100 kDa was mixed with the EV-free fraction including proteins <100 kDa (Supporting Information Figure S1A). The sample was concentrated, digested with trypsin and analyzed by LC–MS/MS (Orbitrap). A total of 61 proteins were identified in two or more replicates with at least two peptides in one of them. The classification of the identified proteins regarding the biological process (Figure 2C) showed that the cell wall-related proteins and the secreted hydrolases and adhesins were the most represented groups with 30 (49%) and 13 proteins (21%), respectively. Regarding the bionformatic analysis of sequences from these proteins secreted into

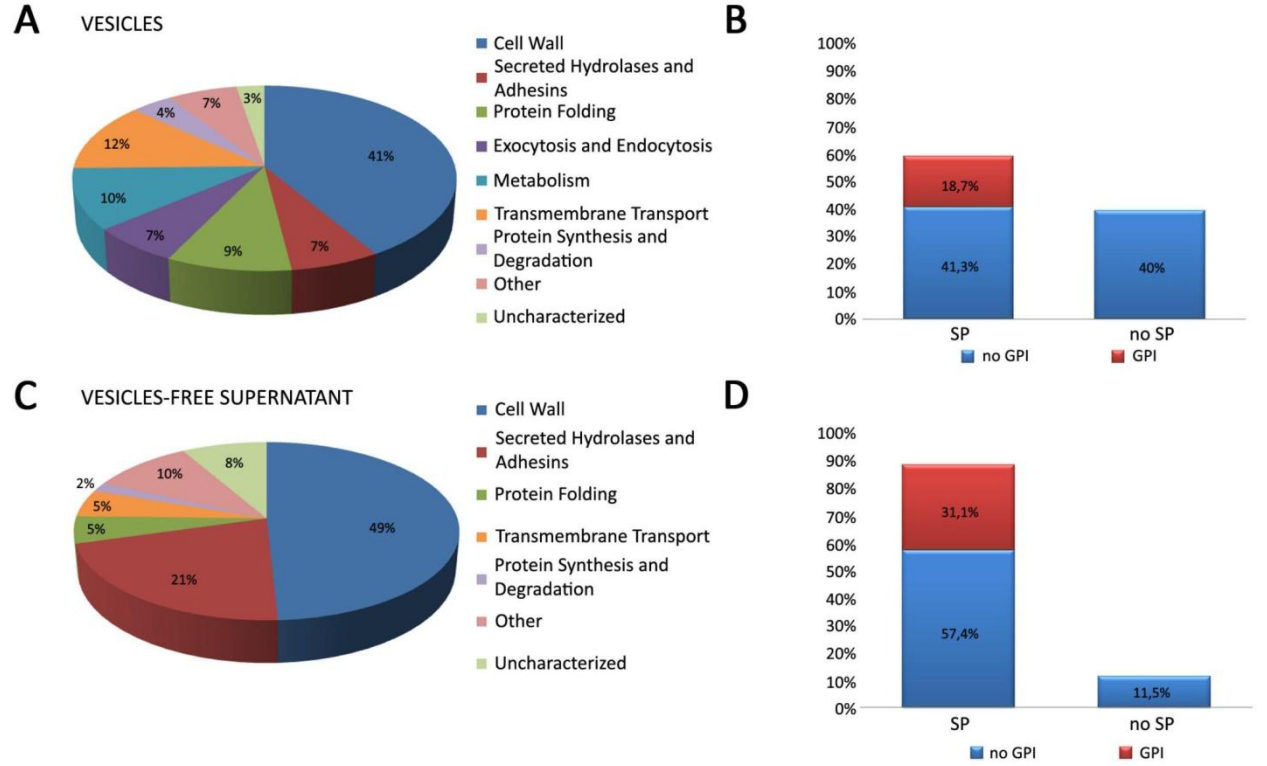


Figure 2. Categorization of the biological process involving (A) EVs proteins and (C) EV-free supernatant proteins. Proportion of proteins in (B) EVs and (D) EV-free supernatant classified as secretory (SP, containing signal peptide) or not secretory (without SP) by SignalP4.1. The proportion of GPI-anchored proteins is also shown.

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142–153

the extracellular medium, the vast majority (55 proteins corresponding to 90%) bear an N-terminal signal peptide to be secreted by the classical secretory pathway; among them, seven are members of the Sap family (secreted aspartyl protease) and 19 have been described as GPI-anchor proteins (Figure 2D and Supporting Information Table S1).

Comparison of the Vesicle and EV-Free Supernatant Proteome: Functions and Immunogenicity

After the proteomic analysis, a total of 96 proteins were identified in the complete extracellular secretome (Supporting Information Table S1). Out of these proteins, 40 were common to both samples, while 21 proteins were identified exclusively in the EV-free supernatant fraction and 35 detected only in the vesicle fraction.

A comparative analysis of the distribution of the different pools of proteins (secreted to the medium or carried by EVs) in the different biological processes in which they are involved is shown in Figure 3A. It is relevant to show that most of the cell

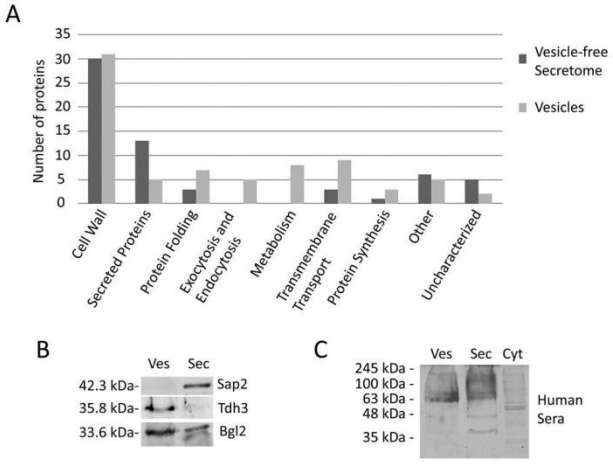


Figure 3. (A) Comparative analysis of the different biological processes in which proteins secreted by the classical pathway (EV-free supernatant) or carried by EVs (vesicles) are involved. (B) Western blot analysis of Sap2p (42.3 kDa), Tdh3p (35.8 kDa) and Bgl2p (33.6 kDa) identified in EVs (Ves), EV-free supernatant (Sec) and in both fractions. (C) Western blot analysis of human sera from patients with candidiasis against the proteins from EVs (Ves) and EV-free supernatant (Sec). A cytoplasmic protein extract was included as a positive control (Cyt).

wall-related proteins are identified in both samples, while there are more secreted hydrolases and adhesins in the EV-free supernatant, contrary to metabolic proteins or the proteins involved in exocytosis and endocytosis that were only carried by vesicles. The subcellular locations detected by the proteomic analysis were confirmed by Western blot analysis performed with specific antibodies against selected proteins identified exclusively in the EV-free supernatant (Sap2p), exclusively in EVs (Tdh3) or in both samples (Bgl2) (Figure 3B). To analyze the relative abundance of the different proteins in each sample, they were shorted according to the averaged normalized spectral abundance factor (NSAF)[52] of each sample (Figure 4). It is noticeable that the most abundant proteins are adhesins and cell wall-related proteins (as the mannoprotein Mp65, the adhesion-like protein Sim1, the cell wall glycosidase Sun41, Tos1, Scw11, Xog1, or the Chitinase Cht3) common to both samples. According to this, just 3 proteins of the top 20 most

Protein	Vesicles	Evs-free Supernantant
MP65	0,1816	0,1171
SIM1	0,1296	0,1180
SUN41	0,0617	0,0982
TOS1	0,0486	0,1336
DAG7	0,0417	0,0457
PLB4.5	0,0307	0,0124
SCW11	0,0287	0,0275
RBE1	0,0224	0,0164
XOG1	0,0223	0,0487
BGL2	0,0207	0,0297
CHT3	0,0154	0,0671
COI1	0,0145	0,0121
CHT2	0,0121	0,0284
ECM33	0,0121	0,0180
TDH3	0,0120	
RHO1	0,0119	
PMA1	0,0112	
CRH11	0,0109	0,0063
RBT4	0,0107	0,0046
PGA4	0,0101	0,0206
MSB2	0,0099	0,0052
ENO1	0,0090	
RHD3	0,0080	0,0498
CYP1	0,0079	
YWP1	0,0073	0,0076
UBI4	0,0073	0,0039
RHO3	0,0072	
PGK1	0,0072	
PHR2	0,0067	0,0068
TEF2	0,0064	
PDC11	0,0061	
orf19.2168.3	0,0053	
PLB1	0,0052	0,0020
SAP9	0,0052	0,0008
SUR7	0,0049	
ENG1	0,0049	0,0074
PGA45	0,0046	0,0075
PIR1	0,0045	0,0091
FET99	0,0042	0,0021
orf19.4952.1	0,0042	0,0023
orf19.6741	0,0037	0,0014
CDC42	0,0035	
POR1	0,0035	
TAL1	0,0034	
CYP5	0,0032	0,0027
FET3	0,0032	0,0017
YPT31	0,0032	
GPM1	0,0031	

(Continued)		
PGA52	0,0031	0,0006
YKT6	0,0029	
orf19.1376	0,0027	
HSP70	0,0027	
SAP7	0,0026	0,0025
NUP	0,0025	0,0107
SAH1	0,0025	
SSA2	0,0022	
CHT1	0,0021	0,0022
FMP45	0,0020	
APR1	0,0020	
UTR2	0,0019	0,0098
MET6	0,0017	
HGT7	0,0016	
ZRT2	0,0016	
ALD5	0,0016	
orf19.6119	0,0015	0,0006
MNT1	0,0014	
FET34	0,0013	
KAR2	0,0011	
EFT2	0,0011	
PDI1	0,0011	
ATC1	0,0009	0,0057
FRE10	0,0008	
YPS7	0,0006	
ALS2	0,0003	0,0010
GSC1	0,0002	
orf19.7596		0,0062
SAP10		0,0034
SSR1		0,0030
RBT5		0,0029
SAP99		0,0024
HEX1		0,0023
ALS4		0,0021
SAP3		0,0018
SAP8		0,0018
PHO112		0,0017
orf19.31		0,0017
GCA2		0,0013
ASC1		0,0012
IFF11		0,0011
SAP2		0,0011
RBT7		0,0010
orf19.4886		0,0010
AXL2		0,0008
DFG5		0,0008
RAX2		0,0006
ERO1		0,0004

Figure 4. Protein ranking according to the averaged normalized spectral abundance factor (NSAF) in the EV sample (vesicles) in comparison to the EV-free supernatant sample (secretome). In red: more abundant (detected) proteins; in green: less abundant proteins.

abundant proteins in vesicles were exclusive: the transmembrane protein Pma1, the glycolytic enzymes Tdh3 and the GTPase Rho1 in vesicles.

From the proteins exclusively present in one of the samples, a GO enrichment analysis of vesicle proteins based on their cellular process showed that 23 out of 35 were annotated in response to stimulus and 9 in carbohydrate metabolic process, among others (Supporting Information Table S2). On the other hand, the most interesting processes, where EV-free supernatant exclusive proteins were enriched, were biological adhesion (6 out of 21) and interaction with host (4 out of 21) (Supporting Information Table S2). Furthermore, the GO enrichment analysis based on the functions of exclusive proteins also showed that these functions were significantly

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142−153

different. Whereas GTPase activity was predominant in the vesicle fraction (17%), aspartic-type endopeptidase activity was predominant in the EV-free supernatant fraction (23.8%) (Supporting Information Table S2). Sequence analysis of the exclusive proteins in each sample, revealed that 9 out of 35 proteins from vesicles carried a predicted N-terminal signal peptide, 6 of them had a transmembrane domain, while 19 out of 21 proteins from EV-free supernatant carried it.

Some of the most abundant proteins found in both type of samples, such as Mp65, are described as highly immunogenic antigens.[59] Therefore, in order to test if the vesicle fraction induced a serological response during a systemic candidiasis similar to the secreted proteins in the medium, human sera from patients with candidiasis were used in immunoblots against the EV proteins, the EV-free supernatant and a cytoplasmic protein extract of *C. albicans* (Figure 3C). Proteins with higher molecular masses underwent reaction against sera in both fractions, which was more intense in the EV-free supernatant than in the EV fraction.

Evaluation of Bgl2 as a Vaccine Candidate

The identification of immunogenic proteins in the two fractions, in addition to the detection of Bgl2 in both fractions with a high number of peptides (6 to 11) and the previous work in our lab describing Bgl2p as immunogenic,[20] prompted an analysis of the immunogenic capacity of Bgl2 as a putative vaccine candidate.

As *C. albicans* Bgl2 is a secreted protein, it was expressed in a *P. pastoris* expression system in order to obtain the glycosylated protein form after its purification from the culture supernatant. No heterologous epitopes were fused to the protein to retain its original conformation. The vaccination assays with the purified protein were performed using five groups of mice to test five doses of Bgl2 (0.003, 0.03, 0.3, 3, and 30 μg) and two control groups (candidemia and adjuvant). Mice were inoculated with Bgl2 protein by intraperitoneal injection on day 1 and day 15 of the experiment and they were infected intravenously with a lethal dose of 5×10^5 cells of *C. albicans* SC5314 strain on day 30 after the first immunization (Figure 5A). The infection caused 100% of mortality between days 8 and 15 postinfection in the mice control groups; however, a delay in the death of mice in the groups vaccinated with 0.03 0.3, 3, and 30 μg of protein was observed. After 28 days of infection, two mice (25%) from the 0.3 μg group were still alive, as well as one mouse (10%) immunized with 30 μg or with 3 μg also survived (being the differences between survival curves for those doses and the control curve statistically significant, except for 3 μg) (Figure 5B). The results suggest that *C. albicans* Bgl2 secreted protein is able to confer a certain level of protection against a subsequent invasive candidiasis infection and that the dose of 0.3 μg seems to be the best for this immunization protocol.

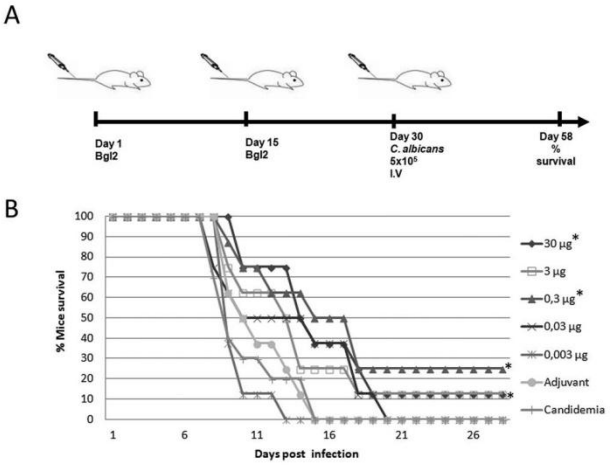


Figure 5. (A) Scheme of vaccination with Bgl2p. Mice (n = 8) were vaccinated in 2-week intervals with different amounts of Bgl2p and with adjuvant as a negative control. All mice, vaccinated and controls, were subsequently infected via the tail vein with SC5314 *C. albicans* 5×10^5 cells. (B) The survival rate of each group is shown: mice vaccinated with 30 μg (white circle), 3 μg (black line), 0.3 μg (asterisk), 0.03 μg (diamond) and 0.003 μg (triangle) of Bgl2p and with adjuvant (black circle). Each experiment was terminated at 28 days postinfection. Doses of 30 and 3 μg showed a $p < 0.05$ in Mantel-Cox test (*).

■ DISCUSSION

Different studies have analyzed the global protein composition of the *C. albicans* secretome under different growing conditions or in *C. albicans* mutants.[24,25,60−62] These works describe the identification of around 80 proteins that mainly comprise cell wall-related proteins, other secreted enzymes involved in pathogenesis or nutrient acquisition, and cytoplasmic proteins. On the other hand, the description of extracellular membrane vesicles as a mechanism of trafficking molecules to the extracellular space in different types of cells including *C. albicans* raises the question of which type of proteins could be carried by them. For this reason we have carried out the first proteomic study of *C. albicans* extracellular proteins that takes into consideration the parallel analyses of EVs and EV-free supernatant preparations. This strategy has rendered the identification of 96 *C. albicans* extracellular proteins, to our knowledge the highest number described to date. Forty of them had never been described in the proteomics analysis of the total extracellular secretome of *C. albicans* under different growth conditions.[24,61,62] They include the presence of 19 proteins detected in EV-free supernatant sample where four cell wall-related proteins (the mannoprotein Dfg5, Iff11, the Orf19.6741 and the predicted GPI-anchored protein Pga52) and five secreted hydrolases or adhesins (Als2, Plb1, Sap2, Sap3 and Sap99) were found among others (Supporting Information Table S1 and S3).

In the EV of *C. albicans* 75 proteins were detected, being the lowest number of EV proteins identified in any fungal species, such as *C. neoformans*, *H. capsulatum*, *S. cerevisiae* and *P. brasiliensis*.[22] This might be the result of different methodological approaches (growing conditions and protein analysis, among others). Interestingly, the types of proteins described as commonly found in at least three fungal species are not largely distinct to the described for *C. albicans* EV: oxidation/reduction components, metabolic proteins, translation-related, stress response, signaling-related and transporting proteins were identified in EVs of all these fungi. However, ribosomal, cytoskeleton and nuclear proteins were not founded in *C. albicans* EV while more adhesins and secreted hydrolases were detected.

EV-Free Supernatant Proteins Are Secreted by the Classical Pathway

A very relevant result of this work is that most of the *C. albicans* proteins (90%) identified in the extracellular medium (free of vesicles) are canonically secreted proteins bearing a signal peptide. A signal peptide was not detected by Signal P in the

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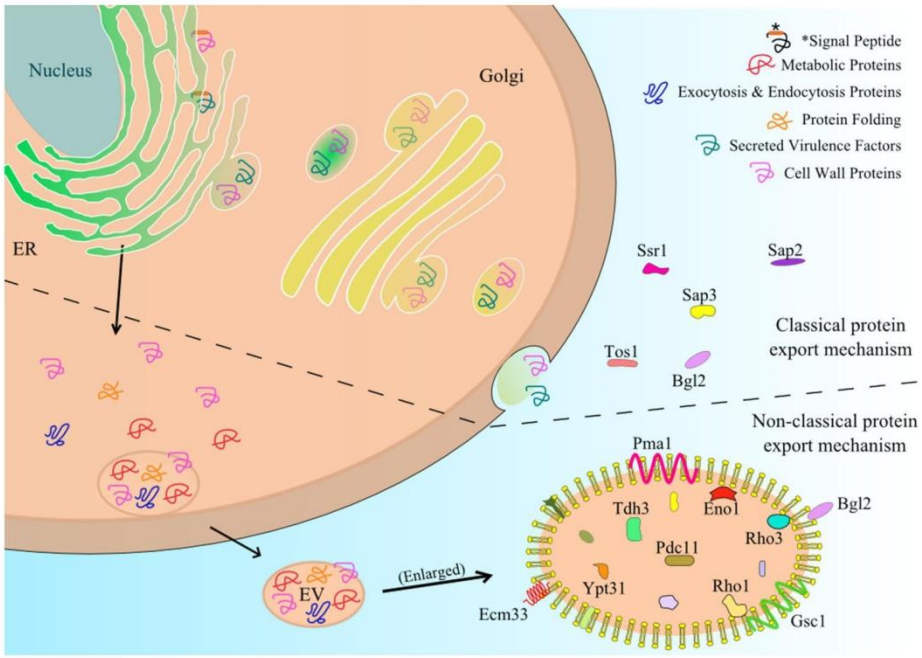

Figure 6. Model of two different *C. albicans* secretory pathways. The classical protein ER−Golgi pathway that secretes proteins with N-terminal signal peptide is shown in the upper part. Underneath, the unconventional secretion mechanism proposed, by which proteins (with and without signal peptide) would be carried to the extracellular medium in vesicles formed at the plasma membrane, is represented. Enlarged view of EV was shown. Representative protein groups and single proteins identified in EVs and EV-free supernatant were shown.

sequence of the other 10% of these proteins (the acid trehalase Atc1, the 40 ribosomal subunit Asc1, the plasma membrane protein Axl2, Orf19.6119, Orf19.6741 and the polyubiquitin Ubi4), but in some of them this is not consistent with previous reports. This inconsistency might be related to bioinformatics clues or sequence errors. In this way, Axl2 was identified exclusively in the EV-free supernatant fraction (not in vesicles), and curiously, it had been previously identified in a genetic screening of *C. albicans* exported proteins. The screening was based on in-frame fusions of N-terminal signal sequences with an intracellular allele of the *S. cerevisiae* invertase gene, a strategy to identify putative secretion signals.[8] In that work, a signal peptide for Axl2 was also detected. Furthermore, the *S. cerevisiae* Axl2 homologue also bears an N-terminal signal sequence.[63] Also, Atc1, detected in the two fractions, has been described as a cell wall-linked acid trehalase containing a hydrophobic N-terminal domain corresponding to a signal peptide.[64] Therefore, the percentage of canonical secreted proteins in the EV-free supernatant would increase to 93% (57 proteins), which suggest that the classical secretory pathway is the general mechanism used for *C. albicans* to secrete EV-free supernatant proteins into the extracellular medium. Interestingly, in the unique report of a different comparative proteomic analysis of vesicles and extracellular vesicles in a pathogenic fungi (*P. brasiliensis*), 70% of proteins are predicted as secretory, but most of them use nonconventional secretory pathways.[22]

It is important to note that the identification of the repertoire of 57 canonically secreted proteins presented in our work represents the most comprehensive analysis of the classical secreted proteins of *C. albicans*. As shown in Supporting Information Table S1, most of the secreted proteins are involved in cell wall synthesis, integrity or remodeling, such as Eng1, Cht1−3 or the glycosidase Phr2, described as located at the cell wall[62,65,66] or at the cell surface.[67,68] From our point of view, some of these proteins might reach the extracellular medium due to the release from the cell wall during its remodeling. On the other hand, some of the identified proteins were categorized as virulence factors.[3] In this way, Als proteins, Ecm33, Mp65, the Saps, glucoamylase (Gca2), Bgl2, Xog1, Plb1, Plb4.5, Phr2 and the protein repressed by Tup1 Rbt5 were included as specialized proteins mediating *C. albicans* adherence to other cells and surfaces or involved in biofilm formation, nutrient acquisition, pH regulators or required for tissue invasion, such as secreted hydrolases and phospholipases.[3,13,69−72]

C. albicans Extracellular Vesicles As Carriers of Cytoplasmic Proteins Including Several Moonlighting Proteins

A very interesting result of our work, shown in Figure 3A and Supporting Information Table S1, is that all of the proteins involved in metabolism were exclusively detected in the vesicle sample (Eno1, the phosphoglycerate mutase Gpm1, Pdc11, Pgk1, the transaldolase Tal1, Tdh3, Met6 and the s-adenosyl-L-homocysteine hydrolase Sah1), as well as all proteins lacking a signal peptide involved in protein folding (the cyclophilin Cyp1, Hsp70, the chaperone Kar2, the protein disulfide-isomerase Pdi1 and the HSP70 family chaperone Ssa2) or protein synthesis (the aspartic proteinase Apr1, the elongation factor Eft2 and the translation elongation factor Tef2). In addition, the metabolic enzymes Tdh3, Eno1, Pdc11 and Pgk1 and the translation elongation factor Tef2 were abundant proteins in vesicles.

The presence of these noncanonical secreted proteins in the cell wall of *C. albicans*, although being controversial, was reported many years ago (revised by Nombela[30] and Chaffin[31]); for example, for the glycolytic enzymes enolase[73] or glyceraldehyde-3-phosphate dehydrogenase[74] and for the

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142−153

chaperones of the Hsp70 family.[75] Interestingly, the identification of glycolytic enzymes, heat shock proteins and elongation factors was also described in the very first proteomic analysis of *C. albicans* cell surface proteins by Urban et al.[19] In this work, the localization of *C. albicans* Tsa1, a secretion-signalless protein, in the cell wall was also correlated to cell morphology. More recently, additional proteomic analyses of *C. albicans* cell-surface proteins (noncovalently attached or extracted by shaving with trypsin) described the identification of these noncanonically secreted proteins.[67,68,76−78] They were also identified in other studies of *C. albicans* or *S. cerevisiae* complete cell wall proteomes.[18,21,79] Furthermore, noncanonically secreted proteins were detected in the extracellular medium, as described by Sorgo[24,61] and in 2D-PAGE-based proteomic studies of proteins secreted by *S. cerevisiae* and *C. albicans* regenerating protoplasts performed many years ago.[17,80] Interestingly, a recent proteomic analysis of glucose-induced changes in the *S. cerevisiae* secretome also described the presence of metabolic enzymes (such as Tdh1, Pgk1 or Pdc1) proteins involved in oxidative stress (such as superoxide dismutase Sod1) and heat shock proteins (such as Ssa1, Hsp82 or Hsp104) in the extracellular medium but associated with membranes in the vesicle-enriched fraction during growth in low glucose,[81] which is in accordance with our data. However, in other pathogenic fungi, such as *P. brasiliensis*, cytoplasmic proteins were detected not only in the vesicle fraction, but also in the extracellular medium in the conditions tested.[22]

The presence of cytoplasmic proteins outside the cell raised two main questions: their mechanism of export and their function in the extracellular space. Some of them could be involved in different roles outside the cell, besides their cytoplasmic function, including Eno1, Pgk1, Tdh3, Gpm1 and Ssa2 among others; thus, they are named moonlighting proteins (a term which, colloquially, means to have more than one job with the second being done at night).[30,82] Interestingly, in bacteria, the most commonly identified moonlighting proteins are also glycolytic and other metabolic enzymes and chaperones having important functions in virulence such as adhesion and modulation of leukocyte activity.[82] Regarding the nonclassical secretion mechanism, several possibilities had been proposed mainly for yeast and mammalian cells.[26,30,83,84] They comprised nonvesicular and vesicular pathways, such as the release of exosomes derived from multivesicular bodies or the export mediated by plasma-membrane shedding of microvesicles.[84] Taking into account our data, at least this last mechanism seems to be used for the leaderless proteins of *C. albicans* to reach the extracellular space. In summary, our data reveal that all the classical cytoplasmic proteins including the moonlighting proteins secreted by *C. albicans* in our growing conditions are cargo of EVs (Figure 6).

Can Determining *C. albicans* Extracellular Vesicles Protein Composition Help to Understand Their Biogenesis?

Taken together, our proteomic data revealed that EVs mainly include the following classes of proteins: conventional secretory proteins, unconventional secretory proteins (classically cytoplasmic) and transmembrane proteins with or without a signal peptide. The most abundant are classical secretory proteins (cell wall related including GPI-anchored and hydrolytic secreted enzymes) also detected in the EV-free supernatant sample (Figures 2A,C and 4). Regarding the integral membrane proteins, in addition to the very abundant plasma membrane ATPase Pma1, there are many more: the essential beta-1,3-glucan synthase subunit Gsc1, transmembrane transporters of ions (Fet3, Fet34, Fet99 and Fre10 for iron and Zrt2 for Zn) or glucose (Hgt7), Pmp45 and Sur7. Furthermore, some of the abundant metabolic enzymes previously mentioned have been described as plasma membrane associated, including Eno1, Pgk1 and Pdc11 as well as the GTPases Rho1 and Rho3.[14,85] Therefore, although different types of *C. albicans* EVs were observed by TEM, the high enrichment in the number and abundance of integral and associated plasma membrane proteins in the purified EVs suggest that at least a relevant part of them are formed from the plasma membrane. They might be formed by shedding (budding directly from the plasma membrane) as proposed for mammalian cells[84,86,87] or by other events involving plasma membrane reshaping during endocytosis as suggested for yeast.[38] Both mechanisms would result (as in the formation of EVs) within the cytoplasmic proteins inside and with the GPI-anchored and periplasmic proteins exposed in the vesicle surface (Figure 6). This model is very suggestive because the proteins needed for cell wall remodeling would be located outside the vesicles and this would allow the degradation and new synthesis of the wall, making easy to traverse. Our data do not definitively support one of these mechanisms. However, five proteins related to exocytosis and endocytosis were identified exclusively in the vesicle sample (Supporting Information Table S1), and further studies to investigate their putative role in the mechanism of EVs would be very interesting. Although we observed the formation of a membrane structure resembling a putative vesicles by TEM that would support the hypothesis of the formation of the EV from the plasma membrane, a relation between these structures and the EV remains to be demonstrated.

C. albicans Vesicles as a Source of New Vaccination Antigens

The relevance of the development of fungal vaccines mainly focused in *Candida* has been recently revised.[88,89] Different entities have been explored for the development of an active vaccine for hematogenously disseminated and mucosal candidiasis. Among them, heat-killed whole organisms or attenuated live organisms render good results. For example the avirulent *C. albicans ecm33* cell wall mutant protected mice from a subsequent lethal infection with virulent strain SC5314 in a systemic candidiasis model.[13,68] However, the use of live, attenuated strains could face relevant challenges regarding safety evaluation by the FDA for approval in humans. Protein based vaccines do not have this limitation and different proteins, peptides or glycopeptides are being tested. Two of them, Als3 and Sap2, are under clinical trials, but none of them is in the market yet.[88,89]

Proteins at the EVs surface are exposed to the host. Furthermore, it is possible that in *C. albicans* infections the EVs were internalized by the macrophage-like cells as it was described for *C. neoformans* infections.[90] Therefore, EVs would be a good source of proteins to be used as vaccine candidates. We selected Bgl2 protein because it was found in the vesicle and the secretome samples and it was previously described as an immunogenic protein by our group.[20] Our results showed that 0.3 μg of Bgl2 protein were able to protect 25% of the mice against hematogenously disseminated candidiasis. Other *C. albicans* proteins evaluated as vaccines candidates, such as

dx.doi.org/10.1021/pr5007944 | J. Proteome Res. 2015, 14, 142−153

Als3, exhibited a slight improvement in survival compared with our data,[46] but different vaccination protocols might improve the percentage of protection obtained in this work. For the recombinant N-terminus of Hyr1p (rHyr1p-N) and Mdh1 a higher level of protection has been also described, but in these cases only 2.2×10^5 or 1.1×10^5 blastospores of *C. albicans* SC5314 were used to produce candidiasis, respectively, making it difficult to compare.[48,91] In any case, Bgl2 protects some of the mice and several of the proteins cargo of *C. albicans* EVs might also represent interesting antigenic proteins.

■ CONCLUSION

Taking into account our data and in accordance with published results, we propose and discuss a model for protein secretion in the studied growing conditions based on the concept that the different types of proteins would be mainly secreted by different mechanisms: proteins in the EV-free medium would be mainly secreted by the classically secretory pathway, and on the other hand, most of the EVs formed from the plasma membrane would carry cytoplasmic and moonlighting proteins, membrane proteins including GPI-anchored proteins and other cell wall-related proteins (Figure 6). These different export mechanisms used in *C. albicans* to secrete proteins are crucial for virulence, host immune response and/or intercellular communication.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1. Strategy followed to recover and identify the EVs and the EV-free supernatant. Table S1. List of proteins identified in *C. albicans* secretome. Table S2. Results of GO Process and Function analysis. Table S3. List of all identified proteins by proteomic analysis of *C. albicans* secretome in different publications. Table S4. Protein identification data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by BIO2012−31767 from the Ministerio de Economía y Competitividad, PROMPT (S2010/BMD-2414) from the Comunidad Autónoma de Madrid, REIPI, Spanish Network for the Research in Infectious Diseases (RD12/0015/0004), and PRB2 (PT13/0001/0038) from the ISCIII. A. Gil-Bona was the recipient of a fellowship from Ministerio de Ciencia e Innovación. C.M.P. was the recipient of a fellowship from the program Continuous training for teachers of the Pontificia Javeriana University (Colombia) in conjunction with COLCIENCIAS-LASPAU University of Harvard. The proteomic analyses were carried out in the Proteomics Unit UCM-Parque Científico de Madrid, a member of ProteoRed network. The Transmision Electron Microscopy was carried out in Centro Nacional de Microscopía Electrónica (ICTS)-UCM. The authors would like to thank M. Monod, Centre Hospitalier Universitaire Vaudois (Switzerland), for the gift of anti-Sap2, to M.L. Gil, Universitat de Vàlencia (Spain), for the anti-Tdh3, and to Fernando Vivanco, Fundación Jiménez Díaz, Universidad Complutense, Madrid (Spain), for the production of Bgl2 antibody. These results are lined up with the Spanish Initiative on the Human Proteome Project (B/DHPP).

