Identification of Candidalysin – a *Candida albicans* peptide toxin involved in epithelial damage

DISSERTATION

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

seit 1558

vorgelegt von Diplom-Biologin

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Date of defence

25.08.2016

This thesis was performed at the “Leibniz-Institute for Natural Product Research and Infection Biology – Hans Knöll Institute” in the Department of Microbial Pathogenicity Mechanisms under the supervision of Prof. Bernhard Hube.

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Summary

Candida albicans is a diploid polymorphic fungus, which can be found as part of the normal microbial flora in the majority of the healthy human population. Normally a harmless coloniser of mucosal surfaces, it can however cause serious disease in case the host's immune system is compromised or epithelial barriers are disrupted, e.g. through gastrointestinal surgery or intravenous catheters. The morphological transition from a budding yeast to a filamentous hyphal growth form is considered as one of the most important virulence traits, as hyphae contribute to epithelial invasion and tissue damage. This transition is accompanied by the expression of a variety of hypha-associated proteins, which strongly contribute to the virulent properties of hyphae. Such a protein is Ece1 (extent of cell elongation 1), whose expression is strictly correlated to the formation of hyphae. While mutants lacking the gene *ECE1* are still able to form proper hyphae, activation of the epithelial danger response pathways by these mutants is impaired, demonstrating that Ece1 may play an important role in the recognition of *C. albicans* by epithelial cells. However, the biological role of Ece1 is unknown. The aim of this study therefore was to elucidate the so far unknown function of this protein. Using a set of *ece1*Δ/Δ mutants, the importance of Ece1 for adherence to, invasion into and damage of epithelial cells was examined. Furthermore, the eight Ece1 peptides that were predicted to result from intracellular processing of the full length protein and to become secreted were examined for their damaging potential on human epithelial cells and erythrocytes.

It was found that only one of the eight Ece1 peptides was highly cytolytic on all cell types tested, i.e. peptide Ece1-III. Closer examination of this short peptide revealed membrane-permeabilising properties and resulted in the identification of this peptide as the first known cytolytic toxin of a human fungal pathogen. Host cell lysis by this toxin, which was named "Candidalysin", was demonstrated to be rapid and efficient, while host cell death was found to be most probably caused by an ion-dependent mechanism. Identifying the phospholipid phosphatidylserine as an interaction partner of Candidalysin within the host cell's membrane allowed the establishment of a theoretical model of pore formation by this toxin.

In summary, this study provides insight into the as yet unknown function and role of Ece1 in infection-associated epithelial damage and demonstrates the importance of the characterisation of all *C. albicans* genes, including those of unknown function, for the identification of further fungal virulence factors.

Zusammenfassung

Candida albicans ist ein diploider polymorpher Pilz, welcher als Bestandteil der normalen Mikroflora im Großteil der gesunden menschlichen Bevölkerung gefunden werden kann. Während dieser Pilz im Regelfall einen harmlosen Besiedler von Schleimhäuten darstellt, kann er im Falle eines geschwächten Immunsystems oder einer Zerstörung der epithelialen Barriere, beispielsweise durch gastrointestinale Eingriffe oder intravenöse Katheter, schwere Infektionen hervorrufen. Der morphologische Wechsel vom Wachstum als sprossender Hefe zu einer filamentösen Hyphenform wird als eine der wichtigsten virulenten Eigenschaften angesehen, da diese Wachstumsform zum Eindringen in Epithelzellen und der Schädigung des Gewebes beiträgt. Der Wechsel von der Hefe- zur Hyphenform wird von der Expression verschiedener hyphenassoziierter Proteine begleitet, welche in großem Ausmaß zu den virulenten Eigenschaften dieser Wachstumsform beitragen. Solch ein Protein ist Ece1 (extent of cell elongation 1), dessen Expression streng mit der Bildung von Hyphen korreliert. Während Mutanten ohne das Gen *ECE1* weiterhin in der Lage sind normale Hyphen auszubilden, führen diese nicht zur Aktivierung des epithelialen „danger response pathway“, was darauf hindeutet, dass Ece1 eine wichtige Rolle bei der Erkennung von *C. albicans* durch Epithelzellen spielen könnte. Die darüber hinaus gehende biologische Rolle von Ece1 ist unbekannt. Das Ziel dieser Arbeit bestand daher darin, die bisher unbekannte Funktion dieses Proteins aufzuklären. Mithilfe eines *ece1*Δ/Δ Mutantensets wurde zunächst untersucht, welche Bedeutung Ece1 während der Adhäsion an, Invasion in sowie der Schädigung von Epithelzellen zukommt. Des Weiteren wurden die acht Peptide, welche durch die intrazelluläre Prozessierung des ganzen Proteins entstehen und anschließend sekretiert werden, auf ihre lytischen Eigenschaften gegenüber humanen Epithelzellen und Erythrozyten hin untersucht.

Die Ergebnisse zeigten, dass nur eines der acht Ece1-Peptide eine hoch lytische Wirkung auf alle getesteten Zelltypen ausübte, nämlich das Peptid Ece1-III. Eine eingehendere Untersuchung dieses kurzen Peptides ließ Membran-permeabilisierende Eigenschaften erkennen und führte zur Identifizierung dieses Peptides als erstes bekanntes zytolytisches Toxin eines humanpathogenen Pilzes. Es konnte gezeigt werden, dass die Schädigung von Wirtzellen durch dieses Peptid, welches als „Candidalysin“ bezeichnet wurde, schnell und effizient erfolgt und dass der Tod der Wirtszelle mit großer Wahrscheinlichkeit durch einen ionenabhängigen Mechanismus bewirkt wird. Die Identifizierung des Phospholipids Phosphatidylserin als Bindungspartner von Candidalysin innerhalb der Wirtszellmembran ermöglichte die Erstellung eines theoretischen Modells für die durch dieses Peptid induzierte Porenbildung.

