Mechanismen der Msb2-vermittelten Virulenz des humanpathogenen Pilzes *Candida albicans*

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1 Einleitung

Weltweit sterben die meisten Menschen an Herz-Kreislauf-Leiden. Auf Platz 2 mit ungefähr 16 % folgen Todesfälle durch Infektionskrankheiten (Datengrundlage World Health Organization; 2008). Einen erheblichen Anteil haben hierbei besonders die Infektionskrankheiten, welche in Entwicklungsländern stark verbreitet sind. So machten 2008 Infektionen und parasitäre Krankheiten in den Entwicklungsländern 34 Prozent aller Todesfälle aus, in den Industrieländern dagegen nur etwa zwei Prozent. Zu den am weitesten verbreiteten Infektionskrankheiten in Entwicklungsländern zählen Tuberkulose und Malaria (Goldberg *et al.*, 2012). Im Gegensatz hierzu werden Pilz-Erkrankungen und die daraus resultierenden Todesfälle oft außer Acht gelassen. So wurde kürzlich berichtet, dass durch invasive Pilzerkrankungen (Mykosen) genauso viele, wenn nicht sogar mehr, Menschen sterben als an Tuberkulose oder Malaria (Brown *et al.*, 2012). Mehr als 90 % aller gemeldeten Todesfälle verursacht durch Pilze resultieren aus Arten, die zu einem von vier Gattungen gehören: *Candida-*, *Aspergillus-, Cryptococcus-* und *Pneumocystis*-Spezies.

Von der Vielzahl an Pilzen, die in der Natur vorkommen, können nach Schätzungen nur etwa 100 Spezies Krankheiten des Menschen verursachen (Moran *et al.*, 2011). Humanpathogene Pilze können in zwei Klassen unterteilt werden: primäre Pathogene und opportunistische Pathogene. Primäre Pathogene sind als „professionelle Pathogene“ in der Lage, gesunde Individuen zu infizieren, während opportunistische Pathogene in erster Linie Erkrankungen in immungeschwächten Patienten hervorrufen (van Burik & Magee, 2001). Zu den primären Pathogenen gehören beispielsweise *Coccidioides immitis* und *Histoplasma capsulatum*, deren natürliches Habitat der Erdboden ist und die durch Einatmen den Menschen infizieren und sich verbreiten (Sil & Andrianopoulos, 2014). Die meisten humanpathogenen Pilze gehören jedoch zu den opportunistischen Krankheitserregern, hierzu zählen beispielsweise *Candida*-Spezies, *Cryptococcus neoformans* und *Aspergillus fumigatus* (Moran *et al.*, 2011). Die opportunistisch humanpathogenen Pilze können entweder in der Umwelt vorkommen, wie z. B. *A. fumigatus*, oder als Kommensale in gesunden Individuen, wie z. B. *Candida albicans* (d'Enfert, 2009).

Das Verständnis der Interaktionen von Pilz- und humanen Zellen ist entscheidend für eine effiziente Behandlung von Mykosen. Die Zellwand zählt durch ihre stabilisierenden und schützenden Eigenschaften zu den wichtigsten Organellen der Pilze. Ihre starre und feste Form schützt die Zelle vor mechanischem Druck, während die aus Mannoproteinen bestehende äußere Zellwandschicht Schutz vor defensiven Enzymen und anderen Molekülen des Wirtes gewährleistet (Heilmann *et al.*, 2012). Somit spielt die Zusammensetzung der Zellwand für die Pathogenität des humanpathogenen Pilzes *C. albicans* eine wichtige Rolle (Chaffin, 2008). Als Reaktion auf die sich schnell ändernde Bedingungen im Wirt werden „mitogen-activated protein kinase“ (MAPK)-Signalwege aktiviert, die präzise Zellantworten und kompensatorische Mechanismen in *C. albicans* auslösen (Ernst & Pla, 2011). Überlebenswichtige Anpassungen der Zelle sind unter anderem die

Regulation von Zellwandkomponenten, Veränderungen in der Verknüpfung von Zellwand-Polymeren und die Zunahme spezifischer Zellwandproteine, sowie deren Relokalisation (Free, 2013).

Im Folgenden wird eine Einführung zur Bedeutung des Membransensors Msb2 bei der Zellwand-Regulation und der Interaktion zwischen *C. albicans* und Wirtszelle gegeben.

1.1 *Candida albicans*- ein opportunistischer Pathogen

Der zu den Ascomycota zählende, opportunistisch pathogene Pilz *C. albicans* besiedelt als harmloser Kommensale asymptotisch den Gastrointestinal- und Urogenitaltrakt, sowie Schleimhäute der Mundhöhle des Menschen (Odds, 1987, Ruhnke, 2006). Bei einem intakten Immunsystem verursacht die Hefe keine Krankheitssymptome, sie kann jedoch unter bestimmten Bedingungen leichte bis schwere Mykosen hervorrufen.

1.1.1 Kommensalismus von *C. albicans*

Die Hefe *C. albicans* gehört zum humanen Mikrobiom und nahezu 70-75 % der gesunden menschlichen Bevölkerung trägt diesen Organismus in sich (Ghannoum *et al.*, 2010). Eine „Drei-Wege-Interaktion“ zwischen Wirt, Pilz und Mikrobiom bestimmt dabei die Rolle von *C. albicans* im menschlichen Wirt (Romani *et al.*, 2014). So erhöht zum Beispiel der Tryptophan-Abbau durch kommensale Lactobazillen und Wirts-Zellen die Resistenz und Toleranz gegenüber *C. albicans* (Romani *et al.*, 2014). Dysbiose, eine Störung der bakteriellen Flora kann zu einer Vielzahl von lokalen oder chronischen Pilzinfektionen führen (Ott *et al.*, 2008, Ghannoum *et al.*, 2010, Li *et al.*, 2014).

Kürzlich wurde gezeigt, dass *C. albicans* in der Lage ist, seine eigene Virulenz bzw. seine Virulenz-Faktoren Umgebungs-spezifisch zu erniedrigen (Doedt *et al.*, 2004, White *et al.*, 2007, Pierce & Kumamoto, 2012, Pande *et al.*, 2013). Bei der Besiedlung des Gastrointestinaltrakts verhindern die Transkriptionsfaktoren Efg1 und Efh1 die Proliferation von *C. albicans* (Doedt *et al.*, 2004, White *et al.*, 2007, Pierce & Kumamoto, 2012). Interessanterweise löste die Passage von wildtypischen *C. albicans*-Zellen durch den murinen Gastrointestinaltrakt die Expression des *WOR1*-Gens, das für einen Transkriptionsfaktor kodiert, aus und führte zur Proliferation der Hefe. Der resultierende GUT (Gastrointestinally-Induced Transition)-Phänotyp unterscheidet sich morphologisch und funktionell von zuvor definierten Zelltypen (Abb. 1.1) (Ernst, 2000, Pande *et al.*, 2013). Als pleomorpher Organismus kommt *C. albicans* entweder einzellig, als Hefe oder Chlamydospore, sowie mehrzellig, als Pseudohyphe oder echte Hyphe vor (Abb. 1.1). Änderungen in der Pilz-Morphologie erfolgen als Reaktion auf eine Vielzahl äußerer Faktoren. So wird beispielsweise hyphales Wachstum bei Temperaturen über 30 °C, bei einem neutralen pH-Wert oder in Anwesenheit von Blutserum induziert (Ernst, 2000). Wie in allen Eukaryoten wird der

Metabolismus bei Hefen durch Sauerstoff reguliert. Der Wechsel zwischen der Hefe- (White) und der Stäbchen-Form (Opaque), der paarungskompetenten *C. albicans*-Form, kann durch Sauerstoff-limitierende Bedingungen induziert werden (Ramirez-Zavala *et al.*, 2008). So konnte gezeigt werden, dass unter Hypoxie, wie z. B. im Darm, dieser morphologische Wechsel ausgelöst wird, um besser an unterschiedliche Wirts-Nischen angepasst zu sein.

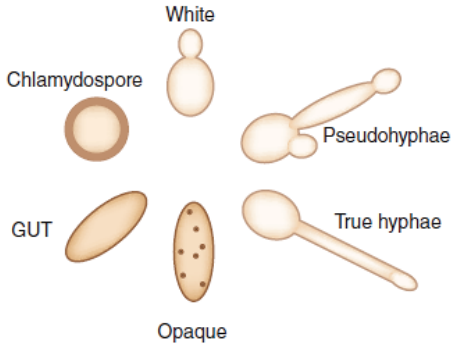

Abb. 1.1- Die unterschiedlichen Zelltypen von *C. albicans*. Hefezellen (White) nehmen eine wichtige Rolle bei der Verbreitung von *Candida*-Infektionen ein. Filamentöse Wachstumsformen, Pseudohyphen und echte Hyphen, spielen eine Rolle bei der Gewebspenetration und Invasion. Opaque-Zellen sind bedeutend bei der Paarung. Die GUT-Zellen ähneln hierbei Opaque-Zellen, unterscheiden sich jedoch morphologisch und transkriptionell. Die *in vivo*-Rolle der Chlamydosporen ist bisher noch nicht beschrieben worden. (Gow, 2013)

1.1.2 Oberflächliche und systemische *C. albicans*-Mykosen

Bei Menschen mit geschwächtem Immunsystem oder einer gestörten bakteriellen Flora kann sich der opportunistische Pilz vermehren und Mykosen auslösen. Grundsätzlich lassen sich zwei Infektionstypen unterscheiden: oberflächliche und invasive Mykosen.

Oberflächenmykosen, wie z.B. oropharyngeale Candidosen, treten häufig bei HIV-infizierten Personen auf (Cassone & Cauda, 2012). 75 % aller Frauen erleiden während ihres Lebens eine durch *Candida* ausgelöste Vaginal-Infektion, auch als Vulvovaginalcandidose (VVC) bezeichnet, wobei 5-8 % der Frauen, mit bis zu vier Vorfällen pro Jahr, eine wiederkehrende VVC haben (Fischer, 2012). Mykosen können oberflächlich auf Haut und Schleimhäuten auftreten, jedoch kann *C. albicans* durch Penetration des Gastrointestinal-Epithels in den Blutstrom gelangen und sich in mehrere innere Organe systemisch verbreiten (Nucci & Anaissie, 2001). Pro Jahr werden weltweit etwa 400.000 systemische Infektionen mit *C. albicans* verzeichnet, wobei die Mortalitätsrate invasiver Mykosen bei 46-75 % liegt (Brown & Netea, 2012). Systemische Mykosen sind somit so gefährlich wie Malaria, eine von einzelligen Parasiten der Gattung *Plasmodium* hervorgerufene Tropenkrankheit (Brown *et al.*, 2012). Risikogruppen für systemische Candidosen sind vor allem Krebs-, Diabetes- oder Transplantationspatienten, sowie Patienten, die eine Chemotherapie oder eine Behandlung mit Breitband-Antibiotika erhalten (Odds *et al.*, 1998, Davies *et al.*, 2002, Pfaller & Diekema, 2007). Interessanterweise fungieren als Quelle einer postoperativen *C. albicans*-Infektion

hauptsächlich der im Patienten siedelnde Kommensale (Eggimann & Pittet, 2006). Somit gehört *C. albicans* zu den häufigsten Erregern von nosokomialen Infektionen. Die Behandlung von oberflächlichen oder systemischen *C. albicans*-Infektionen erfolgt in erster Linie mit Antimykotika der Klasse der Polyene (z.B. Amphotericin-B), Azole (Fluconazol) und Echinocandine (z.B. Caspofungin) (Moudgal & Sobel, 2010).

1.2 Immunabwehr von *C. albicans*

Die Tatsache, dass *C. albicans* als kommensaler Organismus in verschiedensten Bereichen des menschlichen Körpers vorkommt lässt vermuten, dass bereits eine „gesunde" Interaktion zwischen Wirt und dem Pilz auf einem äußerst komplexen Gleichgewichtszustand beruht (Hube, 2004). Durch verschiedene Pathogenitätsmechanismen, wie dem Wechsel zwischen der einzelligen Hefe- und der Hyphen-Form, wird die Komplexität der Interaktion vergrößert, die das Ergebnis einer langen Ko-Evolution des Pathogens des Menschen ist (Cottier & Pavelka, 2012). Hierbei entwickelte *C. albicans* Mechanismen, Veränderungen der Wirtsumgebung zu detektieren und sich daran anzupassen. Im folgenden Abschnitt werden einige dieser Interaktionssysteme, wie die Erkennung und Abwehr von *C. albicans*, näher erläutert.

1.2.1 Angeborene Immunität und *C. albicans*

Das Immunsystem kann als komplexes Zusammenspiel verschiedener Strategien bezeichnet werden, die alle im Dienste der Erhaltung der Individualstruktur stehen. Es kann in ein angeborenes Immunsystem und in ein erworbenes Immunsystem unterteilt werden. Das phylogenetisch ältere, angeborene Immunsystem bildet die erste Verteidigungslinie im Verlauf der Reaktion auf eine Infektion (Janeway & Medzhitov, 2002). Essentiell für die angeborene Immunantwort sind Makrophagen und Granulozyten, welche zur Phagozytose befähigt sind und die antimikrobielle Peptide sezernieren können.

Der erste Schritt zum Aufbau einer schützenden Immunantwort ist die Erkennung der *C. albicans* Zellen durch Zelloberflächenrezeptoren von Immunzellen (Abb. 1.2). Die so genannten „Pattern Recognition Receptors" (PRRs) auf der Oberfläche von Phagozyten erkennen bestimmte Zellwandbestandteile von *C. albicans*, darunter Mannan und einige Mannoproteine (Gazi & Martinez-Pomares, 2009) Chitin und β-Glucan (Gantner *et al.*, 2003, Martinez *et al.*, 2009). Die antigenen Strukturen auf der Pilzzellwand werden „Pathogen-Associated Molecular Patterns" (PAMPs) genannt und umfassen Oberflächendeterminanten, die in Säugetierzellen nicht existieren (Underhill, 2004). Dabei sind verschiedene PRRs in der Lage unterschiedliche PAMPs zu erkennen (Underhill, 2003).

Zu den PRRs gehören die „Toll-like Receptors" (TLRs) und die „C-type Lectin Receptors" (CLRs). TLRs sind entweder mit der Membran assoziiert oder fungieren als intrazelluläre

Rezeptoren. Die CLRs sind hauptsächlich membrangebundene Rezeptoren, die neben Mannan auch Polysaccharidverbindungen der *C. albicans* Zellwand erkennen können (Ezekowitz *et al.*, 1990, McGreal *et al.*, 2006). Dectin-1 und Dectin-2 sind Typ II-Transmembranproteine der C-Typ Lektin-Familie mit einer extrazellulären Kohlenhydrat-Erkennungs-Domäne (CRD). Sie werden hauptsächlich in dendritischen Zellen und Makrophagen exprimiert (Taylor *et al.*, 2007). Dectin-1 erkennt mittels seiner CRD β-Glucan und leitet Signale über das „immunoreceptor-tyrosine-based-inibitory-motif“ (ITAM) an die cytoplasmatische Domäne weiter, während Dectin-2 α-Mannan erkennt und durch die Verbindung mit der Fc-Rezeptor-γ-Kette die Signalweiterleitung erfolgt (Saijo *et al.*, 2007).

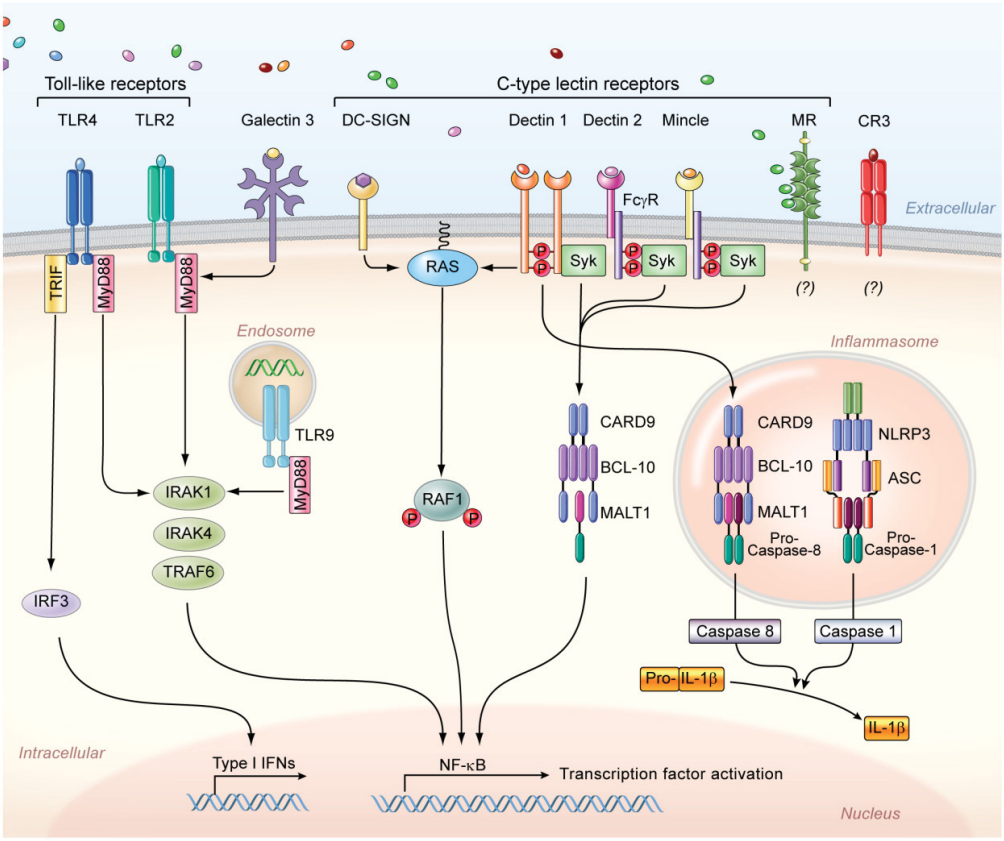

Abb. 1.2- PAMPs / PRRs Interaktion. Der Toll-like receptor 2 (TLR2) und TLR4 erkennen Phospholipomannan- und *O*-glykosidisch verknüpftes Mannan, während TLR9 im Cytosol Pilz-spezifische DNA erkennt. TLR2 bildet Heterodimere mit TLR1 und TLR6; hingegen bildet TLR4 Homodimere. Galectin-3 und TLR2 detektieren β-Mannoside. Das Membran-gebundene C-Typ Lectin DC-SIGN, Mincle und der „macrophage mannose receptor”(MR) erkennen Mannose-haltige *Candida*- Strukturen. Weiterhin detektieren Dectin-1 β-Glucan und Dectin 2 Mannan, zusammen mit dem Fcγ-Rezeptor (FcγR). Der „complement receptor 3” (CR3) auf Neutrophilen erkennt β-Glucan. Der „NOD-like receptor” NLRP3 (nucleotide-binding domain) bildet zusammen mit ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) und der Caspase 1 den Inflammasome-Komplex; dieser bewirkt die Interleukin-1β (IL-1β)-Expression. Weiterhin führt die durch Dectin-1-getriggerte Signalweiterleitung durch die Caspase zur CARD9-Rekrutierung. Dieses hat zur Folge, dass das Nicht-kanonische Inflammasom aktiviert wird und über die Caspase 8 zur IL-1β Produktion führt. (Lionakis & Netea, 2013)

TLR2 erkennt β-Glucan, TLR4 *O*-Mannan der *C. albicans* Zellwand und TLR9 pilzspezifische DNA (Tada *et al.*, 2002, Jouault *et al.*, 2003, Miyazato *et al.*, 2009). Weiterhin haben Makrophagen-

induzierte C-Typ-Lektine (Mincle), dendritisch spezifisch ICAM-3-Grabbing non-Integrin-1 (DC-SIGN), Makrophagen-Mannose-Rezeptor (MR) und Galectin-3 wichtige Rollen bei der Erkennung von Mannan (Miramon *et al.*, 2013).

1.2.2 Humane antimikrobielle Peptide

Antimikrobielle Peptide (AMPs) sind Effektormoleküle des angeborenen Immunsystems mit direkter antimikrobieller Funktion (Huttner & Bevins, 1999, Lehrer & Ganz, 1999, Tossi *et al.*, 2000). Mikroorganismen werden in ihrem Wachstum inhibiert oder abgetötet, indem die Peptide mit ihren Biomembranen oder intrazellulären Zielmolekülen interagieren. Klassifiziert werden AMPs nach Größe und Struktur ihrer enthaltenen Aminosäuren (Hancock, 1997, Andreu & Rivas, 1998) und können basierend auf ihren strukturellen Eigenschaften und der Anzahl an Disulfidbrückenbindungen in fünf Gruppen unterteilt werden. Die erste Gruppe erfasst lineare, α-helikale Peptide, die keine Cysteinreste enthalten, wie z.B. das humane Peptid LL-37, das zu der Gruppe der Cathelicidine gehört. Die AMPs der zweiten Gruppe besitzen ausgeweitete α-Helices, die reich an Prolin, Glycin, Tryptophan, Arginin und/oder Histidin sind. Ein Beispiel für diese Gruppe ist das Indolicidin, das in Rindern gebildet wird. Die dritte Gruppe zeichnet sich durch eine Ringstruktur im Molekül aus, die eine Disulfidbrückenbindung enthält. Beispiele hierfür sind Brevinin und Bactenesin. Die vierte Gruppe ist durch rigide, antiparallele β-Faltblattstrukturen charakterisiert, die durch zwei bis vier Disulfidbrückenbindungen stabilisiert werden. Hierzu gehören z.B. die α- und β-Defensine sowie die Protegrine. Die fünfte strukturelle Gruppe bilden die zyklischen θ-Defensine, die komplex durch post-translationale Zyklisierung von zwei kurzen, α-Defensin-ähnlichen Peptiden modifiziert werden (Tang *et al.*, 1999). AMPs entstehen durch einen oder mehrere proteolytische Prozessierungsschritte aus Vorläuferpeptiden. Daraus folgen kurze Peptide, die aus 10-50 Aminosäuren bestehen und fast immer eine positive Nettoladung sowie einen substanziellen Anteil an hydrophoben Resten besitzen (≥ 30%) (Zasloff, 2002, Hancock & Sahl, 2006).

Histatine werden ausschließlich in der Speicheldrüse des Menschen und höheren Primaten exprimiert und gehören zu einer Familie von kleinen Histidin-reichen kationischen Proteinen, von denen zwölf Mitglieder systematisch als Histatin 1 bis Histatin 12 durchnummeriert wurden. Die wichtigsten sind Histatin 1, 3 und 5, welche insgesamt 85 bis 90 % der Gesamthistatinmenge bilden (Edgerton and Koshlukova, 2000). Histatin 5 besteht aus 24 Aminosäuren und wird proteolytisch aus Histatin 3 gespalten (32 Aminosäuren) und dann freigesetzt (Edgerton & Koshlukova, 2000). Es ist bekannt, dass dieses Peptid weder Poren noch Kanäle in die mikrobielle Plasmamembran bewirkt, da die Membranpermeabilität in Anwesenheit von Histatin 5 unverändert ist.

Das humane Cathelicidin wird als hCAP18 (cDNA-Form), FALL39 (Gen-Form) oder auch LL-37 (Peptidform), bezeichnet. In der Peptidform besteht es aus 37 Aminosäureresten, von denen die

beiden N-terminalen Aminosäuren Leucine sind. Es wurde bisher in Epithelzellen des Hodens, der Haut, des Gastrointestinal- und Respirationstrakts sowie in neutrophilen Granulozyten nachgewiesen. Die von neutrophilen Granulozyten gebildeten Peptide werden in phagozytotischen (primären) und wie beim hCAP18/LL37 in sekretorischen (sekundäre, spezifische) Granula gespeichert (Sorensen *et al.*, 2001). Weiterhin übernimmt LL-37 eine Funktion als Immunmodelator (Bowdish *et al.*, 2005). Bei einer Infektion oder Stimulation mit Entzündungsmediatoren tritt ein Konzentrationsgradient von LL-37 an der Stelle der Infektion auf. Dieser Konzentrationsgradient führt zur Chemotaxis von neutrophilen Granulozyten, Monozyten und anderen Zelltypen (Kahlenberg & Kaplan, 2013).

Eine ausführliche Zusammenfassung zur Interaktion von *C. albicans* und humanen AMPs ist im Manuskript I „Interplay of *Candida albicans* and the antimicrobial peptide armory“ enthalten (Swidergall & Ernst, 2014). Das Review beschreibt evolvierte Resistenzmechanismen von *C. albicans*, Regulation der humanen AMP-Expression, sowie Sekretion und den Einsatz von AMPs als Fungizide.

1.2.3 „Immune escape“ Strategien von *C. albicans*

Während einer Infektion reagiert *C. albicans* auf die angeborene sowie im weiteren Verlauf auf die adaptive Immunantwort. *C. albicans* verwendet eine Batterie von „Evasion“-Faktoren, welche die verschiedenen Stufen des menschlichen Immunangriffs steuern. Zu diesen Strategien gehört das Imitieren von Wirts-Oberflächen Molekülen, den Erwerb von Wirts Komplement-Regulatoren, die Expression von endogenen Komplement-Inhibitoren oder die Sekretion von Proteasen, welche Proteine des Immunsystems abbauen (Abb. 1.3) (Poltermann *et al.*, 2007, Gropp *et al.*, 2009, Luo *et al.*, 2009). Auf diese Weise steuert und entzieht sich *C. albicans* der Immunantwort und inaktiviert neu gebildete fungizide Produkte. So kommt es zur Abschirmung des β-Glucans mit Mannoproteinen, was das Erkennen der Pilzoberfläche durch PRRs verhindert (Chai *et al.*, 2009). Im menschlichen Körper kommt es nach Erkennung einer pathogenen Hefe zur Initiation der Phagozytose, die zur Bildung eines Phagosoms bzw. Phagolysosoms führt. Das antimikrobielle Milieu des Lysosoms geht einher mit einer Senkung des pH-Werts, der Gegenwart von hydrolytischen Enzymen, Defensinen und anderen antimikrobiellen Peptiden und der Erzeugung von toxischen oxidativen Verbindungen. Darüber hinaus weist das Phagolysosom starke Nährstoff- und Spurenelementebegrenzungen auf. Dennoch hat *C. albicans* mehrere Strategien entwickelt um innerhalb von Phagozyten zu überleben. So kann der Reifungsprozess des Phagolysosoms unterdrückt werden, oder toxische Verbindungen abgefangen werden. Außerdem kann es zur Flucht aus dem Phagolysosom oder die Anpassung an die Umgebung innerhalb der Phagozyten kommen (Tavanti *et al.*, 2006). Sobald die Hefezelle phagozytiert ist, zeigt sich eine sofortige Umprogrammierung der Transkription und Translation des Pathogen in Bezug auf die Anpassung als

Überlebensstragegie an die innere Beschaffenheit des Phagozyten. Bei *C. albicans* gehört die Induktion der oxidativen Stressreaktion, DNA-Reparatur, Arginin-Biosynthese und Peptid-Verwertung dazu (Lorenz *et al.*, 2004). Weiterhin reagiert *C. albicans* auf Kohlenstofflimitierung mit einer Hochregulation von Glukose- und Kohlenhydrat-Transporter-Genen, Gene für den Glyoxylatzyklus und die Glukoneogenese (Lorenz *et al.*, 2004, Fan *et al.*, 2005).

Zur Abwehr von AMPs hat *C. albicans* eine „Dreiphasen-Antwort" evolviert, welche sekretierte Proteine, Efflux-Pumpen und die Regulation von Stressantwort-Genen beinhaltet. Diese Mechanismen sind im Manuskript I „Interplay of *Candida albicans* and the antimicrobial peptide armory" (Swidergall & Ernst, 2014) beschrieben.

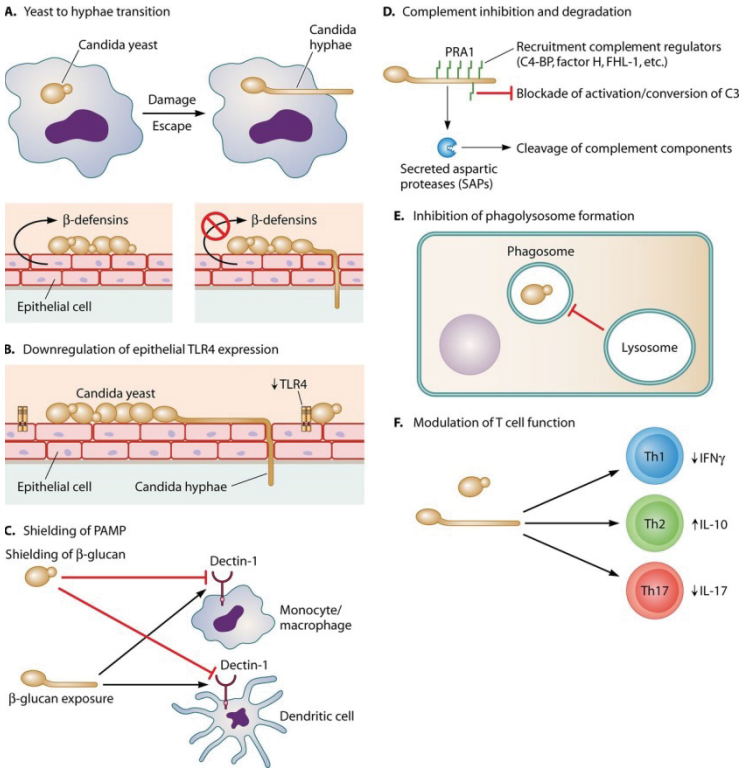

Abb. 1.3- *C. albicans* Strategien zur Überwindung der angeborenen Abwehrabwehr. A Hefe-Hyphe-Morphogenese. **B** Erniedrigung der epithelialen TLR4 Expression. **C** Maskierung von PAMPs. **D** Bindung des Komplement-hemmenden C4-bindende Proteins (C4BP) von Faktor H an der Zellwand. **E** Inhibierung der Phagolysosombildung. **F** Modulation der T-Zell Funktion. (Cheng *et al.*, 2012)

1.3 Zelloberfläche von *C. albicans*

Im Verlauf der Vermehrung von *C. albicans*, trifft die Hefe auf verschiedene Wirtsoberflächen, Gewebebarrieren, sowie humane zelluläre Immunantworten. Dagegen hat der Pilz verschiedene Ausweichstrategien und Virulenzfaktoren entwickelt, um eine Besiedlung oder Neu-Infektion von Geweben auszulösen. Dabei ist die Oberfläche von *C. albicans*, insbesondere die Zellwand, essentiell für die Interaktion zwischen Pathogen und Wirtszelle, sowie die damit einhergehende Kolonisierung von Wirtsgewebe (Rupp, 2004).

Zellwandbestandteile stellen als äußere Komponenten von *C. albicans* antigene Strukturen dar, welche von Immunzellen des Wirts erkannt werden können (Abschn 1.2.1). Die Zellwand besteht zu 90 % aus Polysacchariden und zu 5-10 % aus Proteinen (Chaffin, 2008). Die Hauptkomponenten der Zellwand sind β1,3-Glucan (40 %), β1,6-Glucan (20 %), Chitin (~2 %) und Mannoproteine (40 %) zusammen (Shepherd & Gopal, 1991, Gow *et al.*, 2012). Die Zellwand ist aus mehreren Schichten aufgebaut, wobei die innerste Schicht aus Chitin-Fibrillen gebildet wird, die mit β1,3-Glucanketten verknüpft sind, während β1,6-Glucane mit β1,3-Glucan, Chitin und Mannoproteinen verbunden sind. Die äußerste Schicht besteht hauptsächlich aus Mannoproteinen, welche vielfach *O*- oder *N*-glykosyliert sind (Chaffin *et al.*, 1998, Chaffin, 2008, Hall & Gow, 2013).

1.3.1 Glykosylierung in *C. albicans*

Die Rolle von oberflächengebundenen Glykoproteinen ist für die Pathogenität von *C. albicans* ein wichtiger Aspekt und von großer Bedeutung für die Zellwand für die Pilz-Zelle (Chaffin *et al.*, 1998). Vermutlich wird mehr als die Hälfte aller Proteine durch Glykosylierungsmuster modifiziert (Apweiler *et al.*, 1999). Da die Schritte der Glykosylierung während des Sekretionswegs im Endoplasmatischen Retikulum (ER) und Golgi-Apparat erfolgen, sind glykosylierte Proteine typischerweise sekretorische Proteine, die in der Plasmamembran oder an der Zellwand lokalisiert sind, oder die sezerniert werden. Die hierbei am häufigsten auftretenden Proteinmodifikationen sind die *N*- und *O*-Glykosylierung. Bei der *N*-Glykosylierung wird die Saccharidstruktur über eine *N*-glykosidische Bindung mit der Amidgruppe eines Asparaginrests des Proteins verknüpft (Tanner & Lehle, 1987), wobei nur Asparaginreste der Konsensussequenz Asn-X-Ser/Thr (X darf nicht Prolin sein) erkannt und dabei 30-65 % der im Protein vorkommenden *N*-Glykosylierungsstellen modifiziert werden (Petrescu *et al.*, 2004, Stanley *et al.*, 2009). Der Oligosaccharid-Kern besteht aus $Glc_3Man_9GlcNAc_2$ und wird an Dolichol-Phosphat (Dol-P) synthetisiert. Die ersten sieben Schritte finden auf der cytosolischen Seite des ER statt. Anschließend wird $Man_5GlcNAc_2$-P-Dol in das Lumen des ER transloziert, wo die Synthese fortgesetzt wird. Der vollständige Kern ($Glc_3Man_9GlcNAc_2$-P-Dol) wird *en bloc* auf das Ziel-Protein durch den OST-Komplex transferiert (Shrimal *et al.*, 2014).

Die *O*-Glykosylierung bezeichnet die Modifikation von meist Serin- oder Threonin-Resten durch eine *O*-glykosidische Bindung von Oligosacchariden. Bei der *O*-Mannosylierung von *C. albicans* kommen hauptsächlich lineare α1,2-verknüpfte Mannose-Ketten vor (Abb. 1.4). Bei der initialen Reaktion der *O*-Glykosylierung im ER katalysieren Protein-*O*-Mannosyltransferasen (Pmt1, 2, 4, 5 und 6) die Übertragung der ersten Mannose-Reste auf Serin oder Threonin des Zielproteins (Prill *et al.*, 2005, Lengeler *et al.*, 2008). Das Mannose-Molekül wird von Dolichol-Phosphat-Mannose (Dol-P-Man), welches durch das Enzym Dpm1 aus dem Donor GDP-Man und Dol-P synthetisiert wird, auf die lumenale Seite des ER transloziert. Während der Verknüpfung an das

Ziel-Protein wird die anomerische Konfiguration des Zuckers von β- zu α-glykosidischer Bindung geändert (Ernst & Prill, 2001). Eine defekte Mannosylierung hat für *C. albicans* relevante Auswirkungen. So konnte gezeigt werden, dass Pmt1 und Pmt6 für die Adhäsion an Epithelzellen erforderlich ist (Timpel *et al.*, 1998, Murciano *et al.*, 2011). Weiterhin haben *pmt*-Mutanten einen Defekt in der Hyphen-Morphogenese, sowie eine verringerte Resistenz gegenüber Antimykotika (Prill *et al.*, 2005, Rouabhia *et al.*, 2005). Mnt1 und Mnt2 sind „redundante" α1,2-Mannosyltransferasen, welche die erste und zweite α1,2-Mannose-Einheit in die α-Mannose-Kette einbauen (Munro *et al.*, 2005). Die Deletion von *MNT1* und *MNT2* führt somit zu einer Verkürzung des *O*-Mannan am Zielprotein. Alle untersuchten Mutanten, welche einen Defekt in der Biosynthese von *O*-Mannan aufweisen, zeigen eine veränderte Virulenz im Mausmodel der systemischen Infektion und darüber hinaus Adhäsionsdefekte (Timpel *et al.*, 1998, Munro *et al.*, 2005, Prill *et al.*, 2005, Murciano *et al.*, 2011, Hall & Gow, 2013).

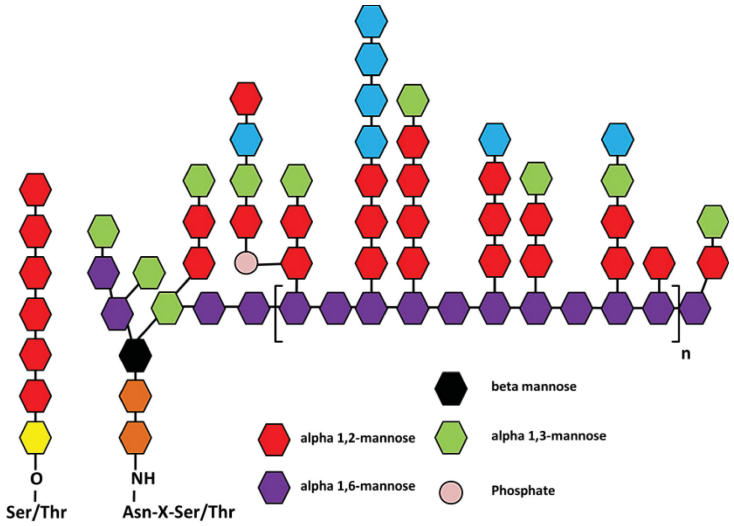

Abb. 1.4- *N*- und *O*-Glykosylierung in *C. albicans*. (nach Hall & Gow, 2013)

1.3.2 Zellwandaufbau und Regulation der Integrität

Die Zellwand der Pilze ist ein dynamisches Organell, welches in eine Vielzahl von Prozessen involviert ist. Zellen sind stetigen Schwankungen des osmotischen Drucks durch äußere Umwelteinflüsse ausgesetzt und müssen dem Tugordruck der Protoplasten zum Schutz der Zelle entgegen wirken (Bowman & Free, 2006).

Während der Proliferation ist der Pilz durch Abwehrmechanismen des menschlichen Wirtes starkem Stress ausgesetzt, welcher sich insbesondere auf die Zellwand auswirkt. Stress-Faktoren sind unter anderem Sauerstoffradikale, AMPs, hydrolytische Enzyme und therapeutische, antimykotische Moleküle wie z.B. Azole (Heilmann *et al.*, 2013). Selbst unter normalen Wachstumsbedingungen muss der Pilz die Zellwandzusammensetzung ständig sicherstellen. Als

Reaktion auf Wachstum oder Schädigung der Zellwand werden „mitogen-activated protein kinases“ (MAPK)-Signalwege aktiviert, die kompensatorische Mechanismen auslösen (Ernst & Pla, 2011). Extrazelluläre Signale werden dabei in zelluläre Antworten umgewandelt, welche essentiell für die Adaption des Pathogens an wechselnde Umweltbedingungen sind. Durch externe Signale kommt es zur Phosphorylierung der Kinase MAPKKK, welche unmittelbar die Kinase MAPKK phosphoryliert. Die terminale MAPK wird anschließend durch MAPKK phosphoryliert und überträgt das Signal auf Transkriptionsfaktoren, welche die spezifische, adaptive Antwort auslösen (Monge *et al.*, 2006). Cek1, Mkc1 und Hog1 gehören zu den wichtigsten MAP-Kinasen in *C. albicans* (Abb. 1.4), welche maßgeblich an der Erhaltung der Zellwandintegrität beteiligt sind (Garcia *et al.*, 2004, Ernst & Pla, 2011).

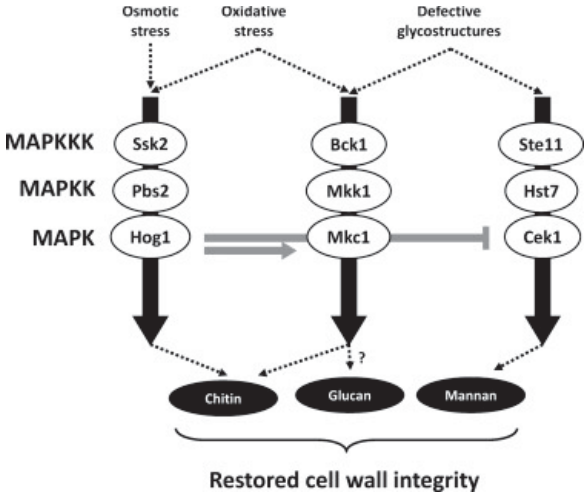

Abb. 1.3- MAPK-Signalwege in *C. albicans* und ihre Rolle bei der Zellwandbildung. Die drei MAPK-Signalwege und deren Stimuli sind schematisch dargestellt. Die hier dargestellten Kaskaden zeigen die MAPK und ihre jeweiligen vorgelagerten Kinasen (MAPKK und MAPKKK). Schäden an Glucan und Chitin (z.B. durch Calcofluor oder Kongo Rot) aktivieren Mkc1, während defekte *N*-Glykosylierung (z.B. durch Tunicamycin) und *O*-Mannosylierung (z.B. durch Rhodamin-Typ-Inhibitoren) Cek1 aktivieren. Osmotischer und oxidativer Stress aktivieren zudem die MAPKs Hog1 und Mkc1 MAPKs. Hog1 reprimiert die Cek1 Phosphorylierung unetr normalen Wachstumsbedingungen und aktiviert Mkc1. Mkc1 und Hog1 sind an der Chitin-Synthese beteiligt sind, während Cek1 Gene der Protein-Glykosylierung induziert. (Ernst & Pla, 2011)

Die MAP-Kinase Cek1 bildet mit der MAPKK Hst7 und der MAPKKK Ste11 den Cek1-Signalweg in *C. albicans*. Glykosylierungs-Defekte führen über die Kinasen MAPKKK Ste11 und MAPKK Hst7 zu einer erhöhten Phosphorylierung von Cek1 (Román *et al.*, 2009, Cantero & Ernst, 2011, Ernst & Pla, 2011). Interessanterweise konnte in einer *pmt2*/*PMT2*-Mutante keine Cek1 Phosphorylierung detektiert werden (Cantero *et al.*, 2007). Dieses Ergebnis deutet darauf hin, dass ein durch Pmt2-mannosyliertes Signalprotein für die Cek1-Aktivierung erforderlich ist. Kürzlich wurde der Sensor Msb2, welcher in dieser Arbeit charakterisiert wurde, als putatives „Upstream-Element“ des Cek1-Signalweges identifiziert (Abschn. 1.3.5), da Msb2 und Komponenten des Cek1-Signalweges für die Regulation von *PMT2* und *PMT4* als Antwort auf defekte Pmt1-*O*-Mannosylierung nötig sind (Cantero & Ernst, 2011). Somit scheint es, dass die *PMT*-Gene „Downstream-Targets“ des Cek1-

Signalweges sind, jedoch der Transkriptionsfaktor, der direkt durch Cek1 aktiviert wird, bisher nicht identifiziert wurde.

Während Mannoproteine die äußere Zellwandschicht bilden, besteht die innere Schicht hauptsächlich aus Glucan und Chitin. Die Chitin-Synthese wird durch vier Chitinsynthasen, Chs1-3 und Chs8 katalysiert (Lenardon *et al.*, 2007, Lenardon *et al.*, 2009). Die *hog1*-Mutante ist sensitiv gegenüber Nikkomycin (Inhibitor der Chitin-Synthese), Kongo Rot und Calcofluor-White (Alonso-Monge *et al.*, 1999); dies führte zur Annahme, dass die Komponenten des Hog1-Signalweges an der Chitin-Reorganisation beteiligt sind. So zeigte sich, dass die Transkription der Chitinsynthasen abhängig vom HOG-Weg sind (Munro *et al.*, 2007). In *C. albicans* konnte gezeigt werden, dass der HOG („high-osmolarity glycerol“) Signalweg, welcher sich aus der MAPKKK Ssk2, der MAPKK Pbs2 und der MAPK Hog1 zusammensetzt, an mindestens drei verschiedenen Prozessen beteiligt ist: der Reaktion auf und Anpassung an osmotischem Stress, bei der Morphogenese und wie oben beschrieben der Biosynthese der Zellwand (Arana *et al.*, 2005). Unter normalen Wachstumsbedingungen werden von der MAPK Cek1 vermittelte Zellantworten durch Hog1 reprimiert (Eisman *et al.*, 2006). So zeigt eine *hog1*-Mutante eine erhöhte Cek1-Phosphorylierung. Zu dem weist eine *hog1*-Mutante eine erhöhte Resistenz gegenüber zellwanddestabilisierender Agenzien wie Kongo Rot und Calcofluor-White auf. Dieses führte zur Schlussfolgerung, dass die erhöhte Resistenz der *hog1*-Mutante gegen bestimmte Zellwandinhibitoren mit der erhöhten Phosphorylierung von Cek1 korreliert.

Mkc1 ist die übergeordnete Kinase des PKC („protein kinase C“)-Signalweges, welcher sich aus der Kinase MAPKKK Bck1, der Kinase MAPKK Mkk1 und der MAPK Mkc1 zusammensetzt. Defekte in der Glucan- und Chitin-Zusammensetzung führten zu einer Aktivierung und somit Phosphorylierung von Mkc1, was die erhöhte Sensitivität von *mkc1*-Mutanten gegenüber Glucanasen verdeutlicht (Navarro-Garcia *et al.*, 1995). Zusätzlich konnte ein Anstieg der Mkc1-Phosphorylierung in α-Glucosidase-defizienten-Stämmen nachgewiesen werden (Mora-Montes *et al.*, 2007). Somit ist neben Hog1 auch Mkc1 an der Chitin-Synthese und damit an der Erhaltung der Zellwand-Integrität beteiligt. Darüber hinaus hat Hog1 bei oxidativem Stress eine induzierende Wirkung auf Mkc1, was durch einen Anstieg der Mkc1-Phosphorylierung in *hog1*-Mutanten deutlich wird (de Dios *et al.*, 2010).

Der Wechsel zwischen der Hefe- und der Hyphenform ist für die *C. albicans*-Virulenz essentiell (Lo *et al.*, 1997). Um Wirts-Immunantworten zu umgehen bildet *C. albicans* filamentöse Wachstumsformen in unterschiedlichen Wirts-Nischen (Gow & Hube, 2012). Durch diesen morphologischen „Switch“ wird die Expression von Zellwand-Proteinen auf trankriptioneller und proteomischer Ebene stark reguliert und beeinflusst somit die Zellwandzusammensetzung (Nantel *et al.*, 2002, Sohn *et al.*, 2003, Heilmann *et al.*, 2011, Gow *et al.*, 2012). Bisher ist jedoch unklar ob

sich die Maskierung der Zellwandproteine durch Mannan in Hefe- und Hyphen-Zellen unterscheidet. Dabei ist jedoch zu beachten, dass Hyphen weniger Phosphodiester-verknüpfte β1,2-Manno-Oligosaccharide besitzen (Shibata *et al.*, 2007). Weiterhin enthalten Hyphen etwa 3-5 mal mehr Chitin als Hefe-Zellen (Nather & Munro, 2008). Im Gegensatz dazu ändert sich der β1,3-Glucan Gehalt bei der Hyphen-Morphogenese nicht (Gantner *et al.*, 2005). Möglicherweise ist jedoch weniger β1,3-Glucan an der hyphalen Zelloberfläche assoziiert, da Glucan wie auch Chitin nur an Sprossnarben eine direkte Oberflächenpräsentation aufweisen und diese nur in Hefe- und nicht in Hyphen-Zellen vorkommen (Cabib & Bowers, 1971). Daher wird, obwohl die grundlegenden Komponenten der *C. albicans* Zellwand sich in der Hefen- und der Hyphen-Form ähneln, das Zelloberflächen-Proteom und die Mengen der einzelnen PAMPs unterschiedlich präsentiert (Gow *et al.*, 2012).

Der morphologische „Switch" wird durch unterschiedliche Mechanismen, wie MAPK, cAMP und pH-abhängigen Kaskaden, gesteuert (Ernst, 2000, Whiteway, 2000). Das Modul der Cek1 MAPK beinhaltet die Kinasen Cst20, Hst7, Cek1 und den Transkriptionsfaktor Cph1 (Román *et al.*, 2007). Es wurde gezeigt, dass Cek1 nur eine untergeordnete Rolle bei der Hyphen-Morphogenese in Flüssigmedium spielt (Csank *et al.*, 1998). So zeigen *cek1*-Mutanten einen Defekt des Hyphen-Wachstums nur auf festen Nährmedien mit Mannitol als Kohlenstoffquelle oder unter Stickstoff-Limitierung (Csank *et al.*, 1998).

Die Proteinkinase A (PKA)-Kaskade ist ein Hauptsignalweg, welcher die Hyphenbildung induziert. Die Aktivierung der Adenylatcyclase durch Umwelteinflüsse führt zu einem erhöhten cAMP-Spiegel, was zur Dissoziation der regulatorischen Untereinheit Bcy1 und den katalytischen Untereinheiten Tpk1 und Tpk2 führt. Die Tpk-Kinasen aktivieren den Transkription Faktor Efg1 um Hyphen-spezifische Gene zu induzieren (Sonneborn *et al.*, 2000, Bockmühl *et al.*, 2001).

1.3.3 Adhäsion von *C. albicans*

Die Adhäsion von Hefen an Oberflächen von Wirts-Zellen oder Fremdmaterialien (z.B. Implantate oder Katheder) wird durch allgemeine und spezifische Parameter beeinflusst. Die Zelloberflächen-Hydrophobizität und elektrostatische Wechselwirkungen zwischen Hefezelle und der Substratoberfläche stellen hierbei allgemeine Faktoren dar (Jones *et al.*, 1997). Zu den spezifischen Faktoren der Adhäsion von *C. albicans* an Oberflächen gehören Oberflächen-assoziierte Adhäsine (Murciano *et al.*, 2012, Lin *et al.*, 2014). Hierzu zählt die *ALS* (Agglutinin-ähnliche Sequenz)-Familie, welche durch acht verschiedene genetische Loci kodiert wird (*ALS1*-*ALS7*, *ALS9*) (Hoyer, 2001, Hoyer *et al.*, 2008). Die *ALS*-Familie zeichnet sich durch hohe Sequenzähnlichkeit und hohe allelische Variabilität aus. Jedes Als-Protein besteht aus einem N-terminalen Signalpeptid, gefolgt von einer 300 AS großen Immunglobulin-ähnlichen Domäne und einer 104 AS Threonin-reichen Domäne. Die zentrale Domäne enthält eine variable Anzahl von 36 AS-„Repeats". Diese Tandem-

„Repeats“ sind reich an Serin- und Threonin-Resten, an der Zelloberfläche lokalisiert und für die Funktion der Als-Proteine essentiell (Liu & Filler, 2011). Deletionen verschiedener *ALS*-Gene in *C. albicans* führten zu verminderter Adhärenz der Hefe an Epithelien. *ALS2*, *ALS4* und *ALS9* vermitteln die Adhäsion der Pilz-Zelle an Endothelzellen, jedoch nicht an Epithelzellen (Zhao *et al.*, 2005, Hoyer *et al.*, 2008). *ALS3* vermittelt zusätzlich zur Adhäsion an Endothelzellen, die Adhäsion an Mundepithelzellen (Zhao *et al.*, 2004, Oh *et al.*, 2005). Die Deletion von *ALS1* hat keinen Einfluss auf die Adhäsion an Epithelien. *ALS5*, *ALS6* und *ALS7* sind „anti-Adhäsine“, deren Deletion zu einer verstärkten Adhäsion an Endothel- und Epithelzellen führt (Murciano *et al.*, 2012).

1.3.4 Das *C. albicans* Sekretom

Die Sekretion einer Reihe von Enzymen hat für die Pathogenität und die Virulenz von *C. albicans* eine bedeutende Rolle. Eine Vielzahl von Studien zu sezernierten hydrolytischen Enzymen zeigen die Bedeutung dieser Proteine bei der Gewebeinvasion und Virulenz (Schaller *et al.*, 2005, Hruskova-Heidingsfeldova, 2008). Dazu gehört die Genfamilie der sauren Aspartatproteasen (Hube, 2000, Naglik *et al.*, 2003, Naglik *et al.*, 2004), Phospholipasen (Ghannoum, 2000, Theiss *et al.*, 2006), N-Acetylglucosaminidasen (Molloy *et al.*, 1995, Niimi *et al.*, 1997), Phosphatasen (Csank *et al.*, 1997, Guhad *et al.*, 1998) und Siderophore, eisenbindende Moleküle (Sweet & Douglas, 1991, Haas, 2003). Die am besten untersuchten sezernierten Enzyme sind die Aspartat-Proteasen Sap1-10. Sap1-8 werden in die extrazelluläre Umgebung sekretiert, während Sap9 und Sap10 in der Zellwand über den Rest eines GPI-Ankers gehalten werden (Albrecht *et al.*, 2006).

Sap-Proteine bewirken durch Proteolyse den Abbau von Wirtsgewebe, um Proteine als Kohlenstoffquelle zu verwerten und um die Gewebe-Invasion zu erleichtern (Naglik *et al.*, 2003, Naglik *et al.*, 2004). Zusätzlich können sie Proteine des Immunsystems, wie die Komponenten des Komplementsystems, abbauen (Gropp *et al.*, 2009). Neben Sap-Proteinen sind Phospholipasen an der Gewebezerstörung und Invasion beteiligt. Alle fünf Phospholipase B Gene in *C. albicans* enthalten eine Signalsequenz für die Sekretion, aber nur Plb3, Plb4.5 und Plb5 einen GPI-Anker (De Groot *et al.*, 2003), jedoch konnte bisher nicht geklärt werden ob die Phospholipasen an der Cytoplasmamembran oder an der Zellwand lokalisiert sind (Theiss *et al.*, 2006).

Abgesehen von hydrolytischen Enzymen sezerniert *C. albicans* eine Vielzahl weiterer Proteine (Sorgo *et al.*, 2010). Mittels LC-Tandem-Massenspektrometrie wurden 44 sekretorische Proteine in *C. albicans* identifiziert. Hierzu zählt das Sensorprotein Msb2, welches in dieser Arbeit charakterisiert wurde.

1.3.5 Das Mucin Msb2 in *C. albicans*

MSB2 auf Chromosom 2 in *C. albicans* umfasst einen 4230 bp-langen ORF („open reading frame"), der für ein 1409 Aminosäuren-langes Protein kodiert. Msb2 enthält eine Signalsequenz, eine Transmembrandomäne, eine cytoplasmatische Region von 89 Aminosauren und eine sehr große extrazellulare Domäne. Weiterhin besitzt das Protein eine Serin-Threonin reiche Domäne (STR), die zu 43 % aus den beiden Aminosäuren Serin und Threonin besteht (Abb. 1.6) (Cottier, 2007).

Msb2 dient als Umgebungssensor in der Plasmamembran. Es identifiziert Zellwanddefekte und reguliert die Zellmorphogenese, Zellwandbiogenese und Stress-Resistenz (Román *et al.*, 2009). Msb2 weist eine sehr ähnliche Struktur wie Msb2 in der apathogenen Hefe *Saccharomyces cerevisiae* auf und teilt 36,1 % Sequenzähnlichkeit und 21,9 %-identität mit ScMsb2 (Cottier, 2007).

```
1       MLANVKLNLVTALYVLSYVSVVNAYQQENEITPADNIDKRAGAIGNFFRDFTNSIFGNDNSEVNQPSTNGATSTGHFFGPSIPSTSTHQQTPGETSNNVN
101     TKSSSQNQSPSTSPTSTVAAAAATSSSPVASTRPASTSEQKQQEETTARQSTSPATTATTSNTPPSPSTSKETPTSNTAQTSSANNNQQSSNTAAPSTSV
201     IQPSTSEVHVQSQQTSTTPNTPTSSPNTPTTSEAAPTTSAAPTTSEAPVTPSTSEVVPNTPTTSXAPNTPTTSEAPVTPSTSEVVPNTPTTSXAPNTPTT
301     SEAPATPTTSEAPNTPTTSEAPVTPTTSEVVPTTSTQGDAVSTSSTSVTEQTTLTSSTQLPPTTASTTQTSTPEASDSPKPSSTSIETPSTSTFEQDPTT
401     TSSVGTPSSEQPQPTTTSESAVTSNSPTQESTSLVEPTTSSLESSNTPTPNPSTSEAQPSTSASQAPPDTTSSAPAPELSSSNADFSNSVLHSSETTSLV
501     NPTDSQIDSSSTTDAVSQATTEPTSENTPTAASSVTANDINSAQSSAPTSNADAETASSPVSEQSLATGSQTSLDTTAGASSTASEATAENLSTFGTDGS
601     SDASQTIAETTSNSPDQSVVTPSASASPDVSTLPTGSESGTSLVSGSETSIDTNTVASGSTVIPESSNIPTQSPSQSVVSSDAAASNVSTGSATTDSLAG
701     SETGVQPISSSATGTSEPVFSSEYNSSEGTTSLVVPTNSELSSTVTGSSETAATAINSESVLTGSSDTAATVTGSESILTGNTETSATAIASESTLTGST
801     TGATDSAATTIASESVLTGTSDASATVIPSESALTGSTTTPIASESVLTGTTSADVSGATTIGSESIFTGTTESTGTPLPTASGTESLDTTVATGTSVSE
901     QSGVETALSTQPTTGTEATVTSGVSQSEQTGTSAVTGVTESSEQIQSGATTPATTTASDATAVTNASEASAESQATTTAASEATTGSQVTTAASEAATES
1001    QATTAASEAATESQATTAASEAATESQATTPASEPATGSQVTSEVTPATVPSSVTAADTAATSIITSPPASAEPSSEVTAVAPSAATTSSTKNWLPSSLV
1101    IAETPSSNASKSTESIVQASATGASTSGLPRAITPETTTTPGFDYQVITVGFKSALNYPFVVENSISSAQIFQYLPRVLKYPFNGDKSLQNVSVRRLIPY
1201    TASNIDYTITVAEVYFPKDSVKALGSFITTPGSAIYRNPDSVLQALASLIDSRIPLTGLVTDDQQVSGSSSDSNPSTNSYGSMDIVSNTKVADKGRIAGI
1301    TIGAAAGCGLYMTLMVLLFRKFRKSNKALELPITDSESNLGFSDEDSSMLESSSGFSAIFSRINHGGVLTGDPNGGGDDMMMMNNNNNNLRPNNISEPVQ
1401    ASNSLGWYH
```

Abb. 1.6- Msb2 Aminosäuresequenz. Dargestellt ist die Aminosäuresequenz im „Single-Letter-Code". Die AS Serin (S), Theronin (T) und die Transmembrandomäne (gelb) wurden farbig markiert. (Nach Cottier,2007)

Eine *msb2*-Mutante ist nicht in der Lage, in Gegenwart von Zellwand-destabilisierenden Agenzien den Cek1-Signalweg zu aktivieren und den Zellwanddefekt zu kompensieren (Abb. 1.7). Weiterhin wurde gezeigt, dass die Mutante weder in der exponentiellen Phase noch durch Zellwanddefekte eine Cek1 Phosphorylierung auslöst (Román *et al.*, 2009).