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Appendix Chapter 2

Supporting Information Figure S1. (**A**) Strategy followed to recover and identify the EVs and the EV-free supernatant from *C. albicans* culture supernatants for TEM and proteomic analysis. (**B**) Silver stained SDS-PAGE from isolated EVs (Ves), and EV-free supernatant (Sec).

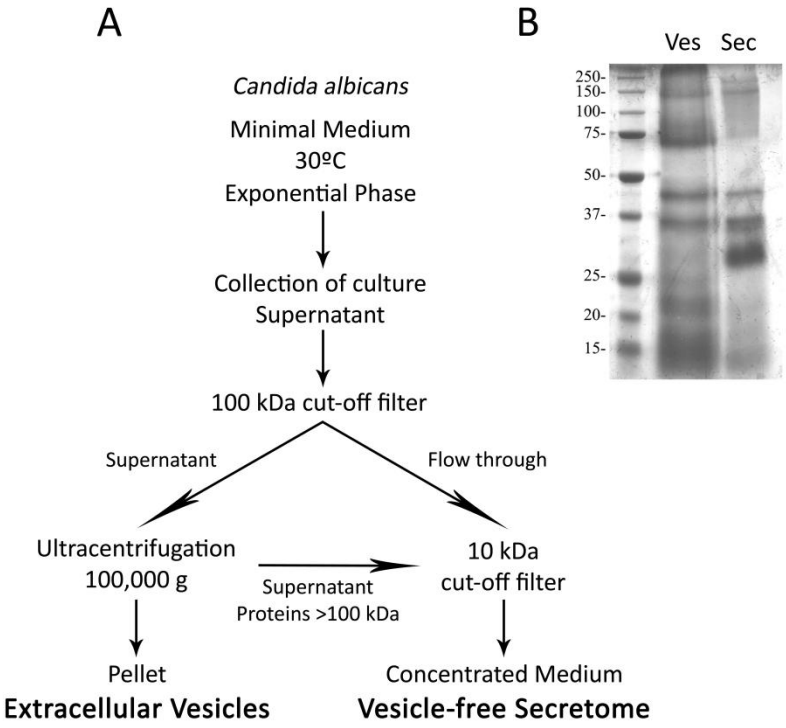


Table S1: List of proteins identified in *C. albicans* SC5314 culture supernatants identified in at least 2 replica with more than 2 peptides in one of them, ordered according their predicted biological process. Extracellular vesicles (vesicles) and extracellular vesicle-free supernatant (secretome) were analyzed separately.

CGD Accesion	Protein name	Description[a]	Replicates with protein (peptides for each replicate)		GPI	Signal Peptide[d]
			Secretome	Vesicles		
CELL WALL-RELATED PROTEINS						
Orf19.6214	ATC1	Cell wall acid trehalase	3 (17,14,17)	2 (5,2)	-	-
Orf19.4565	BGL2	Cell wall 1,3-beta-glucosyltransferase	3 (11,8,7)	3 (6,8,6)	-	Yes
Orf19.7517	CHT1	Chitinase	3 (3,2,3)	2 (3,2)	Yes[c]	Yes
Orf19.3895	CHT2	GPI-linked chitinase	3 (10,9,9)	3 (5,8,7)	Yes[b,c]	Yes
Orf19.7586	CHT3	Major chitinase	3 (7,7,7)	3 (5,6,6)	-	Yes
Orf19.2706	CRH11	GPI-anchored cell wall transglycosylase	3 (14,10,9)	3 (8,9,8)	Yes[b,c]	Yes
Orf19.2075	DFG5	N-linked mannoprotein of cell wall and membrane	2 (4,1)	-	Yes[c]	Yes
Orf19.3010.1	ECM33	GPI-anchored cell wall protein	3 (9,7,7)	3 (5,5,4)	Yes[b]	Yes
Orf19.3066	ENG1	Endo-1,3-beta-glucanase	3 (31,16,21)	3 (6,16,11)	-	Yes
Orf19.6489	FMP45	Predicted membrane protein induced during mating	-	2 (2,1)	-	Yes
Orf19.2929	GSC1	Essential beta-1,3-glucan synthase subunit	-	2 (1,3)	-	-
Orf19.5399	IFF11	Secreted protein required for normal cell wall structure and for virulence	2 (1,2)	-	-	Yes
Orf19.1665	MNT1	Alpha-1,2-mannosyl transferase	-	2 (3,2)	-	Yes
Orf19.1779	MP65	Cell surface mannoprotein	3 (13,11,14)	3 (11,13,12)	-	Yes
Orf19.1490	MSB2	Mucin family adhesin-like protein	3 (9,5,6)	3 (3,7,6)	-	Yes
Orf19.6741	orf19.6741	Putative plasma membrane protein; predicted role in cell wall integrity	3 (6,1,2)	3 (2,3,3)	-	-
Orf19.4035	PGA4	GPI-anchored cell surface protein	3 (15,11,11)	3 (8,7,6)	Yes[b,c]	Yes
Orf19.2451	PGA45	Putative GPI-anchored cell wall protein	3 (7,3,4)	3 (2,5,3)	Yes[b,d]	Yes
Orf19.1911	PGA52	GPI-anchored cell surface protein of unknown function	3 (3,1,1)	2 (4,1)	Yes[c]	Yes
Orf19.6081	PHR2	Glycosidase	3 (11,7,8)	3 (6,8,5)	Yes[b,c]	Yes
Orf19.220	PIR1	1,3-beta-glucan-linked cell wall protein	3 (6,4,3)	3 (1,3,3)	-	Yes
Orf19.7218	RBE1	Pry family cell wall protein	3 (5,4,4)	3 (4,5,5)	-	Yes
Orf19.6202	RBT4	Pry family protein	3 (4,4,4)	3 (4,5,3)	-	Yes

Orf19.5636	RBT5	GPI-linked cell wall protein	3 (2,2,1)	-	Yes[b,c]	Yes
Orf19.5305	RHD3	GPI-anchored yeast-associated cell wall protein	3 (13,9,11)	3 (4,7,3)	Yes[b,c]	Yes
Orf19.3893	SCW11	Cell wall protein	3 (12,9,11)	3 (6,10,9)	-	Yes
Orf19.5032	SIM1	Adhesin-like protein	3 (12,10,11)	3 (9,9,8)	-	Yes
Orf19.7030	SSR1	Beta-glucan associated ser/thr rich cell-wall protein	3 (2,2,2)	-	Yes[b,c]	Yes
Orf19.3642	SUN41	Cell wall glycosidase	3 (9,7,9)	3 (7,6,5)	-	Yes
Orf19.3414	SUR7	Protein required for normal cell wall, plasma membrane	-	3 (4,4,2)	-	Yes
Orf19.1690	TOS1	Protein similar to alpha agglutinin anchor subunit	3 (16,14,14)	3 (12,12,12)	-	Yes
Orf19.1671	UTR2	Putative GPI anchored cell wall glycosidase	3 (14,11,11)	3 (1,3,2)	Yes[b,c]	Yes
Orf19.2990	XOG1	Exo-1,3-beta-glucanase	3 (18,13,12)	3 (4,12,9)	-	Yes
Orf19.6481	YPS7	Putative aspartic-type endopeptidase	-	2 (3,1)	-	Yes
Orf19.3618	YWP1	Secreted yeast wall protein	3 (2,2,2)	2 (2,1)	Yes[b,c]	Yes
SECRETED HYDROLASES AND ADHESINS						
Orf19.1097	ALS2	ALS family protein	3 (5,5,6)	2 (2,2)	Yes[c]	Yes
Orf19.4555	ALS4	GPI-anchored adhesin	3 (3,2,2)	-	Yes[b]	Yes
Orf19.999	GCA2	Predicted extracellular glucoamylase	2 (9,5)	-	-	Yes
Orf19.6673	HEX1	Beta-N-acetylhexosaminidase/chitobiase	3 (12,5,5)	-	-	Yes
Orf19.689	PLB1	Phospholipase B	3 (4,4,6)	2 (6,5)	-	Yes
Orf19.1442	PLB4.5	Phospholipase B	3 (23,12,14)	3 (13,17,14)	Yes[c]	Yes
Orf19.3839	SAP10	Secreted aspartyl protease	3 (7,3,3)	-	Yes[b]	Yes
Orf19.3708	SAP2	Major secreted aspartyl proteinase	3 (5,2,2)	-	-	Yes
Orf19.6001	SAP3	Secreted aspartyl proteinase	3 (4,4,3)	-	-	Yes
Orf19.756	SAP7	Pepstatin A-insensitive secreted aspartyl protease	3 (7,5,5)	3 (2,2,4)	-	Yes
Orf19.242	SAP8	Secreted aspartyl protease	3 (5,3,4)	-	-	Yes
Orf19.6928	SAP9	Secreted aspartyl protease	3 (4,1,2)	3 (7,4,1)	Yes[b,c]	Yes
Orf19.853	SAP99	Putative secreted aspartyl protease	3 (8,3,3)	-	-	Yes
PROTEIN FOLDING						
Orf19.6472	CYP1	Peptidyl-prolyl cis-trans isomerase	-	3 (5,3,3)	-	-
Orf19.7421	CYP5	Putative peptidyl-prolyl cis-trans isomerase	2 (3,3)	2 (2,2)	-	Yes
Orf19.4871	ERO1	Role in formation of disulfide bonds in the endoplasmic reticulum	2 (2,1)	-	-	Yes
Orf19.4980	HSP70	Putative hsp70 chaperone	-	2 (5,2)	-	-
Orf19.2013	KAR2	Similar to Hsp70 family chaperones	-	3 (2,3,1)	-	Yes
Orf19.4952.1	orf19.4952.1	Peptidyl-prolyl cis-trans isomerase activity	2 (2,2)	2 (2,1)	-	Yes

Orf19.5130	PDI1	Putative protein disulfide-isomerase	-	2 (3,2)	-	Yes
Orf19.1065	SSA2	HSP70 family chaperone	-	2 (4,2)	-	-
EXOCYTOSIS AND ENDOCYTOSIS						
Orf19.390	CDC42	Rho-type GTPase	-	2 (2,2)	-	-
Orf19.2843	RHO1	Small GTPase of Rho family	-	3 (5,6,4)	-	-
Orf19.3534	RHO3	Putative Rho family GTPase	-	2 (5,2)	-	-
orf19.2974	YKT6	Putative protein of the vacuolar SNARE complex	-	3 (1,2,1)	-	-
orf19.2622	YPT31	Protein required for resistance to toxic ergosterol analog	-	2 (1,3)	-	-
METABOLISM						
Carbohydrate Metabolism						
Orf19.395	ENO1	Enolase	-	2 (12,1)	-	-
orf19.903	GPM1	Phosphoglycerate mutase	-	3 (2,1,1)	-	-
Orf19.2877	PDC11	Pyruvate decarboxylase	-	2 (9,1)	-	-
Orf19.3651	PGK1	Phosphoglycerate kinase	-	2 (8,1)	-	-
Orf19.4371	TAL1	Transaldolase	-	2 (3,3)	-	-
Orf19.6814	TDH3	NAD-linked glyceraldehyde-3-phosphate dehydrogenase	-	3 (14,1,2)	-	-
Amino Acid Metabolism						
Orf19.2551	MET6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	-	3 (4,1,4)	-	-
Orf19.3911	SAH1	S-adenosyl-L-homocysteine hydrolase	-	2 (6,1)	-	-
TRANSMEMBRANE TRANSPORT						
Ion Transport						
Orf19.4211	FET3	Multicopper oxidase	2 (6,3)	2 (3,5)	-	Yes
Orf19.4215	FET34	Multicopper ferroxidase	-	3 (4,2,1)	-	Yes
Orf19.4212	FET99	Multicopper oxidase family protein	3 (4,4,4)	3 (4,6,5)	-	Yes
Orf19.1415	FRE10	Major cell-surface ferric reductase under low-iron conditions	-	2 (3,1)	-	Yes
Orf19.5383	PMA1	Plasma membrane H(+)-ATPase	-	3 (20,17,14)	-	-
Orf19.1042	POR1	Mitochondrial outer membrane porin	-	2 (3,1)	-	-
Orf19.1585	ZRT2	Zinc transporter	-	2 (2,1)	-	-
Glucose Transport						
Orf19.2023	HGT7	Putative MFS glucose transporter	-	3 (2,1,1)	-	-
Nucleoside Transport						
Orf19.6570	NUP	Nucleoside permease	3 (11,4,4)	3 (1,3,2)	-	Yes

PROTEIN SYNTHESIS						
Orf19.1891	APR1	Vacuolar aspartic proteinase	-	2 (1,3)	-	Yes
Orf19.6906	ASC1	40S ribosomal subunit similar to G-beta subunits	2 (2,2)	-	-	-
Orf19.5788	EFT2	Elongation Factor 2 (eEF2); GTPase	-	2 (3,1)	-	-
orf19.382	TEF2	Translation elongation factor 1-alpha	-	2 (9,3)	-	-
OTHER						
Orf19.5806	ALD5	NAD-aldehyde dehydrogenase	-	2 (3,2)	-	-
Orf19.5292	AXL2	Plasma membrane protein involved in determination of budding pattern	2 (4,2)	-	-	-
Orf19.5063	COI1	Secreted protein	3 (5,4,6)	3 (4,4,3)	-	Yes
Orf19.4688	DAG7	Secretory protein	3 (6,5,6)	3 (5,6,6)	-	Yes
orf19.2168.3	orf19.2168.3	Ortholog(s) have role in cellular protein localization, cytokinesis and other	-	3 (3,1,1)	-	-
Orf19.3727	PHO112	Putative constitutive acid phosphatase	3 (6,2,4)	-	-	Yes
Orf19.3765	RAX2	Plasma membrane protein	3 (8,3,3)	-	-	Yes
Orf19.6771	UBI4	Ubiquitin precursor (polyubiquitin)	3 (4,2,3)	3 (3,3,3)	-	-
UNCHARACTERIZED						
Orf19.1376	orf19.1376	Ortholog(s) have SNAP receptor activity, phosphatidic acid binding activity	-	3 (2,2,2)	-	-
Orf19.31	orf19.31	Uncharacterized	3 (3,2,3)	-	-	Yes
Orf19.4886	orf19.4886	Putative adhesin-like protein	3 (2,1,1)	-	-	Yes
Orf19.6119	orf19.6119	Protein of unknown function	3 (2,2,2)	3 (2,3,1)	-	-
Orf19.7596	orf19.7596	Protein with a phosphoglycerate mutase family domain	3 (9,8,9)	-	-	Yes
Orf19.2681	RBT7	Protein with similarity to RNase T2 enzymes	2 (4,1)	-	-	Yes

[a]Description according to Candida Genome Database (CGD)

[b]GPI-CWP reviewed in Klis *et al.* [89]

[c]Putative GPI proteins that give significant BLAST results by de Groot *et al.* [16]

[d]Signal Peptide predicted by SignalP4.1

*The gray scale indicates proteins never described in the extracellular secretome of *C. albicans*

Remaining Supplemental material is available in the provided CD.

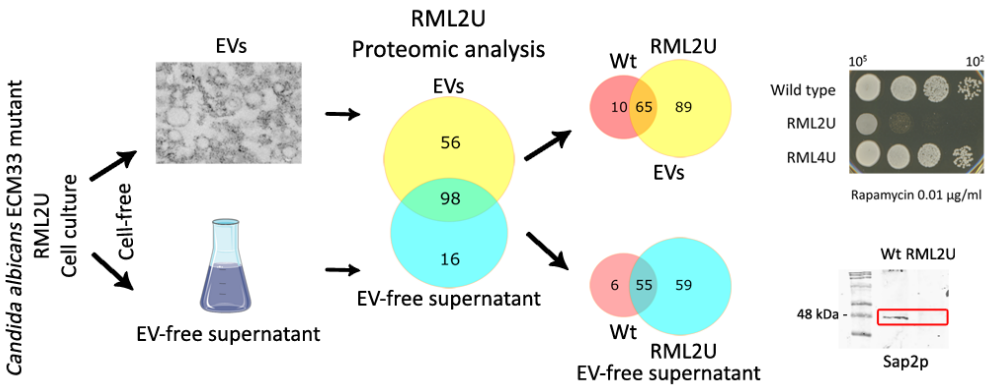
Table S2. Results of GO Process and Function analysis.

Table S3. List of all identified proteins by proteomic analysis of *C. albicans* secretome in different publications

Table S4. Protein identification data.

Chapter 3:

Global proteomic profiling of the secretome of *Candida albicans ecm33* cell wall mutant reveals the involvement of Ecm33 in Sap2 secretion



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Global Proteomic Profiling of the Secretome of *Candida albicans ecm33* Cell Wall Mutant Reveals the Involvement of Ecm33 in Sap2 Secretion

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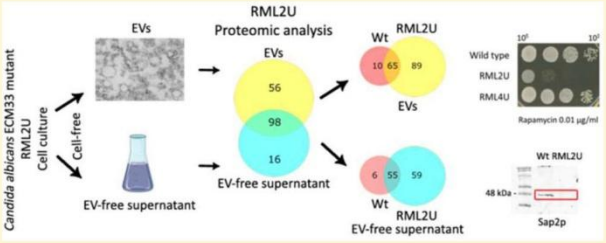
Supporting Information

ABSTRACT: *Candida albicans* secretes numerous proteins related to cell wall remodeling, adhesion, nutrient acquisition and host interactions. Also, extracellular vesicles containing cytoplasmic proteins are secreted into the medium. The *C. albicans ecm33/ecm33* mutant (RML2U) presents an altered cell wall and is avirulent. The proteomic analysis of proteins secreted by RML2U cells identified a total of 170 proteins: 114 and 154 of which correspond to the vesicle-free secretome and extracellular vesicles, respectively. Notably, 98 proteins were common to both samples, and the groups most represented were metabolic and cell wall-related proteins. The results of this study showed that RML2U had an altered pattern of proteins secreted by the classical secretion pathway as well as the formation of extracellular vesicles, including their size, quantity, and protein composition. Specifically, the secretion of aspartic protease 2 (Sap2) was compromised but not its intracellular expression, with bovine serum albumin (BSA) degradation by RML2U being altered when BSA was used as the sole nitrogen source. Furthermore, as recent research links the expression of Sap2 to the TOR (Target Of Rapamycin) signaling pathway, the sensitivity of RML2U to rapamycin (the inhibitor of TOR kinase) was tested and found to be enhanced, connecting Ecm33 with this pathway.



KEYWORDS: *Candida albicans, ECM33, extracellular vesicles, LC−MS/MS analysis, secreted proteins, secreted aspartyl protease (Sap2), rapamycin*

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen of humans that inhabits the microbiota of healthy individuals and can cause superficial infections, such as oral or vaginal candidiasis, and invasive infections in immunocompromised patients.[1] During both infections *C. albicans* uses a wide range of virulence factors and fitness traits, including the formation of biofilms, the morphological transition between yeast and hyphal forms, the expression of adhesins, and the secretion of hydrolytic enzymes.[2,3] Biofilm formation on abiotic or biotic surfaces is connected to mucosal infection and infections that are related to medical devices. Biofilm formation is a very important virulence factor for the establishment of frequent candidiasis highly resistant to antifungal treatment and the host defense mechanism. The adherence of *C. albicans* to medical devices is mediated by cell wall proteins (CWPs) and is the first phase of biofilm formation. One factor that contributes to biofilm formation and to the process of virulence is the expression of hydrolytic enzymes that are most commonly associated with virulence.[4] These enzymes contribute to colonization and infection by degrading host cell membranes to facilitate tissue invasion or to avoid or resist antimicrobial attack by the host. The three most significant families of extracellular hydrolytic enzymes produced by *C. albicans* are the secreted aspartyl proteinases (Saps), phospholipase B enzymes (Plbs), and lipases (Lips). Within the Saps family, only Sap2 is significantly expressed in vitro when *C. albicans* is grown in the presence of bovine serum albumin (BSA) as the sole source of nitrogen, and *sap2* mutants are unable to grow under these conditions.[5] The secretome of *C. albicans*, defined as the fraction of the cell proteome secreted into the medium by the yeast, is composed of a large number of proteins involved in several vital processes. The proteomic analysis of the complete secretome under different conditions showed that *C. albicans* secretes different proteins to adapt to the environment.[6,7] Recent studies have demonstrated that fungal organisms release many molecular components, such as pigments, polysaccharides, lipids, and proteins, into the extracellular medium via extracellular vesicles (EVs).[8−11] These fungal EVs showed a diverse composition and the presence of proteins related to virulence and with immunological activity. Previous studies in

Received: May 13, 2015
Published: August 20, 2015

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

C. albicans showed that EVs transported cytoplasmic and other non-classically secreted proteins as well as cell wall-related proteins.[12,13] Furthermore, inoculation of *Galleria mellonella* larvae with EVs of *C. albicans*, followed by challenge with *C. albicans* yeast cells, enhanced the survival of the larvae.

The fungal cell wall is critical for virulence and pathogenicity, providing adhesive properties and a protective barrier and representing the initial point of interaction between the host and pathogen. The *C. albicans* cell wall is composed of β-1,6-glucan (43−53%), β-1,3-glucan (30−39%), and chitin (2−6%) with different types of attached proteins, including glycosyl-phosphatidylinositol (GPI)-anchored proteins, such as the GPI-linked cell wall protein Ecm33 and Pir proteins.[14,15] Previous studies have demonstrated the importance of Ecm33 in the biogenesis and maintenance of the *C. albicans* cell wall. The *ecm33Δ/ecm33Δ* mutant (RML2U) showed an altered cell wall structure and reduced adherence and damage to host cells. The formation of a catheter-associated biofilm in silicone elastomers was less dense and RML2U was avirulent in a murine model of systemic candidiasis.[16−18] Furthermore, vaccination of mice with the RML2U strain protected them from a subsequent lethal infection with the virulent strain SC5314 in a systemic candidiasis model. The study of the cell surface of RML2U showed fewer cell wall organization- and biogenesis-related proteins at this cellular location.[19]

The lack of virulence of the *ecm33* mutant might be related to a reduced secretion of hydrolytic enzymes, such as Saps and Plbs, among other factors, which contributed to the host-cell damage. To elucidate potential changes in the secretion pattern of virulence proteins from the *ecm33* mutant as well as whether the mutant cell wall was implicated in the release of unexpected cell wall proteins into the medium, the protein composition of the complete RML2U secretome (EV-free medium and extracellular vesicles) was analyzed.

■ MATERIAL AND METHODS

Microorganisms and Culture Conditions

C. albicans SC5314[20] is the wild type of RML2U mutant (*ecm33Δ::hisG/ecm33Δ::hisG ura3Δ::imm434/ura3Δ::imm434::URA3*) and the doubled-complemented strain RML4U (*ecm33Δ::hisG::ECM33-cat/ecm33Δ::hisG::ECM33-cat ura3-Δ::imm434/ura3Δ::imm434::URA3*).[17]The laboratory strain *Saccharomyces cerevisiae* BY474 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (EUROSCARF) was used as control of nonbiofilm formation. Yeast strains were maintained on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar plates at 30 °C. For extracellular proteome experiments, yeast cells were precultured in liquid synthetic defined (SD) medium (20 g/L glucose, 5 g/L ammonium sulfate, 1.7 g/L nitrogen base, and 2.2 g/L amino acids mix) with rotary shaking (200 rpm) at 30 °C, during 7 h. The preculture was used to inoculate flasks containing 1 L of SD medium adjusting OD_{600} to recover the culture at final OD_{600} of 4, 16 h later. For Sap2 activity assays, a single colony from each strain was pregrown overnight at 30 °C in SD medium, washed, and subcultured at an OD_{600} of 0.2 in YCB-BSA medium (23.4 g/L yeast carbon base and 4 g/L BSA, adjusted to pH 4.0 with HCl) and subsequently grown at 30 °C for different times. For biofilm assays Sabouraud liquid medium (1% peptone and 4% glucose) at 37 °C was used.

Isolation of Extracellular Vesicles and Vesicles-Free Secretome

Samples were isolated and analyzed according to Gil-Bona et al.[12] In brief, all of the steps were carried out at 4 °C to avoid proteinase activity and vesicle rupture. Yeast cells were separated from culture supernatants by centrifugation at 5524*g* for 15 min. The resulting supernatants were collected and centrifuged again at 15 344*g* for 30 min to remove smaller debris. The supernatant was collected and concentrated using a Centricon Plus-70 centrifugal filter (cutoff filter 100 kDa, Millipore). The concentrated culture was centrifuged again at 4000*g* for 15 min and 15 000*g* for 30 min to remove smaller debris. The resulting supernatant was ultracentrifuged at 100 000*g* for 1 h. The supernatant was recovered for secretome analysis, and the pellet containing the EVs was washed and centrifuged again at 100 000*g* for 1 h. The pellet was used for the proteomic analysis or it was embedded in a fixative solution (as described later) for electron microscopy analysis. The flow through of the 100 kDa filter and the supernatant recovered from the first ultracentrifugation of the vesicles purification were concentrated approximately 20-fold using a 10 kDa cutoff filter (Millipore) to obtain *C. albicans*-secreted proteins that were soluble in the culture medium. The methanol/chloroform precipitation procedure based on the Wessel et al.[21] protocol was used to precipitate and clean the sample. Three independent biological samples were performed.

Protein Digestion

Two preparations were processed for proteomic analysis: (a) EVs and (b) EV-free supernatant proteome. The concentrated proteins of each sample were resuspended in 0.5 M triethylammonium bicarbonate (TEAB) and quantified using the Bradford protein assay.

Aliquots of 13 μg of vesicles and EV-free supernatant samples were adjusted to the same volume of ammonium bicarbonate (NH_4HCO_3). All samples were reduced by adding 100 mM DTT for 30 min at 37 °C and alkylated with 55 mM iodacetamide for 20 min in the dark. Then, digestion was performed by adding recombinant sequencing-grade trypsin (Roche) 1:20 (w/w) overnight at 37 °C. Subsequently, the produced peptides were cleaned up with a POROS R2 column. The peptides were eluted with 80% acetonitrile (ACN) in 0.1% TFA, dried in a Speed-Vac, and resuspended in 0.1% formic acid. The samples were stored at −20 °C before the nano LC−MS/MS analysis.

LTQ-Orbitrap Velos Analysis

Desalted peptides were concentrated (online) on a C18-A1 ASY 0.1 × 20 mm C18 RP precolumn (Thermo Scientific) and then separated on a Biosphere C18 RP-column [C18, inner diameter 75 μm, 15 cm long, 3 μm particle size (Nano-Separations)] and were eluted using a 150 min gradient (0−140 min from 2 to 35% Buffer B, 140−150 min 35−95% Buffer B. Buffer A: 0.1% formic acid/2% ACN; Buffer B: 0.1% formic acid in ACN) at a flow-rate of 250 nL/min on a nanoEasy HPLC (Proxeon) coupled to a nanoelectrospay ion source (Proxeon). Mass spectra were acquired on the LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) in the positive ion mode. Full-scan MS spectra (m/z 400−1800) were acquired in the Orbitrap with a target value of 1 000 000 at a resolution of 30 000 at m/z 400, and the 15 most intense ions were selected for collision-induced dissociation (CID) fragmentation in the LTQ with a target value of 10 000 and normalized collision energy of 38%. Precursor ion charge-state

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 s.

Protein Identification and Analysis

Protein identification from raw data was carried out using a licensed version of search engine MASCOT 2.3.0 with Proteome Discoverer software version 1.4.1.14 (Thermo Scientific). A database search was performed against the CGD21 database (6221 sequences, 2012). Search parameters were oxidized methionine as variable modification, carbamidomethyl cysteine as fixed modification, peptide mass tolerance 10 ppm, 1 missed trypsin cleavage site, and MS/MS fragment mass tolerance of 0.8 Da. In all protein identification, the FDR was <1%, using a Mascot Percolator, with a q value of 0.01. As an estimation of the relative protein abundances, the normalized spectral abundance factor (NSAF) was used,[22] and the average of the normalized values was calculated.

MS output files were submitted to PeptideAtlas via the PeptideAtlas Submission System (PASS) online submission form with database identifier PASS00408 and to PRIDE through the ProteomeXchange Consortium[23] destock submission tool with identifier PXD000525. The identified proteins have been included into the Proteopathogen Database (http://proteopathogen.dacya.ucm.es).[24]

Bioinformatic Analysis

CGD (www.candidagenome.org) was the database used for the analyses. Proteins identified in at least two replicates with more than two peptides in one of them were used for the analysis. Venn diagrams were prepared using the Venn tool available http://bioinfogp.cnb.csic.es/tools/venny. Signal peptide was predicted using SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP). This program predicts the presence and location of signal peptide cleavage sites in amino acid sequences based on a combination of several artificial neural networks. Selected protein sequences were submitted to Gene Ontology (Go) Term Finder, using the CGD application (http://www.candidagenome.org/cgi-bin/GO/goTermFinder).

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to visualize intact extracellular vesicles from *C. albicans* cells. The pellet obtained by ultracentrifugation was incubated in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 2 h and then fixed in 4% paraformaldehyde, 1% glutaraldehyde, and 0.1% PBS overnight at 4 °C. Samples were incubated for 90 min in 2% osmium tetroxide (TAAB Laboratories, U.K.), serially dehydrated in ethanol, and embedded in EMBed-812 resin (Electron Microscopy Sciences). Thin sections (50−70 nm) were obtained by ultracut and observed in a JEOL JEM 1010 transmission electron microscope operating at 100 kV, and pictures were taken with a Megaview II camera. TEM images were analyzed with Soft Imaging Viewer Software.

SDS-PAGE and Western Blotting

C. albicans cytoplasmic extract was obtained by suspending cells in cold lysis buffer (7 M urea, 2 M tiourea, 2% CHAPs, 1 mM PMSF) and lysed mechanically in a fast-prep cell breaker with glass beads. Cells extracts were clarified by centrifugation, and the supernantants were stored at −80 °C. The protein concentration was measured by Bradford protein assay. Cell extracts, EVs, and EV-free supernatant samples (10 μg of each) were separated by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was stained with Coomassie blue (Quick Coomassie Stain, Generon). For silver staining, the gel was destained, fixed (40% MeOH, 10% acetic acid v/v), and stained by using the Silver Stain Kit (Bio-Rad). For Western blotting, 10% SDS-polyacrylamide gels were transferred to nitrocellulose membranes and blocked in 5% milk. Reversible Ponceau staining was applied to check equal protein quantity loading of gels and transferring to the membranes. Western blots were probed with anti-Sap2 (a gift from M. Monod, Centre Hospitalier Universitaire Vaudois, Switzerland) at 1:3000 and then with fluorescently labeled secondary antibodies: 1/2000 IRDye 800 goat antirabbit IgG (LI-COR Biosciences). The Western blotting was performed with the Odyssey system (LI-COR Biosciences, Nebraska). To detect BSA in the medium, 20 μL of the supernatant was loaded onto a 12% SDS-PAGE gel, and proteins were visualized with Coomassie staining.