Zusammengefasst gibt diese Arbeit Einblicke in die bisher unbekannte Funktion von Ece1 und dessen Rolle in infektions-assoziierter Zellschädigung und demonstriert weiterhin die Wichtigkeit der Charakterisierung aller *C. albicans* Gene, einschließlich derer mit unbekannter Funktion, zur Identifizierung weiterer fungaler Virulenzfaktoren.

1 Introduction

1.1 Human pathogenic fungi

Including approximately 611,000 species, fungi are estimated to account for 7% of all eukaryotic organisms found on earth [1]. Therefore, fungi are ubiquitous in our environment and have a great impact on both plant and animal life. The spectrum of fungal species that we are regularly exposed to consists of mostly innocuous environmental organisms and commensal species. There is, however, among them, a small percentage of opportunistic pathogens that can actively cause disease by infecting warm-blooded animals and humans [2]. The approximately 150 species known to be human pathogens account for only 0.02% of all fungal species [3]. Many of these species only cause harmless, albeit unpleasant infections, e.g. superficial infections such as athlete's foot or ringworm, mainly caused by dermatophytes and *Malassezia* species [2]. On the other hand, several fungi are able to cause life-threatening systemic disease, e.g. *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Candida albicans* and *Cryptococcus neoformans*. These pathogens are able to disseminate into deeper tissue and organs, including the lung, liver, kidney and central nervous system [4]. Such invasive infections, however, go hand in hand with immunosuppression, so while our lungs are constantly exposed to airborne spores of moulds such as *Aspergillus* or *Fusarium* and our skin and mucosal surfaces are densely colonised by *Malassezia* or *Candida* species, only those with predisposing immunosuppressive conditions (e.g. HIV-positive individuals or those undergoing chemotherapy or organ transplantation) are susceptible [2]. Potent antifungal treatments are available today, nevertheless mortality rates often exceed 50%, and it is estimated that more people worldwide are killed by fungal infections every year than by tuberculosis or malaria [5], strongly demonstrating the need for prevention and better treatment of these infections.

1.1.1 The genus *Candida*

The genus *Candida* belongs to the class of hemiascomycetes [4] and is comprised of approximately 200 yeast species, of which only 20 species have been associated with disease in humans [6, 7]. These species are able to cause both superficial infections of the skin and mucosa, as well as systemic infections. While superficial infections are mainly harmless, the establishment of a systemic infection by dissemination of the fungus through the bloodstream and subsequent solid organ colonisation, is life-threatening, which is demonstrated by mortality rates being as high as 40% [8]. *Candida* species represent the fourth-greatest cause of nosocomial bloodstream

infections in the United States, and the most common human fungal pathogens, with an estimated 80% of all fungal infections being caused by *Candida* species [9, 10]. The majority of infections are caused by *C. albicans*, although non-*albicans* species, mostly *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, are also frequently isolated [7, 11]. Species such as *C. krusei*, *C. dubliniensis*, *C. guillermondii*, *C. lusitaniae*, *C. rugosa*, *C. orthopsilosis* and *C. metapsilosis* are less frequently causing infections, while *C. famata*, *C. inconspicua*, *C. kefyr*, *C. lipolytica*, *C. norvegensis*, *C. sake* and *C. zeylanoides* are very rarely found to be the cause of disease. It is noticeable, that many of the medically important species do belong to the so called "CTG-clade", which describes a group of *Candida* species translating the codon CTG as serine instead of leucine [12].

1.1.2 *Candida albicans*

The diploid, polymorphic fungus *C. albicans* is a constituent of the normal human microbiota and can be isolated from the skin, gastrointestinal and urogenital tract of the majority of the human population [10, 13]. A unique characteristic of this fungus is its ability to grow as a unicellular budding yeast, a pseudohyphal or true hyphal form, depending on environmental triggers [14]. True hyphae differ from pseudohyphae in forming long narrow filaments with parallel sides and no constrictions at the site of septation, while pseudohyphae are generally shorter and wider with obvious constrictions showing at the septation sites [15]. Further morphological forms are white and opaque yeast cells and chlamydospores [16, 17]. While the switch from white to opaque cells plays a role in the mating process, chlamydospores can only be observed under *in vitro* conditions when grown on nutrient-poor media and may therefore present a dormant growth form developing under harsh environmental conditions [17, 18]. A phenotypic switch to GUT (Gastrointestinally IndUced Transition) cells is triggered by the exposure of *C. albicans* to the mammalian gut. These cells are better adapted to the digestive tract and promote fungal commensalism [19]. The different morphological forms are depicted in Figure 1.