In *S. cerevisiae* agiert Msb2 früh im Cdc42-abhängigen MAPK Cek1-Signalweg, welcher für die Induktion des filamentösen Wachstums zuständig ist (Cullen, 2007, Tatebayashi *et al.*, 2007) und wird demnach für die Phosphorylierung der MAPK Cek1 benötigt. Der Membransensor Msb2 reguliert in *C. albicans* über die MAP Kinase Cek1 die Antwort auf defekte *O*-Mannosylierung und die daraus resultierende Expression der *PMT*-Gene. Das *PMT1*-Transkript wurde in der *msb2*-Mutante so hoch wie im Wildtyp nach Tunicamycin-Behandlung induziert, somit reprimiert Msb2 die *PMT1*-Expression bei intakter *N*-Glykosylierung (Cantero & Ernst, 2011). Weiterhin wird Msb2 für die basale Expression der *PMT2*- und *PMT4*-Gene und ihrer Induktion als Antwort auf eine defekte Pmt1 *O*-Glykosylierung benötigt (Cantero & Ernst, 2011). Es wird angenommen, dass eine

Interaktion zwischen Msb2, Sho1 und Cdc42 stattfindet und so der Cek1-Weg aktiviert wird (Román *et al.*, 2009). Eine Interaktion zwischen Msb2 und Cdc42 wurde bereits in *S. cerevisiae* nachgewiesen (Cullen *et al.*, 2004), jedoch für *C. albicans* bislang nicht bestätigt (Román *et al.*, 2009). In *S. cerevisiae* wird Msb2 prozessiert, wobei es zur Abspaltung der hochglykosylierten Ektodomäne kommt, welche anschließend von der Zelle ins äußere Milieu sezerniert wird. Die C-terminale Region verbleibt dabei in der Zelle (Vadaie *et al.*, 2008).

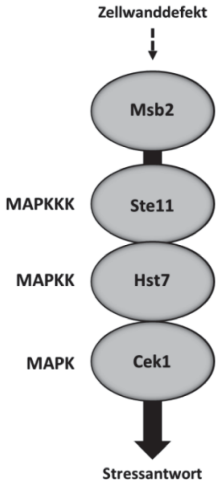

Abb. 1.7- Cek1 MAPK-Signalweg in *C. albicans*. Zellwanddefekte werden durch Msb2 erkannt. Die MAPKKK Ste11 wird phosphoryliert und das Signal über die MAPKK Hst7 an die MAPK Cek1 weitergegeben (modifiziert nach Román *et al.,* 2009)

1.4 Ziel dieser Arbeit

Hefen, aber auch andere Pilze und Bakterien besiedeln eine große Anzahl verschiedener Lebensräume, in denen die Bedingungen sehr variabel sind. Einige Mikroorganismen sind stark an bestimmte Umweltbedingungen angepasst, während andere Mikroorganismen, wie *C. albicans*, in der Lage sind, sich an die schnell ändernden Bedingungen an zu passen. Hierbei bilden verschiedene Sensoren die Grundlage zur Adaption von *C. albicans* an die sich stetig wechselnden Bedingungen.

C. albicans reagiert auf Umwelteinflüsse durch den Membransensor Msb2. So konnte gezeigt werden, dass das Protein für die Hyphenbildung und den Erhalt der Zellwand verantwortlich ist (Román *et al.,* 2009). Msb2 aktiviert dabei zunächst die Proteinkinase Cst20, die dann eine Phosphorylierungskaskade über die Kinasen Ste11 und Hst7 beginnt, in deren Verlauf Cek1 durch Phosphorylierung aktiviert wird (Román *et al.*, 2009). Hierdurch kommt es als Stressantwort der Zelle zu einer veränderten *PMT*-Expression, sowie zur Regulierung der Zellmorphogenese und Stressresistenz (Csank *et al.*, 1998, Eisman *et al.*, 2006, Román *et al.*, 2009). Bei defekter *N*-Glykosylierung, hervorgerufen durch Tunicamycin, wird durch diesen Signalweg eine gesteigerte *PMT1*-Expression induziert, während die *PMT2*- und *PMT4*-Expression bei *O*-Glykosylierungsdefekten erhöht wird. Durch diese Reaktionen kommt es zu einer Kompensation der Defekte (Cantero & Ernst, 2011). Es wird vermutet, dass die Kooperation zwischen Msb2 und dem Msb2-assoziierten Membranprotein Sho1 für die effektive Herstellung der Zellwandintegrität nötig ist (Román *et al.*, 2009).

Im Rahmen dieser Arbeit sollten molekulare Mechanismen der Signalumsetzung durch Msb2 und die phänotypischen Konsequenzen der Msb2-Funktion geklärt werden.

2 Ergebnisse

Der humanpathogene Pilz *C. albicans* muss sich während der Proliferation an die Bedingungen der Zelloberfläche des Wirtes und dessen Immun-Komponenten, sowie an systemische Bedingungen anpassen. Hierzu gehören die Glukose-Anreicherung, limitierte Eisenverfügbarkeit und hohe CO_2-Konzentration im Blut (Brock, 2009), unterschiedlicher Sauerstoffdruck in den Organen, reaktive Sauerstoffspezies und Stickstoffmonoxid-Radikale in Makrophagen (Brown *et al.*, 2009, Brown *et al.*, 2014) und limitierte Nährstoffe in Phagosomen (Brock, 2009). *C. albicans* reagiert auf äußere Einflüsse durch verschiedene Sensoren und infolgedessen durch Adaption der Zellwand. So führen beschädigte Zellwand-Strukturen zu einer Erhöhung der Synthese von Glucan, Chitin und Zellwandproteinen und der Aktivierung von alternativen Mechanismen zur Stabilisierung der Zellwand (Smits *et al.*, 2001, Ernst & Pla, 2011).

Im Rahmen dieser Arbeit sollten molekulare Mechanismen der Signalumsetzung durch den Sensor Msb2 und die phänotypischen Konsequenzen der Msb2-Funktion geklärt werden.

2.1 Manuskriptübersicht

2.1.1 Inhaltsangabe Manuskript I

Im Artikel „Interplay between *Candida albicans* and the antimicrobial peptide armory“ wurde das Zusammenspiel von *C. albicans* und humanen antimikrobielle Peptiden zusammengefasst. AMPs sind lösliche Peptide der angeborenen Immunität, welche Pathogene im Wachstum hemmen oder abtöten können. Die Proliferation des Kommensalen *C. albicans* wird durch ein komplexes Zusammenspiel der humanen Immunantwort und Reaktionen des Pilzes reguliert.

Dieser Artikel fasst menschliche AMP Abwehrsysteme und *C. albicans* Resistenzmechanismen, sowie aktuelle Entwicklungen bei der Verwendung von AMPs als Antimykotika zusammen. Der Übersichtsartikel dient als weiterführende Einleitung und enthält veröffentlichte Daten des Manuskript II „Msb2 shedding protects *Candida albicans* against antimicrobial peptides“ und Manuskript III „*Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides“.

2.1.2 Inhaltsangabe Manuskript II

Im Manuskript II „Msb2 shedding protects *Candida albicans* against antimicrobial peptides“ wurde die Rolle des Sensorprotein Msb2 aus *C. albicans* gegen humane antimikrobielle Peptide aufgeklärt. Hierzu wurden *C. albicans*-Stämme konstruiert, die für eine Msb2-Variante kodieren, in der die große N-terminale extrazelluläre Domäne mit einem HA-Epitop und die kleine C-terminale cytoplasmatische Domäne mit einem V5-Epitop markiert sind. Analysen des synthetisierten Msb2 ergaben, dass die extrazelluläre Domäne quantitativ abgespalten und ins Medium sekretiert wird, während die cytoplasmatische Domäne in der Zelle verbleibt.

Weiterhin konnte für die *O*-Mannosylierung von Msb2 Pmt1 und Pmt2 identifiziert werden. Darüber hinaus wurde gezeigt, dass sezerniertes Msb2 (Msb2*) Pilz-, sowie bakterielle Zellen vor den AMPs Histatin 5 und LL-37 schützt. Diese AMP-Inaktivierung beruht auf Bindung der Msb2-Glykodomäne und den humanen Peptiden.

2.1.3 Inhaltsangabe Manuskript III

Im Manuskript III „*Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides“ wurden die Bindungseigenschaften von Msb2 an humane AMPs weitergehend charakterisiert. Durch Quantifizierung von Msb2/LL-37 Interaktionen mittels Microscale Thermophorese konnte eine hohe Affinität der sezernierten Glykodomäne zu AMPs, abhängig vom Msb2-Glykosylierungsmuster, ermittelt werden. Darüber hinaus wurde gezeigt, dass Msb2 das Reserve-Antibiotikum Daptomycin inaktiviert, welches gegen die bakteriellen Pathogene *Staphylococcus aureus*, *Enterococcus faecalis* und *Corynebacterium pseudodiphtheriticum* wirkt. In Mischkulturen mit *S. aureus* und *C.* albicans wurde dieser protektive Effekt, von Msb2-sezernierenden Zellen und *msb2*-Mutanten, bestätigt.

2.1.4 Inhaltsangabe Manuskript IV

Im Manuskript IV „Signaling domains of mucin Msb2 in *Candida albicans*“ wurden verschiedene Domänen des Glykosensors Msb2 mittels systematischer Deletionen identifiziert. Hierzu wurden verschiedene Msb2-Varianten konstruiert, wodurch eine Sequenz, die essentiell für die Spaltung und Sekretion des Glykoproteins und für alle *MSB2* Phänotypen erforderlich ist, identifiziert wurde.

Die Phosphorylierung der MAP-Kinase Cek1, welche für die Erhaltung der Zellwandintegrität erforderlich ist, wird von drei unterschiedlichen Msb2-Bereichen reguliert: In ungestressten *C. albicans* Zellen reprimieren N-terminale Msb2-Sequenzen die Phosphorylierung, während unter Zellwandstress cytoplasmatische Sequenzen und flankierende Regionen der Transmembrandomäne benötigt werden. Darüber hinaus wurden Sequenzen identifiziert, welche für die Hyphen-Morphogenese, Resistenz gegenüber Antimykotika und die Regulation des *PMT1* Transkripts, die *O*-Mannosyltransferase 1, erforderlich sind. Weiterhin erzeugte die Deletion von zwei Dritteln der Exodomäne einen hyperfilamentösen Phänotypen, abhängig von dem Membranprotein Sho1, der MAPK Cek1 und dem Transkriptionsfaktor Efg1. Ein induzierter Zellwandstress führte zur Relokalisiation des cytoplasmatischen Teils von Msb2 zum Kern und trägt zur Regulation von 117 Genen bei.

2.2 Manuskript I: Interplay between *Candida albicans* and the Antimicrobial Peptide armory

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Erstautor

eigener Anteil an der Arbeit in %: 70

Marc Swidergall schrieb einen Teil des Manuskripts und war korrespondierender Autor.

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MINIREVIEW

Interplay between *Candida albicans* and the Antimicrobial Peptide Armory

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Antimicrobial peptides (AMPs) are key elements of innate immunity, which can directly kill multiple bacterial, viral, and fungal pathogens. The medically important fungus *Candida albicans* colonizes different host niches as part of the normal human microbiota. Proliferation of *C. albicans* is regulated through a complex balance of host immune defense mechanisms and fungal responses. Expression of AMPs against pathogenic fungi is differentially regulated and initiated by interactions of a variety of fungal pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) on human cells. Inflammatory signaling and other environmental stimuli are also essential to control fungal proliferation and to prevent parasitism. To persist in the host, *C. albicans* has developed a three-phase AMP evasion strategy, including secretion of peptide effectors, AMP efflux pumps, and regulation of signaling pathways. These mechanisms prevent *C. albicans* from the antifungal activity of the major AMP classes, including cathelicidins, histatins, and defensins leading to a basal resistance. This minireview summarizes human AMP attack and *C. albicans* resistance mechanisms and current developments in the use of AMPs as antifungal agents.

Understanding host-fungus interactions is crucial for efficient treatment of fungal infections. The human host, upon contact with *Candida albicans*, utilizes an efficient complex immune response that leads to production of soluble effectors, including antimicrobial peptides (AMPs) and cytokines, or to activation of complement, which can directly damage the pathogen (1). Disturbance of human defense systems and activation of fungal virulence traits favor the transition from the commensal stage to the pathogenic stage of fungal infection (2). *C. albicans* defends itself against a multiplicity of innate immune components, including AMPs (3), to remain a successful commensal organism and eventually, to become a human pathogen that causes serious disease. This minireview summarizes human AMP production triggered by fungal recognition and *C. albicans* AMP resistance mechanisms and discusses novel applications in the use of AMPs as antifungal agents.

ACTIVITY OF HUMAN AMPs AGAINST *C. ALBICANS*

AMPs are small soluble defense molecules that kill or block growth of the pathogen by membrane permeabilization of microbial cells and by inactivation of cytoplasmic targets therein (4). AMPs are generated by proteolytic processing of one or more precursor peptides (3). Active peptides consist of 10 to 50 amino acids and are mostly positively charged because of several lysine and arginine residues, but they also contain a substantial proportion of hydrophobic residues (≥30%) (5). Peptide antimicrobial agents that can form an amphipathic, α-helical structure are classified on the basis of their structural characteristics; others, such as the defensins, are classified by the number of disulfide bonds they possess (6). At high AMP concentrations, pores are formed leading to cytoplasmic membrane dysfunction and depolarization by release of ATP and ions; these events effect osmotic dysregulation (Table 1) and finally lead to cell death (7). Beside antimicrobial activity, AMPs also act as immune modulators by promoting migration of neutrophils and monocytes to the site of infection, by upregulating tumor necrosis factor alpha (TNF-α) and by chemoattraction of immature dendritic and T cells to modify the adaptive immune response (reviewed in reference 8). The major antimicrobial peptides in humans include the cathelicidin LL-37, the histatins (Hst), and the defensins (4).

In *C. albicans*, LL-37 initially associates with the cell wall and/or the cytoplasmic membrane (9). Treatment of fungal cells with large amounts of the peptide resulted in a breakdown of the membrane into discrete vesicles and led to rapid efflux of small molecules, such as ATP, as well as larger molecules with molecular masses up to 40 kDa. The *C. albicans* cell wall β-1,3-exoglucanase Xog1 has been identified as a LL-37 receptor (10). Xog1–LL-37 interactions led to cell wall remodeling, and Xog1 enzyme activity was elevated, lowering *C. albicans* adhesion (10, 11). Subsequent to cleavage by a serine protease, processed forms of LL-37 were found at the human skin surface (KS-30 and RK-31) (12). Interestingly, the N-terminally truncated peptide RK-31 accumulated at the cell boundary but migrated into the cytoplasm over time, while the C-terminally truncated peptide was exclusively found in the cytoplasm, inducing leakage of nucleotides and proteins (13).

The fungicidal mechanism of histatin 5 (Hst 5) was reported as a multistep process (14). First, the peptide binds to the ATPase domain of cell envelope proteins Ssa1 and Ssa2 of *C. albicans* (15). Then, Hst 5 utilizes the *C. albicans* polyamine influx transporters Dur3 and Dur31 and accumulates intracellularly (16, 17), where it induces formation of reactive oxygen species (ROS) and efflux of ions and ATP, resulting in cell death (18, 19). Hst 5 downregulates specific mitochondrial proteins involved in *C. albicans* energy metabolism and upregulates biosynthetic proteins (14), which ultimately leads to a drastic decrease in mitochondrial ATP synthesis. The role of the candidacidal mechanism of Hst 5 is also discussed in detail in the accompanying minireview by Puri and Edgerton (20).

In neutrophils, the physiological concentrations of α-de-

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TABLE 1 Mechanisms of human peptide antimicrobials against *C. albicans*

AMP	Mechanism(s)	Reference(s)
LL-37	Association with cell wall and cell membrane by binding to carbohydrates; massive disruption of cell membrane; ATP efflux	7, 9, 11
Histatin 5	Intracellular accumulation; release of cellular ATP in the absence of cytolysis; induced the formation of reactive oxygen species (ROS)	18, 89
hNP-1	Cellular ATP efflux	21
hBD1	Membrane association; increasing the membrane permeability	22
Lactoferrin	Externalization of phosphatidylserine; DNA degradation; increases ROS production; ATP release	61, 90
hGAPDH	Initiating apoptosis; inhibiting *C. albicans* Sap1/2 (CaSap1/2) protease activity; inducing secretion of IL-8 and GM-CSF	28

fensins 1 to 3 (human neutrophil peptide 1 [hNP-1] to -3) are very high (6 mg/ml). hNP-1 acts on energy metabolism by causing depletion of intracellular ATP and increasing extracellular ATP concentrations to kill *C. albicans* (21). The β-defensins hBD1 (human β-defensin 1) to hBD3 cause membrane permeabilization, leading to cell death (22). Interestingly, unlike hBD1 and hBD2, hBD3 kills *C. albicans* by energy-independent mechanisms. In addition to LL-37, hBD3 elevates Xog1 activity, resulting in reduced *C. albicans* adherence (10).

Cationic peptides derived from the N-terminal portion of human mucin MUC7 associate with the fungal plasma membrane but are also internalized to exert fungicidal activity (23). Like hBD3, MUC7-derived peptides induce killing of *Candida* without affecting cellular metabolic activity.

Besides "professional" AMPs dedicated solely to antimicrobial defense, humans produce peptides with AMP activity, which are derived from proteins with different functions. "Moonlighting" proteins include RNase 7, expressed by human keratinocytes, which exhibits antimicrobial activity against *C. albicans* independent of its RNase activity (24). AMP activity of RNase 7 is inhibited by a RNase inhibitor protein expressed in epidermal keratinocytes and is in turn activated, when the inhibitor is cleaved by serine proteases (25). Lactoferrin is a 77-kDa iron-binding glycoprotein present in different body secretions that is active against *C. albicans*. This property is due to a highly basic N-terminal region containing a 25-amino-acid domain termed lactoferricin (LF) that elicits antifungal properties (26). Similar to LL-37, lactoferricin mostly affects membrane morphology, resulting in disintegration of the membrane bilayer, as well as efflux of ATP and proteins in *C. albicans* cells (27).

Recently, a peptide derived from human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was also shown to exhibit antimicrobial activity (28). The hGAPDH peptide killed the fungus by initiating apoptosis and also inhibited Sap1/2 protease activity; in addition, the peptide induced secretion of interleukin 8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) that attracted immune cells to the site of infection (28).

It should be noted that on the one hand, humans produce many more cationic peptides, with yet undefined AMP activity (reviewed in reference 29); on the other hand, a myriad of microbes cohabit the human host, some of which produce AMPs that inhibit *C. albicans* (reviewed in reference 30).

REGULATED AMP ACTIVITY AGAINST *C. ALBICANS*

How does *C. albicans* influence the synthesis of AMPs? Many epithelia produce a basal level of AMPs constitutively, which provides a first line of protection against *C. albicans* and other pathogens (reviewed in references 3 and 31). In a commensal scenario, continuous but relatively infrequent PAMP-PRR interactions trigger low levels of NF-κB activation that drive basal transcription of AMP-encoding genes. Binding of *C. albicans* phospholipomannan by Toll-like receptor 2 (TLR2) may be especially important for this process in keratinocytes (32). Upon increased microbial colonization and in case of epithelial damage or inflammation, AMP transcripts increase dramatically (Fig. 1). This boosted "damage" response is caused predominantly by a strong upregulation of extracellular signal-regulated kinase (ERK)/Jun N-terminal protein kinase (JNK) mitogen-activated protein (MAP) kinase (MAPK) activities and activation of their dedicated transcription factors (e.g., activator protein 1 [AP-1]) (33). In this condition, epithelial cells produce proinflammatory lymphokines, including IL-1β, IL-6, and IL-8 that activate cohabiting immune cells, of which T_H-17 cells producing IL-17, IL-22, and TNF-α are especially relevant for AMP production (34–36). Hyphal formation by *C. albicans* is a decisive factor for the initial release of proinflammatory lymphokines through activation of the inflammosome complex (reviewed in reference 37), but prolonged exposure to hyphae downregulates again the production of β-defensins (38). Inflammatory signaling of epithelial cells is activated further by cohabiting neutrophils, which induce upregulation of TLR4 (39), which in normal human skin may already be upregulated by contact with Gram-positive bacteria (40). TLR4 not only binds *O*-mannans in the *C. albicans* cell wall but also the defensin hBD2, which generates an autostimulatory feedback loop in epithelial cells; in addition, an autocrine IL-1β loop augments transcriptional responses (41). Zones of fungal infection become increasingly inflamed due to the chemoattractive activities of AMPs that recruit more lymphocytes (reviewed in reference 42). Transcriptional activation in epithelial cells is mediated by transcription factors NF-κB, AP-1, and charged vitamin D receptor (VDR) that target specific promoter sequences of genes encoding hBD2 and human CAP18 (18-kDa cationic antimicrobial protein) (hCAP18) (43). TLR signaling increases CYP27B1 (cytochrome p450 27B1) hydroxylase enzyme activity to promote the conversion of 25(OH)D to $1,25(OH)_2D$, which in turn binds to VDR to trigger synthesis of LL-37. The importance of sufficient vitamin D and its maturation for AMP production by human epithelial cells (but surprisingly not by mouse epithelial cells) provide an example for the impact of nutrition on antimicrobial defenses.

It should be noted that transcriptional activation does not proceed equally for all AMPs. For example, increased transcription of the β-defensin 3 (human β-defensin 3 [hBD3])-encoding gene requires TNF-α, which is released from its membrane precursor

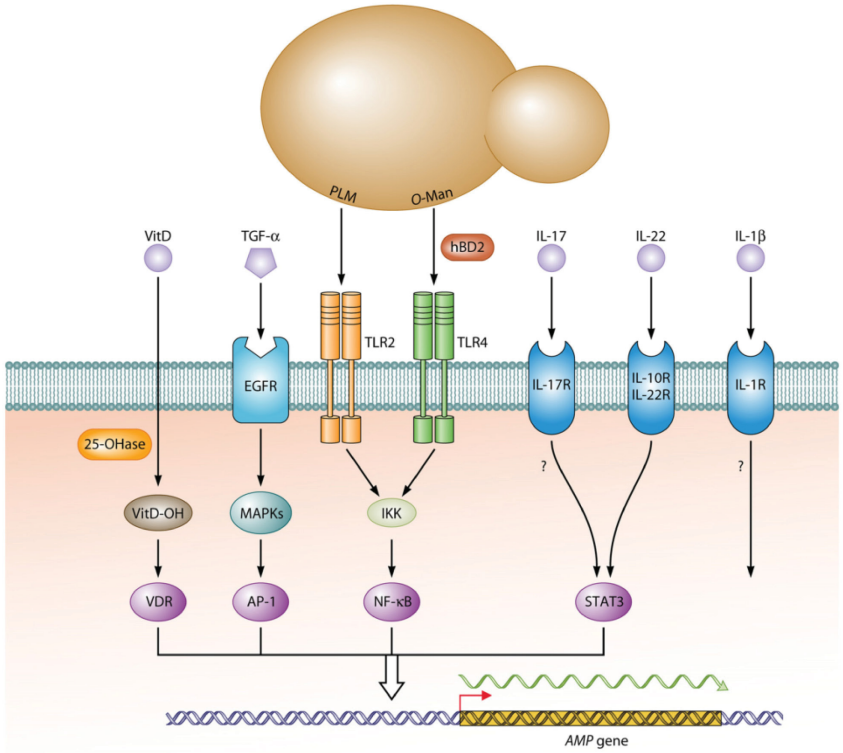

FIG 1 Epithelial signaling pathways directing AMP gene expression. Binding of *C. albicans* PAMPs phospholipomannan (PLM) and *O*-mannan (*O*-Man) to epithelial TLR2/4 proteins leads initially to activation of NF-κB via IkB kinase (IKK). Epithelial proinflammatory lymphokines including IL-1β, IL-6, and IL-8 secreted by epithelial cells trigger cohabiting immune cells to produce IL-1β, IL-17, IL-22, and TNF-α, which boost immune responses of epithelial cells by binding to their dedicated surface receptors IL-1R (IL-1 receptor), IL-17R, IL10R/IL-22R, and EGFR, respectively. MAP kinases (MAPKs) ERK/JNK activate AP-1, which binds with NF-κB and STAT3 transcription factors to promoters of AMP genes to enhance their expression. Production and binding of hBD2, as well as of IL-1β, generates positive-feedback loops in epithelial cells that further increase immune responses. Nutritional input for AMP gene expression is provided by vitamin D (VitD), which is modified by 1α-hydroxylase (25-OHase) to its hydroxylated form (VitD-OH) that binds and activates the promoter of the LL-37-encoding gene.

by the ADAM17 "sheddase"; TNF-α binds to the epidermal growth factor receptor (EGFR) and thereby, through MAP kinases, activates AP-1 (but not NF-κB) (41, 44). While transcriptional activation is the principal mechanism of *C. albicans*-induced AMP induction, AMP processing and/or release is also relevant for cells, including neutrophils, keratinocytes, and Paneth intestinal cells that store precursors or mature AMPs in intracellular vesicles. In neutrophils, azurophil granules accumulate α-defensins, which fuse with phagosomes to attack ingested microbes intracellularly (45). On the other hand, Paneth cells release α-defensins into intestinal crypts, and for unknown reasons, this secretion is enhanced not by fungi but by bacterial cohabitants (46). Another described antimicrobial mechanism of neutrophils is the formation of neutrophil extracellular traps (NETs). These structures are composed of DNA in association with histones as well as granular proteins, such as elastase and myeloperoxidase, and several cytoplasmic proteins (reviewed in reference 47). Interestingly, LL37 stimulates the release of NETs by neutrophils via CD32 (reviewed in reference 48). Immune cells that store the cathelicidin precursor in granules or lamellar bodies include neutrophils, natural killer cells, and mast cells (reviewed in reference 3); upon activation by *C. albicans* or other microbes, these cells degranulate and release the inactive hCAP18 precursor into the extracellular environment, where it is processed and activated. The human skin is known to be hypoxic in deeper layers with oxygen levels ranging between 1.5 and 5.0% (49). Cellular adaptation to low-oxygen environments is orchestrated by the human transcriptional regulator hypoxia-inducible factor 1α (HIF-1α), which also upregulates cathelicidin expression of keratinocytes (50). Interestingly, by inducing c-Fos and AP-1 activity, some antifungal drugs enhance defensin production in keratinocytes, conceivably contributing to the therapeutic success of such antifungals (51). In conclusion, upregulation of AMP production is differentially regulated and is initiated by a variety of PAMP-PRR interactions, as well as by inflammatory signaling and other environmental stimuli.

ESCAPE STRATEGIES FROM ANTIMICROBIAL PEPTIDES

It has been suggested that AMPs and AMP resistance mechanisms have coevolved, leading to a transient host-pathogen balance (5). Several bacterial AMP resistance mechanisms have been reported. Examples include the secretion of AMP-binding proteins that re-

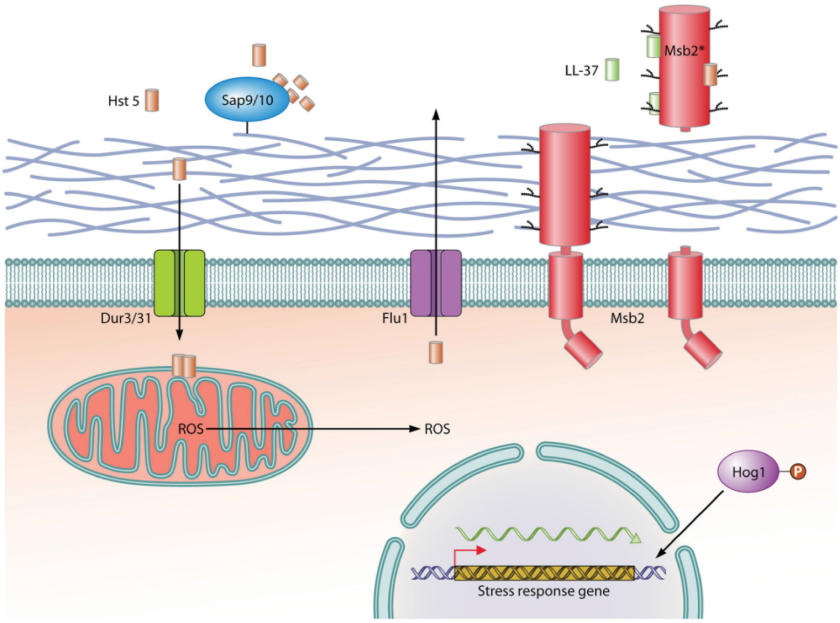

FIG 2 *C. albicans* mechanisms to evade AMP responses. Histatin 5 (Hst 5) is taken up by the *C. albicans* influx transporters Dur3 and Dur31 and induces the formation of reactive oxygen species (ROS); in addition, Hst 5 acts by promoting the efflux of ions and ATP. The Hog1 MAP kinase pathway is activated during AMP stress and upregulates antioxidative and other response mechanisms to overcome AMP activity. The toxicity of Hst 5 is decreased further by its extrusion from fungal cells via the polyamine efflux transporter Flu1. The cell wall-anchored protease Sap9 cleaves and inactivates Hst 5 on the outside of fungal cells. In addition, the shed exodomain fragment of the Msb2 membrane sensor (Msb2*) binds several AMPs extracellularly to provide broad-range protection against AMPs. P, phosphate.

direct peptide antimicrobials away from microbial structures (52). As presented below, similar mechanisms were also shown for *C. albicans* (53–58).

To overcome antifungal activity of AMPs, *C. albicans* has developed a three-phase AMP evasion strategy, including secretion of peptide effectors, AMP efflux pumps, and regulation of signaling pathways. The first line of fungal defense includes secreted proteins that inhibit AMP activity by degradation or binding. To combat antifungal activity of histatin 5, which constitutes the initial barrier against orally invading pathogens, *C. albicans* uses the glycosylphosphatidylinositol (GPI)-anchored proteases Sap9 and Sap10, which are highly and consistently expressed during oral infection; cleavage by Sap9 and Sap10 inactivates the salivary peptide (53) (Fig. 2). Degradation of Hst 5 by purified proteases resulted in complete loss of AMP killing capacity. In addition to this cell surface-associated AMP effector, *C. albicans* secretes the Msb2 glycoprotein, which is a broad-range protectant against peptide antimicrobials (54). The precursor of the plasma membrane protein Msb2 is cleaved, and its extracellular glycodomain is released in considerable amounts into the fungal environment during growth in liquid or on a surface. Extracellularly, it acts to protect the pathogen against AMPs. It is currently debated if secreted aspartic proteases (SAPs) are responsible for the cleavage and release of the secreted Msb2 glycodomain, referred to as Msb2* (55, 59). Msb2* inactivates a wide range of AMPs, including the human cathelicidin LL-37, Hst 5, hNP-1, and hBD1 by tight binding (Fig. 2). By microscale thermophoresis technology, a high affinity (73.1 nM) of LL-37 binding to the shed Msb2* glycodomain was determined (54). In agreement with a protective role of Msb2*, the *C. albicans msb2* mutant was found to be supersensitive to LL-37. It was shown that effective binding and protection under normoxia and hypoxia required native folding and correct *O*-glycosylation of Msb2 by protein-*O*-mannosyltransferases 1 and 2 (Pmt1/Pmt2) (54, 55). This protective effect was not restricted to *C. albicans*, because the mannosylated glycofragment also rescued *Escherichia coli* from the action of LL-37 and Hst 5 (55).

Recently, it was shown that AMPs can be actively extruded from the cytoplasm of fungal cells. The polyamine efflux transporter Flu1, a member of the MDR (*m*ulti*d*rug *r*esistance) family, was found to mediate efflux of Hst 5 in *C. albicans*, resulting in reduction of AMP toxicity (60). Concordantly, deletion of *FLU1* reduced the efflux of Hst 5 and therefore increased sensitivity to the AMP. This finding suggests that various AMPs may represent substrates for fungal efflux transporters, which provide resistance.

Fungal stress response pathways have been shown to be essential for basal resistance to histatins. Physiological levels of Hst 5 activated the mitogen-activated protein kinase (MAPK) Hog1 (61) in wild-type cells, while *hog1* mutants were hypersensitive to the AMP. Nonhuman AMPs (as discussed below) or human β-defensins were shown to trigger Hog1 activity by interaction with the upstream MAPK kinase Pbs2 to orchestrate a cell damage compensatory response (56, 62). Accordingly, both *hog1* and *pbs2* mutants were supersensitive to treatment with the human β-defensins 2 and 3 (56). It has been reported that the high-osmolarity glycerol (HOG) pathway is involved in regulating ROS production and ATP demand by mitochondria (63). On the other hand,

ROS production and ATP efflux are often induced by AMPs (Table 1). Thus, the HOG pathway may function as a key element in survival of *C. albicans* to various AMPs by downregulation of ROS production and of ATP efflux. Some response pathways, which mediate basal resistance to host defense peptides, are distinct from general stress adaptation. The *C. albicans* RNA-binding protein Ssd1, a component of the RAM (regulation of Ace2 and morphogenesis) pathway, and its downstream transcription factor Bcr1 desensitize susceptibility to AMPs by maintaining mitochondrial integrity and by reducing membrane permeabilization (57, 64). Thus, *ssd1* mutants were significantly more susceptible to AMPs. The RAM pathway governs multiple processes such as cell wall integrity (65); therefore, Ssd1 may regulate cell wall and membrane adaptive modification to adapt to specific AMPs, including helical cationic polypeptide protamine and hBD-2 (57). The human salivary mucin MUC7 12-mer activated both the calcineurin pathway and the activity of the 20S and 26S proteasome. Consequently, inactivation of the calcineurin pathway led to hypersensitivity to MUC7 (58). Interestingly, calcineurin signaling is essential for protection against membrane perturbations and conveys tolerance to antifungals (66, 67); therefore, activation of the calcineurin pathway partially protects *C. albicans* from mucin-derived antimicrobial peptides. In *C. albicans*, calcineurin, Hog1, and PKC (protein kinase C) cell wall integrity pathways coordinately regulate chitin synthesis in response to cell wall stress (68). Peptide antimicrobials, including Hst 5, hNP-1, and short lactoferrin peptides, suppress the synthesis of chitin (69), while stimulation of chitin synthesis was shown to rescue *C. albicans* from the action of echinocandin, a glucan synthesis inhibitor (70). Therefore, upregulation of chitin levels by calcineurin, Hog1, and Mkc1 may increase protection against various peptide antimicrobials. Thus, activation and regulation of different signaling pathways mediate a direct fungal response to increase basal AMP resistance.

AMPs are produced by organisms of all types, including insects, vertebrates, plants, and microorganisms (71). Some of these peptides, e.g., daptomycin and vancomycin, are of clinical importance, because they are used as reserve antibiotics for the treatment of multiresistant Gram-positive infections (72). Bacterially produced AMPs, including nisin, contribute to the huge AMP diversity in the human body (73), and continuous AMP interactions may lead to the evolution of resistance mechanisms in pathogens. *C. albicans* exists in many niches in the human body, where fungi may network with many other microbial species, including bacterial pathogens. Fungus-bacterium interactions affect survival, colonization, and pathogenesis of both organisms (74, 75). Such a beneficial situation is represented by the complex polymicrobial biofilm formed by *C. albicans* and *Staphylococcus aureus*, in which the bacterial partner is protected from the action of peptide antibiotics in planktonic and biofilm cohabitation (76). The shed Msb2* from *C. albicans* inactivated the lipopeptide antibiotic daptomycin and provided daptomycin salvage for different bacterial pathogens, including *S. aureus* (54). This action also provided bacterial survival during cohabitation in polymicrobial *C. albicans*-bacterium biofilms. Thus, fungal AMP protectants can improve survival of bacterial pathogens and mediate cross-kingdom resistance against novel human and therapeutic AMPs. This mode of resistance was referred to as "quorum resistance" because it depends on AMP concentrations that depend on *C. albicans* cell numbers. A practical consequence of this finding extends to treatment of multiresistant Gram-positive infections with daptomycin. Thus, multiresistant *S. aureus* infections should be tested for polymicrobial *C. albicans* infestation and consequently treated concurrently with antifungals to guarantee the success of antibacterial antibiotic therapy.

AMPs: NEW ANTIFUNGAL AGENTS FOR THERAPY?

At present, treatment of *C. albicans* infection is primarily based on antifungal agents of the four distinct major classes azoles, polyenes, pyrimidine analogues, and caspofungin/candins (77). Resistance to almost all major antifungal agents, including caspofungin, has been reported in clinical isolates of *C. albicans* (78, 79). Unlike currently used antimicrobial agents, peptide antimicrobials show little or no toxicity toward human cells (80). Besides their direct microbial killing properties, AMPs can neutralize bacterial lipopolysaccharides and regulate host defense mechanisms, as described above (8). Although many AMPs are sensitive to pH and temperature changes, several AMPs are active over a broad range of pH values, and some AMPs are temperature stable up to 100°C (81). The combination of the human broad-spectrum AMP lactoferrin with amphotericin B or fluconazole has been reported to synergistically increase the activity of the antifungals against *Candida* spp. Furthermore, lactoferrin inhibited hypha formation in fluconazole-resistant strains of *C. albicans* (82). Recently, an Hst 5-spermidine conjugate has been developed, which enhanced fungicidal activity compared to nonconjugated Hst 5 (83). It is known that Hst 5 utilizes the polyamine transporters Dur3 and Dur31 for its uptake in *C. albicans* (16); therefore, the Hst 5 peptide (Hst 5 with amino acids 4 to 15 [Hst 5_{4-15}]) conjugated with a GGG linker and spermidine was rapidly taken up, leading to higher *in vitro* and *in vivo* candicidal activity than with nonconjugated Hst 5 (83). Collectively, these results suggest that the development and investigation of AMP conjugates could lead to promising new antifungals.

Some antimicrobial peptides are currently in clinical trials or under development (reviewed in reference 84). Part of commercial AMPs are derived from human peptides such as lactoferrin or human defensins modified in their structure, e.g., cyclization to prevent degradation. Alternative AMP-based approaches include plant defensins, which are active both against phytopathogenic and also against human-pathogenic fungi such as *C. albicans*. Besides their antifungal activity, plant AMPs are advantageous because they are nontoxic to human cells (85). Plant defensins have a wide variety of functions and differ in their mechanisms of antifungal activity. It was shown that antifungal protein 2 of radish (*Raphanus sativus* AFP2 [RsAFP2]) interacts with glucosylceramides in the *C. albicans* cell wall and induces an intracellular signaling pathway, which leads to apoptosis of the fungal pathogen (86). The defensin NaD1 from *Nicotiana alata* interacts with the fungal cell surface and causes membrane permeabilization, which leads to entry of AMPs into the cytoplasm and to increased production of ROS (62). The modes of action of plant AMPs compared to human AMP peptides could also reveal new fungal "Achilles' heels" for antifungal compounds to be used in therapy.

Recently, synthetic peptide mimics of transmembrane regions (TMPMs) of the ABC (ATP-binding cassette) efflux pump Cdr1 (*Candida* drug resistance 1) were found to bind to Cdr1 and to block the efflux of antifungal agents (87). By optimizing TMPMs to avoid aggregation of the highly hydrophobic peptides, azole-resistant *C. albicans* cells were chemosensitized to azoles. This approach may possibly be applied to other targets, e.g., secreted as-

partyl proteases or to the MDR family member Flu1, to inhibit essential fungal resistance functions. In this scenario, AMPs are used not to kill the fungal pathogen directly but to inhibit essential functions in growth, morphogenesis, and resistance of fungi.

CONCLUSIONS

During the last years, the numbers of fungal morbidity and mortality cases caused by *C. albicans* have increased, not only because of diagnostics of invasive infections but also because of limited efficacy of current antifungals. Humans express a large number of various peptides with antimicrobial and, specifically, antifungal action that are key effectors of innate immunity. In addition to their role in combating microbial pathogens by direct killing or recruitment of immune cells to the site of infection, AMPs are involved in the processes of wound healing and angiogenesis. AMPs have many potential uses in treatment of complex infections, and their mode of action can be exploited for the generation of novel antifungal molecules. Various applications of human and nonhuman AMPs show the effectiveness of small peptides against the human-pathogenic fungus *C. albicans* (62, 83, 86, 87). Recently, different AMP resistance mechanisms that allow *C. albicans* to become either a successful commensal or a human fungal pathogen were discovered (15, 17, 54–56, 61, 88). These fungal AMP resistance mechanisms include secreted AMP-blocking proteins and proteins regulating signaling pathways, which collectively may prevent AMP-mediated killing. The molecular understanding of AMP activity may reveal novel antifungal targets and aid in the design of new strategies and agents in the fight against drug-resistant fungi. In future research, it will important to clarify the molecular details of *C. albicans* AMP resistance to fully understand interactions between the host and pathogen in fungal disease. Importantly, novel therapeutic peptide antifungals should be evaluated in the light of fungal AMP-resistance mechanisms.

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REFERENCES

1. **Cheng SC, Joosten LA, Kullberg BJ, Netea MG.** 2012. Interplay between *Candida albicans* and the mammalian innate host defense. Infect. Immun. **80:**1304–1313. http://dx.doi.org/10.1128/IAI.06146-11.
2. **Hube B.** 2004. From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. Curr. Opin. Microbiol. **7:**336–341. http://dx.doi.org/10.1016/j.mib.2004.06.003.
3. **Vandamme D, Landuyt B, Luyten W, Schoofs L.** 2012. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell. Immunol. **280:**22–35. http://dx.doi.org/10.1016/j.cellimm.2012.11.009.
4. **Lehrer RI, Ganz T.** 1999. Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol. **11:**23–27. http://dx.doi.org/10.1016/S0952-7915(99)80005-3.
5. **Peschel A, Sahl HG.** 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4:**529–536. http://dx.doi.org/10.1038/nrmicro1441.
6. **van't Hof W, Veerman EC, Helmerhorst EJ, Amerongen AV.** 2001. Antimicrobial peptides: properties and applicability. Biol. Chem. **382:**597–619.
7. **Vylkova S, Sun JN, Edgerton M.** 2007. The role of released ATP in killing *Candida albicans* and other extracellular microbial pathogens by cationic peptides. Purinergic Signal **3:**91–97. http://dx.doi.org/10.1007/s11302-006-9040-0.
8. **Steinstraesser L, Kraneburg U, Jacobsen F, Al-Benna S.** 2011. Host defense peptides and their antimicrobial-immunomodulatory duality. Immunobiology **216:**322–333. http://dx.doi.org/10.1016/j.imbio.2010.07.003.
9. **den Hertog AL, van Marle J, van Veen HA, Van 't Hof W, Bolscher JG, Veerman EC, Nieuw Amerongen AV.** 2005. Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. Biochem. J. **388:**689–695. http://dx.doi.org/10.1042/BJ20042099.
10. **Chang HT, Tsai PW, Huang HH, Liu YS, Chien TS, Lan CY.** 2012. LL37 and hBD-3 elevate the beta-1,3-exoglucanase activity of *Candida albicans* Xog1p, resulting in reduced fungal adhesion to plastic. Biochem. J. **441:**963–970. http://dx.doi.org/10.1042/BJ20111454.
11. **Tsai PW, Yang CY, Chang HT, Lan CY.** 2011. Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. PLoS One **6:**e17755. http://dx.doi.org/10.1371/journal.pone.0017755.
12. **Murakami M, Lopez-Garcia B, Braff M, Dorschner RA, Gallo RL.** 2004. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J. Immunol. **172:**3070–3077. http://dx.doi.org/10.4049/jimmunol.172.5.3070.
13. **den Hertog AL, Van Marle J, Veerman ECI, Valentijn-Benz M, Nazmi K, Kalay H, Grün CH, Van't Hof W, Bolscher JGM, Amerongen AVN.** 2006. The human cathelicidin peptide LL-37 and truncated variants induce segregation of lipids and proteins in the plasma membrane of *Candida albicans*. Biol. Chem. **387:**1495–1502.
14. **Komatsu T, Salih E, Helmerhorst EJ, Offner GD, Oppenheim FG.** 2011. Influence of histatin 5 on *Candida albicans* mitochondrial protein expression assessed by quantitative mass spectrometry. J. Proteome Res. **10:**646–655. http://dx.doi.org/10.1021/pr100861k.
15. **Sun JN, Li W, Jang WS, Nayyar N, Sutton MD, Edgerton M.** 2008. Uptake of the antifungal cationic peptide histatin 5 by *Candida albicans* Ssa2p requires binding to non-conventional sites within the ATPase domain. Mol. Microbiol. **70:**1246–1260. http://dx.doi.org/10.1111/j.1365-2958.2008.06480.x.
16. **Kumar R, Chadha S, Saraswat D, Bajwa JS, Li RA, Conti HR, Edgerton M.** 2011. Histatin 5 uptake by *Candida albicans* utilizes polyamine transporters Dur3 and Dur31 proteins. J. Biol. Chem. **286:**43748–43758. http://dx.doi.org/10.1074/jbc.M111.311175.
17. **Mayer FL, Wilson D, Jacobsen ID, Miramon P, Grosse K, Hube B.** 2012. The novel *Candida albicans* transporter Dur31 is a multi-stage pathogenicity factor. PLoS Pathog. **8:**e1002592. http://dx.doi.org/10.1371/journal.ppat.1002592.
18. **Helmerhorst EJ, Troxler RF, Oppenheim FG.** 2001. The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species. Proc. Natl. Acad. Sci. U. S. A. **98:**14637–14642. http://dx.doi.org/10.1073/pnas.141366998.
19. **Koshlukova SE, Araujo MW, Baev D, Edgerton M.** 2000. Released ATP is an extracellular cytotoxic mediator in salivary histatin 5-induced killing of *Candida albicans*. Infect. Immun. **68:**6848–6856. http://dx.doi.org/10.1128/IAI.68.12.6848-6856.2000.
20. **Puri S, Edgerton M.** 2014. How does it kill?: understanding the candidacidal mechanism of salivary histatin 5. Eukaryot. Cell **13:**958–964.
21. **Edgerton M, Koshlukova SE, Araujo MW, Patel RC, Dong J, Bruenn JA.** 2000. Salivary histatin 5 and human neutrophil defensin 1 kill *Candida albicans* via shared pathways. Antimicrob. Agents Chemother. **44:**3310–3316. http://dx.doi.org/10.1128/AAC.44.12.3310-3316.2000.
22. **Krishnakumari V, Rangaraj N, Nagaraj R.** 2009. Antifungal activities of human beta-defensins HBD-1 to HBD-3 and their C-terminal analogs Phd1 to Phd3. Antimicrob. Agents Chemother. **53:**256–260. http://dx.doi.org/10.1128/AAC.00470-08.
23. **Situ H, Wei G, Smith CJ, Mashhoon S, Bobek LA.** 2003. Human salivary MUC7 mucin peptides: effect of size, charge and cysteine residues on antifungal activity. Biochem. J. **375:**175–182. http://dx.doi.org/10.1042/BJ20030779.
24. **Harder J, Schroder JM.** 2002. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. J. Biol. Chem. **277:**46779–46784. http://dx.doi.org/10.1074/jbc.M207587200.
25. **Abtin A, Eckhart L, Mildner M, Ghannadan M, Harder J, Schroder JM, Tschachler E.** 2009. Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking antimicrobial activities of

RNase 5 and RNase 7. J. Investig. Dermatol. **129:**2193–2201. http://dx.doi.org/10.1038/jid.2009.35.
26. **Lupetti A, Paulusma-Annema A, Welling MM, Senesi S, van Dissel JT, Nibbering PH.** 2000. Candidacidal activities of human lactoferrin peptides derived from the N terminus. Antimicrob. Agents Chemother. **44:**3257–3263. http://dx.doi.org/10.1128/AAC.44.12.3257-3263.2000.
27. **Bolscher J, Nazmi K, van Marle J, van 't Hof W, Veerman E.** 2012. Chimerization of lactoferricin and lactoferrampin peptides strongly potentiates the killing activity against *Candida albicans*. Biochem. Cell Biol. **90:**378–388. http://dx.doi.org/10.1139/o11-085.
28. **Wagener J, Schneider JJ, Baxmann S, Kalbacher H, Borelli C, Nuding S, Kuchler R, Wehkamp J, Kaeser MD, Mailander-Sanchez D, Braunsdorf C, Hube B, Schild L, Forssmann WG, Korting HC, Liepke C, Schaller M.** 2013. A peptide derived from the highly conserved protein GAPDH is involved in tissue protection by different antifungal strategies and epithelial immunomodulation. J. Investig. Dermatol. **133:**144–153. http://dx.doi.org/10.1038/jid.2012.254.
29. **Mehra T, Koberle M, Braunsdorf C, Mailander-Sanchez D, Borelli C, Schaller M.** 2012. Alternative approaches to antifungal therapies. Exp. Dermatol. **21:**778–782.
30. **Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F.** 2012. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J. Appl. Microbiol. **113:**723–736. http://dx.doi.org/10.1111/j.1365-2672.2012.05338.x.
31. **Selsted ME, Ouellette AJ.** 2005. Mammalian defensins in the antimicrobial immune response. Nat. Immunol. **6:**551–557. http://dx.doi.org/10.1038/ni1206.
32. **Li M, Chen Q, Shen Y, Liu W.** 2009. *Candida albicans* phospholipomannan triggers inflammatory responses of human keratinocytes through Toll-like receptor 2. Exp. Dermatol. **18:**603–610. http://dx.doi.org/10.1111/j.1600-0625.2008.00832.x.
33. **Moyes DL, Runglall M, Murciano C, Shen C, Nayar D, Thavaraj S, Kohli A, Islam A, Mora-Montes H, Challacombe SJ, Naglik JR.** 2010. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of *Candida albicans* in epithelial cells. Cell Host Microbe **8:**225–235. http://dx.doi.org/10.1016/j.chom.2010.08.002.
34. **Eyerich S, Wagener J, Wenzel V, Scarponi C, Pennino D, Albanesi C, Schaller M, Behrendt H, Ring J, Schmidt-Weber CB, Cavani A, Mempel M, Traidl-Hoffmann C, Eyerich K.** 2011. IL-22 and TNF-alpha represent a key cytokine combination for epidermal integrity during infection with *Candida albicans*. Eur. J. Immunol. **41:**1894–1901. http://dx.doi.org/10.1002/eji.201041197.
35. **Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R.** 2004. IL-22 increases the innate immunity of tissues. Immunity **21:**241–254. http://dx.doi.org/10.1016/j.immuni.2004.07.007.
36. **Kao CY, Chen Y, Thai P, Wachi S, Huang F, Kim C, Harper RW, Wu R.** 2004. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. J. Immunol. **173:**3482–3491. http://dx.doi.org/10.4049/jimmunol.173.5.3482.
37. **Rehaume LM, Jouault T, Chamaillard M.** 2010. Lessons from the inflammasome: a molecular sentry linking *Candida* and Crohn's disease. Trends Immunol. **31:**171–175. http://dx.doi.org/10.1016/j.it.2010.01.007.
38. **Lu Q, Jayatilake JA, Samaranayake LP, Jin L.** 2006. Hyphal invasion of *Candida albicans* inhibits the expression of human beta-defensins in experimental oral candidiasis. J. Investig. Dermatol. **126:**2049–2056. http://dx.doi.org/10.1038/sj.jid.5700346.
39. **Weindl G, Naglik JR, Kaesler S, Biedermann T, Hube B, Korting HC, Schaller M.** 2007. Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. J. Clin. Invest. **117:**3664–3672.
40. **Kawai K, Shimura H, Minagawa M, Ito A, Tomiyama K, Ito M.** 2002. Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. J. Dermatol. Sci. **30:**185–194. http://dx.doi.org/10.1016/S0923-1811(02)00105-6.
41. **Pahl R, Brunke G, Steubesand N, Schubert S, Bottner M, Wedel T, Jurgensen C, Hampe J, Schafer H, Zeissig S, Schreiber S, Rosenstiel P, Reiss K, Arlt A.** 2011. IL-1beta and ADAM17 are central regulators of beta-defensin expression in *Candida* esophagitis. Am. J. Physiol. Gastrointest. Liver Physiol. **300:**G547–G553. http://dx.doi.org/10.1152/ajpgi.00251.2010.
42. **Wuerth K, Hancock RE.** 2011. New insights into cathelicidin modulation of adaptive immunity. Eur. J. Immunol. **41:**2817–2819. http://dx.doi.org/10.1002/eji.201142055.
43. **Campbell Y, Fantacone ML, Gombart AF.** 2012. Regulation of antimicrobial peptide gene expression by nutrients and by-products of microbial metabolism. Eur. J. Nutr. **51:**899–907. http://dx.doi.org/10.1007/s00394-012-0415-4.
44. **Steubesand N, Kiehne K, Brunke G, Pahl R, Reiss K, Herzig KH, Schubert S, Schreiber S, Folsch UR, Rosenstiel P, Arlt A.** 2009. The expression of the beta-defensins hBD-2 and hBD-3 is differentially regulated by NF-kappaB and MAPK/AP-1 pathways in an in vitro model of *Candida* esophagitis. BMC Immunol. **10:**36. http://dx.doi.org/10.1186/1471-2172-10-36.
45. **Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N.** 1997. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood **90:**2796–2803.
46. **Rumio C, Besusso D, Palazzo M, Selleri S, Sfondrini L, Dubini F, Menard S, Balsari A.** 2004. Degranulation of Paneth cells via Toll-like receptor 9. Am. J. Pathol. **165:**373–381. http://dx.doi.org/10.1016/S0002-9440(10)63304-4.
47. **Brinkmann V, Zychlinsky A.** 2007. Beneficial suicide: why neutrophils die to make NETs. Nat. Rev. Microbiol. **5:**577–582. http://dx.doi.org/10.1038/nrmicro1710.
48. **Mantovani A, Cassatella MA, Costantini C, Jaillon S.** 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. **11:**519–531. http://dx.doi.org/10.1038/nri3024.
49. **Stewart FA, Denekamp J, Randhawa VS.** 1982. Skin sensitization by misonidazole: a demonstration of uniform mild hypoxia. Br. J. Cancer **45:**869–877. http://dx.doi.org/10.1038/bjc.1982.139.
50. **Peyssonnaux C, Boutin AT, Zinkernagel AS, Datta V, Nizet V, Johnson RS.** 2008. Critical role of HIF-1alpha in keratinocyte defense against bacterial infection. J. Investig. Dermatol. **128:**1964–1968. http://dx.doi.org/10.1038/jid.2008.27.
51. **Kanda N, Kano R, Ishikawa T, Watanabe S.** 2011. The antimycotic drugs itraconazole and terbinafine hydrochloride induce the production of human beta-defensin-3 in human keratinocytes. Immunobiology **216:**497–504. http://dx.doi.org/10.1016/j.imbio.2010.08.008.
52. **Frick IM, Åkesson P, Rasmussen M, Schmidtchen A, Björck L.** 2003. SIC, a secreted protein of Streptococcus pyogenes that inactivates antibacterial peptides. J. Biol. Chem. **278:**16561–16566. http://dx.doi.org/10.1074/jbc.M301995200.
53. **Meiller TF, Hube B, Schild L, Shirtliff ME, Scheper MA, Winkler R, Ton A, Jabra-Rizk MA.** 2009. A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. PLoS One **4:**e5039. http://dx.doi.org/10.1371/journal.pone.0005039.
54. **Swidergall M, Ernst AM, Ernst JF.** 2013. *Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides. Antimicrob. Agents Chemother. **57:**3917–3922. http://dx.doi.org/10.1128/AAC.00862-13.
55. **Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Roman E, Pla J, Ernst JF.** 2012. Msb2 shedding protects *Candida albicans* against antimicrobial peptides. PLoS Pathog. **8:**e1002501. http://dx.doi.org/10.1371/journal.ppat.1002501.
56. **Argimon S, Fanning S, Blankenship JR, Mitchell AP.** 2011. Interaction between the *Candida albicans* high-osmolarity glycerol (HOG) pathway and the response to human beta-defensins 2 and 3. Eukaryot. Cell **10:**272–275. http://dx.doi.org/10.1128/EC.00133-10.
57. **Jung SI, Finkel JS, Solis NV, Chaili S, Mitchell AP, Yeaman MR, Filler SG.** 2013. Bcr1 functions downstream of Ssd1 to mediate antimicrobial peptide resistance in *Candida albicans*. Eukaryot. Cell **12:**411–419. http://dx.doi.org/10.1128/EC.00285-12.
58. **Lis M, Liu TT, Barker KS, Rogers PD, Bobek LA.** 2010. Antimicrobial peptide MUC7 12-mer activates the calcium/calcineurin pathway in *Candida albicans*. FEMS Yeast Res. **10:**579–586.
59. **Puri S, Kumar R, Chadha S, Tati S, Conti HR, Hube B, Cullen PJ, Edgerton M.** 2012. Secreted aspartic protease cleavage of Candida albicans Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis. PLoS One **7:**e46020. http://dx.doi.org/10.1371/journal.pone.0046020. (Author Correction, 8:10.1371/annotation/13ef5e14-c192-4bb2-af91-886024500b4b, 2013. http://dx.doi.org/10.1371/annotation/13ef5e14-c192-4bb2-af91-886024500b4b.)
60. **Li R, Kumar R, Tati S, Puri S, Edgerton M.** 2013. *Candida albicans* Flu1-mediated efflux of salivary histatin 5 reduces its cytosolic concentration and fungicidal activity. Antimicrob. Agents Chemother. **57:**1832–1839. http://dx.doi.org/10.1128/AAC.02295-12.
61. **Vylkova S, Jang WS, Li W, Nayyar N, Edgerton M.** 2007. Histatin 5

initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell **6:**1876–1888. http://dx.doi.org/10.1128/EC.00039-07.