Biofilm Quantification by Crystal Violet Assay (CV assay)

Peeters et al.[25] method was used. In brief, overnight Sabouraud cultures of strains were washed and diluted in 10^7 CFUs/mL. Twenty-four wells of a round-bottomed polystyrene 96-well microtiter plate (Greiner Bio-One, GBO) were inoculated with 100 μL of these dilutions, and 24 control wells were filled with sterile medium. Following 4 h of adhesion, the supernatant (containing nonadhered cells) was removed from each well, and plates were rinsed using 100 μL of PBS. Subsequently, 100 μL of fresh medium was added to each well, and the plates were further incubated for 24 h. After incubation time, biofilms were fixed by adding 100 μL of 99% methanol (15 min), after which supernatants were removed and the plates were air-dried. Then, 100 μL of CV 3 M was added to all wells. After 20 min, the excess of CV was removed by washing the plates with water. Finally, bound CV was released by adding 150 μL of 33% acetic acid. The absorbance was measured at 595 nm.

Cell Susceptibility to Rapamycin

After growing overnight in YPD broth, cells were collected and washed. Five microliters of 10-fold serial dilutions were spotted onto YPD agar plates containing 0.01 μg/mL rapamycin prepared as a 10 μg/mL stock by dissolution in methanol or YPD plates made with the same volume of methanol (as a control). Cell viability was observed after incubation at 30 °C for 24 h. This assay was performed independently three times.

Sensitivity to Zymolyase

Exponentially growing cells were harvested and resuspended at an OD_{600} between 0.4 and 0.5 in 10 mM Tris-HCl, pH 7.5, 5% glucose, containing 60 units of zymolyase 100T to measure the inhibition of growth caused by zymolyase. The same conditions were used by the control wells without the enzyme. The assay was performed using duplicate rows of a 96-well plate, and cells were incubated at 37 °C. Decrease in optical density was monitored over 180 min period. The graph represents the percentage of growth taking as 100% the value at the beginning of the experiment. Experiments were performed in triplicate.

■ RESULTS

Proteomic Analysis of the RML2U Secretome

Studies of the *ecm33Δ/ecm33Δ* mutant (RML2U) have revealed cell wall defects and fewer proteins involved in cell wall organization and biogenesis on the cell surface than the corresponding wild-type strain.[16,17,19] The potential migration

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

Table 1. Categorization of Proteins Identified in the *C. albicans* RML2U Extracellular Secretome (EV-Free Supernatant and EVs)[a]

categories	common in EV-free supernatant and EVs	only identified in EV-free supernatant	only identified in EVs	total proteins
cell-wall-related proteins	30	2	8	40
	Atc1, Bgl2, Cht1, Cht2, Cht3, Crh11, Eng1, Kre9, Mp65, Msb2, Orf19.6741, Pga4, Pga45, Pga52, Phr2, Pir1, Rbe1, Rbt4, Rhd3, Scw11, Sim1, Ssr1, Sun41, Sur7, Tos1, Tsa1, Utr2, Xog1, Yps7, Ywp1	Dfg5, Rbt5	Dcw1, Exg2, Fmp45, Gsc1, Pga17, Rot1, Srb1, Tma19	
secreted hydrolases and adhesins	9	7	0	16
	Als4, Hex1, Orf19.1765, Orf.19.3499, Plb1, Plb4.5, Sap3, Sap7, Sap9	Als1, Als2, Gca2, Orf.5126, Sap10, Sap8, Sap99		
protein folding	3	1	3	7
	Cyp1, Cyp5, Ssa2	Ero1	Hsp90, Kar2, Ssb1	
exocytosis and endocytosis	0	0	2	2
			Ras1, Rho1	
metabolism	25	0	14	39
	Adh1, Ahp1, Cdc19, Eno1, Fba1, Fdh1, Fdh3, Gdh3, Glx3, Gnd1, Gpm1, Grp2, Hxk2, Ipp1, Mdh1−1, Met6, Pdc11, Pgk1, Sah1, Sam2, Spe3, Tal1, Tdh3, Tpi1, Ynk1		Aco1, Act1, Adh2, Ado1, Bmh1, Car2, Cit1, Hom6, Ilv5, Lys22, Pgi1, Snz1, Thr4, Tkl1	
transmembrane transport	8	0	8	16
	Atp1, Atp2, Fet3, Fet34, Fet99, Nup, Pet9, Pma1		Ctr1, Fre10, Gnp1, Het1, Hgt7, Nce102, Por1, Zrt2	
protein synthesis	9	2	12	23
	Apr1, Arf2, Asc1, Orf19.3572.3, Rpl12, Rpl6, Rpl82, Rps19a, Tef2	Rpl14, Rpp2a	Cef3, Eft2, Kex2, Pmm1, Rpl15a, Rpl35, Rps14b, Rps18, Rps24, Rps6a, Tif, Yst1	
other	9	1	5	15
	Ald5, Axl2, Cof1, Coi1, Crd2, Dag7, Rax2, Trx1, Ubi4	Pho113	Acb1, Ntf2, Orf19. 2168.3, Pho112, Pho8	
unknown	5	3	4	12
	Orf19.3378, Orf19.4886, Orf19.6119, Orf19.6484, Orf19. 7596	Orf19.31, Orf19.6160, Pbr1	Orf19.1376, Orf19.2460, orf19.4150, Orf19.86	
total proteins	98	16	56	170

[a]Proteins were classified into the nine designated categories according to their predicted biological process.

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DOI: 10.1021/acs.jproteome.5b00411
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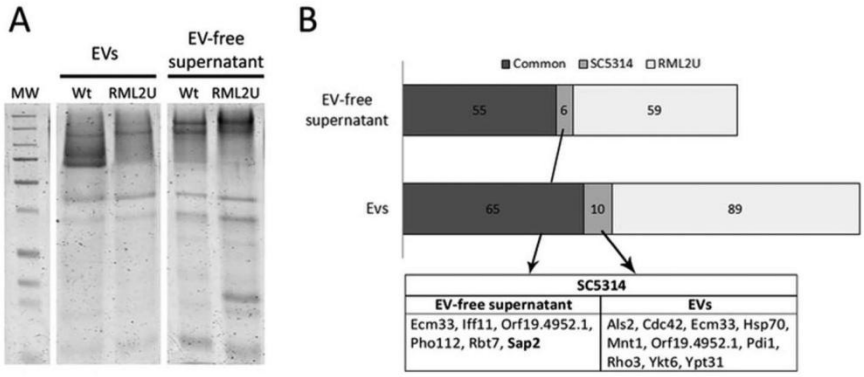

Figure 1. Comparison of the extracellular proteomes of RML2U and SC5314 (A) 1-DE of EVs and EV-free supernatant samples from SC5314 (wild type) and RML2U. Proteins were separated using SDS-PAGE and stained with Coomassie blue. (B) Total number of identified proteins in two or more replicates with at least two peptides in one of them in the different samples (EV-free supernatant and EVs) and strains (dark gray: common proteins identified in both strains; light gray: proteins identified only in SC5314; white: proteins identified only in RML2U). Proteins identified exclusively in strain SC5314 are listed below.

of these proteins into the extracellular medium through the weak cell wall and the potential alterations in the pattern of secretion of virulence proteins were the objectives of this study. The proteomic analysis of RML2U extracellular vesicles and vesicle-free supernatant was performed according to the procedure published by Gil-Bona et al.[12] The strain was grown in SD medium at 30 °C, which were optimal conditions for this strain because it grows slowly at 37 °C (data not shown). Secreted proteins were first separated from the cells by centrifugation. The EVs secreted by RML2U were subsequently recovered from the culture supernatant and separated from the rest of the secreted material using a 100 kDa cutoff filter and by ultracentrifugation. The ultracentrifuged supernatant and the filter flow-through were concentrated using a 10 kDa cutoff filter and represented the EV-free supernatant. The EVs and EV-free supernatant samples were digested with trypsin, and three independent biological replicates were analyzed by LC–MS/MS (Orbitrap). In addition, EVs were observed by TEM. Spherical and bilayer structures with a heterogeneous size were detected (Figure S1A), having a mean observed size of 60–150 nm.

The proteomic study identified a total of 170 single proteins in two or more replicates with at least two peptides in one of them, corresponding to 114 proteins for the EV-free supernatant and 154 proteins for the EVs (Table S1). The classification of the identified proteins concerning the biological processes in which they are involved showed that cell wall-related proteins and metabolic proteins were the most represented in both fractions (Table 1). No exocytosed- or endocytosed-related proteins were detected in the EV-free supernatant, but secreted hydrolases and adhesins were more abundant in this fraction. Classic cytoplasmic proteins, such as those involved in metabolism, protein synthesis and degradation, transmembrane transport, and protein folding were predominantly, but not exclusively, identified in EVs. Nonetheless, Gene Ontology (GO) Term Finder by biological process enrichment analysis performed at CGD showed that EVs contained more proteins involved in processes such as host interaction, carbohydrate metabolism, cell wall organization or biogenesis, pathogenesis, and growth. Biological adhesion-related proteins were more represented in the EV-free supernatant sample, and proteins related to biofilm formation were enriched only in this sample (Table S2).

The comparative analysis of proteins performed by Venny analysis showed that 98 of the detected proteins were identified in both fractions, with only 16 exclusive proteins remaining in the EV-free supernatant and 56 proteins in the EVs (Table 1). Notably, among the proteins that were exclusively detected in the EV-free supernatant, seven were secreted hydrolases and adhesins (Als1, Als2, Gca2, Orf19.5126, Sap10, Sap8, and Sap99), two were cell wall-related proteins (Dfg5 and Rbt5), and three proteins had an unknown function (Orf19.31, Orf19.6160, and Pbr1). In contrast, eight cell wall-related proteins (Dcw11, Exg2, Fmp45, Gsc1, Pga17, Rot1, Srb1, and Tma19), including three GPI proteins, were detected in EVs and not in the EV-free supernatant. Furthermore, classical cytoplasmic proteins were common in both fractions or were exclusive to EVs; with the exception of Rpl14, which was only identified in the EV-free extract (Table 1).

The prediction of signal peptides using the bioinformatics tool SignalP 4.1 showed that 53% of the proteins detected in the EV-free supernatant (60 proteins) contained a predicted N-terminal signal peptide, whereas only 38% of EVs proteins (60 proteins) possessed a predicted N-terminal signal peptide for secretion via the classical secretory pathway; 47 proteins were common to both samples (Table S1).

Comparison of the Extracellular Proteomes of RML2U and SC5314

The secretome, including EVs, is a key component of the infection strategy. Because the RML2U strain was described as avirulent in a murine model of infection, the second goal of this study was to analyze the differences in the pattern of secreted proteins between the wild type, SC5314, and RML2U under the same growth conditions. The recently published SC5314 data from our laboratory[12] were used to compare the protein profile of both samples (EV and EV-free supernatant). In that study, a total of 96 proteins were identified in the complete extracellular secretome. To identify differences between the extracellular secretome of the wild-type strain and the mutant strain, a comparative analysis was carried out and a 1-DE analysis of the overall protein profile of the samples was performed. The protein profile differed between the strains (Figure 1A): high molecular proteins were detected in SC5314 and RML2U EVs, whereas more bands were detected in the

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270–4281

RML2U EV-free supernatant than in SC5314 within the lower molecular weight range.

Comparison of RML2U EVs Proteins and Those of SC5314. The SC5314 and RML2U EVs were compared by TEM, and similar structures were detected; however, the number and size of EVs differed considerably between the strains. The RML2U EVs were more abundant and had a different size distribution in the samples observed (Figure S1B). The RML2U strain had 38% more EVs than SC5314 under the same growth conditions. The sizes observed were heterogeneously distributed, but the mutant strain had larger EVs.

The number of proteins detected by LC−MS/MS was significantly different in EVs from both strains. Proteomic analysis identified a total of 164 single proteins: 75 and 154 in SC5314 and RML2U, respectively (Figure 1B and Table S3). From these, 89 proteins were detected only in the RML2U sample, 65 proteins were identified in both strains, and only 10 proteins were exclusive to SC5314 (Figure 1B and Table S3). Among these 10 proteins, Ecm33 was identified, as expected, together with another cell wall related protein, Mnt1p, the alpha-1,2-mannosyl transferase implicated in cell-wall mannoprotein biosynthesis. The other eight proteins were related to exocytosis and endocytosis (Cdc42, Rho3, Ykt6, and Ypt31) or were folding-related proteins (Hsp70, Pdi1, and the Orf19.4952.1, an orthologue of *S. cerevisiae FPR2* that binds to the drugs FK506 and rapamycin[26]), and Als2, a secreted hydrolase involved in adhesion and biofilm formation, which was, however identified in the mutant secretome.

The number of classical intracellular proteins was much higher in EVs from RML2U than those from SC5314, including metabolic, transmembrane transport, and protein synthesis and degradation proteins (Figure S2A). A large number of proteins of unknown function was present among the RML2U proteins (nine proteins). Furthermore, the differential proteins in the RML2U EVs included five secreted hydrolases and adhesins (Als4, Hex1, Orf19.1765, Orf3499, and Sap3), 59 classical cytoplasmic proteins related to protein folding, metabolism, transmembrane transport, and protein synthesis and nine cell wall-related proteins (Dcw1, Exg2, Kre9, Pga17, Rot1, Srb1, Ssr1, Tma19, and Tsa1). Some of these cell wall-related proteins are involved in β-glucan synthesis and assembly. In addition, the predicted presence of signal peptide cleavage sites revealed that 62% (96 proteins) from the vesicle fractions of the mutant lacked signal peptide, which was close to the estimated value for SC5314 (60% proteins without predicted signal peptide).

Comparison of RML2U EV-Free Supernatant Proteins with Those of SC5314. Bioinformatic analysis revealed that 120 proteins were identified in total in the soluble extracellular secretomes of SC5314 and RML2U, which contained 61 and 114 proteins, respectively; 55 proteins were common to both strains (Figure 1B and Table S3). A comparison of the involvement of the identified proteins in different biological processes showed that cell wall-related proteins and secreted hydrolases and adhesins were the most-represented categories in both strains (Figure S2B); however, metabolic proteins comprised the second most-abundant group in the RML2U sample, and no proteins from this category were detected in the SC5314 strain. Furthermore, all categories were represented by more proteins in RML2U and fewer proteins in SC5314. Six proteins were unique for the SC5314 sample, including two cell wall-related (Ecm33 and Iff11) and four additional proteins (Orf19.4952.1, Pho112, Rbt7, and Sap2) (Figure 1B and Table S3), two of which, Sap2 and Ecm33, are related to biological adhesion and pathogenesis.[17,27] In contrast, four cell-wall-related proteins were only detected in RML2U (Kre9, Sur7, Tsa1, and Yps7).

The GO Biological process enrichment analysis showed that the EV-free supernatant from RML2U was statistically enriched in proteins involved in more processes, including those related to biological adhesion, host interaction, pathogenesis and carbohydrate metabolism (Table S4). The RML2U fraction was also enriched in proteins from other categories, including glucose metabolism, catabolic processes and glycolysis, which are not represented in the SC5314 GO Term Finder analysis. Furthermore, proteins related to biofilm formation were enriched, with nine proteins in SC5314, including Ecm33 exclusively and 12 in RML2U, including Sur7, Adh1, Als1, and Pbr1.

Sap2 Extracellular and Intracellular Analysis

The major secreted aspartyl proteinase has been described as Sap2, which is the main contributor to endothelial cell damage,[28,29] although the relative contribution of Saps to the pathogenicity of *C. albicans* is controversial.[30] Sap2 was detected in the SC5314 EV-free supernatant but not in the RML2U extracellular medium. To confirm the LC−MS/MS data, the absence of this protein in the EV-free supernatant sample was confirmed by Western blot with antibodies raised against Sap2, which specifically recognized this protein in the EV-free supernatant samples of SC5314 and RML2U (Figure 2A). The protein was detected in the SC5314 sample and not in that of RML2U, confirming that this enzyme was not secreted by the mutant.

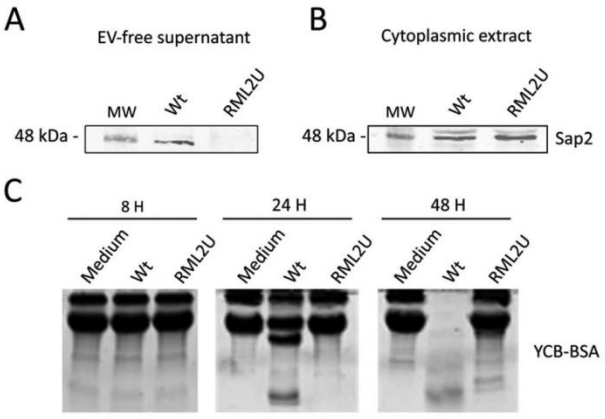

Figure 2. Sap2 is produced intracellularly but is not secreted by RML2U, preventing growth in medium with BSA as the major nitrogen source. Expression of extracellular (A) and intracellular (B) Sap2 (42.3 kDa) in SC5314 (wild type) and RML2U under the same growth conditions was detected by Western blotting. (C) BSA degradation after 8, 24, and 48 h of strain incubation in YCB-BSA medium. Each cell supernatant was analyzed by SDS-PAGE, and proteins were visualized by Coomassie blue staining. Intact BSA (66 kDa) in medium without yeast was used as a control.

Because Sap2 is the major secreted protease in vitro, intracellular Sap2 expression was analyzed to eliminate problems of protein expression. Intracellular protein production was analyzed by Western blotting under the same growth conditions used for proteomic samples. The presence of intracellular Sap2 in *C. albicans* wild type and mutant was

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

confirmed. This suggests that under this growth condition RML2U produced Sap2 but did not secrete it into the extracellular medium (Figure 2B).

The protease Sap2 is required by *C. albicans* for growth in medium with BSA as the sole nitrogen source, and this medium induces its expression.[5,31,32] The method described by Staib et al.[5] was used to correlate the secretion of Sap2 by RML2U with BSA degradation. Cells of *C. albicans* were grown in yeast carbon base (YCB) medium supplemented with BSA, and the supernatants were analyzed by SDS-PAGE. The degradation of BSA by RML2U was almost undetectable after 24 h of incubation compared with that by SC5314 (Figure 2C). After 48 h of incubation, BSA was digested by the SC5314 strain and peptides or low-molecular-weight proteins derived from BSA degradation were still apparent, whereas BSA was only slightly degraded by RML2U. Experiments were performed at least three times with identical outcomes. These results suggest that the BSA utilization of the RML2U strain is not efficient as that of the SC5314 strain.

Rapamycin Sensitivity Study

The TOR (target of rapamycin) signaling pathway has been described to regulate *SAP2* expression in *C. albicans*.[33] Rapamycin is an inhibitor of Tor kinase and forms a complex with FKBP12, a 12 kDa protein.[34] To analyze a putative relationship between RML2U, which is defective in Sap2 secretion, and a deficiency in the TOR signaling pathway due to the *C. albicans ECM33* deletion, the SC5314, RML2U, and *ECM33*-reintegrant (RML4U) strains were spotted onto agar plates with or without rapamycin. The RML2U strain showed rapamycin hypersensitivity compared with SC5314 (Figure 3A) and the RML4U strain could rescue the rapamycin hypersensitivity phenotype, showing the same rapamycin resistance phenotype as the SC5314 strain.

Biofilm Formation in RML2U

The cell wall is the initial point of contact in the interaction between *C. albicans* and the inert material and host tissues.[35] An important factor in *C. albicans* virulence is its ability to form biofilms on abiotic or biotic surfaces.[3] The *SAP2* gene is highly up-regulated in biofilms grown in vitro, as shown by real-time PCR expression profiling.[4] The secretion data showed that cell wall proteins are increased in the extracellular medium of RML2U and the secretion of Sap2 was affected, and considering the modestly reduced adherence of the mutant to endothelial cells and to the FaDu oral epithelial cell line,[17] the ability of this strain to form biofilms in polystyrene material was evaluated using microtiter plates. For this purpose, SC5314 was used as a wild-type strain that was capable of forming biofilms, and the strain BY4741 of *S. cerevisiae* was used as a negative control of biofilm formation. Three different experiments were performed. The mean absorbances obtained confirm the inability of RML2U to form biofilms (Figure 3B) because the absorbances shown by the mutant were similar to those of the *S. cerevisiae* strain, which was unable to form biofilms.[36] The RML4U strain recovered the ability to form biofilms.

Effect of Zymolyase on RML2U

Several cell wall proteins were detected only in RMLU2 samples and not in SC5314 (Table S3), such as Sur7 (required for normal cell walls) or Kre9, Exg2, Rot1, and Ssr1 (β-glucan associated proteins). To relate the presence of these proteins in the extracellular medium of the mutant with its aberrant cell wall organization,[16,17] a sensitivity assay to zymolyase, an

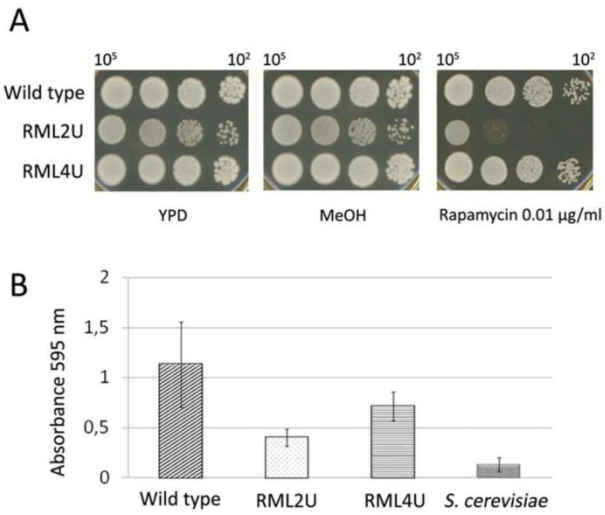

Figure 3. Deletion of the *ECM33 C. albicans* gene increases susceptibility to rapamycin and prevents biofilm formation. (A) 10-fold serial dilutions of strains SC5314 (wild type), RML2U, and RML4U (*ecm33Δ::ECM33/ecm33Δ::ECM33*) were spotted onto YPD with 0.01 μg/mL rapamycin or with MeOH (as control). Plates were incubated at 30 °C for 24 h. Data are representative of at least three independent experiments with identical results. Cell numbers are shown at the top of the panels. (B) Quantification of biofilm biomass formation. Mean absorbance (DO_{595}) obtained for SC5314 (wild type), RML2U, RML4U, and *S. cerevisiae* BY4741 (control for no biofilm formation). Error bars indicate standard deviations of three independent experiments using the CV assay. All show a $p < 0.05$.

enzymatic cocktail mainly consisting of β-1,3-glucanase, which acts on fungal cell walls, was performed. The RML2U strain displayed a higher sensitivity to zymolyase than the wild type (Figure 4A). The control wells, which contained the strains in the same buffer without zymolyase, showed a slight increase in the growth of both strains (data not shown). Cells were also observed by inverted microscopy at different times of treatment. A large amount of debris was observed after 15 min of treatment of RML2U with the enzyme and from 150 min onward, no cells were observed, whereas no debris were observed in the control wells. A slight increase in debris was observed after 30 min for SC5314, and no damaged cells were observed at any of the studied time points (Figure 4B).

■ DISCUSSION

RML2U Extracellular Proteome Is Enriched in Metabolic and Cell Wall-Related Proteins

This study analyzed the complete secretome of the *C. albicans ecm33Δ/ecm33Δ* cell wall mutant strain RML2U by proteomic analysis. For this purpose, the analysis of EVs and EV-free supernatant was performed separately. In total, 170 single proteins were identified and studied. The majority of the proteins detected had no predicted signal-peptide sequence (99 out of 170) and were usually annotated as being cytoplasmic, corresponding to 50 proteins detected in both samples and 45 or 3 proteins detected only in EVs or in the EV-free supernatant, respectively. These results differ from those of previous studies of secreted proteins and vesicles from wild-type strains of *C. albicans*, in which most proteins without signal peptide were described as being secreted by nonclassical pathways, specifically by extracellular vesicles, and were not

DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

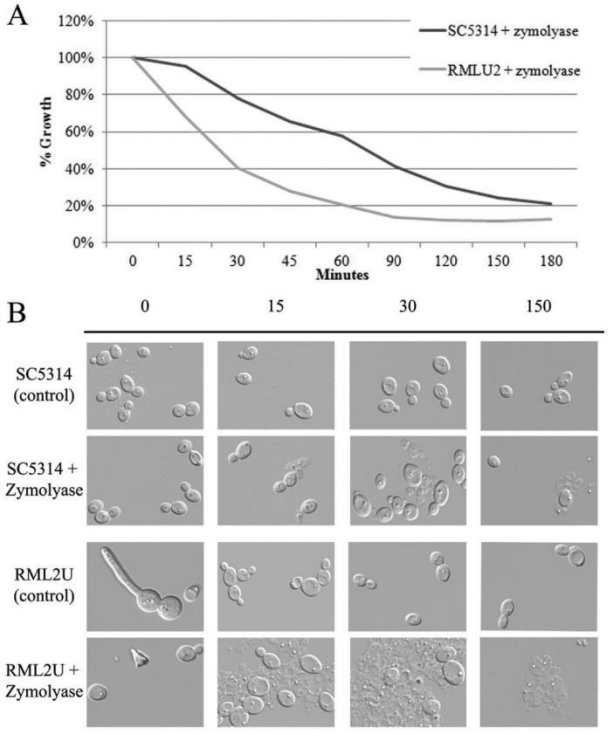

Figure 4. Susceptibility to zymolyase. (A) Strains SC5314 and RML2U were grown in the presence of Zymolyase 100T for 180 min starting with an OD_{600} between 0.4 and 0.5. OD_{600} was monitored every 15 min, and growth is depicted as the percentage of growth compared with growth without the enzyme. (B) Nomarski images at different time points (0, 15, 30, and 150 min). SC5314 (wild type) and RML2U, marked as control, correspond to the cell culture without zymolyase.

detected in the EV-free supernatant.[12,13] The classically intracellular proteins detected in the RML2U secretome correspond to ribosomal proteins, such as those in *RPL*, *RPS*, and *RPP* families and metabolic proteins, such as Atp1−2, Eno1, and Fba1, among others. Some of these ribosomal and metabolic proteins were identified previously in SC5314 on the surface,[37] and defects in the cell wall integrity of RML2U were also observed previously,[16,17] and here the sensitivity to zymolyase was verified. This aberrant cell wall organization might cause problems in the anchoring of these proteins to the cell wall, thus enriching their presence outside the cell. Furthermore, in a previous analysis of the RML2U surfome,[19] the total number of proteins identified on the RML2U surface was twice as many as that on SC5314 (as was observed in the EV-free supernatant and similar to the EVs results), including many nontypical cell surface proteins, which agrees with this hypothesis. A comparison of the surfome data and those of this study showed that only 28 proteins were common to all fractions (data not shown) and most of them were metabolic proteins. Notably, only three cell wall-related proteins that were detected in the RML2U EVs and EV-free supernatant were found in the surfome (Eng1, Msb2, and Tsa1), with a remaining 38 cell wall-related proteins detected in the complete extracellular secretome (EVs and EV-free supernatant) and not in the surfome. Cell wall proteins that were detected on the surface of wild-type *C. albicans*, such as Bgl2, Cht1−3, Crh11, or Utr2, among others, were not detected in the surface of the mutant and were identified in its extracellular secretome. It is reasonable to assume that proteins that were present in the extracellular medium were initially localized at the cell wall, as they need to pass through this structure; however, the amount of these proteins at the cell wall might be low. Thus, one possible explanation for the nondetectability of these proteins on the cell surface is that the proteomic analysis in the previous study was less sensitive and has been improved in this study by using LTQ-Orbitrap Velos, an ultra-high-resolution mass analyzer. This reason together with a potentially minor presence of these proteins on the RML2U surface might explain why they were not detected. In addition, other studies that analyzed the effect of fluconazole on the secretome of *C. albicans* observed that the secretome of the treated cells had an increased number of proteins with a predicted intracellular localization;[7] however, although some of these proteins were also found in this study, such as Ald5, Arf2, Fba1, Het1, or Pgi1, RML2U secretes more classical cytoplasmic proteins, suggesting that the traffic through the cell wall is higher than in cells treated with antifungal drugs.

ECM33 Deletion Strongly Influences the Composition of the Extracellular Proteome

Changes in the Pattern of Immunoreactive and Virulence Proteins. Several immunogenic proteins were recognized by antibodies from a murine model of candidiasis (with *C. albicans* wild type), but antibodies in sera from mice vaccinated with the RML2U mutant recognized even more immunogenic proteins. Many of these immunoreactive proteins were detected in vesicles or in the EV-free supernatant, and some are involved in virulence, such as Asc1, Cdc19, Eno1, Fba1, and Hsp90, and might be useful diagnostic and prognostic biomarkers.[38−46] These proteins were more exposed to the medium in the RML2U strain; this might explain why they were more accessible to cells of the host immune system and the ability of the RML2U strain to generate a protective response in a vaccination assay against a lethal dose of *C. albicans* in mice.[19] Different proteomic approaches identified Fba1 as an antigen that induces the protective IgG2a antibody isotype in the sera from mice vaccinated with RML2U and detected Fba1p antibodies in immune sera from mice with systemic candidiasis and also in the serum of systemic candidiasis patients during human infection.[19,47−49]

Als1 and Ssa2, proteins that are related to virulence, were also detected only in RML2U EV-free supernatant sample. The cell-surface adhesion and biofilm-induced protein Als1 is abnormally distributed on the surface of RML2U[17] and was detected in the RML2U EV-free supernatant but not in SC5314, probably as a result of its abnormal trafficking. The presence of Ssa2 at the cell wall was previously described.[50] As previously discussed, more proteins were identified in the RML2U samples, and among them, several virulence-related proteins have been detected, which might be due to a general problem in their anchorage to the cell wall. However, considering the increased secretion in the RML2U strain, the fact that some proteins were not secreted into the medium might relate to the deleted protein in the mutant. In the EV-free supernatant, six proteins that were identified in SC5314 were not detected in RML2U, and this number increased to 10 for EVs, including proteins related to endocytosis and exocytosis such as Cdc42, Rho3, Ykt6, and Ypt31. The absence of this group of proteins might be related to the alteration of the mechanism of EV formation in RML2U described in this

DOI: 10.1021/acs.jproteome.5b00411
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study. Other proteins that were not detected in RML2U but that were identified in both samples of SC5314 included the Orf19.4952.1, discussed later. Two additional virulence-related proteins, Ecm33 and Sap2, were identified only in the wild type.

Ecm33 and Sap2 Secretion and Their Relationship to the TOR Signaling Pathway. Sap2 is the most highly expressed secreted protease in vitro, and the *sap2* mutant is less virulent in a murine model of infection;[31] however, its relative contribution to the pathogenicity of *C. albicans* is controversial,[30] and thus its role as well as that of other Sap proteins still needs to be clarified. Deletion of the *ECM33* gene results in a strain that is deficient in Sap2 secretion, although it can secrete more proteins than the SC5314 strain. Proteomic analysis and Western blotting has revealed the complete absence of extracellular Sap2; however, Western blotting using the cytoplasmic extract of *C. albicans* SC5314 and RML2U strains indicated a normal expression of intracellular Sap2 (Figure 2B), suggesting a problem in the Sap2 secretion pathway. Furthermore, no biological extracellular proteolytic activity of Sap2 was demonstrated when strains were grown on BSA. Staib et al.[5] described that only Sap2 is significantly expressed under these conditions and mutation of the *SAP2* gene caused the inability to utilize BSA as a sole nitrogen source. In this study, BSA was not degraded by RML2U after 48 h of incubation, whereas it was completely digested by the SC5314 strain (Figure 2C), demonstrating the reduced proteolytic activity of the RML2U strain. Collectively, these data show that *ECM33* does not act at the level of *SAP2* expression but affects its secretion. One recent study associates Sap2 secretion with GTPase activity of Vps1.[51] The repressed tetR-*VPS1* strain (a conditional mutant strain, with a tetracycline-regulatable promoter) demonstrated a lower extracellular protease activity and is defective in filamentation, which are characteristics shared by RML2U. A deficiency in Saps secretion was also observed in the *C. albicans VPS11* mutant[52] as well as in other vacuolar mutants *vps34* and *vps4*;[53,54] however, the mechanism by which *VPS* mediates Sap2 secretion remains unknown, which relates the prevacuolar secretory pathway to the amount of secreted Saps. A putative link between Ecm33 and this pathway would explain the RML2U defects in Sap2 secretion. The secretion of other members of the Sap family is not decreased in the *ecm33* mutant, which, in fact, secretes more proteins by the classical secretory pathway. This suggests a specific mechanism of Sap2 secretion affected in RML2U and that might be related to the prevacuolar secretory pathway and to the nondetected exocytosis- and endocytosis-related proteins already mentioned.