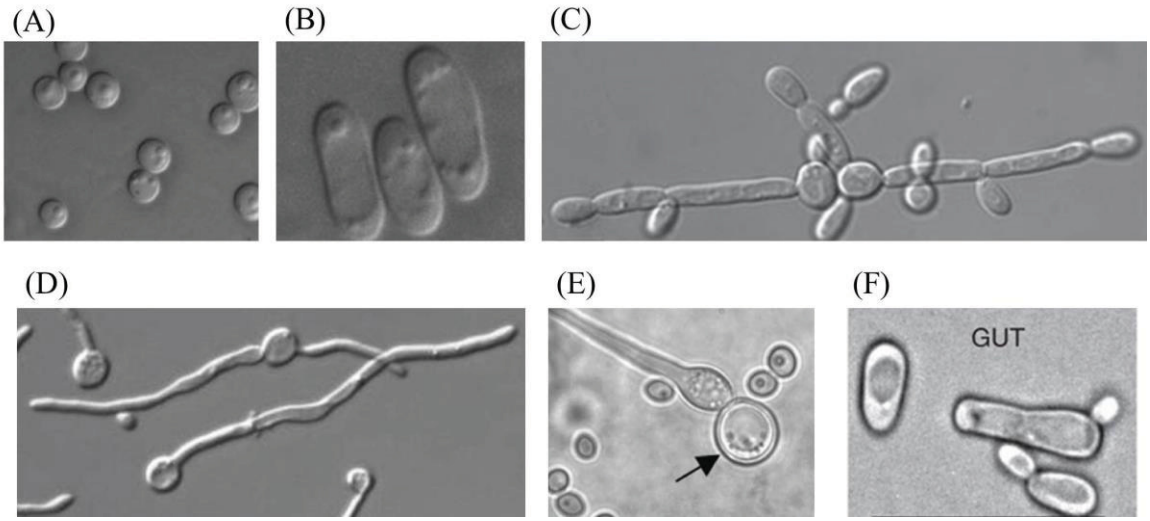


Figure 1: *C. albicans* morphologies. (A) yeast cells, (B) opaque cells, (C) pseudohyphal cells, (D) hyphal cells, (E) chlamydospores, (F) GUT cells. Pictures (A), (B), (D) and (E) taken from Hickman *et al*. 2013 [20]; picture (C) taken from Sudbery 2011 [15]; picture (F) taken from Pande *et al*. 2014 [19].

C. albicans is one of the most important human fungal pathogens and therefore the genome of the clinical isolate SC5314 was the first genome of a human pathogenic fungus to be sequenced [21]. The genome is made up of 14.88 Mb in total size and the > 6,000 genes are distributed over eight different chromosomes, labelled chromosome 1-7 and chromosome R [22]. Based on data provided by the *Candida* Genome Database (CGD) as of January 2016, the *C. albicans* genome contains a total number of 6,218 open reading frames (ORFs), of which only 1,581 ORFs (~25%) are defined as characterised. While 152 ORFs (~2.5%) are listed as dubious, the vast majority of *C. albicans* ORFs (~72%) is stated as uncharacterised. In contrast, only 660 ORFs (10%) of the well-studied model-organism *Saccharomyces cerevisiae* remain uncharacterised (Figure 2). The genomes of *C. albicans* and *S. cerevisiae* can be accessed through CGD [23] or the *Saccharomyces* Genome Database, respectively.

In the United States and most European countries, *C. albicans* is responsible for about 50% of invasive *Candida* infections [24]. The prevalence of this fungal pathogen is supposed to be connected to a stronger adherence potential of both *C. albicans* yeast and hyphal cells in comparison to non-*albicans Candida* species and only *C. dubliniensis* has been reported to exhibit an adherence comparable to that of *C. albicans* [25, 26].

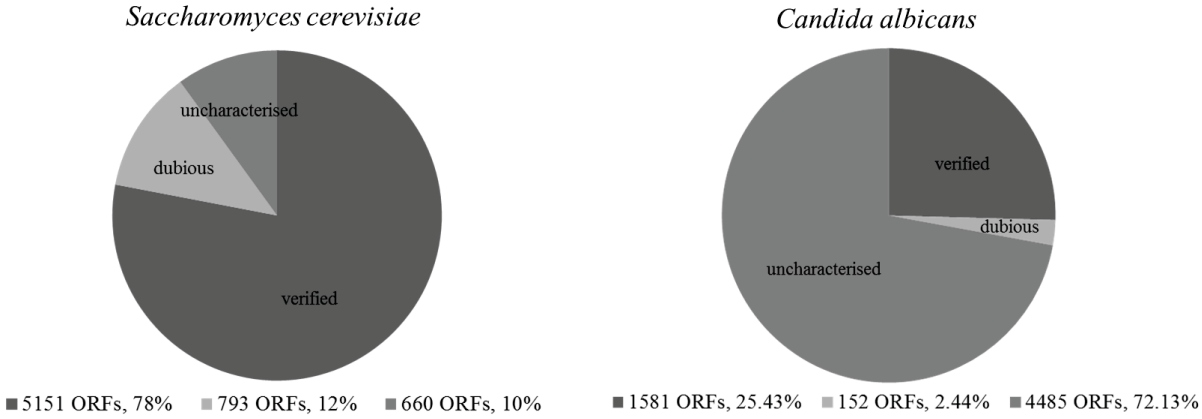


Figure 2: ***S. cerevisiae*** **and** ***C. albicans*** **genomes.** Total number of *S. cerevisiae* ORFs: 6604, verified ORFs: 5151, dubious ORFs: 793, uncharacterised ORFs: 660. Total number of *C. albicans* ORFs: 6218, verified ORFs: 1581, dubious ORFs: 152, uncharacterised ORFs: 4485. Based on data provided by the *Saccharomyces* Genome Database and *Candida* Genome Database, respectively (January 2016).

1.2 *C. albicans* infection types

In addition to skin infections, there are three major infection types in humans that are caused by *C. albicans*: infection of the oral cavity (oral candidiasis), infections of the vaginal tract (vulvovaginal candidiasis) and infection of the bloodstream and organs (invasive candidiasis).