62. **Hayes BM, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL.** 2013. Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against *Candida albicans*. Antimicrob. Agents Chemother. **57:**3667–3675. http://dx.doi.org/10.1128/AAC.00365-13.
63. **Alonso-Monge R, Carvaihlo S, Nombela C, Rial E, Pla J.** 2009. The Hog1 MAP kinase controls respiratory metabolism in the fungal pathogen *Candida albicans*. Microbiology **155:**413–423. http://dx.doi.org/10.1099/mic.0.023309-0.
64. **Gank KD, Yeaman MR, Kojima S, Yount NY, Park H, Edwards JE, Jr, Filler SG, Fu Y.** 2008. SSD1 is integral to host defense peptide resistance in *Candida albicans*. Eukaryot. Cell **7:**1318–1327. http://dx.doi.org/10.1128/EC.00402-07.
65. **Bharucha N, Chabrier-Rosello Y, Xu T, Johnson C, Sobczynski S, Song Q, Dobry CJ, Eckwahl MJ, Anderson CP, Benjamin AJ, Kumar A, Krysan DJ.** 2011. A large-scale complex haploinsufficiency-based genetic interaction screen in *Candida albicans*: analysis of the RAM network during morphogenesis. PLoS Genet. **7:**e1002058. http://dx.doi.org/10.1371/journal.pgen.1002058.
66. **Cruz MC, Goldstein AL, Blankenship JR, Del Poeta M, Davis D, Cardenas ME, Perfect JR, McCusker JH, Heitman J.** 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. EMBO J. **21:**546–559. http://dx.doi.org/10.1093/emboj/21.4.546.
67. **Sanglard D, Ischer F, Marchetti O, Entenza J, Bille J.** 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. **48:**959–976. http://dx.doi.org/10.1046/j.1365-2958.2003.03495.x.
68. **Lenardon MD, Lesiak I, Munro CA, Gow NA.** 2009. Dissection of the *Candida albicans* class I chitin synthase promoters. Mol. Genet. Genomics **281:**459–471. http://dx.doi.org/10.1007/s00438-009-0423-0.
69. **Tanida T, Okamoto T, Ueta E, Yamamoto T, Osaki T.** 2006. Antimicrobial peptides enhance the candidacidal activity of antifungal drugs by promoting the efflux of ATP from *Candida* cells. J. Antimicrob. Chemother. **57:**94–103.
70. **Walker LA, Munro CA, De Bruijn I, Lenardon MD, McKinnon A, Gow NAR.** 2008. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. PLoS Pathog. **4:**e1000040. http://dx.doi.org/10.1371/journal.ppat.1000040.
71. **Zasloff M.** 2002. Antimicrobial peptides of multicellular organisms. Nature **415:**389–395. http://dx.doi.org/10.1038/415389a.
72. **Moise PA, North D, Steenbergen JN, Sakoulas G.** 2009. Susceptibility relationship between vancomycin and daptomycin in Staphylococcus aureus: facts and assumptions. Lancet Infect. Dis. **9:**617–624. http://dx.doi.org/10.1016/S1473-3099(09)70200-2.
73. **Akerey B, Le-Lay C, Fliss I, Subirade M, Rouabhia M.** 2009. In vitro efficacy of nisin Z against *Candida albicans* adhesion and transition following contact with normal human gingival cells. J. Appl. Microbiol. **107:**1298–1307. http://dx.doi.org/10.1111/j.1365-2672.2009.04312.x.
74. **Harriott MM, Noverr MC.** 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob. Agents Chemother. **53:**3914–3922. http://dx.doi.org/10.1128/AAC.00657-09.
75. **Mallick EM, Bennett RJ.** 2013. Sensing of the microbial neighborhood by *Candida albicans*. PLoS Pathog. **9:**e1003661. http://dx.doi.org/10.1371/journal.ppat.1003661.
76. **Harriott MM, Noverr MC.** 2010. Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. Antimicrob. Agents Chemother. **54:**3746–3755. http://dx.doi.org/10.1128/AAC.00573-10.
77. **Moudgal V, Sobel J.** 2010. Antifungals to treat *Candida albicans*. Expert Opin. Pharmacother. **11:**2037–2048. http://dx.doi.org/10.1517/14656566.2010.493875.
78. **Katiyar S, Pfaller M, Edlind T.** 2006. *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob. Agents Chemother. **50:**2892–2894. http://dx.doi.org/10.1128/AAC.00349-06.
79. **Sanglard D, Odds FC.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2:**73–85. http://dx.doi.org/10.1016/S1473-3099(02)00181-0.
80. **Velden WJ, van Iersel TM, Blijlevens NM, Donnelly JP.** 2009. Safety and tolerability of the antimicrobial peptide human lactoferrin 1–11 (hLF1–11). BMC Med. **7:**44. http://dx.doi.org/10.1186/1741-7015-7-44.
81. **Tokle T, McClements DJ.** 2011. Physicochemical properties of lactoferrin stabilized oil-in-water emulsions: effects of pH, salt and heating. Food Hydrocolloids **25:**976–982. http://dx.doi.org/10.1016/j.foodhyd.2010.09.012.
82. **Venkatesh MP, Rong L.** 2008. Human recombinant lactoferrin acts synergistically with antimicrobials commonly used in neonatal practice against coagulase-negative staphylococci and *Candida albicans* causing neonatal sepsis. J. Med. Microbiol. **57:**1113–1121. http://dx.doi.org/10.1099/jmm.0.2008/001263-0.
83. **Tati S, Li R, Puri S, Kumar R, Davidow P, Edgerton M.** 2014. Histatin 5-spermidine conjugates have enhanced fungicidal activity and efficacy as a topical therapeutic for oral candidiasis. Antimicrob. Agents Chemother. **58:**756–766.
84. **Zhang L, Falla TJ.** 2010. Potential therapeutic application of host defense peptides. Methods Mol. Biol. **618:**303–327. http://dx.doi.org/10.1007/978-1-60761-594-1_19.
85. **Thevissen K, Kristensen HH, Thomma BP, Cammue BP, Francois IE.** 2007. Therapeutic potential of antifungal plant and insect defensins. Drug Discov. Today **12:**966–971. http://dx.doi.org/10.1016/j.drudis.2007.07.016.
86. **Thevissen K, de Mello Tavares P, Xu D, Blankenship J, Vandenbosch D, Idkowiak-Baldys J, Govaert G, Bink A, Rozental S, de Groot PW, Davis TR, Kumamoto CA, Vargas G, Nimrichter L, Coenye T, Mitchell A, Roemer T, Hannun YA, Cammue BP.** 2012. The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*. Mol. Microbiol. **84:**166–180. http://dx.doi.org/10.1111/j.1365-2958.2012.08017.x.
87. **Maurya IK, Thota CK, Verma SD, Sharma J, Rawal MK, Ravikumar B, Sen S, Chauhan N, Lynn AM, Chauhan VS, Prasad R.** 2013. Rationally designed transmembrane peptide mimics of the multidrug transporter protein Cdr1 act as antagonists to selectively block drug efflux and chemosensitize azole-resistant clinical isolates of *Candida albicans*. J. Biol. Chem. **288:**16775–16787. http://dx.doi.org/10.1074/jbc.M113.467159.
88. **Jang WS, Bajwa JS, Sun JN, Edgerton M.** 2010. Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. Mol. Microbiol. **77:**354–370. http://dx.doi.org/10.1111/j.1365-2958.2010.07210.x.
89. **Edgerton M, Koshlukova SE.** 2000. Salivary histatin 5 and its similarities to the other antimicrobial proteins in human saliva. Adv. Dent. Res. **14:**16–21. http://dx.doi.org/10.1177/08959374000140010201.
90. **Andres MT, Viejo-Diaz M, Fierro JF.** 2008. Human lactoferrin induces apoptosis-like cell death in *Candida albicans*: critical role of K+-channel-mediated K+ efflux. Antimicrob. Agents Chemother. **52:**4081–4088. http://dx.doi.org/10.1128/AAC.01597-07.

2.3 Manuskript II: Msb2 Shedding Protects *Candida albicans* against Antimicrobial Peptides

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Zweitautor

eigener Anteil an der Arbeit in %: 40

Marc Swidergall hat folgende Experimente geplant, durch geführt und interpretiert: Sensitivitätstest verschiedener *C. albicans*-Stämme gegenüber LL-37 und Histatin 5; Lokalisation von TAMRA markiertem LL-37; Aufreinigung der extrazellulären Msb2-Domäne mittels Immunaffinitätschromatographie (IAC); Dot-Blot Analysen; AMP-Assays mit bakteriellen Zellen; Protease-Assay; Bindungsanalysen von Msb2 und LL-37.

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Msb2 Shedding Protects *Candida albicans* against Antimicrobial Peptides

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Abstract

Msb2 is a sensor protein in the plasma membrane of fungi. In the human fungal pathogen *C. albicans* Msb2 signals via the Cek1 MAP kinase pathway to maintain cell wall integrity and allow filamentous growth. Msb2 doubly epitope-tagged in its large extracellular and small cytoplasmic domain was efficiently cleaved during liquid and surface growth and the extracellular domain was almost quantitatively released into the growth medium. Msb2 cleavage was independent of proteases Sap9, Sap10 and Kex2. Secreted Msb2 was highly *O*-glycosylated by protein mannosyltransferases including Pmt1 resulting in an apparent molecular mass of >400 kDa. Deletion analyses revealed that the transmembrane region is required for Msb2 function, while the large N-terminal and the small cytoplasmic region function to downregulate Msb2 signaling or, respectively, allow its induction by tunicamycin. Purified extracellular Msb2 domain protected fungal and bacterial cells effectively from antimicrobial peptides (AMPs) histatin-5 and LL-37. AMP inactivation was not due to degradation but depended on the quantity and length of the Msb2 glycofragment. *C. albicans msb2* mutants were supersensitive to LL-37 but not histatin-5, suggesting that secreted rather than cell-associated Msb2 determines AMP protection. Thus, in addition to its sensor function Msb2 has a second activity because shedding of its glycofragment generates AMP quorum resistance.

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Introduction

Crosstalk between pathogens and the human host determines the outcome of microbial colonization and disease [1]. Pathogen-host communication occurs between cells and secreted proteins of both organisms. Surface structures of the important human fungal pathogen *Candida albicans* bind to dectin receptors on immune cells and trigger responses inhibiting fungal proliferation including the production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS) (for a review, see [2,3]. In addition, binding to immunoglobulins and complement factors by the fungal pathogen facilitate its phagocytosis and killing (for a review, see [4]). Conversely, *C. albicans* partially overcomes host defenses by secreting hydrolytic enzymes and proteins that block the complement system (for a review, see [4,5]). Furthermore, by switching its growth from a yeast to a hyphal growth form *C. albicans* is able to evade immune cells and to penetrate into host niches less accessible to the immune system.

Survival of fungal pathogens in the human host requires that their cell surfaces are intact. Defects in the cell wall of *C. albicans* that occur under immune attack or by treatment with antifungals are sensed and activate compensatory activities [6]. Reduced glucan content leads to the activation of the protein kinase C (PKC) pathway that includes the Mkc1 MAPK module, which activates the glucan synthase activity and stimulates the transcription of genes involved in glucan and chitin biosynthesis [7,8]. In addition, defective *N*- or *O*-glycosylation activates the Cek1 MAPK module and recent results indicate that *PMT* genes encoding protein-*O*-mannosyltransferases are downstream regulatory targets [9,10]. Sensing through this pathway is accomplished by the Msb2 and Sho1 cytoplasmic membrane proteins, which signal via the Cdc42 GTPase to Cek1. Intact *N*-glycosylation is detected by Msb2 and represses *PMT1* transcription, while defective *N*-glycosylation induces Cek1 phosphorylation and derepresses *PMT1* transcription [9,10]. In a different mode of regulation, defective Pmt1-type *O*-glycosylation is sensed by Msb2, activates Cek1 and induces *PMT2* and *PMT4* expression. Induction of *PMT2/PMT4* genes by inhibition of Pmt1 and damage of β1,3-glucan also requires Msb2 and Cek1 suggesting that cell wall damage is reported to Cek1 via Msb2 [10]. This function of Msb2 is supported by its associated partner membrane protein Sho1 [9]. Defects in either Mkc1 or Cek1 pathways lead to defective hypha formation on some semi-solid media, supersensitivity against antifungals and other stressors and reduce the virulence of *C. albicans* [9,11,12].

Msb2 is a type I membrane protein containing a single transmembrane region that separates a large extracellular from a small cytoplasmic domain; this structure is conserved in several fungal species [13–16]. Msb2 in the yeast *Saccharomyces cerevisiae* has been shown to be continuously cleaved by the Yps1 yapsin

Author Summary

Microbial pathogens are attacked by antimicrobial peptides (AMPs) produced by the human host. AMPs kill pathogens and recruit immune cells to the site of infection. In defense, the human fungal pathogen *Candida albicans* continuously cleaves and secretes a glycoprotein fragment of the surface protein Msb2, which protects against AMPs. The results suggest that shed Msb2 allows fungal colonies to persist and avoid inflammatory responses caused by AMPs. Msb2 shedding and its additional role in stabilizing the fungal cell wall may be considered as novel diagnostic tools and targets for antifungal action.

protease, releasing the extracellular domain into the growth medium [17]. This property, coupled with the high level of *N*- and *O*-glycosylation of the extracellular domain has led to the concept that fungal Msb2 proteins represent functional analogs of the mammalian MUC1/2 signaling mucins, which by proteolytic cleavage generate highly hydrated mucous glycoprotein layers around cells and at the same time confer transcriptional regulation by the cleaved cytoplasmic domain [18]. In fungi, intertwining of Msb2 hydrated glycostructures with cell wall components may be related to the sensing function of Msb2. Cleavage of the ScMsb2 cytoplasmic domain has not been reported and its presence may be required for Cdc42 binding, which is an essential upstream element of the Kss1 MAPK pathway [13]. Here we report that the glycosylated extracellular domain of *C. albicans* Msb2 is released into the growth medium in considerable amounts and we show that the shed protein has the function to protect against AMPs produced by the host. In humans, the most prominent AMPs exhibiting strong antimicrobial and immunostimulatory activities are the histatins, which are produced by salivary glands and secreted into saliva and the cathelicidins and defensins, which are produced by neutrophils and macrophages (for a review, see [19–21]). The human cathelicidin LL-37 occurs on mucosal surfaces at a concentration of 2–5 μg/ml but its concentration rises to 1.5 mg/ml in acute inflammation [22]. Histatin-5 and LL-37 are cationic AMPs that damage the cytoplasmic membranes of *C. albicans* [23–25] and histatin-5 also attacks intracellular targets [26]. The combined findings of this study suggest that shed Msb2 is a glycoprotein that effectively protects *C. albicans* against killing by AMPs LL-37 and histatin-5, allowing *C. albicans to* evade immune reactions and to allow its persistence as a commensal.

Results

Construction and activity of epitope-tagged Msb2

To immunologically detect Msb2 we constructed a strain producing a variant Msb2 protein carrying an HA-epitope within the large extracellular domain and in addition a V5-epitope in the middle of the short cytoplasmic domain (Figure 1 A). *MSB2* was expressed either under the control of the constitutive *ACT1* promoter when plasmid pES11a was integrated in the *LEU2* locus (strain ESCa3) or by the authentic *MSB2* promoter when pES11a was integrated in the partially deleted *msb2Δ1* allele of strain FCCa28 (strain ESCa10). The *msb2Δ1* allele encoding 406 N-terminal residues of Msb2 was found to be completely non-functional in all phenotypic assays (see below) and it was fully complemented in transformants containing pES11a integrated in both genomic loci; complementation efficiencies were equal between transformants carrying singly HA-tagged or doubly HA-V5-tagged Msb2 versions. Thus, while several *msb2Δ1* mutant strains were as supersensitive to caspofungin and tunicamycin as the *pmt4* control strain [10] complementation by the epitope-tagged versions of Msb2 restored normal resistance (Figure 1 B). While tunicamycin-supersensitivity indicates that *msb2Δ1* mutants require intact *N*-glycosylation for growth, *O*-mannosylation by Pmt1 appears not relevant since mutants grew normally in the presence of the Pmt1 inhibitor. The tagged versions of Msb2 were also fully active to reverse the hyphal growth defects of the *msb2Δ1* mutants [9] (Figure 1 C).

In addition, we constructed plasmid pES11c, which encodes the HA-tagged Msb2 variant carrying the V5 epitope at its C-terminal end (allele $MSB2^{\text{HA-V5 end}}$). The phenotypic results for pES11a- and pES11c-transformants were identical (data not shown).

Secretion and processing of Msb2

Release of a Msb2 subfragment into the growth medium has been observed in *S. cerevisiae* and other fungi [13–16]. When we examined cells and growth medium of *C. albicans* transformants producing tagged Msb2 by immunoblotting we discovered that the majority of HA-carrying Msb2 was present in the medium and migrated as a diffuse band of >460 kDa (Figure 2 A). No significant difference regarding the amount of immunoreactive protein was detected in strains either transcribing *MSB2* from the *ACT1* or *MSB2* promoters (compare lanes 3 and 5) suggesting that both promoters are of comparable strength. As expected, the tagged ER-membrane protein $Pmt1^{HA}$ was associated only with cells (lane 2). In contrast to HA immunodetection the V5-tagged Msb2 protein was found exclusively in association with cells and not in the medium, similar to the $Pmt2^{V5}$ control protein (Figure 2 B). The V5-tagged Msb2 protein migrated as a doublet of about 15 kDa and thus corresponded in size to the cytoplasmic domain of Msb2. Thus, it appears that during growth in liquid culture the Msb2 full-length protein is mostly cleaved proteolytically into the large extracellular (HA-tagged) and the small cytoplasmic (V5-tagged) subfragments. Importantly, release of the $Msb2^{HA}$ fragment was almost quantitative during growth in complex YPD growth medium and was not altered significantly in YEPG medium containing galactose as in *S. cerevisiae* [17] or during hypha formation in YP medium containing 10% serum (data not shown). The released extracellular fragment or Msb2 will now be referred to as Msb2*.

To examine if Msb2* secretion would also occur during growth on a semisolid agar surface we used a double sandwich system consisting of a PVDF membrane used for immunoblotting topped by a membrane filter precluding the passage of cells, which were both placed on YPD agar (Figure 2 C, a). Cells grew on the membrane filter (Figure 2 C, b) and immunoanalysis of the PVDF filter detected HA-proteins only released from cells producing Msb2* (Figure 2 C, c 3, 4) but not from cell producing tagged $Pmt1^{HA}$ protein. This result indicates that the extracellular Msb2 fragment is also detected in surface growth of *C. albicans*.

Considering the possibility that Msb2 is cleaved immediately upstream of the transmembrane region it was expected that Msb2* has an approximate molecular mass of 131 kDa but the heterogeneity and apparent molecular mass in immunoblotting (Figure 2 A) suggested extensive glycosylation. To estimate its molecular mass more accurately we carried out fractionation of culture fluid containing Msb2* by gel filtration, using a column previously calibrated with standard proteins (Figure 2 D, a, b). Fractions eluted from the column were examined by immunodetection and yielded a major peak from 468–614 kDa (Figure 2 D, c) in agreement with the above immunoblotting results. A minor peak in the void volume, presumably representing aggregated

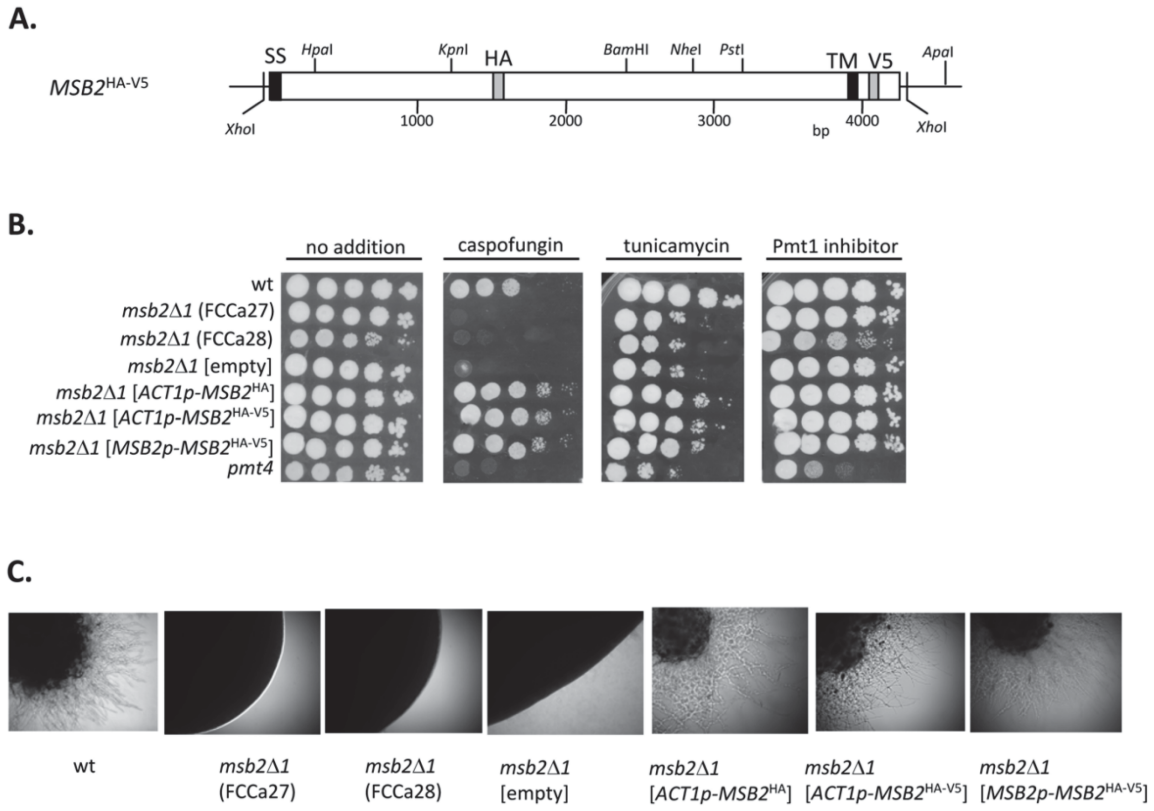

Figure 1. Structure and activity of epitope-tagged Msb2. A. Structure of *MSB2* alleles encoding Msb2 variants. The *MSB2* coding region with sequences encoding the signal sequence (SS), the transmembrane region (TM), HA- /V5-epitopes and relevant restriction sites used for the construction of *MSB2* variant alleles are shown. **B.** Tagged *MSB2* alleles confer inhibitor resistance. Sensitivity of strains to caspofungin (125 ng/ml), tunicamycin (2 μg/ml) and Pmt1 inhibitor (12 μM) was tested by a drop dilution test. **C.** Tagged *MSB2* alleles reconstitute formation of hyphae on YPM agar. Colonies of strains were photographed following growth for 2 d at 37°C. Strains CAF2-1 (wt), FCCa27 (*msb2Δ1 URA3*) and FCCa28 (*msb2Δ1 ura3*) were compared to FCCa28 transformants. Transformants contained empty plasmid pDS1044-1 (ESCa7; *msb2Δ1*[empty]), pES10 (ESCa8; *msb2Δ1*[*ACT1p-MSB2*^{HA}]) or pES11a (ESCa3; *msb2Δ1*[*ACT1p-MSB2*$^{HA-V5}$]) integrated in the *LEU2* locus. The tagged *MSB2* allele was also placed under transcriptional control of the authentic *MSB2* promoter by directing integration of *Hpa*I-cut pES11a into the *msb2Δ1* allele of FCCa28 (ESCa10; *msb2Δ1* [*MSB2p-MSB2*$^{HA-V5}$]). Strain CAP4-2164 (*pmt4*) was used as a supersensitive control strain [27].
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Msb2* of >1000 kDa, was also detected. Since this result suggested that glycosylation contributed equally to the mass of Msb2* as its protein content we attempted to clarify the type of protein glycosylation. Extensive treatment of the growth medium (and of purified Msb2*, see below) with PNGase F did not result in a significant alteration of its apparent molecular mass (data not shown), while β-elimination led to a mass reduction to about 300 kDa (Figure 2 E, a) indicating that Msb2* is significantly *O*- but not *N*-glycosylated. On the other hand, complete chemical deglycosylation by trifluoromethanesulfonic acid (TFMS) reduced the mass of Msb2* to about 117–130 kDa (Figure 2 E, b) consistent with the proteolytic cleavage of the Msb2 precursor protein immediately upstream of the transmembrane region (expected molecular mass of unmodified 1291 residue fragment is 130 kDa). It is yet unclear if the different deglycosylation results obtained for β-elimination and TFMS treatments is due to residual *O*-glycosylation not removable by β-elimination, by residual *N*-glycosylation, which is not removed by PNGase F or by yet unknown modifications. However, because clear evidence for *O*-glycosylation of secreted Msb2 was obtained we produced epitope-tagged Msb2 in *C. albicans* mutants lacking each of the 5 isoforms of protein-*O*-mannosyltransferases. Immunoanalysis of secreted Msb2* showed faster electrophoretic mobility in the *pmt1* mutant, while in the *pmt4*, *pmt5* and *pmt6* homozygous mutants no difference to the control strain was detected (Figure 2 E, c). We conclude that Pmt1 is at least partially involved in Msb2 *O*-glycosylation, although the contribution of Pmt2 (only testable in a heterozygous *PMT2/pmt2* strain since it is essential for growth [27]) cannot be excluded. Compensatory upregulation of other Pmt isoforms in a *pmt1* mutant [10,28] may also account for remaining Msb2 *O*-glycosylation, which showed a very broad mobility distribution corresponding to apparent molecular masses from 240–480 kDa.

It has been reported that in *S. cerevisiae* the yapsin-type protease Yps1 is responsible for cleavage and secretion of Msb2 [17]. In *C. albicans* the closest homolog to Yps1 is Sap9 (21.9% identity), while Sap10 is also structurally similar because it is GPI-anchored in the cytoplasmic membrane [29]. When we expressed the tagged *MSB2*$^{HA-V5}$ allele in the *sap9* mutant (ESCa33), the *sap10* mutant (ESCa34) or the *sap9 sap10* double mutant (ESCa35) we did not observe any difference in amounts and molecular masses of Msb2* (data not shown). We also observed normal secretion of Msb2 in a

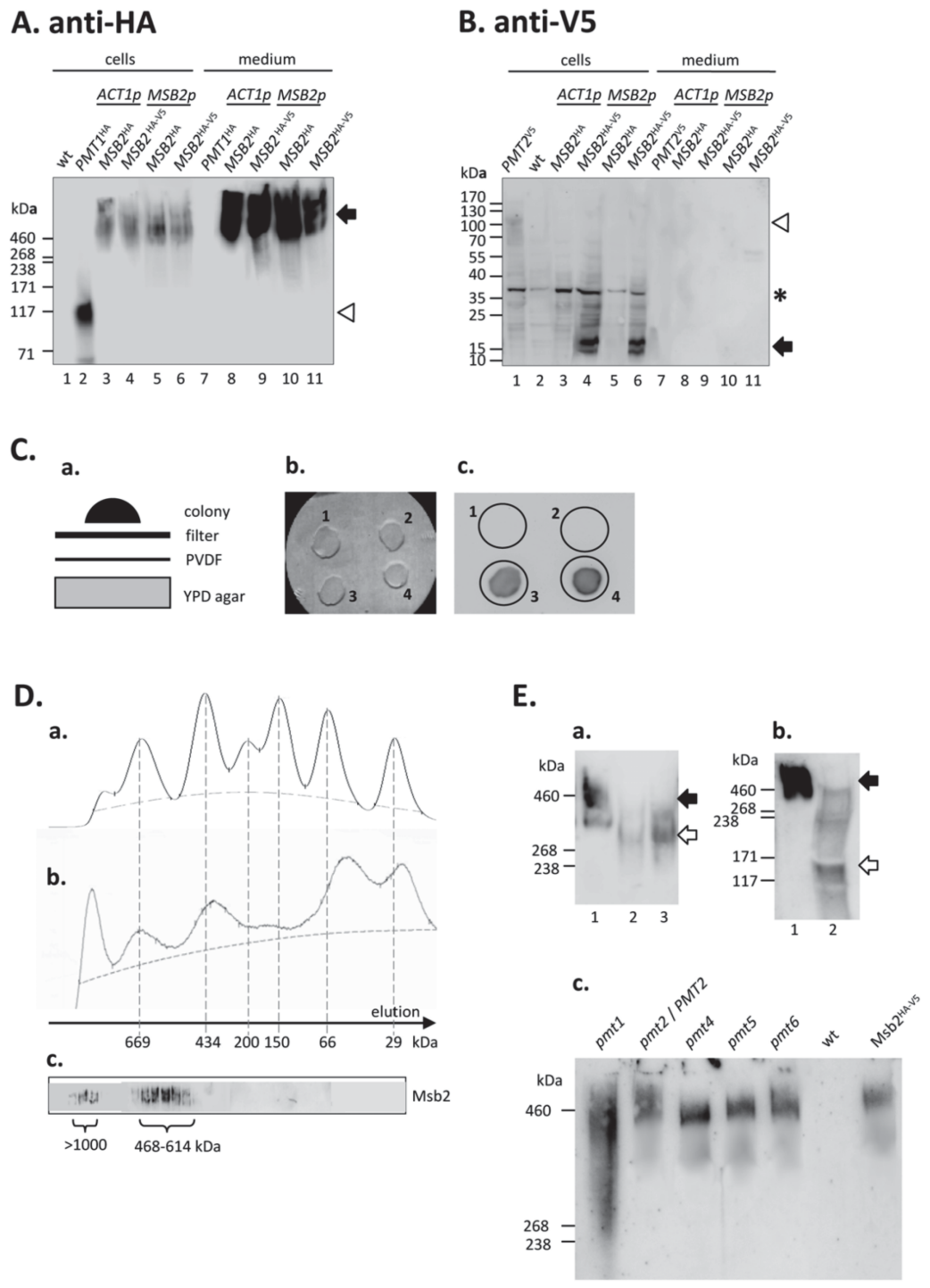

Figure 2. Secretion and processing of Msb2. *C. albicans* strains grown in YPD medium to $OD_{600} = 6$, centrifuged and cell extracts (50 µg protein derived from cells in 90 µl of medium) or medium (20 µl) were analyzed for epitope-tagged Msb2 protein. **A.** Immunoblot to detect HA-tagged Msb2. Proteins were separated by a 8% SDS-PAGE gel and immunoblots were reacted with rat anti-HA antibody. Strains tested included ESCa8 (*ACT1p-MSB2*^{HA}; lanes 3 and 8), ESCa3 (*ACT1p-MSB2*$^{HA\text{-}V5}$; lanes 4 and 9), ESCa9 (*MSB2p-MSB2*^{HA}; lanes 5 and 10) and ESCa10 (*MSB2p-MSB2*$^{HA\text{-}V5}$; lanes 6 and 11). Strains CAF2-1 (wt) and CIS23 (*PMT1*^{HA}) were used as negative and positive control strains, respectively. The migration of HA-tagged Msb2 and Pmt1 are indicated by the arrow and triangle, respectively. **B.** Immunoblot to detect V5-tagged Msb2. Proteins were separated by a 4–20% gradient

SDS-PAGE gel and immunoblots were reacted with mouse monoclonal anti-V5 antibody. Identical strains and fractions as in (A) were tested. The migration of V5-tagged Msb2 and Pmt2 (strain CIS29) are indicated by the arrow and the triangle, respectively; a protein cross-reacting with the anti-V5 antibody is marked by the asterisk. **C.** Secretion of HA-tagged Msb2 protein during growth on agar. Cell suspensions were dropped on a membrane filter (pore diameter 0.45 μm) situated on a PVDF membrane, which had been placed on YPD agar (a). Colonies were allowed to grow for 15 h at 30°C (b). The membrane filter was removed and the PVDF membrane was probed by immunoblotting using rat anti-HA antibody (c). Strains tested were (1) CAF2-1 (wild-type), (2) CIS23 (*PMT1*^{HA}), (3) ESCa3 (*ACT1p-MSB2*$^{HA\text{-}V5}$) and ESCa10 (*MSB2p-MSB2*$^{HA\text{-}V5}$). **D.** Gel filtration chromatography of secreted Msb2. A Superdex 200 10/300 GL column was (a) calibrated using standard proteins of the indicated sizes (dotted lines) and (b) used to fractionate 500 μl of the medium of strain ESCa3 (Msb2$^{HA\text{-}V5}$), which had been grown at 30°C in SD medium to $OD_{600} = 10$. The protein elution profiles were recorded by absorption at 280 nm. 200 μl fractions were collected and (c) tested by immunoblotting for the presence of HA-tagged Msb2. Fractions tested are placed at a position corresponding to the elution profile in b). **E.** Glycosylation of secreted Msb2. (a) Growth medium of strain ESCa3 (Msb2$^{HA\text{-}V5}$) was not treated (1) or treated with β-elimination reagent mixture over night (2,3); the sample in lane 3 was heated to 80°C before reagent addition in an attempt to increase deglycosylation. (b) The medium was not treated (1) or treated with TFMS (2). Samples were tested by immunoblotting as in A. The migration of glyosylated and deglycosylated Msb2* are indicated by the filled and open arrows, respectively. (c) Msb2 secreted by *pmt* mutants defective in protein-*O*-mannosyltransferases carrying carried pES11a (Msb2$^{HA\text{-}V5}$). Strains included ESca18 (*pmt1*), ESCa19 (*PMT2/pmt2*), ESCa20 (*pmt4*), ESCa21 (*pmt5*) and ESCa22 (*pmt6*) and were tested by immunblotting as in A.
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mutant (ESCa36) lacking the furin-type and Golgi-resident Kex2 serine endoproteinase, which in *S. cerevisiae* is required for cleavage and shedding of the Flo11 protein [30]. Furthermore, we repeatedly added high concentrations (15 μg/ml) of the aspartyl protease inhibitor pepstatin, of the metalloprotease inhibitor amastatin (15 μg/ml) or of a commercial mix of inhibitors for serine- and cysteine proteases (complete mini tablets; Roche) to growing cultures of ESCa3 but we did not find any effect on Msb2* release (data not shown). We conclude that the processing mechanism of Msb2 in *C. albicans* requires an as yet unidentified protease and that Sap9, Sap10 and Kex2 proteases are not involved.

Structure-function relationship of Msb2

We constructed several *C. albicans* strains producing deleted Msb2 variants under the control of the *ACT1* promoter in a *msb2* mutant background and tested Msb2-dependent phenotypes including secretion of Msb2, hypha formation and resistance to caspofungin; furthermore, the ability of variants to activate the Cek1 MAP kinase was examined. The results are summarized in Figure 3 A and presented in Figure 3 B and Figure S1.

Two major deletion variants either lacking 449 residues of the extracellular domain (Msb2-ΔN) or lacking the complete cytoplasmic tail of 103 residues (Msb2-ΔC) were fully able to complement all *msb2* mutant phenotypes. In contrast, strains only

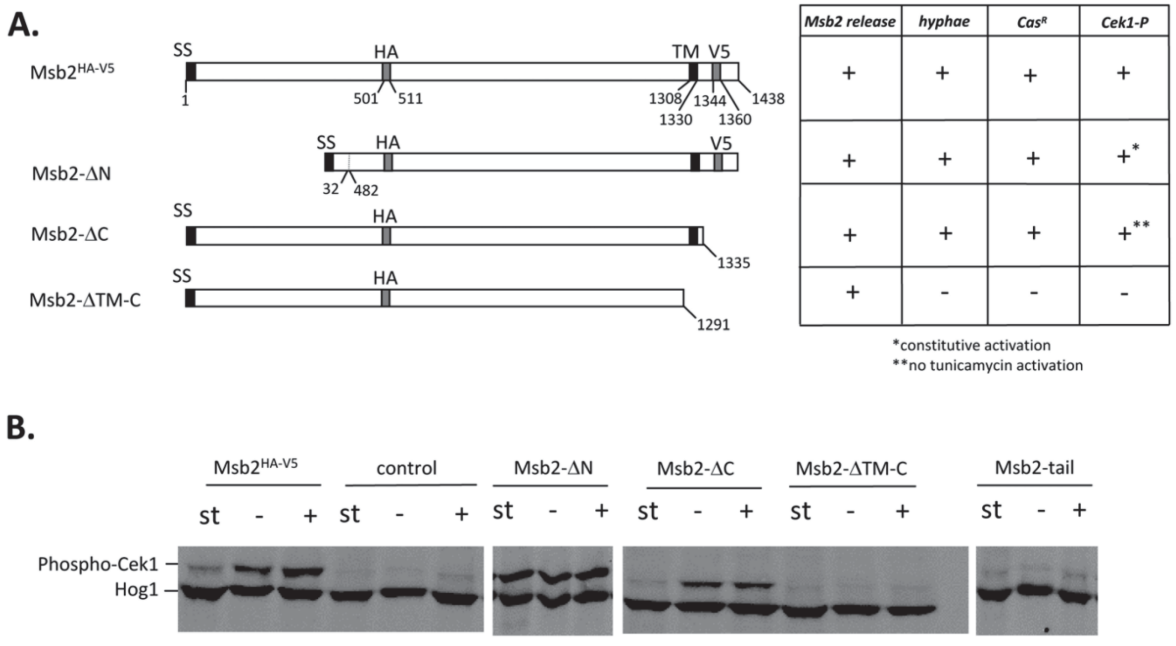
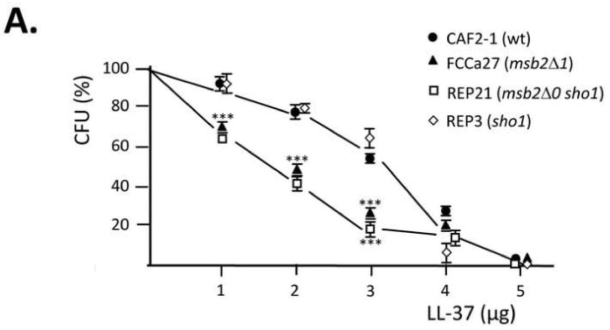
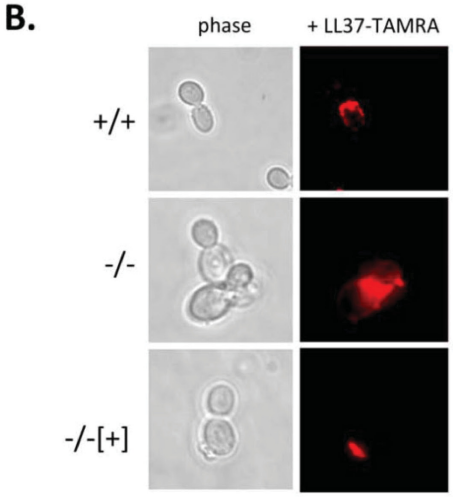
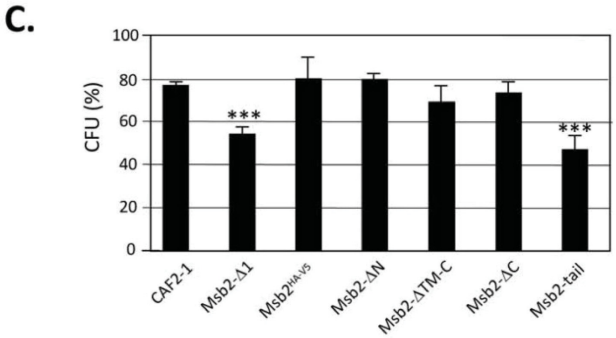


Figure 3. Activity of Msb2 variants. A. Structure of Msb2 protein variants. The positions of signal sequence (SS), transmembrane region (TM) and HA- and V5-epitope tags are indicated. Plasmids encoding variants were chromosomally integrated into strain FCCa28, which produces the inactive Msb2-Δ1 variant by the *msb2Δ1* allele. Resulting transformants (encoded variants) were strains ESCa3 (Msb2$^{HA\text{-}V5}$), ESCa25 (Msb2-ΔN), ESCa38 (Msb2-ΔC) and ESCa39 (Msb2-ΔTM-C). Corresponding phenotypes are summarized in the table and are presented in Figure S1. +, wild-type phenotype; −, *msb2* mutant phenotype with regard to Msb2* release, hypha formation, caspofungin resistance (Cas^R) and Cek1 phosphorylation (Cek1-P). **B.** Cek1 activation by strains producing variant Msb2 proteins. Cells were grown to stationary phase (st), diluted in fresh YPD medium, grown to $OD_{600} = 0.8$ at 37°C and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 μg/ml). Cells in stationary phase (st) and after 1 h incubation were harvested and assayed for the activation of MAPK Cek1 by immunoblottings; the Hog1 MAPK protein signal was used as the loading control. Strains as in A., in addition strains ESCa37 encoding the Msb2-tail variant and strain ESCa7 carrying an empty vector (control) were tested.
doi:10.1371/journal.ppat.1002501.g003


A.
CAF2-1 (wt)
FCCa27 (msb2Δ1)
REP21 (msb2Δ0 sho1)
REP3 (sho1)
CFU (%)
LL-37 (µg)


B.
phase
+ LL37-TAMRA
+/+
-/-
-/-[+]


C.
CFU (%)
CAF2-1
Msb2-Δ1
Msb2HA-V5
Msb2-ΔN
Msb2-ΔTM-C
Msb2-ΔC
Msb2-tail

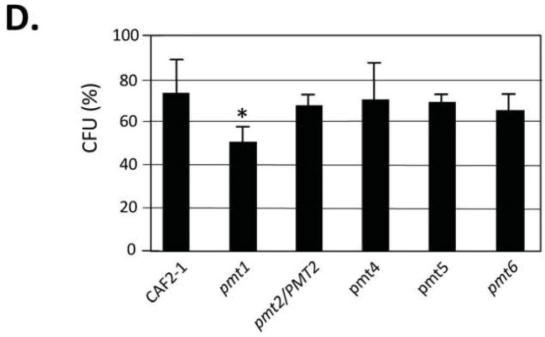

D.
CFU (%)
CAF2-1
pmt1
pmt2/PMT2
pmt4
pmt5
pmt6

Figure 4. Msb2 synthesis protects *C. albicans* against LL-37. A. Basal LL-37-resistance of *C. albicans* depends on Msb2. The indicated strains were incubated with different LL-37 amounts for 1.5 h at 37°C before plating of cells to determine colony-forming units (CFUs). Standard deviations of triplicate measurements are indicated; statistical differences of mutant versus control strain cfu values were evaluated by a t-test. **B.** Staining of *C. albicans* by TAMRA-labelled LL-37. 50 µl of cells were resuspended in PBS were incubated for 5 min with 5 µg LL-37-TAMRA before visualization using phase contrast and fluorescence microscopy. CAF2-1. +/+; FCCa27, −/−; ESCa3, −/−[+]. **C, D.** LL-37 sensitivity of *C. albicans* strains producing Msb2 variants (C) and of *pmt* mutants producing undeleted Msb2 (D). Transformants producing variant Msb2 proteins are described in Figures 2 and 3. Strain suspensions (5 µl) were co-incubated with 2 µg LL-37 for 1.5 h before CFU determination. Means and standard deviations of triplicate assays are shown. Statistical significance using a t-test is indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).
doi:10.1371/journal.ppat.1002501.g004

producing the N-terminal region of Msb2 up to the transmembrane region (variant Msb2-ΔTM-C) or solely the 108 cytoplasmic variant Msb2 tail residues were as defective for Msb2 phenotypes as mutants REP18 carrying a complete deletion of the *MSB2* ORF or strain FCCa27 only producing N-terminal residues 1–406 of Msb2 (Msb2-Δ1). Inactivity of the Msb2-ΔTM-C variant was not caused by lack of protein biosynthesis since amounts of Msb2* released into the medium were comparable for all HA-tagged variants (Figure S1). However, with regard to the activation of Cek1 a particular phenotype of these deletion variants was observed. The wild-type strain ESCa3 showed low levels of phosphorylation in stationary phase and phosphorylation was increased during logarithmic growth, which was stimulated further in the presence of tunicamycin [9] (Figure 3 B). In contrast, strain ESCa25 producing the Msb2-ΔN variant activated Cek1 not only in stationary phase but also in the absence of tunicamycin to high levels. In addition, strain ESCa38 carrying the Msb2-ΔC variant was impaired in its ability to activate Cek1 in response to tunicamycin. Strains producing the Msb2-ΔTM-C and the Msb2-tail were completely unable to activate Cek1 phosphorylation. Thus, it appears that the Msb2 N-terminal, transmembrane and cytoplasmic domains region convey different functions in Cek1 phosphorylation.

C. albicans msb2 are supersensitive to LL-37

C. albicans ESCa3 expressing *ACT1p-MSB2*$^{HA\text{-}V5}$ released considerable amounts of the Msb2* glycoprotein into the complex YPD growth medium, amounting to 76 µg/ml and 150 µg/ml in logarithmic growth ($OD_{600} = 1$) and in stationary phase ($OD_{600} = 6$). Msb2* was quantitated immunologically by a dot-blot procedure, because its high glycosylation status prevented quantitation by standard methods. We considered that this glycoprotein could contribute to defense against immunological responses of the human host, in particular to the attack by AMPs [20]. To verify this concept we first tested if the presence of Msb2 would contribute to basal levels of AMP resistance of *C. albicans*. Wild-type strains were significantly more LL-37-resistant than *msb2* mutants (Figure 4 A). Sensitivity of a *msb2 sho1* double mutant was only slightly increased compared to a *msb2Δ1* single mutant and a *sho1* single mutant showed wild-type resistance indicating that Msb2 but not Sho1 mediates LL-37 resistance. The increased LL-37 sensitivity of *msb2* mutant strains versus a wild-type strain was also correlated with increased fluorescent staining of mutant cells [26,27] by TAMRA-labelled LL-37 (Figure 4 B). We also observed that in the presence of LL-37 the *msb2* mutant tended to aggregate more readily than wild-type cells [31].

We next tested the LL-37 sensitivity of the above series of transformants producing truncated Msb2 variants. Interestingly, while the transformant only synthesizing the C-terminal tail of Msb2 was as sensitive as the *msb2Δ1* mutant all other transformants showed wild-type sensitivity (Figure 4 C). Even the transformant producing Msb2 deleted for its transmembrane region and C-tail was not supersensitive, although as described above this Msb2 variant was inactive in complementing *msb2* mutant phenotypes (Figure 3). It was concluded that the basal resistance of *C. albicans* to LL-37 depended on the secreted extracellular domain of Msb2 but its N-terminal domain was not required for this action. Since full-length and N-terminally deleted Msb2* are *O*-glycosylated to a large part by Pmt1 (Figure 2) transformants were constructed producing doubly tagged Msb2 in *C. albicans* strains defective in each of the 5 Pmt proteins (a heterozygous strain was used in case of *PMT2* because of its essentiality for growth). Among these transformants only the *pmt1* mutant was LL-37 supersensitive supporting the notion that Pmt1-directed *O*-glycosylation of Msb2* is required to provide resistance to LL-37.

In conclusion, these results suggest that the secreted extracellular Msb2* domain is required for LL-37 basal resistance of *C. albicans*.

Secreted Msb2 protects against AMPs

Several mechanisms are possible to explain the requirements of Msb2 (and Sho1) for LL-37 resistance and one mechanism is inactivation of LL-37 by the secreted Msb2*. To verify this concept we first purified Msb2* fragment from the growth medium by affinity chromatography using anti-HA antibody and verified that the purified material consisted solely of the heterogeneous >460 kDa protein by silver staining and immunoblotting (Figure 5 A). Next we asked if the purified Msb2* would proteolytically attack cathelicidin LL-37. Msb2* and AMPs were co-incubated and then assayed AMPs on a 18% SDS-PAGE gel (which excludes Msb2*). Msb2* co-incubation did not diminish amounts of LL-37 and no degradation products were observed (Figure 5 B) even if a 22.5% SDS-PAGE gel was used (data not shown). Furthermore, long term incubations (16 h) of Msb2* preparations with substrates of a protease detection kit able to detect a wide variety of protease did not detect any protease activity (data not shown). Therefore, it was concluded that Msb2* preparations had no general proteolytic activity. In additional pre-tests we bound Msb2* (or Msb2-ΔN*) to wells of microtiter dishes and checked if TAMRA-labelled LL-37 would absorb to these wells. Msb2* coating did indeed stimulate binding of LL-37-TAMRA significantly, while preincubation with unlabelled LL-37 reduced subsequent binding (Figure 5 C). This result indicates that LL-37 has a specific binding site on Msb2*.

To test a potential function of Msb2* in AMP protection we set up an AMP activity assay, in which we treated *C. albicans* for 1.5 h with AMPs in the absence or presence of purified Msb2* and then assessed fungal viability by determination of colony-forming units (CFU). The results show that added Msb2* rescued *C. albicans* from LL-37 killing, which was obvious for the wild-type strain and even more significant for *msb2* and *msb2 sho1* mutants; even an *E.coli* strain was protected against LL-37 by Msb2* (Figure 5 D). Interestingly, even the shortened Msb2*-ΔN fragment secreted and purified from strain ESCa25 was able to provide protection, although a concentration dependence of its activity revealed that it is slightly less active in AMP inactivation compared to the full-length Msb2* protein (Figure 5 E). AMP inactivating activity was

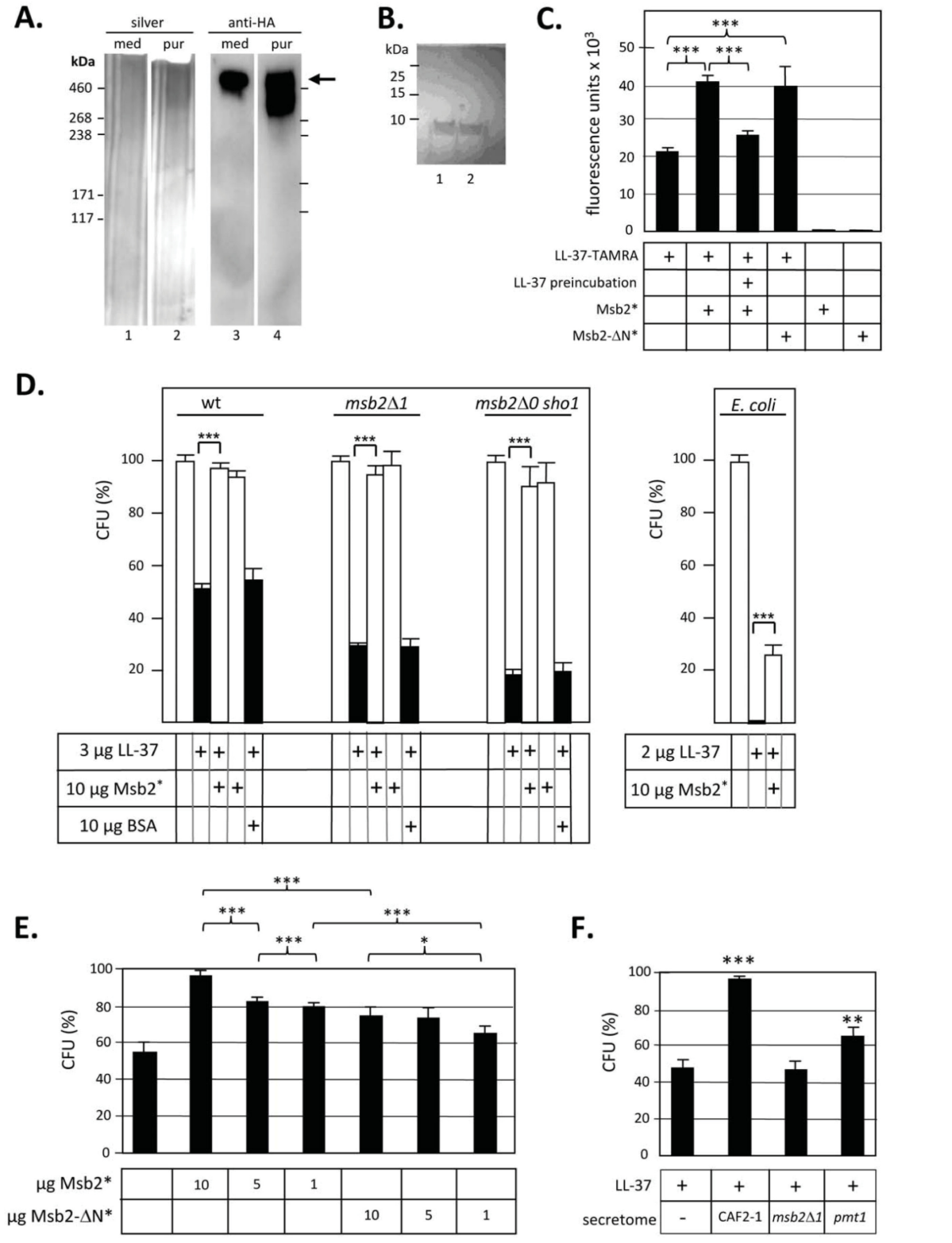

A.
silver
anti-HA
med
pur
med
pur
kDa
460
268
238
171
117
1
2
3
4
B.
kDa
25
15
10
1
2
C.
fluorescence units x 10³
50
40
30
20
10
0
***
***
***
LL-37-TAMRA
LL-37 preincubation
Msb2*
Msb2-ΔN*
D.
wt
msb2Δ1
msb2Δ0 sho1
CFU (%)
100
80
60
40
20
***
3 µg LL-37
10 µg Msb2*
10 µg BSA
E. coli
2 µg LL-37
10 µg Msb2*
E.
***
***
***
***
*
µg Msb2*
µg Msb2-ΔN*
10
5
1
F.
***
**
LL-37
secretome
-
CAF2-1
msb2Δ1
pmt1

Figure 5. Msb2*-mediates protection of *C. albicans* and *E. coli* against LL-37. A. Purification of Msb2*. Msb2* in culture medium of strain ESCa3 (lanes 1, 3) was affinity-purified using an anti-HA column (lanes 2, 4) and samples were separated by SDS-PAGE (4–20% acrylamide gel). For silver staining 50 µl of medium/purified (med/pur) fractions (lanes 1, 2) and for immunoblotting using an anti-HA antibody 15 µl of medium/purified fractions (lanes 3, 4) was analyzed. **B.** Msb2 does not degrade LL-37. 3 µg of LL-37 were co-incubated without (lane 1) or with 10 µg of Msb2* (lane 2) for 1.5 h at 37°C. Samples were separated on a 18% SDS-PAGE gel; the migration of standard proteins is indicated. **C.** Immobilized Msb2* binds LL-37. 10 µg Msb2* or Msb2-ΔN* were allowed to attach to each well of polystyrene microtiter plates over night at 4°C. Wells were washed with PBST and unspecific binding sites were removed by incubation with skim milk solution. 5 µl (5 µg) of TAMRA-labelled LL-37 was allowed to bind for 1 h, wells were washed and TAMRA emission was recorded at 590 nm. As a control, coated wells were incubated first with 3 µg of unlabelled LL-37 for 1 h before addition of LL-37-TAMRA. **D.** Msb2*-mediated protection of *C. albicans* and *E. coli* against LL-37. Strain suspensions (5 µl) were co-incubated with LL-37 in the absence or presence of 10 µg Msb2* or 10 µg of its deleted variant Msb2*-ΔN for 1.5 h before determination of viable cell counts (CFUs). As a control, 10 µg BSA was used to replace Msb2*. *C. albicans* strains CAF2-1 (wt), FCCa27 (*msb2Δ1*), REP21 (*msb2Δ0 sho1*) and *E. coli* DH5αF′ were tested; means and standard deviations of triplicate assays are shown. **E.** Msb2* concentration dependence. *C. albicans* strain CAF2-1 was incubated with 3 µg LL-37 in the absence and presence of the indicated amounts of purified Msb2* and the deleted Msb2*-ΔN variant. In addition, the activity of HA peptides used for affinity purification of Msb2* was tested. **F.** Protection of *C. albicans* against LL-37 by medium proteins (secretome). 3 µg LL-37 was added to cells of strain CAF2-1 (5 µl; OD_{600} = 0.3) in the absence or presence of culture medium (17 µl) of *C. albicans* strains grown to stationary phase. Following incubation for 1.5 h at 37°C cell viability (CFU) was tested. Secretome of control strain CAF2-1, FCCa27 (*msb2Δ1*) and SPCa2 (*pmt1*) was used. Means and standard deviations of triplicate assays are shown. Statistical significance was evaluated using a t-test (*, $p<0.05$; **, $p<0.01$; ***; $p<0.001$).
doi:10.1371/journal.ppat.1002501.g005

also detected by merely using medium (secretome) of a *C. albicans* wild-type strain (CAF2-1) for co-incubation with LL-37 (Figure 5 F). As expected, medium of the *msb2Δ1* strain (FCCa27) had no protective effect, while medium of the *pmt1* mutant (SPCa2) had reduced inactivating activity.

These findings demonstrate that the extracellular Msb2 domain has an additional function in *C. albicans* biology, e. g. in LL-37 defense, which is different from its roles in cell wall integrity and filamentation.

Secreted Msb2 protects *C. albicans* against histatin-5

C. albicans is known to be sensitive to low levels of histatin-5 [23–26,32,33]. We considered the possibility that higher Msb2* levels occurring in the vicinity of *C. albicans* colonies in the human host could protect against histatin-5 as we had found for LL-37. Although we did not observe a significant higher sensitivity to histatin-5 in *msb2* mutants (as for LL-37) we found that added purified Msb2* did indeed protect *C. albicans* strains significantly against histatin-5 (Figure 6). As expected, HA peptide used for elution of Msb2* from the anti-HA antibody in affinity chromatography did not provide protection. The protective action of Msb2* was not restricted to *C. albicans* because even an *E. coli* strain was rescued from histatin-5 killing (Figure 6). Thus, we conclude that protection by the secreted Msb2 glycofragment is not specific for LL-37 but extends to other AMPs including histatin-5 and affects microorganisms other than *C. albicans*.

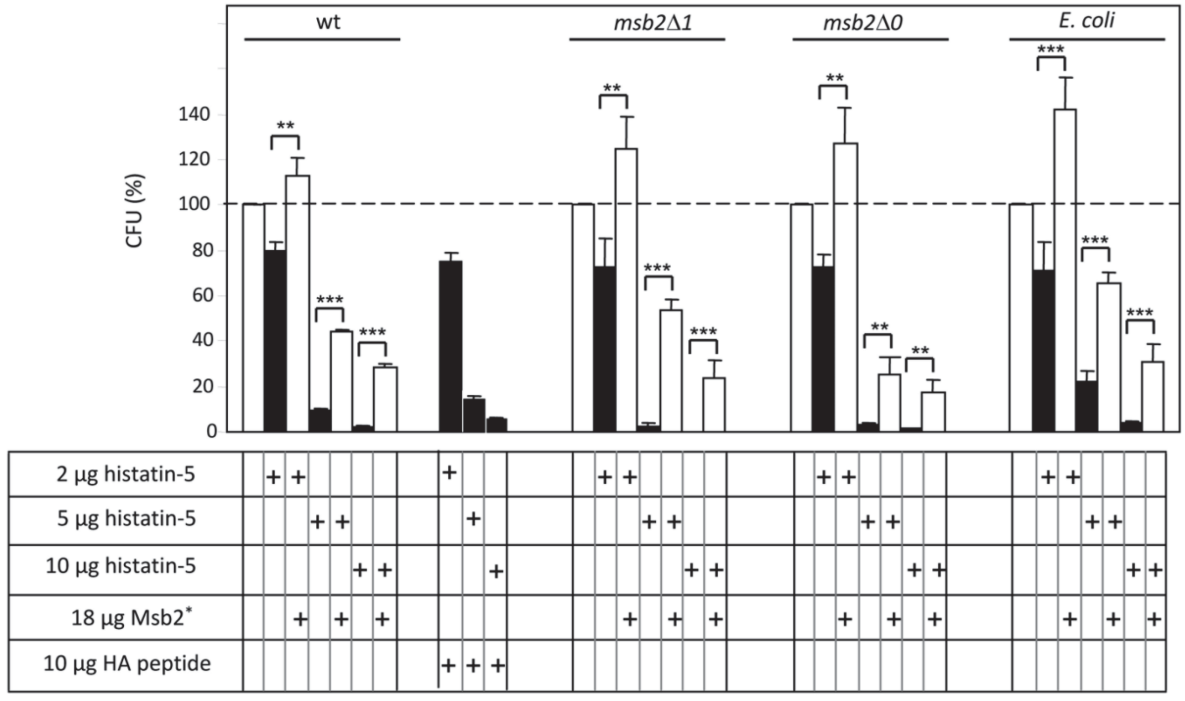


Figure 6. Msb2*-mediates protection of *C. albicans* and *E. coli* against histatin-5. *C. albicans* strains CAF2-1 (wt), FCCa27 (*msb2Δ1*), REP18 (*msb2Δ0*) and *E. coli* DH5αF′ were allowed to react with the indicated amounts of histatin-5 for 1.5 h at 37°C, in the absence or presence of the affinity-purified secreted Msb2* protein. Colony-forming units were determined on YPD (*C. albicans* strains) or on LB medium (*E. coli*).
doi:10.1371/journal.ppat.1002501.g006

Discussion

A complex interplay of responses and counter-responses characterizes the encounter of microbial pathogens with the human host. Opportunistic pathogens including *C. albicans* may be commensals, held in check by the immune system and supported by actions of the pathogen that favour a commensal life-style [1,34]. Conversely, immunological impairment or other conditions can favour propagation of pathogens and result in disease through microbial virulence traits and/or immune hyperstimulation causing autoimmune damage [35] Immune cells detect surface structures of *C. albicans* including glucan and mannoproteins and trigger IL-17-dependent reactions [2,3] including the production of AMPs, which kill the pathogen and attract immune cells [19,20]. The *C. albicans* protein Msb2 has a dual function to stabilize the fungal cell wall and we show here that it is also required to block an important aspect of the immune response by inactivating AMPs (Figure 7).