The *vps4* and *vps1 C. albicans* mutants are also hypersensitive to rapamycin as well as the *rhb1*-deleted mutant.[33] Rapamycin is an inhibitor of TOR and forms a complex with the cytosolic peptidyl-prolyl cis−trans isomerase FKBP12 (FK506-binding protein 12).[55−57] Rhb1 is involved in *SAP2* expression and Sap2 secretion via the TOR signaling pathway. Furthermore, Rhb1 mediates cross-talk between the TOR signaling pathway and the cell wall integrity (CWI) pathway and is involved in Tor activation.[58] On the basis of these findings and keeping in mind that RML2U has the CWI pathway activated, the increased sensitivity of this mutant to rapamycin (Figure 3A) could suggest a role of Ecm33 in the CWI and the TOR signaling pathways. The *S. cerevisiae* orthologue of orf19.4952.1, *FPR2*, binds to the drugs FK506 and rapamycin.[26,59] This protein was not identified in RML2U (EV and EV-free supernatant) samples, although it was identified in the SC5314 EVs and EV-free supernatant. Furthermore, Rot1, which was detected only in EVs of RML2U, is similar to *S. cerevisiae* Rot1p, which can reverse Tor2 lethality.[60] Thus, this proteomic study has revealed several lines of evidence that relate Ecm33 with the TOR pathway, a relationship that was verified by testing the sensitivity of the *ecm33* mutant to the rapamycin.

SAP2, as well as other members of the *SAP*, *ALS*, *PLB*, and *LIP* genes families, is up-regulated in biofilms formed in vitro.[4] The enrichment in proteins related to biofilm formation was observed in the RM2LU EV-free supernatant (Table S2), and 4 out of 12 proteins that were related to this process according to GO Term Finder were detected only in the mutant sample: Adh1, Als1, Pbr1, and Sur7. Of these four proteins, Adh1 restricts the ability of *C. albicans* to form a biofilm, and Sur7 possibly acts as a negative regulator;[61,62] the other two proteins, Pbr1 (pheromone-induced biofilm regulator) and Als1, promote biofilms formation.[4,63] The abnormal secretion and filamentation defect of RML2U on some liquid and solid media and its reduced adherence to endothelial and epithelial cells[16,17] suggest that it cannot form biofilms. This hypothesis was corroborated by this study, where RML2U was unable to form biofilms in polystyrene material (Figure 3B). Other studies have additionally found that *C. albicans* requires both Als1 and Als3 surface proteins for biofilm formation.[64] The abnormal distribution of Als1 on the surface of RML2U and the abnormal cell wall architecture of RML2U[19] possibly contribute to its inability to form biofilms; however, biofilm formation is a complex process, and the marked alteration in cell wall organization probably affects the ability of RML2U to form biofilms.

■ CONCLUSIONS

Collectively, the data presented here highlight the importance of Ecm33 in cell wall integrity, the classical secretion pathway, and EVs morphology and content. Furthermore, these data draw attention to the significance of Ecm33 in the CWI and TOR pathways. The changes resulting from mutation of this gene contribute to the secretion of more proteins, either via the classical secretion pathway or by EVs, which might explain why mice are protected in a systemic candidiasis model with SC5314 after vaccination with RML2U. Other mechanisms via which Ecm33 might contribute to virulence include its involvement in the secretion of important virulence proteins, such as Sap2, which was confirmed in this study by several techniques. More studies are necessary to elucidate the role of *ECM33* in these cellular pathways.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00411.

Table S1. List of proteins identified in RML2U secretome. (XLSX)

Table S2. GO Term analysis by Biological Process of RML2U secretome. (XLSX)

Table S3. Proteins identified by proteomic analysis in SC5314 and RML2U secretomes. (PDF)

Table S4. GO Term analysis by Biological Process of SC5314 and RML2U EV-free supernatant proteins. (XLSX)

DOI: 10.1021/acs.jproteome.5b00411
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Figure S1. Characterization of RML2U EVs. (PDF)
Figure S2. Comparative analysis of the different biological processes in which proteins from SC5314 and RML2U are involved. (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by BIO2012-31767 from the Ministerio de Economía y Competitividad, PROMPT (S2010/BMD-2414) from the Comunidad Autónoma de Madrid, REIPI, Spanish Network for the Research in Infectious Diseases (RD12/0015/0004), and PRB2-ISCIII (PT13/0001/0038) funded by ISCIII and FEDER. A. Gil-Bona was the recipient of a fellowship from Ministerio de Ciencia e Innovación. The proteomic analyses were carried out in the Proteomics Unit UCM-Parque Científico de Madrid, a member of ProteoRed network. The transmission electron microscopy was carried out in Centro Nacional de Microscopía Electrónica (ICTS)-UCM. We thank M. Monod, Centre Hospitalier Universitaire Vaudois (Switzerland), for the gift of anti-Sap2 and M. A. de la Torre-Ruiz, Institut de Recerca Biomedica, Universidad de Lleida (Spain) for the rapamycin. These results are lined up with the Spanish Initiative on the Human Proteome Project (B/DHPP).

ABBREVIATIONS

BSA, bovine serum albumin; CWI, cell wall integrity; CWP, cell wall proteins; EV, extracellular vesicle; GPI, glycosylphosphatidylinositol; TOR, target of rapamycin

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DOI: 10.1021/acs.jproteome.5b00411
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DOI: 10.1021/acs.jproteome.5b00411
J. Proteome Res. 2015, 14, 4270−4281

Appendix Chapter 3

Reduced version of the Table S1.

Table S1. List of identified proteins in *C. albicans* RML2U culture supernatants, ordered according to their predicted biological processes.

CGD Accesion	Protein name	Description[a]	Replicates with protein (total peptides for each replicate)[b]		GPI[c]	Signal Peptide[d]
			EV-free supernatant	Vesicles		
		CELL WALL-RELATED PROTEINS				
orf19.6214	Atc1	Cell wall acid trehalase	3 (15,14,18)	2 (2,2)	-	-
orf19.4565	Bgl2	Cell wall 1,3-beta-glucosyltransferase	3 (10,9,9)	3 (9,8,7)	-	Yes
orf19.7517	Cht1	Chitinase	3 (3,3,3)	3 (3,4,3)	Yes	Yes
orf19.3895	Cht2	GPI-linked chitinase	3 (7,7,8)	3 (9,9,8)	Yes	Yes
orf19.7586	Cht3	Major chitinase	3 (7,7,7)	3 (8,7,6)	-	Yes
orf19.2706	Crh11	GPI-anchored cell wall transglycosylase	3 (12,8,9)	3 (11,11,9)	Yes	Yes
orf19.1989	Dcw1	Protein with predicted GPI modification		2 (1,3)	Yes	Yes
orf19.2075	Dfg5	N-linked mannoprotein of cell wall and membrane	3 (4,2,4)		Yes	Yes
orf19.3066	Eng1	Endo-1,3-beta-glucanase	3 (28,17,23)	3 (22,25,15)	-	Yes
orf19.2952	Exg2	GPI-anchored cell wall protein		2 (3,1)	Yes	Yes
orf19.6489	Fmp45	Predicted membrane protein induced during mating		2 (2,4)	-	Yes
orf19.2929	Gsc1	Essential beta-1,3-glucan synthase subunit		3 (21,16,5)	-	-
orf19.5861	Kre9	Protein of beta-1,6-glucan biosynthesis	3 (1,1,2)	3 (2,2,1)	-	Yes
orf19.1779	Mp65	Cell surface mannoprotein	3 (13,11,11)	3 (14,14,12)	-	Yes
orf19.1490	Msb2	Mucin family adhesin-like protein	3 (6,6,6)	3 (9,6,7)	-	Yes
orf19.6741	Orf19.6741	Putative plasma membrane protein; predicted role in cell wall integrity	2 (5,2)	3 (6,7,2)	-	-
orf19.893	Pga17	Putative GPI-anchored protein		2 (2,1)	Yes	Yes
orf19.4035	Pga4	GPI-anchored cell surface protein	3 (13,12,12)	3 (9,10,7)	Yes	Yes
orf19.2451	Pga45	Putative GPI-anchored cell wall protein	3 (6,3,3)	3 (5,4,3)	Yes	Yes
orf19.1911	Pga52	GPI-anchored cell surface protein of unknown function	3 (5,2,3)	3 (5,3,2)	Yes	Yes
orf19.6081	Phr2	Glycosidase	3 (12,9,10)	3 (9,9,9)	Yes	Yes
orf19.220	Pir1	1,3-beta-glucan-linked cell wall protein	3 (5,4,5)	3 (3,3,3)	-	Yes
orf19.7218	Rbe1	Pry family cell wall protein	3 (5,5,4)	3 (4,4,4)	-	Yes
orf19.6202	Rbt4	Pry family protein	3 (4,4,3)	3 (5,4,6)	-	Yes
orf19.5636	Rbt5	GPI-linked cell wall protein	3 (2,2,2)		Yes	Yes
orf19.5305	Rhd3	GPI-anchored yeast-associated cell wall protein	3 (12,11,12)	3 (6,8,7)	Yes	Yes
orf19.6029	Rot1	Similar to S. cerevisiae Rot1p, which is involved in cell wall 1,6-beta-glucan biosynthesis		2 (1,2)	-	Yes
orf19.3893	Scw11	Cell wall protein	3 (12,10,10)	3 (11,10,10)	-	Yes

orf19.5032	Sim1	Adhesin-like protein	3 (12,11,10)	3 (12,12,9)	-	Yes
orf19.6190	Srb1	Essential GDP-mannose pyrophosphorylase		2 (2,1)	-	-
orf19.7030	Ssr1	Beta-glucan associated ser/thr rich cell-wall protein with a role in cell wall structure	3 (2,2,2)	2 (1,2)	Yes	Yes
orf19.3642	Sun41	Cell wall glycosidase	3 (9,7,7)	3 (10,8,6)	-	Yes
orf19.3414	Sur7	Protein required for normal cell wall, plasma membrane	2 (3,2)	3 (4,3,3)	-	Yes
orf19.3268	Tma19	Cell wall protein		2 (2,2)	-	-
orf19.1690	Tos1	Protein similar to alpha agglutinin anchor subunit	3 (15,12,14)	3 (14,14,12)	-	Yes
orf19.7417	Tsa1	TSA/alkyl hydroperoxide peroxidase C (AhPC) family protein	3 (3,1,6)	3 (5,5,4)	-	-
orf19.1671	Utr2	Putative GPI anchored cell wall glycosidase	3 (14,13,11)	3 (4,6,3)	Yes	Yes
orf19.2990	Xog1	Exo-1,3-beta-glucanase	3 (21,15,16)	3 (21,15,12)	-	Yes
orf19.6481	Yps7	Putative aspartic-type endopeptidase	3 (7,4,4)	3 (6,6,3)	-	Yes
orf19.3618	Ywp1	Secreted yeast wall protein	3 (2,2,2)	3 (1,2,2)	Yes	Yes
SECRETED HYDROLASES AND ADHESINS						
orf19.5741	Als1	Cell-surface adhesin	3 (1,2,6)		Yes	Yes
orf19.1097	Als2	ALS family protein	3 (5,2,5)		Yes	Yes
orf19.4555	Als4	GPI-anchored adhesin	3 (3,3,3)	3 (3,1,2)	Yes	Yes
orf19.999	Gca2	Predicted extracellular glucoamylase	2 (7,5)		-	Yes
orf19.6673	Hex1	Beta-N-acetylhexosaminidase/chitobiase	3 (11,4,9)	2 (3,3)	-	Yes
orf19.1765	Orf19.1765	Secreted protein	3 (3,3,5)	2 (2,4)	-	Yes
orf19.3499	Orf19.3499	Secreted potein	3 (4,8,8)	2 (7,7)	-	Yes
orf19.5126	Orf19.5126	Putative adhesin-like protein	2 (1,2)		-	-
orf19.689	Plb1	Phospholipase B	2 (1,2)	3 (2,5,5)	-	Yes
orf19.1442	Plb4.5	Phospholipase B	3 (19,15,17)	3 (15,20,16)	Yes	Yes
orf19.3839	Sap10	Secreted aspartyl protease	3 (6,4,5)		Yes	Yes
orf19.6001	Sap3	Secreted aspartyl proteinase	3 (3,5,5)	3 (5,5,1)	-	Yes
orf19.756	Sap7	Pepstatin A-insensitive secreted aspartyl protease	3 (6,4,6)	3 (7,8,4)	-	Yes
orf19.242	Sap8	Secreted aspartyl protease	3 (5,5,5)		-	Yes
orf19.6928	Sap9	Secreted aspartyl protease	3 (6,3,4)	3 (8,4,2)	Yes	Yes
orf19.853	Sap99	Putative secreted aspartyl protease	3 (7,6,8)		-	Yes
PROTEIN FOLDING						
orf19.6472	Cyp1	Peptidyl-prolyl cis-trans isomerase	2 (2,4)	3 (3,3,5)	-	-
orf19.7421	Cyp5	Putative peptidyl-prolyl cis-trans isomerase	2 (3,1)	3 (1,3,2)	-	Yes
orf19.4871	Ero1	Role in formation of disulfide bonds in the endoplasmic reticulum	3 (2,1,1)		-	Yes
orf19.6515	Hsp90	Essential chaperone		2 (3,4)	-	-
orf19.2013	Kar2	Similar to Hsp70 family chaperones		2 (4,3)	-	Yes
orf19.1065	Ssa2	HSP70 family chaperone	3 (2,1,15)	3 (10,6,5)	-	-
orf19.6367	Ssb1	HSP70 family heat shock protein		3 (6,3,2)	-	-
EXOCYTOSIS AND ENDOCYTOSIS						

orf19.1760	Ras1	RAS signal transduction GTPase		2 (1,2)	-	-
orf19.2843	Rho1	Small GTPase of Rho family		3 (8,8,5)	-	-
METABOLISM						
orf19.6385	Aco1	Aconitase		2 (5,1)	-	-
orf19.5007	Act1	Actin		3 (6,4,2)	-	-
orf19.3997	Adh1	Alcohol dehydrogenase	3 (2,1,8)	3 (8,6,5)	-	-
orf19.5113	Adh2	Alcohol dehydrogenase		2 (1,2)	-	-
orf19.5591	Ado1	Adenosine kinase		3 (3,5,1)	-	-
orf19.2762	Ahp1	Alkyl hydroperoxide reductase	2 (1,5)	2 (5,2)	-	-
orf19.3014	Bmh1	Sole 14-3-3 protein in *C. albicans*		2 (3,3)	-	-
orf19.5641	Car2	Ornithine aminotransferase		2 (4,1)	-	-
orf19.3575	Cdc19	Pyruvate kinase at yeast cell surface	2 (1,9)	3 (9,7,2)	-	-
orf19.4393	Cit1	Citrate synthase		2 (2,1)	-	-
orf19.395	Eno1	Enolase	3 (9,6,17)	3 (13,13,9)	-	-
orf19.4618	Fba1	Fructose-bisphosphate aldolase	3 (1,1,9)	3 (7,5,4)	-	-
orf19.638	Fdh1	Formate dehydrogenase	2 (1,5)	2 (4,5)	-	-
orf19.7600	Fdh3	Glutathione-dependent formaldehyde dehydrogenase	2 (2,2)	2 (2,3)	-	-
orf19.4716	Gdh3	NADP-glutamate dehydrogenase	2 (2,7)	3 (4,9,3)	-	-
orf19.251	Glx3	Glutathione-independent glyoxalase	2 (1,7)	3 (1,4,2)	-	-
orf19.5024	Gnd1	6-phosphogluconate dehydrogenase	2 (3,14)	3 (7,7,4)	-	-
orf19.903	Gpm1	Phosphoglycerate mutase	3 (1,1,6)	3 (2,4,4)	-	-
orf19.4309	Grp2	Methylglyoxal reductase	2 (2,8)	3 (4,8,2)	-	-
orf19.2951	Hom6	Putative homoserine dehydrogenase		2 (2,1)	-	-
orf19.542	Hxk2	Hexokinase II	2 (2,4)	2 (2,2)	-	-
orf19.88	Ilv5	Ketol-acid reductoisomerase		3 (1,3,1)	-	-
orf19.3590	Ipp1	Putative inorganic pyrophosphatase	3 (2,1,7)	3 (5,3,2)	-	-
orf19.4506	Lys22	Putative homocitrate synthase		3 (2,2,1)	-	-
orf19.4602	Mdh1-1	Predicted malate dehydrogenase precursor	2 (1,12)	2 (3,4)	-	-
orf19.2551	Met6	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	3 (4,2,19)	3 (17,16,16)	-	-
orf19.2877	Pdc11	Pyruvate decarboxylase	2 (3,14)	3 (9,11,4)	-	-
orf19.3888	Pgi1	Glucose-6-phosphate isomerase		3 (2,2,1)	-	-
orf19.3651	Pgk1	Phosphoglycerate kinase	3 (2,2,18)	3 (11,12,6)	-	-
orf19.3911	Sah1	S-adenosyl-L-homocysteine hydrolase	2 (2,7)	3 (6,6,5)	-	-
orf19.657	Sam2	S-adenosylmethionine synthetase	2 (2,2)	3 (2,3,1)	-	-
orf19.2947	Snz1	Stationary phase protein		3 (2,3,1)	-	-
orf19.2250	Spe3	Putative spermidine synthase	2 (1,3)	2 (2,1)	-	-
orf19.4371	Tal1	Transaldolase	3 (14,7,11)	3 (16,16,15)	-	-
orf19.6814	Tdh3	NAD-linked glyceraldehyde-3-phosphate dehydrogenase	3 (9,6,17)	3 (17,13,13)	-	-
orf19.4233	Thr4	Putative threonine synthase		2 (1,2)	-	-

orf19.5112	Tkl1	Putative transketolase		3 (2,4,2)	-	-
orf19.6745	Tpi1	Triose-phosphate isomerase	3 (2,1,4)	2 (4,6)	-	-
orf19.4311	Ynk1	Nucleoside diphosphate kinase (NDP kinase)	2 (1,5)	3 (5,3,3)	-	-
TRANSMEMBRANE TRANSPORT						
orf19.6854	Atp1	ATP synthase alpha subunit	2 (2,18)	3 (7,6,2)	-	-
orf19.5653	Atp2	F1 beta subunit of F1F0 ATPase complex	2 (1,15)	2 (7,3)	-	-
orf19.3646	Ctr1	Copper transporter		2 (4,1)	-	-
orf19.4211	Fet3	Multicopper oxidase	3 (5,5,6)	3 (4,8,6)	-	Yes
orf19.4215	Fet34	Multicopper ferroxidase	3 (2,3,4)	3 (3,4,3)	-	Yes
orf19.4212	Fet99	Multicopper oxidase family protein	3 (4,7,7)	3 (4,6,6)	-	Yes
orf19.1415	Fre10	Major cell-surface ferric reductase under low-iron conditions		3 (1,2,1)	-	Yes
orf19.1193	Gnp1	Similar to asparagine and glutamine permease		2 (2,1)	-	-
orf19.6327	Het1	Putative sphingolipid transfer protein		2 (2,1)	-	-
orf19.2023	Hgt7	Putative MFS glucose transporter		2 (4,4)	-	-
orf19.5960	Nce102	Non classical protein export protein		2 (1,2)	-	-
orf19.6570	Nup	Nucleoside permease	3 (5,2,4)	3 (3,4,3)	-	Yes
orf19.930	Pet9	Mitochondrial ADP/ATP carrier protein involved in ATP biosynthesis	2 (2,7)	2 (6,4)	-	-
orf19.5383	Pma1	Plasma membrane H(+)-ATPase	2 (1,11)	3 (25,15,16)	-	-
orf19.1042	Por1	Mitochondrial outer membrane porin		2 (3,2)	-	-
orf19.1585	Zrt2	Zinc transporter		3 (3,3,1)	-	-
PROTEIN SYNTHESIS						
orf19.1891	Apr1	Vacuolar aspartic proteinase	3 (1,2,5)	3 (2,3,2)	-	Yes
orf19.5964	Arf2	Putative ADP-ribosylation factor	2 (2,2)	2 (3,1)	-	-
orf19.6906	Asc1	40S ribosomal subunit similar to G-beta subunits	3 (3,1,5)	3 (4,4,1)	-	-
orf19.4152	Cef3	Translation elongation factor 3		2 (7,2)	-	-
orf19.5788	Eft2	Elongation Factor 2 (eEF2); GTPase		3 (11,3,2)	-	-
orf19.4755	Kex2	Subtilisin-like protease (proprotein convertase)		3 (2,2,2)	-	Yes
orf19.3572.3	Orf19.3572.3	Ribosomal 60S subunit protein L31B	2 (1,3)	2 (1,2)	-	-
orf19.2937	Pmm1	Phosphomannomutase		3 (1,4,2)	-	-
orf19.1635	Rpl12	Ribosomal protein L12, 60S ribosomal subunit	2 (2,3)	3 (3,3,2)	-	-
orf19.4931.1	Rpl14	Ribosomal protein L14	2 (1,4)		-	-
orf19.493	Rpl15a	Putative ribosomal protein		2 (3,2)	-	-
orf19.5964.2	Rpl35	Ribosomal protein		2 (1,2)	-	-
orf19.3003.1	Rpl6	Ortholog of S. cerevisiae ribosomal subunit, Rpl6B	2 (1,5)	2 (2,2)	-	-
orf19.2311	Rpl82	Predicted ribosomal protein	2 (1,5)	2 (2,1)	-	-
orf19.6403.1	Rpp2a	Acidic ribosomal protein	2 (1,2)		-	Yes
orf19.6265.1	Rps14b	Putative ribosomal protein		2 (2,2)	-	-
orf19.7018	Rps18	Predicted ribosomal protein		3 (4,1,1)	-	-
orf19.5996.1	Rps19a	Putative ribosomal protein S19	2 (1,4)	3 (2,1,2)	-	-

orf19.5466	Rps24	Predicted ribosomal protein		2 (1,2)	-	-
orf19.4660	Rps6a	Ribosomal protein 6A		2 (2,1)	-	-
orf19.382	Tef2	Translation elongation factor 1-alpha	3 (5,2,15)	3 (13,9,8)	-	-
orf19.3324	Tif	Translation initiation factor		2 (5,2)	-	-
orf19.6975	Yst1	Ribosome-associated protein		2 (3,1)	-	-
OTHER						
orf19.7043.1	Acb1	Protein similar to a region of acyl-coenzyme-A-binding protein		3 (2,1,2)	-	-
orf19.5806	Ald5	NAD-aldehyde dehydrogenase	2 (1,14)	3 (4,3,2)	-	-
orf19.5292	Axl2	Plasma membrane protein involved in determination of budding pattern	3 (4,3,4)	2 (2,1)	-	-
orf19.953.1	Cof1	Putative cofilin	2 (2,1)	2 (3,1)	-	-
orf19.5063	Coi1	Secreted protein	3 (5,4,4)	3 (2,5,5)	-	Yes
orf19.4674.1	Crd2	Metallothionein	3 (1,1,2)	2 (1,2)	-	-
orf19.4688	Dag7	Secretory protein	3 (6,6,6)	3 (8,5,6)	-	Yes
orf19.4879.2	Ntf2	Putative nuclear envelope protein		2 (1,2)	-	-
orf19.2168.3	Orf19.2168.3	Ortholog(s) have role in cellular protein localization, cytokinesis and other		3 (2,2,1)	-	-
orf19.3727	Pho112	Putative constitutive acid phosphatase		3 (3,5,1)	-	-
orf19.2619	Pho113	Putative constitutive acid phosphatase	2 (10,8)		-	Yes
orf19.984	Pho8	Putative repressible vacuolar alkaline phosphatase		2 (2,1)	-	Yes
orf19.3765	Rax2	Plasma membrane protein	3 (5,2,2)	3 (2,2,1)	-	Yes
orf19.7611	Trx1	Thioredoxin	3 (2,2,4)	3 (3,5,3)	-	-
orf19.6771	Ubi4	Ubiquitin precursor (polyubiquitin)	2 (4,4)	2 (4,4)	-	-
UNKNOWN						
orf19.1376	Orf19.1376	Ortholog(s) have SNAP receptor activity, phosphatidic acid binding activity		3 (3,2,3)	-	-
orf19.2460	Orf19.2460	Protein of unknown function		3 (4,6,2)	-	Yes
orf19.31	Orf19.31	Uncharacterized	3 (4,4,4)		-	Yes
orf19.3378	Orf19.3378	Protein of unknown function	2 (3,3)	3 (2,2,3)	-	Yes
orf19.4150	Orf19.4150	Putative glutaredoxin		2 (2,1)	-	Yes
orf19.4886	Orf19.4886	Putative adhesin-like protein	3 (3,3,3)	2 (3,2)	-	Yes
orf19.6119	Orf19.6119	Protein of unknown function	3 (4,1,3)	3 (3,5,1)	-	-
orf19.6160	Orf19.6160	Ortholog(s) have role in eisosome assembly and eisosome, membrane raft, mitochondrion	2 (1,2)		-	-
orf19.6484	Orf19.6484	Uncharacterized	3 (2,2,2)	3 (1,1,2)	-	Yes
orf19.7596	Orf19.7596	Protein with a phosphoglycerate mutase family domain	3 (9,8,11)	3 (1,8,3)	-	Yes
orf19.86	Orf19.86	Putative glutathione peroxidase		3 (2,1,1)	-	-
orf19.6274	Pbr1	Protein of unknown function	3 (3,5,4)		-	Yes

[a]Description according to Candida Genome Database (CGD)

[b]Outside the parentheses: number of replicas in which the protein was identified. Inside the parenthesis: number of total peptides detected in each replica.

[c]GPI-CWP reviewed in Richard and Plaine[1]

[d]Signal Peptide predicted by SignalP4.1

*The gray background highlights proteins described previously in the extracellular secretome of *C. albicans* (EV-free supernatant and/or EVs)[2]

[1]Richard, M. L.; Plaine, A. Eukaryotic cell 2007, 6 (2), 119-33.

[2]Gil-Bona, A.; Llama-Palacios, A.; Parra, C. M.; Vivanco, F.; Nombela, C.; Monteoliva, L.; Gil, C. *Journal of proteome research* 2015, *14* (1), 142-53.

Table S3. Identified proteins in *C. albicans* SC5314 and RML2U EV-free supernatant and EVs samples. SC5314 proteins were obtained from Gil-Bona et al. 2015. The gray background highlights the number of proteins described in each group.

Sample/Strain	SC5314	RML2U	SC5314 and RML2U	Total proteins
EV-free supernatant	6	59	55	120
	Ecm33, Iff11, Orf19.4952.1, Pho112, Rbt7, Sap2.	Adh1, Ahp1, Ald5, Als1, Apr1, Arf2, Atp1, Atp2, Cdc19, Cof1, Crd2, Cyp1, Eno1, Fba1, Fdh1, Fdh3, Fet34, Gdh3, Glx3, Gnd1, Gpm1, Grp2, Hxk2, Ipp1, Kre9, Mdh1-1, Met6, Orf19.1765, Orf19.3378, Orf19. 3499, Orf19. 3572.3, Orf19. 5126, Orf19.6160, Orf19.6484, Pbr1, Pdc11, Pet9, Pgk1, Pho113, Pma1, Rpl12, Rpl14, Rpl6, Rpl82, Rpp2a, Rps19a, Sah1, Sam2, Spe3, Ssa2, Sur7, Tal1, Tdh3, Tef2, Tpi1, Trx1, Tsa1, Ynk1, Yps7.	Als2, Als4, Asc1, Atc1, Axl2, Bgl2, Cht1, Cht2, Cht3, Coi1, Crh11, Cyp5, Dag7, Dfg5, Eng1, Ero1, Fet3, Fet99, Gca2, Hex1, Mp65, Msb2, Nup, Orf19.31, Orf19.4886, Orf19.6119, Orf19.6741, Orf19.7596, Pga4, Pga45, Pga52, Phr2, Pir1, Plb1, Plb4.5, Rax2, Rbe1, Rbt4, Rbt5, Rhd3, Sap10, Sap3, Sap7, Sap8, Sap9, Sap99, Scw11, Sim1, Ssr1, Sun41, Tos1, Ubi4, Utr2, Xog1, Ywp1.	
EVs	10	89	65	164
	Als2, Cdc42, Ecm33, Hsp70, Mnt1, Orf19.4952.1, Pdi1, Rho3, Ykt6, Ypt31.	Acb1, Aco1, Act1, Adh1, Adh2, Ado1, Ahp1, Als4, Arf2, Asc1, Atp1, Atp2, Axl2, Bmh1, Car2, Cdc19, Cef3, Cit1, Cof1, Crd2, Ctr1, Dcw1, Exg2, Fba1, Fdh1, Fdh3, Gdh3, Glx3, Gnd1, Gnp1, Grp2, Het1, Hex1, Hom6, Hsp90, Hxk2, Ilv5, Ipp1, Kex2, Kre9, Lys22, Mdh1-1, Nce102, Ntf2, Orf19.1765, Orf19. 2460, Orf19.3378, Orf19.3499, Orf19.3572.3, Orf19.4150, Orf19.4886, Orf19.6484, Orf19.7596, Orf19.86, Pet9, Pga17, Pgi1, Pho112, Pho8, Pmm1, Ras1, Rax2, Rot1, Rpl12, Rpl15a, Rpl35, Rpl6, Rpl82, Rps14b, Rps18, Rps19a, Rps24, Rps6a, Sam2, Sap3, Snz1, Spe3, Srb1, Ssb1, Ssr1, Thr4, Tif, Tkl1, Tma19, Tpi1, Trx1, Tsa1, Ynk1, Yst1.	Ald5, Apr1, Atc1, Bgl2, Cht1, Cht2, Cht3, Coi1, Crh11, Cyp1, Cyp5, Dag7, Eft2, Eng1, Eno1, Fet3, Fet34, Fet99, Fmp45, Fre10, Gpm1, Gsc1, Hgt7, Kar2, Met6, Mp65, Msb2, Nup, Orf19.1376, Orf19.2168.3, Orf19.6119, Orf19.6741, Pdc11, Pga4, Pga45, Pga52, Pgk1, Phr2, Pir1, Plb1, Plb4.5, Pma1, Por1, Rbe1, Rbt4, Rhd3, Rho1, Sah1, Sap7, Sap9, Scw11, Sim1, Ssa2, Sun41, Sur7, Tal1, Tdh3, Tef2, Tos1, Ubi4, Utr2, Xog1, Yps7, Ywp1, Zrt2.	