1.2.1 Oral candidiasis

C. albicans is responsible for different types of oral infections, including oropharyngeal candidiasis (OPC) or thrush, and denture stomatitis (DS) [27]. OPC is particularly common in HIV patients and often one of the first signs of an underlying HIV infection [4]. Although a number of different immunocompromising conditions may result in the development of OPC, the probability for an HIV-infected person to get OPC is as high as 90% [28]. OPC manifests as infection of the hard and soft palate, tongue, buccal mucosa, and floor of the mouth [4]. These infections can present as either reddened patches (erythematous) or white curd-like lesions (pseudomembranous) [29]. Besides HIV infection, the existence of a genetic disorder may be the reason for recurrent *Candida* infections of the mouth, skin and other mucosal surfaces. Mutations in the gene “signal transducer and activator of transcription 1” (*STAT1*) as well as diseases resulting in the presence of specific auto-antibodies against Th17 cytokines can lead to the

development of chronic mucocutaneous candidiasis (CMC) [30–34]. Both OPC and CMC generally do not progress to life-threatening fungaemia [27].

DS is the most common form of oral candidiasis and affects approximately 50-75% of denture wearers, with xerostomia being an additional risk factor, as saliva does contain various proteins which exhibit direct antifungal activity [35–38]. DS manifests as an infection of tissues underlying the maxillary denture-bearing surfaces [37]. In comparison to healthy denture wearers, the saliva of DS patients was found to show increased levels of cystatins, immunoglobulins, and lactotransferrin [39]. While the presence of cystatins and immunoglobulins may be indicative for a host defence reaction in response to *Candida*, lactotransferrin has both antifungal and antibacterial activity [27]. Furthermore, the presence of bacterial species in denture biofilms differs between DS patients and healthy denture wearers, most probably elicited by the interplay of *Candida* with specific bacterial species of the oral microbiota [40]. To study the interactions taking place between *C. albicans* and oral epithelia, several *in vitro* infection models have been established, including the commonly used cell line for monolayer infection experiments TR-146, a buccal epithelial cell line originally derived from a human squamous cell carcinoma.

1.2.2 Vulvovaginal candidiasis

Affecting almost 75% of reproductive-age women, vulvovaginal candidiasis (VVC) is one of the most frequent forms of *Candida* infections, and with up to 7-8% of the patients suffering from the most severe form, known as recurrent vulvovaginal candidiasis (RVVC), it presents major quality of life issues for these women [41, 42]. Around 90% of all cases are caused by *C. albicans*, while the remaining 10% can mainly be attributed to *C. glabrata* [43]. VVC is the second most common cause of vaginal infections, only exceeded by bacterial vaginosis [44]. While the yeast form of *C. albicans* can be found in the vagina of >50% of healthy, asymptomatic women, the hyphal form is invariably connected to pathologic conditions and can be isolated from invaded tissues [45]. In contrast to other mucosal or invasive *Candida* infections, which mostly occur in patients with immunological deficits or underlying severe illnesses, predisposing factors for women to develop VVC rather include conditions like uncontrolled diabetes, use of antibiotics, oral contraception, pregnancy and hormone replacement therapy [45, 46]. It is now clear, that an overwhelming rather than a deficient immune response is the main cause of symptoms [47, 48]. VVC involves infections of the vaginal lumen as well as the vulva and typical symptoms and clinical signs include burning, itching, an abnormal vaginal discharge, an erythematous vulva and dyspareunia [29]. VVC infections are generally treated via the administration of antifungal drugs, such as clotrimazole, fluconazole, or ketoconazole. Regarding the high incidence of RVVC, however, the ultimate aim for immunotherapy would be the development of an anti-*Candida*

vaccine. The two most promising vaccines up to date are directed against the Als3 adhesin and Sap2, a member of the secreted aspartic protease family [49, 50]. Both vaccines have been successfully tested in mouse and rat models of vaginal infection and have passed phase I clinical trials, with one of them entering a phase II clinical trial [45].

1.2.3 Invasive candidiasis (IC)

When *C. albicans* penetrates and breaches the epithelial barriers and enters the bloodstream, infections can become life-threatening [7, 51]. From the bloodstream, the fungus can disseminate to virtually every organ, e.g. kidney, liver, but also the central nervous system [4]. Most cases of systemic *Candida* infection are believed to arise from an endogenous source, as the gastrointestinal tract harbours a variety of *Candida* species [52, 53]. Indeed, commensal and infecting isolates of *C. albicans* are most often genetically similar, supporting the notion that infecting strains generally originate from the patient's own flora [54, 55]. There are, however, exceptions, in which a systemic infection may also arise by introducing *C. albicans* via external routes, e.g. via central venous catheters [56–58]. Further risk factors include gastrointestinal surgery and parenteral nutrition. Physical alterations of the anatomical barrier function may result in damage of the gut, thus easing the transfer of *C. albicans* from the gut into the bloodstream, while parenteral nutrition may cause alterations of the commensal organism burden [59]. Even supposedly minor health issues, such as the use of antibiotics, can result in an increased risk for developing systemic candidiasis due to the overgrowth of bacterial members of the gut microflora by *Candida* [60]. The mortality rate of systemic candidiasis is significantly higher than that of other bloodstream infections (e.g. by *Staphylococcus aureus*) [61]. However, regarding the fact that most patients who develop systemic candidiasis are already severely ill before, the mortality rate that can directly be attributed to IC cannot be determined exactly, but is estimated to range from 14.5% [62] – 49% [61]. An important factor contributing to the outcome of an IC infection also lies in a timely diagnosis. Fungal diagnostics are generally difficult, due to a high rate of false-negative blood cultures [4]. Particularly in case of *Candida*, colonisation and infection have to be discriminated and the infectious *Candida* species specified, often leading to a late diagnosis and delayed start of antifungal treatment or an inappropriate choice of antifungals (e.g. in the case of *C. glabrata* or *C. krusei*, which both tolerate high azole concentrations) [63]. With mortality rates increasing from 15% (antifungal treatment initiated immediately after a positive blood culture) to 40% when treatment is delayed by only 72 hours, a fast and reliable diagnosis is of utmost importance [64]. Similar to the other forms of candidiasis, *C. albicans* is most frequently identified as the infectious species, being responsible for an estimated 50 to 60% of IC cases [24, 65]. Besides *C. albicans*, only *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* are