Fungal pathogens have a relatively high ability to resist attack by hydrolytic enzymes or small toxic molecules including antifungals in the human host. Cell wall damage is restored or compensated for by signaling pathways that sense the defect and initiate appropriate rescue responses [6]. In *C. albicans* defects in glucan or chitin are sensed especially by pathways containing the Mkc1 or Hog1 MAP kinases that trigger enhanced glucan or chitin biosynthesis [7,36]. Defects in protein glycosylation are transmitted mainly via the Cek1 MAP kinase pathway and lead to activation of individual isoforms of protein-*O*-mannosyltransferases [9,10]. Blockage of *N*-glycosylation by tunicamycin depends on Cek1 and upregulates *PMT1* transcription, while inhibition of Pmt1-*O*-glycosylation stimulates transcription of *PMT2* and *PMT4* genes. Interestingly, we found that the Msb2 membrane sensor protein functioning at the head of the Cek1 pathway is itself a highly glycosylated protein as in other fungal species. Despite the presence of 5 potential acceptor sites no evidence for *N*-glycosylation of Msb2 was obtained but the secreted Msb2 migrated faster in a *pmt1* mutant (not in other homozygous *pmt* mutants) indicating that Pmt1 is partially responsible for Msb2 *O*-mannosylation. Residual *O*-chains in a *pmt1* strain were removed by chemical treatment suggesting that they are contributed by the Pmt2 isoform, which is essential for growth [27]. Lack of Pmt1 glycosylation was previously shown to increase phosphorylation of Cek1 and to activate *PMT2/4* transcription [9,10] and we add here that lack of the N-terminal Msb2 glycodomain leads to constitutive Cek1 phosphorylation. Conceptually, lack of Msb2 *O*-glycosylation could trigger Cek1 phosphorylation but other *O*-glycosylated proteins interacting with Msb2 could also provide the triggering signal. Signaling by proteins interacting with Msb2 is suggested by the finding that tunicamycin-treatment induces Cek1 phosphorylation, although Msb2 does not appear to be *N*-glycosylated itself. In *S. cerevisiae*, however, Msb2 is *N*-glycosylated and *O*-mannosylated by the Pmt1, 2 and 4 isoforms; furthermore, activation of the Cek1 homolog Kss1 occurred only in cells lacking Pmt4 and inhibited for *N*-glycosylation by tunicamycin [37,38]. Thus, Msb2 glycosylation and resulting MAP kinase activation proceed differently in *C. albicans* and *S. cerevisiae*.

The single transmembrane region of Msb2 divides the protein in a large glycosylated extracellular and a small cytoplasmic domain in *C. albicans*, *S. cerevisiae* and other fungi. A *S. cerevisiae* Msb2-GFP fusion has been shown to get efficiently cleaved leading to release of the extracellular domain into the medium [17]. This processing occurs at a yet undefined site and requires the Yps1 yapsin-type protease suggesting that it is directly or indirectly involved in the cleavage. Similarly, using doubly epitope-tagged Msb2 we found that in *C. albicans* Msb2 is cleaved almost quantitatively, which sheds the extracellular domain into the medium and retains the cytoplasmic domain in the cells. However, in *C. albicans* the closest homologs of ScYps1, Sap9, Sap10 [29], and serine endoproteinase Kex2 [30] were not required for CaMsb2 processing. Cleavage/release was found to occur both in liquid and on surfaces and the amount of secreted Msb2 depended on the number of growing *C. albicans* cells. Thus, importantly, the level of released Msb2 is a measure of *C. albicans* propagation. In agreement, Msb2 peptides were recently identified in the secretome of *C. albicans* yeast and hyphal cultures; peptides corresponded to the extracellular domain including residue 1290 upstream of the transmembrane region [39].

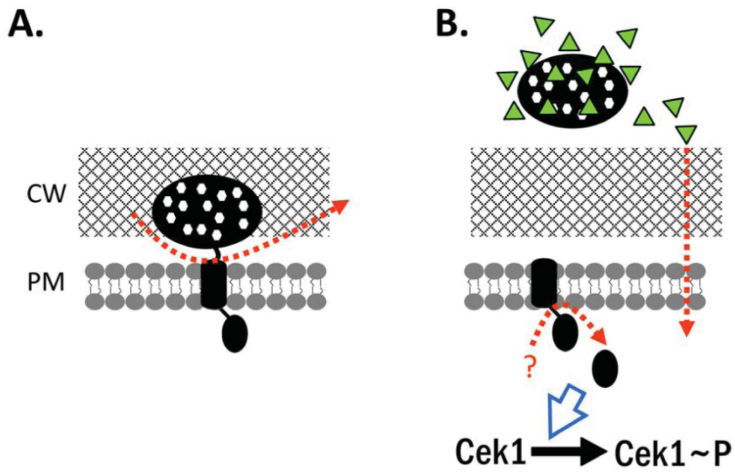


Figure 7. Model for Msb2 functions. The dual function of *C. albicans* Msb2 protein is shown. The precursor protein (A) is cleaved during growth and the extracellular domain, which is highly O-glycosylated (indicated by white dots), is shed into the medium (B). Msb2 has an intracellular function in activating the Cek1 MAP kinase and the secreted exodomain is able to protect cells against AMPs (triangles).
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The relationship between Msb2 structure, processing/secretion and Cek1 phosphorylation was studied using *C. albicans* strains producing Msb2 variants. A large deletion of 450 N-terminal residues adjacent to the signal sequence (Msb2-ΔN) led to functional Msb2 able to complement defects of the *msb2* mutant; this variant differed from the native protein, however, in that the Cek1 MAP kinase was constitutively phosphorylated. In agreement, *S. cerevisiae* Msb2 deletions of the extracellular domain have been found to hyperactivate the dedicated MAP kinase Kss1 [17]. Different phenotypes were obtained for C-terminal deletions of *C. albicans* Msb2. While a Msb2 variant deleted for its C-terminal end and the transmembrane region (Msb2-ΔTM-C) was completely inactive, a deletion retaining the transmembrane region (Msb2-ΔC) was fully functional in complementing *msb2* phenotypes. Unexpectedly, however, the latter variant did not respond to tunicamycin-treatment by induction of Cek1 phosphorylation, in agreement with results obtained for a similar *S. cerevisiae* Msb2 variant [38]. We conclude that the transmembrane region of Msb2 is absolutely required for Msb2 functions and furthermore, that tunicamycin-regulated signaling to the Cek1 MAP kinase requires the cytoplasmic domain. Conceivably, the cytoplasmic domain could be directly involved in regulation of Cek1 kinase activity or it could participate in gene regulation as has been reported for signaling mucins and the Notch protein in higher eukaryotes [18,40].

In the human host *C. albicans* contacts surfaces of body cells including immune cells, which may phagocytose the pathogen and elicit a wave of antifungal activities. Resident or induced soluble defense molecules such as immunoglobulins, complement factors and AMPs kill or block the growth of the pathogen. AMPs have a wide range of antiviral, antibacterial and antifungal activities and provide an antimicrobial barrier on mucosal surfaces such as histatins produced and secreted by salivary glands or they are components of the antimicrobial armory of neutrophils that produce cathelicidins (LL-37) and defensins [20]. Furthermore, AMPs act as chemoattractants recruiting leukocytes to sites of infection [19,21]. *C. albicans* is known to be sensitive to histatins, LL-37 and defensins, which inhibit fungal growth by cytoplasmic membrane disruption, interference with mitochondrial activity or yet undefined mechanisms [23–26]. Furthermore, binding of LL-37 or histatins to cell wall carbohydrates prevents adhesion of *C. albicans* to host cells and plastic surfaces [31]. It should be noted also that bacterially-produced AMPs such as the lantibiotic nisin secreted by *Lactobacillus lactis* contribute to the diversity and high concentration of AMPs in the human body [41]. Nevertheless, a myriad of microbial commensals including some opportunistic pathogens persist as cohabitants because they are at least partially AMP-resistant. Several AMP-resistance mechanisms have been reported. Cleavage of AMPs by soluble or membrane-bound proteases has been described for many bacterial species and it has been shown that *C. albicans* is also able to cleave histatin-5 by the yapsin-type protease Sap9 [42,43]. Another evasion mechanism known in bacteria is the secretion of AMP-binding proteins that act as decoys deflecting AMPs from their dedicated action at microbial cell surfaces. Examples include the secreted SIC, staphylokinase and FAF proteins by *Streptococcus pyogenes*, *Staphylococcus aureus* and the commensal *Finegoldia magna*, respectively [44–46]. Here we describe that an analogous mechanism is relevant also for fungal pathogens since shedding of a large glycosylated fragment of the Msb2 sensor protein renders *C. albicans* AMP-resistant. Msb2 shedding reached high levels during liquid growth (about 150 μg/ml in stationary phase) and was also observed during surface growth. Purified Msb2 fragment effectively blocked the fungicidal activity of histatin-5 and LL-37 even at a >20 fold molar excess of AMPs suggesting multiple binding sites. Interestingly, a *C. albicans msb2* mutant was supersensitive to LL-37 but not to histatin-5 suggesting that the relatively small amount of cell-associated Msb2 suffices to protect against LL-37 but not against histatin-5. This finding agrees with the recent finding that LL-37 but not histatin-5 binds to *C. albicans* cell-wall carbohydrates [31]. The underlying molecular mechanisms for AMP binding to Msb2* remain to be determined. We found that the Pmt1-type of *O*-mannosylation is partially required for Msb2 glycosylation, its binding to LL-37 and for LL-37 resistance of wild-type cells, which raises the question if the glycostructures of Msb2* directly or indirectly affect LL-37 binding. Previous work has established the binding of LL-37 to various glycostructures including bacterial lipopolysaccaride [47], bacterial exopolysaccharides [48], human glycosaminoglycans [49] and fungal cell-wall polysaccharides [31]. These glycostructures may provide anionic contact sites for cationic AMPs such as LL-37 and histatin-5, which are enriched for basic amino acids (net charge +6 and, respectively, +12 at physiological pH). Since *O*-mannosyl side chains of Msb2* do not add net charge (unless they carry as yet undefined modifications) they do not allow ionic interactions with cationic AMPs, although non-ionic interactions cannot be excluded. Possibly, the functional role of *O*-mannosylation is indirect by providing an extended, bottle-brush conformation of the protein, as it is often observed in highly *O*-glycosylated protein domains [50]; this conformation could help to expose carboxylate side groups of aspartate and glutamate residues in Msb2* that could interact with basic residues of AMPs. Other *C. albicans* components including members of the Hog1 MAP kinase pathway are also involved in basal AMP resistance [51]; since Msb2 is not an upstream element in the Hog1 pathway of *C. albicans* [52] it probably regulates AMP resistance independently of Hog1. In a process that is analogous to functions of Msb2, the Pra1 protein of *C. albicans* is partially shed and impairs immune responses, in this case by binding of human factor H in solution leading to downregulation of the complement system in the vicinity of fungal cells [53].

We reported previously that in the standard mouse model of systemic infection (tail vein injection) no significant attenuation of virulence was detected for a *msb2* mutant [9]. However, the systemic infection model may not appropriately reflect growth of *C. albicans* in the form of biofilms or foci of infection within organs, which are expected to be surrounded by a diffusion cloud of shed Msb2 at high levels that cause quorum resistance depending on fungal cell numbers. Shedding of Msb2 may also be important for *C. albicans* commensal growth, e. g. survival in the gut, where it is confronted with AMPs of other microbial commensals such as nisin produced by *Lactobacillus* [41]. On the other hand, shed Msb2 is able to provide cross-protection for other species as we have shown for protection of *E. coli* against LL-37 and histatin-5. Therefore, we propose that novel models for virulence and commensalism are needed to test the biological relevance of Msb2 and its shedding. Shed Msb2 may be of diagnostic value since its levels reflect fungal growth in the human host. Shed Msb2 is highly soluble and proteolytically stable because of its extensive glycosyl modifications and its presence in body fluids may be indicative of hidden localized fungal infections.

Materials and Methods

Strains and media

C. albicans strains are listed in Table 1. In *C. albicans* strain REP18 the *MSB2* ORF of both alleles is completely removed [9]; this *msb2* mutant allele is referred to as *msb2*Δ*0*. Strain FCCa27/28 contains partially deleted alleles designated *msb2*Δ*1* (encoding the

Table 1. *C. albicans* strains.

Strain	Genotype	Reference/Source
CAF2-1	*ura3Δ::imm434/URA3*	[55]
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	[55]
FCCa27	as CAI4 but *msb2Δ1::hisG/msb2Δ1::hisG-URA3-his*G	this study
FCCa28	as CAI4 but *msb2Δ1::hisG/msb2Δ1::hisG*	this study
REP3	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG sho1::FRT/sho1::FRT*	[9]
REP18	*ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG msb2Δ0::FRT/msb2Δ0::FRT*	[9]
REP21	*ura3Δ::imm434/ura3Δ:imm434 his1Δ::hisG/his1Δ::hisG sho1::hisG/sho1::hisG-URA3-hisG msb2Δ0::FRT/ msb2Δ0::FRT*	[9]
CAP1-3121	as CAI4 but *pmt1Δ::hisG/pmt1Δ::hisG*	[27]
SPCa2	as CAP1-3121, but *ura3Δ::imm434/ URA3*	[27]
P2-22	as CAI4 but *PMT2/pmt2Δ::hisG*	[27]
CAP4-2161	as CAI4 but *pmt4Δ::hisG/pmt4Δ::hisG*	[27]
P5-5711	as CAI4 but *pmt5Δ::hisG /pmt5Δ::hisG*	[27]
CAP2-2311	as CAI4 but *pmt6Δ::hisG/pmt6Δ::hisG*	[27]
CNA4	as CAI4 but *kex2::hisG/kex2::hisG*	[56]
Δsap9	as CAI4 but *sap9::hisG/sap9::hisG*	[29]
Δsap10	as CAI4 but *sap10::hisG/sap10::hisG*	[29]
Δsap9 Δsap10	as CAI4 but *sap9::hisG/sap9::hisG sap10::hisG/sap10::hisG*	[29]
CIS23	as CAI4 but *PMT1/PMT1^{HA}::SAT1*	Schmidt and Ernst, unpublished
CIS29	as CAI4 but *PMT2/PMT2^{V5}::URA3*	Schmidt and Ernst, unpublished
ESCa3 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa5 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES11c (*ACT1p-MSB2$^{HA\text{-}V5\ end}$*)	this study
ESCa7 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pDS1044-2 (*ACT1p*)	this study
ESCa8 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES10 (*ACT1p-MSB2^{HA}*)	this study
ESCa9 (−1,2,3)	as FCCa28 but *msb2Δ1/msb2Δ1*::pES10 (*MSB2p-MSB2^{HA}*)	this study
ESCa10 (−1,2,3)	as FCCa28 but *msb2Δ1/msb2Δ1*::pES11a (*MSB2p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa11 (−1,2,3)	as FCCa28 but *msb2Δ1/msb2Δ1*::pES11c (*MSB2p-MSB2$^{HA\text{-}V5\ end}$*)	this study
ESCa18 (−1,2,3)	as CAP1-3121 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa19 (−1,2,3)	as P2-22 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa20 (−1,2,3)	as CAP4-2164 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa21 (−1,2,3)	as P5-5744 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa22 (−1,2,3)	as CAP2-2311 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa25 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa26 (−1,2,3)	as CAP1-3121 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa27 (−1,2,3)	as P2-22 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa28 (−1,2,3)	as CAP4-2164 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa29 (−1,2,3)	as P5-5744 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa30 (−1,2,3)	as CAP2-2311 but *LEU2/LEU2*::pES14 (*ACT1p-MSB2-ΔN$^{HA\text{-}V5}$*)	this study
ESCa33 (−1,2,3)	as Δsap9 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa34 (−1,2,3)	as Δsap10 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa35 (−1,2,3)	as Δsap9 Δsap10 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa36 (−1,2,3)	as CNA4 but *LEU2/LEU2*::pES11a (*ACT1p-MSB2$^{HA\text{-}V5}$*)	this study
ESCa37 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES15 (*PCK1p-MSB2-tail*)	this study
ESCa38 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES16 (*ACT1p-MSB2-ΔC^{HA}*)	this study
ESCa39 (−1,2,3)	as FCCa28 but *LEU2/LEU2*::pES17 (*ACT1p-MSB2-ΔTM-C^{HA}*)	this study

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406 N-terminal residues of Msb2), which were constructed using the URA-blaster method. A 3.8 kb genomic fragment encompassing *MSB2* was PCR-amplified using primers IPF6003-*Not*I and IPF6003-*Sac*II and cloned into pUK21 (*Not*I, *Sac*II). The large *Bam*HI-*Kpn*I fragment of the resulting plasmid was ligated to the *hisG-URA3-hisG* blaster cassette of p5921 to generate pUK-

6003.ko.Urab. The *Not*I-*Sac*II disruption cassette of this plasmid was used according to the standard URA blaster protocol to partially delete both *MSB2* alleles in *C. albicans* CAI4 generating FCCa27 (Ura^+) and FCCa28 (Ura^-). Strain FCCa28 allows integration of *MSB2* expression vectors in the *MSB2* locus by transformation with *Hpa*I-cleaved plasmid and ectopically in *LEU2* after digestion with *Eco*RV, which place *MSB2* alleles under transcriptional control of the *MSB2* and *ACT1* promoter, respectively. The disruption was verified by colony PCR using primers IPF6003-3verif/ i-p2-Ura3ver and by Southern blottings (data not shown). *E. coli* strain DH5αF′ was used for plasmid constructions and for AMP protection experiments.

Strains were grown on/in standard YPD or SD media. Pmt1-inhibitor OGT2599 was resuspended in DMSO to prepare a stock solution of 10 mM [54]. Standard drop dilution tests (10 fold dilutions to 10^{-5}) were used to determine sensitivity to inhibitors. Hyphal formation was induced by growth at 37°C on YPM medium containing 2% mannitol as sole carbon source or in liquid YP medium containing 10% serum [27].

MSB2 expression vectors

Relevant restriction site used for the construction of *MSB2* variant alleles are shown in Figure 1A. A *MSB2* allele encoding heme agglutinin (HA)-tagged Msb2 was constructed by first PCR-amplifying the 5′-end of the *MSB2* coding region using primers Msb2-ATG-*Xho*I and IPF6003-3′ (all oligonucleotides are listed in Table S1). The PCR fragment contained a novel *Xho*I site upstream of the ATG and extended to bp position 3227 of the ORF, 50 bp downstream of the *Pst*I site. The *Xho*I-*Pst*I subclone in pUC21 was mutagenized using the Quikchange kit (Stratagene) and primers HA-hin and HA-her were used to insert the sequence encoding a single HA epitope (11 amino acids) 1500 bp downstream of the ATG start codon sequence. The 3′-end of the *MSB2* ORF was then amplified by genomic PCR using primers Msb2-int2 und Msb2-Stopp-*Xho*I-*Not*I, which generated a fragment containing a *MSB2* sequence from 61 bp upstream of the *Pst*I site to the *Xho*I site downstream of the stop codon sequence that was generated in the PCR reaction. This 3′ PCR fragment was mixed with the above 5′ *Xho*I-*Pst*I fragment and the full-length modified *MSB2* allele was generated by overlap PCR using the flanking primers Msb2-ATG-*Xho*I und Msb2-Stopp-*Xho*I-*Not*I. The resulting *Xho*I fragment was cloned downstream of the *ACT1* promoter in *C. albicans* expression vector pDS1044-1 to generate plasmid pES10.

To insert the V5 epitope-encoding sequence into *MSB2* a 1037-bp region from upstream of the *Pst*I site to the middle of cytoplasmic domain sequence was PCR amplified using pES10 as template and primers PCR1 Hin und PCR1 Mitte Her, the latter primer added V5 sequences to the PCR product. In addition, a second PCR fragment (712 bp) was generated by PCR using primers PCR2 Mitte Hin (containing the V5 sequence) und PCR2 Her (downstream of the *Apa*I site in the 3′-UTR). Because both fragments contained the V5 sequence an overlap PCR using flanking primers PCR1 Hin und PCR2 Her generated a 1695 bp PCR fragment that was cut with *Nhe*I and *Apa*I and then inserted into pES10 to replace the corresponding unmodified fragment. The resulting expression plasmid encoding the $MSB2^{HA\text{-}V5}$ allele was designated pES11a. In a similar approach, an expression vector encoding a Msb2 variant carrying the V5 epitope at the C-terminal end of Msb2 was constructed using primers PCR1 Hin, PCR1 Ende Her, PCR2 Ende Hin and PCR2 Her; the resulting plasmid was designated pES11c ($MSB2^{HA\text{-}V5\ end}$).

Expression vectors encoding Msb2 variants were constructed by primer-directed mutagenesis of plasmid pES11a, using the Quikchange kit (Stratagene). Plasmid pES14 encoding Msb2-ΔN lacking residues 33–481 of Msb2 was constructed using primers *Cla*1 Del1 next1/-2, plasmid ES16 encoding the Msb2-ΔC variant lacking the cytoplasmic tail of Msb2 was constructed using oligonucleotides *MSB2* Stopp nach TM Hin/-Her and plasmid ES17 encoding the Msb2-ΔTM-C variant lacking transmembrane region and cytoplasmic tail was constructed using oligonucleotides *MSB2* Stopp vor TM Hin/-Her. Plasmid ES15 encoding the Msb2-tail variant was constructed by PCR-amplification of sequences encoding the cytoplasmic tail by primers C-Tail vor/ -rück and inserting it into downstream of the *PCK1* promoter in plasmid pBI-1. Plasmids were integrated into the *LEU2* locus of strain FCCa28 as described above.

Protein methods

Strains were grown in 50 ml YPD or SD medium at 30°C to $OD_{600} = 6–10$ and cells were harvested by centrifugation. Cells were washed with water and resuspended in lysis buffer (50 mM HEPES/pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100) containing protease inhibitors (Complete, Mini, Roche). Cells were broken by shaking with glass beads at 4°C for 2×10 min on a vibrax (Janke & Kunkel, 2200 rpm) or with a FastPrep homogenizer (MP Biochemicals). Cell debris and glass beads were separated from the crude cell extract by centrifugation. For immunoblottings proteins were separated by SDS-PAGE (8%, 18% or 4–20% acrylamide) and blotted to PVDF membranes. Protein standards used were the PageRuler set (Fermentas; 11–170 kDa) or the HiMark set (Invitrogen; 31–460 kDa) of proteins. Membranes were probed using rat anti-HA monoclonal antibody (1:2000; Roche) or mouse monoclonal anti-V5 antibody (1:2000; Serotec) and visualized using peroxidase-coupled goat anti-rat or anti-mouse antibodies (1:10000; Thermo) and the SuperSignal West Dura chemiluminescent substrate (Pierce).

Gel filtration chromatography was done on a Superdex 200 10/300 GL column (GE healthcare) equilibrated with SD medium. Elution characteristics were established using a set of standard proteins (Sigma) containing carboanhydrase (23 kDa), BSA (66 kDa), ADH (150 kDa), β-amylase (200 kDa), apoferritin (434 kDa) and thyroglobulin (669 kDa); the void volume (V_0) was determined using Blue dextran (2000 kDa). Protein elution volumes (V_e) were monitored at 280 nm and fractions were collected by an ÄKTA prime plus (GE Healthcare) at a flow speed of 0.4 ml/min. To determine the molecular mass of secreted Msb2, strain ESCa3 ($Msb2^{HA\text{-}V5}$) was grown in SD medium to $OD_{600} = 10$. Cells were removed by centrifugation and 500 µl of the medium was degassed, sterile-filtered and applied to the Superdex column. 200 µl fractions were collected and 20 µl per fraction were tested for the presence of $Msb2^{HA}$ by immunoblotting. The approximate molecular mass of $Msb2^{HA}$ was calculated from the standard protein graph using the equation $y = 62258e-3{,}695x$ (x: Ve/Vo; y: molecular mass).

Deglycosylation reactions using PNGase F and α-mannosidase (jack bean) were carried out according to the instructions of the manufacturers (Roche; Sigma). To remove *O*-glycosylation the GlycoProfile β-elimination kit (Sigma) was used, either without or with pretreatment of the sample at 80°C. 200 µl of the ESCa3 growth medium was acetone-precipitated and resuspended in the same volume of water. 40 µl of the reagent mixture was added and the sample was incubated over night at 4°C. The sample was neutralized with HCl and 20 µl were analyzed by immunoblotting. The GlycoProfile IV kit (Sigma) was used to remove all forms of protein glycosylation by trifluoromethanesulfonic acid (TFMS). 1.5 ml of the growth medium of strain ESCa3 was lyophilized and 150 µl of TFMS was added and the proteins incubated at 4°C for 25 min. 4 µl of 0.2% bromophenol blue was added and

neutralization by precooled pyridine (added drop-wise) was monitored by the yellowish coloring. This latter step was carried out in a bath of dry ice in ethanol. Reagents in the samples were removed by dialysis against PBS using Slide-A-Lyzer cassettes (Thermo).

The secreted $Msb2^{HA}$ domain was purified by affinity chromatography from cultures grown in SD medium containing 2% casamino acids to an $OD_{600} = 10$ using a column (1 ml) containing agarose beads covalently coupled to 3.5 mg of monoclonal anti-HA high affinity antibody (Roche). The column equilibrated with buffer (20 mM Tris/HCl, pH 7.5; 0.1 M NaCl; 0.1 mM EDTA) and 50–400 ml of the culture medium containing $Msb2^{HA}$ were loaded and the column was washed with 20 bed volumes of wash buffer (20 mM TrisHCl/pH 7.5; 0.1 M NaCl; 0.1 mM EDTA; 0,05% Tween 20). The $Msb2^{HA}$ protein was eluted twice by 1 ml (1 mg) of HA peptide (Roche) in Tris-buffered saline.

Proteins on SDS-PAGE gels were routinely visualized by Coomassie blue or silver staining and protein concentrations were determined by the Bradford assay using a commercial assay kit (BioRad). Because of the high glycosylation status of Msb2* its concentration could not be determined reliably by any of these methods. Therefore, we developed a dot blot procedure, in which known molar concentrations of HA peptide were compared to Msb2* (or Msb2-ΔN*) signals resulting from reaction with the anti-HA antibody. Dilutions of a HA peptide solution (Roche) were spotted on an activated PDVF membrane and a dilution series of the sample containing unknown amounts of Msb2* was spotted alongside. The membrane was processed as for immunoblottings and the resulting signals were recorded using a Fujifilm LAS400 mini image analyzer and evaluated with the Fujifilm Multi Gauge program. The standard curve derived from the HA peptide were used to calculate molar amounts of the Msb2* sample.

Msb2* samples were assayed for protease contamination using the Protease Detection Kit (Jena Bioscience) that detects a wide variety of proteases, including serine proteases, cysteine proteases and acid proteases. Substrate solution (50 µl) and incubation buffer (50 µl) were mixed with 100 µl (50 µg) of Msb2* in TBS and incubated at 37°C for 16 h. 120 µl precipitation reagent was added and samples were incubated at 37°C for 30 min. Tubes were centrifuged at 12.000× *g* for 5 min and 50 µl of the supernatant was transferred to a flat bottom 96 well plate, 150 µl assay buffer was added and absorbance at 492 nm was measured using a plate spectrophotometer (Biotek).

MAPK activation assay

Strains were grown over night to stationary phase in YPD medium and diluted into YPD medium to an $OD_{600} = 0.1$. Cells were grown to $OD_{600} = 0.8$ at 37°C and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 µg/ml). Immunoblots were prepared as described previously verifying equal loading by Ponceau red staining of the membranes [9]. Blots were probed with anti-phospho-p44/42 MAP kinase (Cell Signaling Technology) to detect phosphorylated Cek1 protein and ScHog1 polyclonal antibody (Santa Cruz Biotechnology) was used to detect all forms of Hog1 [9].

Antimicrobial peptide assays

Over night cultures of *C. albicans* and *E. coli* DH5αF′ were diluted and grown in YPD at 30°C to an $OD_{600} = 0.3$. Cells were harvested by centrifugation and washed with and resuspended in PBS. Triplicate assays containing 5 µl cell suspension and 0–10 µg LL-37 (Sigma) or histatin-5 (AnaSpec Inc.) in a total volume 25 µl were incubated 1.5 h at 37°C, diluted 500 fold and plated on YPD. Colony forming units were determined after 2 d of growth at 30°C. The action of LL-37 on cells was visualized by fluorescence microscopy using LL-37-TAMRA (Innovagen).

To assay binding of LL-37 to Msb2* a microtiter plate assay was used. 10 µg Msb2* or Msb2-ΔN* in 200 µl PBS were allowed to bind wells of a 96 well flat bottom polystyrene plate over night at 4°C. The wells were washed three times with PBST (PBS containing 0.05% Tween 20). Then 200 µl of blocking buffer (5% w/v nonfat dry milk in PBST) was added for 2 hours at room temperature. Wells were washed three times and incubated with 5 µg LL-37 5-TAMRA for one hour. After washing three times, the fluorescence was measured on a Tecan infinite 200 plate reader (excitation 560 nm, emission wavelength 590 nm). In a competition experiment, following Msb2* binding, 3 µg LL-37 was added to wells and incubated for one hour before cells were washed and LL-37-TAMRA was added.

Supporting Information

Figure S1 Phenotypes of *C. albicans* strains producing deleted Msb2 variants. **A.** Antifungal sensitivity. Sensitivities of strains to caspofungin (125 ng/ml) and tunicamycin (2 µg/ml) were tested by a drop dilution test on YPD agar. **B.** Hypha formation. Colonies of strains were photographed following growth for 2 d at 37°C on YPM agar. **C.** Detection of Msb2* in the growth medium. Strains were grown in YPD medium to $OD_{600} = 6$, centrifuged and the medium (20 µl) was analyzed by immunoblotting using rat anti-HA antibody. Strains included CAF2-1 (wt), ESCa3 ($Msb2^{HA\text{-}V5}$), ESCa25 (Msb2-ΔN), ESCa37 (Msb2-tail), ESCa37 (Msb2-ΔC), ESCa39 (Msb2-ΔTM-C) and control strains FCCa27/28 (Msb2-Δ1) and CAP4-2164 (*pmt4*). The following *pmt* mutant strains carrying plasmid pES14 encoding the Msb2-ΔN variant were also tested by immunoblotting: ESca26 (*pmt1*), ESCa27 (*PMT2/pmt2*), ESCa28 (*pmt4*), ESCa29 (*pmt5*) and ESCa30 (*pmt6*).
(PDF)

Table S1 List of oligonucleotides.
(PDF)

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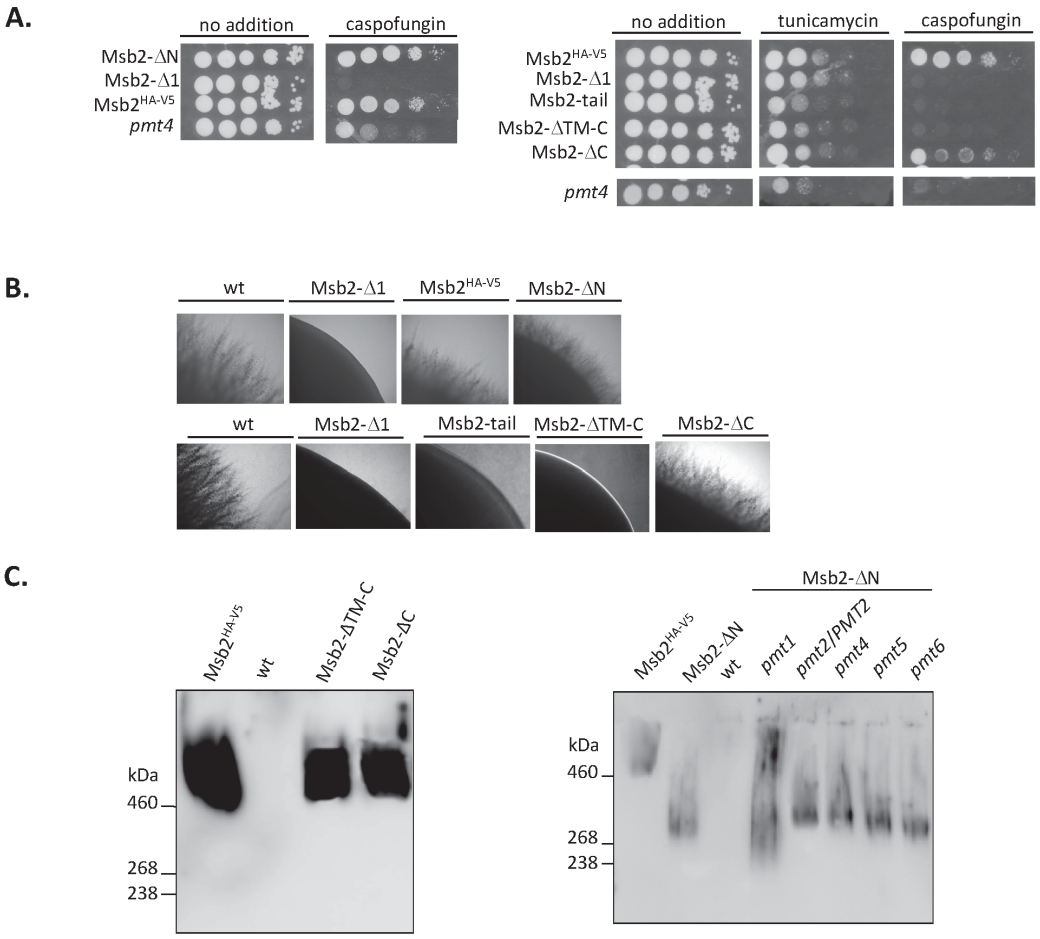
Author Contributions

Conceived and designed the experiments: JFE JP. Performed the experiments: ES-S MS FC DT ER. Analyzed the data: JFE JP ES-S MS FC DT ER. Contributed reagents/materials/analysis tools: JFE ES-S MS FC DT JP ER. Wrote the paper: JFE.

References

1. Lee YK, Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330: 1768–1773.
2. Jouault T, Sarazin A, Martinez-Esparza M, Fradin C, Sendid B, et al. (2009) Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by *Candida albicans*. Cell Microbiol 11: 1007–1015.
3. van der Meer JW, van de Veerdonk FL, Joosten LA, Kullberg BJ, Netea MG (2010) Severe Candida spp. infections: new insights into natural immunity. Int J Antimicrob Agents 36 Suppl 2: S58–S62.
4. Zipfel PF (2009) Complement and immune defense: from innate immunity to human diseases. Immunol Lett 126: 1–7.

5. Zhu W, Filler SG (2010) Interactions of *Candida albicans* with epithelial cells. Cell Microbiol 12: 273–282.
6. Ernst JF, Pla J (2011) Signaling the glycoshield: maintenance of the *Candida albicans* cell wall. Int J Med Microbiol 301: 378–383.
7. Navarro-García F, Sánchez M, Pla J, Nombela C (1995) Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. Mol Cell Biol 15: 2197–2206.
8. Navarro-García F, Eisman B, Fiuza SM, Nombela C, Pla J (2005) The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. Microbiology 151: 2737–2749.
9. Román E, Cottier F, Ernst JF, Pla J (2009) Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. Eukaryot Cell 8: 1235–1249.
10. Cantero PD, Ernst JF (2011) Damage to the glycoshield activates *PMT*-directed O-mannosylation via the Msb2-Cek1 pathway in *Candida albicans*. Mol Microbiol 80: 715–725.
11. Diez-Orejas R, Molero G, Navarro-García F, Pla J, Nombela C, et al. (1997) Reduced virulence of *Candida albicans* MKC1 mutants: a role for mitogen-activated protein kinase in pathogenesis. Infect Immun 65: 833–837.
12. Csank C, Schröppel K, Leberer E, Harcus D, Mohamed O, et al. (1998) Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun 66: 2713–2721.
13. Cullen PJ, Sabbagh Jr. W, Graham E, Irick MM, van Olden EK, et al. (2004) A signaling mucin at the head of the Cdc42- and MAPK-dependent filamentous growth pathway in yeast. Genes Dev 18: 1695–1708.
14. Lanver D, Mendoza-Mendoza A, Brachmann A, Kahmann R (2010) Sho1 and Msb2-related protein regulate appressorium development in the smut fungus *Ustilago maydis*. Plant Cell 22: 2085–2101.
15. Liu W, Zhou X, Li G, Li L, Kong L, et al. (2011) Multiple plant surface signals are sensed by different mechanisms in the rice blast fungus for appressorium formation. PLoS Pathog 7: e1001261.
16. Pérez-Nadales E, Di Pietro A (2011) The membrane mucin Msb2 regulates invasive growth and plant infection in *Fusarium oxysporum*. Plant Cell 23: 1171–1185.
17. Vadaie N, Dionne H, Akajagbor DS, Nickerson SR, Krysan DJ, et al. (2008) Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. J Cell Biol 181: 1073–1081.
18. Cullen PJ (2007) Signaling mucins: the new kids on the MAPK block. Crit Rev Eukaryot Gene Expr 17: 241–257.
19. Dürr M, Peschel A (2002) Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. Infect Immun 70: 6515–6517.
20. Peschel A, Sahl H-G (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature Rev Microbiol 4: 529–536.
21. Oudhoff MJ, Blaauboer ME, Nazmi K, Scheres N, Bolscher JG, et al. (2010) The role of salivary histatin and the human cathelicidin LL-37 in wound healing and innate immunity. Biol Chem 391: 541–548.
22. Nijnik A, Hancock REW (2009) The roles of cathelicidin LL-37 in immune defences and novel clinical applications. Curr Opin Hematol 16: 41–47.
23. Mochon AB, Liu H (2008) The antimicrobial peptide histatin-5 causes a spatially restricted disruption on the *Candida albicans* surface, allowing rapid entry of the peptide into the cytoplasm. PLoS Pathog 4: e1000190.
24. den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, et al. (2005) Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. Biochem J 388: 689–695.
25. den Hertog AL, van Marle J, Veerman ECI, Valentijn-Benz M, Nazmi K, et al. (2006) The human cathelicidin peptide LL-37 and trucated variants induce segregation of lipids and proteins in the plasma membrane of *Candida albicans*. Biol Chem 387: 1495–1502.
26. Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, et al. (1999) The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. J Biol Chem 274: 7286–7291.
27. Prill SK, Klinkert B, Timpel C, Gale CA, Schröppel K, et al. (2005) PMT family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. Mol Microbiol 55: 546–60.
28. Cantero PD, Lengsfeld C, Prill SK, Subanović M, Román E, et al. (2007) Transcriptional and physiological adaptation to defective protein-*O*-mannosylation in *Candida albicans*. Mol Microbiol 64: 1115–1128.
29. Albrecht A, Felk A, Pichova I, Naglik JR, Schaller M, et al. (2006) Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. J Biol Chem 281: 688–694.
30. Karunanithi S, Vadaie N, Chavel CA, Birkaya B, Joshi J, et al. (2010) Shedding of the mucin-like flocculin Flo11p reveals a new aspect of fungal adhesion regulation. Curr Biol 20: 1389–1395.
31. Tsai PW, Yang CY, Chang HT, Lan CY (2011) Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. PLoS One 6: e17755.
32. Veerman EC, Valentijn-Benz M, Nazmi K, Ruissen AL, Walgreen-Weterings E, et al. (2007) Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. J Biol Chem 282: 18831–18841.
33. Jang WS, Bajwa JS, Sun JN, Edgerton M (2010) Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. Mol Microbiol 77: 354–370.
34. Kumamoto CA, Pierce JV (2011) Immunosensing during colonization by *Candida albicans*: does it take a village to colonize the intestine? Trends Microbiol 19: 263–267.
35. Kumamoto CA (2011) Inflammation and gastrointestinal *Candida* colonization. Curr Opin Microbiol 14: 386–391.
36. Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, et al. (2007) The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. Mol Microbiol 63: 1399–1413.
37. Tatebayashi K, Tanaka K, Yang H-Y, Yamamoto K, Matsushita Y, et al. (2007) Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branc of yeast HOG pathway. EMBO J 26: 3521–3533.
38. Yang H-Y, Tatebayashi K, Yamamoto K, Saito H (2008) Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK. EMBO J 28: 1380–1391.
39. Sorgo AG, Heilmann CJ, Dekker HL, Brul S, de Koster CG, et al. (2010) Mass spectrometric analysis of the secretome of *Candida albicans*. Yeast 27: 661–672.
40. Carson DD (2008) The cytoplasmic tail of MUC1: a very busy place. Sci Signal 1: pe35.
41. Akerey B, Le-Lay C, Fliss I, Subirade M, Rouabhia M (2009) *In vitro* efficacy of nisin Z against *Candida albicans* adhesion and transition following contact with normal human gingival cells. J Appl Microbiol 107: 1298–1307.
42. Ruissen AL, Groenink J, Krijtenberg P, Walgreen-Weterings E, van 't Hof W, et al. (2003) Internalisation and degradation of histatin 5 by *Candida albicans*. Biol Chem 384: 183–190.
43. Meiller TF, Hube B, Schild L, Shirtliff ME, Scheper MA, et al. (2009) A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. PLoS One 4: e5039.
44. Frick IM, Akesson P, Rasmussen M, Schmidtchen A, Björck L (2003) SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. J Biol Chem 278: 16561–1656.
45. Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, et al. (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J Immunol 172: 1169–1176.
46. Frick I-M, Karlsson C, Mörgelin M, Olin AI, Janjusevic R, et al. (2008) Identification of a novel protein promoting the colonization and survival of *Finegoldia magna*, a bacterial commensal and opportunistic pathogen. Mol Microbiol 70: 695–708.
47. Scott A, Weldon S, Buchanan PJ, Schock B, Ernst RK, et al. (2011) Evaluation of the ability of LL-37 to neutralise LPS in vitro and ex vivo. PLoS One 6: e26525.
48. Foschiatti M, Cescutti P, Tossi A, Rizzo R (2009) Inhibition of cathelicidin activity by bacterial exopolysaccharides. Mol Microbiol 72: 1137–1146.
49. Baranska-Ryback W, Sonesson A, Nowicki R, Schmidtchen A (2006) Glycosaminoglycans inibit the antibacterial activity of LL-37 in biological fluids. J Antimicrob Chemother 57: 260–265.
50. Van den Steen P, Rudd PM, Dwek RA, Opdenakker G (1998) Concepts and principles of O-linked glycosylation. Crit Rev Biochem Mol Biol 33: 151–208.
51. Argimón S, Fanning S, Blankenship JR, Mitchell AP (2011) Interaction between the *Candida albicans* high-osmolarity glycerol (HOG) pathway and the response to human beta-defensins 2 and 3. Eukaryot Cell 10: 272–275.
52. Cheetham J, Smith DA, da Silva Dantas A, Doris KS, Patterson MJ, et al. (2007) A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus *Candida albicans*. Mol Biol Cell 18: 4603–4614.
53. Luo S, Poltermann S, Kunert A, Rupp S, Zipfel PF (2009) Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. Mol Immunol 47: 541–550.
54. Orchard MG, Neuss JC, Galley CM, Carr A, Porter DW, et al. (2004) Rhodanine-3-acetic acid derivatives as inhibitors of fungal protein mannosyl transferase 1 (PMT1). Bioorg Med Chem Lett 14: 3975–3978.
55. Fonzi WA, Irwin MY (1993) Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134: 717–728.
56. Newport G, Agabian N (1997) KEX2 influences *Candida albicans* proteinase secretion and hyphal formation. J Biol Chem 272: 28954–28961.



Supplemental Fig. S1. Phenotypes of *C. albicans* strains producing deleted Msb2 variants. **A.** Antifungal sensitivity. Sensitivities of strains to caspofungin (125 ng/ml) and tunicamycin (2 µg/ml) were tested by a drop dilution test on YPD agar. **B.** Hypha formation. Colonies of strains were photographed following growth for 2 d at 37 °Con YPM agar. **C.** Detection of Msb2* in the growth medium. Strains were grown in YPD medium to OD_{600}= 6, centrifuged and the medium (20 µl) was analyzed by immunoblotting using rat anti-HA antibody. Strains included CAF2-1 (wt), ESCa3 (Msb2^{HA-V5}), ESCa25 (Msb2-ΔN), ESCa37 (Msb2-tail), ESCa37 (Msb2-ΔC), ESCa39 (Msb2-ΔTM-C) and control strains FCCa27/28 (Msb2-Δ1) and CAP4-2164 (*pmt4*). The following *pmt* mutant strains carrying plasmid pES14 encoding the Msb2-ΔN variant were also tested by immunoblotting: ESca26 (*pmt1*), ESCa27 (*PMT2/pmt2*), ESCa28 (*pmt4*), ESCa29 (*pmt5*) and ESCa30 (*pmt6*).

Supplemental Table 1. List of oligonucleotides

name	sequence
IPF6003-NotI	5´-ATCTAGCGGCCGCGTCTATTTTGATACCCACCCC-3´
IPF6003-SacII	5´-TCAGTACCGCGGCTTGATGGCTCAGCTGATGC-3´
IPF6003-3verif	5´-CTGCTGAAGGAGCAACTGCG-3´
i-p2-Ura3ver	5´-TTACAATCAAAGGTGGTCC-3´
Msb2-ATG-*Xho*I	5´-TGAATCTCGAGATGTTGGCCAACGTTAAATTG-3´
IPF6003-3´	5´-CTTGATGGCTCAGCTGATGC-3´
HA-hin	5´-GAAACCACTTCATTAGTGAGATCTTACCCATACGATGTT CCTGACTATGCGAACCCTACCGATTCCCAAATTG-3´
HA-her	5´-CAATTTGGGAATCGGTAGGGTTCGCATAGTCAGGAACAT CGTA TGGGTAAGATCTCACTAATGAAGTGGTTTC-3´
Msb2-int2	5´-GCTACTGGTTCTCAAGTTAC-3´
Msb2-Stopp-*Xho*I-*Not*I	5´-ATTCAGCGGCCGCCTCGAGCTAATGATACCAACCCAA TG-3´
PCR1 Hin	5´-CAACAGCTGCTAGCGAG-3´
PCR1 Mitte Her	5´-CCCGGGCGTAGAATCGAGACCGAGGAGAGGGTTAGG GATAGGCTTACCACCACCTTCTAATGCCTTATTAC-3´
PCR1 Ende Her	5´-CCCGGGCTACGTAGAATCGAGACCGAGGAGAGGGTT AGGGATAGGCTTACCACCACCATGATACCAACCCAATG-3´
PCR2 Her	5´-GGGTACCGGGCCC-3´
PCR2 Mitte Hin	5´-GGTGGTGGTAAGCCTATCCCTAACCCTCTCCTCGGTC TCGATTCTACGCCCGGGTTACCAATTACTGATTC-3´
PCR2 Ende Hin	5´-GGTGGTGGTAAGCCTATCCCTAACCCTCTCCTCGGTCT CGATTCTACGTAGCCCGGGCTCGAGGCGAGTG-3´
*Cla*1 Del1 next1	5´-CCATCGATGAAATCTCTGAAAAAATTACCAATAGC-3´
*Cla*1 Del1 next2	5´-CCATCGATGATTTCTCCAATCTGGTATTGCATAGTTC-3´
C-Tail vor (*Xho*I)	5´-CCGCTCGAGATGAGAAAGTTTAGAAAGAG-3´
C-Tail rück (*Xho*I)	5´-CCGCTCGAGTAAAGTTCTCTAATGATACC-3´
MSB2 Stopp nach TM Hin	5´-GAAAGTTTAGAAAGTAGTAAGTAGCTAGCTTAAGAGTA ATAAGGCATTAG-3´
MSB2 Stopp nach TM Her	5´-CTAATGCCTTATTACTCTTAAGCTAGCTACTTACTACTT TCTAAACTTTC-3´
MSB2 Stopp vor TM Hin	5´-CGATAAAGGAAGATAGTAAGTAGCTAGCTTAAGATTGCT GGTATAAC-3´
MSB2 Stopp vor TM Her	5´-GTTATACCAGCAATCTTAAGCTAGCTACTTACTATC TTCCTTTATCG-3´
C-Tail vor (*Bam*HI)	5´-CGCGGATCCATGAGAAAGTTTAGAAAGAG-3´
C-Tail rück (*Bgl*II)	5´-GGAAGATCTTAAAGTTCTCTAATGATACC-3´

2.4 Manuskript III: *Candida albicans* Mucin Msb2 Is a Broad-Range Protectant against Antimicrobial Peptides

Marc Swidergall, Andreas M. Ernst, and Joachim F. Ernst

Erstautor

eigener Anteil an der Arbeit in %: 85

Marc Swidergall hat folgende Experimente geplant, durchgeführt und interpretiert: Aufreinigung von Msb2-Varianten; AMP-Assays mit verschiedenen Peptiden unter unterschiedlichen Bedingungen; MST Messungen; Daptomycin-Assays; Polymikrobielle Assays.
Marc Swidergall schrieb einen Teil des Manuskripts.

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Candida albicans Mucin Msb2 Is a Broad-Range Protectant against Antimicrobial Peptides

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The human fungal pathogen *Candida albicans* releases a large glycofragment of the Msb2 surface protein (Msb2*) into the growth environment, which protects against the action of human antimicrobial peptides (AMPs) LL-37 and histatin-5. Quantitation of Msb2*/LL-37 interactions by microscale thermophoresis revealed high-affinity binding (dissociation constant [K_D] = 73 nM), which was lost or greatly diminished by lack of *O*-glycosylation or by Msb2* denaturation. Msb2* also interacted with human α- and β-defensins and protected *C. albicans* against these AMPs. In addition, the lipopeptide antibiotic daptomycin was bound and inactivated by Msb2*, which prevented the killing of bacterial pathogens *Staphylococcus aureus*, *Enterococcus faecalis*, and *Corynebacterium pseudodiphtheriticum*. In coculturings or mixed biofilms of *S. aureus* with *C. albicans* wild-type but not *msb2* mutant strains, the protective effects of Msb2* on the bactericidal action of daptomycin were demonstrated. These results suggest that tight binding of shed Msb2* to AMPs that occurs during bacterial coinfections with *C. albicans* compromises antibacterial therapy by inactivating a relevant reserve antibiotic.

Antimicrobial peptides (AMPs) are major weapons of the human immune system against microbial pathogens, which kill microbes and attract immune cells to the site of infection (1, 2). Both bacteria and fungi are inhibited by AMPs, but they can nevertheless survive if they contain defense mechanisms that limit AMP uptake (3) or inactivate AMPs, e.g., by proteases (4). In addition, microbes may secrete AMP binding proteins as decoys to lower effective AMP concentrations in their vicinity (5–7).

The *Candida albicans* Msb2 membrane protein has been designated a signaling mucin (8) because of its characteristic structure consisting of a short cytoplasmic C terminus, a transmembrane region, and a large, highly *O*-glycosylated exodomain, which is released in considerable amounts into the growth environment during planktonic and surface growth (9–11). Msb2 fulfills at least two functions to allow *C. albicans* survival and growth in the infected human host. First, external stimuli, including surface growth and cell wall integrity, are signaled via Msb2 to trigger corresponding response or rescue pathways required for growth and hyphal morphogenesis (10, 12–14). Second, we recently found that the secreted Msb2 glycodomain (Msb2*) acts to protect *C. albicans* and *Escherichia coli* against toxicity of AMPs LL-37 and histatin-5 (10). The evidence suggested that Msb2* mediates its protective function by binding of AMPs and that this activity requires intact *O*-mannosylation by protein *O*-mannosyltransferases (Pmt proteins). However, binding constants reflecting the affinity of AMPs for Msb2* or any other binding partner have not yet been determined, and the range of AMP substrates bound by Msb2*is still unclear.

Here we report that *C. albicans* Msb2* binds not only to multiple AMPs produced by the human host, including α- and β-defensins (15, 16), but also to daptomycin, an AMP produced by the bacterium *Streptomyces roseosporus*. The lipopeptide antibiotic daptomycin and the glycopeptide antibiotic vancomycin are of special importance in current anti-infectious therapy because these compounds serve as reserve antibiotics for the treatment of multiresistant Gram-positive bacteria (17–19). We demonstrate that the presence of *C. albicans* Msb2* protects the important bacterial pathogens *Staphylococcus aureus*, *Enterococcus faecalis*, and *Corynebacterium pseudodiphtheriticum* against the inhibitory activity of daptomycin. These results suggest that mixed infections of *S. aureus* and other important bacterial pathogens with *C. albicans* are of particular risk because bacteria are cross-protected by the shed fungal Msb2* protein against peptide antimicrobials, including daptomycin.

MATERIALS AND METHODS

Strains and media. Strains are listed in Table 1. *C. albicans* strain ESCa3 produces a full-length hemagglutinin (HA) and V5 epitope-labeled Msb2 protein. Strain FCCa27 contains a partially deleted allele designated *msb2Δ1* (encoding the 406 N-terminal residues of Msb2), which was found to be completely nonfunctional (10). YPD (1% yeast extract, 2% peptone, 2% glucose) or SD (0.67% yeast nitrogen base, 2% glucose) medium was used for growth of *C. albicans* strains on agar or in liquid cultures.

Antimicrobial peptides. The sensitivity of *C. albicans* wild-type strain CAF2-1 to various AMPs was determined as previously described (10) using the AMPs LL-37 (Sigma), histatin-5 (AnaSpec Inc.), human β-defensin-1 (hBD1) (Innovagen), and human α-defensin-1 (hNP-1) (AnaSpec Inc.). Bacteria were tested for their sensitivity against daptomycin (Sigma) or vancomycin (Hikma Pharmaceuticals PLC).

Proteins. Strain ESCa3 produces full-length Msb2 doubly epitope tagged in its large extracellular and small cytoplasmic domains (HA and V5 epitopes, respectively); strain ESCa25 encodes Msb2 variant Msb2-ΔN, which lacks residues 33 to 481 of Msb2 (10). The extracellular domains of both Msb2 proteins (functional in AMP binding) were almost quantitatively released into the growth medium and are referred to as Msb2* and Msb2ΔN*, respectively. The secreted HA-tagged Msb2* or

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TABLE 1 Strains

Strain	Genotype	Reference
C. albicans		
CAI4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	27
CAF2-1	*ura3*Δ::*imm434*/*URA3*	27
FCCa27	As CAI4 but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG-URA3-hisG*	10
ESCa3	As CAI4 but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG LEU2*/*LEU2*::pES11a (*ACT1p-MSB2*$^{HA\text{-}V5}$)	10
ESCa18	As CAI4 but *pmt1*Δ::*hisG*/*pmt1*Δ::*hisG LEU2*/*LEU2*::pES11a (*ACT1p-MSB2*$^{HA\text{-}V5}$)	10
ESCa25	As CAI4 but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG LEU2*/*LEU2*::pES14 (*ACT1p-MSB2-*Δ*N*$^{HA\text{-}V5}$)	10
Bacteria		
S. aureus ATCC 35556 (Sa113)	As NCTC 8325 but restriction deficient	30
E. faecalis ATCC 29212	Wild type	31
C. pseudodiphtheriticum ATCC 10701	Wild type	32
B. subtilis ATCC 6051	Wild type	29

Msb2-ΔN* protein fragments were purified by affinity chromatography and quantified immunologically, as previously described (10). Mucin from porcine stomach (type III, 0.5 to 1.5% bound sialic acid, partially purified powder; Sigma) was solubilized in water and autoclaved (20); the solubilized mucin was centrifuged (4,000 rpm, 30 min), and the supernatant was used in assays. Mucin concentrations were calculated assuming a molecular weight of 2,000,000 (21).

MST. Microscale thermophoresis (MST) analysis was performed using a NanoTemper Monolith NT.115 apparatus (22, 23). A constant amount of 94 nM carboxyfluorescein-tagged LL-37 (5-FAM-LL-37; AnaSpec, Fremont, CA) was incubated for 30 min at 37°C in the dark with different concentrations of Msb2 protein in HEPES-KOH (pH 7.4 or 8) or morpholineethanesulfonic acid (MES) (pH 6) buffer containing 0.005% SDS. Then, 10 μl of the samples was loaded into standard glass capillaries (Monolith NT Capillaries) and thermophoresis analysis was performed (settings for the light-emitting diode and infrared [IR] laser were 80% and 50%, respectively). In a competition experiment, 4.25 μM unlabeled LL-37 or 50 μM daptomycin was preincubated for 15 min at 37°C with the samples before addition of FAM-LL-37.

Antimicrobial peptide assay. *C. albicans* was grown in YPD medium at 30°C to an optical density at 600 nm (OD_{600}) of 0.3. Cells were harvested by centrifugation and washed with and resuspended in phosphate-buffered saline (PBS). Triplicate assays contained 5 μl cell suspension and different concentrations of AMPs in a total volume of 25 μl. Assay mixtures were incubated for 1.5 h at 37°C, diluted 500-fold, and plated on YPD agar. CFU were determined after 2 days of growth at 30°C. Bacteria were grown and diluted in LB (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) or Loeffler medium (0.25% peptone, 0.15% beef extract, 0.125% sodium chloride, 0.5% dextrose) containing horse serum (Sigma) at 37°C to an OD_{595} of 0.3. Cells were harvested by centrifugation, washed with PBS, and resuspended in growth medium. Triplicate assay mixtures of different concentration of antibiotics in a total volume of 1 ml were incubated for 30 min (10 μg/ml daptomycin) and 4 h (4 μg/ml vancomycin) at 37°C, diluted 10,000-fold, and plated on LB or Loeffler agar. CFU were determined after 1 day of growth at 37°C. In the case of hBD1, this assay was carried out in a hypoxic chamber (0.2% O_2, 0.2% CO_2) because hBD1 is activated in this environment (24).