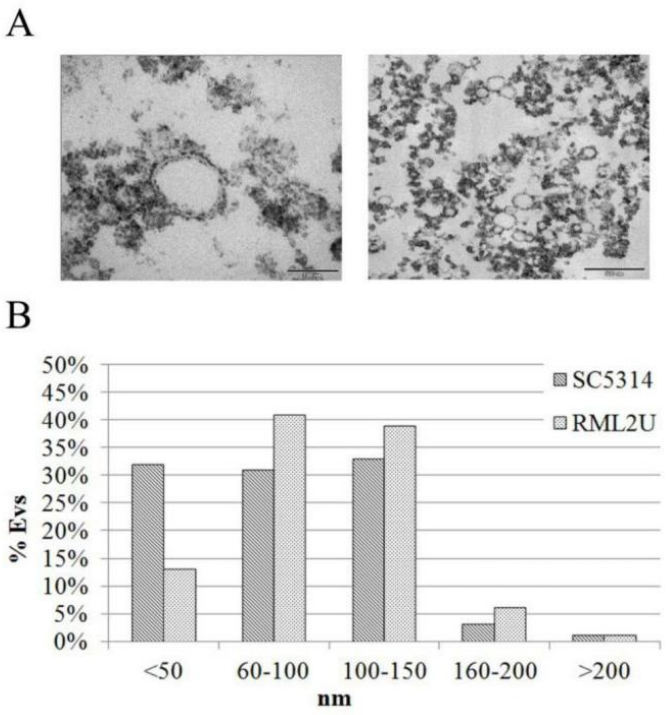


Figure S1. Characterization of RML2U EVs obtained by ultracentrifugation of culture supernatants. (**A**) TEM images of purified EVs from the ecm33 mutant. Scale bars: left: 500 nm and right: 100 nm. (**B**) Percentage sizes of SC5314 and RML2U EVs observed by TEM.

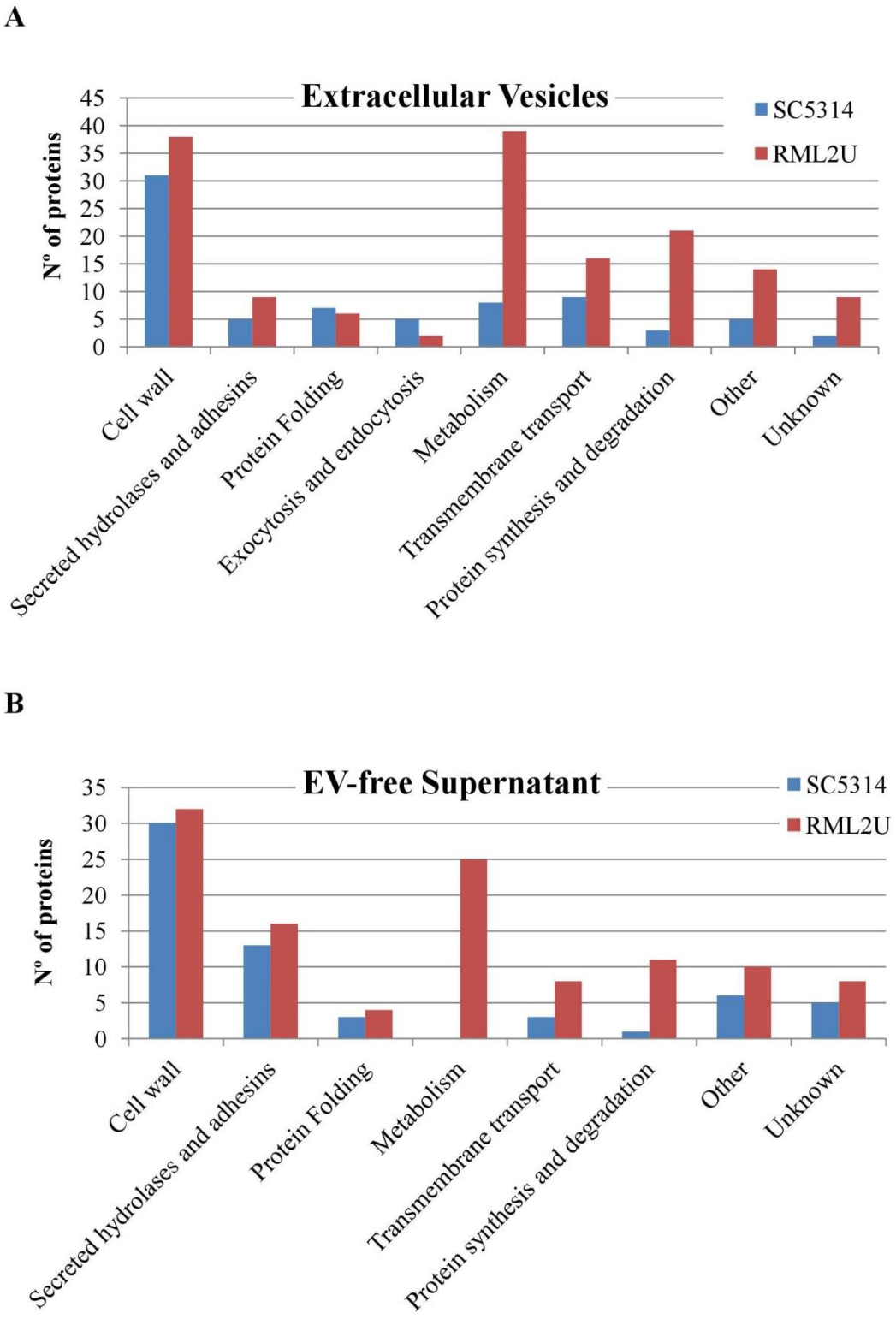


Figure S2. Comparative analysis of the different biological processes in which proteins from SC5314 and RML2U that are (**A**) carried by extracellular vesicles or (**B**) secreted by the classical pathway (EV-free supernatant) are involved.

Remaining Supplemental material is available in the provided CD.

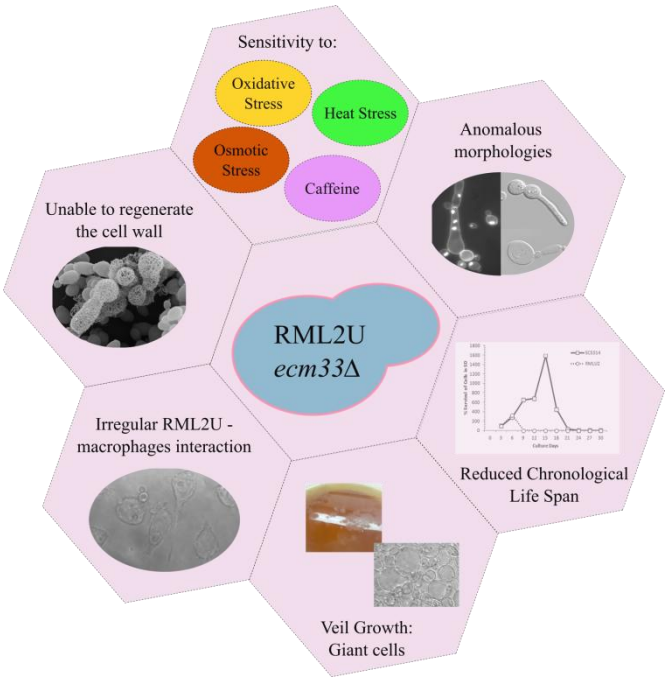
Complete Table S1. List of proteins identified in RML2U secretome.

Table S2. GO term analysis by Biological Process of RML2U secretome.

Table S4. GO Term analysis by Biological Process of SC5314 and RML2U EV-free supernatant proteins.

Chapter 4:

The cell wall protein Ecm33 of *Candida albicans* is involved in chronological life span, morphogenesis, cell wall regeneration, multi-stress tolerance and host-cell interaction



*In this chapter, the participation of Gil-Bona includes most of the work presented with the exception of the cell wall regeneration assay

Research Article

The cell wall protein Ecm33 of *Candida albicans* is involved in chronological life span, morphogenesis, cell wall regeneration, multi-stress tolerance and host-cell interaction

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Ecm33 is a glycosylphosphatidylinositol (GPI)-anchored protein in the human pathogen *Candida albicans*. This protein is known to be involved in fungal cell wall integrity and is also critical for normal virulence in the mouse model of hematogenously disseminated candidiasis, but its function remains unknown. In this work, several phenotypic analyses of the *C. albicans ecm33/ecm33* mutant (RML2U) were performed. We observed that RML2U displays the inability of protoplast to regenerate the cell wall, activation of the cell wall integrity pathway, hypersensitivity to temperature, osmotic and oxidative stresses and a shortened chronological lifespan. During the exponential and stationary culture phases, nuclear and actin staining revealed the possible arrest of the cell cycle in RML2U cells. Interestingly, a "veil growth", never previously described in *C. albicans*, was serendipitously observed under static stationary cells. The cells that formed this structure were also observed in cornmeal liquid cultures. These cells are giant, round cells, without DNA, and contain large vacuoles, similar to autophagic cells observed in other fungi. Furthermore, RML2U was phagocytozed more than the wild-type strain by macrophages at earlier time points, but the damage caused to the mouse cells was less than with the wild-type strain. Additionally, the percentage of RML2U apoptotic cells after interaction with macrophages, was fewer than in the wild-type strain.

Keywords: *Candida albicans*, Ecm33, multi-stress response, cell wall regeneration, veil growth, chronological life span, host-cell interaction.

INTRODUCTION

The fungal cell wall is an essential organelle that is required for the maintainance of cell integrity and also plays an important role in primary interactions between pathogenic fungi and their hosts. Different analyses have demonstrated its importance as a protective barrier against a wide range of environmental conditions, such as temperature, oxidative and osmotic stresses. The cell wall is essential for the virulence of pathogenic fungi, since it provides adhesive properties and protection against host defense mechanisms. The composition of the *Candida albicans* cell wall consists of β-1,6-glucan, β-1,3-glucan and chitin, as well as different attached proteins, including glycosylphosphatidylinositol (GPI) proteins (Chaffin, 2008;Free, 2013). These GPI proteins contain a C-terminal domain that allows for linkage to a GPI anchor and might target proteins to the membrane or the cell wall.

The *C. albicans ECM33* gene encodes a GPI-anchored protein of this human pathogen. The Ecm33 protein was detected in the *C. albicans* cell wall, plasma membrane, extracellular vesicles and soluble extracellular medium by proteomic analysis, revealing

its high abundance (Castillo et al., 2008;Cabezón et al., 2009;Gil-Bona et al., 2015a) and it has also been detected by cell surface shaving analysis of yeast, hyphae and biofilms (Vialas et al., 2012;Gil-Bona et al., 2015c). Although its function is unknown, it is highly important in cell wall morphology and virulence. The *ecm33*Δ null mutant (RMLU2) displays cell-wall defects such as an enhanced sensitivity to cell wall-perturbing agents such as Calcofluor white, Congo red and hygromycin B, an abnormal electron-dense outer mannoprotein layer and an aberrant surface localization of the adhesin Als1, together with defects during the yeast-to-hyphae transition (Martínez-Lopez et al., 2004;Martinez-Lopez et al., 2006). Recent proteomic analysis of the extracellular medium of RML2U relates Ecm33 to the proper functioning of the classical secretion pathway and to the composition, shape and quantity of extracellular vesicles (Gil-Bona et al., 2015b). The secretory aspartyl proteinases, particularly Sap2, play important roles in vaginitis in mice (Pericolini et al., 2015). Sap2 secretion was compromised in the *ecm33*Δ mutant and negatively affects bovine serum albumin (BSA) degradation when BSA is used as the sole nitrogen source. Additionally, RML2U causes an irregular protein trafficking to the medium that might contribute to the avirulence of RML2U in a mouse model of systemic infection and to the reduced capacity to invade and damage endothelial cells and oral epithelial cells (Martínez-Lopez et al., 2004;Martinez-Lopez et al., 2006). RML2U cells are also sensitive to rapamycin, the inhibitor of the Target Of Rapamycin (TOR) pathway, suggesting a relationship between the TOR pathway and Ecm33 (Gil-Bona et al., 2015b). The TOR kinase mediates important cellular responses that are implicated in extended longevity, metabolism and morphogenesis, including stress responses, autophagy and actin organization, among others (Wullschleger et al., 2006;Kaeberlein et al., 2007). Moreover, there is evidence of crosstalk between the TOR and cell wall integrity (CWI) pathways (Fuchs and Mylonakis, 2009).

The connection of Ecm33 in fungi with CWI is known (Martínez-Lopez et al., 2004;Pardo et al., 2004), but its contribution to multi-stress tolerance is largely unknown in *C. albicans*. Recently a characterization study of two Ecm33 orthologues in *Beauveria bassiana* and *Metarhizium robertsii* was published, in which the relationship of this protein with multi-stress tolerance was demonstrated (Chen et al., 2014). However, in contrast with previous studies in *C. albicans*, these orthologs showed no relationship with virulence in a larvae assay. To clarify these results and to extend the study of Ecm33, this study characterizes *C. albicans* Ecm33 functions via a range of phenotypic analyses of the *ecm33Δ* mutant, RML2U, and its involvement in longevity and in the engulfment by macrophages.

MATERIALS AND METHODS

Microorganisms and culture conditions

C. albicans SC5314 (wild type) (Gillum et al., 1984) was used to generate the RML2U mutant strain (*ecm33*Δ*::hisG/ecm33*Δ*::hisG ura3*Δ*::imm434/ura3*Δ*-::imm434::URA3*) and the doubled-complemented strain RML4U (*ecm33*Δ*::hisG::ECM33-cat/ecm33*Δ*::hisG::ECM33-cat ura3*Δ*::imm434-/ura3*Δ*::imm434::URA3*) (Martinez-Lopez et al., 2006). *C. albicans* cell wall mutants used in the rapamycin assay were acquire from Noble collection (Noble et al., 2010) stored in the Fungal Genetics Stock Center (Kansas City, Missouri USA) (McCluskey et al., 2010). *C. albicans* strains were maintained on YPD (1% yeast extract, 2% peptone, and 2% glucose) agar plates at 30°C. For chronological life span assays yeast cells were grown in synthetic defined (SD) cultures (20 g/L glucose, 5 g/L ammonium sulfate, 1.7 g/L nitrogen base and 2.2 g/L amino acids mix) at 30 °C. Cornmeal growth was in cornmeal media (2 % commercial cornmeal) at 37 °C 200 rpm shaking. For interaction and phagocytosis assays, RAW 264.7 murine macrophages were cultured in RPMI 1640 medium supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml), L-Glutamine (2 mM) and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cel wall regeneration

Candida protoplast yeast cells were prepared according to previous work (Pitarch et al., 2006). Cells were grown in YPD medium until OD_{600} 0.8-1.2, washed and incubated at 30 °C 80 rpm in a pretreatment solution (10 mM Tris-HCl, pH 9.0, 5 mM EDTA, 1% v/v 2-mercaptoethanol) for 30 min. Then, $5x10^8$ cells/ml were resuspended in a solution containing 1 M sorbitol and 30 µg/ml glusulase (Du Pont®) and maintained with gentle shaking until protoplast were obtained. After five washes, protoplasts were induced to regenerate their cell walls in Lee medium (Lee et al., 1975) containing 1 M sorbitol at 30 °C with gentle shaking (80 rpm) for 30 min and 3 and 24 hours. The cell wall regeneration was observed by Scanning Electron Microscopy and

osmotic fragility of regenerating protoplast were determined as reported previously (Nishiyama et al., 1995) with several modification. Briefly, the osmotic fragility of regenerating protoplast were determined at 0 min, 30 min, 3 h and 24 h by counting colony-forming units (CFUs) after resuspending a portion of the cells on phosphate buffer (PBS) with or without 1 M sorbitol for 3 min, and the resulting suspension was diluted and plated in triplicate on YPD agar containing 1 M sorbitol. After 72 h incubation at 30 °C, visible colonies were counted. The percentage of CFUs was calculated using like 100% the number of CFUs counted from the solution with sorbitol. Three biological replicates were done.

Protein extracts and immunoblot analysis

Yeast strains were grown overnight at 37 °C in 20 ml YPD cultures. Cells extracts were obtained by suspending cells in cold lysis buffer (50 mM Tris/HCl, pH 7.5, 10% glycerol (v/v), 1% Triton X-100, 0.1% SDS, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, pH 8, 1 mM PMSF, 25 µg ml^{-1} N-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 25 µg ml^{-1} N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 25 µg ml^{-1} pepstatin A, 25 µg ml^{-1} leupeptin, 25 µg ml^{-1} antipain and 25 µg ml^{-1} aprotinin) and lysed mechanically using glass beads in a fast-prep cell breaker applying three 35 s rounds at 5.5 speed with intermediate ice coolings. Cells extracts were clarified by centrifugation, and the supernatants were collected and stored at -80 °C. The protein concentration was measured by Bradford protein assay. Equal amounts of proteins (80 µg per lane), were loaded onto 10% SDS-polyacrylamide gel, separated and transferred to nitrocellulose membranes and blocked with 5% milk. Reversible Ponceau red staining was applied to check protein transferring to the membranes. Western blots were probed with anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (Cell Signaling Technology Inc.) that recognizes the phosphorylated form of Mkc1 and Cek1 kinases, and anti-Hog1 (Santa Cruz Biotechnology) using 1:2000 dilutions. Primary antibodies were detected using fluorescently labeled secondary antibody IRDye 800 goat antirabbit IgG (LI-COR Biosciences) at 1:5000. The Western blotting was performed with the Odyssey system (LI-COR Biosciences, Nebraska).

Stress-related phenotypic assays

Drop tests were performed by spotting serial dilutions of cells onto agar plates and YPD agar plates supplemented with sorbitol (1.5 M), KCl (1 M), NaCl (1 M), caffeine, diamide (2 mM), menadione (0.2 mM) and rapamycin (0.1µg/ml). Hydrogen peroxide stress was tested adding H_2O_2 100 mM to *C. albicans* growing cells in YPD medium at an OD_{600} 0.8 at 30 °C and spotted onto YPD plates at different times: 2, 5, 10, 15, 30, 60 and 90 minutes. Plates were incubated 24-48 h at 30 °C. For heat-shock stress YPD plates were incubated at different temperatures (30 °C, 37 °C, 42 °C and 45 °C).

Growth curve and growth rate determination

Overnight YPD cultures were used to inoculate 100 ml YPD at an OD_{600} of 0.1. Cultures were grown at 30 °C with shaking. OD measurements were taken every hour to generate growth curves. Doubling times of each strain were calculated using time points within the logarithmic phase of growth. This assay was repeated three times.

Staining and fluorescent image analysis

For viability assay, Propidium Iodide was added to cultures to a final concentration 10 µg/ml and incubated for 10 minutes at room temperature. Cells were washed and resuspended in PBS. For chitin staining, Calcofluor white was added directly to the culture medium to a final concentration 5 µg/ml. After 10 min cells were washed with PBS and observed by fluorescent microscopy. For DNA-specific fluorescent probe, DAPI (4',6-diamidino-2-phenylindole) was added to the cell culture at a final concentration 0.5 µg/ml, incubated 5 min in the dark and then rinsed with PBS and observed by fluorescent microscopy. To detect actin, cells were stained as described previously (Hausauer et al., 2005). Briefly, *C. albicans* cells were fixed in 3.7% formaldehyde for 30 min, followed by incubation in PK buffer (50 mM potassium phosphate, pH 3.6) containing 3.7% formaldehyde for an additional 60 minutes. Formaldehyde-fixed cells were harvested by centrifugation, incubated in PK buffer containing 0.1% Triton X-100 for 30 minutes, washed twice in phosphate-buffered saline (PBS), and incubated overnight in PBS containing 2 U of Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR) at 4 °C. Cells were harvested by centrifugation and resuspended in PBS prior to fluorescence microscopy. For vacuolar membrane staining, 0.2 µl FM4-64 dye (Invitrogen T3166 1 mg) were added and incubated o/n at 37 ºC shaking. Cells were washed and resuspended in PBS.

Chronological life span

Yeast chronological life span was measured as previously described Wei et al. 2008. Overnight liquid

SD cultures were diluted 1:200 in 10 ml fresh SD medium and were maintained at 30 °C with shaking. This time point was considered day 0. Every 3 days, aliquots from the culture were properly diluted and plated on to YPD plates. The YPD plates were incubated at 30 °C for 3 days and viability was checked by Colony Forming Units (CFUs). Viability at day 3, when the yeast had reached the stationary phase, was considered to be the initial survival (100%). For extreme Calorie Restriction (CR)/starvation, cells from 3 days old SD culture were washed three times with water, and resuspended in 10 ml water. This time point was considered day 0. Water cultures were maintained at 30 °C with shaking. Every 3 days, cells from the water cultures were washed to remove nutrients released from dead cells and plated onto YPD plates. For CR modeled by glucose reduction, overnight SD culture was diluted (1:200) into fresh 0.5% glucose SD medium. The protocol followed is the same as for yeast chronological life span. All dilutions were plated 3 times and several dilutions were made. The complete experiment was repeated 3 times. The values represented correspond to the average of the 3 replicates.

Induced apoptosis by interaction with macrophages

Macrophages were grown in 6-well plates at a cell density of $6x10^6$ cells/well. *C. albicans* cells were added at a ratio 1:1 and co-incubated at 37 °C with 5% CO_2. Different apoptotic markers were analyzed as previously described Cabezon *et al.* (Submitted). Each experiment was repeated 3 times for each interaction time point. Briefly, intracellular reactive oxygen species (ROS) were detected in control *Candida* cells and cells co-incubated with macrophages (3, 6 and 8 hours) by adding 5 µg/ml dihydrorhodamine (DHR) 123 (Sigma-Aldrich) 30 min before the end of each experiment. *In vivo* measurement of caspase-like enzymatic activity was performed washing the cells in PBS and resuspended in 10 µM/ml of staining solution containing fluorescein isothiocyanate FITC-VAD-FMK (CaspACET, Promega) for 20 min at 37 °C after each interaction time point (3, 6 and 8 hours). After each interaction time point, cells were washed with water in order to lysate the macrophages and cells were recovered and evaluated using a fluorescence microscopy.

***C. albicans* phagocytosis assay**

For the phagocytosis assay, macrophages were plated onto 18-mm glass sterile coverslips placed in 24-well plates. *C. albicans* strains were pre-labeled with 1 µM Oregon Green 488 (Molecular Probes) in the dark with gentle shaking at 30 °C for 1h. Macrophages were confronted with the yeast at a ratio 1:1 at 37 ºC and 5% CO_2. Interaction was stopped after 45 minutes, 1.5 and 3h and cells were then washed with ice-cold PBS and fixed in 4% paraformaldehyde for 30 min. To distinguish between internalized and attached/non-ingested yeasts, *C. albicans* cells were counterstained with 2.5 M calcofluor white (Sigma) for 15 min in the dark. The number of ingested cells (green fluorescence) and/or adhered/non-ingested (calcofluor white blue fluorescence) were quantified by fluorescence microscopy with FITC and UV (Fernández-Arenas et al., 2007). Three different replicates with two different slides were prepared for each time point. At least 400 *C. albicans* cells were counted per slide, and results were expressed as the percentage of yeasts internalized by macrophages.

Cytotoxicity measurement

RAW 264.7 cells were seeded onto 24-well plastic plates at a density of $1x10^6$ cells/well in RPMI 1640 complete medium and incubated 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then, macrophages were co-incubated (in a new complete media without phenol red (pH indicator) to avoid the background in the LDH test) with *C. albicans* yeast strains at a ratio 1:1 during 3 and 8h. Staurosporine 5mM was used as a positive control. After the incubation, LDH was measured with the Cytotoxicity Detection Kit^{PLUS} (Roche) according to the manufacturer`s protocol.

Microscopy techniques

For fluorescence microscopy, a fluorescence microscope NIKON ECLIPSE TE2000-U, connected to a high resolution HAMAMATSU ORCA-ER camera was used. For Scanning Electron Microscopy (SEM), used to visualize protoplast cell wall regeneration, cells were fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer containing 1M sorbitol at 4 °C, overnight. Postfixation was carried out for 2 h at room temperature with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). Initial dehydration was accomplished in a graded ethanol series. Then, samples were dehydrated with acetone until dried by the critical point method in liquid CO_2 (Balzers® CPD 030). Subsequently, the specimens of the different strains were coated with graphite and gold in a vacuum evaporator (EMITECH SCD 004) and examined with a SEM JEOL Observations were carried out in JEOL JSM-6400 microscope scanning electorn microscope. For

Transmission Electron Microscopy (TEM), used to observe cell wall, cells were fixed in 4% paraformaldehyde, 1%, glutaraldehyde and 0.1% PBS overnight at 4 °C. Samples were incubated for 90 min in 2 % osmium tetroxide and then serially dehydrated in ethanol and embebbed in EMBed-812 resin (Electron Microscopy Sciences). Thin sections (50-70 nm) were obtained by ultracut and observed in JEOL JEM 1010 transmission electron microscope.

RESULTS

RML2U has the cell integrity pathway activated and its protoplast are unabled to regenerate the cell wall

The ability of RML2U to regenerate the protoplast cell wall was examined to study Ecm33 involvement in the synthesis and organization of the cell wall (Martínez-Lopez et al., 2004). Cells were treated with glusulase to obtain more than 90% protoplasts and these were incubated in regeneration conditions for 0, 3 or 24 h. The osmotic fragility of the regenerating protoplast cell wall was determined by colony counts on agar plates in the presence of stabilizer (1 M sorbitol) after resuspending the cells in PBS in the presence or absence of 1 M sorbitol. More than half of the SC5314 cells (52%) were osmotically stable after 3 h of regeneration, whereas only 10% of RML2U cells started to proliferate (Figure 1A). At the end of the study, after 24 h of regeneration, 98% of the SC5314 cells showed osmotic stability, compared with 32% of RML2U cells.

Protoplast regeneration was examined by scanning electron microscopy (SEM). Fresh protoplasts appeared as round cells with a smooth surface (Figure 1B). After 30 min of regeneration, a net of thick fibrils were irregularly distributed over the surface of most SC5314 protoplasts. After 3 h, most cells showed filamentous material and some cells showed complete protoplast regeneration. After 24 h of regeneration, all cells exhibited complete regeneration of the cell wall; however, RML2U was unable to regenerate the cell

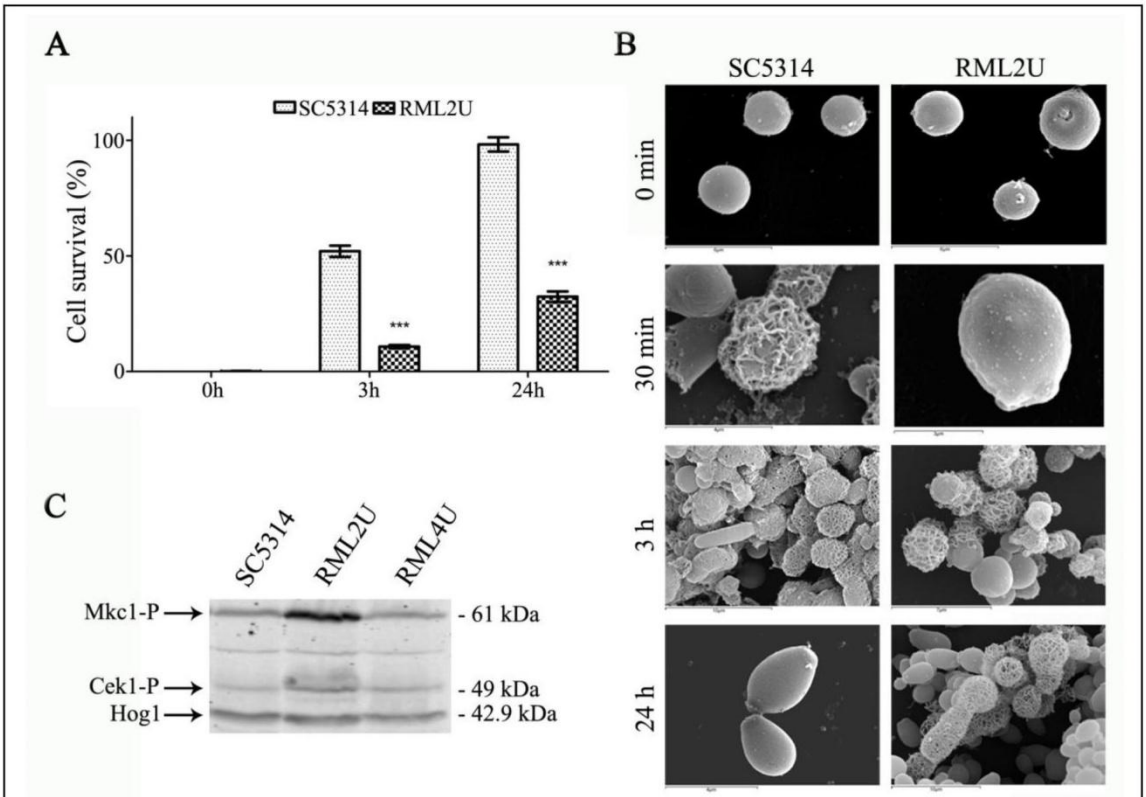

Figure 1. Protoplast regeneration and CWI activation. (**A**) Comparison of the percentage of viable counts of osmotically stabilized shocked cultures of *C. albicans* cells. (**B**) Scanning Electron Microscopy (SEM) of freshly prepared protoplast after 0 min, 30 min, 3 h and 24 h of cell wall regeneration in Lee medium supplemented with sorbitol. (**C**) Mkc1 is activated in the RML2U strain. Western blotting analysis of protein extracts from the indicated strains.

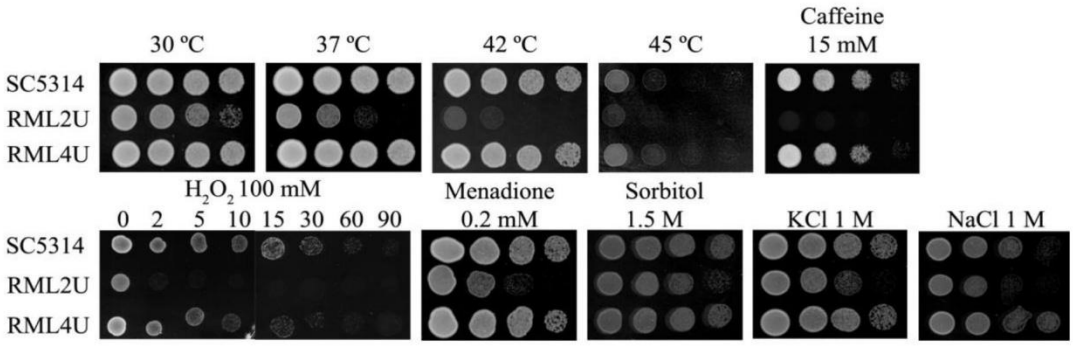

Figure 2. Stress response. Strains were grown in the presence of 100 mM hydrogen peroxide (H_2O_2), 0.2 mM menadione, 1.5 M sorbitol, 1 M KCl, 1 M NaCl and 15 mM Caffeine, and were heat shocked at 37 °C, 42 °C and 45 °C. Control plate was also performed at 30 °C in YPD.

wall. A network of filamentous materials started to cover several protoplasts after 3 h and 24 h of regeneration, some cells possessed complete cell wall but regeneration was incomplete for other protoplasts. The SEM images showed that some cells probably began to generate the cell wall, but were unable to complete the process (Figure 1B). Protoplast regeneration of RML2U appeared to be greatly delayed compared with that of the SC5314 strain and most cells were unable to complete the process, according to osmotic fragility data (Figure 1).