regularly isolated from IC patients, with the second most common species responsible for IC noticeably varying depending on the country [24, 65]. While in North America and Europe *C. glabrata* is the second most common species, in South America *C. parapsilosis* is most commonly isolated after *C. albicans* [66–71].

1.3 Virulence factors

C. albicans possesses a number of virulence factors and fitness attributes that contribute to its virulence when shifting from a commensal to a pathogenic state. Virulence traits of great importance include the ability of the fungus to reversibly change its morphology from a budding yeast to a filamentous form, expression of adhesins and invasins on the cell surface, the ability to damage host cells, the formation of biofilms, thigmotropism (contact sensing), phenotypic switching, and the secretion of hydrolytic enzymes [72]. Fitness attributes on the other hand allow the fungus to physiologically adapt to changing environments within the host during colonisation and infection. Therefore, the ability of *C. albicans* to adapt to changing environmental conditions, such as pH, and an elaborate stress response, are prerequisites to successfully colonise a host, disseminate throughout the bloodstream, tissues and organs, and evade and resist the host's immune response [73].

1.3.1 Morphogenesis

The morphological transition from a budding yeast to a filamentous growth form is considered to represent the most important virulence factor of *C. albicans* [74]. Therefore, mutants which are unable to form hyphae under *in vitro* conditions are generally attenuated in virulence [75]. Nevertheless, both morphological forms have been ascribed an important role in pathogenicity [76]. While hyphae are more invasive and therefore contribute to the gross of tissue damage, yeast cells are believed to play a role in dissemination [76–78]. There are a number of environmental stimuli leading to the yeast-to-hypha transition *in vitro*, which include the presence of serum at 37°C, *N*-acetyl-*D*-glucosamine (GlcNAc), neutral pH, 5% CO_2, starvation, and microaerophilic conditions [79–83]. These stimuli induce either the cAMP/PKA or MAPK signalling pathway via stimulation by the GTPase Ras1, eventually resulting in the expression of master activators of hyphal formation, i.e. the transcription factors Efg1 and Cph1 [15, 84–86]. Further transcription factors which promote the yeast-to-hypha transition are Cph2, Tec1, Flo8, Czf1, Rim101 and Ndt80, while a complex consisting of the general transcriptional co-repressor Tup1 in association with Nrg1 or Rox1p-like regulator of filamentous growth (Rfg1) has been shown to repress the morphological change [15]. The *C. albicans* adenylyl cyclase *CYR*1 plays a central role in

integrating and forwarding hypha-inducing signals. Cyr1 is either stimulated in a Ras1-dependent manner by serum, or directly and Ras1-independent by bacterial peptidoglycan, which may be present in the serum [87, 88]. Direct binding of either CO_2 or HCO_3 to the catalytic domain will also lead to Cyr1 activation [89]. The quorum sensing molecule farnesol, on the contrary, exhibits repressing effects on hypha formation via the repression of Cyr1 [90]. Furthermore, farnesol may act through the negative regulators Tup1 and Nrg1, as deletion mutants lacking the respective genes do not respond to the presence of farnesol and maintain their characteristic, constitutively pseudohyphal phenotype [91]. Environmental cues and subsequent pathways leading to the induction of hypha formation are schematically shown in Figure 3. Hypha formation is characterised by the expression of specific hypha-associated proteins, namely the hyphal wall protein Hwp1, the agglutinin-like sequence protein Als3, the secreted aspartic proteases Sap4, Sap5 and Sap6, and the hypha-associated proteins Ece1 and Hyr1 [15, 72]. These proteins are not required for hypha formation and maintenance, but contribute to the special properties of hyphae that are important for virulence, e.g. by aiding in adhesion to and invasion into host cells. Therefore, genes controlling morphogenesis are transcriptionally co-regulated with genes encoding virulence factors [75, 92]. It might be possible, that some of these hypha-associated genes contribute to the modulation of the hyphal structure, e.g. by increasing the chitin content in the cell wall, which is doubled during hyphal growth [93]. The ability of *C. albicans* hyphae to exhibit a directional growth when encountering a solid surface (thigmotropism) facilitates the invasion of an epithelium, as the fungus can specifically identify and invade intercellular junctions as well as small ridges and grooves associated with weakened integrity of the epithelial cells [83, 94–96].