AMP coculturing assay. *C. albicans* strains (CAF2-1, FCCa27, and ESCa3) were grown in 5 ml YPD medium to an OD_{600} of 8. *S. aureus* (Sa113) cells (3×10^7/ml) were added, and incubation was continued for 30 min at 37°C. Daptomycin (5 μg/ml) was added, and *S. aureus* cells (CFU) were determined by plating on LB agar containing caspofungin (100 ng/ml) at different times of coincubation in the presence or absence of daptomycin.

AMP biofilm assay. *C. albicans* was pregrown in YPD and *S. aureus* was pregrown in LB medium overnight at 37°C. Cells were harvested by centrifugation and resuspended in PBS; *C. albicans* cellular aggregates were then disassembled in a bath sonifier (10 min), pelleted, and resuspended again in PBS. Numbers of cells were estimated by measuring the optical density at 600 nm (*C. albicans*) or 595 nm (*S. aureus*) using a standard curve, and cells were resuspended in RPMI 1640 medium at a concentration of 10^6 cells per ml. Strains were inoculated, separately or in combination, at a concentration of 10^6 cells per ml into wells of 24-well polystyrene cell culture plates, and cells were allowed to form homogenous or mixed biofilms by growth in RPMI 1640 medium at 37°C for 24 h or 48 h. Biofilms were treated for 5 h with daptomycin (10 μg/ml) or vancomycin (4 μg/ml) at 37°C before removal of biofilms using a sterile scalpel. Biofilms were disassembled in RPMI 1640 medium by strong vortexing, diluted, and plated on LB plates containing caspofungin (100 ng/ml) to prevent fungal growth. *S. aureus* CFU were determined after 24 h at 37°C. Experiments were repeated three times with each strain. The statistical significance of differences between groups of data was analyzed using an unpaired *t* test calculating two-tailed *P* values (GraphPad Prism 5 program).

RESULTS

MST reveals high-affinity interaction of Msb2* and LL-37. Microscale thermophoresis (MST) is an effective method to assess dissociation constants for the interaction of a fluorescent ligand with its unlabeled binding molecule in solution (22, 23). In this method, the avidity of binding is typically demonstrated by the rate of disappearance of the fluorescent signal from a zone of heating, a process termed "thermophoresis." We used MST technology to precisely quantitate binding of the human cathelicidin LL-37 to the secreted N-terminal fragment of the Msb2 surface protein of the fungal pathogen *C. albicans*. In previous experiments, it had been shown that this Msb2 fragment (referred to as Msb2*) is released during fungal growth into the environment and is able to neutralize the toxic effects of LL-37 on *C. albicans* and *E. coli* (10).

A series of binding assays with a constant amount of 5-FAM-LL-37 and various concentrations of affinity-purified Msb2* showed concentration-dependent thermophoresis reaching a half-maximal rate at 73 nM, which corresponds to the dissociation constant (K_D) of the binding reaction (Fig. 1A and B). This high binding affinity was retained in an Msb2 variant lacking 450 residues in the N-terminal domain (Msb2-ΔN*) (Table 2), consistent with AMP inactivation functions of the deleted variant (10). Furthermore, binding of Msb2* to LL-37 was not affected greatly between pH 6 and 8. On the other hand, binding was abolished by heat inactivation of Msb2* and also by preincubation of Msb2* with unlabeled LL-37 (Table 2), demonstrating that LL-37 bind-

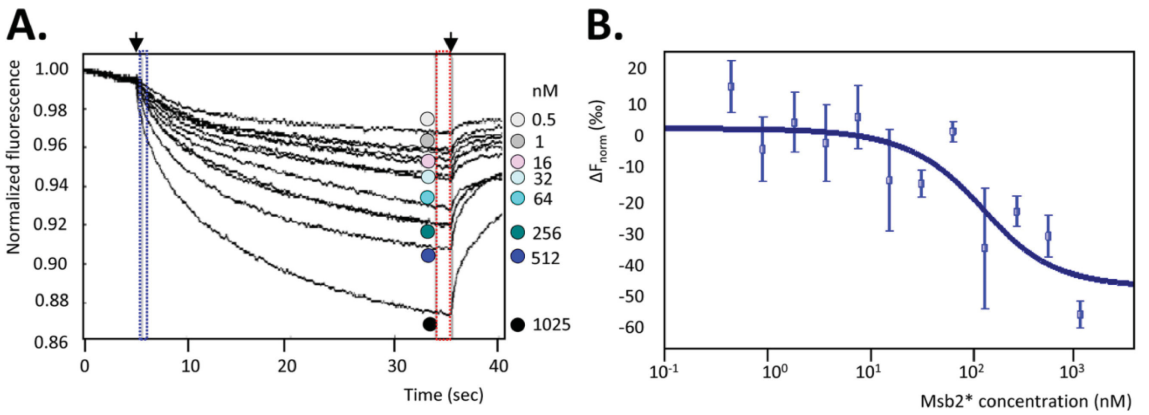

FIG 1 Microscale thermophoresis assay to quantitate Msb2/LL-37 interaction. (A) 5-FAM-LL-37 (94 nM) and Msb2* were allowed to interact and heated locally to generate a temperature gradient; the rate of fluorescence disappearance as a function of Msb2* concentration was recorded. Results are from a representative set of assays done in triplicate; concentrations are indicated by colored dots, and arrows indicate the begin and end of heating. (B) The degree of thermophoresis was plotted against Msb2* concentration and yielded a half-maximal rate at 73 nM. Mean values of three measurements ± standard deviations are shown.

ing occurs on a specific site of Msb2 in its native protein conformation. Interestingly, *in vitro* binding of 5-FAM-LL-37 was also highly dependent on *O*-glycosylation of Msb2*, since binding was lost using an Msb2* preparation from a *pmt1* mutant host strain (ESCa18). This finding is in agreement with lowered AMP inactivation by the *pmt1* mutant (10).

Human cells produce several mucins, which reside on cellular surfaces and in the intercellular space of tissues (25). Human mucins, like the *C. albicans* Msb2 mucin, are highly *O*-glycosylated proteins, which conceptually could compete for the binding of AMPs. As a model for human mucin, we assayed binding of 5-FAM-LL-37 to porcine gastric mucin, which is structurally similar to human mucin (20, 26). MST measurements revealed a much lower affinity of binding to porcine mucin (K_D = 876 nm) than to *C. albicans* Msb2* (Table 2). Thus, it appears that unlike mammalian mucin, Msb2* exhibits a remarkable affinity for the cathelicidin LL-37.

Msb2* protects against different AMPs, including the antibiotic daptomycin. In previous studies, we reported that the secreted glycofragment of the signaling mucin Msb2 (Msb2*) protects *C. albicans* and *E. coli* against the toxicity of human AMPs LL-37 and histatin-5 (10). It is shown here that Msb2* also prevents toxicity of two other human AMPs, human α-defensin-1 (hNP-1) and human β-defensin-1 (hBD1) (Table 3). This Msb2* activity was demonstrated by exposing *C. albicans* CAF2-1 (27) for 1.5 h to AMPs in the absence or presence of purified Msb2* (10 μg), followed by assessment of fungal viability by determination of CFU. For all tested AMPs, a clear protective effect of Msb2* against *C. albicans* killing was observed. Note that in the assay for hBD1 activity, the incubation was carried out not only under normoxia but also in an hypoxic chamber (0.2% O_2, 0.2% CO_2), because under hypoxia, reduction of disulfide bonds leads to activation of this AMP (24). We indeed observed increased killing by hBD1 under hypoxia compared to normoxia, but importantly, Msb2* provided protection under both conditions.

AMPs not only are of human origin but are also produced by numerous microorganisms (28). Some microbial AMPs, including daptomycin and vancomycin, are of special importance for antibiotic therapy of infectious diseases, because they serve as reserve antibiotics for the treatment of multiresistant Gram-positive bacteria (17–19). Here we tested if the presence of *C. albicans* Msb2* affects the antibiotic activity of daptomycin, and we could

TABLE 2 Dissociation constants for binding of 5-FAM-LL-37

Protein	pH	K_D (nM, mean ± SD)
Msb2*	7.4	73.1 ± 23.3
	6.0	70.7 ± 14.7
	8.0	86.1 ± 17.9
Msb2* + unlabeled LL-37[a]	7.4	328 ± 46.9
Msb2* boiled[b]	7.4	418 ± 33.7
Msb2ΔN*	7.4	86.4 ± 17.6
	6.0	58.1 ± 17.5
	8.0	55.3 ± 6.34
Msb2* from *pmt1*Δ	7.4	951 ± 80.9
Mucin (porcine stomach)	7.4	876 ± 48.9
Msb2* + daptomycin[c]	7.4	498 ± 16.8

[a] Preincubation for 15 min with 4.25 μM unlabeled LL-37.
[b] 95°C, 15 min.
[c] Preincubation for 15 min with 50 μM daptomycin.

TABLE 3 Msb2* protects *C. albicans* against different AMPs

Antimicrobial peptide and condition	Net charge	Concn (μM)	CFU (%, mean ± SD)	
			Without Msb2*	With Msb2*
LL-37	+6	26.7	51.7 ± 0.7	97.5 ± 0.9
Histatin-5	+12	26.4	79.6 ± 2.1	114.6 ± 3.7
hNP-1	+3	23.2	72.7 ± 4.4	98.8 ± 6.7
hBD1				
Normoxia	+6	37.2	82.1 ± 7.8	96.3 ± 1.6
Hypoxia[a]	+6	37.2	75.4 ± 2.0	107.1 ± 4.0

[a] 0.2% O_2, 0.2% CO_2.

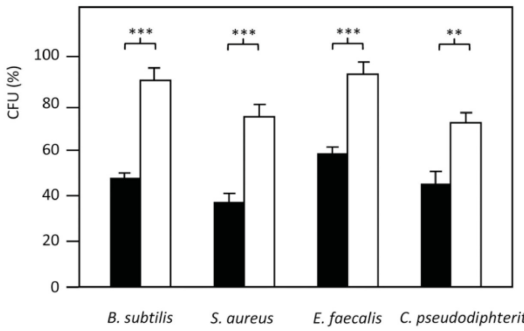

FIG 2 Msb2 protects bacterial pathogens against daptomycin. Bacteria were incubated with 10 μg/ml daptomycin for 30 min in the absence (black bars) or presence (white bars) of Msb2* (50 μg/ml). Numbers of surviving bacterial cells were determined by plating (CFU counts), with values for a control incubation without daptomycin set as 100%. Mean values of three measurements ± standard deviation are shown. ***, $P < 0.0001$. The bacterial strains were *S. aureus* Sa113, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6051, and *C. pseudodiphtheriticum* ATCC 10701.

indeed show that Msb2* protected the Gram-positive bacteria *Bacillus subtilis* (29), *S. aureus* (30), *E. faecalis* (31), and *C. pseudodiphtheriticum* (32) against the inhibitory action of daptomycin (Fig. 2). Furthermore, daptomycin competed with 5-FAM-LL37 for binding to Msb2* (Table 2), suggesting binding of daptomycin to a specific site on the Msb2* protein, which also binds LL-37. In additional experiments we also tested Msb2* for its protective effects against the glycopeptide vancomycin, but we did not observe significant protection by Msb2* using this antibiotic (data not shown).

Collectively, these results suggest that the shed *C. albicans* Msb2 glycofragment protects *C. albicans* and several Gram-positive bacteria, including important human bacterial pathogens, against a wide range of human AMPs and the relevant current antibiotic daptomycin.

Cross-species protection mediated by Msb2 in coculturings. The Gram-positive bacterium *S. aureus* and the fungus *C. albicans* are currently among the leading nosocomial pathogens, often coinfecting patients and leading to high mortality (33). It is known that *C. albicans* and *S. aureus* are able to form complex polymicrobial biofilms (34), in which *S. aureus* is protected from the action of vancomycin (35). We considered the possibility that Msb2* produced by live *C. albicans* cells could also help to protect cohabitating cells of *S. aureus* against daptomycin. In a first series of experiments, mixed planktonic *C. albicans*-*S. aureus* cultures were generated by adding bacteria to stationary cultures of *C. albicans* strains either producing or not producing Msb2*. In the absence of daptomycin, bacterial growth occurred equally with all *C. albicans* strains (Fig. 3A, left panel). However, in the presence of daptomycin, killing of *S. aureus* was maximal in cohabitation with the *msb2* mutant (strain FCCa27), which is unable to produce functional Msb2, while cohabitation with Msb2*-producing strains led to significantly reduced killing of bacteria (Fig. 3A, right panel). This finding was confirmed in a second experimental approach using cohabitation in mixed biofilms. In triplicate experiments, Msb2-producing strains of *C. albicans* (CAF2-1 and ESCa3) or the *msb2* mutant (strain FCCa27) were allowed to form a mixed biofilm with *S. aureus* (Sa 113) for 24 or 48 h at 37°C in

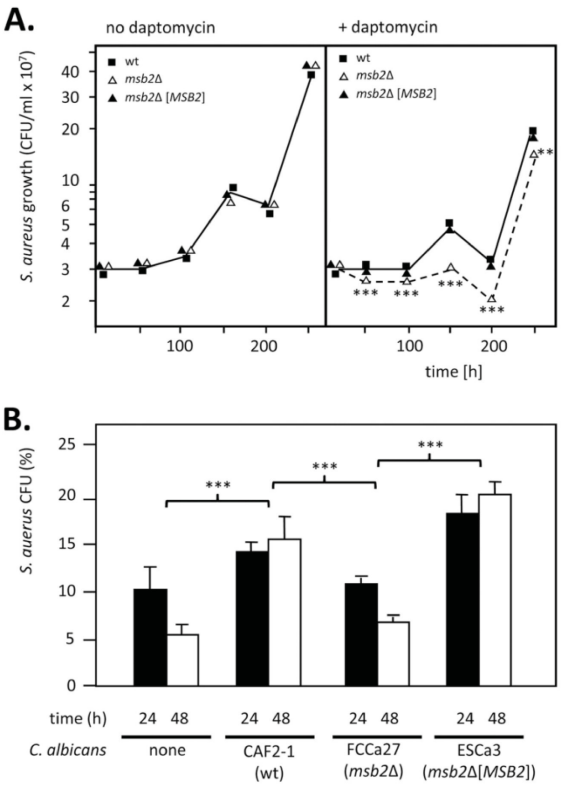

FIG 3 Cocultured *C. albicans* protects *S. aureus* against daptomycin. (A) Planktonic coculturing. *C. albicans* strains CAF2-1 (wild type [wt]), FCCa27 (*msb2Δ1*), and ESCa3 (*msb2Δ1* [*MSB2*]) were grown in 5 ml YPD medium to an OD_{600} of 8. *S. aureus* (Sa113) cells (3×10^7/ml) were added, and incubation was continued for 30 min at 37°C. No daptomycin (left panel) or 5 μg/ml daptomycin (right panel) was added, and numbers of viable *S. aureus* (Sa113) cells (CFU) were determined by plating on LB-caspofungin agar at different times of coincubation. (B) Biofilm coculturing. Mixed biofilms of *C. albicans* and *S. aureus* were allowed to form for 24 or 48 h before addition of daptomycin (10 μg/ml). Biofilms were disassembled following 24 h (black bars) or 48 h (white bars), and numbers of bacterial survivors were determined by plating on LB-caspofungin agar. CFU of a single-species *S. aureus* biofilm without added daptomycin were set as 100%.

RPMI 1640 medium; in a control experiment, *C. albicans* cells were omitted to allow growth of an *S. aureus* monoculture biofilm. Following growth, 10 μg/ml daptomycin was added and cells were incubated further for 5 h. Biofilms were then disassembled, and live cell counts were determined on LB agar containing caspofungin to select for bacterial survivors.

The results indicate that *S. aureus* growth was inhibited to the same extent by daptomycin in the control culture and in cohabitation with the *msb2* mutant, while significantly greater numbers of *S. aureus* survivors were found in the coculture with the *C. albicans* Msb2* strain (Fig. 3B). It is concluded that production of Msb2* by *C. albicans* can partially overcome daptomycin toxicity and protect cohabitating *S. aureus* cells.

DISCUSSION

Disease caused by *C. albicans* manifests itself in the form of massive fungal growth on skin and mucous layers, as well as at multi-

ple systemic body sites. Approximately one-fourth of all *C. albicans* bloodstream infections appear to be polymicrobial (33), and coinfection with *C. albicans* and the important bacterial pathogen *S. aureus* in mice is synergistic to increase mortality (36). *C. albicans* and *S. aureus* were shown to interact directly by forming a mixed biofilm (34) that depended to a considerable extent on the *C. albicans* Als3 cell wall protein (37). Importantly, mixed biofilm formation with *C. albicans* enhanced the resistance of *S. aureus* to vancomycin (35), which, like the more recent antibiotic daptomycin, is being used as a reserve-type antibiotic to combat multiresistant Gram-positive bacterial pathogens, including *Staphylococcus*, *Enterococcus*, and *Corynebacterium* species. Here we describe a novel molecular strategy explaining how the *C. albicans*-*S. aureus* cohabitation can improve survival of the bacterial pathogen during antibiotic treatment. It is shown that a fragment of the Msb2 surface protein, which is secreted in considerable amounts during planktonic and surface growth of *C. albicans* (10), is able to protect *S. aureus*, *E. faecalis*, and *C. pseudodiphtheriticum* against the toxic activity of daptomycin. This protection was detected using purified Msb2* protein but also in coculturing with Msb2*-producing *C. albicans* strains and is expected to occur by binding of daptomycin to Msb2*, because daptomycin competed with LL-37 in MST measurements. The Msb2* binding activity lowers the effective concentration of antibiotic in liquid culture and around mixed microbial colonies and biofilms occurring in the host. Thus, these data indicate that for treatment of *S. aureus* infections, attention should be given to *C. albicans* coinfection, which should be treated simultaneously to ensure the success of antibacterial antibiotic therapy.

Here we have quantitated for the first time the affinity of a fungal secretory protein for a human AMP by using MST technology (22, 23). This method allows measurement of the affinity of protein-protein interactions in solution, which more closely reflects native conditions than binding assays using surface-bound molecules. The average dissociation constant (K_D) of 73 nM indicates a very high affinity of the cathelicidin LL-37 for the Msb2* glycoprotein, whereas the abundant glycoprotein mucin, on the other hand, binds to LL-37 only weakly (K_D = 876 nM). Thus, mammalian mucins and possibly other human glycostructures (38) may loosely bind or store AMPs, but they lack tight AMP binding activity (like *C. albicans* mucin Msb2*), because such activity could prevent release and accessibility of human AMPs, which would be counterproductive during microbial attack. MST technology may be used in the future to determine dissociation constants of AMPs with other body or microbial glycostructures (5–7, 38–42).

The structural requirements leading to tight binding of AMPs to Msb2* remain to be determined in detail. We previously determined that AMP interactions still occurred with an Msb2* variant lacking 40% of the N-terminal residues (variant Msb2-ΔN*); furthermore, the presence of *O*-mannosyl residues (43) was found to be important for efficient AMP regulation (10). Here we verified by MST technology the importance of *O*-mannosylation for AMP binding by demonstrating that Msb2* isolated from a *pmt1* mutant showed low affinity for LL-37. Denatured Msb2*, however, had minor AMP binding activity, indicating that glycostructures *per se* are not sufficient for binding. Regarding the types of AMPs bound by Msb2*, a surprising variety of structures was found, including the human AMPs LL-37, histatin-5 (10), and α- and β-defensins, as well as the microbial AMP daptomycin. Net charges of AMPs bound by Msb2* vary greatly and range from −1 (daptomycin) to +12 (histatin-5), indicating that charge interactions may contribute to but are not essential for these interactions. Interestingly, binding of LL-37 to Msb2* was competed by daptomycin, suggesting one or more specific binding sites for various AMPs on Msb2*. It will be of great interest to clarify the molecular details of these interactions and to establish why some peptides, including human AMPs and daptomycin, bind strongly to Msb2* while other peptides, including vancomycin, interact only weakly.

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REFERENCES

1. **Dürr M, Peschel A.** 2002. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. Infect. Immun. **70:**6515–6517.
2. **van der Meer JW, van de Veerdonk FL, Joosten LA, Kullberg BJ, Netea MG.** 2010. Severe *Candida* spp. infections: new insights into natural immunity. Int. J. Antimicrob. Agents **36:**S58–62.
3. **Peschel A, Sahl H-G.** 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4:**529–536.
4. **Meiller TF, Hube B, Schild L, Shirtliff ME, Scheper MA, Winkler R, Ton A, Jabra-Rizk MA.** 2009. A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. PLoS One **4:**e5039. doi:10.1371/journal.pone.0005039.
5. **Frick IM, Akesson P, Rasmussen M, Schmidtchen A, Björck L.** 2003. SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. J. Biol. Chem. **278:**16561–16566.
6. **Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, Tarkowski A.** 2004. Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J. Immunol. **172:**1169–1176.
7. **Frick IM, Karlsson C, Mörgelin M, Olin AI, Janjusevic R, Hammarström C, Holst E, de Château M, Björck L.** 2008. Identification of a novel protein promoting the colonization and survival of *Finegoldia magna*, a bacterial commensal and opportunistic pathogen. Mol. Microbiol. **70:**695–708.
8. **Cullen PJ.** 2007. Signaling mucins: the new kids on the MAPK block. Crit. Rev. Eukaryot. Gene Expr. **17:**241–257.
9. **Sorgo AG, Heilmann CJ, Dekker HL, Brul S, de Koster CG, Klis FM.** 2010. Mass spectrometric analysis of the secretome of *Candida albicans*. Yeast **27:**661–672.
10. **Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Román E, Pla J, Ernst JF.** 2012. Msb2 shedding protects *Candida albicans* against antimicrobial peptides. PLoS Pathog. **8:**e1002501. doi:10.1371/journal.ppat.1002501.
11. **Puri S, Kumar R, Chadha S, Tati S, Conti HR, Hube B, Cullen PJ, Edgerton M.** 2012. Secreted aspartic protease cleavage of *Candida albicans* Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis. PLoS One **7:**e46020. doi:10.1371/journal.pone.0046020.
12. **Román E, Cottier F, Ernst JF, Pla J.** 2009. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. Eukaryot. Cell **8:**1235–1249.
13. **Cantero PD, Ernst JF.** 2011. Damage to the glycoshield activates *PMT*-directed O-mannosylation via the Msb2-Cek1 pathway in *Candida albicans*. Mol. Microbiol. **80:**715–725.
14. **Ernst JF, Pla J.** 2011. Signaling the glycoshield: maintenance of the *Candida albicans* cell wall. Int. J. Med. Microbiol. **301:**378–383.
15. **Ganz T.** 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. **3:**710–720.
16. **Ericksen B, Wu Z, Lu W, Lehrer RI.** 2005. Antibacterial activity and specificity of the six human α-defensins. Antimicrob. Agents Chemother. **49:**269–275.

17. **Fowler VG Jr, Boucher HW, Corey GR, Abrutyn E, Karchmer AW, Rupp ME, Levine DP, Chambers HF, Tally FP, Vigliani GA, Cabell CH, Link AS, DeMeyer I, Filler SG, Zervos M, Cook P, Parsonnet J, Bernstein JM, Price CS, Forrest GN, Fätkenheuer G, Gareca M, Rehm SJ, Brodt HR, Tice A, Cosgrove SE.** 2006. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. N. Engl. J. Med. **355:**653–665.
18. **Vilhena C, Bettencourt A.** 2012. Daptomycin: a review of properties, clinical use, drug delivery and resistance. Mini Rev. Med. Chem. **12:**202–209.
19. **Otto M.** 2012. MRSA virulence and spread. Cell. Microbiol. **14:**1513–1521.
20. **Glenister DA, Salamon KE, Smith K, Beighton D, Keevil CW.** 1988. Enhanced growth of complex communities of dental plaque bacteria in mucin-limited continuous culture. Microb. Ecol. Health Dis. **1:**31–38.
21. **Fogg FJ, Hutton DA, Jumel K, Pearson JP, Harding SE, Allen A.** 1996. Characterization of pig colonic mucins. Biochem. J. **316:**937–942.
22. **Wienken CJ, Baaske P, Rothbauer U, Braun D, Duhr S.** 2010. Protein-binding assays in biological liquids using microscale thermophoresis. Nat. Commun. **1:**100.
23. **Zillner K, Jerabek-Willemsen M, Duhr S, Braun D, Längst G, Baaske P.** 2012. Microscale thermophoresis as a sensitive method to quantify protein: nucleic acid interactions in solution. Methods Mol. Biol. **815:**241–252.
24. **Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J.** 2011. Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1. Nature **469:**419–423.
25. **Bansil R, Turner BS.** 2006. Mucin structure, aggregation, physiological functions and biomedical applications. Curr. Opin. Colloid Interface Sci. **11:**164–170.
26. **Herp A, Wu AM, Moschera J.** 1979. Current concepts of the structure and nature of mammalian salivary mucous glycoproteins. Mol. Cell. Biochem. **23:**27–44.
27. **Fonzi WA, Irwin MY.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
28. **Hegedüs N, Marx F.** 2013. Antifungal proteins: more than antimicrobials? Fungal Biol. Rev. **26:**132–145.
29. **Kabisch J, Thürmer A, Hübel T, Popper L, Daniel R, Schweder T.** 2013. Characterization and optimization of *Bacillus subtilis* ATCC 6051 as an expression host. J. Biotechnol. **163:**97–104.
30. **Iordanescu S, Surdeanu M.** 1976. Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. J. Gen. Microbiol. **96:**277–281.
31. **Kim EB, Kopit LM, Harris LJ, Marco ML.** 2012. Draft genome sequence of the quality control strain *Enterococcus faecalis* ATCC 29212. J. Bacteriol. **194:**6006–6007.
32. **Díez-Aguilar M, Ruiz-Garbajosa P, Fernández-Olmos A, Guisado P, Del Campo R, Quereda C, Cantón R, Meseguer MA.** 2013. Non-diphtheriae *Corynebacterium* species: an emerging respiratory pathogen. Eur. J. Clin. Microbiol. Infect. Dis. **32:**769–772.
33. **Klotz SA, Chasin BS, Powell B, Gaur NK, Lipke PN.** 2007. Polymicrobial bloodstream infections involving Candida species: analysis of patients and review of the literature. Diagn. Microbiol. Infect. **59:**401–406.
34. **Harriott MM, Noverr MC.** 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. Antimicrob. Agents Chemother. **53:**3914–3922.
35. **Harriott MM, Noverr MC.** 2010. Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. Antimicrob. Agents Chemother. **54:**3746–3755.
36. **Carlson E, Johnson G.** 1985. Protection by *Candida albicans* of *Staphylococcus aureus* in the establishment of dual infection in mice. Infect. Immun. **50:**655–659.
37. **Peters BM, Ovchinnikova ES, Krom BP, Schlecht LM, Zhou H, Hoyer LL, Busscher HJ, van der Mei HC, Jabra-Rizk MA, Shirtliff ME.** 2012. Staphylococcus aureus adherence to *Candida albican*s hyphae is mediated by the hyphal adhesin Als3p. Microbiology **158:**2975–2986.
38. **Baranska-Ryback W, Sonesson A, Nowicki R, Schmidtchen A.** 2006. Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids. J. Antimicrob. Chemother. **57:**260–265.
39. **Foschiatti M, Cescutti P, Tossi A, Rizzo R.** 2009. Inhibition of cathelicidin activity by bacterial exopolysaccharides. Mol. Microbiol. **72:**1137–1146.
40. **Luo S, Poltermann S, Kunert A, Rupp S, Zipfel PF.** 2009. Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a factor H, FHL-1 and plasminogen binding surface protein. Mol. Immunol. **47:**541–550.
41. **Tsai PW, Yang CY, Chang HT, Lan CY.** 2011. Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. PLoS One **6:**e17755. doi:10.1371/journal.pone.0017755.
42. **Scott A, Weldon S, Buchanan PJ, Schock B, Ernst RK, McAuley DF, Tunney MM, Irwin CR, Elborn JS, Taggart CC.** 2011. Evaluation of the ability of LL-37 to neutralise LPS in vitro and ex vivo. PLoS One **6:**e26525. doi:10.1371/journal.pone.0026525.
43. **Prill SK, Klinkert B, Timpel C, Gale CA, Schröppel K, Ernst JF.** 2005. PMT family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. Mol. Microbiol. **55:**546–560.

2.5 Manuskript IV: Signaling domains of mucin Msb2 in *Candida albicans*

Marc Swidergall, Lasse van Wijlick, and Joachim F. Ernst

Erstautor

eigener Anteil an der Arbeit in %: 85

Marc Swidergall hat folgende Experimente geplant, durchgeführt und interpretiert: Konstruktion von Msb2-Varianten; Phänotypisierung der *C. albicans*-Stämme; qPCR; Phosphorylierungs-Blots der Kinasen Mkc1 und Cek1; Immunfluoreszenz, AMP-Assays; Transkriptom-Analysen.
Marc Swidergall schrieb einen Teil des Manuskripts.

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Signaling Domains of Mucin Msb2 in *Candida albicans*

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Candida albicans **adapts to the human host by environmental sensing using the Msb2 signal mucin, which regulates fungal morphogenesis and resistance characteristics. Msb2 is anchored within the cytoplasmic membrane by a single transmembrane (TM) region dividing it into a large N-terminal exodomain, which is shed, and a small cytoplasmic domain. Analyses of strains carrying deleted Msb2 variants revealed an exodomain segment required for cleavage, shedding, and all functions of Msb2. Phosphorylation of the mitogen-activated protein kinase (MAP kinase) Cek1 was regulated by three distinct regions in Msb2: in unstressed cells, N-terminal sequences repressed phosphorylation, while its induction under cell wall stress required the cytoplasmic tail (C-tail) and sequences N-terminally flanking the TM region, downstream of the proposed cleavage site. Within the latter Msb2 region, overlapping but not identical sequences were also required for hyphal morphogenesis, basal resistance to antifungals, and, in unstressed cells, downregulation of the *PMT1* transcript, encoding protein *O*-mannosyltransferase-1. Deletion of two-thirds of the exodomain generated a truncated Msb2 variant with a striking ability to induce hyperfilamentous growth, which depended on the presence of the Msb2-interacting protein Sho1, the MAP kinase Cek1, and the Efg1 transcription factor. Under cell wall stress, the cytoplasmic tail relocalized partially to the nucleus and contributed to regulation of 117 genes, as revealed by transcriptomic analyses. Genes regulated by the C-tail contained binding sites for the Ace2 and Azf1 transcription factors and included the *ALS* cell wall genes. We concluded that Msb2 fulfills its numerous functions by employing functional domains that are distributed over its entire length.**

Upon proliferation and dissemination in a host tissue or target cell, pathogens encounter different types of host cells, extracellular matrices, and molecules that participate in pathogen defense. Upon contact with fungal pathogens, the human host utilizes an efficient and complex immune response that acts in multiple layers, including pathogen recognition and production of soluble innate effectors (1). To survive and overcome host immunity, pathogenic fungi have evolved sophisticated strategies which include two major steps: immune evasion and tissue invasion (2). Polysaccharides and glycoproteins in the fungal cell wall are the first contact points between the fungus and human immune cells, representing pathogen-associated molecular patterns (PAMPs). PAMPs are detected by human pattern recognition receptors (PRRs) and respond to a variety of innate immune components (3), including antimicrobial peptides (AMPs), which constitute major weapons of innate immunity that can directly kill fungal pathogens (4, 5). In response to the host, modification of PAMPs by cell wall remodeling or by hypha formation alters their availability for PRRs in many fungal species (6). The cellular morphology of the important human fungal pathogen *Candida albicans* is determined by host contact, which regulates morphogenetic signaling pathways depending on the type and intensity of environmental cues in body niches (7).

Fungal signaling mucins are large, transmembrane glycoproteins that undergo posttranslational modifications, such as glycosylation and proteolytic processing, which are crucial for their cellular functions (8–13). The *C. albicans* mucin Msb2 triggers responses and rescue pathways required for hyphal morphogenesis and growth in the presence of antifungal compounds (8, 14). Msb2 is cleaved to release and shed its large glycosylated exodomain, which is able to bind AMPs with a high affinity and thereby to protect fungal cells (15). The yeast-to-hypha transition is regulated by several signal transduction cascades containing protein kinase A (PKA), mitogen-activated protein kinases (MAP kinases), and pH-responsive modules (16). Msb2 triggers hyphal morphogenesis via the Cek1 MAP kinase module. Defects in cell wall glycostructures affecting protein glycosylation and β1,3-glucan levels are sensed by Msb2 and transmitted to the Cek1 MAP kinase cascade regulating target genes, e.g., *PMT* genes, encoding protein-*O*-mannosyltransferases (14, 17). Intact *N*-glycosylation is detected by Msb2 and represses *PMT1* transcription, while defective *N*-glycosylation induces Cek1 phosphorylation and derepresses *PMT1* transcription. The responses to defective *O*-glycosylation also require Msb2 to upregulate *PMT2* and *PMT4* expression (17). Recently, the transcription factor Ace2 was implicated in the regulation of *PMT* genes (17).

The structure of signaling mucins consists of a large, highly glycosylated, rod-shaped extracellular domain that is connected to a small, cytosolic carboxy-terminal domain via a transmembrane (TM) region (9). Previous results indicated that the cytoplasmic tail (C-tail) of Msb2 in *C. albicans* is needed to activate Cek1 in response to cell wall defects (8). In the present work, a systematic deletion analysis of Msb2 identified a mosaic of functional regions in the Msb2 structure, which contribute to various

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TABLE 1 *C. albicans* strains used for this study

Strain	Genotype	Reference
AS1	CAI4, but *tpk2*Δ::*hisG*/*tpk2*Δ::*hisG*	41
CAF2-1	*ura3*Δ::*imm434*/*URA3*	42
CAI4	*ura3*::*imm434*/*ura3*::*imm434*	43
CK43B-16L	*ura3*/*ura3 cek1*Δ::*hisG*/*cek1*Δ::*hisG*	44
CKY157	*ura3*/*ura3 czf1*::*hisG*/*czf1*::*hisG*	45
HLC67	CAI4, but *efg1*::*hisG*/*efg1*::*hisG*	46
JKC18	CAI4, but *cph1*::*hisG*/*cph1*::*hisG*	47
IIHH6-4a	CAI4, but *tpk1*Δ::*hisG*/*tpk1*Δ::*hisG*	48
FCCa27	CAI4, but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG-URA3-hisG*	8
FCCa28	CAI4, but *msb2*Δ::*hisG*/*msb2*Δ::*hisG*	8
ESCa3	CAI4, but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG LEU2*/*LEU2*::pES11a(*ACT1p-MSB2*$^{HA\text{-}V5}$)	8
ESCa25	CAI4, but *msb2*Δ*1*::*hisG*/*msb2*Δ*1*::*hisG LEU2*/*LEU2*::pES14(*ACT1p-MSB2-*Δ*N*$^{HA\text{-}V5}$)	8
ESCa38	FCCa28, but *LEU2*/*LEU2*::pES16(*ACT1p-MSB2-*ΔC^{HA})	8
ESCa39	FCCa28, but *LEU2*/*LEU2*::pES17(*ACT1p-MSB2-*Δ*TM-*C^{HA})	8
REP22	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG-msb2*Δ::*FRT*/*msb2*Δ::*FRT sho1*::*hisG*/*sho1*::*hisG*	14
LvW1000	CAI4, but *LEU2*/*LEU2*::pDS1044-1 (empty vector)	This study
MSCa1	FCCa28, but *LEU2*/*LEU2*::pSM4(*ACT1p-MSB2*$^{\Delta 1293–1307}$)	This study
MSCa3	FCCa28, but *LEU2*/*LEU2*::pSM4(*ACT1p-MSB2*$^{\Delta 1278–1292}$)	This study
MSCa8	FCCa28, but *LEU2*/*LEU2*::pSM6(*ACT1p-MSB2*Δ$N^{514–680}$)	This study
MSCa29	FCCa28, but *LEU2*/*LEU2*::pSM7(*ACT1p-MSB2*Δ$N^{682–780}$)	This study
MSCa30	FCCa28, but *LEU2*/*LEU2*::pSM9(*ACT1p-MSB2*$^{\Delta 788–853}$)	This study
MSCa31	FCCa28, but *LEU2*/*LEU2*::pSM10(*ACT1p-MSB2*$^{\Delta 855–939}$)	This study
MSCa34	FCCa28, but *LEU2*/*LEU2*::pSM11(*ACT1p-MSB2*$^{\Delta 920–1087}$)	This study
MSCa35	FCCa28, but *LEU2*/*LEU2*::pSM12(*ACT1p-MSB2*$^{\Delta 1085–1282}$)	This study
MSCa37	FCCa28, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa38	CAI4, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2 MSB2*Δ$N^{514–1087}$)	This study
MSCa39	CK43B-16L, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa40	CKY157, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa41	HLC67, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa42	JKC18, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa43	IIHH6-4a, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa44	AS1, but *LEU2*/*LEU2*::pSM14(*ACT1p-MSB2*Δ$N^{514–1087}$)	This study
MSCa45	FCCa28, but *LEU2*/*LEU2*::pSM15(*ACT1p-MSB2*Δ*N-*$C^{514–1087}$)	This study
MSCa46	FCCa28, but *LEU2*/*LEU2*::pSM16(*ACT1p-MSB2*Δ*N-*$TMC^{514–1087}$)	This study
MSCa47	REP22, but *LEU2*/*LEU2*::pSM14(*ACT1p- MSB2*Δ$N^{514–1087}$)	This study
MSCa48	FCCa28, but *LEU2*/*LEU2*::pSM17(*ACT1p-MSB2 MSB2*Δ$^{514–1087}$)	This study

cellular processes, including hypha formation, activation of the MAP kinase Cek1, basal antifungal resistance, and regulation of the transcriptome, including regulation of *PMT1*. Thus, the combined Msb2 activities help to protect *C. albicans* from immune reactions and treatment with antifungal compounds.

MATERIALS AND METHODS

Strains and media. *C. albicans* strains used for this study are listed in Table 1. *MSB2* expression vectors digested with EcoRV were integrated ectopically into the *LEU2* locus of strain FCCa28, which places *MSB2* alleles under transcriptional control of the *ACT1* promoter (8). Strains were grown on/in complex yeast extract-peptone-dextrose (YPD) or minimal supplemented SD medium (8). Standard drop dilution tests (10-fold dilutions to 10^{-5}) were used to determine sensitivity to inhibitors on YPD agar. Hypha formation was induced by growth at 37°C on YPM medium (1% yeast extract, 2% peptone, 2% mannitol).

***MSB2* expression vectors.** Expression vectors encoding Msb2 variants were constructed by primer-directed mutagenesis of plasmid pES11a (8), using a QuikChange kit (Stratagene). Sequences of oligonucleotide primers are listed in Table S1 in the supplemental material. Designations of deletion variants (with the encoding plasmids and mutagenic primers) were as follows: ΔD3 (plasmid pSM3; primer pair Msb2-15ASDel1 Fwd/Rev), ΔD4 (pSM4; Msb2-15ASDel2 Fwd/Rev), ΔD5 (pSM12; Msb2-Del12 Fwd/Rev), ΔD6 (pSM11; Msb2-Del11 Fwd/Rev), ΔD7 (pSM10; Msb2-Del10 Fwd/Rev), ΔD8 (pSM9; Msb2-Del9 Fwd/Rev), ΔD9 (pSM17; Msb2-Del6 Rev/Msb2-Del11 Fwd), ΔD11 (pSM14; Msb2-Del6 Rev/Msb2-Del11), ΔD14 (pSM7; Msb2-Del7 Fwd/Rev), and ΔD15 (pSM6; Msb2-Del6 Fwd/Rev). pSM15/16 was based on construct pSM14. pES16/17 (8) and pSM14 were cut with ApaI and Bsu36I. Fragments including stop codons upstream or downstream of the TM domain were ligated into pSM14, encoding the ΔD12 and ΔD13 variants. Plasmids were integrated into the *LEU2* locus of strain FCCa28. Integration into the *LEU2* locus was verified by colony PCR with primers Kolo AMP Fwd and Kolo LEU2 Rev.

RNA methods. RNAs were isolated from all cultures, and transcriptomic analyses were performed essentially as described previously (18). Cy3- and Cy5-labeled cDNAs generated from RNAs of strains ESCa3 and ESCa38 were cohybridized to *C. albicans* genomic arrays (Eurogentec, Belgium). The arrays were read and evaluated using GeneSpring software as described previously. Genes were considered to be significantly regulated ($P < 0.05$) if the expression ratios for strains ESCa3 and ESCa38 were ≥1.5 during caspofungin treatment (50 ng/ml; 30 min).

For quantitative PCR (qPCR) analyses, total RNA was isolated, treated with DNase I (Turbo DNase kit; Ambion), and purified using an RNA cleanup kit (Zymo Research). One to 2 μg of purified RNA was reverse transcribed into cDNA (Maxima First Strand cDNA synthesis kit; Thermo Scientific), using nuclease-free water (protocols of the suppliers were used for all steps). A parallel sample was not reverse transcribed as a control for

contaminated DNA. The qPCR assay was done using a model Mx3000P (Stratagene) machine, with 10 μl of cDNA sample (1:10), 4 μl EvaGreen QPCR-mix II (Bio-Budget), and 3 μl each of forward and reverse oligonucleotide primers (400 pmol/μl) in each reaction mixture. AmpliTaq polymerase was activated at 95°C for 10 min, and annealing was performed at 60°C for 20 s. The extension step was performed at 72°C for 30 s, and the denaturation step was performed at 95°C for 30 s; a total of 40 cycles were completed.

Protein methods. Strains were grown in 50 ml YPD or SD medium at 30°C to an optical density at 600 nm (OD_{600}) of 0.8 or 6 to 10, respectively, and cells were harvested by centrifugation. Cells were washed with water and resuspended in lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100) containing protease inhibitors (Complete Mini; Roche). Cells were broken by shaking with glass beads at 4°C with a FastPrep homogenizer (MP Biochemicals). Cell debris and glass beads were separated from the crude cell extract by centrifugation. For immunoblotting, proteins were separated by SDS-PAGE (12% SDS, 4 to 20% acrylamide) and blotted to polyvinylidene difluoride (PVDF) membranes. Protein standards used were the PageRuler set (11 to 170 kDa; Fermentas) and the HiMark set (31 to 460 kDa; Invitrogen) of proteins. Membranes were probed using a rat anti-hemagglutinin (anti-HA) monoclonal antibody (1:2,000; Roche) and visualized using peroxidase-coupled goat anti-rat or anti-mouse antibody (1:10,000; Thermo Scientific) and the SuperSignal West Dura chemiluminescent substrate (Pierce). The membrane was processed as for immunoblotting, and the resulting signals were recorded using a Fujifilm LAS400 mini-image analyzer and evaluated with the Fujifilm Multi Gauge program. Signals were quantitated using ImageJ software (http://imagej.nih.gov/ij/).

MAP kinase activation assay. Strains were grown overnight to stationary phase in YPD medium and diluted in YPD medium to an OD_{600} of 0.1. Cells were grown to an OD_{600} of 0.8 at 37°C and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 μg/ml). Immunoblots were prepared as described previously, with verification of equal loading by Ponceau red staining of the membranes. Blots were probed with anti-phospho-p44/42 MAP kinase (Cell Signaling Technology) to detect phosphorylated Cek1 protein (8) or with a polyclonal Cek1 antibody to detect total Cek1 (14).

Fluorescence microscopy. Strains were grown in YPD at 30°C to an OD_{600} of 1 and were stained with calcofluor white (0.1% calcofluor white solution; Sigma) to visualize chitin. Cells (OD_{600} = 0.8) used for immunofluorescence microscopy were fixed with 4% formaldehyde, and 1 ml of cell suspension was treated with Zymolyase T100 (100 μg; 50 μl), glucuronidase (30 μl), and 10 mM dithiothreitol (DTT) for 30 min at 30°C. Cells were pelleted and treated with 0.1% Triton X-100 for 5 min at room temperature. Cells (20 μl) were fixed to polylysine-coated glass slides and washed with phosphate-buffered saline (PBS), followed by blocking of unspecific binding sites by use of 2% milk powder in PBS. The blocking solution was removed, and 40 μl of rat anti-V5 antibody (1:100; AbD Serotec) was allowed to react for 90 min at room temperature or overnight at 4°C in a wet chamber. Cells were washed, and fluorescein isothiocyanate (FITC)-coupled anti-mouse antibody (1:100; Sigma-Aldrich) in 0.2% milk powder was added and allowed to react for 90 min at room temperature. For nuclear staining, 20 μl 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) was added for 15 min at room temperature. Slides were washed with PBS, and a drop of antifade reagent (Pro-Long; Sigma) was added before covering the specimen with a coverslip, which was sealed with nail polish. Microscopic inspection of FITC and DAPI fluorescence was done using an Axioskop 40 microscope (Carl Zeiss, Goettingen, Germany), and the ImageJ program (http://imagej.nih.gov/ij/) was used for evaluation of fluorescence signals.

Antimicrobial peptide assays. Overnight cultures of *C. albicans* were diluted and grown in YPD at 30°C to an OD_{600} of 0.3. Cells were harvested by centrifugation and washed with and resuspended in PBS. Triplicate assay mixtures containing 5 μl cell suspension and 2 μg LL-37 (Sigma) in a total volume of 25 μl were incubated for 1.5 h at 37°C, diluted 500-fold, and plated on YPD. Numbers of CFU were determined after 2 days of growth at 30°C. Sensitivity to long-term exposure to LL-37 was determined by growing strains in the presence or absence of LL-37 (1 μg/200 μl; final concentration, 5 μg/ml). Triplicate assay mixtures were inoculated to an OD_{600} of 0.1 and were incubated for 7 h at 37°C on a rotary shaker at 115 rpm. To assess killing, the OD_{600} was measured and compared to that of untreated cells.

Statistical analysis. Statistical significance was determined using the unpaired *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

RESULTS

***C. albicans* strains producing Msb2 deletion variants.** The precursor of the membrane sensor protein Msb2 in *C. albicans* contains a single transmembrane (TM) region that separates a large, N-terminal glycosylated exodomain from a short, C-terminal cytoplasmic tail. The precursor is cleaved and the exodomain released in substantial amounts into the environment during planktonic and surface growth (8, 15, 19). Previous studies suggested that the N-terminal, TM, and cytoplasmic domains of Msb2 convey different cellular functions (8). To further define Msb2 functional domains, strains were constructed to produce additional Msb2 deletions; variants were designated ΔD1 to ΔD15 and included the previously described variants ΔN (ΔD10), ΔC (ΔD1), and ΔTM-C (ΔD2) (8) (Fig. 1). The respective *MSB2* alleles were chromosomally integrated into an *msb2* mutant strain and expressed under the control of the constitutive *ACT1* promoter (8), because preliminary results revealed that *MSB2* transcription is autoregulated via the Msb2-Cek1 pathway (L. van Wijlick and J. F. Ernst, unpublished results). Transformants produced all Msb2 variants at similar levels, although amounts of cell-associated/secreted proteins were different for certain variants (see below).

Hyphal morphogenesis and basal resistance to glycostress require specific Msb2 domains. As previously described, the *msb2* mutant and a strain producing an Msb2 variant lacking both the TM region and the cytoplasmic tail (ΔD2 variant) were not able to form hyphae on YPM agar, while removal solely of the cytoplasmic tail (ΔD1 variant) had no effect (8). Interestingly, a series of small deletions directly upstream of the TM region (ΔD3, ΔD4, and ΔD5 variants) led to defective hypha formation, indicating that the respective sequences (residues 1084 to 1308) collectively contribute to hyphal development (Fig. 2A). Defective hypha formation was not observed for any of the deletions further upstream, assigning Msb2 requirements for hyphal morphogenesis to a specific internal Msb2 segment.

Previous work indicated that *msb2* mutants are supersensitive to caspofungin and to other agents generating glycostress, including Congo red and Zymolyase (14). To define functional domains of Msb2 required for basal resistance, we tested the sensitivity of strains producing Msb2 variants to caspofungin and to low concentrations of tunicamycin, which blocks *N*-glycosylation. As expected, the *msb2* mutant was more sensitive than the wild-type strain to tunicamycin. Similarly, the Msb2 deletion series encompassing sequences immediately N-terminal to the TM region (ΔD3, ΔD4, and ΔD5 variants) was also more sensitive, while further upstream deletions (ΔD6 to ΔD10 variants) restored basal resistance (Fig. 1 and 2B). Collectively, the results indicate that basal resistance to glycostress requires specific internal Msb2 sequences, which coincide essentially with sequences required for hypha formation.

Shedding of the exodomain requires a specific Msb2 segment. To establish if specific sequences of Msb2 are needed for its

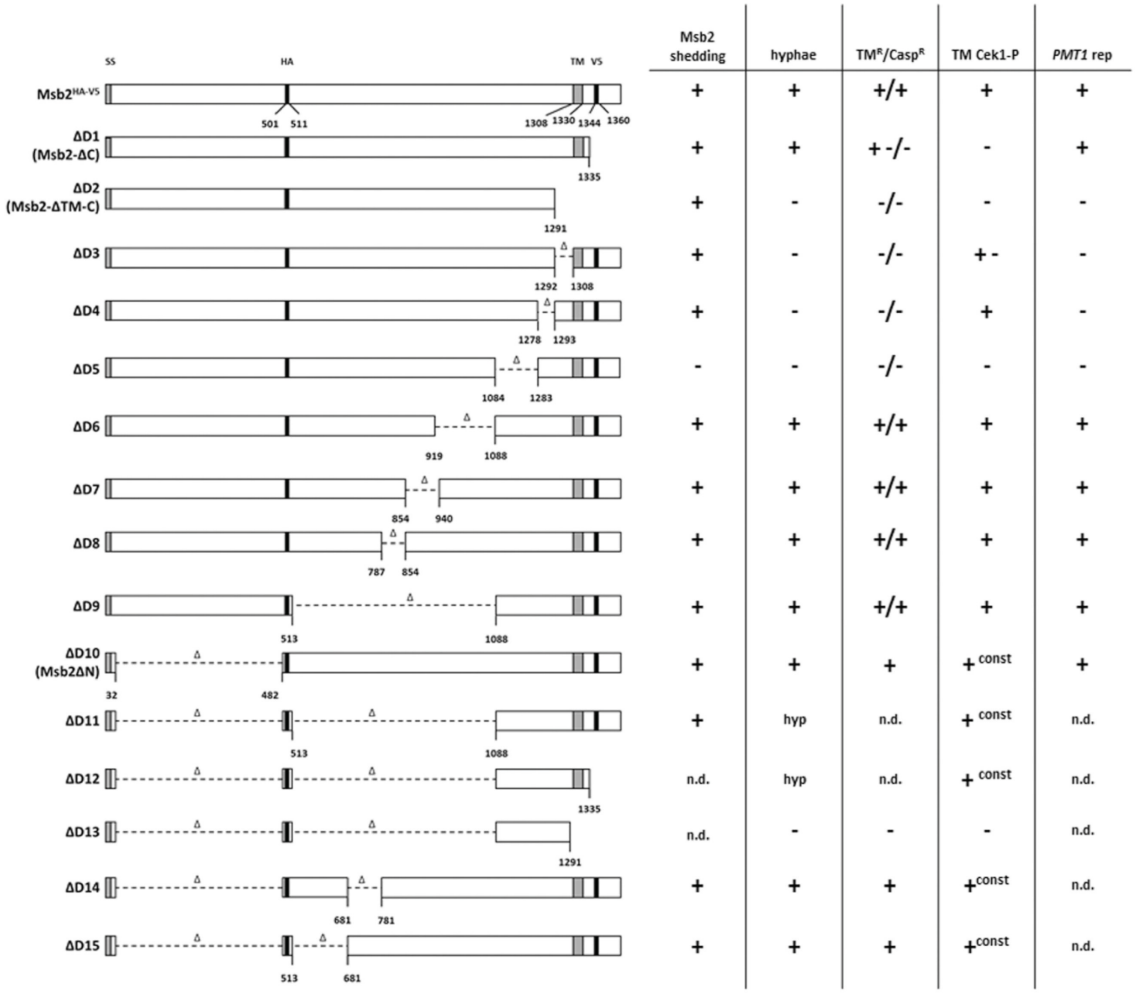


FIG 1 Structures and functions of Msb2 protein variants. Plasmids encoding Msb2 variants were chromosomally integrated into strain FCCa28, which produces the inactive Msb2-Δ1 variant via the *msb2Δ1* allele (8). The positions of the signal sequence (SS), the transmembrane region (TM), and HA and V5 epitope tags in Msb2 are indicated. The transformants (encoded variants) were strains ESCa3 ($Msb2^{HA-V5}$), ESCa38 (ΔD1), ESCa39 (ΔD2), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa34 (ΔD6), MSCa35 (ΔD5), MSCa30 (ΔD8), MSCa31 (ΔD7), MSCa48 (ΔD9), ESCa25 (ΔD10), MSCa37 (D11), MSCa45 (ΔD12), MSCa46 (ΔD13), MSCa29 (ΔD14), and MSCa8 (ΔD15). Data for strains ESCa25 (ΔD10; Msb2ΔN), ESCa38 (ΔD1; Msb2-ΔC), and ESCa39 (ΔD2; Msb2-ΔTM-C) have been reported previously (8). Listed Msb2 phenotypes include Msb2 shedding, hypha formation on YPM agar at 37°C, tunicamycin (TM^R) and caspofungin ($Casp^R$) resistances, phosphorylation of Cek1 induced by tunicamycin treatment (TM Cek1-P), and repression of *PMT1* expression (*PMT1* rep) under normal growth conditions. Hyperfilamentous growth (hyp) and constitutive phosphorylation of Cek1 during growth in YPD at 37°C (const) are listed. n.d., not determined.

shedding from *C. albicans* cells, we examined the presence of Msb2 in strains producing variants both in the growth medium and in association with cells. Because all Msb2 variants retained the HA epitope (Fig. 1), immunoblotting using an anti-HA antibody was performed. We discovered that only a single deletion variant (ΔD5), lacking 199 residues (residues 1085 to 1282), was not shed by such *C. albicans* cells, since the Msb2 signal was detected only in association with cells, not in the medium (Fig. 3). For all other Msb2 variants, evidence of shedding was observed by detecting both cell-associated Msb2 and Msb2 in the medium. These results suggest that proteolytic cleavage of Msb2 occurs between residues 1085 and 1282 as a precondition to allow shedding of the exodomain. This sequence is also required for all other Msb2-related phenotypes, suggesting that Msb2 shedding is essential for its functions.

Separate Msb2 sequences activate or suppress phosphorylated Cek1 levels. Msb2 has been described as an upstream element of a signaling pathway regulating levels of the phosphorylated form of the MAP kinase Cek1 (14). *msb2* mutants are unable to respond to glycostress provoked by defects in *N*- and *O*-protein glycosylation by increasing phosphorylated Cek1 (17). To identify the functional domains of Msb2 that mediate Cek1 regulation, we

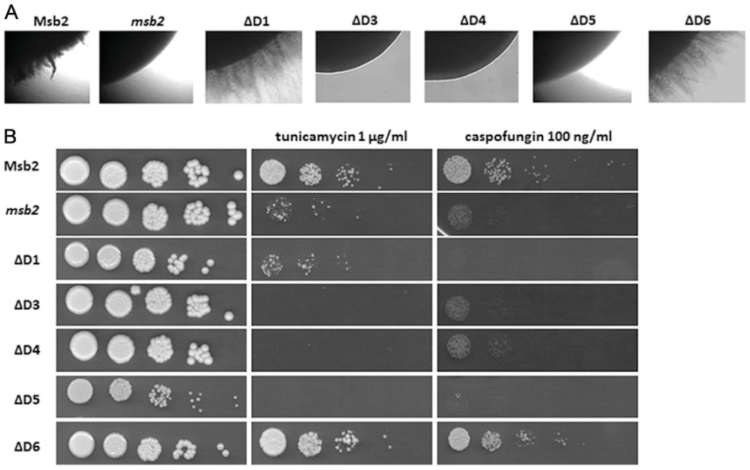


FIG 2 Msb2 variants affect morphogenesis and glycostress resistance. (A) Msb2 variants are defective in formation of hyphae on YPM agar. Colonies of strains were photographed following growth for 2 days at 37°C. Strains FCCa27 (*msb2*), ESCa38 (ΔD1), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa34 (ΔD6), and MSCa35 (ΔD5) were compared to ESCa3 ($Msb2^{HA\text{-}V5}$). (B) Sensitivities of strains to tunicamycin (1 μg/ml) and caspofungin (100 ng/ml) were tested by a drop dilution test.

examined the phosphorylation status of Cek1 by immunoblotting crude extracts of cells grown either without stress or in the presence of tunicamycin. Interestingly, under both conditions, separate Msb2 domains were found to be responsible for Cek1 regulation. First, under tunicamycin stress, the control strain (Msb2) revealed a strong upregulation of phosphorylated Cek1 and total Cek1, which did not occur in the *msb2* mutant and occurred at reduced levels in strains producing the ΔD3, ΔD4, and ΔD5 variants (defective in Msb2 shedding) or the ΔD13 variant (Fig. 4). In addition, the C-terminal deletion variants ΔD1 and ΔD2 were reported to be defective for upregulation of phosphorylated Cek1 with tunicamycin treatment (8). All other Msb2 variants were able to produce phosphorylated Cek1 as in control cells (Fig. 4; see Fig. S1 in the supplemental material). We concluded that the C-terminal region surrounding the transmembrane region of Msb2 is required to increase levels of phosphorylated Cek1 in response to glycostress by tunicamycin and also by caspofungin (see below).

The second, repressive function of the functional domains in Msb2 was detected by analyzing Cek1 in unstressed cells. Under these conditions, Cek1 and Cek1-P levels were low in both the control strain (Msb2) and the *msb2* mutant but were as high as those with tunicamycin induction in cells with the ΔD11, ΔD12, ΔD14, and ΔD15 Msb2 variants; no other deleted Msb2 variant showed this characteristic (Fig. 4). Collectively, the results indicate that N-terminal sequences of Msb2 prevent upregulation of Cek1 levels in unstressed cells, while its C-terminal sequences are crucial for increasing the levels of Cek1 and its phosphorylated form under glycostress conditions.