To understand whether the absence of *ECM33* is sensed by the MAP K pathway, the phosphorylation of Mkc1, the MAP kinase of the cell integrity pathway, and Cek1, the protein kinase part of a MAPK regulatory cascade, were analyzed by examining growing cells at 37 °C in YPD. Commercial antibodies raised against the phosphorylated forms of p44-42 MAP kinases recognize the simultaneous phosphorylation of the threonine and tyrosine residues of the TEY signature of the Mkc1 and Cek1 proteins in *C. albicans* (Navarro-Garcia et al., 2005). Differences were clearly observed concerning the intensity of phosphorylation (Figure 1C). The intensity of the signal of the upper band (corresponded to Mkc1 61 kDa) was greater compared to that of the wild type strain. Notably, the signal decreased in the reintegrated strain, RML4U. The lower band corresponds to the phosphorylated form of Cek1(49 kDa), and the signal was slightly increased compared to that in wild type and RML4U strains. Therefore, Mkc1 is phosphorylated in the absence of *ECM33*.

RML2U is hypersensitive to temperature, osmotic and oxidative stresses

The activation of cellular protection mechanisms represents an important survival strategy in yeast (Longo and Fabrizio, 2002). Furthermore, the CWI pathway crosstalks with other stress-response pathways, such as the TOR pathway (Fuchs and Mylonakis, 2009). To examine the potential role of Ecm33 in cellular protection and its relationship with other pathways, the cellular responses of RML2U to different types of stresses were compared with those of SC5314.

Deletion of *ECM33* affects the growth of *C. albicans* under oxidative, temperature and osmotic stress. Oxidative stress induced by different exposure times to 100 mM hydrogen peroxide, resulted in a slight sensitivity in the RML2U strain and importantly, this phenotype was reversed upon reintroduction of the wild type *ECM33* gene into the *ecm33Δ/ecm33Δ* strain (RML4U) (Figure 2), which was performed by growing *C. albicans* in the exponential phase (OD_{600} 0.8). Time 0 represents the culture before addition of the stress factor. The oxidative stress response was also tested by growing the strains on YPD agar supplemented with 0.2 mM menadione, another oxidative stress-inducing agent, which can generate ROS by redox cycling which leads to stress (Figure 2). No difference was observed in response to diamide, a thiol oxidant (data not shown).

Growth at high temperatures, from 37 °C to 45 °C, seriously affected the growth of RML2U compared with SC5314 (Figure 2), which suggests that the mutant begins to display compromised growth at human body temperature. Notably, the deletion of *ECM33* resulted in impaired resistance to the osmotic stress-inducing agents sorbitol (1.5 M), NaCl (1 M) and KCl (1 M) (Figure 2). The sensitivity to caffeine, a compound related to the cell wall integrity pathway, as well as to TOR function, was also tested. The RML2U strain showed an increase in the sensitivity to caffeine compared with SC5314 (Figure 2).

Ecm33 is necessary for normal morphology

The observation of several cultures of RML2U in standard growth conditions (YPD 30 °C, 200 rpm shaking), showed a variety of different morphologies ranging from relatively normal to aberrant cells (Martínez-Lopez et al., 2004). Therefore, the morphology of this mutant was studied under several conditions using SC5314 as a control strain. Firstly, growth rates were determined to corroborate the data of Martinez-Lopez et al. 2006. The RML2U growth rate was approximately 1.5 times slower than that of SC5314.

Previous study showed that RML2U cells had an aberrant morphology, which varied depending on the

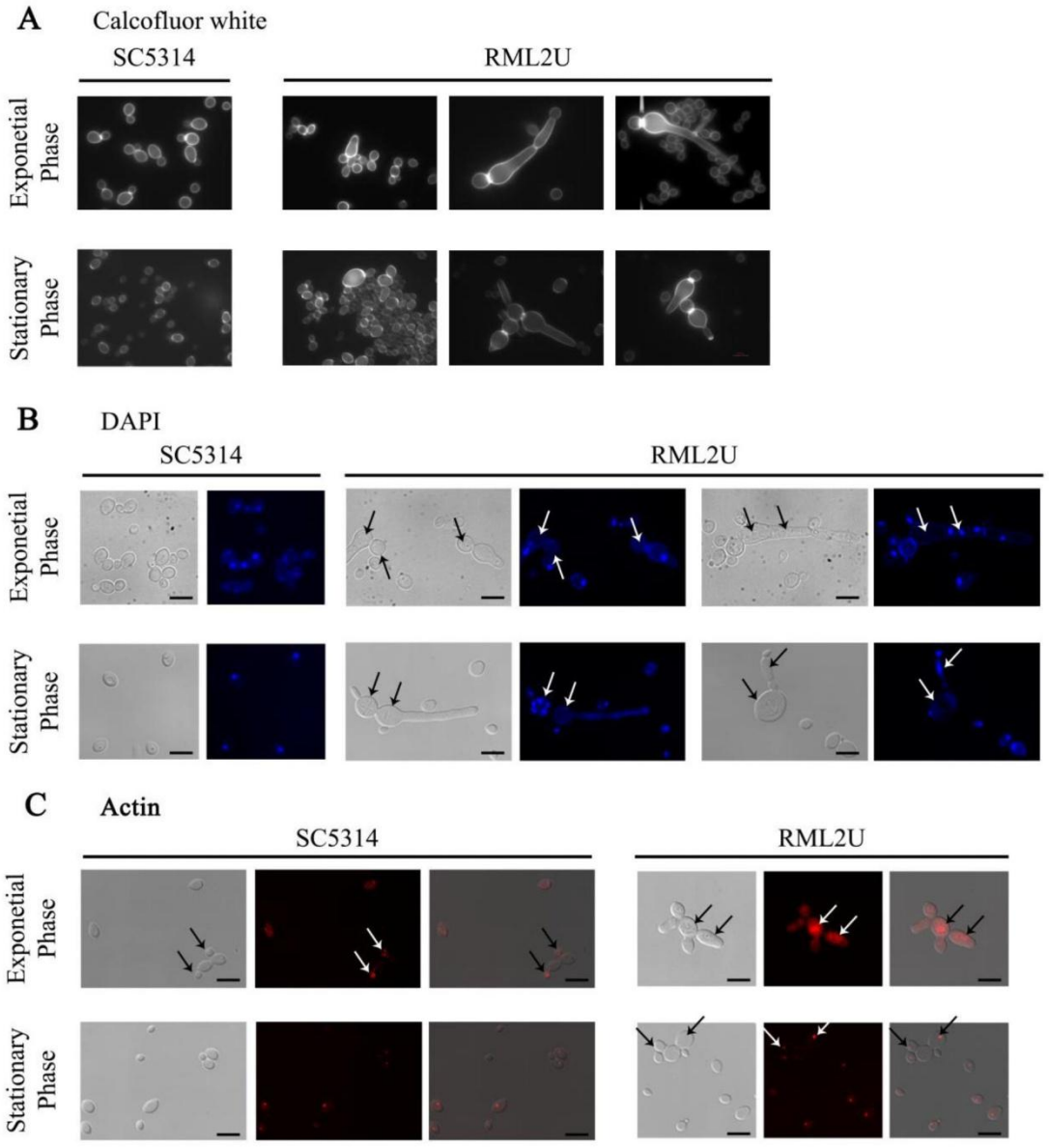

Figure 3. Ecm33 is important for nuclear DNA division, septa distribution and actin polarization during *C. albicans* growth. (**A**) Calcofluor staining was used to visualize the chitin localized in the septum. (**B**) The nuclear DNA was observed by labeling with DAPI, a highly specific stain for DNA. Arrows indicate defects in the number of nuclei. (**C**) *C. albicans* strains were fixed and stained with Alexa phalloidin to visualize actin. Arrows in SC5314 indicate normal actin polarization in buds, compared to RML2U.

growth conditions and the culture medium. Staining with Calcofluor white, a compound that binds to chitin or glucan polymer of the cell wall, showed uniformly stained material in wild type and large aggregates in RML2U (Martínez-Lopez et al., 2004). Different morphologies during growth in YPD medium at 30 °C resulted from compromised *ECM33* function (Figure 3 and Supplemental Figure S1A). The cell morphology of the exponential and stationary growth phases was analyzed to examine the response and phenotype of RML2U under these conditions in depth. For this purpose, Calcofluor white was used to examine the distribution of septa by fluorescence microscopy. Some pseudohyphal-like RML2U cells were observed in the exponential phase (Figure 3A). Regular cells and large cells that were not separated from the mother cell were observed and the amount of chitin appeared to be higher in RML2U cells. These data were confirmed by flow cytometry and were twice as high as the values detected in SC5314 cells (data not shown). In addition, cells with aberrant morphologies, including non-separated cells and large cells, were counted and 10% showed some type of aberrant morphology (Supplemental Table S1). On the other hand, the localization of DNA and distribution of actin in the cells in each stage of growth were observed by specific dyes (Figure 3B and C). In the exponential phase, fewer than 1% of the total cells counted contained more than two nuclei between the septa and no DNA was detected in 2% of the observed cells (Figure 3B and Supplemental Table S1). Nuclear division was uncoupled in RML2U cells. Phalloidin staining revealed that actin was mislocalized in RML2U, and appeared large actin patches in the center and surrounding cells (Figure 3C). These results reveal a defect in the cell cycle. Futhermore, the altered morphology and cell wall organization of RML2U was analyzed in more detail by TEM. Sections of SC5314 cells showed a smooth cell wall layer with a thickness between 126 nm and 88 nm (Supplemental Figure S1C), but the RML2U cell wall was thicker than in SC5314, at 334.21-79.63 nm, corroborating the cytometry data.

The proportion of aberrant cells increased during the stationary phase and the different morphologies observed in these cultures are shown in Figure 3, including elongated cells and aggregates. Calcofluor staining also showed unseparated cells and approximately 5% of non-nuclear cells were stained with DAPI (Figure 3A and B, and Supplemental Table S1). Moreover, a "veil growth", never previously described for *C. albicans*, was serendipitously observed in RML2U stationary and static cultures and not in those of SC5314 at room temperature after

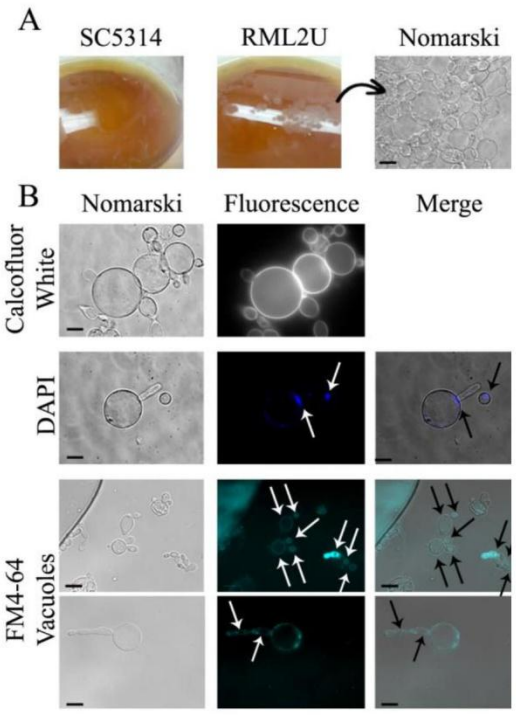

Figure 4. Novel C. albicans "veil growth". (**A**) "Veil growth" was observed in stationary and static RML2U cultures and not in those of SC5314. (**B**) Different cell stainings were performed to clarify chitin distribution (calcofluor white staining), nuclear DNA composition (DAPI labeling) and to locate each cell vacuole (FM4-64).

weeks (Figure 4A). The microscopic analysis of the cells within this structure revealed giant cells with a round morphology and DAPI stain showed only slight fluorescence on the side of the cell (Figure 4A and B). Calcofluor staining showed a homogenous distribution of chitin around the cells, which increased in amount, which also occurs in other RML2U conditions (Figure 4B). In addition, FM 4-64 staining was performed, which is a vital stain for the vacuolar membrane in yeast (Vida and Emr, 1995). A large cellular compartment was observed in cells that recovered from the "veil", which completely occupied the cell (Figure 4B). In several cells, an irregular allocation of this marker was observed, suggesting aberrant internal structures or no living cells.

Similar cells with this shape were observed on cornmeal agar (data not shown), a medium used to study chlamydospore production in *C. albicans* (Pollack and Benham, 1957). Cells that grew in liquid 10% cornmeal were large and, as in "veil growth",

contained large vacuoles (Figure 5A and B) and no DAPI or propidium iodide-positive cells were observed (data not shown). SC5314 cells displayed hyphal growth in liquid cornmeal medium. Supplementing the medium with amino acids, ammonium sulphate or yeast nitrogen base did not result in cells with a normal phenotype, moreover, is toward more visible even in the wild type. Cells that grew in this medium were observed by TEM and showed abnormal structures, with a large cell wall, massive vacuolization, translucent cytoplasm and heterogeneous sizes compared with SC5314 cells

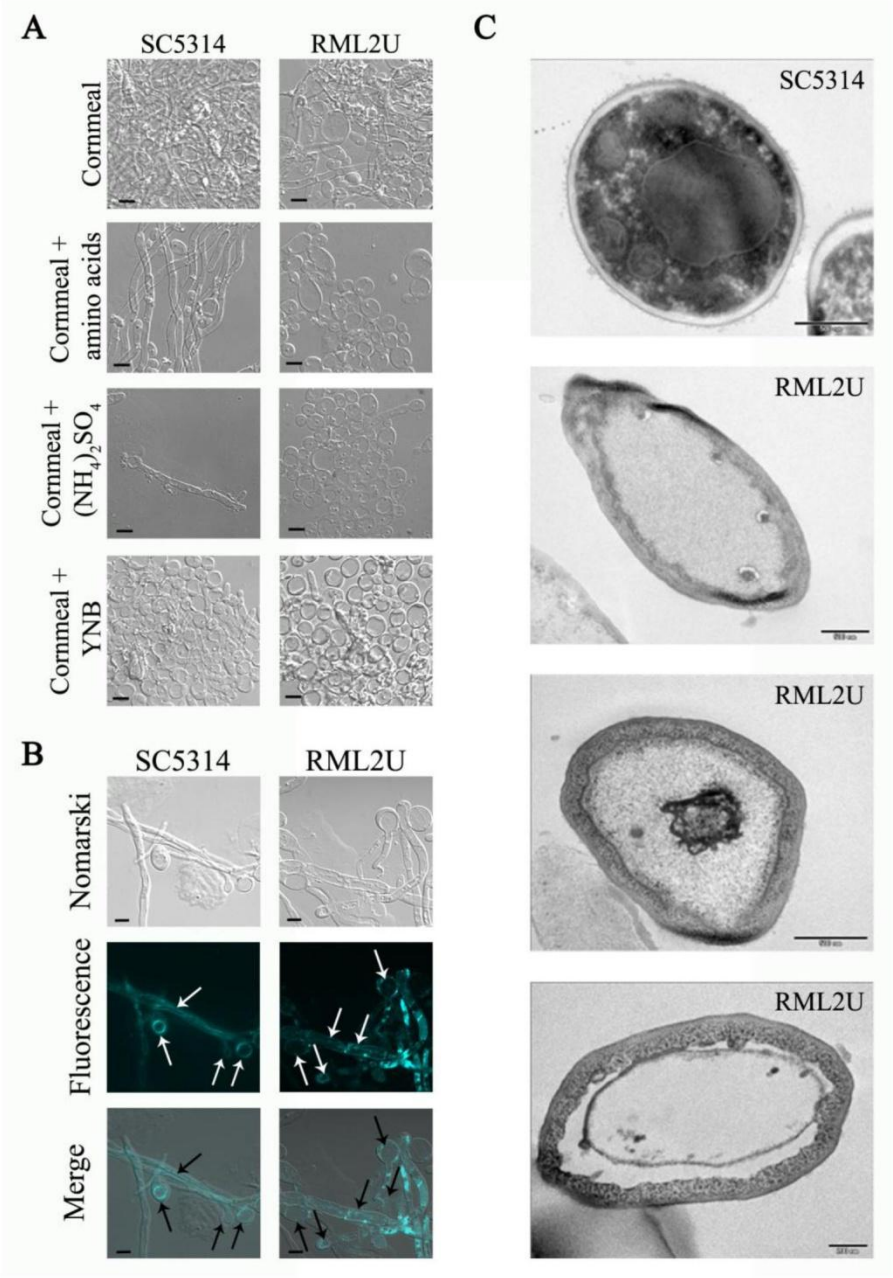

Figure 5. Cornmeal liquid medium mimics the "veil growth" cell phenotype. (**A**) Amino acids, ammonium $(NH_4)_2SO_4$ and yeast nitrogen base (YNB) were added to the cornmeal medium to observe their influence on the RML2U cell phenotype. (**B**) The cell vacuole was observed by FM4-64 labeling. (**C**) TEM images of SC5314 and RML2U cells from cornmeal culture. Scale bar: 500 nm and 200 nm (the last image of RML2U).

(Figure 5C).

RML2U cells show a shorter chronological life span

Previous studies showed that defects in the TOR signaling pathway can extend the chronological life span (CLS) of *Saccharomyces cerevisiae* (Wei et al., 2008). Recently, it has been shown that Ecm33 is related to the TOR pathway because of its sensitivity to rapamycin (Gil-Bona et al., 2015b). To determine whether the CLS is affected in the RML2U strain, growth in SD medium plus amino acids and glucose was compared with that of SC5314. The CLS measures the length of time that cells remain viable in the stationary phase. The number of colonies on day 3 was considered to represent the initial survival rate (100%). Cells of each strain were collected every 3 days and were plated on YPD agar for 48 h to determine the percentage survival. The CLS was different in RML2U cells than in SC5314 cells. Both strains contained an almost similar percentage of surviving cells by day 30, but the behavior of both strains differed considerably during the period of study (Figure 6A and Supplemental Table S2). On day 9, the percentage survival of RML2U cells decreased to 4%, whereas that of SC5314 increased to day 18 and then decreased more gradually. These data suggest that Ecm33 is necessary for cell survival.

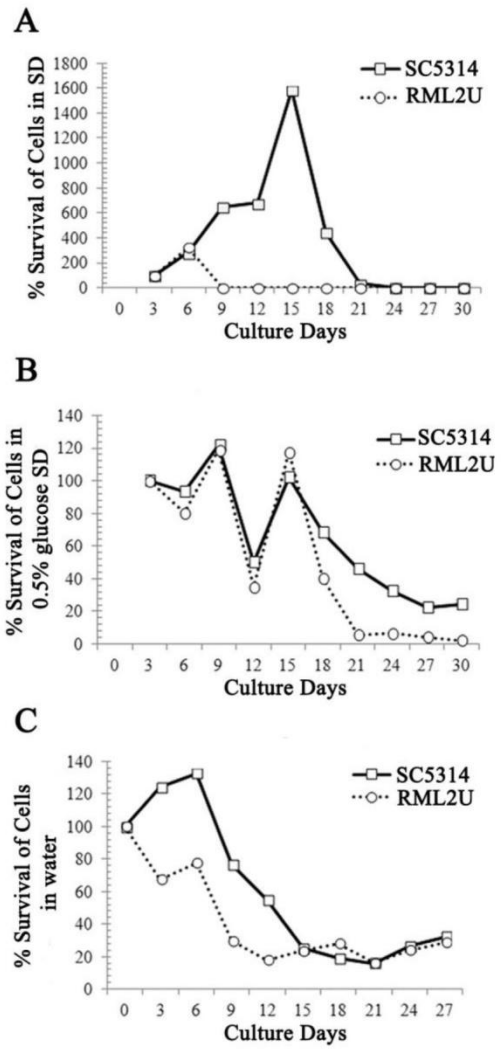

Figure 6. Ecm33 possitively influences the chronological life span of *C. albicans*. (**A**) SC5314 and RML2U were logarithmically growth in SD medium at 30 °C. Samples were taken at the indicated times to determine CLS, as described in Materials and Methods. (**B**) CLS under calorie restriction by glucose reduction (0.5% glucose SD). (**C**) Extreme calorie restriction in which cultures were transferred to water on day 3 further extends the life span of RML2U.

In addition, extreme Calorie Restriction (CR)/starvation, in which stationary phase cells were transferred to water, doubled the mean life span of wild type yeast (Fabrizio et al., 2004;Fabrizio et al., 2005). To understand the role of Ecm33 in CR, we monitored RML2U survival in CLS under CR modeled by glucose reduction (0.5% glucose SD medium) and in water. The behavior of the strains under glucose reduction CR (0.5% glucose) was different from that under CLS (Figure 6B). At the end of the study, on day 30, 10-fold fewer RML2U cells survived (2.4%) than SC5314 cells (24%). The decrease and increase in the survival rate of both strains was gradual, but the percentage of cells was lower in RML2U from day 18 (40%) to 30 (2.4%) compared to SC5314 (68% and 24%). Unexpectedly, the percentage of surviving cells of SC5314 and RML2U by day 30 in starvation/extreme CR conditions was almost similar, at about 30% (Figure 6C). At day 3, the percentage survival of RML2U decreased to 70%, then increased to 77% at day 6 and decrease more gradually from day 9 (29%) to day 30 (24%). The decrease in the percentage survival of SC5314 cells was more gradual from days 9 to 30. Taken together, these data suggest that Ecm33 is required for longevity, whereas it is dispensable for survival in conditions of extreme CR.

Macrophage-*C. albicans* interaction: phagocytosis, cytotoxicity and apoptosis

To understand the effect of *C. albicans ECM33* on the immunological response, some effector functions were

assessed in mouse macrophages. Phagocytosis by RAW 264.7 macrophages were evaluated at a ratio of 1:1 and at different interaction times. Phagocytosis was measured at 45 min, 90 min and 3 h after the start of the interaction. Figure 7A shows that the recognition and engulfment of RML2U at 45 min was statistically significantly higher than for SC5314 cells and no difference was observed at later time points.

To determine the ability of the SC5314 and RML2U *C. albicans* strains to damage RAW 264.7 macrophages, the lactate dehydrogenase (LDH) cytotoxicity detection kit was used to measure the amount of LDH released into the medium from damaged cells. Figure 7B shows that RML2U consistently caused significantly less damage to the macrophages than SC5314 and was more than 30% less cytotoxic, at the different time points studied.

We propose a model of *C. albicans*-macrophage interaction in which more than 30% of SC5314

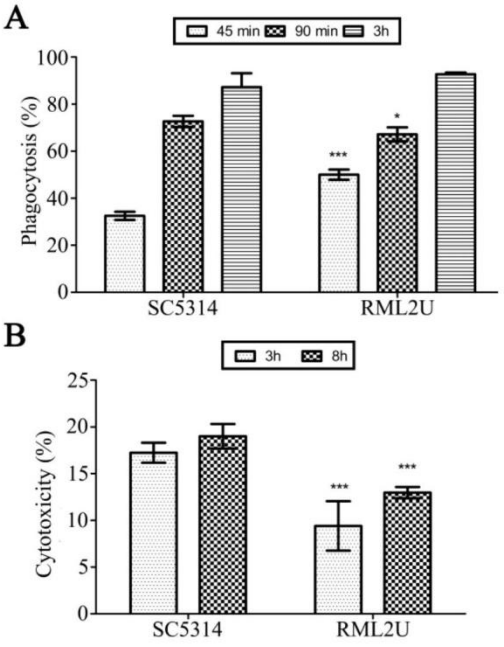

Figure 7. Murine macrophage response to *C. albicans* strains. (**A**) Quantification of phagocytosis of *C. albicans* yeast at 45 min, 90 min and 3 h following co-incubation. (**B**) Cytotoxicity of the SC5314 and RML2U strains in RAW 264.7 macrophages at 3 and 8 h following co-incubation. Data are represented as the mean ± SD (n = 3), and statistically significant differences relative to the wild-type are indicated (*, $p < 0.05$; ***, $p < 0.001$).

C. albicans cells ingested by RAW264.7 died through apoptosis after 12 h of interaction (Cabezon et al. Submitted). Therefore, the apoptotic status of RML2U at different time points of interaction with macrophages was analyzed. Two apoptotic markers were analyzed: the accumulation of ROS and caspase-like enzymatic activity. As shown in Figure 8A, significantly more cells in the RML2U interaction samples (16%) accumulated ROS than the control cells (7%) or *C. albicans* RML2U control cells without macrophages. The percentage of RML2U cells containing activated caspase increased after 3 h of interaction and this percentage (>15%) was maintained throughout the time-period studied (Figure 8B).

DISCUSSION

Fungal cell walls are vital organelles that determine cell shape, protect against physical injury and other stresses imposed by the external environment, and are essential for host-pathogen interactions (Klis et al., 2009). The integrity of the cell wall is very important for the survival of the microorganism and stress damage actives mechanisms developed by the cell to repair and reinforce the wall through cell wall biosynthesis and the integration of cell wall components into the wall, via activation of the CWI pathway (Lagorce et al., 2003). The activation of this pathway is not restricted to an individual stimulus and can be elicited by a number of events. Crosstalk between the CWI pathway and other stress-response pathways enable the CWI pathway to respond to diverse stress events with responses that appropriately alleviate cellular stress (Fuchs and Mylonakis, 2009). Here, the activation of the CWI pathway was studied by the phosphorylation of the 42-44 MAK kinases. The results agree with those of previous studies, which showed that Ecm33 is required for cell wall integrity and morphogenesis in *S. cerevisiae* and *C. albicans* (Martínez-Lopez et al., 2004;Pardo et al., 2004;Gil-Bona et al., 2015b). However, there is evidence for crosstalk between TOR-mediating signaling and the CWI pathway in *S. cerevisiae* (Torres et al., 2002). Previous studies showed the sensitivity of RML2U to rapamycin, the inhibitor of TOR kinase, which connected Ecm33 with this pathway (Gil-Bona et al., 2015b). In addition, to rule out that this sensitivity was due to cell wall damage, the sensitivity to rapamycin was tested in other *C. albicans* cell wall protein mutants, such as *mkc1/mkc1* and *cht2/cht2*. These mutants showed no difference in growth compared with the wild type strain (Supplemental Figure S2).

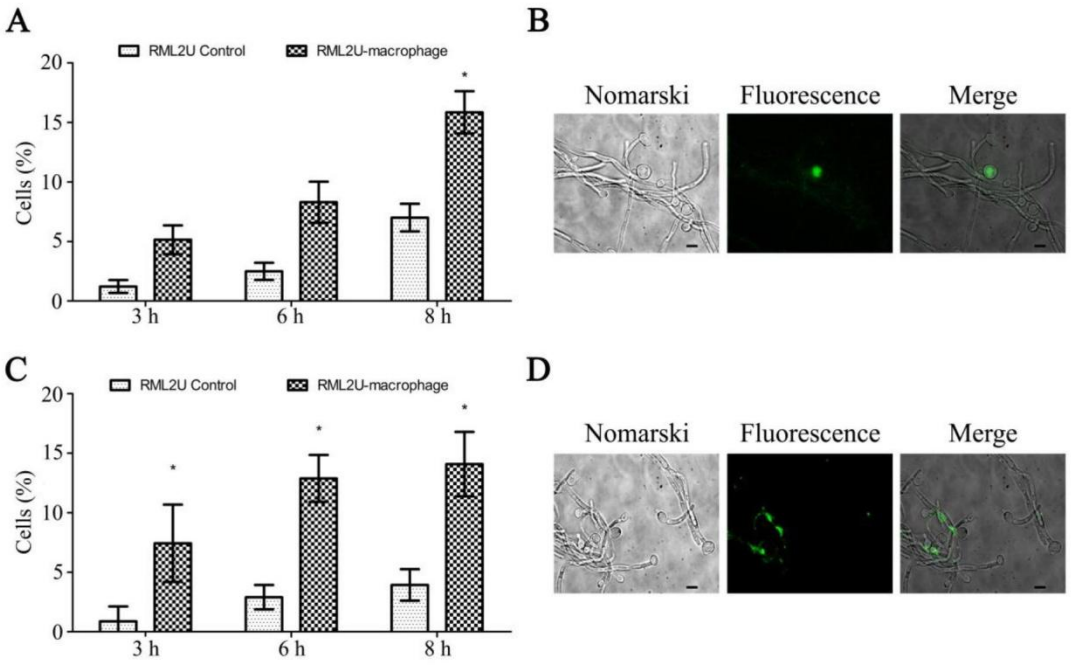

Figure 8. Measurement of the apoptotic status of *C. albicans* strains after the interaction with murine macrophages. (**A**) Percentage of *C. albicans* cells that contain ROS after 3 h, 6 h and 8 h of macrophage interaction. (**B**) Representative fluorescence images of *C. albicans* culture stained with DHR 123. (**C**) Percentage of *C. albicans* cells with active caspase revealed by the caspase inhibitor VAD-FMK-FITC. (**D**) Representative fluorescence micrographs showing caspase activity in *C. albicans* cells. Statistically significant differences relative to the RML2U control are indicated (*, $p < 0.05$).

Thus, these results confirm the role of Ecm33 in the TOR pathway, but not via a direct effect of cell wall damage. This study also tested the involvement of Ecm33 to respond to different types of stress. RML2U showed hypersensitivity to heat stress (from 37 ˚C to 45 ˚C), oxidative stress (Menadione and H_2O_2) and osmotic stress, demonstrating a role for Ecm33 in resistance to these different stresses.

Ecm33 function appears to be linked with the secretion of Sap2 through the TOR signaling pathway (Gil-Bona et al., 2015b). This pathway is an important mediator of survival in a population of non-dividing cells, the CLS, in addition to other cellular responses implicated in aging, growth and morphogenesis (Wullschleger et al., 2006;Kaeberlein et al., 2007;Fontana et al., 2010). Calorie restriction increases the life span of yeast and also of yeast with decreased TOR pathway signaling (Kaeberlein et al., 2005;Powers et al., 2006;Bonawitz et al., 2007;Wei et al., 2008). In optimum nutrient conditions, TOR kinase activity is stimulated, which activate growth processes and inactivate starvation responses. However, in starvation conditions, the activation of growth responses is prevented and starvation responses are activated by the inhibition of TOR kinase activity. Due to the hypersensitivity of RML2U to rapamycin, the role of Ecm33 in the TOR pathway was analyzed by CLS and CR assays. The mean life span of RML2U was reduced compared to that of SC5314 (Figure 6). These results agree with those observed in a large-scale survey in *S. cerevisiae,* in which the CLS of the *ecm33*Δ mutant decreased (Marek and Korona, 2013). In *C. albicans*, only three genes have been implicated in a reduced CLR to date: a protein required for respiratory growth, Growth and Oxidant Adaptation (Goa1), and two protein kinases related to the TOR pathway, Rim15 and Sch9 (Li et al., 2011;Stichternoth et al., 2011). Stichternoth et al. 2011 demonstrated that Sch9 is not involved in regulating CLS in *C. albicans*, and proposed a regulatory pathway for the long-term survival of *C. albicans* that was different from that in *S. cerevisiae*. These data suggest that the reduced survival of RML2U is more related to the cell wall damage than to the TOR pathway. According to these differences in regulation, the High-Osmolarity

Glycerol (HOG) protein is directly involved in the sensitivity of *C. albicans* to rapamycin, whereas it is not in *S. cerevisiae,* but the link between the TOR and HOG pathways remains unclear (Li et al., 2008). It is uncertain how Tor1 activity is regulated in *C. albicans*, but a recent study published a role in hyphal elongation (Su et al., 2013). Moreover, in a large-scale study, genetic and physical interactions were observed between Ecm33 and the two components of the TOR pathway present in *S. cerevisiae*, Tor1 and Tor2 (Aronova et al., 2007;Costanzo et al., 2010). Further genetic and proteomic analysis will further clarify the relationship between Tor1 and Ecm33 in *C. albicans.*

Caffeine (1,3,7-trimethylxanthine) is an analogue of purine bases that is used as a phenotypic criterion to evaluate the CWI, because several mutants that are defective in components of CWI pathway are caffeine-sensitive (Martin et al., 2000;Park et al., 2005). In addition, caffeine interferes with TOR function, inhibiting the function of the kinase and extending the life span of *S. cerevisiae* (Martin et al., 2000;Park et al., 2005;Kuranda et al., 2006;Reinke et al., 2006). For these reasons, and based on previous results, the sensitivity of RML2U to caffeine was tested. As expected, RML2U was hypersensitive to caffeine. Taken together, these phenotypes together with those previously published, such as sensitivity to zymolyase and cell wall-perturbing agents, link Ecm33 function with cell wall integrity and the structure of the β-1,3-glucan network (Martínez-Lopez et al., 2004;Gil-Bona et al., 2015b). In addition, taking into account the importance of Ecm33 for "*de novo*" cell wall biosynthesis, the results suggest that Ecm33 also has a role in cell wall reconstruction.