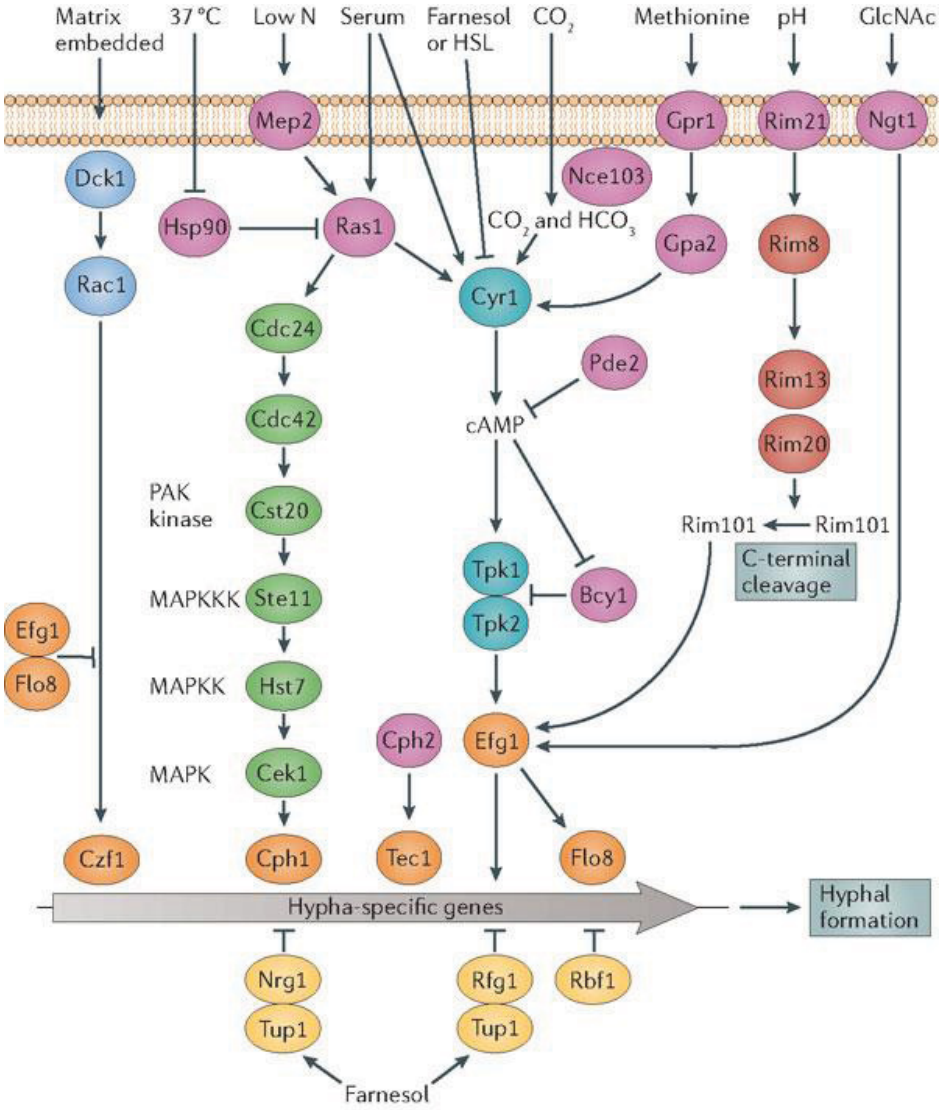


Figure 3: Signalling pathways involved in the regulation of hypha formation. Environmental cues and triggers are sensed via specific receptors and forwarded by different signalling pathways, finally resulting in the expression of activating transcription factors (Efg1, Cph1, Cph2, Tec1, Flo8, Czf1, Rim101, Ndt80) or repressors of hyphal growth (Tup1/Nrg1 or Tup1/Rfg1). Picture taken from Sudbery 2011 [15].

1.3.2 Adhesion, invasion and damage

During both commensalism and pathogenicity, adhesion of *C. albicans* to host cells, other microorganisms or abiotic surfaces is essential for colonisation and survival within the host. As opposed to initial cell-cell contact, which is mediated by passive forces such as van der Waals forces and hydrophobic interactions, active adhesion requires specific proteins, adhesins, which are able to interact with receptors on the host cell's surface [97, 98]. Although the initial adhesion is likely to occur between yeast cells and epithelial surfaces, many adhesins of dominant

significance are specifically associated with the hyphal form, which is induced after the first contact with a host cell surface [97]. The group of hypha-associated adhesins include the Als (agglutinin-like sequence) family, consisting of eight members that are encoded by the *ALS* gene family (*ALS1-7* and *ALS9*) and which present the best studied group of all known *C. albicans* adhesins [72]. The *ALS* genes encode for glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins, which are characterised by three distinctive domains: a substrate-binding N-terminal domain containing a signal peptide, an immunoglobulin-like domain and a threonine-rich domain, a central serine/threonine-rich domain consisting of several 36-amino acid tandem repeat sequences, and a C-terminal domain that is predicted to be cleaved when the protein is exported to the cell surface due to its GPI-anchorage sequence [99–103]. Of the eight Als proteins, Als3 does play the most significant role in the adhesion process. This protein directly promotes adhesion, presumably by binding of the immunoglobulin-domains in its N-terminal region to the extracellular immunoglobulin-like domain of either E-cadherin on epithelial cells or N-cadherin on endothelial cells, thereby mimicking the mechanism responsible for cadherin-cadherin binding [104].

Another key protein for mediating adhesion to host cells is the hyphal wall protein (Hwp1), a hypha-associated GPI-linked protein [105]. Via cross-linking of glutamine residues of the N-terminal region of Hwp1 to as yet unidentified host proteins, *C. albicans* is able to covalently attach to host epithelial cells [97, 106]. While Hwp1 does not seem to play a direct role in disseminated infection, this adhesin is evidently important for the colonisation of the oral cavity, as *C. albicans* strains defective in *HWP1* do not cause oropharyngeal candidiasis in mice [107].