Msb2 domains repress transcription of *PMT1* in unstressed cells. Previous results indicated that the Msb2-Cek1 pathway regulates transcription of *PMT* genes, encoding protein *O*-mannosyltransferases (17). Specifically, the presence of Msb2 and Cek1 proteins was found to downregulate transcript levels of *PMT1*, encoding Pmt isoform 1. When *PMT1* transcript levels were ana-

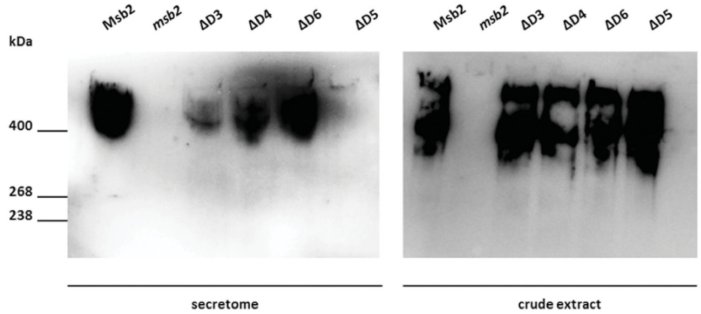


FIG 3 Msb2 shedding. Immunoblotting was performed to detect HA-tagged Msb2. Fifty-microgram samples of proteins in cell extracts and 15-μl aliquots of the secretome were separated in 4 to 20% SDS-PAGE gels, and immunoblots were incubated with rat anti-HA antibody. Strains tested included ESCa3 (Msb2; $MSB2^{HA\text{-}V5}$), FCCa27 (*msb2*), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa34 (ΔD6), and MSCa35 (ΔD5).

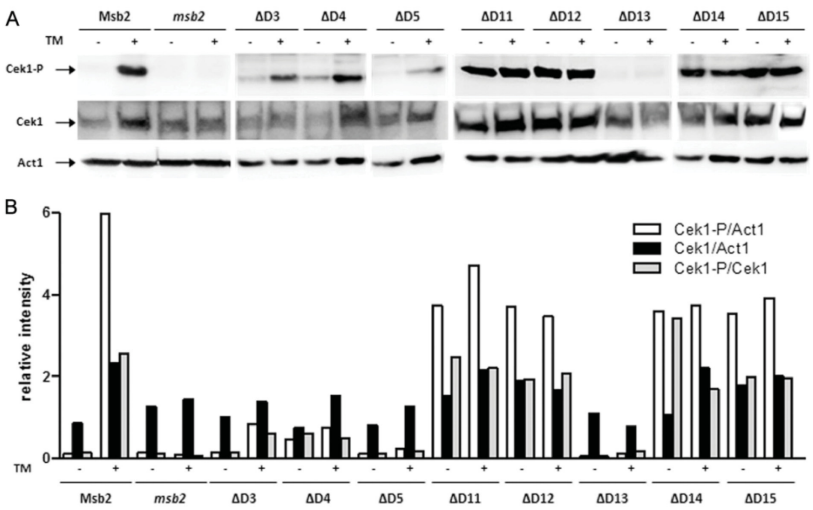


FIG 4 Levels of MAP kinase Cek1 in strains producing Msb2 variants. (A) Cells were grown to stationary phase, diluted in YPD medium, grown to an OD_{600} of 0.8 at 37°C, and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 μg/ml). Cell extracts (50 μg) were separated by SDS-PAGE, and immunoblots were incubated with anti-phospho-p44/42 MAP kinase antibody, which detects phosphorylated Cek1 (Cek1-P), or with a polyclonal antibody to detect total Cek1 (Cek1). The actin protein (Act1), detected by anti-Act1 antibody, was used as the loading control. Strains tested included ESCa3 (Msb2; $MSB2^{HA\text{-}V5}$), FCCa27 (*msb2*), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa35 (ΔD5), MSCa37 (D11), MSCa45 (ΔD12), MSCa46 (ΔD13), MSCa29 (ΔD14), and MSCa8 (ΔD15). (B) Relative intensities of Cek1-P and total Cek1 compared to Act1 and of Cek1-P compared to total Cek1. Band intensities were quantified using ImageJ software (http://imagej.nih.gov/ij/).

lyzed in tunicamycin-stressed cells, high levels were detected in the control strain, in the *msb2* mutant, and in all strains producing Msb2 variants (Fig. 5), suggesting that Msb2 is dispensable for upregulating *PMT1* transcripts under tunicamycin stress conditions. In unstressed cells, however, Msb2 appeared to have a repressive function, since the *PMT1* transcript level was low in the control strain but was upregulated in the *msb2* mutant strain. Furthermore, the ΔD2 to ΔD5 variants upregulated the *PMT1* transcript in unstressed cells, suggesting that the encompassed sequences (residues 1084 to 1308) mediate repression of *PMT1*. Low levels of phosphorylated Cek1 (Fig. 4) but derepressed high transcript levels of its downstream element *PMT1* in the *msb2* mutant and the *cek1* mutant (17) suggest that transcriptional repression of *PMT1* depends on Cek1 in its unphosphorylated form. The identified Msb2 sequences may transmit information on the intactness of glycostructures to Cek1 to permit downregulation of *PMT1* transcription.

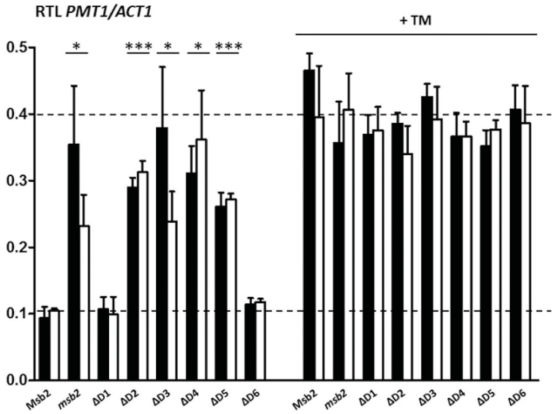


FIG 5 Regulation of *PMT1* transcripts in strains producing Msb2 variants. The level of the *PMT1* transcript relative to the *ACT1* transcript (RTL) was determined by qPCR for cells grown to an OD_{600} of 0.8 in the presence or absence of tunicamycin (0.5 μg/ml). Strains tested included ESCa3 (Msb2; $MSB2^{HA\text{-}V5}$), FCCa27 (*msb2*), ESCa38 (ΔD1), ESCa39 (ΔD2), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa34 (ΔD6), and MSCa35 (ΔD5). Means and standard deviations of results for two biological replicates (black and white bars) in triplicate assays are shown. Statistical significance was determined using the *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Msb2 sequences involved in basal resistance to LL-37. In previous studies, it was shown that the basal resistance of *C. albicans* to the AMP LL-37 depends on the shed Msb2 glycofragment (8, 15). In this study, we tested the ability of Msb2 variants to mediate resistance during short- and long-term exposures to LL-37. In experiments employing high LL-37 concentrations for short exposure times, we found that only the *msb2* mutant, not the strains producing Msb2 deletion variants, was supersensitive to LL-37 (Fig. 6A). Physiological concentrations of LL-37 are low but persist at sites of infection and inflammation (20). Therefore, we also incubated strains for 7 h in the presence or absence of low levels of LL-37 (5 μg/ml). In this experimental setting, the elevated sensitivity of the *msb2* mutant was confirmed (Fig. 6B). Remarkably, Msb2 variants lacking C-terminal sequences (ΔD1 to ΔD5 variants) were also more sensitive to LL-37. Thus, it appears that Msb2 sequences involved in basal glycostress resistance (Fig. 2B) also contribute to basal LL-37 resistance. In these experiments, we also examined the involvement of Cek1 in LL-37 resistance by including a *cek1* mutant. This mutant was supersensitive to LL-37

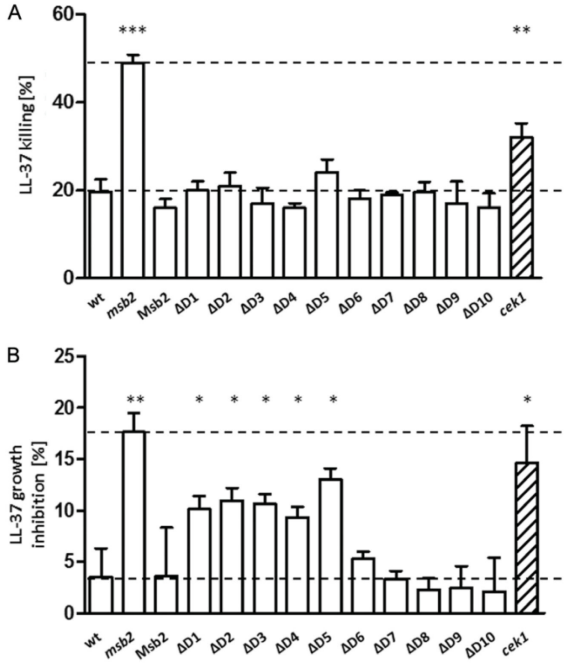


FIG 6 Shed Msb2 and Cek1 regulate basal LL-37 resistance. (A) Killing by short-term exposure to LL-37. Cells (OD_{600} = 0.3) were incubated with 2 μg LL-37 in a total volume of 25 μl for 1.5 h, followed by CFU determination. Killing by LL-37 is indicated as a percentage relative to the killing of untreated cultures. (B) Growth inhibition by long-term exposure to LL-37. Cells (OD_{600} of 0.1) were incubated with 1 μg LL-37 (5 μg/ml) in wells of a microtiter plate (total volume, 200 μl) for 7 h at 37°C before measurement of the OD_{600} by use of a microplate reader. The ratios of OD_{600} values for untreated and treated cultures are given as percentages. Strains tested included CAF2-1 (wt), FCCa27 (*msb2*), ESCa3 ($Msb2^{HA\text{-}V5}$), ESCa38 (ΔD1), ESCa39 (ΔD2), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa34 (ΔD6), MSCa35 (ΔD5), MSCa30 (ΔD8), MSCa31 (ΔD7), MSCa48 (ΔD9), ESCa25 (ΔD10), and CK43B-16 (*cek1*) (hatched bar). Dotted lines indicate values for the wild-type and *msb2* mutant strains. Means and standard deviations of results for triplicate assays are shown. Statistical significance was determined using the *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 6) and did not prevent Msb2 shedding (data not shown), indicating that basal resistance to LL-37 is mediated not only by binding of LL-37 to the shed Msb2 exodomain (15) but also by other cell-associated Msb2 sequences that are required for Cek1 signaling.

Hyperfilamentous phenotype generated by Msb2 variants. Transformants producing the ΔD11 variant, in which most N-terminal sequences of the Msb2 exodomain are deleted (Fig. 1), were remarkably hyperfilamentous under all growth conditions, including those environments that normally do not induce hyphal morphogenesis (YPD and 30°C) (Fig. 7A). This phenotype was not observed with the ΔD9 and ΔD10 variants, in which only one of the two deleted segments of the ΔD11 variant is missing. Two partial deletions of the segment missing in the ΔD9 variant (ΔD14 and ΔD15 variants) did not reproduce the ΔD11 phenotype, indicating that other sequences in this region are involved in generating hyperfilamentation. To investigate if the cytoplasmic tail is required for hypha formation, we constructed the ΔD12 variant, lacking 103 C-terminal residues, and the ΔD13 variant, which also lacks the TM region. Because the ΔD12 variant but not the ΔD13 variant allowed hypha formation, it appears that the TM region but not the cytoplasmic tail of Msb2 is involved in triggering exaggerated hyphal induction.

Next, we investigated the activity of the ΔD11 variant in an $Msb2^{+}$ wild-type strain and in several mutant strains carrying known defects in hypha formation. The hyperfilamentation phenotype of the ΔD11 variant was dominant, since transformants of the wild-type strain showed extensive filamentation (Fig. 7B) and were able to shed Msb2 (see Fig. S1 in the supplemental material). Among mutants hosting the ΔD11 variant, only the *cek1* and *efg1* mutants were defective in hypha formation, while mutants lacking either PKA isoform (*tpk1* and *tpk2* mutants) or the transcription factor Czf1 or Cph1 showed strong filamentation. Thus, hyperfilamentation induced by the ΔD11 variant requires some elements that normally are required for hyphal morphogenesis. The Sho1 membrane protein, which controls the activity of Cek1 in cooperation with Msb2 (14), also appears to be required for ΔD11 hyperfilamentation, because in the *msb2 sho1* double mutant genetic background, only yeast growth was observed (Fig. 7B).

Cleavage and nuclear localization of the cytoplasmic tail of Msb2. The above-described experiments indicated that the cytoplasmic tail of Msb2 is needed for basal resistance against caspofungin (Fig. 2B) and to stimulate phosphorylation of Cek1 in response to stress (Fig. 4) (8). To explore the intracellular fate of the cytoplasmic tail of Msb2, we performed immunofluorescence microscopy of cells producing doubly tagged $Msb2^{HA\text{-}V5}$ (the V5 epitope is located within the middle of the cytoplasmic domain of Msb2) (Fig. 1). In unstressed cells, the cytoplasmic domain was detected on the cellular surface, most likely reflecting the localization in the plasma membrane of cells (Fig. 8A). Short-term exposure to caspofungin (50 ng/ml for 30 min) led to the appearance of the V5 epitope in the cytoplasm and in the nucleus (Fig. 8A, merged signals). Quantification revealed that about 70% of the Msb2 C-terminal domain localized to intracellular locations upon caspofungin treatment (Fig. 8B). These results suggested that under glycostress by caspofungin, the cytoplasmic tail of Msb2 is partially cleaved and enters the nucleus.

The cytoplasmic tail of Msb2 regulates transcriptional responses to glycostress. To further investigate the role of the Msb2 cytoplasmic tail, we first analyzed the activation of Cek1 under caspofungin stress conditions (Fig. 9A). We first tested different caspofungin concentrations, ranging from 10 to 100 ng/ml, during short-term exposure to determine conditions under which <10% of *C. albicans* cells were killed for both strains (data not shown). As in the case of glycostress by tunicamycin (Fig. 4), a strain producing the ΔD1 variant showed strongly reduced levels of phosphorylated Cek1 with treatment by caspofungin (50 ng/ml for 30 min) compared to those of the control strain. Genome-wide transcript profiling was then carried out on strain ESCa38, producing the ΔD1 variant, compared to the Msb2 wild-type strain ESCa3 grown under identical conditions in the presence of caspofungin. Genes for transcripts regulated differently in ESCa38 cells producing deleted Msb2 compared to ESCa3 cells producing full-length Msb2 are listed in Table S2 in the supplemental material. The deletion of the cytoplasmic tail of Msb2 altered the expression of 117 genes >1.5-fold, with 80 genes being upregulated and 37 being downregulated. Categorization of the regulated

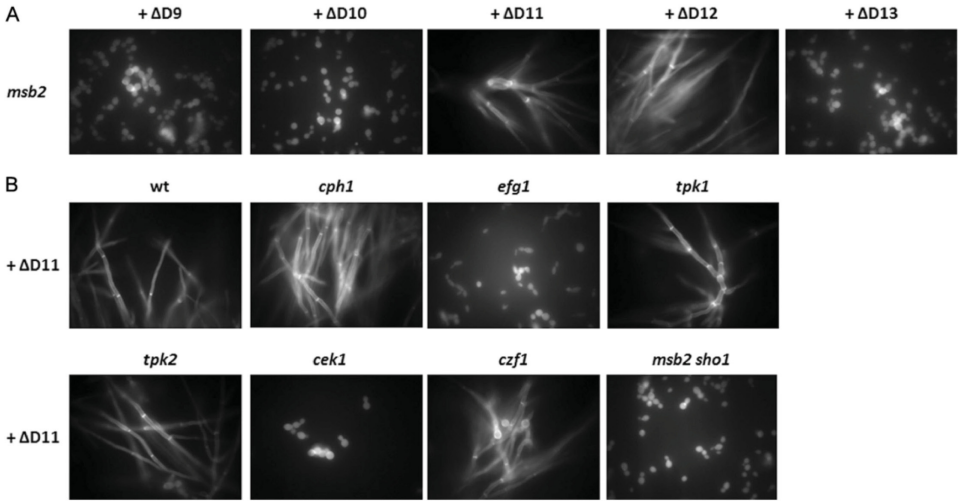
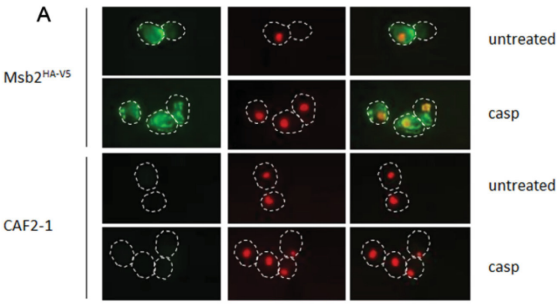


FIG 7 Msb2-Sho1 interactions induce hypha formation via Cek1 and Efg1. (A) Hyperfilamentous growth of strains producing Msb2 protein variants ΔD11 and ΔD12. Transformants (encoded variants) included strains ESCa25 (ΔD10), MSCa37 (ΔD11), MSCa45 (ΔD12), MSCa46 (ΔD13), and MSCa48 (ΔD9). (B) Hyphal induction induced by the ΔD11 Msb2 variant in different genetic backgrounds. Resulting transformants included MSCa37 (*msb2* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa38 (wt [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa39 (*cek1* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa40 (*czf1* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa41 (*efg1* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa42 (*cph1* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa43 (*tpk1* [*ACT1p-MSB2*ΔN$^{514–1087}$]), MSCa44 (*tpk2* [*ACT1p-MSB2*ΔN$^{514–1087}$]), and MSCa47 (*msb2 sho1* [*ACT1p-MSB2*ΔN$^{514–1087}$]). All cells were grown at 30°C in YPD before staining with calcofluor white and microscopic inspection.

genes according to their functional categories (21; http://www.candidagenome.org/cgi-bin/GO/goTermFinder) revealed that deletion of the Msb2 C-tail had a significant effect on *ALS* genes (*ALS1*, *ALS3*, and *ALS5*), which are responsible for the entry of *C. albicans* into host cells (see Table S3A). GO analysis (Slim Mapper) of the complete regulated gene set assigned 19 genes to stress responses, 16 to cellular protein modification processes, and 15 to responses to chemicals (see Table S3B). To confirm the transcriptional profiling data, we performed qPCR on two selected Msb2 target genes. Transcript levels for the *ALS1* and *ALS3* genes, encoding cell wall proteins, were increased by a factor of 4 or 6 (Fig. 9B). The results confirmed the increased levels of *ALS* gene transcripts in cells producing the ΔD1 variant under caspofungin stress conditions.



B

condition	CM [%]	intracellular [%]
untreated	98	2
casp	28,6	70,2

FIG 8 Cellular localization of the Msb2 cytoplasmic tail. (A) Fluorescence microscopy was performed on wild-type strain CAF2-1 (wt) and strain ESCa3, producing Msb2^{HA-V5} containing the V5 epitope in its cytoplasmic tail, without and with treatment with 50 ng/ml caspofungin (casp) for 30 min. Fixed cells were treated with mouse anti-V5 antibody, which was detected with FITC-labeled anti-mouse antibody (green signals); staining by DAPI was used to detect nuclei (red signals); and merged signals are shown in yellow. (B) Quantification of cellular localization. Fifty cells were analyzed for V5-FITC staining in the cytoplasmic membrane (CM) or for intracellular localization in the cytoplasm or the nucleus. Fluorescence signals were quantitated using ImageJ software and are indicated as percentages.

The promoter regions of the regulated genes were analyzed using dyad analysis and YeTFaSCo (22, 23), which revealed the following two highly significant transcription factor motifs: GA_2GA_5 (101 sites) and CAC_2AC_2 (46 sites) (Fig. 9C). The identified GA_2GA_5 motif matches the motif for binding of the glucose-induced transcriptional regulator Azf1 in *Saccharomyces cerevisiae* (24). The identified CAC_2AC_2 motif matches the Ace2 binding site in *S. cerevisiae* (23) and in *C. albicans* (L. van Wijlick and J. F. Ernst, unpublished results). Collectively, the transcriptomic results support the notion that the cytoplasmic portion of Msb2 is functional in the nucleus to regulate transcriptional circuits alleviating glycostress. Ace2 was previously identified as contributing to regulation of *PMT* genes (17).

DISCUSSION

The fungal pathogen *C. albicans* is able to adapt to and proliferate in various complex environments of the human host (25). Fungal survival requires stress response pathways to restore cell wall integrity during attack of host immune effectors (26). The signaling mucin Msb2 has dual functions: sensing environmental stress signals and blocking an important aspect of immune defenses by inactivating AMPs (5, 14). Here we showed that different se-

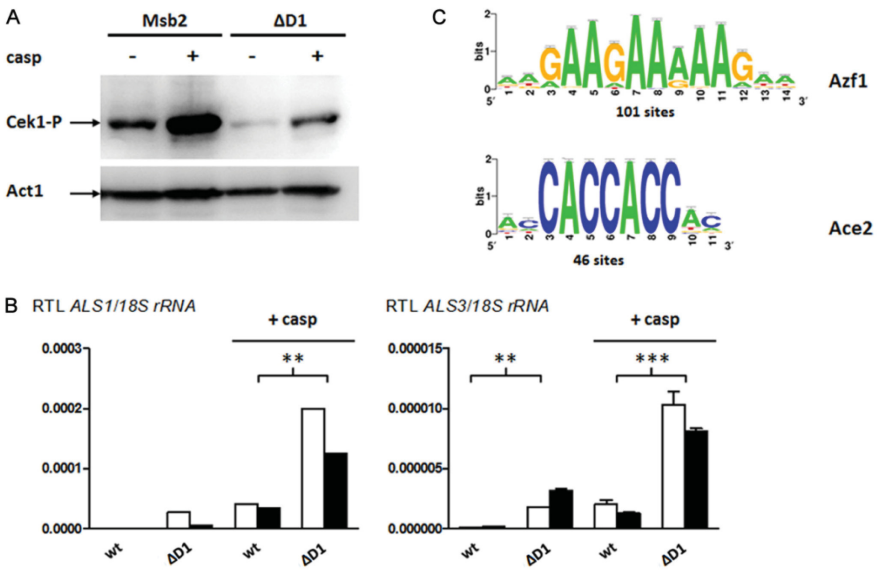


FIG 9 Phosphorylation of Cek1 and regulation of *ALS1* and *MSB2* gene expression by caspofungin treatment. (A) Msb2 C-tail is required for Cek1 phosphorylation during caspofungin treatment. Strains tested include ESCa3 (Msb2; $MSB2^{HA\text{-}V5}$) and mutant strain ESCa38, producing Msb2 lacking the cytoplasmic tail (ΔD1). Cells were grown to stationary phase, diluted in fresh YPD medium, grown to an OD_{600} of 0.8 at 37°C, and incubated further for 30 min in the presence (+) or absence (−) of caspofungin (50 ng/ml). Immunoblot detection of Cek1 and Act1 proteins was performed as described in the legend to Fig. 4. (B) *ALS1* and *ALS3* expression. Transcript levels relative to the 18S rRNA transcript levels in wild-type and ESCa38 mutant cells left untreated or treated with caspofungin (50 ng/ml; 30 min) were determined by qPCR. Means and standard deviations of results for two biological replicates (black and white bars) in triplicate assays are shown. Statistical significance was determined using the *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Consensus sequences for transcription factors in promoters of genes regulated by the C-tail of Msb2. Genome-wide expression analysis revealed 117 genes regulated by the presence of the Msb2 C-tail (see Table S2 in the supplemental material). Promoter regions were analyzed using dyad analysis (22) and YeTFaSCo (23) to define consensus sequences and revealed binding sites for the Azf1 and Ace2 transcription factors.

quences of Msb2 provide different functions in its proteolytic maturation and in hyphal morphogenesis, basal antifungal resistance, and gene regulation. A scheme depicting the established functional domains of Msb2 is shown in Fig. 10.

Mammalian signaling mucins are known to become proteolytically processed to generate a large extracellular glycodomain as well as a cytoplasmic domain conveying regulatory functions (9). Autoproteolytic cleavage of the MUC1 mucin depends on its internal SEA module, comprising about 120 residues, which cleaves the precursor protein at a G ↑ SVVV motif; the VVV sequence is replaced by other bulky hydrophobic residues in homologs (27). The *C. albicans* Msb2 protein lacks SEA-homologous sequences but contains a $G^{1232}SAIY^{1235}$ sequence N-terminal to its TM region; however, deletion of residues 1231 to 1233 did not abolish shedding of Msb2 (data not shown). Thus, the present evidence suggests that the Msb2 precursor is processed by a separate dedicated protease, as for some mammalian mucins and for the Msb2 protein of *S. cerevisiae*, which is cleaved by the yapsin Yps1 (13). It is currently unclear if, in *C. albicans*, secreted aspartyl proteases (Sap proteins) initiate cleavage and shedding of Msb2 and/or contribute to subsequent degradation of its shed exodomain (8, 19). Here we defined a segment in the Msb2 exodomain, between residues 1084 and 1283, which is essential for the cleavage and shedding of Msb2. This sequence contains several predicted proteolytic cleavage sites (28; http://web.expasy.org/peptide_cutter/) and may contain the relevant site for shedding, or it may ectopically direct the cleavage event. Importantly, we found that the presence of this Msb2 segment is essential for all Msb2-dependent functions.

The Msb2 sensor regulates the activity of the MAP kinase Cek1, which is required for hypha formation on semisolid surfaces (8). Here we showed that glycosylation defects upregulate Cek1 levels, in agreement with the finding that the *CEK1* transcript level is upregulated by tunicamycin (L. van Wijlick and J. F. Ernst, unpublished results). We identified two modes of Msb2 function on Cek1 phosphorylation, which are conveyed by three separate functional Msb2 domains. In unstressed cells, N-terminal sequences (encompassed by the ΔD10 variant) repressed the appearance of phosphorylated Cek1, while under tunicamycin stress, C-terminal sequences flanking the TM region (encompassed by the ΔD1 and ΔD3 variants) mediated induction of Cek1 and its phosphorylated form. Previously, the Mkc1 MAP kinase was described to become activated by surface growth, oxidative stress, and cell wall defects, including caspofungin glycostress (29–32); thus, Msb2 may mediate basal caspofungin resistance by activating both Mkc1 and Cek1. In contrast, increased Mkc1-P levels were not consistently reported for tunicamycin treatment conditions (14, 33), suggesting that defective protein *N*-glycosylation triggers upregulation of Mkc1-P only under as yet undefined conditions (possibly upon prolonged tunicamycin treatment, when cell wall proteins become significantly underglycosylated). The ability to generate hyphae was not correlated with phosphorylated

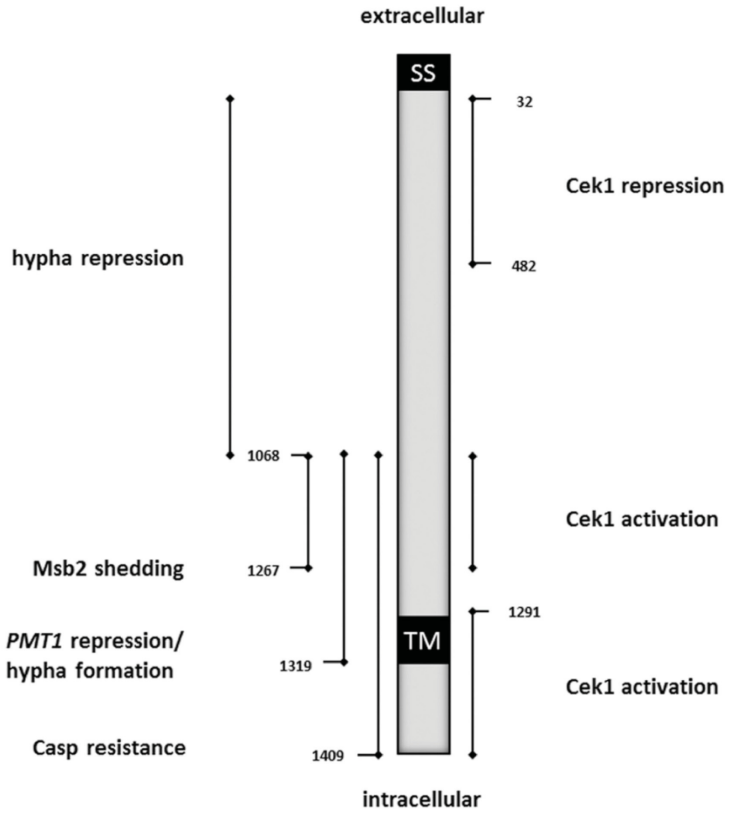


FIG 10 Msb2 domains involved in cellular processes. The schematic of Msb2 indicates the positions of the signal sequence (SS) and the transmembrane region (TM) in the extracellular (shed) and intracellular domains. Bars indicate overlapping subdomains involved in activating/repressing hypha formation and repressing *PMT1* transcripts in unstressed cells. Furthermore, different Msb2 subdomains are indicated that are required for repressing or inducing Cek1 phosphorylation. In unstressed cells, N-terminal sequences mediate repression of Cek1 phosphorylation and activity, while C-terminal sequences allow activation of Cek1 in cells stressed by caspofungin (Casp) and/or tunicamycin.

Cek1, since the ΔD1 variant showed low levels of phosphorylated Cek1 but formed hyphae, while the ΔD4 variant produced high levels of phosphorylated Cek1 but did not undergo filamentation. This result suggests that Msb2 triggers hypha formation, at least partially, independently of Cek1 and/or its state of phosphorylation. Surprisingly, a remarkable hyperfilamentation phenotype was observed when about two-thirds of the N-terminal sequences of Msb2 were deleted (ΔD11 and ΔD12 variants). Strong filamentation occurred in liquid media and led to large hyphal aggregates. Hyperfilamentation was still observed when the cytoplasmic tail of Msb2 was removed but did not occur when the TM region was deleted (ΔD13 variant). Thus, the presence of the TM region and/or its close flanking sequences appears to be sufficient to strongly induce hyphal morphogenesis. Interestingly, the Msb2-generated abnormal hypha formation also occurred in the wild-type genetic background, suggesting the dominance of the Msb2 fragment over full-length Msb2. On the other hand, hyphal formation was not induced in cells carrying the *sho1*, *cek1*, or *efg1* mutation. These results indicate that hyperfilamentation induced by Msb2 sequences in the ΔD11 variant requires a known Msb2 interactor (Sho1) (14) and its downstream kinase (Cek1), but also a transcription factor essential for hypha formation (Efg1) (34). However, the contribution of Msb2 to regular hyphal induction under physiological conditions requires further analysis.

Previous results have implicated the Msb2-Cek1 pathway in fungal basal resistance and cell wall integrity (8, 14, 17). Shed Msb2 binds human AMPs and a peptide antibiotic with high affinity, thereby generating a protective cloud surrounding cells (15). Here we showed that Msb2 sequences that probably are not shed (C-terminal to the proposed cleavage region) also contribute to AMP resistance. C-terminal sequences extending from the cytoplasmic tail to residue 1085 are necessary to increase basal resistance not only to the AMP LL-37 but also to the cell wall-damaging compounds caspofungin and tunicamycin. Although sequences comprising residues 1278 to 1308 are not essential for either Msb2 shedding or Cek1 phosphorylation, they are relevant for basal resistance. The underlying mechanisms are not yet clear, but it appears that extracellular Msb2 sequences that remain after cleavage of the exodomain regulate nuclear activities, as observed in the case of mammalian N-CAM and β-dystroglycan proteins (35, 36).

The transcriptional output of Msb2-Cek1 signaling consists of altered gene expression that restores cell wall integrity. We previ-

ously found that *PMT1* expression is repressed by Msb2-Cek1 proteins during normal growth, while the induction of *PMT2* and *PMT4* genes by inhibition of Pmt1 requires both proteins (17). Since no significant phosphorylation of Cek1 occurs in unstressed cells, it appears that *PMT1* repression requires the presence of Cek1, but not in its phosphorylated form. Msb2 sequences upstream of the TM region, which are also needed to provide basal resistance to caspofungin and tunicamycin, were found to be required for *PMT1* repression, although the latter process was unaffected by absence of the cytoplasmic tail (ΔD1 variant). With regard to the mechanism by which Msb2-Cek1 signaling alters gene expression, we obtained evidence by immunofluorescence microscopy that the C terminus of Msb2 (possibly the cytoplasmic domain, including the TM region) partially relocates to the cytoplasm and the nucleus if cells are stressed by caspofungin. Thus, the carboxy end of Msb2 may enter the nucleus either by itself or as a passenger of the MAP kinase modules and be involved in target gene regulation. Cleavage of type I transmembrane proteins in the membrane, releasing an intracellular fragment capable of transducing nuclear signals, has been described for the Notch-1 protein, ErbB-4, and CN14 (37–39). In two fungal species, the subcellular localization of the Msb2 (*Ustilago maydis*) and MsbA (*Aspergillus nidulans*) C-terminal ends fused to a fluorescent protein is a dynamic process in which the mucin is constantly removed from the membrane and recruited to the vacuole (10, 40). Transcriptomic comparisons of strains containing Msb2 with and without its cytoplasmic tail indeed revealed 117 differentially regulated genes. Intriguingly, the list of genes includes five cell wall genes (*ALS1*, *ALS3*, *ALS5*, *CHT2*, and *PGA14*). Previously, it was shown that caspofungin-induced cell aggregation activates an Efg1-dependent signaling pathway to regulate *ALS1* expression (41). We suggest that Msb2 is an upstream signaling molecule for Efg1-mediated hyphal morphogenesis and for upregulation of *ALS1*. Promoter comparisons of all regulated genes revealed binding motifs for two transcription factors (Ace2 and Azf1) known to activate transcription of genes involved in the maintenance of cell wall integrity. Interestingly, previous results identified Ace2 as an essential protein for regulation of *PMT* genes and for basal antifungal resistance (17). Future research should be directed at clarifying the details of Msb2 processing and its influence in directing environment-regulated gene expression.

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We declare that we have no conflicts of interest.

REFERENCES

1. **Van Der Meer JWM, Van De Veerdonk FL, Joosten LAB, Kullberg BJ, Netea MG.** 2010. Severe *Candida* spp. infections: new insights into natural immunity. Int J Antimicrob Agents **36**(Suppl 2):S58–S62. http://dx.doi.org/10.1016/j.ijantimicag.2010.11.013.
2. **Zipfel PF, Skerka C, Kupka D, Luo S.** 2011. Immune escape of the human facultative pathogenic yeast *Candida albicans*: the many faces of the *Candida* Pra1 protein. Int J Med Microbiol **301:**423–430. http://dx.doi.org/10.1016/j.ijmm.2011.04.010.
3. **Jouault T, Sarazin A, Martinez-Esparza M, Fradin C, Sendid B, Poulain D.** 2009. Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by *Candida albicans*. Cell Microbiol **11:**1007–1015. http://dx.doi.org/10.1111/j.1462-5822.2009.01318.x.
4. **Mehra T, Koberle M, Braunsdorf C, Mailander-Sanchez D, Borelli C, Schaller M.** 2012. Alternative approaches to antifungal therapies. Exp Dermatol **21:**778–782. http://dx.doi.org/10.1111/exd.12004.
5. **Swidergall M, Ernst JF.** 2014. Interplay between *Candida albicans* and the antimicrobial peptide armory. Eukaryot Cell **13:**950–957. http://dx.doi.org/10.1128/EC.00093-14.
6. **Collette JR, Lorenz MC.** 2011. Mechanisms of immune evasion in fungal pathogens. Curr Opin Microbiol **14:**668–675. http://dx.doi.org/10.1016/j.mib.2011.09.007.
7. **Ernst JF.** 2000. Transcription factors in *Candida albicans*—environmental control of morphogenesis. Microbiology **146:**1763–1774.
8. **Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Roman E, Pla J, Ernst JF.** 2012. Msb2 shedding protects *Candida albicans* against antimicrobial peptides. PLoS Pathog **8:**e1002501. http://dx.doi.org/10.1371/journal.ppat.1002501.
9. **Cullen PJ.** 2011. Post-translational regulation of signaling mucins. Curr Opin Struct Biol **21:**590–596. http://dx.doi.org/10.1016/j.sbi.2011.08.007.
10. **Lanver D, Mendoza-Mendoza A, Brachmann A, Kahmann R.** 2010. Sho1 and Msb2-related proteins regulate appressorium development in the smut fungus *Ustilago maydis*. Plant Cell **22:**2085–2101. http://dx.doi.org/10.1105/tpc.109.073734.
11. **Liu W, Zhou X, Li G, Li L, Kong L, Wang C, Zhang H, Xu JR.** 2011. Multiple plant surface signals are sensed by different mechanisms in the rice blast fungus for appressorium formation. PLoS Pathog **7:**e1001261. http://dx.doi.org/10.1371/journal.ppat.1001261.
12. **Perez-Nadales E, Di Pietro A.** 2011. The membrane mucin Msb2 regulates invasive growth and plant infection in *Fusarium oxysporum*. Plant Cell **23:**1171–1185. http://dx.doi.org/10.1105/tpc.110.075093.
13. **Vadaie N, Dionne H, Akajagbor DS, Nickerson SR, Krysan DJ, Cullen PJ.** 2008. Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. J Cell Biol **181:**1073–1081. http://dx.doi.org/10.1083/jcb.200704079.
14. **Roman E, Cottier F, Ernst JF, Pla J.** 2009. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. Eukaryot Cell **8:**1235–1249. http://dx.doi.org/10.1128/EC.00081-09.
15. **Swidergall M, Ernst AM, Ernst JF.** 2013. *Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides. Antimicrob Agents Chemother **57:**3917–3922. http://dx.doi.org/10.1128/AAC.00862-13.
16. **Whiteway M.** 2000. Transcriptional control of cell type and morphogenesis in *Candida* albicans. Curr Opin Microbiol **3:**582–588. http://dx.doi.org/10.1016/S1369-5274(00)00144-2.
17. **Cantero PD, Ernst JF.** 2011. Damage to the glycoshield activates PMT-directed O-mannosylation via the Msb2-Cek1 pathway in *Candida albicans*. Mol Microbiol **80:**715–725. http://dx.doi.org/10.1111/j.1365-2958.2011.07604.x.
18. **Doedt T, Krishnamurthy S, Bockmuhl DP, Tebarth B, Stempel C, Russell CL, Brown AJ, Ernst JF.** 2004. APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. Mol Biol Cell **15:**3167–3180. http://dx.doi.org/10.1091/mbc.E03-11-0782.
19. **Puri S, Kumar R, Chadha S, Tati S, Conti HR, Hube B, Cullen PJ, Edgerton M.** 2012. Secreted aspartic protease cleavage of *Candida albicans* Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis. PLoS One **7:**e46020. http://dx.doi.org/10.1371/journal.pone.0046020.
20. **Nijnik A, Hancock REW.** 2009. The roles of cathelicidin LL-37 in immune defences and novel clinical applications. Curr Opin Hematol **16:**41–47. http://dx.doi.org/10.1097/MOH.0b013e32831ac517.
21. **Inglis DO, Arnaud MB, Binkley J, Shah P, Skrzypek MS, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G.** 2012. The *Candida* genome database incorporates multiple *Candida* species: multispecies search and analysis tools with curated gene and protein information for *Candida albicans* and *Candida glabrata*. Nucleic Acids Res **40:**D667–D674. http://dx.doi.org/10.1093/nar/gkr945.
22. **van Helden J, Rios AF, Collado-Vides J.** 2000. Discovering regulatory elements in non-coding sequences by analysis of spaced dyads. Nucleic Acids Res **28:**1808–1818. http://dx.doi.org/10.1093/nar/28.8.1808.
23. **de Boer CG, Hughes TR.** 2012. YeTFaSCo: a database of evaluated yeast transcription factor sequence specificities. Nucleic Acids Res **40:**D169–D179. http://dx.doi.org/10.1093/nar/gkr993.
24. **Newcomb LL, Hall DD, Heideman W.** 2002. AZF1 is a glucose-

dependent positive regulator of CLN3 transcription in *Saccharomyces cerevisiae*. Mol Cell Biol **22:**1607–1614. http://dx.doi.org/10.1128/MCB.22.5.1607-1614.2002.

25. **Yan L, Yang C, Tang J.** 2013. Disruption of the intestinal mucosal barrier in *Candida albicans* infections. Microbiol Res **168:**389–395. http://dx.doi.org/10.1016/j.micres.2013.02.008.
26. **de Dios CH, Roman E, Monge RA, Pla J.** 2010. The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans*: implications in virulence. Curr Protein Pept Sci **11:**693–703. http://dx.doi.org/10.2174/138920310794557655.
27. **Maeda T, Inoue M, Koshiba S, Yabuki T, Aoki M, Nunokawa E, Seki E, Matsuda T, Motoda Y, Kobayashi A, Hiroyasu F, Shirouzu M, Terada T, Hayami N, Ishizuka Y, Shinya N, Tatsuguchi A, Yoshida M, Hirota H, Matsuo Y, Tani K, Arakawa T, Carninci P, Kawai J, Hayashizaki Y, Kigawa T, Yokoyama S.** 2004. Solution structure of the SEA domain from the murine homologue of ovarian cancer antigen CA125 (MUC16). J Biol Chem **279:**13174–13182. http://dx.doi.org/10.1074/jbc.M309417200.
28. **Gasteiger E, Hoogland C, Gattiker A, Duvaud Se Wilkins M, Appel R, Bairoch A.** 2005. Protein identification and analysis tools on the ExPASy server, p 571–607. *In* Walker J (ed), The proteomics protocols handbook. Humana Press, Totowa, NJ.
29. **Navarro-Garcia F, Alonso-Monge R, Rico H, Pla J, Sentandreu R, Nombela C.** 1998. A role for the MAP kinase gene *MKC1* in cell wall construction and morphological transitions in *Candida* albicans. Microbiology **144:**411–424. http://dx.doi.org/10.1099/00221287-144-2-411.
30. **Navarro-Garcia F, Eisman B, Fiuza SM, Nombela C, Pla J.** 2005. The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. Microbiology **151:**2737–2749. http://dx.doi.org/10.1099/mic.0.28038-0.
31. **Kumamoto CA.** 2005. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. Proc Natl Acad Sci U S A **102:**5576–5581. http://dx.doi.org/10.1073/pnas.0407097102.
32. **Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA.** 2008. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. PLoS Pathog **4:**e1000040. http://dx.doi.org/10.1371/journal.ppat.1000040.
33. **Cantero PD, Lengsfeld C, Prill SK, Subanovic M, Roman E, Pla J, Ernst JF.** 2007. Transcriptional and physiological adaptation to defective protein-*O*-mannosylation in *Candida albicans*. Mol Microbiol **64:**1115–1128. http://dx.doi.org/10.1111/j.1365-2958.2007.05723.x.
34. **Stoldt VR, Sonneborn A, Leuker CE, Ernst JF.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J **16:**1982–1991. http://dx.doi.org/10.1093/emboj/16.8.1982.
35. **Oppizzi ML, Akhavan A, Singh M, Fata JE, Muschler JL.** 2008. Nuclear translocation of beta-dystroglycan reveals a distinctive trafficking pattern of autoproteolyzed mucins. Traffic **9:**2063–2072. http://dx.doi.org/10.1111/j.1600-0854.2008.00822.x.
36. **Kleene R, Mzoughi M, Joshi G, Kalus I, Bormann U, Schulze C, Xiao MF, Dityatev A, Schachner M.** 2010. NCAM-induced neurite outgrowth depends on binding of calmodulin to NCAM and on nuclear import of NCAM and fak fragments. J Neurosci **30:**10784–10798. http://dx.doi.org/10.1523/JNEUROSCI.0297-10.2010.
37. **De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ, Goate A, Kopan R.** 1999. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature **398:**518–522. http://dx.doi.org/10.1038/19083.
38. **Lammich S, Okochi M, Takeda M, Kaether C, Capell A, Zimmer AK, Edbauer D, Walter J, Steiner H, Haass C.** 2002. Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. J Biol Chem **277:**44754–44759. http://dx.doi.org/10.1074/jbc.M206872200.
39. **Ni CY, Murphy MP, Golde TE, Carpenter G.** 2001. γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science **294:**2179–2181. http://dx.doi.org/10.1126/science.1065412.
40. **Brown NA, Dos Reis TF, Goinski AB, Savoldi M, Menino J, Almeida MT, Rodrigues F, Goldman GH.** 8 October 2014. The *Aspergillus nidulans* signalling mucin MsbA regulates starvation responses, adhesion and affects cellulase secretion in response to environmental cues. Mol Microbiol http://dx.doi.org/10.1111/mmi.12820.
41. **Gregori C, Glaser W, Frohner IE, Reinoso-Martin C, Rupp S, Schuller C, Kuchler K.** 2011. Efg1 controls caspofungin-induced cell aggregation of *Candida albicans* through the adhesin Als1. Eukaryot Cell **10:**1694–1704. http://dx.doi.org/10.1128/EC.05187-11.
42. **Sonneborn A, Bockmuhl DP, Gerads M, Kurpanek K, Sanglard D, Ernst JF.** 2000. Protein kinase A encoded by *TPK2* regulates dimorphism of *Candida albicans*. Mol Microbiol **35:**386–396. http://dx.doi.org/10.1046/j.1365-2958.2000.01705.x.
43. **Fonzi WA, Irwin MY.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
44. **Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas DY, Whiteway M.** 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun **66:**2713–2721.
45. **Brown DH, Jr, Giusani AD, Chen X, Kumamoto CA.** 1999. Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique *CZF1* gene. Mol Microbiol **34:**651–662. http://dx.doi.org/10.1046/j.1365-2958.1999.01619.x.
46. **Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949. http://dx.doi.org/10.1016/S0092-8674(00)80358-X.
47. **Liu H, Kohler J, Fink GR.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726. http://dx.doi.org/10.1126/science.7992058.
48. **Bockmuhl DP, Krishnamurthy S, Gerads M, Sonneborn A, Ernst JF.** 2001. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. Mol Microbiol **42:**1243–1257. http://dx.doi.org/10.1046/j.1365-2958.2001.02688.x.

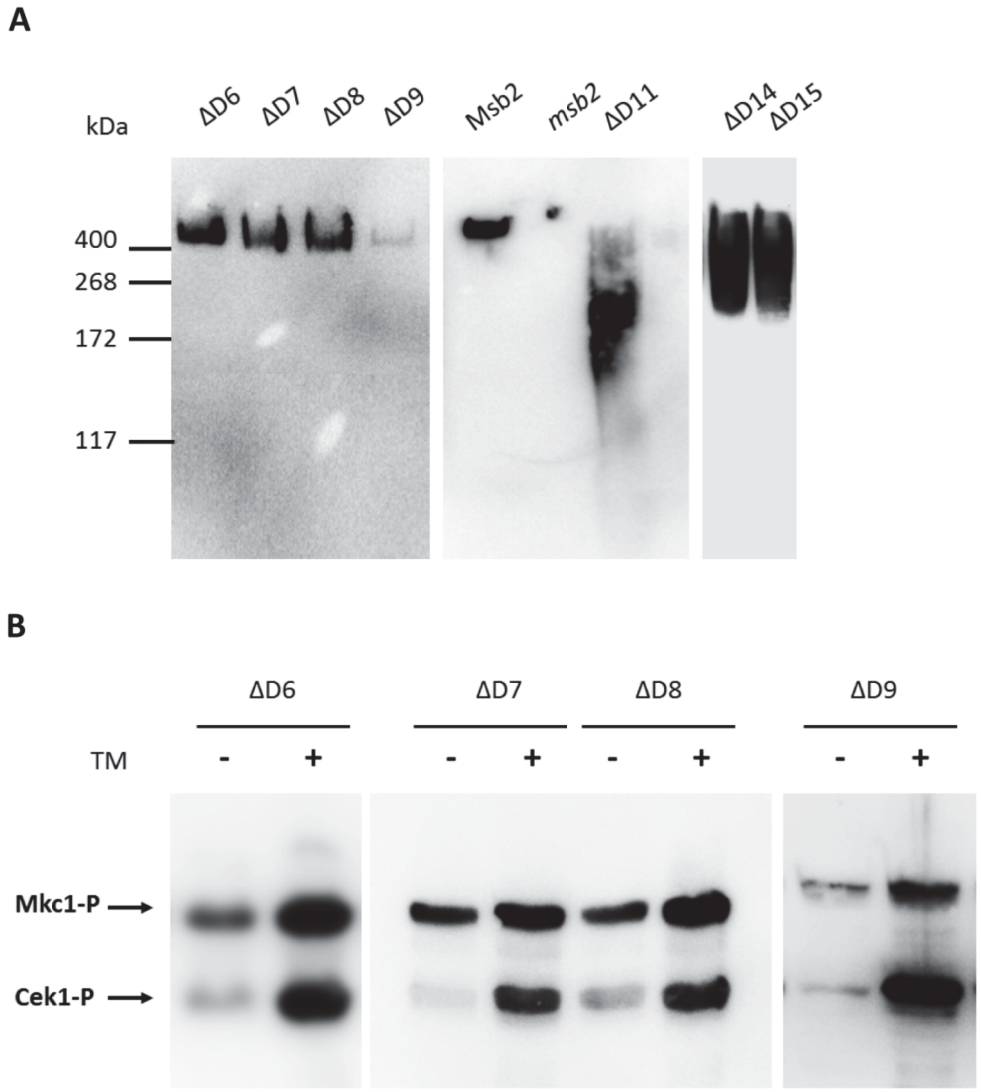


Figure S1. A. Shedding of Msb2 variants. Immunoblot to detect shed HA-tagged Msb2. Proteins in the growth medium (15 µl) were separated by a 4-20 % SDS-PAGE gel and immunoblots were reacted with rat anti-HA antibody. Strains tested included ESCa3 (Msb2; *MSB2*$^{HA\text{-}V5}$), FCCa27 (*msb2*), MSCa34 (ΔD6), MSCa30 (ΔD8), MSCa31 (ΔD7), MSCa48 (ΔD9), MSCa37 (ΔD11), MSCa29 (ΔD14) and MSCa8 (ΔD15). **B. Cek1 activation triggered by Msb2 variants.** Cek1 activation by strains producing variant Msb2 proteins. Cells were grown to stationary phase, diluted in fresh YPD medium, grown to OD_{600} = 0.8 at 37°C and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 µg/ml). Cell extracts (50 µg) were separated by SDS-PAGE and immunoblots were reacted with with anti-phospho-p44/42 MAP kinase antibody, which detects phosphorylated Cek1 (Cek1-P) and Mkc1 (Mkc1-P) proteins. Strains tested included MSCa34 (ΔD6), MSCa30 (ΔD8), MSCa31 (ΔD7) and MSCa48 (ΔD9).

Supplemental Table 1- Oligonucleotides

Name	Sequence
Msb2-15ASDel1 Fwd	5'-GAT TAG TTA CTG ATG ATC AAC AAG TTT CAA TGG ATA TAG TTT CCA ATA C-3'
Msb2-15ASDel1 Rev	5'-GTA TTG GAA ACT ATA TCC ATT GAA ACT TGT TGA TCA TCA GTA ACT AAT C-3'
Msb2-15ASDel2 Fwd	5'-AGT TGC CGA TAA AGG AAG AAT TGC TGG TAT AAC CAT TGG TGC AGC AGC AG-3'
Msb2-15ASDel2 Rev	5'-CTG CTG CTG CAC CAA TGG TTA TAC CAG CAA TTC TTC CTT TAT CGG CAA CT-3'
Msb2 Seq C	5'-CGC CTC GAG CTA ATG ATA CC-3'
Msb2 Seq N	5'-CGC TGT TCT CAC CAG GAT TTA TTG C-3'
Kolo AMP Fwd	5'-GTT GGC CGC AGT GTT ATC-3'
Kolo LEU2 Rev	5'-CACA CTG ATG CTG CTC AGG-3'
Msb2-Del6 Fwd	5'-CCC ACC CAA TCA CCA TCA CAA-3'
Msb2-Del6 Rev	5'- AGG GTT CGC ATA GTC AGG AAC-3'
Msb2-Del7 Fwd	5'-GCT ACA GTT ACT GGT AGT GAA TC-3'
Msb2-Del7 Rev	5'-GGG AAT ATT ACT GCT TTC TG-3'
Msb2-delta PTS Fwd	5'-GTG AAA GCT TTA GGT AGT TTT ATA ACT ACT GCT ATC TAT AGA AAT CCT GAC AGT GTT TTA CAA G-3'
Msb2-delta PTS Rev	5'-CTT GTA AAA CAC TGT CAG GAT TTC TAT AGA TAG CAG TAG TTA TAA AAC TAC CTA AAG CTT TCA C-3'
Msb2-Del9 Fwd	5'-GCT GAG CCA TCA AGT GAA GTT ACC-3'
Msb2-Del9 Rev	5'-GAT TTG TTC AGA TGA TTC TGT AAC-3'
Msb2-Del10 Fwd	5'-CAA ACC GGA ACT TCA GCT GTC-3'
Msb2-Del10 Rev	5'-GGC AAT TGG AGT GGT GGT AGA-3'
Msb2-Del11 Fwd	5'-GAA GTT ACC GCA GTT GCT CCT TC-3'
Msb2-Del11 Rev	5'-CAA TGC AGT TTC AAC TCC ACT TTG-3'
Msb2-Del12 Fwd	5'-GAT AGT AAT CCA TCT ACT AAT TC-3'
Msb2-Del12 Rev	5'-CTC AGC TGA TGC TGG TGG AGA TG-3'
RTACT1 Fwd	5'-CAA CTG GGA CGA TAT GGA AAA AA-3'
RTACT1 Rev	5'-TTC GGT CAA CAA AAC TGG ATG T-3'
RTACT1long Fwd	5'-GAC TTG ACC AAC CAT TTG TCC-3'
RTACT1long Rev	5'-TCA TGA TGG AGT TGA AAG TGG-3'
RTPMT1 Fwd	5'- GCT GCT GAA CCT GTT GAA GA-3'
RTPMT1 Rev	5'- CAT CAG CAA CTT GTG GGT CT-3'
RTALS1 Fwd	5'-GAC TAG TGA ACC AAC AAA TAC CAG A-3'
RTALS1 Rev	5'-CCA GAA GAA ACA GCA GGT GA-3'
18S-rRNA(RT)-f	5'-CGA TGG AAG TTT GAG GCA AT-3'
18S-rRNA(RT)-r	5'-CAC GAC GGA GTT TCA CAA GA-3'

Table S2. Transcriptomal analysis of genes regulated by the C-tail of Msb2. Genes up/down-regulated in response to caspofungin treatment in strain ESCa38 (ΔD1) compared to control strain ESCa3 (Msb2) are listed. Fold regulation is shown for downregulated transcripts (negative values) and upregulated transcripts (positive values). **MSB2* gene expressed from *ACT1*-promoter.