The RML2U mutant displays an anomalous morphology in standard growth conditions (Martínez-Lopez et al., 2004). In this study, an extensive phenotypic characterization of RML2U was performed, to try to understand the pathways that are altered by the absence of *ECM33* function. The observed morphologies suggest a role for Ecm33 in cell division and cell cycle progression. A previous study, in which the function of the molecular chaperone heat shock protein 90 (Hsp90) was compromised, resulted in a similar morphology, which was attributed to cell cycle arrest (Senn et al., 2012). The same study also showed that the F-actin staining was highly polarized, whereas in this study, RML2U showed actin patches and an unusual distribution in the cell during both growth phases. Polarization of the actin cytoskeleton is required for normal cell shape, thus, Ecm33 is required for the polarized localization of actin patches. The Sur7 transmembrane protein is also required for normal actin localization and cell wall synthesis (Alvarez et al., 2008) and was also detected in the extracellular secretome of RML2U and following fluconazole treatment (Sorgo et al., 2011;Gil-Bona et al., 2015a;Gil-Bona et al., 2015b). However, the role of Sur7 is different following infection by murine macrophages, and *sur7*Δ cells were phagocytozed less efficiently than wild-type cells (Douglas et al., 2012); in this context, RML2U was more engulfed than SC5314 at early time points, but caused less damage to macrophages than SC5314. These data agree with those of previous study (Keppler-Ross et al., 2010), which showed that both murine and bone marrow macrophages have a high preference for yeast cells that contain mannan in their cell wall to be uptaken. Martinez-Lopez et al. 2006 showed that RML2U had an abnormally electron dense outer mannoprotein layer, which might be related to the increased preference for RML2U cells by macrophages. The low damage caused to macrophages by RML2U compared to SC5314 might be due to several reasons, which relate to the phenotypes observed in the mutant, such as the sensitivity to oxidative stress, the delay in forming filaments, or temperature, among others. Apoptosis induced in *C. albicans* by macrophages was measured by two different apoptotic markers: ROS accumulation and caspase-like activity, because the combined evaluation of apoptotic methods is required, to be able to conclude that a cell is undergoing apoptosis (Carmona-Gutierrez et al., 2010). A previous study proposed a model based on combined proteome and transcriptome data obtained from SC5314 cells ingested by RAW264.7 macrophages, which indicated that some of the changes observed at the genomic and proteomic levels might be associated with apoptosis (Fernández-Arenas et al., 2007). In fact, the study of different apoptotic markers in SC5314 cells ingested by macrophages demonstrated that approximately 30% of cells showed apoptotic death after 16 h of interaction with murine macrophages. These SC5314 data were repeated in the present study (Cabezon et al. Submitted and Supplemental Figure S3). In contrast with the published data, 12% of cells contained activated caspase in RML2U cells at the final two time-points studied (6 and 8 h), and ROS accumulation was observed in 16% of cells, 8 h following interaction. Apoptosis in yeast is an essential function of the cell to maintain a balance between young and old cells,

and release compounds to the medium to promote the growth of the most stable and healthy cells (Sharon et al., 2009). These data suggest that the cell death of RML2U within the macrophage is the result of different mechanisms, including apoptosis, because the percentage of cells expressing apoptotic markers is very low, and the hostile environment and the lack of available nutrients, as well as all the disadvantages caused by the lack of Ecm33 function, contribute to the inability of the mutant to cause damage to the immune system.

To conclude, the appearance of "veil growth" in static and stationary RML2U cultures is a new phenomenon. A similar structure was observed during aging of certain white wines, in which yeasts develop a film on the wine surface after alcoholic fermentations, which is known as "flor velum" yeast (Alexandre, 2013). More than 95% of the velum is composed of *S. cerevisiae* and yeasts have acquired the ability to float as an adaptative mechanism to tolerate environmental constraints. A comparative study of a *S. cerevisiae* strain that was unable to form a velum, and one that could, revealed that the main difference between both strains was the presence of a mannoprotein in the cell wall of the flor forming yeast (Alexandre et al., 2000). However, other processes observed in RML2U and not in SC5314 might be responsible for, or participate in, velum formation, such as the flocculation process, aging or aberrant morphology (Figures 4 and 5) (Martínez-Lopez et al., 2004). In an attempt to connect the previous results, if the TOR pathway was affected because Ecm33 potentially functions upstream to the pathway, nutrient starvation due to growth on cornmeal should enhance autophagy. In fact, cornmeal medium is used to stimulate chlamydospore formation in *C. albicans*, an adaptation to survive unfavourable conditions. The function of this process is similar to autophagy, which is the adaptative response to stress and promotes cellular survival, but in some cases, promotes cell death (Kroemer et al., 2009). Possibly, RML2U cells enter the survival stage and show phenotypes similar to both autophagy and chlamydospore formation: round cells similar to chlamydospores and vacuolization and translucent cytoplasm similar to during phagocytosis. Furthermore, the RML2U cells observed by TEM are similar to those observed in a GPI-anchor synthesis mutant of *Aspergillus fumigates,* which showed morphological evidence of autophagy (Yan et al., 2013). Massive vacuolization and translucent cytoplasm were observed by TEM in this mutant after growth for 24 h and 36 h, similar to RMLU2 cells grown in cornmeal medium. Although, the nitrogen source and addition of amino acids did not rescue the wild type phenotype of RML2U cells, this morphology was even more extreme in SC5314.

CONCLUSIONS

The data presented in this study implicate Ecm33 in cell wall biogenesis, morphogenesis, tolerance to stress-induced agents, host-pathogen interactions and survival. The exact role of this protein is still unknown, but previous published data, together with those here, suggest a role in two different pathways: the CWI and TOR pathways. In addition, the "veil growth" observed in this mutant represents a form of growth that has never been described for *C. albicans,* and is probably related to a role to adapt and survive in extreme environmental conditions.

ACKNOWLEDGMENTS

This work was supported by BIO2012−31767 from the Ministerio de Economía y Competitividad, PROMPT (S2010/BMD-2414) from the Comunidad Autónoma de Madrid, REIPI, Spanish Network for the Research in Infectious Diseases (RD12/0015/0004), and PRB2-ISCIII (PT13/0001/0038) founded by ISCIII and FEDER. A. Gil-Bona was the recipient of a fellowship from Ministerio de Ciencia e Innovación. CM. Parra-Giraldo was the recipient of a fellowship from the program Continuous training for teachers of the Pontificia Javeriana University (Colombia) in conjunction with COLCIENCIAS-LASPAU University of Harvard. The electron microscopy was carried out in Centro Nacional de Microscopía Electrónica (ICTS)-UCM. These results are lined up with the Spanish Initiative on the Human Proteome Project (B/DHPP).

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Appendix Chapter 4

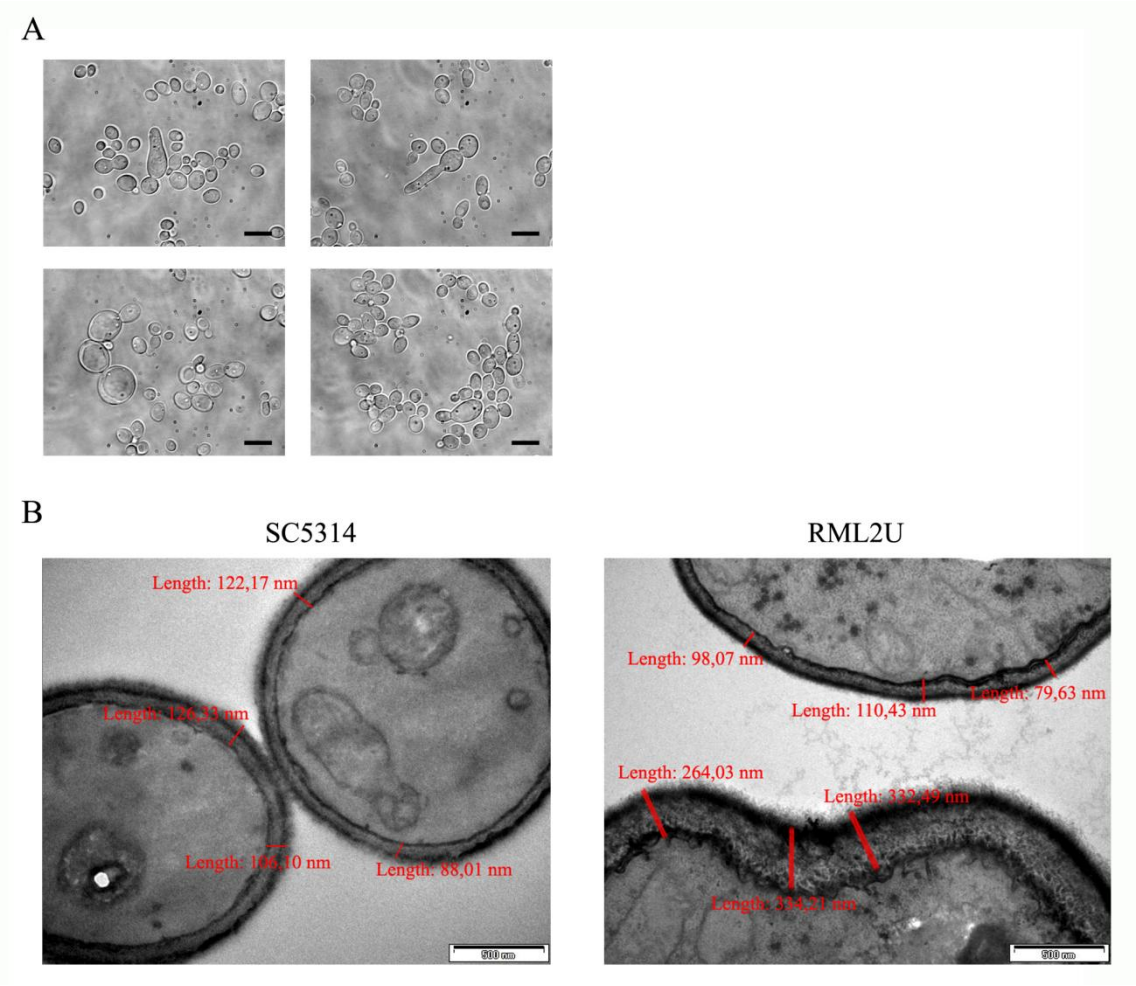

Figure S1. RML2U cells show abnormal morphologies in standard growth conditions. (**A**) Different morphologies of RML2U cells growing in YPD medium at 30 ˚C in exponential phase. (**B**) Representative TEM images of SC5314 and RML2U cells obtained from YPD cultures at 30 °C during the exponential phase. Red lines indicate the thickness of the cell wall. Scale bars: 500 nm

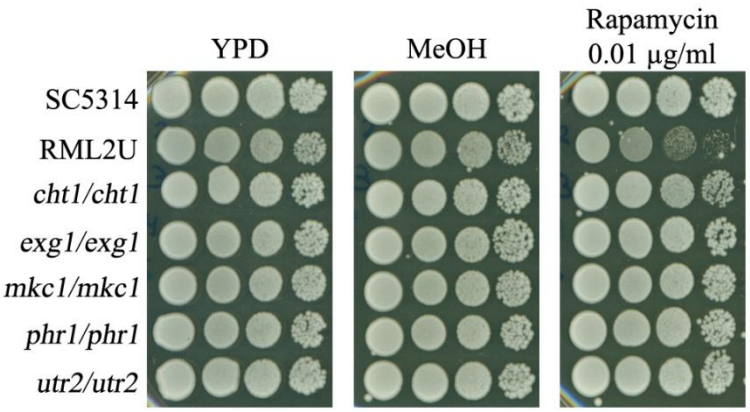

Figure S2. Deletion of different genes related to cell wall not increase susceptibility to rapamycin. 10-fold serial dilutions of strains SC5314 (wild type), RML2U (*ecm33/ecm33*), *cht1/cht1*, *exg1/exg1*, *mkc1/mkc1*, *phr1/phr1* and *utr2/utr2* of *C. albicans* were spotted onto YPD with 0.01 µg/mL rapamycin or with MeOH (as control). Plates were incubated at 30 °C for 24 h. Data are representative of at least three independent experiments with identical results.

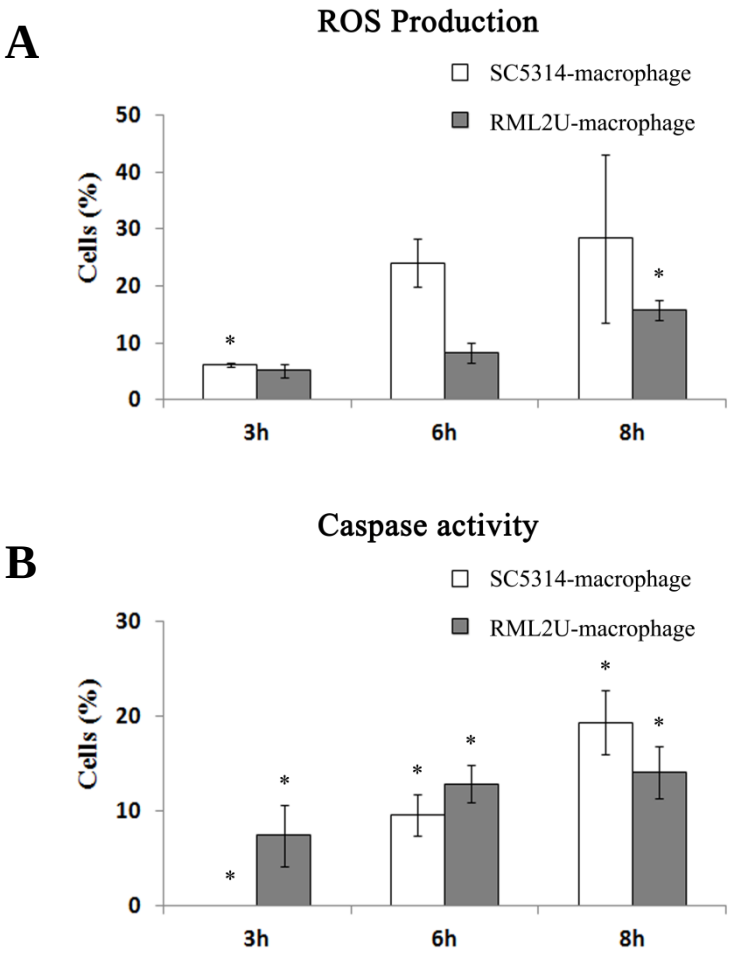

Figure S3. Measurement of the apoptotic status of SC5314 and RML2U *C. albicans* strains after the interaction with murine macrophages. (**A**) Percentage of *C. albicans* cells that contain ROS after 3 h, 6 h and 8 h macrophage interaction. (**B**) Percentage of *C. albicans* cells with active caspasa after 3 h, 6 h and 8 h macrophage interaction. (*, $p < 0.05$).

Supplemental Table 1. Number of RML2U aberrant cells. Different morphologies resulted from compromised *ECM33* function were observed in cells growing in YPD medium at 30 °C by nomarski microscopy. The number of aberrant cells was counted and the percentage was obtained.

		Aberrant morphology	No DNA detected	More than 1 nucleus	Total aberrant cells (total cells observed)
Exponential Phase	**Number of observed cells**	94	20	4	118 (922)
	Percentage	10%	2%	0.43%	12.43%
Stationary Phase	**Number of observed cells**	106	23	3	132 (509)
	Percentage	21%	4.51%	0.58%	25.82%

Supplemental Table 2. Survival rate after CLS. The number of colonies at day 3 was considered to be the initial survival (100%). Cells of each strain were collected every 3 days and plated on YPD agar for 48 h to determine % survival. The percentages showed in the table correspond to the end of the study (day 30).

	Survival rate after treatment	
	SC5314	**RML2U**
Chronological Life Span (CLS)	0.7%	0.08%
0.5 % Glucose SD	24.7%	2.4%
CLS under Extreme Calorie Restriction – Water	32.26%	28.83%

General discussion

As commensal, *C. albicans* is harmless, however, when the balance of the normal flora is disrupted or the immune defenses are compromised, this fungus becomes pathogenic. In these conditions, *C. albicans* can colonize mucosal surfaces and cause symptoms of disease, whereas in healthy individuals, it belongs to the normal microbiota of skin and mucosal surfaces, detected in up to 50% of the healthy population. Although the host-immune state is the primary determinant of the severity of candidiasis, *C. albicans* ability to switch between different morphologies clearly contributes to its virulence in susceptible individuals. In addition, the secretome of this fungal pathogen help it in tissue invasion, immune evasion, cell wall integrity, biofilm formation and nutrient acquisition. For these reasons, the objectives of this thesis were the proteomic study of proteins secreted by this opportunistic fungal pathogen and the functional analysis of the proteins identified at the cell surface and the secretome, in particular the Ecm33 mutant.

1. Relevant roles of proteins identified by shaving *C. albicans* yeast and hypha cell surface

Previous published works in our laboratory studied the identification of *C. albicans* surface proteins under different conditions: live yeast, hypha and biofilms (Hernáez et al. 2010, Vialas et al. 2012). The methodology used to identify these proteins were based on a non-gel proteomic approach that used a short period of trypsin treatment followed by peptide separation and identification using nano-LC followed by off-line MS/MS. Based on this methodology, CM. Parra-Giraldo (Parra-Giraldo 2013) (Chapter 1) carried out the study of the surface proteome of *C. albicans* yeast and hypha cells. The improvement of this work was the use of a LTQ-Orbitrap Velos, an ultra-high resolution mass analyzer with increased sensitivity, versus the MALDI TOF/TOF used in previous works. A total of 438 and 928 proteins were identified in yeast and hypha morphology, respectively, versus the smaller number of detected proteins reported in previous works (Ebanks et al. 2006, Martinez-Gomariz et al. 2009, Hernáez et al. 2010, Heilmann et al. 2011, Sosinska et al. 2011, Vialas et al. 2012). These previous studies performed no characterization of the identified proteins. For this reason, 17 proteins identified in the global surfome analysis of *C. albicans* yeast and hypha-surface by cell shaving carried out by CM. Parra-Giraldo, were selected to investigate their function; in

particular, their involvement in cell wall maintenance, stress resistance and yeast to hypha transition.

The selection of these 17 proteins was based on their mutant availability in the Noble collection (Noble et al. 2010), obtained from Fungal Genetics Stock Center, and their unknown function. Other cell wall-related proteins identified, such as Phr1, Phr2 or Pir1, identified in both morphologies, were studied previously (Fonzi 1999, Martinez et al. 2004, De Virgilio and Loewith 2006). In addition, Ecm33 was identified in both morphological states and it was also thoroughly studied and it is discussed ahead. The resistance of the 17 selected mutants to cell wall, oxidative and osmotic stresses was analyzed, as well as the sensitivity to high temperatures and the dimorphic transition process. Out of the 17 mutants, eight did not show any sensitivity to the compounds or temperature studied (*ihdΔ*, *orf19.3335Δ*, *orf19.6553Δ*, *orf19.7196Δ*, *orf19.7238Δ*, *pga45Δ*, *ptp3Δ* and *ycp4Δ*). Two of these proteins were GPI proteins, Ihd1 and Pga45, with a presumably role in cell wall, but it was not detected in our study conditions. Both proteins are members of protein families. In this context, other members of the same family with similar functions in the cell could compensate their absence. Ihd family is composed by two proteins, Ihd1 and Ihd2. The function of Ihd2 is unknown but there are evidences of its implication during hyphae development (Carlisle and Kadosh 2013). Pga proteins are more than 30 GPI proteins with a large variety of functions. Little is known about the functions of these 8 proteins, which mutant strains did not show sensitivity to the stressor tested, and slso regarding Orf19.3290, which mutant was only sensitive to osmotic stress. The other 8, out of the 17 initial mutants, had a marked increased susceptibility to some of the stresses tested, *ali1Δ, mci4Δ, orf19.287Δ, orf19.3060Δ, orf19.5352Δ, orf19.7590Δ, pst3Δ* and *tos1Δ*. From those, only Tos1 was detected in both morphologies. The other 7 proteins were identified only in hypha (Figure 10).

Seven of the mutants presented sensitivity to cell wall-disturbing agents, *ali1Δ, orf19.287Δ, orf19.3060Δ, orf19.5352Δ, orf19.7590Δ, pst3Δ* and *tos1Δ*, which was suppressed in osmotic medium stabilized with sorbitol 1 M. Three of them, *orf19.3060Δ, orf19.5352Δ* and *tos1Δ* mutant strains, only presented defects when they grew in the presence of the cell wall-disturbing agents, showing to have a cell wall-specific defect. In addition, *pst3*Δ and *orf19.3060*Δ mutants showed an increase in their

ability to cause damage in the murine macrophages. This damage could be explained for the higher rate of mutant cells engulfed and their normal hyphal formation. The mutation of these proteins could affect the composition of specific components of the *C. albicans* cell wall, leading to an increase in recognition by macrophages, as occurs with different O-linked and N-linked mannan-deficient strains (McKenzie et al. 2010). The *orf19.3060*Δ mutant also showed to be of significant importance in regulating *C. albicans* interaction with oral epithelia cells *in vitro*. As mentioned above, the

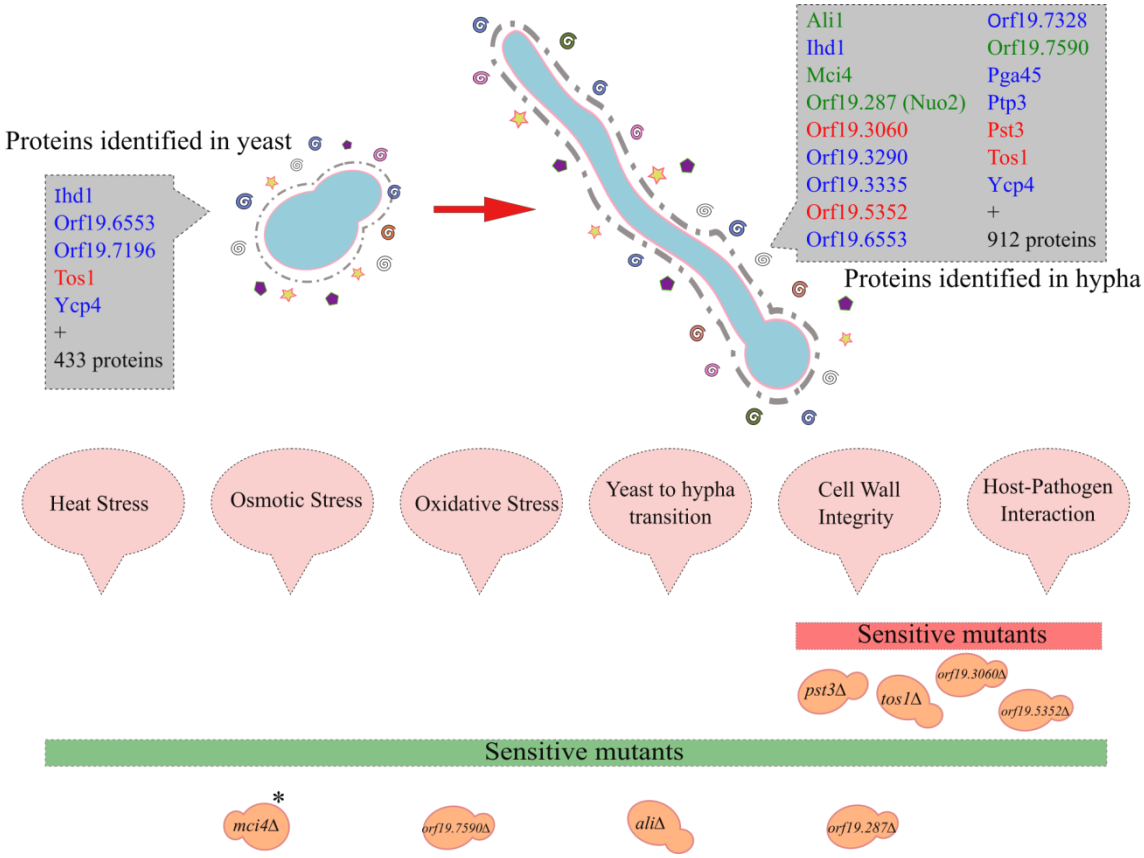

Figure 10. Summary of the main findings of the phenotypic and virulence analysis of interesting proteins identified in *C. albicans* cell surface. Blue proteins correspond to the mutants that did not show any sensitivity to the compounds or temperature studied. Green and pink proteins correspond to the mutants which showed different phenotypes than the wild-type strain. * The *mci4*Δ mutant did not present sensitivity to cell wall disturbing agents. See Chapter 1 for further details.

glycosylation status of the *C. albicans* cell wall is critical in host-pathogen interaction. Besides being involved in phagocytosis, it is also critical for the stimulus and regulation of epithelial responses (Wagener et al. 2012). Moreover, *tos1*Δ and *orf19.5352*Δ showed high variability in both assays with FaDu cells, showing slightly increased endocytosis and acting similarly to the wild-type strain in the damage assays, suggesting that are not essential for adhesion and endocytosis by FaDu cells. The *orf19.3060Δ, orf19.5352Δ, pst3Δ* and *tos1Δ* strains did not present difference in virulence in the mouse model of OPC, showing that they are not necessary for virulence during oropharyngeal candidiasis.

On the other hand, the other four mutants that showed sensitivity to several stresses, *ali1Δ, mci4Δ, orf19.287Δ* and *orf19.7590Δ,* also presented filamentation defects in 10% serum agar plates and growing defects in the Spider plates containing mannitol. In fact, these mutants were incapable of growing using this C-source. Recently, Orf19.287 was named as Nuo2, and it was verified as NADH:ubiquinone oxidoreductase (She et al. 2015). The data obtained in that study are in agreement with the results showed in the present thesis, including a reduced colonization of tissues and a corresponding avirulence in an invasive model of candidiasis. The other three proteins are described in CGD also as putative NADH-ubiquinone oxidoreductase (Ali1 and Orf19.7590) and as putative NADH-ubiquinone dehydrogenase (Mci4). The first two, Ali1 and Orf19.7590, were previously identified by 2-D gel in the DTT/SDS-extraction of the cell wall-enriched fraction in the yeast and hyphae, more expressed in hyphae fraction (Ebanks et al. 2006). The interaction assays of these four mutants with RAW 264.7 macrophages at a MOI 1:1 displayed that *mci4Δ* and *orf19.287Δ* mutants were more phagocytized and showed an increased in their ability to cause damage in the host cells. In particular, the *orf19.287Δ* mutant was the most cytotoxic and it was highly phagocytized. We hypothesized that the large number of phagocytized cells could cause the increased damage by other mechanism, such as apoptotic signals or high secretion of proteins or enzymes involved in virulence. Furthermore, macrophages phagocytized and killed more *ali1Δ* and *orf19.7590Δ* mutants. This phenotype and their sensitivity to oxidative stress suggest that these proteins could have a role in avoiding the damage produced by reactive oxidative species inside the macrophages. In addition, as for the interaction with oral epithelial cells *in vitro*, these four mutants showed decreased endocytosis and

reduced capacity to cause damage, probably due to their slow generation time and their incapacity in yeast to hypha transition.

All of this data focuses on the analysis of these four proteins, Ali1, Mci4, Nuo2 (Orf19.287) and Orf19.7590, relevant in cellular processes, such as cell wall maintenance, osmotic and oxidative stress resistance and host-pathogen interplay. The other four proteins commented before, are related to cell wall maintenance. This is an important success because the goal of this work was to find proteins involved in cell wall maintenance and proteins involved in host-cell interaction. However, many proteins were detected in the surfome study and they could have important roles in pathogenicity. For this reason, and considering that fungal cell wall is the part of the cell that acts as protective structural shield and also contributes to interactive contacts with the human host during the infection, the analysis of the proteins exposed on the fungal surface and the fact that they are different from the host proteins highlights the importance to find potential targets for new drugs. Among these exposed proteins, we found an important number of classical cytoplasmic proteins. The results for the proteomic analysis of EVs of *C. albicans* could explain this finding as commented in the next section.

2. Extracellular vesicles transport metabolic proteins to the extracellular media in *C. albicans* and non vesicular secreted proteins were secreted by the classical secretory-pathway

Recently, a number of studies about the protein secretion of various pathogenic fungi have been published. In case of the *C. albicans* secretome, several published studies analyzed the global protein composition under different growth conditions (Sorgo et al. 2010, Sorgo et al. 2011, Ene et al. 2012, Sorgo et al. 2013). *C. albicans* secretes proteins with functions related to biofilm formation, tissue invasion, cell wall and nutrient acquisition. Also, classical cytoplasmic proteins were detected in the growth medium. Release of EVs into culture supernatants of fungi has been investigated in detail in the last few years (Albuquerque et al. 2008, Rodrigues et al. 2008, Oliveira et al. 2010, Vallejo et al. 2012). Fungal EVs contain a complex molecular mixture, including many cytoplasmic proteins, polysaccharides, lipids and pigments (Rodrigues et al. 2007, Albuquerque et al. 2008, Rodrigues et al. 2008, Vallejo et al. 2012). In

C. albicans, the presence of EVs was observed by TEM in a previous study (Albuquerque et al. 2008). These authors demonstrated the presence of bilayered structures similar to those described for other fungi, such as *C. neoformans* or *H. capsulatum*. In the present thesis, the proteomic study of *C. albicans* EVs was carried out in parallel with the EV-free supernatant proteomic analysis. A total of 96 extracellular proteins were identified, being 75 proteins identified in EVs and 61 in EV-free supernatant. A relevant result of this work is that 93% (57) of the proteins identified in the extracellular medium (61) are canonically secreted proteins bearing signal peptide, which suggest that the classical secretory pathway is the general mechanism used for *C. albicans* to secrete proteins into the medium. The other four proteins in which a signal peptide is not detected by Signal P are the 40 ribosomal subunit Asc1, the polyubiquitin Ubi4 and two Orfs, Orf19.6119 with unknown function and Orf19.6741, a putative plasma membrane protein with predicted role in cell wall integrity. Some of the secreted identified proteins were categorized as virulence factors or involved in biofilm formation, nutrient acquisition, pH regulators or required for tissue invasion, such as Als proteins, Ecm33, Mp65, Sap proteins or Phr2, among others (Martínez-Lopez et al. 2004, Mayer et al. 2013, Pericolini et al. 2015).