In addition to hypha-associated adhesins, there are several morphology-independent proteins that may contribute to adhesion. These include Eap1, Iff4, Ecm33 and Mp65. The putative ß-glucanase Mp65 facilitates adhesion of *C. albicans* by mediating attachment to various substrates. Furthermore, this protein can modify the cell wall structure via its glucanase activity, which may have a positive effect on the expression or function of other adhesins [108, 109].

During both commensalism and pathogenicity a certain degree of host cell invasion is required in order to maintain a foothold on the cell surface or to invade an epithelium, respectively [56]. For the purpose of invasion, it is of importance that the hyphae invade the epithelium at a rate faster than the rate of desquamation, to prevent being shed along with the epithelial cells [110, 111]. *C. albicans* is able to invade host cells by utilisation of two distinct mechanisms: induced endocytosis and active penetration [112]. Induced endocytosis is a completely passive, host cell-driven process independent of the viability of the *Candida* cell and can mainly be observed during the early stages of invasion (~4h) [92, 113–115]. The process itself is actin-dependent and clathrin-mediated [116, 117]. Invasins localised on the hyphal surface interact with target host cell receptors, thereby triggering engulfment of the fungal cell into the host cell [72, 104, 109]. Two

invasins that have been identified so far are the before-mentioned Als3 (which also functions as an adhesin) and Ssa1. Ssa1 belongs to the Hsp70 family of heat shock proteins, containing an N-terminal ATPase domain and a C-terminal peptide binding domain [118]. Both *als3*Δ/Δ and *ssa1*Δ/Δ mutants showed attenuated endocytosis due to the inability to correctly bind E- and N-cadherin [119–121]. Besides E-cadherin on epithelial cells and N-cadherin on endothelial cells, the receptor tyrosine kinases, EGF receptor (EGFR) and HEGF receptor 2 (HER2) have been identified to serve as receptors on the host side [104, 120, 122]. After the establishment of a strong cell-cell contact due to receptor-ligand interaction, clathrin, dynamin and cortactin are recruited to the site of hyphal entry, resulting in cytoskeleton reorganisation such as filament assembly and polymerisation to surround the fungus during the endocytic uptake [119].
The alternative invasion route of active penetration is a fungal-driven process mediating penetration of both killed and living host cells. As *C. albicans* frequently encounters stratified, squamous epithelium with terminally differentiated, non-proliferative cells forming the uppermost layer, host-driven induced endocytosis is not possible, while active penetration allows the fungus to directly invade into a cell or pass through the intercellular junction between epithelial cells [56, 115]. Furthermore, enterocytes are only invaded by active penetration [123]. This process requires different factors, e.g. the elongation of hyphae, physical force exerted by extending hyphae and the secretion of hydrolytic enzymes [112, 121, 124]. It still remains unclear which proteins are involved in this invasion route, but secreted aspartic proteases (Saps) have been implicated as being crucial contributors [97]. These proteases may be involved in the degradation of E-cadherin within the intercellular junctions between epithelial cells, eventually resulting in the loss of epithelial integrity [124–126]. However, the two other groups of *C. albicans* hydrolytic enzymes, namely phospholipases and lipases, have not yet been shown to be involved in the process of active penetration. The invasion route ultimately utilised by this fungus to gain access to the host cell is dependent on many different factors, e.g. the invasion stage, fungal morphology and the epithelial lineage to be invaded.

Upon invasion of the tissue by *C. albicans*, damage can occur. Proteolytic degradation of E-cadherin results in the loss of epithelial integrity and finally leads to the destruction and loss of the superficial epithelium [124, 125]. Induced endocytosis is not *per se* responsible for damage to the host cell, as dead *C. albicans* cells are as readily endocytosed as living cells without host cell damage, suggesting further processes to be involved in inducing damage [113]. Extensive damage is mainly associated with filamentation and active penetration and therefore it is most likely that damage occurring during induced endocytosis is mediated by active penetration of the endocytic compartment into which the *C. albicans* cell has been taken up [97]. Mutant strains reverting back to yeast cell growth after the initial germ tube formation and invasion of the host cell (e.g. *eed1*Δ/Δ) thereby get trapped inside the host cell, demonstrating that not only hypha formation

but also the maintenance of hyphal growth is of significance for host cell damage [127]. Damage to the host cell may be caused by necrosis, induced by the mechanical disruption of host cell integrity by piercing hyphae, or via the activation of specific epithelial cell-death pathways by *C. albicans* [128]. The anti-apoptotic proteins Bcl-2 and Bcl-xL, present in macrophages and neutrophils, can be inactivated by the fungus, thereby inducing apoptosis [129, 130]. It is estimated that approximately 50% of epithelial cells induce apoptosis in response to *C. albicans*. However, only 15% of these cells move from early to late apoptotic events, suggesting that epithelial cells may possess specific response pathways which contribute to limiting the pathways that cause progression of apoptosis [97, 131]. Considering these numbers and the fact that anti-apoptotic signalling pathways are activated at later time points of a *C. albicans* infection, it can be assumed that final epithelial cell death is caused by necrosis [114, 131].

1.3.3 Biofilm formation and drug resistance

Biofilms are defined by structured microbial communities, which are attached to a surface and surrounded by a self-produced extracellular matrix [132]. *C. albicans* is able to form biofilms on both abiotic surfaces, e.g. dentures and catheters, and on biotic substrates, such as mucosal cell surfaces [133]. The process of biofilm formation can be divided into four different stages, which are (1) cell-wall mediated adherence of yeast cells to the substrate, (2) proliferation of these cells into a thin layer, (3) maturation of the biofilm by development of both pseudohyphal and hyphal cells and secretion/accumulation of extracellular matrix material, and finally (4) the dispersion of yeast cells from the mature biofilm, possibly with the purpose to disseminate into different niches within the host [134–137]. Important transcription factors that have been identified to play a role in controlling biofilm formation are Bcr1, Tec1, Efg1, Ndt80, Rob1 and Brg1, and mutants being deficient in any of these major regulators exhibit a defective biofilm formation, as was shown *in vivo* using a rat infection model [138].