Name	fold regulation	Gene	Description
NOVEL-C121chr7-051	-4.19	*NOVEL TRANSCRIPT 73*	
ORF19.1150	-3.44		Predicted ORF in Assemblies 19, 20 and 21; regulated by Gcn2p and Gcn4p
ORF19.1105	-3.28		
NOVEL-Ca21chr3-021	-2.94	*NOVEL TRANSCRIPT 329*	
ORF19.4575	-2.64		*S. cerevisiae* ortholog YPL109C localizes to mitochondrion
ORF19.7265	-2.56		Hap43p-repressed gene
ORF19.7301	-2.54		
NOVEL-C121chr5-011(+)	-2.49	*NOVEL TRANSCRIPT 58*	
*ORF19.1490**	-2.46	*MSB2**	Adhesin-like protein; mucin family; cell wall damage sensor; N-terminal secretion signal; required for Cek1p phosphorylation in response to cell wall stress; Rim101p-repressed; activation releases extracellular domain into medium
ORF19.3774	-2.41	*PPG1*	Putative protein phosphatase of the Type 2A-related family (serine/threonine-specific), similar to *S. cerevisiae* Ppg1p; possibly an essential gene, disruptants not obtained by UAU1 method
ORF19.5606	-2.36		
ORF19.2201	-2.32		*S. cerevisiae* ortholog CBP6 localizes to mitochondrion
ORF19.3158	-2.32		*S. cerevisiae* ortholog RMD1 localizes to cytoplasm
NOVEL-C121chr2-076	-2.21	*NOVEL TRANSCRIPT 33*	
ORF19.4097	-2.15		
ORF19.7609	-2.10	*PGA11*	Putative GPI-anchored protein
ORF19.3607	-2.06		
ORF19.2436	-2.03		Ortholog of *C. glabrata* CAGL0F03905g
ORF19.5565	-2.02		Putative 3-hydroxyisobutyrate dehydrogenase
NOVEL-Ca21chr1-130	-2.01	*NOVEL TRANSCRIPT 210*	
ORF19.3622	-2.01	*ANP1*	Putative mannosyltransferase of Golgi; member of Mnn9p family; similar to *S. cerevisiae* Anp1p; fungal-specific (no human or murine homolog)
ORF19.6674	-1.91	*BTS1*	Putative geranylgeranyl diphosphate synthase; decreased transcription is observed upon benomyl treatment
NOVEL-Ca21chrR-091	-1.90	*NOVEL TRANSCRIPT 656*	
NOVEL-Ca21chr7-040	-1.90	*NOVEL TRANSCRIPT 570*	
NOVEL-Ca21chr2-025	-1.90	*NOVEL TRANSCRIPT 238*	
ORF19.3229	-1.89		Predicted ORF in Assemblies 19, 20 and 21; transcription detected in high-resolution tiling array experiments
ORF19.2131	-1.86		*S. cerevisiae* ortholog TUL1 has ubiquitin-protein ligase activity, has role in protein ubiquitination, ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway and localizes to Golgi apparatus
ORF19.4279	-1.86	*MNN1*	Putative alpha-1,3-mannosyltransferase, a component of mannosyltransferase complex; transcription negatively regulated by Rim101p; transcription elevated in chk1 and nik1 null mutants, but not in sln1 null mutant; biofilm-induced gene
ORF19.2809	-1.85	*CTN3*	Predicted peroxisomal carnitine acetyl transferase; Ura+ deletion strain has no obvious metabolic, hyphal, or virulence defects; transcription induced by macrophage

			engulfment, hyphal growth, starvation, or nonfermentable carbon sources
ORF19.1913	-1.82		Late-stage biofilm-induced gene
NOVEL-Ca21chr6-040	-1.81	*NOVEL TRANSCRIPT 530*	
NOVEL-Ca21chr4-004(+)	-1.80	*NOVEL TRANSCRIPT 381*	
ORF19.5622	-1.79	*GLC3*	Putative 1,4-glucan branching enzyme; fluconazole-induced; shows colony morphology-related gene regulation by Ssn6p; stationary phase enriched protein; planktonic growth-induced gene
ORF19.3690.2	-1.78		*S. cerevisiae* ortholog RPL26B has RNA binding, structural constituent of ribosome, has role in translation and localizes to cytosolic large ribosomal subunit
ORF19.644	-1.69	*HGT9*	Putative glucose transporter of the major facilitator superfamily; the C. albicans glucose transporter family comprises 20 members; 12 probable membrane-spanning segments; induced at low (0.2%, compared to 2%) glucose in rich media; intron
ORF19.7660	-1.67	*VPS52*	Protein required for hyphal growth; has similarity to *S. cerevisiae* Vps52p
ORF19.1959	-1.66		*S. cerevisiae* ortholog *OTU2* localizes to ribosome, cytoplasm
C11lfMt26	-1.64		
NOVEL-Ca21chrR-042	-1.62	*NOVEL TRANSCRIPT 614*	
ORF19.5665	-1.60		*S. cerevisiae* ortholog ARA2 has D-arabinose 1-dehydrogenase [NAD(P)+] activity and has role in dehydro-D-arabinono-1,4-lactone biosynthetic process
NOVEL-Ca21chr5-001	-1.58	*NOVEL TRANSCRIPT 447*	
NOVEL-Ca21chr5-042	-1.58	*NOVEL TRANSCRIPT 484*	
ORF19.1636	-1.57	*STE50*	Protein with sterile alpha motif (SAM) and Ras-associated domain (RAD); similar to *S. cerevisiae* Rad50p, which is involved in signal transduction via interaction with and regulation of MAPKKK
ORF19.6462	-1.57		*S. cerevisiae* ortholog AIM27 has role in protein folding in endoplasmic reticulum and localizes to ER membrane protein complex
ORF19.3785	-1.57		
ORF19.4676	-1.56		
ORF19.7370	-1.56		Member of family of putative 7-transmembrane, PQ loop-family of G-protein-coupled receptors (GPCRs) similar to S. pombe Stm1p
ORF19.1536	-1.51		Putative vacuolar transporter; Hap43p-induced gene, required for normal filamentous growth; mRNA binds to She3p and is localized to hyphal tips
ORF19.5736	-1.51	*ALS5*	ALS family adhesin; highly variable; expression in *S. cerevisiae* causes adhesion to human epithelium, endothelium or ECM, endothelial invasiveness by endocytosis and, at high abundance, ECM-induced aggregation; can form amyloid fibrils
ORF19.1428	-1.50	*DUO1*	Subunit of the Dam1 (DASH) complex, which acts in chromosome segregation by coupling kinetochores to spindle microtubules
ORF19.344	-1.50		Predicted ORF in Assemblies 19, 20 and 21; increased transcription is observed upon fluphenazine treatment or in an azole-resistant strain that overexpresses CDR1 and CDR2; possibly transcriptionally regulated by Tac1p
ORF19.4491	3.05	*ERG20*	Putative farnesyl pyrophosphate synthetase involved in isoprenoid and sterol biosynthesis, based on similarity to *S. cerevisiae* Erg20p; likely to be essential for growth, based on an insertional mutagenesis strategy
ORF19.548	2.85	*CDC10*	Septin, required for wild-type cell, hyphal, or chlamydospore morphology; role in virulence and kidney tissue invasion in a mouse model of systemic infection; forms ring at sites of cell division and filaments in mature chlamydospore
NOVEL-Ca21chr4-027	2.59	*NOVEL TRANSCRIPT 401*	

ORF19.1868	2.53	*RNR22*	Putative ribonucleoside diphosphate reductase; shows colony morphology-related gene regulation by Ssn6p; RNA abundance regulated by tyrosol and cell density; Hap43p-repressed gene; biofilm- and planktonic growth-induced
ORF19.5741	2.51	*ALS1*	Adhesin; ALS family of cell-surface glycoproteins; adhesion, virulence roles; immunoprotective; band at hyphal base; amyloid domain; biofilm-induced; Rfg1p, Ssk1p; strain background affects expression; N-term binds fucose-containing glycans
ORF19.6022	2.46	*NRM1*	Transcriptional regulator of cell cycle gene expression; regulates expression of genes involved in DNA replication stress; interacts with Swi4 and Swi6; repressed during core stress response; Spider biofilm repressed
ORF19.2529.1	2.44		
*ORF19.280*_Antisense	2.43	*ANTISENSE-ORF19.280*	
ORF19.257	2.34		Transcription is negatively regulated by Sfu1p; repressed by nitric oxide
ORF19.5419	2.33	*ATP5*	Putative F0-ATP synthase FO subunit B; caspofungin repressed; protein level decreased in stationary phase yeast cultures
ORF19.2640	2.31	*FUR1*	Uracil phosphoribosyltransferase; predicted tetrameric enzyme of pyrimidine salvage; mutations associated with flucytosine resistance in clade I clinical isolates; flucytosine, macrophage-induced protein; levels decrease in stationary phase
ORF19.5586	2.30		*S. cerevisiae* ortholog FIG4 has phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity, has role in phosphatidylinositol dephosphorylation and localizes to fungal-type vacuole membrane, extrinsic to membrane, PAS complex
ORF19.2093	2.28	*RFA1*	Putative DNA replication factor A; RNA abundance regulated by cell cycle, tyrosol and cell density
ORF19.4979	2.24	*KNS1*	*S. cerevisiae* ortholog KNS1 has protein tyrosine kinase activity, protein serine/threonine kinase activity and has role in protein autophosphorylation
ORF19.6753	2.22		Predicted ORF in Assemblies 19, 20 and 21; possibly an essential gene, disruptants not obtained by UAU1 method
ORF19.1353	2.21		Biofilm-and planktonic growth-induced gene; transcription downregulated upon yeast-hyphal switch; Ras1p-regulated
ORF19.4026	2.18	*HIS1*	ATP phosphoribosyl transferase; enzyme of histidine biosynthesis; fungal-specific (no human, murine homolog); upregulated in biofilm; acid upregulated/alkaline downregulated by Rim101p; regulated by Gcn2p, Gcn4p; strain CA9 is a his1 mutant
ORF19.2364	2.18	*MIS11*	Similar to precursor of mitochondrial C1-tetrahydrofolate synthase; putative protein of glycine catabolism; downregulated by Efg1p; fluconazole-induced; stationary phase enriched protein
ORF19.2180	2.14		Ortholog of C. glabrata CAGL0K04433g
ORF19.3055	2.13		Putative RNA polymerase II mediator complex subunit; possibly an essential gene, disruptants not obtained by UAU1 method
ORF19.1678	2.12		Predicted ORF in Assemblies 19, 20 and 21; transcription detected in high-resolution tiling array experiments
ORF19.3583	2.09		
ORF19.3541	2.09	*ERF1*	Putative translation release factor 1, which interacts with stop codons and promotes release of nascent peptides from ribosomes; Hap43p-induced gene
ORF19.4436	2.09	*GPX3*	Planktonic growth-induced gene
ORF19.497	2.09	*EAF7*	Subunit of the NuA4 histone acetyltransferase complex
ORF19.6634	2.08	*VMA2*	Vacuolar H(+)-ATPase; protein present in exponential and stationary growth phase yeast cultures; plasma membrane localized; amphotericin B repressed, caspofungin repressed
ORF19.2947	2.07	*SNZ1*	Stationary phase protein; soluble in hyphae; induced on yeast to hyphal switch, in response to 3-aminotriazole, or in azole-resistant strain overexpressing MDR1; regulated by Gcn4p, macrophage; no human/murine homolog

ORF19.2183	2.06	*KRE30*	YEF3-subfamily ABC family protein, predicted not to be a transporter; downregulated during core stress response; mutation confers hypersensitivity to amphotericin B
ORF19.1652	2.05	*POX1-3*	Predicted acyl-CoA oxidase; farnesol regulated; stationary phase enriched protein
ORF19.7183	2.03		*S. cerevisiae* ortholog EMC4 has role in protein folding in endoplasmic reticulum and localizes to ER membrane protein complex
ORF19.6882	2.02	*OSM1*	Putative flavoprotein subunit of fumarate reductase; soluble protein in hyphae; fungal-specific (no human or murine homolog); caspofungin repressed; stationary phase enriched protein; biofilm-induced gene
ORF19.2309.2	1.99	*RPL2*	Putative 60S ribosomal protein L2; Hap43p-induced gene; shows downregulation in infected rabbit kidney in SC5314, but not NGY152, strain background
ORF19.1816	1.98	*ALS3*	ALS family cell wall adhesin; role in epithelial adhesion, endothelial invasiveness; allelic variation in adhesiveness; immunoprotective in mice; promotes biofilm formation by binding to SspB adhesin of *S. gordonii*; fluconazole-repressed
ORF19.5812	1.94		*S. cerevisiae* ortholog *ETT1* has role in translational termination and localizes to nucleus
ORF19.5747	1.90		*S. cerevisiae* ortholog *MRP4* has structural constituent of ribosome and localizes to mitochondrial small ribosomal subunit
ORF19.825	1.90	*GCD7*	Putative translation initiator; downregulated in the presence of human whole blood or polymorphonuclear (PMN) cells
ORF19.3895	1.87	*CHT2*	GPI-linked chitinase required for normal filamentous growth; downregulated in core caspofungin response; induced in yeast cells; fluconazole, Cyr1p-, Efg1p-, pH-regulated; mRNA binds She3p, is localized to yeast-form buds and hyphal tips
ORF19.3426	1.87	*ANB1*	Translation initiation factor eIF-5A; decreased expression in hyphae vs to yeast-form cells; genes encoding ribosomal subunits, translation factors, tRNA synthetases are downregulated upon phagocytosis by murine macrophage; Hap43p-induced
ORF19.2560	1.87	*CDC60*	Cytosolic leucyl tRNA synthetase; conserved amino acid and ATP binding class I signature, tRNA binding, proofreading motifs; likely essential for growth; interacts with benzoxaborole antifungals; present in exponential and stationary phase
ORF19.5911	1.85	*CMK1*	Putative calcium/calmodulin-dependent protein kinase II; expression regulated upon white-opaque switching; biochemically purified Ca2+/CaM-dependent kinase is soluble, cytosolic, monomeric, and serine-autophosphorylated; Hap43p-repressed
ORF19.4851	1.83	*TFA1*	Protein with polyglutamate motifs and abundant Ser/Thr residues; described as a subunit of TFIIE, which is a basal transcription initiation factor of RNA Polymerase II; possibly an essential gene, disruptants not obtained by UAU1 method
ORF19.1064	1.83	*ACS2*	Acetyl-CoA synthetase; antigenic during human and murine infection; upregulated by Efg1p; macrophage-induced protein; soluble protein in hyphae; gene contains an intron
ORF19.862	1.82		
ORF19.211	1.82		
ORF19.3616	1.81	*ERG9*	Putative farnesyl-diphosphate farnesyl transferase (squalene synthase) involved in the sterol biosynthesis pathway; likely to be essential for growth; regulated by fluconazole and lovastatin; amphotericin B, caspofungin repressed
ORF19.2023	1.81	*HGT7*	Putative glucose transporter, major facilitator superfamily; glucose-, fluconazole-, Snf3p-induced, expressed at high glucose; upregulated in biofilm; C. albicans glucose transporter family comprises 20 members; 12 TM regions predicted
ORF19.5660.1	1.80		*S. cerevisiae* ortholog *TIM11* has structural molecule

			activity, proton-transporting ATPase activity, rotational mechanism and has role in ATP synthesis coupled proton transport, cristae formation, protein oligomerization
ORF19.4558	1.80		ORF|Deleted from Assembly 20
ORF19.5074	1.80		*S. cerevisiae* ortholog *UBA2* has SUMO activating enzyme activity, has role in protein sumoylation and localizes to SUMO activating enzyme complex
ORF19.4865	1.77		*S. cerevisiae* ortholog *SAC1* has role in phosphatidylinositol dephosphorylation
ORF19.5801	1.76	*RNR21*	Putative ribonucleoside-diphosphate reductase; regulated by tyrosol and cell density; transcription upregulated in response to treatment with ciclopirox olamine; fluconazole or flucytosine induced; Hap43p-induced; planktonic growth-induced
ORF19.899	1.76		
ORF19.1505	1.75		
ORF19.4406	1.74		*S. cerevisiae* ortholog *NIF3* localizes to mitochondrion
ORF19.2876	1.74	*CBF1*	Transcription factor that binds upstream of ribosomal protein genes and the rDNA locus, with Tbf1p; also regulates sulfur starvation-response, respiratory, and glycolytic genes; does not bind to centromeres as does *S. cerevisiae* Cbf1p
ORF19.6766	1.74		Hap43p-induced gene; *S. cerevisiae* ortholog *NOP13*, a nucleolar protein found in preribosomal complexes
NOVEL-Ca21chrR-053	1.73	*NOVEL TRANSCRIPT 624*	
ORF19.7178	1.72	*PRE5*	Alpha6 subunit of the 20S proteasome; regulated by Gcn4p; induced in response to amino acid starvation (3-aminotriazole treatment)
ORF19.3861	1.72	*SIS1*	Putative Type II HSP40 co-chaperone; macrophage/pseudohyphal-repressed; heavy metal (cadmium) stress-induced; heterozygous null mutant displays sensitivity to virgineone
ORF19.2328	1.71		
ORF19.88	1.67	*ILV5*	Putative ketol-acid reductoisomerase; antigenic during human/murine infection; regulated by Gcn4p; amino acid starvation (3-AT)-induced; biofilm induced; macrophage-downregulated protein; protein present in exponential and stationary phase
ORF19.968	1.67	*PGA14*	Putative GPI-anchored protein; induced during cell wall regeneration; regulated by Ssn6p
ORF19.5425	1.66		*S. cerevisiae* ortholog *TRZ1* has 3'-tRNA processing endoribonuclease activity, has role in tRNA 3'-trailer cleavage, endonucleolytic and localizes to nucleus, mitochondrion
ORF19.954	1.64		Hap43p-repressed gene
ORF19.5495	1.63		Putative RNA-binding protein; not essential for viability; transcription is induced in response to alpha pheromone in SpiderM medium
ORF19.2728	1.62		*S. cerevisiae* ortholog *RAD24* has DNA clamp loader activity, has role in reciprocal meiotic recombination, DNA damage checkpoint, nucleotide-excision repair and localizes to nucleus, Rad17 RFC-like complex
ORF19.3949	1.61		*S. cerevisiae* ortholog *YTA7* has role in positive regulation of isoprenoid metabolic process, positive regulation of transcription from RNA polymerase II promoter, cellular protein localization
ORF19.7284	1.61	*ASR2*	Gene regulated by cAMP and by osmotic stress; greater mRNA abundance observed in a cyr1 or ras1 homozygous null mutant than in wild type; stationary phase enriched protein
ORF19.6893	1.60		*S. cerevisiae* ortholog *RUD3* has role in ER to Golgi vesicle-mediated transport
ORF19.5982	1.60	*RPL18*	Predicted ribosomal protein; Plc1p-regulated, Tbf1p-activated; genes encoding cytoplasmic ribosomal subunits, translation factors, tRNA synthetases are downregulated upon phagocytosis by murine macrophage; Hap43p-induced gene

ORF19.5943	1.59		*S. cerevisiae* ortholog *PEX28* has role in peroxisome organization and localizes to peroxisomal membrane
ORF19.5285	1.57	*PST3*	Putative flavodoxin; biofilm induced; fungal-specific (no human or murine homolog); stationary phase enriched protein
ORF19.7292	1.57	*ARP2*	Putative component of the Arp2/3 complex; required for virulence, hyphal growth, and cell wall and cytoskeleton organization, but not for endocytosis; mutation confers hypersensitivity to cytochalasin D; regulated by Gcn2p and Gcn4p
ORF19.5854	1.56	*SBP1*	Similar to RNA binding proteins; downregulated upon adherence to polystyrene; stationary-phase enriched protein
ORF19.6403	1.55		*S. cerevisiae* ortholog SIL1 has adenyl-nucleotide exchange factor activity, has role in SRP-dependent cotranslational protein targeting to membrane, translocation and localizes to endoplasmic reticulum
ORF19.6773	1.55	*ECM29*	Protein similar to *S. cerevisiae* Ecm29p; transposon mutation affects filamentous growth
ORF19.3182	1.55	*GIS2*	Putative transcription factor; expression is increased in high iron and reduced upon yeast-hyphal switch; null mutant exhibits sensitivity to sorbitol, 5-fluorocytosine, and cold temperatures
ORF19.4520	1.54		*S. cerevisiae* ortholog YDR248C localizes to cytoplasm
ORF19.1142	1.52		Putative vacuolar transporter of large neutral amino acids; transcription is induced in response to alpha pheromone in SpiderM medium
ORF19.4432	1.51	*KSP1*	Putative serine/threonine protein kinase; mRNA binds to She3p and is localized to hyphal tips; mutation confers hypersensitivity to amphotericin B
ORF19.5467	1.50	*TLO7*	Member of a family of telomere-proximal genes of unknown function; may be spliced in vivo
ORF19.5963	1.50		*S. cerevisiae* ortholog *NUS1* has role in protein glycosylation and localizes to endoplasmic reticulum, lipid particle, nuclear envelope
ORF19.4468	1.50		Putative succinate dehydrogenase; localized to the mitochondrial membrane; Hap43p-repressed gene

Table S3. GO analysis of genes regulated by the C-tail of Msb2. Regulated genes listed in Table S2 were categorized for their functions using GO programs **A.** Term Finder (www.candidagenome.org/cgi-bin/GO/goTermFinder) and **B.** Slim Mapper (www.candidagenome.org/cgi-bin/GO/goTermMapper). In cells lacking the Msb2 C-tail upregulated genes are in red, while downregulated genes are in blue color.

A

GO term	Cluster frequency	Background frequency	Corrected P-value	False discovery rate	Gene(s) annotated to the term
entry into host cell	3 out of 117 genes, 2.6%	4 out of 6473 background genes, 0.1%	0.01352	4.00%	*ALS5*, *ALS1*, *ALS3*
entry into cell of other organism involved in symbiotic interaction	3 out of 117 genes, 2.6%	4 out of 6473 background genes, 0.1%	0.01352	2.00%	*ALS5*, *ALS1*, *ALS3*

B

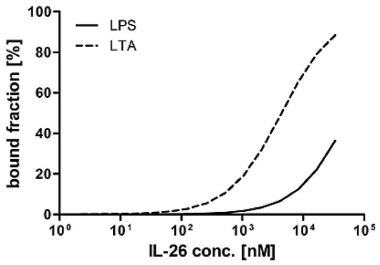
GO term	Frequency	Gene(s)
response to stress	19 out of 117 genes, 16.2%	*GPX3*, *ORF19.2728*, *STE50*, *MSB2*, *RFA1*, *SIS1*, *ORF19.211*, *GIS2*, *ECM29*, *SBP1*, *CMK1*, *ARP2*, *CHT2*, *AVT4*, *PGA14*, *ORF19.1536*, *VPS52*, *EAF7*, *ERF1*
cellular protein modification process	16 out of 117 genes, 13.6%	*PPG1*, *GPX3*, *ORF19.2436*, *ORF19.5963*, *RFA1*, *KNS1*, *KSP1*, *CMK1*, *ORF19.2131*, *ORF19.1959*, *ACS2*, *MNN1*, *ANP1*, *ANB1*, *UBA2*, *EAF7*
response to chemical	15 out of 117 genes, 12.7%	*GPX3*, *ORF19.2436*, *HGT7*, *STE50*, *NRM1*, *CBF1*, *SIS1*, *GIS2*, *KSP1*, *CMK1*, *KRE30*, *ARP2*, *PGA14*, *ORF19.1536*, *FUR1*

2.6 Weiterführende Ergebnisse zur Interaktion von AMPs mit Zelloberflächen Komponenten von Bakterien

Im Rahmen einer Kooperation mit Prof. Dr. med. Bernhard Homey von der Hautklinik des Universitätsklinikums Düsseldorf wurde die Rolle des Interleukin-26 (IL-26) bei einer Infektion charakterisiert.

Das kationische und amphipathische Cytokin IL-26 wird zusammen mit IL-22, durch aktivierte T- Zellen, insbesondere Th17-Zellen, ko-exprimiert (Fickenscher & Pirzer, 2004). Th17-Zellen spielen eine wichtige Rolle beim Schutz gegen Infektionen, sowie bei Autoimmunerkrankungen (Wilson *et al.*, 2007). Die von Th17-Zellen sezernierten Cytokine triggern Epithel-Zellen antimikrobielle Effektor-Moleküle zu produzieren um eine Proliferation von Pathogenen, wie z.B. *C. albicans* und *S. aureus*, zu verhindern. IL-26 bindet an einen heterodimeren Rezeptor-Komplex bestehend aus IL-20R1 und IL-10R2. IL-26-Rezeptoren werden in erster Linie auf nicht-hämatopoetischen Zell-Typen, insbesondere Epithelzellen, exprimiert. Die durch IL-26 induzierte Signaltransduktion führt zur Aktivierung von STAT1 und STAT3. In Kooperation konnte gezeigt werden, dass IL-26 Bakterien, wie z.b. *Pseudomonas aeruginosa* und *S. aureus*, jedoch nicht *C. albicans*, abtötet.

Mittels MST wurde gezeigt, dass IL-26 an Bestandteile der bakteriellen Zellwand binden kann. Durch Quantifizierung wurde eine höhere Affinität des Cytokins zu Lipoteichonsäuren (LTA) im Vergleich zu Lipopolysacchariden (LPS) bestimmt (Abb. 2.1). Desweiteren zeigten sich IL-26-DNA-Komplexe, bakteriellen und humanen Ursprungs, wodurch es zu einer Produktion von Typ-I-Interferonen in plasmacytoiden dendritischen Zellen (pDCs) über die TLR9-Aktivierung kam. Diese Ergebnisse liefern neue Erkenntnisse über die antimikrobielle und entzündungsfördernde Funktion der Th17-Zellen, sowie IL-26, ein Cytokin mit direkter antimikrobieller Wirkung.



Protein/ ligand	IL-26/ LPS	IL-26/ LTA	LL-37/ LPS	LL-37/ LTA	IL-26/ LL-37	IL-22/ LPS	IL-22/ LTA
K_D (mean ± SD)	58.9 µM ± 2.8	4.42 µM ± 0.5	6.57 nM ± 2.2	7.33 nM ± 4.6	NI	NI	NI

Abb. 2.1- IL-26 bindet LPS and LTA. Gebundenes (%) FITC-LPS (45 nM) und LTA-Alexa Fluor 488 (38 nM) in Anhängigkeit von IL-26 mittels „Microscale thermophoresis“. Die Tabelle zeigt die gemessenen Dissoziationskonstanten (KD) der Proteine und der Liganden; (NI) Keine Interaktion.

3 Diskussion

Der opportunistisch humanpathogene Pilz *C. albicans* stellt mit bis zu 66 % aller Fälle die häufigste Ursache von invasiven Candidosen dar (Pfaller & Diekema, 2007). Die Sterberate von *C. albicans*-Infektionen des Blutstromes liegt bei bis zu 50 % (Eggimann *et al.*, 2003). Ein komplexes Wechselspiel von Reaktionen charakterisiert die Begegnung von mikrobiellen Erregern mit dem menschlichen Wirt. Die *C. albicans*-Zellwand ist eine essentielle und speziell aufgebaute Organelle des Pathogens, die im Rahmen der Immunabwehr durch den Wirt angegriffen wird. Mittels unterschiedlicher Sensoren löst die Hefe verschiedene Mechanismen aus um Defekte der Zellwand zu kompensieren (Ernst & Pla, 2011). Eine wichtige Rolle übernimmt hierbei der Cek1-Signalweg, bestehend aus dem Kinase-Modul Ste11, Hst7 und der MAPK Cek1, welcher durch Glykosylierungs-Defekte aktiviert wird. Als möglicher Rezeptor wurde das Mucin-ähnliche Protein Msb2 identifiziert (Román *et al.*, 2009). Msb2 enthält 43% Serin- und Threonin-Reste und besteht aus einem großen N-terminalen extrazellulären Bereich, einer Transmembrandomäne und einem kurzen C-terminalen cytoplasmatischen Teil.

Im Rahmen dieser Arbeit wurde die Rolle des Membransensors Msb2 für die Morphogenese und Resistenzen gegenüber Antimykotika bzw. AMPs charakterisiert; außerdem wurden molekulare Mechanismen der Signalumsetzung durch Msb2 und die phänotypischen Konsequenzen der Msb2-Funktion untersucht. Hierzu wurden *C. albicans*-Stämme konstruiert, die für eine Msb2-Variante kodieren, in der die große N-terminale extrazelluläre Domäne mit einem HA-Epitop und die kleine C-terminale cytoplasmatische Domäne mit einem V5-Epitop markiert sind. Hiermit konnten die Lokalisation, die Sekretion und Glykosylierungsmuster von Msb2 untersucht werden.

3.1 CaMsb2 wird sekretiert und in die Umgebung abgegeben

Eine der maßgeblichen posttranslationalen Modifikationen von Mucinen ist die proteolytische Prozessierung, wobei die Mucine in eine extrazelluläre und eine cytoplasmatische Domäne gespalten werden können (Cullen, 2011).

Bei *S. cerevisiae* wird Msb2 gespalten und der extrazelluläre Bereich in das Wachstumsmedium sekretiert, während der cytoplasmatische Bereich innerhalb der Zelle verbleibt und in der Vakuole angereichert wird (Vadaie *et al.*, 2008). Bei *C. albicans* wird das Mucin-ähnliche Protein Msb2 in der Hefe-, wie auch in der Hyphen-Form ins Medium sekretiert (Msb2*; Manuskript II). Analysen der Epitop-markierten Msb2-Variante, in der die große N-terminale extrazelluläre Domäne mit einem HA-Epitop und die kleine C-terminale cytoplasmatische Domäne mit einem V5-Epitop markiert sind, ergaben, dass die extrazelluläre Domäne quantitativ abgespalten und ins Medium sekretiert wird, während die cytoplasmatische Domäne in der Zelle verbleibt. Weiterhin wurde festgestellt, dass Msb2* nicht nur in Flüssigmedium sezerniert wird, sondern auch auf Festmedium (Manuskript II). Sorgo *et al.* (2010) konnten ebenfalls nachweisen,

dass Msb2 unter Standardwuchsbedingungen sekretiert wird, da die folgenden Peptide mittels Q-TOf identifiziert wurden: Msb2-Aminosäuren 1212-1219, 1223-1237, 1238-1253 und 1254-1290. Vadaie *et al.* (2008) konnten einen Bereich für die Prozessierung in Msb2 in *S. cerevisiae* eingrenzen, an dessen Spaltung die Yapsin-Aspartylprotease Yps1 beteiligt ist. Das zu ScYps1 homologe Protein in *C. albicans* Sap9, wie auch die Aspartylprotease Sap10, welche durch einen GPI-Anker in der Plasmamembran assoziiert ist, haben bei *C. albicans* jedoch keinen Einfluss auf die Sekretion von Msb2 (Manuskript II). Untersuchungen des *S. cerevisiae* Flocculins Flo11, eines großen GPI-verankerten Glykoproteins, zeigten, dass dieses durch die Furin-Protease Kex2 mehrfach prozessiert wird (Karunanithi *et al.*, 2010). Die CaMsb2-Prozessierung ist jedoch unabhängig von Kex2 (Manuskript II). Kürzlich veröffentlichte Arbeiten zeigten, dass die Prozessierung von Msb2 in *C. albicans* abhängig von der Protease Sap8 ist (Puri *et al.*, 2012). Diese Ergebnisse konnten in unserer Arbeitsgruppe jedoch nicht bestätigt werden, da keine Unterschiede von sezerniertem oder Zell-assoziiertem Msb2 in der *sap8*-Mutante im Vergleich zum Wildtypen festgestellt wurden (Daten nicht gezeigt). Darüber hinaus wurde die Msb2-Prozessierung in Gegenwart von Protease-Inhibitoren untersucht (Manuskript II). Inhibitoren von Metallo-, Serin-, Cystein- und Aspartylproteasen, wie z.B. Sap8, beeinflussten die Spaltung und somit die Sekretion der extrazellulären Domäne nicht, anders als es von Puri *et al.* (2012) beschrieben wurde. Die Identifizierung der Msb2-spaltenden Protease steht daher noch aus, ist aber von großer Relevanz, da die Spaltung essentiell für alle *MSB2*-Phänotypen ist (siehe unten). Eine weitere Möglichkeit der Msb2-Prozessierung ist eine autoproteolytische Spaltung. Die autoproteolytische Spaltung des humanen Mucins MUC1 ist abhängig von der SEA-Domäne, welche das Vorläuferprotein im G↑SVVV-Motiv spaltet (Hattrup & Gendler, 2008). Wie an homologen Proteinen gezeigt wurde kann die VVV-Sequenz durch andere hydrophobe Reste ersetzt werden (Maeda *et al.*, 2004). Dem Msb2 Protein in *C. albicans* fehlen homologe SEA-Sequenzen, jedoch enthält das Protein eine $G^{1232}SAIY^{1235}$ Sequenz N-terminal von der Transmembrandomäne. Die Deletion dieser Aminosäuren (AS-Reste 1231-1233) beeinflusste jedoch nicht die Msb2-Sekretion (Manuskript IV; Daten nicht gezeigt). Somit ist davon auszugehen, dass Msb2 in *C. albicans* weder autoproteolytisch, noch durch die Proteasen Sap8-10 oder Kex2 gespalten und somit in die Umgebung sezerniert wird.

Um die Region einzugrenzen, in der die extrazelluläre Msb2-Domäne geschnitten wird, wurden Msb2-Varianten mittels Mutagenese-PCR konstruiert, die Deletionen stromaufwärts der Transmembrandomäne aufweisen (Manuskript IV). Mittels Immunoblotanalysen wurde ein Msb2-Segment, in dem die AS-Reste zwischen den Positionen 1084 und 1283 der Exodomäne deletiert sind, identifiziert, welches für die Spaltung und Sekretion verantwortlich ist (Manuskript IV). Diese Sequenz enthält mehrere theoretisch vorhergesagte proteolytische Schnittstellen, die direkt oder

indirekt, die Spaltung beeinflussen könnte. Dieses Msb2-Segment ist für die Spaltung und Freisetzung der Exodomäne und damit verbundenen *MSB2*-abhängigen Funktionen essentiell.

Um die proteolytische Schnittstelle von Msb2 zu identifizieren müsste als nächstes das Segment, in dem die Aminosäure-Reste zwischen den Positionen 1084 und 1283 der Exodomäne deletiert sind, weitergehend untersucht werden. Hierzu müsste dieses Fragment in kleinere Segmente unterteilt und im wildtypischen Msb2-Protein deletiert werden. Weiterhin wäre eine Msb2-Expression in *E.coli* möglich. Nach anschließender Aufreinigung und Zugabe des Msb2 aus *E. coli* zu wildtypischen *C. albicans*-Zellen und/oder Rohextrakten könnte durch Immunoblot-Analysen die Msb2-Prozessierung untersucht werden. Anschließend würde durch Edmann-Abbau die Sequenzierung am N-terminalen Ende des prozessierten Proteins (Edman, 1949) und mittels Schlack-Kumpf-Abbau die C-terminalen Sequenzierung des Fragments (Schlack & Kampf, 1926, Li & Liang, 2002) durchgeführt um die proteolytische Schnittstelle zu identifizieren.

3.2 Glykosylierung von Msb2 in *C. albicans*

Defekte in der *O*-Glykosylierung resultieren in einer veränderten Zusammensetzung der Chitin-, Glukan- und Mannoproteingehalte der *C. albicans*-Zellwand (Prill *et al.*, 2005). Hierbei zeigte sich, dass die *pmt1*-, wie auch die *pmt4*-Mutante einen höheren Anteil an β1,3-Glucan in der Zellwand enthielt. Desweiteren wiesen die Mutanten einen niedrigeren Gehalt an Mannoproteinen auf.

Msb2 wird in *S. cerevisiae* durch die Protein-*O*-Mannosyltransferasen Pmt1, Pmt2 und Pmt4 in der STR-Domäne (Serin/Threonin-reiche Domäne) stark glykosyliert (Yang *et al.*, 2009). Mittels Gelfiltration konnten für CaMsb2 ebenfalls posttranslationale Modifikationen festgestellt werden, da eine molekulare Masse von ~ 468–614 kDa ermittelt wurde und die theoretische Größe mit 141 kDa ~4-fach geringer ist (Manuskript II). Die Behandlung des sekretierten Msb2 mit PNGase F führte zu keiner signifikanten Änderung der molekularen Masse, wohingegen die β-Elimination zu einer Reduktion auf 300 kDa führte. Dieses Ergebnis deutet darauf hin, dass das sezernierte Msb2 *O*- und nicht *N*-glykosyliert ist. Die komplette chemische Deglykosylierung mittels TMFS reduzierte die Masse von Msb2* auf ~ 117–130 kDa, welches der ungefähren Masse von Msb2 entspräche, wenn das Protein direkt stromaufwärts der Transmembrandomäne geschnitten wird. Für *C. albicans* konnten Prill *et al*. (2005) fünf Pmt-Isoformen identifizieren, welche den ersten Schritt der *O*-Glykosylierung im ER katalysieren. Pmt1 und Pmt5 gehören jeweils zur Pmt1-Familie, Pmt2 und Pmt6 zur Pmt2-Familie und als einziger Vertreter der Pmt4-Familie konnte Pmt4 identifiziert werden (Prill *et al.*, 2005, Lengeler *et al.*, 2008). Durch Immunoblotanalysen wurde gezeigt, dass Msb2 durch Pmt1 mannosyliert wird (Manuskript II). Aufgrund der hohen molekularen Masse von Msb2 ist es jedoch schwierig, geringe Unterschiede im Glykosylierungsmuster festzustellen. Möglicherweise modifizieren einige Pmt-Isoformen, wie z.B. Pmt4, nur wenige, aber strukturell wesentliche AS-Reste. Der Einfluss von Pmt2 auf die Msb2-Glykosylierung konnte nicht

ausgeschlossen werden, da Pmt2 essentiell für *C. albicans* ist und eine homozygote *pmt2*-Mutante nicht lebensfähig ist (Prill *et al.*, 2005). Desweiteren könnten kompensatorische Regulationen der Pmt-Isoformen die *O*-Mannosylierung von Msb2 in *pmt*-Mutanten beeinflussen (Cantero *et al.*, 2007, Cantero & Ernst, 2011).

3.3 C-terminale Sequenzen vermitteln basale Resistenz gegenüber Zellwand-Stress

C. albicans ist als kommensaler Organismus einer sich stetig verändernden Umwelt ausgesetzt. Der Pathogen ist durch Abwehrmechanismen des Wirtes starken Stress ausgesetzt, welcher sich besonders auf die Zellwand auswirkt. Stressfaktoren sind beispielsweise Sauerstoffradikale, antimikrobielle Peptide, hydrolytische Enzyme und therapeutische, antimykotische Verbindungen (Heilmann *et al.*, 2013).

Um zu überprüfen, welchen Einfluss die Disruption des *MSB2*-Gens bei Zellwand-Stress hat wurde die Sensitivität gegenüber Zellwand-destabilisierender Agenzien, wie Caspofungin und Tunicamycin, sowie dem Pmt1-Inhibitor untersucht (Manuskript II). Die *msb2*-Mutante wächst in Gegenwart von Tunicamycin im Vergleich zum Wildtyp schlechter und ist in Anwesenheit von Caspofungin nicht lebensfähig; der Pmt1-Inhibitor hatte jedoch keinen Einfluss auf das Wachstumsverhalten der Mutante. In vorangegangenen Arbeiten konnte bereits gezeigt werden, dass die *msb2*-Mutante sensitiv gegenüber Kongo Rot und Zymolyase ist (Román *et al.*, 2009). Somit ist Msb2 für die basale Resistenz von *C. albicans* gegenüber Zellwand-Stress notwendig.

Um funktionelle Regionen von Msb2 gegenüber basalem Zellwand-Stress zu identifizieren, wurden Msb2-Varianten mittels Mutagenese-PCR konstruiert und die transformierten *C. albicans*-Stämme auf deren Sensitivität gegenüber Caspofungin und niedrigen Konzentrationen von Tunicamycin getestet (Manuskript IV). Es zeigte sich, dass N-terminale Sequenzen nahe der Transmembrandomäne (ΔD3, ΔD4, ΔD5) essentiell für Funktion von Msb2 für die basale Zellwand-Resistenz sind.

3.4 Interne Msb2-Sequenzen regulieren den MAP Kinase Cek1 Spiegel

Der Cek1-Signalweg kompensiert defekte Glykostrukturen in der *C. albicans* Zellwand (Ernst & Pla, 2011). Der Sensor Msb2 reguliert dabei die Aktivität der MAP-Kinase Cek1, welche auch für die Hyphenbildung auf festen Medien erforderlich ist (Román *et al.*, 2009).

In dieser Arbeit konnte gezeigt werden, dass die *msb2*-Mutante nicht in der Lage ist, unter Tunicamycin-bewirktem *N*-Glykosylierungsstress Cek1 zu phosphorylieren (Manuskript II). Des Weiteren wurden an Hand der konstruierten Msb2-Varianten zwei Funktionen bei der Cek1 Phosphorylierung identifiziert, die durch drei getrennte Domänen vermittelt werden (Manuskript IV). Unter normalen Wachstumsbedingungen reprimieren N-terminale Sequenzen die Cek1 Phosphorylierung (Manuskript II; IV), während unter Tunicamycin-Stress C-terminale Sequenzen,

welche die TM-Domäne flankieren, die Cek1 Phosphorylierung induzieren (Manuskript II; IV). Somit können wildtypische *C. albicans*-Zellen in Gegenwart von Zellwand-destabilisierenden Agenzien den Cek1-Weg über Msb2 aktivieren und diese Schäden kompensieren. Die Cek1-Signalweg-Aktivierung bei Glykosylierungsdefekten dient unter anderem der Regulation der *PMT*-Genexpression (Cantero & Ernst, 2011). So wurde nachgewiesen, dass der basale *PMT1* Transkriptspiegel in der *msb2*- und *cek1*-Mutante deutlich erhöht war, woraus geschlossen werden kann, dass er unter normalen Wachstumsbedingungen durch Msb2 und Cek1 reprimiert wird. Da keine signifikante Cek1 Phosphorylierung in ungestressten *C. albicans*-Zellen erfolgt, lässt dies darauf schließen, dass die Repression von *PMT1* die Anwesenheit von Cek1 erfordert, jedoch nicht in seiner phosphorylierten Form. Interessanterweise werden interne Msb2-Sequenzen stromaufwärts der TM-Domäne (ΔD3 und ΔD4; Manuskript IV), welche ebenfalls für die Basalresistenz gegen Caspofungin und Tunicamycin erforderlich sind (Abschn. 3.3), benötigt, um den *PMT1* Transkriptspiegel zu reprimieren. Dieser Mechanismus der Regulation der *PMT*-Genexpression bei Glykosylierungsdefekten ist unabhängig vom cytoplasmatischen Msb2-Teil (ΔD1; Manuskript IV).

Somit lässt sich schlussfolgern, dass eine defekte *O*-Mannosylierung von Msb2 (Abschn. 3.2; Manuskript II) oder anderen defekten *O*- oder *N*-glykosylierten Proteinen, welche mit Msb2 interagieren, die MAP Kinase Cek1 aktivieren, um den Defekt zu kompensieren. Die Annahme einer Proteininteraktion entsteht aus der Tatsache, dass Msb2 nicht *N*-glykosyliert wird (Abschn. 3.2; Manuskript II), jedoch essentiell für die Cek1 Phosphorylierung unter Tunicamycin-Stress ist (Manuskript II; IV). Für *S. cerevisiae* konnte dagegen gezeigt werden, dass Msb2 sowohl *N*- als auch *O*-glykosyliert ist. Das Cek1 Homolog Kss1 wird in dieser Hefe nur in *pmt4*-Mutanten unter Tunicamycin-Stress aktiviert (Tatebayashi *et al.*, 2007, Yang *et al.*, 2008). Daraus folgt, dass die Msb2-Aktivierung bei defekter Protein-Glykosylierung und die resultierende MAPK-Aktivierung in *C. albicans* und *S. cerevisiae* unterschiedlich ablaufen.

3.5 Die Beteiligung von Msb2 an der Hyphen-Morphogenese

Der Wechsel zwischen der Hefe- und der Hyphenform ist für die *C. albicans*-Virulenz essentiell (Csank *et al.*, 1997). Dieser Phänotypenwechsel wird in *C. albicans* über mehrere Signalwege gesteuert, wobei die MAPK-Kaskade sowie der cAMP-abhängige Proteinkinase A (PKA)-Weg am besten untersucht sind. Die Zielproteine dieser Signalwege sind die Transkriptionsfaktoren Cph1 und Efg1. Eine *cph1 efg1*-Doppelmutante ist afilamentös, sowie avirulent (Lo *et al.*, 1997, Sonneborn *et al.*, 2000) Dies führte zu der Annahme, dass beide Signalwege parallel verlaufen und gemeinsam die Regulation der Hyphenbildung kontrollieren, wobei der PKA-Weg von größerer Bedeutung für den morphologischen „Switch“ ist. Die Aktivierung der GTPase Ras1 führt im Falle der MAPK-Kaskade zur Signalweiterleitung über die GTPase Cdc42, welche wiederum die Kinase

Cst20 aktiviert. Hierdurch kommt es zur Aktivierung des MAP-Kinase-Moduls, das aus den drei Kinasen Ste11, Hst7 und Cek1 besteht; dabei stellt Cek1 die zentrale MAPK-Komponente dar. Die Weiterleitung des Signals erfolgt durch Phosphorylierung und resultiert in der Aktivierung des Transkriptionsfaktors Cph1, welcher die Hyphenbildung anregt (Liu *et al.*, 1994, Csank *et al.*, 1998). Interessanterweise führen Mutationen der einzelnen MAPK-Komponenten nur unter bestimmten Bedingungen, wie z.B. auf festen Nährmedien mit Mannitol als Kohlenstoffquelle, zu Defekten in der Filamentierung. Hingegen bewirken Mutationen im PKA-Signalweg zu einem vollständigen Defekt der Filamentierung (Sonneborn *et al.*, 2000, Bockmühl *et al.*, 2001, Rocha *et al.*, 2001).

In vorangegangenen Arbeiten konnte gezeigt werden, dass der Sensor Msb2 oberhalb der MAPK Cek1 an der Hyphen-Morphogenese beteiligt ist (Román *et al.*, 2009). In dieser Arbeit wurde beschrieben, dass der morphologische Wechsel von der Hefe- in die Hyphenform unabhängig von der Cek1 Phosphorylierung ist (Manuskript IV). Die Msb2-Variante, welcher der cytoplasmatische Msb2-Teil fehlt, ist nicht in der Lage Cek1 zu aktivieren, bildet jedoch Hyphen aus (Manuskript II; IV), während die Variante ΔD4 (Deletion stromaufwärts der TM-Domäne) Cek1 phosphoryliert aber nicht filamentiert. Dies lässt darauf schließen, dass die durch Msb2-ausgelöste Filamentierung teilweise unabhängig von der Phosphorylierung der Kinase Cek1 ist.

Interessanterweise konnte durch spezifische Deletionen ein hyperfilamentöser Phänotyp beobachtet werden (Manuskript IV). Durch Deletion von Zweidritteln der N-terminalen Msb2-Sequenz (ΔD11) zeigten *C. albicans*-Zellen in nicht induzierten Bedingungen filamentöse Strukturen. Dieser Phänotyp war unabhängig vom cytoplasmatischen Teil, jedoch abhängig von der TM-Domäne. Die Hyperfilamentierung war nicht in Zellen, denen das Membranprotein Sho1, die Kinase Cek1 oder der Transkriptionsfaktor Efg1 fehlen, zu beobachten. Diese Ergebnisse zeigen, dass für die Msb2-induzierte Hyphenbildung eine Interaktion mit Sho1, der stromabwärts befindlichen Kinase Cek1 und dem Regulator Efg1 erforderlich sind. Eine direkte Interaktion bzw. „Cross-talk“ des MAPK- und des PKA-Signalwegs, also eine direkte Interaktion zwischen der Kinase Cek1 und Efg1 als direktes „Target“ ist somit möglich, bedarf jedoch weiterer Analysen, da bisher von zwei parallelen Signalwegen ausgegangen wurde (Gow *et al.*, 2012).

3.6 Die cytoplasmatische Msb2-Domäne wird bei Zellwandstress in das Cytoplasma/Kern transloziert

Neben der Prozessierung der Exodomäne können Mucine zusätzlich im cytoplasmatischen Teil gespalten werden (Cullen, 2011). Beispiele hierfür finden sich bei dem Notch-Rezeptor, ErbB-4 und CN14 (De Strooper *et al.*, 1999, Ni *et al.*, 2001, Lammich *et al.*, 2002). So konnte gezeigt werden, dass der cytoplasmatische Teil des membranständigen Notch-Rezeptors durch Präsenilin-1 prozessiert wird und somit in den Zellkern diffundiert, wo das Fragment in einem Komplex die Expression von Notch-Response-Genen reguliert.

In zwei Pilz-Spezies zeigte sich die subzelluläre Lokalisation von Msb2 (*Ustilago maydis*) und MsbA (*Aspergillus nidulans*), welche am C-terminalen Ende mit einem Fluoreszenzprotein fusioniert waren, als dynamischer Prozess. Hierbei wurde das Mucin stetig von der Membran entfernt und in die Vakuole rekrutiert (Lanver *et al.*, 2010, Brown *et al.*, 2014). Der cytoplasmatische Msb2-Teil bei *C. albicans* ist essentiell für die basale Resistenz vor allem gegenüber Caspofungin, jedoch weniger für das Wachstum unter Glykosylierungs-Stress (Manuskript IV). Weiterhin konnte gezeigt werden, dass die Abwesenheit der C-Domäne zu einer verminderten Cek1 Phosphorylierung unter Zellwand-Stress führt. Mittels Immunfluoreszenz wurde der cytoplasmatische Teil von Msb2 an der Cytoplasmamembran lokalisiert (Manuskript IV). Unter Zellwand-Stress wird das Msb2-Verläuferprotein vermutlich durch Entfernung der TM-Region prozessiert, wodurch die C-Domäne in dem Cytoplasma und dem Kern detektiert werden konnte. Somit unterliegt Msb2 bei *C. albicans* zwei Prozessierungs-Schritten. Zum einen wird die große N-terminale Exodomäne gespalten und in die Pilz-Umgebung sezerniert (Manuskript II), zum anderen wird die C-terminale Domäne bei Zellwand-Stress prozessiert und transloziert (Manuskript IV). Genomweite Expressionsanalyse ergaben 117 differentiell regulierte Gene durch die Abwesenheit der C-terminalen Msb2-Domäne, darunter fünf Zellwand-Gene (*ALS1*, *ALS3*, *ALS5*, *CHT2* und *PGA14*). Der anschließende Vergleich der Promotorregionen aller regulierten Gene ergab zwei signifikante Transkriptionsfaktor-Konsensus-Bindesequenzen (Ace2 und Azf1), welche an der Aufrechterhaltung der Zellwandintegrität beteiligt sind (Kurischko *et al.*, 2005, de Boer & Hughes, 2012, Saputo *et al.*, 2012). In vorangegangenen Arbeiten wurde Ace2 als wesentliches Protein für die Regulierung der *PMT*-Gene und Antimykotika-Resistenz identifiziert (Cantero & Ernst, 2011). Diese Ergebnisse führen zu der Annahme, dass der cytoplasmatische Teil von Msb2 prozessiert und passiv in den Kern diffundiert oder aktiv mittels eines Komplexes transportiert wird, um Gene der Zellwandintegrität zu regulieren.

3.7 Die Rolle des Msb2-Sensorproteins bei der Interaktion mit antimikrobiellen Peptiden

Im menschlichen Wirt kommt *C. albicans* in Kontakt mit Zelloberflächen wie beispielsweise von Immunzellen, welche daraufhin eine Welle antimykotischer Aktivitäten auslösen. Induzierte lösliche Abwehrmoleküle wie Immunglobuline, Komplementfaktoren und AMPs töten oder blockieren infolgedessen das Wachstum des Pathogens *C. albicans*. AMPs wirken antiviral, antibakteriell und antimykotisch. Sie bilden z.B. durch sezerniertes Histatin eine antimikrobielle Barriere auf Schleimhäuten oder sie sind Bestandteile der Erreger-abtötenden Granula von Neutrophilen, wie z.B. das Cathelicidin LL-37 (Peschel & Sahl, 2006). Darüber hinaus wirken AMPs als chemotaktische Stoffe und rekrutieren Leukozyten zu Infektionsstellen. *C. albicans* ist sensitiv gegenüber Histatin 5, LL-37 und Defensinen, welche das Wachstum durch Disruption der Cytoplasmamembran, Hemmung der mitochondrialen Aktivität und weitere unbekannte Mechanismen hemmen

(Manuskript I). Darüber hinaus führt die Bindung von LL-37 an Zuckerstrukturen der Zellwand zu einer verringerten Adhäsion der Pilz-Zellen an Kunststoffoberflächen oder Wirtszellen (Tsai *et al.*, 2011).

Eine Vielzahl von kommensalen Mikroben und auch einige opportunistische Erreger überleben im menschlichen Wirt, da sie zumindest teilweise AMP-resistent sind. Mehrere AMP-Resistenzmechanismen wurden bereits für Pathogene beschrieben. Dazu gehört zum Beispiel die Spaltung von antimikrobiellen Peptiden durch lösliche oder membrangebundene Proteasen, wie die Yapsin Protease Sap9 von *C. albicans*, welche Histatin 5 degradiert und somit inaktiviert (Meiller *et al.*, 2009). Ein weiterer, in Bakterien aufgeklärter Mechanismus ist die Sekretion von AMP-bindenden Proteinen. Diese dienen als Köder und lenken somit die AMPs von der mikrobiellen Zelloberfläche ab. Beispiele hierfür sind die sekretierte SIC-Staphylokinasen und FAF-Proteine aus *Streptococcus pyogenes*, *S. aureus* und den kommensalen Bakterium *Finegoldia magna* (Frick *et al.*, 2003, Jin *et al.*, 2004, Frick *et al.*, 2008).

Im Rahmen dieser Arbeit konnte gezeigt werden, dass die *msb2*-Mutante sensitiver gegenüber dem Cathelicidin LL-37 ist (Manuskript II), da es nach Behandlung mit LL-37 zu einer Reduktion der Kolonie-bildenden Einheiten von 20 % im Vergleich zum Wildtypstamm CAF2-1 kam. Dieser Effekt von LL-37 konnte für Histatin 5 (Hst 5) jedoch nicht bestätigt werden. Es ist bekannt, dass Hst 5 weder Poren noch Kanäle in die mikrobiellen Plasmamembran verursacht, sondern intrazellulär auf die Funktion der Mitochondrien wirkt (Komatsu *et al.*, 2011). Dies deutet darauf hin, dass die relativ geringen Mengen von zellassoziiertem Msb2 ausreichen, *C. albicans* gegenüber LL-37, nicht jedoch gegenüber Histatin 5, zu schützen. Mehrere Mechanismen könnten die erhöhte Sensitivität der *msb2*-Mutante gegenüber LL-37 erklären. Einer dieser Mechanismen ist die Sekretion von AMP-bindenden Proteinen. Es konnte gezeigt werden, dass die Sekretion des hochglykosylierten extrazellulären Teils von Msb2 eine basale AMP Resistenz hervorruft (Manuskript II). Somit wurde ein analoger Mechanismus der AMP-Resistenz für den Pathogen *C. albicans*, wie in den erwähnten bakteriellen Beispielen, festgestellt. Die Sekretion des extrazellulären Msb2-Fragments führt zu einem hohen Msb2-Proteinspiegel im Medium (Manuskript II), der die AMP-Resistenz effektiv beeinflussen kann. Es wurde tatsächlich nachgewiesen, dass sekretiertes aufgereinigtes Msb2 (Msb2*) die antimikrobiellen Peptide (LL-37, Hst 5, hNP-1 und hBD1) blockiert und somit die *C. albicans*-Zellen vor der antimikrobiellen Wirkung schützt (Manuskript II, III). Mittels Protease-Assays wurde eine proteolytische Wirkung von Msb2* gegenüber AMPs ausgeschlossen (Manuskript II), während eine direkte Bindung mittels „Microscale Thermophorese“ gezeigt wurde (Manuskript III). Die Quantifizierung der Msb2*/LL-37 Interaktionen ergab eine hochaffine Bindung (Dissoziationskonstante [K_D] = 73 nM). Interessanterweise wurde die hohe Bindungsaffinität in einer Msb2-Variante (ΔD10), der 450 AS-

Reste der N- terminalen Domäne fehlen, beibehalten (Manuskript III). Aufgrund der positiven Ladung der AMPs und der hohen Anzahl an negativen Aminosäure-Resten von Msb2 (Abb. 3.1) könnten elektrostatische Wechselwirkungen die molekulare Interaktion erklären. Ebenfalls zeigte die Msb2-Variante bei der Zweidrittel der N-terminalen Sequenz (ΔD11; Manuskript IV) deletiert wurde eine hochaffine Bindung (23 nM) zu LL-37 (Daten nicht gezeigt). Dieses führte zur Schlussfolgerung, dass elektrostatische Wechselwirkungen allein nicht für die Bindung verantwortlich sind (siehe unten). Durch Denaturierung von aufgereinigtem Msb2 ging die Affinität zu LL-37 verloren. Weiterhin zeigte sich, dass das von wildtypischen Zellen sezernierte Msb2 *C. albicans*-Zellen vor AMPs schützt, die Msb2 nicht produzieren (Daten nicht gezeigt).

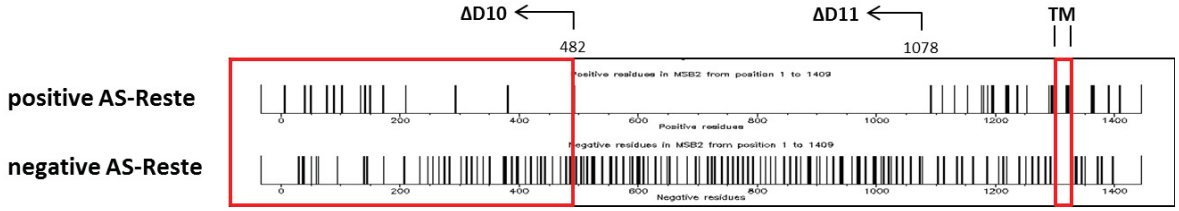


Abb. 3.1 Übersicht der positiven und negativen Aminosäure-Reste von Msb2. Schematische Darstellung positiver Aminosäuren (Lysin, Arginin und Histidin) und negativer Aminosäuren (Asparaginsäure und Glutaminsäure). Eingezeichnet sind die Positionen der N-terminalen Deletionen von den Msb2-Varianten ΔD10 und ΔD11 (Manuskript IV), sowie die Transmembrandomöne (TM).

In vorangegangenen Arbeiten wurde beschrieben, dass LL-37 an verschiedene Glykostrukturen bindet, einschließlich bakterielle Lipopolysaccharide, bakterielle Exopolysaccharide und menschliche Glykosaminoglykane, sowie Pilz-Zellwand-Polysaccharide (Barańska-Rybak *et al.*, 2006, Foschiatti *et al.*, 2009, Scott *et al.*, 2011). Im Rahmen dieser Arbeit wurden Dissoziationskonstanten für LL-37, dem Cytokin IL-26 und bakteriellen Zellwandstrukturen bestimmt (Abschn. 2.5). LL-37 bindet an Lipopolysaccharide (LPS) (Larrick *et al.*, 1995). Diese direkte Interaktion konnte durch „Microscale Thermophorese“ bestätigt werden. Es zeigte sich eine hochaffine Bindung [6,57 nM]; darüber hinaus konnte ebenfalls eine Bindung von LL-37 an Lipoteichonsäuren (LTA) bestimmt werden [7,33 nM]. IL-26, ein Cytokin mit direkter antimikrobieller Wirkung (persönliche Mitteilung B. Homey; Hautklinik Düsseldorf), wies hingegen nur eine geringe Affinität zu LPS [58 µM] und LTA [4 µM] auf (Abschn. 2.5). Somit sind die Hauptbestandteile der äußeren Bakterien-Zellwand direkte „Targets“ des AMPs LL-37 und des Cytokins IL-26. Jedoch sollte man nicht außer Acht lassen, dass diese Bestandteile oft nicht direkt der Umgebung ausgesetzt sind, anders als das von *C. albicans* sezernierte Msb2 (Manuskript II, III, IV), da viele natürlich vorkommende Gram-positive und -negative Bakterien mit einer dicken Polysaccharidkapsel ausgestattet sind und somit LPS und LTA maskieren (Roberts, 1996).

Interessanterweise ist die korrekte *O*-Mannosylierung von Msb2* durch Pmt1 für die Bindung (Manuskript III) und die basale Resistenz gegenüber LL-37 (Manuskript II) notwendig. Eine

pmt1-Mutante wies eine deutlich erhöhte Sensitivität gegenüber dem Cathelicidin LL-37 auf und das Sekretom (Medium) einer *pmt1*-Mutante zeigte keine Schutzwirkung gegenüber dem AMP LL-37 (Manuskript II). Glykostrukturen könnten anionische Kontaktstellen für kationische Peptide, wie LL-37 und Histatin-5 ausbilden, die eine Anreicherung von basischen Aminosäuren (Nettoladung +6 bzw. +12 bei physiologischem pH-Wert) aufweisen. Bisher ist noch nicht beschrieben worden, dass die *O*-Mannosyl-Seitenketten allgemein oder von Msb2* modifiziert werden. Sollte dieses nicht der Fall sein, so fügen die Seitenketten keine Nettoladung hinzu und erlauben somit keine ionische Wechselwirkungen mit kationischen AMPs, obwohl nicht-ionische Wechselwirkungen nicht ausgeschlossen werden können. Die Bindung von Msb2 an Daptomycin, mit einer Nettoladung von -1 (Manuskript III; siehe unten) weist darauf hin, dass Ladungswechselwirkungen zur Msb2/AMP-Bindung beitragen können, jedoch nicht wesentlich für die Wechselwirkung ist. Möglicherweise trägt die korrekte *O*-Mannosylierung auch zur Proteinkonformation bei, so dass die Carboxylat-Seitengruppen von Aspartat- und Glutamat-Resten in Msb2* mit basischen Aminosäuren der antimikrobiellen Peptiden interagieren können, so dass indirekt durch die *O*-Mannosylierung eine verlängerte „Flaschenbürsten-Konformation“ des Msb2-Proteins unterstützt wird, wie es oft in stark *O*- glykosylierten Proteindomänen beschrieben wurde (Van den Steen *et al.*, 1998).