The proteomic analysis of *C. albicans* EVs showed that all of the proteins involved in metabolism identified in the secretome were exclusively detected in this sample, such as Eno1, Gpm1, Pdc11, Pgk1, Tal1, Tdh3 and Met6, as well as proteins involved in protein folding or protein synthesis. This mechanism of protein transport could explain the problem of non-conventional secretion of proteins lacking typical secretory signal peptide and their incorporation into cell wall or exposition at the cell surface. In the present work, 60% (45) of the proteins detected in EVs have the secretory signal peptide. This list includes GPIs (14), cell wall-related proteins (17), transmembrane transport proteins (5) and other proteins related to secretion, protein synthesis and secreted hydrolases. Recently, a compositional and immunological analysis of *C. albicans* EVs was published, at the same time that we published our work (Vargas et al. 2015). This work contains the proteomic analysis of EVs from strains 11 and ETCC 90028, other *C. albicans* clinical isolates different from SC5314. The identification of the proteins was performed using a different database and 57 unique proteins were identified versus our 75 proteins detected in EVs. Thus, the work published in the present thesis includes the highest number of proteins described up to date in

C. albicans. The functions of the proteins identified in the two studies are the same: cell adhesion, cell wall organization or metabolism of carbohydrates among others. As it was discussed in Chapter 2, Vargas et al. also conclude that vesicle transportation could explain why some nuclear and cytosolic proteins are detected in the cell wall and the extracellular medium. Finally, they also suggested that EVs might correspond to cytoplasmic subtractions derived from plasma membrane reshaping, which is in agreement with our hypothetical model published in Chapter 2 and displayed again in Figure 11, based on our results and previously published observation (Rodrigues et al. 2013). In addition, Vargas et al. investigated the kinetics of internalization of EVs by

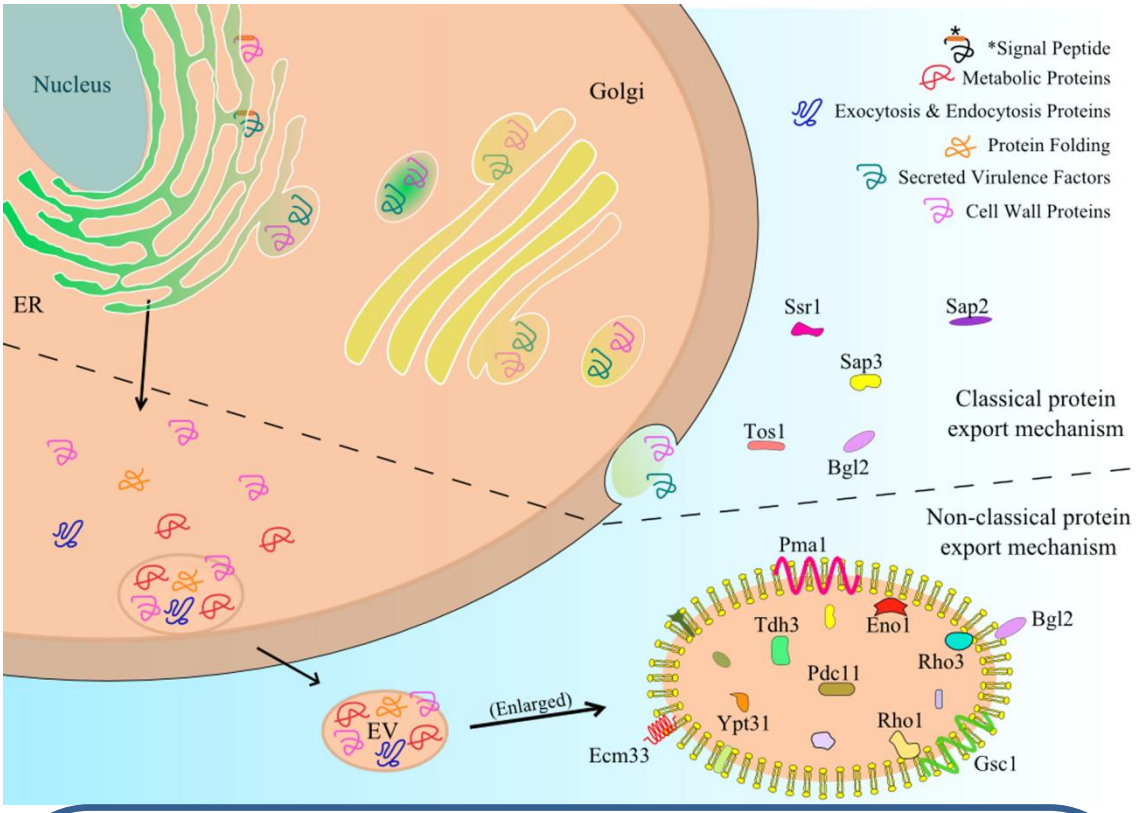

Figure 11. Model of two different *C. albicans* secretory pathways (Chapter 2). The classical protein ER-Golgi pathway that secretes proteins with N-terminal signal peptide is shown in the upper part. Underneath, the unconventional secretion mechanism proposed, by which proteins (with and without signal peptide) would be carried to the extracellular medium in vesicles formed at the plasma membrane, is represented. Enlarged view of EV was shown. Representative protein groups and single proteins identified in EVs and EV-free supernatant were shown.

bone-marrow derived murine macrophages and by dendritic cells, and the immunomodulatory activity of these vesicles, as well as the impact of them on fungal virulence using the *Galleria mellonella* larvae model. As we observed in Chapter 2, the EVs contain seroreactive proteins, and as we supposed and discussed in the Chapter 2, EVs shown to be immunologically active and to have the potential to interfere with the host responses in the setting of invasive candidiasis. It would be appropriate to carry out future investigations of their role in stimulating disease control in mammalian models of invasive candidiasis or their role in enhancing fungal pathogenesis.

As it has been mentioned before, the study of EVs is a hot topic in fungal research. Besides these two studies discussed above (Gil-Bona et al. 2015, Vargas et al. 2015), other two works has been recently published about *C. albicans* EVs. One of them characterized EVs as carriers of fungal RNA to the extracellular media (Peres da Silva et al. 2015). The implication of these findings for fungal pathogenesis remains unclear, but opens up new possibilities for investigating how these nucleic acids could interfere with gene expression in host cells. The most recent study published observes the relationship between *C. albicans* lipid biosynthetic genes and EVs (Wolf et al. 2015). It showed that the phosphatidylserine decarboxylase (*PSD1* and *PSD2*) mutant releases EVs larger than those from the wild-type and activated the NFkB in bone marrow-derived macrophage and murine macrophage-like cell lines showed no statistical difference from wild-type strain. However, the mutant of phosphatidylserine synthase (*CHO1*) releases EVs comparable in size to wild-type EVs and they did not activate the NFkB. These mutants also exhibit defects in cell wall integrity (Chen et al. 2010), as the mutant studied in the present thesis, RML2U which showed large EVs than the SC5314 strain and defects in the cell wall, as discussed below. The conclusion of this study highlights a complex interplay between lipid metabolism and vesicle production. Therefore, all of these data together showed that the *C. albicans* EVs are a very complex field of study that just starts to take shape. There are many points remain unclear, such as its implication in *C. albicans* pathogenicity, the process of EVs formation or their function in the cell. However, the rate at which the publications are coming out suggests that many of these questions would be resolved soon.

3. The importance of Ecm33 in cell wall, cell morphology and its relation to TOR pathway in the human pathogen *C. albicans*

Ecm33 is a GPI-anchored cell wall protein of the human pathogen *C. albicans* as well other fungi species, such as *S. cerevisiae, Schizosaccharomyces pombe, M. robertsii* and *B. bassiana.* In *C. albicans*, this protein is involved in fungal cell wall integrity, host cell adhesion, host cell damage and endocytosis. In addition, mutant shows morphology defects during the yeast to hypha transition under different filament-inducing conditions and it is avirulent in a mouse model of systemic infection (Martínez-Lopez et al. 2004). Ecm33 has been detected in all the proteomic studies published in the present thesis, including *C. albicans* yeast and hyphae cell surface (Parra-Giraldo 2013) (Chapter 1), yeast EVs and yeast EV-free extracellular secretome analysis (Chapter 2). Other proteomic studies published previously also identified Ecm33 in *C. albicans* cell wall, plasma membrane and extracellular secretome under different growth conditions (de Groot et al. 2004, Castillo et al. 2008, Cabezón et al. 2009, Sorgo et al. 2011). Despite its identification in different cell and extracellular cell locations, its molecular function remains unknown.

The analysis of extracellular secretome of RML2U unravels several important issues. As commented in Chapters 2 and 3, the EVs were separated from the rest of the soluble proteins by ultracentrifugation. The proteomic analysis of EVs and the soluble fraction was done separately. Interesting data was obtained. A total of 170 single proteins were identified, 114 proteins for the EV-free supernatant and 154 proteins for the EVs. Of them 98 were common for both fractions. Interestingly, most of the proteins secreted by RML2U strain to the extracellular medium had no predicted signal-peptide sequence and were annotated as being intracellular location. Several of these proteins are included in ribosomal families, such as *RPL*, *RPS* and *RPP*, or are metabolic proteins such as Eno1, Gdh3, Met6 or Tdh3, among others. These metabolic proteins were not detected in the EV-free supernatant of SC5314 strain. Remarkably, this data is in agreement with a study published several years ago in which the effect of fluconazole on the wall integrity and extracellular proteome of *C. albicans* was analyzed (Sorgo et al. 2011). Fluconazole is an antifungal drug which causes increased membrane fluidity and drug permeability. In the mentioned study, the number of proteins with predicted intracellular localization was increased in the secretome of the treated cells. However, RML2U

secretes more classical cytoplasmic proteins than SC5314 growth with fluconazole, suggesting that its cell wall aberrant organization might cause higher traffic of proteins through the cell wall, thus enriching their presence outside the cell. However, other possible explanation for the increased number of proteins detected in RML2U is that the proteomic analysis in the fluconazole study was less sensitive than ours and a new study with a high-resolution mass analyzer could elucidate these differences.

When the EV-free supernatant and EVs from SC5314 and RML2U were compared, just 10 and 6 proteins identified in SC5314 were not identified in RML2U samples, respectively. Two of these proteins undoubtedly are relevant, the Orf19.4952.1 and Sap2, both related directly or indirectly with Target of Rapamycin (TOR) pathway. The *S. cerevisiae* orthologue of *ORF19.4952.1*, *FPR2*, binds to the drugs FK506 and rapamycin (Nielsen et al. 1992, Partaledis et al. 1992). Its absence could influence on sensitivity to rapamycin, although *fkb2* (*fpr2*) mutation looks as sensitive as the wild-type strain to rapamycin. RML2U strain showed increased sensitivity to rapamycin, whereas the complementing strain RML4U resulted in a phenotype that was indistinguishable from the wild-type strain (Chapter 3). However, there is no evidence that the absence of this protein outside the cell could influence the sensitivity to the drug. Other protein not detected in RML2U extracellular proteome was Sap2. *C. albicans* Saps are considered to play important roles in the pathogenicity of this fungus. The pathological implication of Sap expression by this fungus in mucosal infections was observed by experimental and clinical evidences (Hube et al. 1997, Schaller et al. 1999), although the experimental systemic *C. albicans* infections with *sap*-null mutants have produced some contradictory findings (Lermann and Morschhauser 2008, Correia et al. 2010). In particular, Sap2 is dominantly expressed under various *in vitro* and *in vivo* conditions, including experimental infected rats and in women affected by vulvovaginal candidiasis (Schaller et al. 1998, Naglik et al. 2003). Also, a recently publication showed that it is able to cause vaginitis in mice (Pericolini et al. 2015). In the Chapter 3, proteomic and western blotting analysis confirmed the absence of extracellular Sap2 in RML2U cultures but its intracellular presence indicated a normal expression of Sap2, suggesting a problem in Sap2 secretion pathway. Furthermore, RML2U is not able to degrade BSA in a medium with BSA as a sole nitrogen source in which medium Sap2 is significantly expressed (Staib et al. 2008). This reduced proteolytic activity of the RML2U strain together with the fact that

RML2U secretes more proteins by the classical secretory pathway, including other members of the Sap family, suggests a different or specific mechanism of Sap2 secretion which could have been compromised by the absence of Ecm33. Other *C. albicans* mutants that showed hypersensitivity to rapamycin, also results in an altered Sap2 secretion, *vps1*, *vps4* and *rhb1*-deleted mutants (Bernardo et al. 2008, Lee et al. 2009, Chen et al. 2012). Furthermore, Rhb1 mediates cross-talk between the TOR signaling pathway and the CWI pathway (Tsao et al. 2009). However, the mechanism of *VPS* mediated Sap2 secretion and Rhb1-TOR signaling remains unknown. Based in all of these results, a possible protein regulated secretion mechanism different for Sap2 could be used by *C. albicans*. In yeast, this type of particular secretion has not been described until now.

The incapacity to secrete Sap2 and the hypersensitivity to rapamycin of RML2U (Chapter 3) lead us to study if Ecm33 have an indirect role in TOR pathway. TOR is a growth regulator that senses and integrates diverse nutritional and environmental cues, including growth factors, energy levels and cellular stress, and also limits autophagy (Wullschleger et al. 2006, Kaeberlein et al. 2007, Fontana et al. 2010). This pathway is not well known in *C. albicans*. While in *S. cerevisiae* the central component of the TOR pathway contains two *TOR* genes codifying the closely related kinases, Tor1 and Tor2, in *C. albicans* only one *TOR* gene has been identified, *TOR1* (Cruz et al. 2001, Loewith and Hall 2011). Besides, to confirm that the RML2U sensitivity to rapamycin was not only related to damage in the cell wall, the sensitivity to rapamycin was also tested in other *C. albicans* mutants of cell wall proteins. These mutants did not show difference of growth compared with the wild type strain (Chapter 4. Supplemental Figure S2). In addition, RML2U showed sensitivity to caffeine, a compound used to evaluate problems in CWI pathway as well in TOR function (Martin et al. 2000, Park et al. 2005, Kuranda et al. 2006, Reinke et al. 2006), which supports previous results.

Recently, it has been published that the transmembrane protein Mtl1 is a key regulator of TOR and Protein Kinase A (PKA) signaling pathways in *S. cerevisiae* (Sundaram et al. 2015). The absence of Mtl1 shortens CLS while its over expression increases CLS, suggesting a positive role in the chronological life span. Moreover, the values in the *mtl1tor1* double mutant were similar to those in *tor1*, suggesting that Mtl1 might function upstream of TORC1, negatively regulating Tor1 in the progression though

stationary phase. These authors also studied the CLS in conditions of glucose restriction and *mtl1* deletion decreased life span compared with wild type values but the life span increased compared to the other condition. They concluded that Mtl1 acts as inhibitor of the AGC family protein kinase Sch9 activity under their study conditions and Sch9 is also an inhibitor of the MAP kinase Slt2 activity in stationary phase and in the absence of glucose or in glucose restrictive conditions. However, a previous study displayed that Sch9 is not involved in the *C. albicans* CLS regulation (Stichternoth et al. 2011). Based on these results, together with previous data, the Figure 12 shows the hypothetical model for TOR and CWI signaling pathways in *S. cerevisiae* in order to compare it with the *C. albicans* model proposed. In the present thesis, it was observed that RML2U has the life span reduced compared to the wild type in all the studied conditions (Chapter 4), and, as observed in *mtl1* mutant, the values under calorie restriction and water, are higher than the CLS condition. Besides, Mkc1 is phosphorylated in RML2U under basal growth conditions, showing the activation of the CWI pathway (Chapter 4). However, although these data is similar in both cases, there is no clear evidence that relates directly Ecm33 to the TOR signaling pathway.

Bioinformatics analysis done in *C. albicans* Ecm33 protein and other family members described a domain similar to "L-Domain receptor" that is usually present in the linking region to ligand of important receptors with great physiological relevance as the insulin-like receptor, or the epidermal growth factor receptor. Besides, TOR pathway responds to growth factors (insulin or insulin-like growth factors) in mammals (Wullschleger et al. 2006). In this way and taking previous data together, Ecm33 could be presented as a possible receptor of some physiological ligands, to coordinate the proper cell wall organization with cell cycle progression. Besides, the *Candida albicans* database (CGD) describes the presence of a leucine rich region ("Leucine Rich Repeat" o LRR) in Ecm33, which are typical regions for protein-protein interactions. During the present thesis, a dot blot was done to determine if Ecm33 was able to bind insulin. No clear results were obtained. Further analysis would be needed to determine the role on this pathway. Figure 12 shows a hypothetical and simplified scheme of how Ecm33 could be involved in TOR and CWI pathways. The analysis of *tor1ecm33* double mutant in *C. albicans* would be interest to perform. Also, the complementation of rapamycin-sensitive phenotype of RML2U with the *TOR1-1* mutant allele could give an idea of the role of Ecm33 in TOR pathway. *TOR1-1* mutant allele carries a point mutation

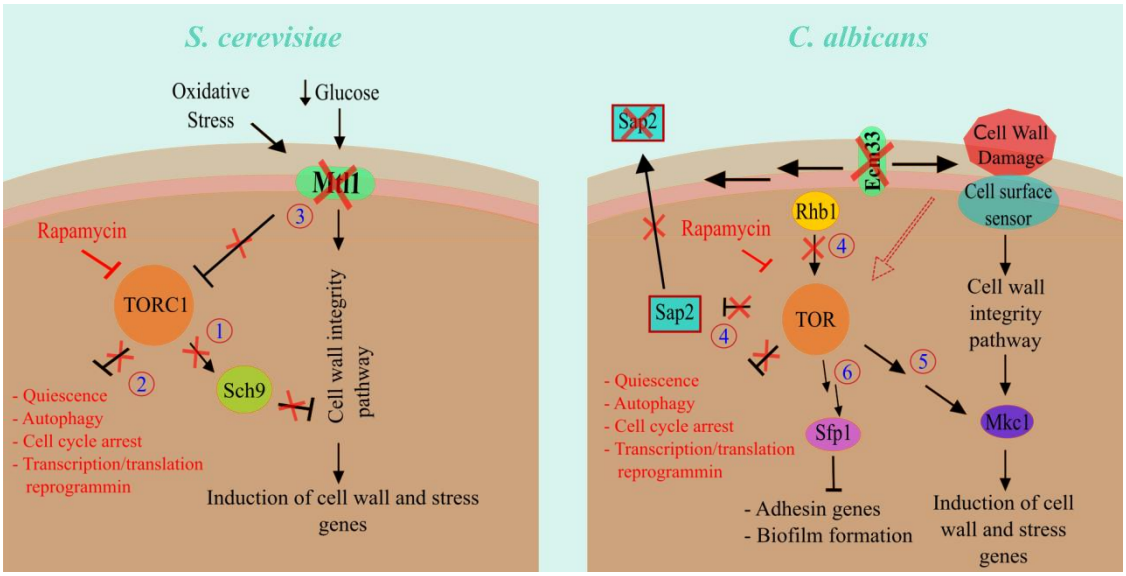

Figure 12. Hypothetical model for TOR and CWI signaling pathways in *S. cerevisiae* and *C. albicans*. Right: Hypothetical model in *S. cerevisiae* based on previous works: 1 (Soulard et al. 2010) 2 (Jacinto and Hall 2003) (De Virgilio and Loewith 2006) 3 (Sundaram et al. 2015). **Left**: Hypothetical model in *C. albicans* based on previous works and the data obtained in this thesis: 4 (Chen et al. 2012) 5 (Tsao et al. 2009) 6 (Chen and Lan 2015). The irregular red arrow indicates a possible link between Ecm33 and TOR pathway.

(TOR1^{S1984I}) in the FKBP12 rapamycin-binding domain of the Tor kinase, giving rapamycin resistance to the strain that carries the allele (Cruz et al. 2001).

Previous work showed that RM2LU have altered cell wall structure, aberrant morphology and fail to produce true hyphae (Martínez-Lopez et al. 2004, Martinez-Lopez et al. 2006). In the present thesis, several stress tolerance assays were done to know in depth its contribution in the cell. The data obtained in Chapter 4 showed that RML2U displays sensitivity to osmotic stress, heat stress and oxidative stress. This data complement previous results in which RML2U showed sensitivity to cell wall-perturbing agents, such as calcofluor white or congo red (Martínez-Lopez et al. 2004). Besides, its incapacity to form biofilms and its hypersensitivity to zymolyase (Chapter 3) confirm cell wall defects. Also, the incapacity of the RML2U protoplast to regenerate the cell wall is an important finding of this work because it gives evidence for Ecm33 as fundamental for "*de novo*" cell wall reconstruction or biosynthesis (Chapter 4).

In *S. cerevisiae*, TOR plays a fundamental role in morphogenesis by regulating numerous biological processes, including autophagy, translation and ribosome biogenesis (Wullschleger et al. 2006). In *C. albicans*, TOR pathway was related to hyphal development and cell morphogenesis (Zacchi et al. 2010, Su et al. 2013). Several abnormal morphological phenotypes could relate Ecm33 to this pathway and also to cell wall integrity and biogenesis. RML2U displays abnormal cell shapes in standard grow conditions (Martínez-Lopez et al. 2004). An important analysis of different phenotypes was developed in this thesis. Among the observed phenotypes, it is important to highlight the unusual distribution of actin, related to abnormal cell shape, the increasing chitin in the cell wall, multinucleated or no nucleated cells and cell like pseudohyphae. These observations suggest problems in cell cycle and cell division. Other mutants of cell surface or cell wall proteins, such as Hsp90 or Mkc1, showed similar phenotypes and are attributed to cell cycle arrest or CWI activation pathway (Navarro-Garcia et al. 1998, Senn et al. 2012). However, the presence of the "veil growth" in RML2U static and stationary cultures has been never described before. In Chapter 4, the relation with the "flor velum" developed on the wine surface after the alcoholic fermentations (Alexandre 2013) and the RML2U veil was discussed. In *S. cerevisiae*, the difference between strains able or unable to form a velum was the presence of mannoprotein in the cell wall of the yeast (Alexandre et al. 2000), a similarity with RML2U which presented an abnormally electron-dense outer mannoprotein layer, which may represent a compensatory response to reduced cell wall integrity (Martinez-Lopez et al. 2006). Also, the cells observed in the "flor velum" have acquired the capacity to float as an adaptive mechanism to cope the environmental limitations. Then, we could associate the RML2U rounded cells observed in the veil and in corn meal growth conditions as the adaptive phenotype used by RML2U to survive. In fact, cornmeal medium is used to stimulate the chlamydospore formation in *C. albicans*. As discussed in Chapter 4, the RML2U cells obtained from cornmeal cultures and observed by TEM are similar to the *Aspergillus fumigatus* autophagic cells (Yan et al. 2013). This phenotype could be the RML2U strategy to survive, showing phenotypes similar to both stages, round cells like chlamydospores and vacuolization and translucent cytoplasm like phagocytosis. All this data together could explain the low damage caused by RML2U to the macrophages. The aberrant phenotypes observed, such as the sensitivity to oxidative stress or temperature, could influence in the RML2U incapacity to survive and cause damage to murine

macrophages. The apoptotic cells observed upon macrophage interaction by two apoptotic markers, ROS accumulation and caspase-like activity, presents other type of death different from necrosis.

All the different results obtained from the RML2U study were summarized in Figure 13. The function of this protein remains unknown but all of these results focus on the fact that this protein could have a direct or an indirect role in TOR and CWI pathways. For that reason, the *ECM33* mutation affects the cell morphology, cell viability and host-cell interaction.

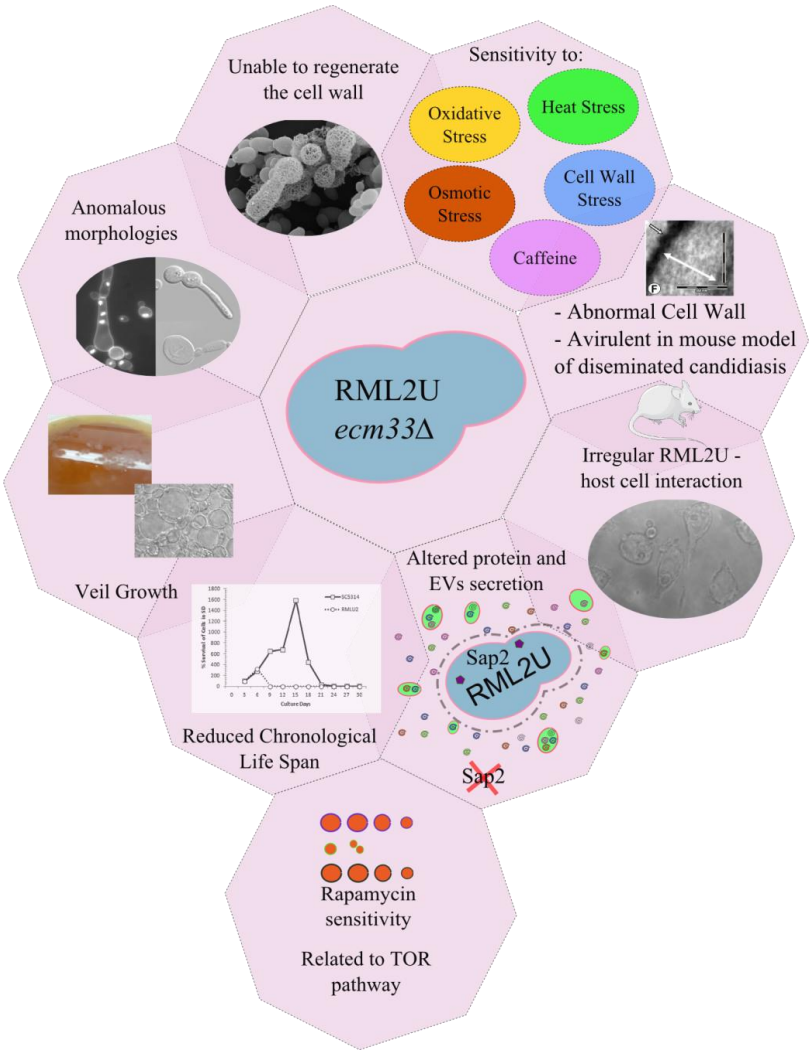

Figure 13. Summary of the main findings of the RML2U strain. The data presented in the figure come from previous studies from our group (Martínez-Lopez et al. 2004, Martinez-Lopez et al. 2006) and from the results published in the present thesis (Chapters 3 and 4).

Conclusions

1. The phenotypic analysis of 17 mutants of *C. albicans* cell surface proteins detected by cell shaving, revealed four new proteins that are involved in the maintenance of cell wall integrity and in the *C. albicans* engulfment by macrophages: Pst3, Tos1, Orf19.3060 and Orf19.5352. However, these proteins are not relevant for virulence during oropharyngeal candidiasis.

2. Four putative NADH-ubiquinone-related proteins, Ali1, Mci4, Orf19.287 and Orf19.7590, identified in the same cell shaving study, are involved in cell wall, osmotic and oxidative stress resistance, in the yeast-to-hypha transition and in the interaction with oral epithelial cells.

3. The proteomic analysis of *C. albicans* extracellular proteins revealed that *C. albicans* is able to use at least two different mechanisms to secrete different types of proteins: the classical secretory pathway and the extracellular vesicles.

4. More than 90% of the proteins identified in the extracellular vesicle-free secretome were classical secretory proteins with predicted N-terminal signal peptide. These proteins were enriched in cell wall- and secreted pathogenesis-related proteins.

5. The extracellular vesicles are the most important mechanism used by *C. albicans* to secrete proteins without predicted N-terminal signal peptide. All of the secreted metabolic or heat-shock proteins (including some moonlighting proteins) and secreted proteins involved in the exocytosis and endocytosis process are carried by extracellular vesicles. In addition, membrane proteins, including GPI-anchored proteins, and other cell wall-related proteins are transported by the extracellular vesicles.

6. The deletion of *ECM33* affects the classical secretion pathway and the extracellular vesicles morphology and its protein content. This results in the secretion of more proteins by both secretory mechanisms.

7. The secretion of the secreted aspartyl proteinase 2 (Sap2) is compromised in the RML2U mutant. Because the secretion of other members of the Sap family is not affected in the RML2U mutant, this result suggests a different mechanism of Sap2 secretion.

8. The defects in Sap2 secretion joined to the hypersensitivity to rapamycin and the reduced chronological life span showed by RML2U mutant point to a relation between Ecm33 and the TOR pathway.

9. RML2U mutant is sensitive to high temperatures, oxidative stress-inducing agents and osmotic stress-inducing agents. In addition, RML2U presents morphological defects: abnormal cell morphologies, irregular septa distribution, nuclear disorganization and actin patches with unusual distribution in the cell in absence of external stress.

10. The "veil growth" observed in RML2U stationary static cultures after long periods, and not in those of SC5314, represents a form of growth that has never been described for *C. albicans* and it was also observed in cornmeal medium. The cells observed are giant, round, with a large cell wall, large vacuoles and translucent cytoplasm, and it is probably related to a role to adapt and survive in extreme environmental conditions.

Conclusiones

1. Mediante el análisis fenotípico de 17 mutantes carentes de proteínas detectadas en la superficie celular de *C. albicans* se ha descubierto la presencia de cuatro nuevas proteínas implicadas en el mantenimiento de la integridad de la pared celular y en la fagocitosis por los macrófagos: Pst3, Tos1, Orf19.3060 y Orf19.5352. Sin embargo, estas proteínas no son relevantes para el desarrollo de candidiasis orofaríngea.

2. Cuatro proteínas descritas como posibles NADH-ubiquinona oxidorreductasas o deshidrogenasas, Ali1, Mci4, Orf19.287 y Orf19.7590, identificadas en el mismo estudio de superficie celular, están relacionadas con la resistencia a estrés osmótico, oxidativo y de pared celular, con la transición levadura-hifa y con la interacción con células de epitelio oral.

3. El análisis proteómico del secretoma de *C. albicans* ha demostrado que *C. albicans* puede usar al menos dos mecanismos diferentes para secretar distintos tipos de proteínas: la ruta clásica de secreción y las vesículas extracelulares.

4. Más del 90% de las proteínas identificadas en el secretoma libre de vesículas son proteínas clásicas de secreción que presentan un péptido señal en su secuencia. Este grupo de proteínas está enriquecido en proteínas relacionadas con la pared celular y proteínas de secreción relacionadas con patogénesis.

5. Las vesículas extracelulares constituyen el mecanismo más importante utilizado por *C. albicans* para secretar proteínas que no presentan péptido señal. Todas las proteínas de choque térmico y metábolicas que son secretadas (incluyendo proteínas multifuncionales) son transportadas por las vesículas extracelulares, así como las proteínas secretadas relacionadas con procesos de exocitosis y endocitosis. Además, las proteínas de membrana, incluyendo proteínas de anclaje GPI, y otras proteínas relacionadas con pared celular son también transportadas por las vesículas extracelulares.

6. La deleción de *ECM33* afecta a la ruta clásica de secreción y a la morfología y al contenido proteico de las vesículas extracelulares. Esta alteración provoca que el mutante RML2U secrete más proteínas mediante ambos mecanismos de secreción.

7. El mutante RML2U no es capaz de secretar la aspartil proteasa 2 (Sap2). Dado que la secreción del resto de los miembros de la familia Sap no está afectada en el mutante, este resultado apunta a un mecanismo de secreción distinto para Sap2.

8. La incapacidad de secretar Sap2 unido a la hipersensibilidad de RML2U a rapamicina y su reducida longevidad sugieren que la función de Ecm33 está relacionada con la ruta TOR.

9. El mutante RML2U es sensible a las altas temperaturas, al estrés osmótico y al estrés oxidativo. Además, RML2U presenta defectos en su morfología en ausencia de estrés: células con morfologías anómalas, distribución irregular de los septos, desorganización nuclear y parches de actina con distribución inusual en la célula.

10. El "velo" observado en cultivos estacionarios y estáticos de RML2U tras largos periodos de tiempo, representan una forma de crecimiento no descrita anteriormente en *C. albicans*, con células grandes, redondas, con pared celular gruesa, grandes vacuolas y citoplasma traslúcido. Estas células que no se observan en la cepa SC5314 son similares a las observadas en el mutante creciendo en medio de cultivo de maíz, y su papel podría estar relacionado con la adaptación y supervivencia en condiciones ambientales extremas.

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