Das angeborene Immunsystem besitzt eine Vielzahl von AMPs, zu diesen gehören die α- und β-Defensine. In dieser Arbeit konnte gezeigt werden, dass das Msb2* nicht nur LL-37 bindet sondern *C. albicans* auch vor den menschlichen α- und β-Defensinen, hNP-1 und hBD1, schützt (Manuskript III). Dieser protektive Effekt ist unabhängig von einer reduzierenden Umgebung (Manuskript III), da diese die Ladung und antimikrobielle Wirkung von AMPs beeinflussen kann (Schroeder *et al.*, 2011). Durch gleichzeitige Inkubation mit unterschiedlichen Mengen von Msb2* und konstanten Konzentrationen von LL-37 konnte zudem gezeigt werden, dass die Inaktivierung von LL-37 abhängig von der Msb2* Konzentration ist (Manuskript II). Ein analoger Mechanismus wurde für das CaPra1 Protein beschrieben. Das Oberflächenprotein Pra1 bindet konzentrationsabhängig den Komplementfaktor H, FHL-1 und Plasminogen (Luo *et al.*, 2011). Ein 20-fach molarer Überschuss des AMP (440 pmol LL-37 zu 25 pmol Msb2*) deutet darauf hin, dass Msb2* mehrere AMP-Bindungsstellen besitzt und somit ein Msb2-Protein mehrere LL-37 Peptide binden kann (Manuskript II).

Untersuchungen zeigten, dass weitere Komponenten bei der basalen Resistenz von *C. albicans* gegenüber AMPs involviert sind (Manuskript I). Zu diesen Komponenten gehört der Hog1 MAP Kinase Signalweg (Argimón *et al.*, 2011). Dieser-Signalweg ist für die Reaktion auf osmotischen und oxidativen Stress verantwortlich. In *C. albicans* ist Sho1 als Adapterprotein beschrieben, der als Sensor oberhalb des HOG MAPK-Signalwegs fungiert und essentiell für die Aktivierung der Cek1-MAPK ist. Sho1 spielt somit eine Rolle bei der Adaption an oxidativen Stress sowie der

Morphogenese (Román *et al.*, 2005). Da Msb2 kein „Upstream"-Element im Hog1-Signalweg ist, ist davon auszugehen, dass die Msb2 vermittelte AMP-Resistenz unabhängig von Hog1 reguliert wird oder in Kooperation mit Sho1, welches zusammen mit Msb2 an der Hyphenmorphogenese beteiligt ist (Abschn. 3.5; Manuskript IV).

Weiterhin konnten durch systematische Msb2-Deletionsvarianten essentielle Sequenzen für die basale AMP-Resistenz ermittelt werden (Manuskript IV). Hierbei zeigte sich, dass es Unterschiede zwischen der kurzzeitigen AMP-Resistenz gegenüber hohen LL-37 Konzentrationen und der langen Inkubation mit niedrigen Peptid Konzentrationen gibt. Die Exposition mit hohen LL-37 Konzentrationen zeigte, dass nur die *msb2*-Mutante sensitiv gegenüber dem AMP war. Im menschlichen Wirt sind die physiologischen Konzentrationen von LL-37 niedrig, erhöhen sich jedoch an Entzündungs- und Infektionsherden (Nijnik & Hancock, 2009). In Anwesenheit geringer LL-37 Mengen (5 µg/ml) zeigte sich das C-terminale Msb2 Sequenzen für die basale AMP-Resistenz von Nöten sind (Manuskript IV). Somit scheint es, dass Sequenzen die für die Glykostress-Resistenz essentiell sind (Abschn. 3.3; Manuskript IV), ebenfalls eine wichtige Rolle in der basalen AMP-Resistenz übernehmen. Weiterhin konnte gezeigt werden, dass unter den untersuchten Bedingungen die *cek1*-Mutante ebenfalls LL-37 sensitiv ist und somit nicht nur das sekretierte Glykofragment (Manuskript II; III) sondern auch für die Cek1-Aktivierung zellassoziierte Msb2-Sequenzen für die AMP-Resistenz erforderlich sind (Manuskript IV).

In einem Standard Mausmodell einer systemischen Infektion wies eine *msb2*-Mutante keine signifikante Abschwächung der Virulenz auf (Román *et al.*, 2009). Jedoch beinhaltet das systemische Infektionsmodell nur partiell typische *C. albicans* Wachstumsformen, wie zum Beispiel die Ausbildung von Biofilmen oder die Besiedelung von Organen. Bei Puri *et al*. (2012) wies die *msb2*-Mutante eine verringerte Virulenz im murinen Model für die orale Candidose auf. Weiterhin ist Msb2 essentiell für die Kolonisierung des Gastrointestinaltrakts in der Maus (persönliche Mitteilung J. Pla; Madrid, Spanien). Die Tatsache, dass Msb2* sowohl in der Hefe- als auch in der Hyphenform und auch im Verband eines Biofilms kontinuierlich prozessiert wird (Manuskript II; Puri *et al.*, 2012), spricht dafür, dass sich *C. albicans* mit einer Art Msb2*-„Wolke" umgibt (Abb. 3.2) und bei einer hohen Zellzahl, wie in einem Biofilm, eine Resistenz gegenüber humanen antimikrobiellen Peptiden ausbildet. Die Msb2-vermittelte Aktivierung der MAP Kinase Cek1 und die AMP-bindende „Glyko-Wolke" ermöglichen es *C. albicans*-Zellen als Kommensale im menschlichen Wirt zu überleben.

3.8 Msb2 bindet und inaktiviert das Reserveantibiotikum Daptomycin

Eine beträchtliche Anzahl von Infektionskrankheiten beinhalten mehrere mikrobielle Spezies, welche im menschlichen Wirt ko-existieren und interagieren (Klotz *et al.*, 2007). In mikrobiellen Biofilmen spielt die Kommunikation zwischen den verschiedenen Arten eine wichtige Rolle. Hierfür

werden beispielsweise kleine chemische Verbindungen abgesondert, sogenannte „Quorum Sensing“ (QS) Moleküle (Blankenship & Mitchell, 2006). Rund ein Viertel aller *C. albicans* Blutstrom-Infektionen sind polymikrobieller Natur. Hierbei sind die häufigsten isolierten Bakterienarten, *Staphylococcus* und *Enterococcus*-Spezies (Bouza *et al.*, 2013). Eine polymikrobielle Sepsis zeigt im Vergleich zu einer monomikrobiellen Sepsis eine deutlich höhere Sterblichkeitsrate (Harriott & Noverr, 2009). Harroitt und Noverr konnten weiterhin zeigten, dass *C. albicans* und *S. aureus* komplexe polymikrobielle Biofilme ausbilden können. Diese polymikrobielle Umgebung erhöht die Resistenz von *S. aureus* gegen das Reserveantibiotikum Vancomycin. Diese Resistenz wird teilweise durch die *Candida*-Matrix vermittelt und erfordert einen direkten bakteriellen Kontakt mit *C. albicans* (Harriott & Noverr, 2010). Neben der Auswirkung auf Antibiotika, können Wechselwirkungen zwischen mehreren Arten auch die Anfälligkeit für Komponenten des Wirts-Immunsystem verringert werden (Ramsey & Whiteley, 2009).

So schützt das von *C. albicans* sekretierte Msb2* nicht nur Pilz-Zellen, sondern auch Bakterien vor der antimikrobiellen Wirkung der Peptide (Manuskript II) und übernimmt somit eine wichtige Stellung im Kommensalismus. Darüber hinaus ist Msb2* in der Lage das Lipopeptid-Antibiotikum Daptomycin, ein klinisch relevantes Reserve-Antibiotikum, zu binden, und verhindert dadurch die Abtötung der pathogenen Bakterien *S. aureus*, *Enterococcus faecalis* und *Corynebacterium pseudodiphtheriticum* (Manuskript III). Durch Ko-Kulturen und polymikrobielle Biofilme von *S. aureus* mit *C. albicans* konnte die protektive Wirkung gegenüber Daptomycin bestätigt werden; hierbei bewirkte der *C. albicans* Wildtyp, nicht aber eine *msb2*-Mutante, die schützende Wirkung gegenüber der bakteriziden Wirkung von Daptomycin (Abb. 3.2; Manuskript III).

Diese Ergebnisse legen nahe, dass bei einer Infektion und Daptomycin-Therapie von multi-resistenten bakteriellen Erregern, ebenfalls auf eine *C. albicans*-Infektion getestet und gegebenenfalls therapiert werden sollte, aufgrund der hohen Affinität von Msb2* zu Daptomycin und der daraus resultierenden Inaktivierung des Antibiotikums.

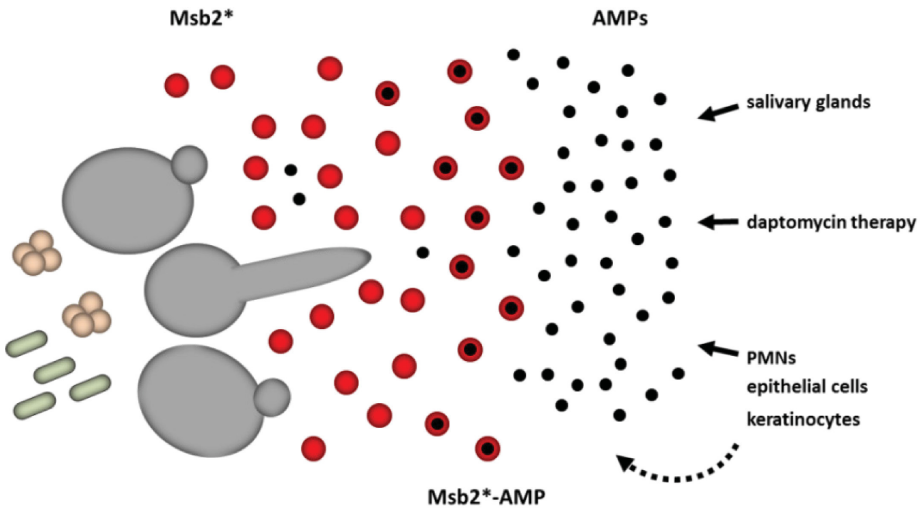


Abb. 3.2 Msb2 vermittelt eine Spezies-übergreifende AMP-Resistenz. Msb2* (rot) wird von *C. albicans* (grau) in die Umgebung sezerniert. Dort bindet und inaktiviert es verschiedene AMPs (schwarz) wie LL-37 und das Reserve-Antibiotikum Daptomycin. Hierdurch werden Pilz- und bakterielle Zellen (braun/grün) direkt vor der mikrobiellen AMP-Wirkung geschützt und verhindern zusätzlich, dass Immunzellen rekrutiert werden.

4 Zusammenfassung

Der fakultativ humanpathogene Pilz *Candida albicans* hat die Fähigkeit, sich an wechselnde Bedingungen im menschlichen Wirt anzupassen. Das Sensorprotein Msb2 in der Plasmamembran erkennt Veränderungen in der Pilzzellwand und reguliert daraufhin die Zellmorphogenese, Zellwandbiogenese und Stressresistenz über den Cek1 „mitogen-activated protein kinase“ (MAPK)-Signalweg. Hierdurch kompensiert der Pathogen Defekte, die durch die Aktivitäten des menschlichen Immunsystems verursacht werden.

C. albicans-Stämme, die eine zweifach Epitop-markierte Msb2-Variante produzieren, sekretierten die große N-terminale extrazelluläre Domäne quantitativ ins Medium, während die kleine C-terminale cytoplasmatische Domäne in der Zelle verblieb. Die sezernierte Exodomäne wurde durch die Protein-Mannosyltransferasen Pmt1 und Pmt2 hoch *O*-glykosyliert und erhielt eine molekulare Masse von > 400 kDa. Die proteolytische Spaltung des Msb2 Vorläuferproteins war dabei unabhängig von den sekretorischen Aspartylproteinasen Sap9 und Sap10, sowie der Subtilisin-ähnlichen Serinprotease Kex2. Durch Analysen verschiedener Msb2-Varianten wurde eine Sequenz identifiziert, die essentiell für die Spaltung und Sekretion des Glykoproteins, sowie für alle Msb2-abhängigen Phänotypen ist. Die Ergebnisse zeigten, dass die Phosphorylierung und die damit verbundene Aktivierung der MAPK Cek1 von drei unterschiedlichen Msb2-Bereichen reguliert wird: in ungestressten Zellen reprimieren N-terminale Sequenzen die Cek1-Phosphorylierung, während diese bei Zellwandstress über Sequenzen im Cytoplasma und Bereiche flankierend zur Transmembrandomäne stimuliert wird. Weitere Sequenzen waren für die basale Resistenz gegen Antimykotika, die Regulation des *PMT1*-Transkriptspiegels und die Hyphen-Morphogenese erforderlich. Die Deletion von zwei Dritteln der Exodomäne führte zu einem hyperfilamentösen Phänotypen, welcher von dem Membranprotein Sho1, der MAPK Cek1 und dem Transkriptionsfaktor Efg1 abhängig war. Bei Zellwandschädigungen konnte weiterhin die Translokation der cytoplasmatischen Msb2-Domäne in das Cytoplasma und teilweise in den Kern nachgewiesen werden. Das Fehlen der cytoplasmatischen Region in Msb2 führte zu einer veränderten transkriptomalen Regulation von 117 Genen. Dieses Ergebnis zeigt, dass die cytoplasmatische Msb2-Domäne eine aktive Rolle bei der transkriptionellen Zellwand-Stressantwort spielt.

Eine wichtige Verteidigungsstrategie des Wirts gegen *C. albicans* besteht in der Produktion von antimikrobiellen Peptiden (AMPs). AMPs sind Effektormoleküle des angeborenen Immunsystems mit direkter antimikrobieller Funktion. Die Produktion von Msb2 war für die basale Resistenz von *C. albicans* gegen AMPs erforderlich. Die anschließende Charakterisierung der Interaktion des Msb2-Proteins mit verschiedenen AMPs (LL-37, Histatin 5, hBD1 und hNP-1) zeigte, dass das sekretierte Msb2-Protein als Schutzprotein gegen AMPs fungiert. Die schützende Wirkung von Msb2 war nicht auf proteolytischen Abbau der AMPs, sondern auf eine direkte hoch-affine Bindung von AMPs an die Msb2-Exodomäne (K_D =73 nM) zurückzuführen. Die Schutzfunktion von Msb2 war abhängig von dessen Konzentration, sowie der durch Pmt1-vermittelten *O*-Glykosylierung von Msb2.

Infektionskrankheiten werden oft nicht durch eine, sondern mehrere interagierende mikrobielle Spezies verursacht. Diese Interaktionen bewirken Antibiotika-Resistenzen, sowie eine verringerte Empfindlichkeit gegenüber Immunkomponenten. Es wurde festgestellt, dass das von *C. albicans* sekretierte Msb2 nicht nur Pilz-Zellen, sondern auch Bakterien vor der antimikrobiellen Wirkung von AMPs schützt. Auch das klinisch relevante Reserve-Peptidantibiotikum Daptomycin wurde durch Msb2 blockiert und schützte dadurch pathogene Bakterien in Ko-Kulturen und polymikrobiellen Biofilmen. Somit begünstigt das Msb2-Protein nicht nur das Überleben von *C. albicans*, sondern auch von begleitenden bakteriellen Pathogenen im Menschen und erschwert die antibiotische Therapie.

5 Summary

The facultative pathogenic yeast *Candida albicans* has the ability to adapt to changing conditions in the human host. The sensor protein Msb2 in the plasma membrane detects cell wall alterations and regulates subsequently morphogenesis, cell wall biogenesis and stress resistance via the Cek1 mitogen-activated protein kinase (MAPK) pathway. Hereby, the pathogen is able to compensate defects, which are caused by activities of the human immune system.

C. albicans strains that produce a double epitope-labeled Msb2 variant, quantitatively secreted the extracellular domain into medium, while the cytoplasmic domain remained within the cell. The shed exodomain was highly *O*-glycosylated by protein mannosyltransferases Pmt1 and Pmt2 leading to a molecular mass of > 400 kDa. The proteolytic processing of the Msb2 precursor protein was independent of the secreted aspartyl proteases Sap9 and Sap10, and the subtilisin-like protease Kex2. Analyses of different Msb2 variants identified a sequence, which is essential for cleavage and secretion of the glycoprotein, as well as for all Msb2-dependent phenotypes. The results showed that phosphorylation of the MAP kinase Cek1 is regulated by three distinct Msb2 regions: in unstressed cells, N-terminal sequences repress Cek1 phosphorylation, while its induction under cell wall stress require the cytoplasmic tail and sequences flanking the transmembrane region. Further Msb2 sequences were required for basal resistance to antifungals, the regulation of *PMT1* transcript levels, and for hyphal morphogenesis. The deletion of two-thirds of the exodomain induced a hyperfilamentous phenotype, which depended on the presence of the Msb2 interacting protein Sho1, MAPK Cek1 and the Efg1 transcription factor. During cell wall damage the cytoplasmic Msb2 domain was shown to become translocated to the cytoplasm and partially to the nucleus. Deletion of the cytoplasmic region in Msb2 led to transciptomal regulation of 117 genes. This result indicates that the cytoplasmic domain of Msb2 has an active role in the transcriptional response to cell wall stress.

An important defense mechanism of the host is the production of antimicrobial peptides (AMPs). AMPs are effector molecules of the innate immune system with direct antimicrobial function. The production of Msb2 was required for the basal AMP resistance. The subsequent characterization of the interaction of the Msb2 protein and various AMPs (LL-37, Histatin 5, hBD1 and hNP-1) revealed that the shed Msb2 acts as a protectant against AMPs. The protective action is not due to proteolytic degradation of AMPs but depends on direct high-affinity binding to the Msb2 exodomain (K_D =73 nM). The protective function of Msb2 depended on its concentration and Pmt1-mediated *O*-glycosylation of Msb2.

Many infectious diseases are not caused by a single but multiple interacting microbial species. These interactions provide antibiotic resistance and reduced susceptibility to components of the immune system. It was found that the shed Msb2 protects fungal and bacterial cells against the antimicrobial activity of AMPs. Futhermore, the clinically relevant reserve peptide antibiotic daptomycin was blocked by Msb2 and protected pathogenic bacteria in co-cultures and polymicrobial biofilms. Thus, the Msb2 protein not only favors the survival of *C. albicans* but also of accompanying bacterial pathogens in the human host, thereby interfering with antibiotic therapy.

6 Abkürzungsverzeichnis

%	Prozent
°C	Grad Celsius
Abb.	Abbildung
Abschn.	Abschnitt
AIDS	Acquired Immune Deficiency Syndrome
ALS	Agglutinin -ähnliche Sequenz
AMP	Antimikrobielles Peptid
AS	Aminosäure
Asn	Asparagin
Bp	Basenpaare
bzw.	Beziehungsweise
Ca	*Candida albicans*
cAMP	cyclisches denosinmonophosphat
CLRs	C-type Lectin Receptors
CRD	Kohlenhydrat-Erkennungs-Domäne
DC-SIGN	dendritisch spezifisch ICAM-3-Grabbing non-Integrin-1
FcγR	Fcγ-Rezeptor
FG	Filamentous growth
FITC	Fluorescein isothiocyanate
G	Glycin
GUT	Gastrointestinally-Induced Transition
HA	Hämagglutinin
hBD1	Human beta-defensin-1
hNP-1	neutrophil defensin 1
HOG	high-osmolarity glycerol
Hst 5	Histatin 5
ITAM	immunoreceptor-tyrosine-based-inibitory-motif
K_D	Dissoziationskonstante
kDa	Kilodalton
LPS	Lipopolysaccharide
LTA	Lipoteichonsäuren
m	Milli
MAPK	Mitogen-aktivierte Proteinkinasen
mg	Milligramm
Mincle	Makrophagen-induzierte C-Typ-Lektine
MR	Makrophagen-Mannose-Rezeptor
ORF	Open reading frame
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase-Ketten-Reaktion
PKA	Proteinkinase A
Pmts	Protein-*O*-Mannosyltransferasen
PRRs	Pattern Recognition Receptors
QS	Quorum Sensing
Q-TOf	Quadrupole time-of-flight mass spectrometer
S/Ser	Serin
Sc	*Sacharomyces cerevisiae*
SEA	Sperm protein, Enterokinase and Agrin
STR	Serin/Threonin-reiche
T/Thr	Threonin
TF	Transkriptionsfaktor
TLRs	Toll-like Receptors
TM	Transmembran
TMFS	trifluoromethanesulfonic acid
V	Valine
VVC	Vulvovaginalcandidose
z.B.	Zum Beispiel
Δ	Delta (Mutation/Deletion)
µg	Mikrogramm

7 Literatur

Albrecht A, Felk A, Pichova I*, et al.* (2006) Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J Biol Chem* **281**: 688-694.

Alonso-Monge R, Navarro-Garcia F, Molero G, Diez-Orejas R, Gustin M, Pla J, Sanchez M & Nombela C (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* **181**: 3058-3068.

Andreu D & Rivas L (1998) Animal antimicrobial peptides: an overview. *Biopolymers* **47**: 415-433.

Apweiler R, Hermjakob H & Sharon N (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* **1473**: 4-8.

Arana DM, Nombela C, Alonso-Monge R & Pla J (2005) The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen *Candida albicans*. *Microbiology* **151**: 1033-1049.

Argimón S, Fanning S, Blankenship JR & Mitchell AP (2011) Interaction between the *Candida albicans* high-osmolarity glycerol (HOG) pathway and the response to human β-defensins 2 and 3. *Eukaryot Cell* **10**: 272-275.

Barańska-Rybak W, Sonesson A, Nowicki R & Schmidtchen A (2006) Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids. *J Antimicrob Chemother* **57**: 260-265.

Blankenship JR & Mitchell AP (2006) How to build a biofilm: a fungal perspective. *Curr Opin Microbiol* **9**: 588-594.

Bockmühl DP, Krishnamurthy S, Gerads M, Sonneborn A & Ernst JF (2001) Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. *Mol Microbiol* **42**: 1243-1257.

Bouza E, Burillo A, Munoz P, Guinea J, Marin M & Rodriguez-Creixems M (2013) Mixed bloodstream infections involving bacteria and *Candida* spp. *J Antimicrob Chemother* **68**: 1881-1888.

Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG & Hancock RE (2005) Impact of LL-37 on anti-infective immunity. *J Leukoc Biol* **77**: 451-459.

Bowman SM & Free SJ (2006) The structure and synthesis of the fungal cell wall. *Bioessays* **28**: 799-808.

Brock M (2009) Fungal metabolism in host niches. *Curr Opin Microbiol* **12**: 371-376.

Brown AJ, Haynes K & Quinn J (2009) Nitrosative and oxidative stress responses in fungal pathogenicity. *Curr Opin Microbiol* **12**: 384-391.

Brown AJ, Budge S, Kaloriti D*, et al.* (2014) Stress adaptation in a pathogenic fungus. *J Exp Biol* **217**: 144-155.

Brown GD & Netea MG (2012) Exciting developments in the immunology of fungal infections. *Cell Host Microbe* **11**: 422-424.

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG & White TC (2012) Hidden killers: human fungal infections. *Sci Transl Med* **4**: 165rv113.

Brown NA, Dos Reis TF, Goinski AB, Savoldi M, Menino J, Almeida MT, Rodrigues F & Goldman GH (2014) The *Aspergillus nidulans* signalling mucin MsbA regulates starvation responses, adhesion and affects cellulase secretion in response to environmental cues. *Mol Microbiol* doi: 10.1111/mmi.12820

Cabib E & Bowers B (1971) Chitin and yeast budding. Localization of chitin in yeast bud scars. *J Biol Chem* **246**: 152-159.

Cantero PD & Ernst JF (2011) Damage to the glycoshield activates PMT-directed *O*-mannosylation via the Msb2-Cek1 pathway in *Candida albicans*. *Mol Microbiol* **80**: 715-725.

Cantero PD, Lengsfeld C, Prill SK, Subanovic M, Román E, Pla J & Ernst JF (2007) Transcriptional and physiological adaptation to defective protein-*O*-mannosylation in *Candida albicans*. *Mol Microbiol* **64**: 1115-1128.

Cassone A & Cauda R (2012) *Candida* and candidiasis in HIV-infected patients: where commensalism, opportunistic behavior and frank pathogenicity lose their borders. *AIDS* **26**: 1457-1472.

Chaffin WL (2008) *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev* **72**: 495-544.

Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D & Martinez JP (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol Mol Biol Rev* **62**: 130-180.

Chai LY, Netea MG, Vonk AG & Kullberg BJ (2009) Fungal strategies for overcoming host innate immune response. *Med Mycol* **47**: 227-236.

Cheng SC, Joosten LA, Kullberg BJ & Netea MG (2012) Interplay between *Candida albicans* and the mammalian innate host defense. *Infect Immun* **80**: 1304-1313.

Cottier F (2007) Funktionen von Msb2 und anderer putativer Sensoren bei der Umweltanpassung von *Candida albicans*. Dissertation, Heinrich-Heine-Universität Düsseldorf.

Cottier F & Pavelka N (2012) Complexity and dynamics of host-fungal interactions. *Immunol Res* **53**: 127-135.

Csank C, Makris C, Meloche S, Schroppel K, Rollinghoff M, Dignard D, Thomas DY & Whiteway M (1997) Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen *Candida albicans*. *Mol Biol Cell* **8**: 2539-2551.

Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas DY & Whiteway M (1998) Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect Immun* **66**: 2713-2721.

Cullen PJ (2007) Signaling mucins: the new kids on the MAPK block. *Crit Rev Eukaryot Gene Expr* **17**: 241-257.

Cullen PJ (2011) Post-translational regulation of signaling mucins. *Curr Opin Struct Biol* **21**: 590-596.

Cullen PJ, Sabbagh Jr W, Graham E, Irick MM, Van Olden EK, Neal C, Delrow J, Bardwell L & Sprague Jr GF (2004) A signaling mucin at the head of the Cdc42- and MAPK-dependent filamentous growth pathway in yeast. *Genes and Development* **18**: 1695-1708.

d'Enfert C (2009) Hidden killers: persistence of opportunistic fungal pathogens in the human host. *Curr Opin Microbiol* **12**: 358-364.

Davies A, Brailsford S, Broadley K & Beighton D (2002) Resistance amongst yeasts isolated from the oral cavities of patients with advanced cancer. *Palliat Med* **16**: 527-531.

de Boer CG & Hughes TR (2012) YeTFaSCo: a database of evaluated yeast transcription factor sequence specificities. *Nucleic Acids Res* **40**: D169-179.

de Dios CH, Román E, Monge RA & Pla J (2010) The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans*: implications in virulence. *Curr Protein Pept Sci* **11**: 693-703.

De Groot PW, Hellingwerf KJ & Klis FM (2003) Genome-wide identification of fungal GPI proteins. *Yeast* **20**: 781-796.

De Strooper B, Annaert W, Cupers P*, et al.* (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**: 518-522.

Doedt T, Krishnamurthy S, Bockmuhl DP, Tebarth B, Stempel C, Russell CL, Brown AJ & Ernst JF (2004) APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. *Mol Biol Cell* **15**: 3167-3180.

Edgerton M & Koshlukova SE (2000) Salivary histatin 5 and its similarities to the other antimicrobial proteins in human saliva. *Adv Dent Res* **14**: 16-21.

Edman P (1949) A method for the determination of amino acid sequence in peptides. *Arch Biochem* **22**: 475.

Eggimann P & Pittet D (2006) Postoperative fungal infections. *Surg Infect (Larchmt)* **7 Suppl 2**: S53-56.

Eggimann P, Garbino J & Pittet D (2003) Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect Dis* **3**: 685-702.

Eisman B, Alonso-Monge R, Román E, Arana D, Nombela C & Pla J (2006) The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot Cell* **5**: 347-358.

Ernst JF (2000) Transcription factors in *Candida albicans* - environmental control of morphogenesis. *Microbiology* **146 (Pt 8)**: 1763-1774.

Ernst JF & Prill SK (2001) *O*-glycosylation. *Med Mycol* **39 Suppl 1**: 67-74.

Ernst JF & Pla J (2011) Signaling the glycoshield: maintenance of the *Candida albicans* cell wall. *Int J Med Microbiol* **301**: 378-383.

Ezekowitz RA, Sastry K, Bailly P & Warner A (1990) Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. *J Exp Med* **172**: 1785-1794.

Fan J, Whiteway M & Shen SH (2005) Disruption of a gene encoding glycerol 3-phosphatase from *Candida albicans* impairs intracellular glycerol accumulation-mediated salt-tolerance. *FEMS Microbiol Lett* **245**: 107-116.

Fickenscher H & Pirzer H (2004) Interleukin-26. *Int Immunopharmacol* **4**: 609-613.

Fischer G (2012) Chronic vulvovaginal candidiasis: what we know and what we have yet to learn. *Australas J Dermatol* **53**: 247-254.

Foschiatti M, Cescutti P, Tossi A & Rizzo R (2009) Inhibition of cathelicidin activity by bacterial exopolysaccharides. *Mol Microbiol* **72**: 1137-1146.

Free SJ (2013) Fungal cell wall organization and biosynthesis. *Adv Genet* **81**: 33-82.

Frick IM, Åkesson P, Rasmussen M, Schmidtchen A & Björck L (2003) SIC, a secreted protein of Streptococcus pyogenes that inactivates antibacterial peptides. *J Biol Chem* **278**: 16561-16566.

Frick IM, Karlsson C, Mörgelin M, Olin AI, Janjusevic R, Hammarström C, Holst E, De Château M & Björck L (2008) Identification of a novel protein promoting the colonization and survival of *Finegoldia magna*, a bacterial commensal and opportunistic pathogen. *Mol Microbiol* **70**: 695-708.

Gantner BN, Simmons RM & Underhill DM (2005) Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. *EMBO J* **24**: 1277-1286.

Gantner BN, Simmons RM, Canavera SJ, Akira S & Underhill DM (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med* **197**: 1107-1117.

Garcia R, Bermejo C, Grau C, Perez R, Rodriguez-Pena JM, Francois J, Nombela C & Arroyo J (2004) The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. *J Biol Chem* **279**: 15183-15195.

Gazi U & Martinez-Pomares L (2009) Influence of the mannose receptor in host immune responses. *Immunobiology* **214**: 554-561.

Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* **13**: 122-143, table of contents.

Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A & Gillevet PM (2010) Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog* **6**: e1000713.

Goldberg DE, Siliciano RF & Jacobs WR, Jr. (2012) Outwitting evolution: fighting drug-resistant TB, malaria, and HIV. *Cell* **148**: 1271-1283.

Gow NA (2013) A developmental program for *Candida* commensalism. *Nat Genet* **45**: 967-968.

Gow NA & Hube B (2012) Importance of the *Candida albicans* cell wall during commensalism and infection. *Curr Opin Microbiol* **15**: 406-412.

Gow NA, van de Veerdonk FL, Brown AJ & Netea MG (2012) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol* **10**: 112-122.

Gropp K, Schild L, Schindler S, Hube B, Zipfel PF & Skerka C (2009) The yeast *Candida albicans* evades human complement attack by secretion of aspartic proteases. *Mol Immunol* **47**: 465-475.

Guhad FA, Csank C, Jensen HE, Thomas DY, Whiteway M & Hau J (1998) Reduced pathogenicity of a *Candida albicans* MAP kinase phosphatase (*CPP1*) mutant in the murine mastitis model. *Apmis* **106**: 1049-1055.

Haas H (2003) Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. *Appl Microbiol Biotechnol* **62**: 316-330.

Hall RA & Gow NA (2013) Mannosylation in *Candida albicans*: role in cell wall function and immune recognition. *Mol Microbiol* **90**: 1147-1161.

Hancock RE (1997) Peptide antibiotics. *Lancet* **349**: 418-422.

Hancock RE & Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* **24**: 1551-1557.

Harriott MM & Noverr MC (2009) *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: Effects on antimicrobial resistance. *Antimicrob Agents Chemother* **53**: 3914-3922.

Harriott MM & Noverr MC (2010) Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. *Antimicrob Agents Chemother* **54**: 3746-3755.

Hattrup CL & Gendler SJ (2008) Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol* **70**: 431-457.

Heilmann CJ, Sorgo AG & Klis FM (2012) News from the fungal front: wall proteome dynamics and host-pathogen interplay. *PLoS Pathog* **8**: e1003050.

Heilmann CJ, Sorgo AG, Siliakus AR, Dekker HL, Brul S, de Koster CG, de Koning LJ & Klis FM (2011) Hyphal induction in the human fungal pathogen *Candida albicans* reveals a characteristic wall protein profile. *Microbiology* **157**: 2297-2307.

Heilmann CJ, Sorgo AG, Mohammadi S, Sosinska GJ, de Koster CG, Brul S, de Koning LJ & Klis FM (2013) Surface stress induces a conserved cell wall stress response in the pathogenic fungus *Candida albicans*. *Eukaryot Cell* **12**: 254-264.

Hoyer LL (2001) The *ALS* gene family of *Candida albicans*. *Trends Microbiol* **9**: 176-180.

Hoyer LL, Green CB, Oh SH & Zhao X (2008) Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family--a sticky pursuit. *Med Mycol* **46**: 1-15.

Hruskova-Heidingsfeldova O (2008) Secreted proteins of *Candida albicans*. *Front Biosci* **13**: 7227-7242.

Hube B (2000) Extracellular proteinases of human pathogenic fungi. *Contrib Microbiol* **5**: 126-137.

Hube B (2004) From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. *Curr Opin Microbiol* **7**: 336-341.

Huttner KM & Bevins CL (1999) Antimicrobial peptides as mediators of epithelial host defense. *Pediatr Res* **45**: 785-794.

Janeway CA, Jr. & Medzhitov R (2002) Innate immune recognition. *Annu Rev Immunol* **20**: 197-216.

Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J & Tarkowski A (2004) *Staphylococcus aureus* Resists Human Defensins by Production of Staphylokinase, a Novel Bacterial Evasion Mechanism. *J Immunol* **172**: 1169-1176.

Jones DS, McGovern JG, Woolfson AD & Gorman SP (1997) The effects of hexetidine (Oraldene) on the adherence of *Candida albicans* to human buccal epithelial cells *in vitro* and *ex vivo* and on i*n vitro* morphogenesis. *Pharm Res* **14**: 1765-1771.

Jouault T, Ibata-Ombetta S, Takeuchi O, Trinel PA, Sacchetti P, Lefebvre P, Akira S & Poulain D (2003) *Candida albicans* phospholipomannan is sensed through toll-like receptors. *J Infect Dis* **188**: 165-172.

Kahlenberg JM & Kaplan MJ (2013) Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol* **191**: 4895-4901.

Karunanithi S, Vadaie N, Chavel CA, Birkaya B, Joshi J, Grell L & Cullen PJ (2010) Shedding of the mucin-like flocculin flo11p reveals a new aspect of fungal adhesion regulation. *Curr Biol* **20**: 1389-1395.

Klotz SA, Chasin BS, Powell B, Gaur NK & Lipke PN (2007) Polymicrobial bloodstream infections involving *Candida* species: analysis of patients and review of the literature. *Diagn Microbiol Infect Dis* **59**: 401-406.

Komatsu T, Salih E, Helmerhorst EJ, Offner GD & Oppenheim FG (2011) Influence of histatin 5 on *Candida albicans* mitochondrial protein expression assessed by quantitative mass spectrometry. *J Proteome Res* **10**: 646-655.

Kurischko C, Weiss G, Ottey M & Luca FC (2005) A role for the *Saccharomyces cerevisiae* regulation of Ace2 and polarized morphogenesis signaling network in cell integrity. *Genetics* **171**: 443-455.

Lammich S, Okochi M, Takeda M, Kaether C, Capell A, Zimmer AK, Edbauer D, Walter J, Steiner H & Haass C (2002) Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. *J Biol Chem* **277**: 44754-44759.

Lanver D, Mendoza-Mendoza A, Brachmann A & Kahmann R (2010) Sho1 and Msb2-related proteins regulate appressorium development in the smut fungus *Ustilago maydis*. *Plant Cell* **22**: 2085-2101.

Larrick JW, Hirata M, Balint RF, Lee J, Zhong J & Wright SC (1995) Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun* **63**: 1291-1297.

Lehrer RI & Ganz T (1999) Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* **11**: 23-27.

Lenardon MD, Lesiak I, Munro CA & Gow NA (2009) Dissection of the *Candida albicans* class I chitin synthase promoters. *Mol Genet Genomics* **281**: 459-471.

Lenardon MD, Whitton RK, Munro CA, Marshall D & Gow NAR (2007) Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the *Candida albicans* cell wall. *Mol Microbiol* **66**: 1164-1173.

Lengeler KB, Tielker D & Ernst JF (2008) Protein-*O*-mannosyltransferases in virulence and development. *Cell Mol Life Sci* **65**: 528-544.

Li J & Liang S (2002) C-terminal sequence analysis of peptides using triphenylgermanyl isothiocyanate. *Anal Biochem* **302**: 108-113.

Li Q, Wang C, Tang C, He Q, Li N & Li J (2014) Dysbiosis of gut fungal microbiota is associated with mucosal inflammation in Crohn's disease. *J Clin Gastroenterol* **48**: 513-523.

Lin J, Oh SH, Jones R, Garnett JA, Salgado PS, Rusnakova S, Matthews SJ, Hoyer LL & Cota E (2014) The peptide-binding cavity is essential for Als3-mediated adhesion of *Candida albicans* to human cells. *J Biol Chem* **289**: 18401-18412.

Lionakis MS & Netea MG (2013) *Candida* and host determinants of susceptibility to invasive candidiasis. *PLoS Pathog* **9**: e1003079.

Liu H, Kohler J & Fink GR (1994) Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. *Science* **266**: 1723-1726.

Liu Y & Filler SG (2011) Candida albicans Als3, a multifunctional adhesin and invasin. *Eukaryot Cell* **10**: 168-173.

Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A & Fink GR (1997) Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**: 939-949.

Lorenz MC, Bender JA & Fink GR (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**: 1076-1087.

Luo S, Poltermann S, Kunert A, Rupp S & Zipfel PF (2009) Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Mol Immunol* **47**: 541-550.

Luo S, Blom AM, Rupp S, Hipler UC, Hube B, Skerka C & Zipfel PF (2011) The pH-regulated antigen 1 of *Candida albicans* binds the human complement inhibitor C4b-binding protein and mediates fungal complement evasion. *J Biol Chem* **286**: 8021-8029.

Maeda T, Inoue M, Koshiba S*, et al.* (2004) Solution structure of the SEA domain from the murine homologue of ovarian cancer antigen CA125 (MUC16). *J Biol Chem* **279**: 13174-13182.

Martinez RC, Seney SL, Summers KL, Nomizo A, De Martinis EC & Reid G (2009) Effect of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 on the ability of *Candida albicans* to infect cells and induce inflammation. *Microbiol Immunol* **53**: 487-495.

McGreal EP, Rosas M, Brown GD, Zamze S, Wong SY, Gordon S, Martinez-Pomares L & Taylor PR (2006) The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* **16**: 422-430.

Meiller TF, Hube B, Schild L, Shirtliff ME, Scheper MA, Winkler R, Ton A & Jabra-Rizk MA (2009) A novel immune evasion strategy of *Candida albicans*: Proteolytic cleavage of a salivary antimicrobial peptide. *PLoS ONE* **4**.

Miramon P, Kasper L & Hube B (2013) Thriving within the host: *Candida* spp. interactions with phagocytic cells. *Med Microbiol Immunol* **202**: 183-195.

Miyazato A, Nakamura K, Yamamoto N*, et al.* (2009) Toll-like receptor 9-dependent activation of myeloid dendritic cells by Deoxynucleic acids from *Candida albicans*. *Infect Immun* **77**: 3056-3064.

Molloy C, Shepherd MG & Sullivan PA (1995) Differential extraction of N-acetylglucosaminidase and trehalase from the cell envelope of *Candida albicans*. *Exp Mycol* **19**: 178-185.

Monge RA, Román E, Nombela C & Pla J (2006) The MAP kinase signal transduction network in *Candida albicans*. *Microbiology* **152**: 905-912.

Mora-Montes HM, Bates S, Netea MG*, et al.* (2007) Endoplasmic reticulum alpha-glycosidases of *Candida albicans* are required for *N*-glycosylation, cell wall integrity, and normal host-fungus interaction. *Eukaryot Cell* **6**: 2184-2193.

Moran GP, Coleman DC & Sullivan DJ (2011) Comparative genomics and the evolution of pathogenicity in human pathogenic fungi. *Eukaryot Cell* **10**: 34-42.

Moudgal V & Sobel J (2010) Antifungals to treat *Candida albicans*. *Expert Opin Pharmacother* **11**: 2037-2048.

Munro CA, Selvaggini S, De Bruijn I, Walker L, Lenardon MD, Gerssen B, Milne S, Brown AJP & Gow NAR (2007) The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol Microbiol* **63**: 1399-1413.

Munro CA, Bates S, Buurman ET*, et al.* (2005) Mnt1p and Mnt2p of *Candida albicans* are partially redundant alpha-1,2-mannosyltransferases that participate in *O*-linked mannosylation and are required for adhesion and virulence. *J Biol Chem* **280**: 1051-1060.

Murciano C, Moyes DL, Runglall M, Tobouti P, Islam A, Hoyer LL & Naglik JR (2012) Evaluation of the role of *Candida albicans* agglutinin-like sequence (Als) proteins in human oral epithelial cell interactions. *PLoS One* **7**: e33362.

Murciano C, Moyes DL, Runglall M, Islam A, Mille C, Fradin C, Poulain D, Gow NA & Naglik JR (2011) *Candida albicans* cell wall glycosylation may be indirectly required for activation of epithelial cell proinflammatory responses. *Infect Immun* **79**: 4902-4911.

Naglik J, Albrecht A, Bader O & Hube B (2004) *Candida albicans* proteinases and host/pathogen interactions. *Cell Microbiol* **6**: 915-926.

Naglik JR, Challacombe SJ & Hube B (2003) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* **67**: 400-428, table of contents.

Nantel A, Dignard D, Bachewich C*, et al.* (2002) Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* **13**: 3452-3465.

Nather K & Munro CA (2008) Generating cell surface diversity in *Candida albicans* and other fungal pathogens. *FEMS Microbiol Lett* **285**: 137-145.

Navarro-Garcia F, Sanchez M, Pla J & Nombela C (1995) Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. *Mol Cell Biol* **15**: 2197-2206.

Ni CY, Murphy MP, Golde TE & Carpenter G (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**: 2179-2181.

Niimi K, Niimi M, Shepherd MG & Cannon RD (1997) Regulation of N-acetylglucosaminidase production in *Candida albicans*. *Arch Microbiol* **168**: 464-472.

Nijnik A & Hancock REW (2009) The roles of cathelicidin LL-37 in immune defences and novel clinical applications. *Current Opinion in Hematology* **16**: 41-47.

Nucci M & Anaissie E (2001) Revisiting the source of candidemia: skin or gut? *Clin Infect Dis* **33**: 1959-1967.

Odds FC (1987) *Candida* infections: an overview. *Crit Rev Microbiol* **15**: 1-5.

Odds FC, Van Gerven F, Espinel-Ingroff A, Bartlett MS, Ghannoum MA, Lancaster MV, Pfaller MA, Rex JH, Rinaldi MG & Walsh TJ (1998) Evaluation of possible correlations between antifungal susceptibilities of filamentous fungi in vitro and antifungal treatment outcomes in animal infection models. *Antimicrob Agents Chemother* **42**: 282-288.

Oh SH, Cheng G, Nuessen JA, Jajko R, Yeater KM, Zhao X, Pujol C, Soll DR & Hoyer LL (2005) Functional specificity of *Candida albicans* Als3p proteins and clade specificity of *ALS3* alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology* **151**: 673-681.

Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, Drews O, Weichert W, Timmis KN & Schreiber S (2008) Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scand J Gastroenterol* **43**: 831-841.

Pande K, Chen C & Noble SM (2013) Passage through the mammalian gut triggers a phenotypic switch that promotes *Candida albicans* commensalism. *Nat Genet* **45**: 1088-1091.

Peschel A & Sahl HG (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* **4**: 529-536.

Petrescu AJ, Milac AL, Petrescu SM, Dwek RA & Wormald MR (2004) Statistical analysis of the protein environment of *N*-glycosylation sites: implications for occupancy, structure, and folding. *Glycobiology* **14**: 103-114.

Pfaller MA & Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**: 133-163.

Pierce JV & Kumamoto CA (2012) Variation in *Candida albicans EFG1* expression enables host-dependent changes in colonizing fungal populations. *MBio* **3**: e00117-00112.

Poltermann S, Kunert A, von der Heide M, Eck R, Hartmann A & Zipfel PF (2007) Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of *Candida albicans*. *J Biol Chem* **282**: 37537-37544.

Prill SK, Klinkert B, Timpel C, Gale CA, Schroppel K & Ernst JF (2005) *PMT* family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance. *Mol Microbiol* **55**: 546-560.

Puri S, Kumar R, Chadha S, Tati S, Conti HR, Hube B, Cullen PJ & Edgerton M (2012) Secreted aspartic protease cleavage of *Candida albicans* Msb2 activates Cek1 MAPK signaling affecting biofilm formation and oropharyngeal candidiasis. *PloS one* **7**: e46020.

Ramirez-Zavala B, Reuss O, Park YN, Ohlsen K & Morschhauser J (2008) Environmental induction of white-opaque switching in *Candida albicans*. *PLoS Pathog* **4**: e1000089.

Ramsey MM & Whiteley M (2009) Polymicrobial interactions stimulate resistance to host innate immunity through metabolite perception. *Proc Natl Acad Sci U S A* **106**: 1578-1583.

Roberts IS (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu Rev Microbiol* **50**: 285-315.

Rocha CR, Schroppel K, Harcus D, Marcil A, Dignard D, Taylor BN, Thomas DY, Whiteway M & Leberer E (2001) Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol Biol Cell* **12**: 3631-3643.

Román E, Arana DM, Nombela C, Alonso-Monge R & Pla J (2007) MAP kinase pathways as regulators of fungal virulence. *Trends Microbiol* **15**: 181-190.

Román E, Nombela C & Pla J (2005) The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. *Mol Cell Biol* **25**: 10611-10627.

Román E, Cottier F, Ernst JF & Pla J (2009) Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. *Eukaryot Cell* **8**: 1235-1249.

Romani L, Zelante T, Palmieri M, Napolioni V, Picciolini M, Velardi A, Aversa F & Puccetti P (2014) The cross-talk between opportunistic fungi and the mammalian host via microbiota's metabolism. *Semin Immunopathol* **37:** 163-171.

Romani L, Zelante T, De Luca A, Iannitti RG, Moretti S, Bartoli A, Aversa F & Puccetti P (2014) Microbiota control of a tryptophan-AhR pathway in disease tolerance to fungi. *Eur J Immunol* **44**: 3192-3200.

Rouabhia M, Schaller M, Corbucci C, Vecchiarelli A, Prill SKH, Giasson L & Ernst JF (2005) Virulence of the fungal pathogen *Candida albicans* requires the five isoforms of protein mannosyltransferases. *Infect Immun* **73**: 4571-4580.

Ruhnke M (2006) Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. *Curr Drug Targets* **7**: 495-504.

Rupp S (2004) Proteomics on its way to study host-pathogen interaction in *Candida albicans*. *Curr Opin Microbiol* **7**: 330-335.

Saijo S, Fujikado N, Furuta T*, et al.* (2007) Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat Immunol* **8**: 39-46.

Saputo S, Chabrier-Rosello Y, Luca FC, Kumar A & Krysan DJ (2012) The RAM network in pathogenic fungi. *Eukaryot Cell* **11**: 708-717.

Schaller M, Borelli C, Korting HC & Hube B (2005) Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**: 365-377.

Schlack P & Kampf W (1926) Über eine neue Methode zur Ermittlung der Konstitution von Peptiden. Vol. 154 p.^pp. 125.

Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF & Wehkamp J (2011) Reduction of disulphide bonds unmasks potent antimicrobial activity of human β 2-defensin 1. *Nature* **469**: 419-423.

Scott A, Weldon S, Buchanan PJ, Schock B, Ernst RK, McAuley DF, Tunney MM, Irwin CR, Elborn JS & Taggart CC (2011) Evaluation of the ability of LL-37 to neutralise LPS *in vitro* and *Ex vivo*. *PLoS ONE* **6**.

Shepherd MG & Gopal PK (1991) Candida Albicans: Cell Wall Physiology and Metabolism. *Candida and Candidamycosis,* Vol. 50 (Tümbay E, Seeliger HR & Anğ Ö, eds.), p.^pp. 21-33. Springer US.

Shibata N, Suzuki A, Kobayashi H & Okawa Y (2007) Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. *Biochem J* **404**: 365-372.

Shrimal S, Cherepanova NA & Gilmore R (2014) Cotranslational and posttranslocational *N*-glycosylation of proteins in the endoplasmic reticulum. *Semin Cell Dev Biol*.

Sil A & Andrianopoulos A (2014) Thermally Dimorphic Human Fungal Pathogens-Polyphyletic Pathogens with a Convergent Pathogenicity Trait. *Cold Spring Harb Perspect Med*.

Smits GJ, van den Ende H & Klis FM (2001) Differential regulation of cell wall biogenesis during growth and development in yeast. *Microbiology* **147**: 781-794.

Sohn K, Urban C, Brunner H & Rupp S (2003) EFG1 is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Mol Microbiol* **47**: 89-102.

Sonneborn A, Bockmühl DP, Gerads M, Kurpanek K, Sanglard D & Ernst JF (2000) Protein kinase A encoded by TPK2 regulates dimorphism of *Candida albicans*. *Mol Microbiol* **35**: 386-396.

Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS & Borregaard N (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* **97**: 3951-3959.

Sorgo AG, Heilmann CJ, Dekker HL, Brul S, De Koster CG & Klis FM (2010) Mass spectrometric analysis of the secretome of *Candida albicans*. *Yeast* **27**: 661-672.

Stanley P, Schachter H & Taniguchi N (2009) *N*-Glycans. *Essentials of Glycobiology,*(Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW & Etzler ME, eds.), p.^pp. Cold Spring Harbor Laboratory Press

Sweet SP & Douglas LJ (1991) Effect of iron concentration on siderophore synthesis and pigment production by *Candida albicans*. *FEMS Microbiol Lett* **64**: 87-91.

Swidergall M & Ernst JF (2014) Interplay between *Candida albicans* and the Antimicrobial Peptide Armory. *Eukaryot Cell* **13**: 950-957.

Tada H, Nemoto E, Shimauchi H*, et al.* (2002) *Saccharomyces cerevisiae*- and *Candida albicans*-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiol Immunol* **46**: 503-512.

Tang YQ, Yuan J, Miller CJ & Selsted ME (1999) Isolation, characterization, cDNA cloning, and antimicrobial properties of two distinct subfamilies of alpha-defensins from rhesus macaque leukocytes. *Infect Immun* **67**: 6139-6144.

Tanner W & Lehle L (1987) Protein glycosylation in yeast. *Biochim Biophys Acta* **906**: 81-99.

Tatebayashi K, Tanaka K, Yang HY, Yamamoto K, Matsushita Y, Tomida T, Imai M & Saito H (2007) Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J* **26**: 3521-3533.

Tavanti A, Campa D, Bertozzi A, Pardini G, Naglik JR, Barale R & Senesi S (2006) *Candida albicans* isolates with different genomic backgrounds display a differential response to macrophage infection. *Microbes Infect* **8**: 791-800.

Taylor PR, Tsoni SV, Willment JA*, et al.* (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* **8**: 31-38.

Theiss S, Ishdorj G, Brenot A, Kretschmar M, Lan CY, Nichterlein T, Hacker J, Nigam S, Agabian N & Kohler GA (2006) Inactivation of the phospholipase B gene *PLB5* in wild-type *Candida albicans* reduces cell-associated phospholipase A2 activity and attenuates virulence. *Int J Med Microbiol* **296**: 405-420.

Timpel C, Strahl-Bolsinger S, Ziegelbauer K & Ernst JF (1998) Multiple functions of Pmt1p-mediated protein *O*-mannosylation in the fungal pathogen *Candida albicans*. *J Biol Chem* **273**: 20837-20846.

Tossi A, Sandri L & Giangaspero A (2000) Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers* **55**: 4-30.

Tsai PW, Yang CY, Chang HT & Lan CY (2011) Human antimicrobial peptide LL-37 inhibits adhesion of *Candida albicans* by interacting with yeast cell-wall carbohydrates. *PLoS ONE* **6**.

Underhill DM (2003) Toll-like receptors: networking for success. *Eur J Immunol* **33**: 1767-1775.

Underhill DM (2004) Toll-like receptors and microbes take aim at each other. *Curr Opin Immunol* **16**: 483-487.

Vadaie N, Dionne H, Akajagbor DS, Nickerson SR, Krysan DJ & Cullen PJ (2008) Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. *J Cell Biol* **181**: 1073-1081.

van Burik JA & Magee PT (2001) Aspects of fungal pathogenesis in humans. *Annu Rev Microbiol* **55**: 743-772.

Van den Steen P, Rudd PM, Dwek RA & Opdenakker G (1998) Concepts and principles of *O*-linked glycosylation. *Crit Rev Biochem Mol Biol* **33**: 151-208.

White SJ, Rosenbach A, Lephart P, Nguyen D, Benjamin A, Tzipori S, Whiteway M, Mecsas J & Kumamoto CA (2007) Self-regulation of *Candida albicans* population size during GI colonization. *PLoS Pathog* **3**: e184.

Whiteway M (2000) Transcriptional control of cell type and morphogenesis in *Candida albicans*. *Curr Opin Microbiol* **3**: 582-588.

Wilson NJ, Boniface K, Chan JR*, et al.* (2007) Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* **8**: 950-957.

Yang HY, Tatebayashi K, Yamamoto K & Saito H (2008) Glycosylation defects activate filamentous growth Kss1 MAPK and inhibit osmoregulatory Hog1 MAPK. *EMBO J* **28**: 1380-1391.

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* **415**: 389-395.

Zhao X, Oh SH, Yeater KM & Hoyer LL (2005) Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151**: 1619-1630.

Zhao X, Oh SH, Cheng G, Green CB, Nuessen JA, Yeater K, Leng RP, Brown AJ & Hoyer LL (2004) *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150**: 2415-2428.

8 Übersicht: Lebenslauf, Publikationen, Präsentationen und Preise

Lebenslauf

Geburtsdatum	28.11.1983
Geburtsort	Wesel, Deutschland
Staatsangehörigkeit	Deutsch
Ausbildung	
seit 07/2012	Heinrich-Heine-Universität Düsseldorf Doktorarbeit, Molekular Mykologie
10/2010-07/2012	Heinrich-Heine-Universität Düsseldorf Biologie Abschluss: Master of Science (ausgezeichnet) Thema: Functional characterization of the Msb2 protein in *Candida albicans*
10/2007-08/2010	Heinrich-Heine-Universität Düsseldorf Biologie Abschluss: Bachelor of Science (gut) Thema: Function of the Efg1 regulatory protein from *Candida albicans* in the yeast *Saccharomyces cerevisiae*
09/2005-07/2007	Rheinische Akademie Köln e.V. Abschluss: staatl. geprüfter Biologisch-technischer Assistent (BTA)
08/2000-06/2004	Sekundarstufe II Andreas-Vesalius-Gymnasium Wesel, (Abitur)

Publikationen

Swidergall, M., van Wijlick, L., and Ernst, J. F. (2015) Signaling domains of mucin Msb2 in *Candida albicans*. *Eukaryotic Cell* Apr 14(4):359-70.

Swidergall M. and Ernst, J. F. (2014) Interplay between *Candida albicans* and the antimicrobial peptide armory. *Eukaryotic Cell* Aug 2014 13(8): 950-957

Swidergall, M., Ernst, A. M. and Ernst, J. F. (2013) *Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides. *Antimicrobial Agents Chemotherapy* vol. 57 no. 8 3917-3922

Szafranski-Schneider, E., Swidergall, M., Cottier, F., Tielker, D., Román, E., Pla, J., and Ernst, J. F. (2012) Msb2 shedding protects *Candida albicans* against antimicrobial peptides. *PLoS Pathogens* 8: e1002501

Präsentationen

Swidergall, M., van Wijlick, L., and Ernst, J. F. (2015) Signaling domains of mucin Msb2 in *Candida albicans*. 6h FEBS Advanced Lecture Course HFP2015, La Colle sur Loup, France (Poster)

Swidergall, M., van Wijlick, L., and Ernst, J. F. (2015) Glycoshield regulation in *Candida albicans* by the Ace2 signaling pathway. Workshop of the section "Eukaryontische Krankheitserreger" (Eukaryotic Pathogens) of the German Society for Hygiene and Microbiology (DGHM), Erlangen, Germany (Talk)

Swidergall, M. and Ernst, J. F. (2014). Functional mapping of the *Candida albicans* signaling mucin Msb2. 12th ASM Conference on *Candida* and Candidiasis, New Orleans, USA (Talk)

Swidergall, M. and Ernst, J. F. (2014). Insights into signaling mucins: Msb2 of *Candida albicans*. Workshop of the section "Eukaryontische Krankheitserreger" (Eukaryotic Pathogens) of the German Society for Hygiene and Microbiology (DGHM), Göttingen, Germany (Talk)

Swidergall, M. and Ernst, J. F. (2013) Msb2 of *Candida albicans*: Environmental sensor and protectant. 8th BMFZ annual retreat, Wermelskirchen, Germany (Talk)

Swidergall, M. and Ernst, J. F. (2013) Cross-species protection mediated by *Candida albicans* Msb2 protein. 5th FEBS Advanced Lecture Course HFP2013, La Colle sur Loup, France (Talk & Poster)

Swidergall, M., Szafranski-Schneider, E., and Ernst, J. F. (2012) Msb2 shedding protects *Candida albicans* against antimicrobial peptides. 7th BMFZ annual retreat, Bergisch-Gladbach, Germany (Talk)

Swidergall, M., Szafranski-Schneider, E., Cottier, F., Tielker, D., Román, E., Pla, J., and Ernst, J. F. (2012) Msb2 shedding protects *Candida albicans* against antimicrobial peptides. VAAM conference, Tübingen, Germany (Poster)

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in ihrer jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht. Ich habe zuvor keine erfolglosen Promotionsversuche unternommen.

Düsseldorf,

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(Ort, Datum) (Marc Swidergall)

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Swidergall, M., van Wijlick, L., and Ernst, J. F. (2015) Signaling domains of mucin Msb2 in *Candida albicans*. *Eukaryotic Cell* Apr 14(4):359-70.

Swidergall M. and Ernst, J. F. (2014) Interplay between *Candida albicans* and the antimicrobial peptide armory. *Eukaryotic Cell* Aug 13(8): 950-957

Swidergall, M., Ernst, A. M. and Ernst, J. F. (2013) *Candida albicans* mucin Msb2 is a broad-range protectant against antimicrobial peptides. *Antimicrobial Agents Chemotherapy* vol. 57 no. 8 3917-3922

Szafranski-Schneider, E., **Swidergall, M.**, Cottier, F., Tielker, D., Román, E., Pla, J., and Ernst, J. F. (2012) Msb2 shedding protects *Candida albicans* against antimicrobial peptides. *PLoS Pathogens* 8: e1002501