One of the most challenging characteristics of a *C. albicans* biofilm is the high resistance of the cells against antifungal compounds, making treatment rather difficult and putting hospitalised patients at high risk of establishing a *Candida* infection, as biofilms are estimated to establish on 30% of catheters [133, 139]. There are numerous factors responsible for this increased resistance, including a reduced growth rate of the cells, up-regulation of drug efflux pumps, the presence of persister cells, and an impermeable extracellular matrix [140–146]. ATP-binding cassette (ABC) transporters encoded by the *CDR* (*Candida* drug resistance) genes and the *MDR1* (multidrug resistance) gene of the major facilitator (MF) superfamily are involved in conferring drug resistance to *C. albicans* and several members of these gene families could be shown to be up-regulated in the presence of azoles [147–150]. Persister cells are phenotypically variant cells

within a biofilm and present a difficult target to even high concentrations of antifungal drugs due to their metabolically dormant state [151]. Even though they account for only approximately 1% of all cells within a biofilm, killing these cells is crucial for treatment of a *Candida* infection [144]. Mature *C. albicans* biofilms are embedded in an extracellular matrix. While the thickness of the extracellular matrix only seems to play a minor role in restricting penetration of drugs, the composition of the matrix material seems to be significant, as ß-1,3-glucans are able to bind fluconazole in biofilm structures [143, 152]. Mutants lacking one of several genes (*BGL2*, *PHR1*, *XOG1*) involved in ß-1,3-glucan production, delivery to the matrix and accumulation into the matrix material show an increased susceptibility to fluconazole [153]. The two transcriptional regulators Rlm1 and Zap1 are also known to contribute to matrix production. Rlm1 is involved in antifungal drug resistance via the regulation of *FKS1* expression, a gene encoding for a ß-1,3-glucan synthase [154]. Correspondingly, heterozygous deletion of *FKS1* results in a 30% reduction of ß-1,3-glucan [152].

1.3.4 Metabolic adaptation

The colonisation of different host organs and dissemination throughout the body during infection are accompanied by a dramatic change of available nutrients. As indicated by the ability of *C. albicans* to proliferate in numerous niches of the human host, this fungus is metabolically flexible [155]. While the bloodstream is relatively rich of glucose (6 – 8 mM), which is the preferred carbon source of *C. albicans*, other niches harbour nutrient-starving conditions, e.g. inside phagosomes of phagocytic cells, or *C. albicans* has to compete for the nutrients with other microbes, e.g. in the gastrointestinal tract [155–157]. In addition, essential micronutrients, such as iron, are only available in very low concentrations and are almost completely associated with host proteins in sterile body niches [158]. Therefore, *C. albicans* needs to quickly adapt to the local nutrients provided in a specific niche, and also needs mechanisms to actively sequester micronutrients from host proteins. Following uptake into a phagolysosome after ingestion by a macrophage or neutrophil, *C. albicans* switches from glycolysis to gluconeogenesis and activates fatty acid ß-oxidation and the glyoxylate cycle [159–161]. In order to provide non-glucose carbon sources, *C. albicans* secretes a number of hydrolases. Lipases and phospholipases B are involved in the degradation of host lipids, while secreted aspartic proteases (Saps) break down host proteins, resulting in the release of amino acids and oligopeptides [155]. Interestingly, *C. albicans* cells grown on lactate as an alternative carbon source, were found to be more resistant to environmental stresses and showed a higher virulence potential [162]. Furthermore, in contrast to the diauxic growth, observed e.g. in the non-pathogenic yeast *Saccharomyces cerevisiae*, *C. albicans* is able to assimilate lactate even in the presence of glucose [163]. Therefore, the

diauxic shift, which is characterised by a lag phase of microbial growth during which the enzymes needed for the catabolisation of the second carbon source are produced, cannot be observed in *C. albicans*. This allows the fungus a faster adaptation to changing carbon sources and provides advantageous benefits during both commensal and pathogenic growth [155]. The growth on unfavourable carbon sources can also result in *C. albicans* actively changing the surrounding environmental conditions. The utilisation of amino acids and polyamines as carbon sources will inevitably lead to an increased excretion of nitrogen in the form of ammonia. Thus, the outside environment will ultimately become more alkaline, thereby providing a trigger for hypha formation [164]. Besides macronutrients, *C. albicans* also needs to acquire trace metals such as iron, zinc, manganese and copper from its host, as these serve as essential cofactors of many enzymes required for fungal growth [165]. Inside the host, those trace metals are associated with proteins, due to the toxicity of free metals and to prevent microbial growth, a process which has been given the name of "nutritional immunity" [166, 167]. To effectively overcome this nutrient withdrawal and access the micronutrients bound within the host proteins, *C. albicans* has evolved elaborate strategies. For iron, the most abundant trace metal, three different uptake systems have been described: the siderophore-uptake system, the reductive pathway, and the haemoglobin uptake and degradation system [168]. In contrast to many other microbial species, *C. albicans* lacks the ability to produce iron-binding siderophores. However, siderophores produced by other species can be taken up by *C. albicans* via the Sit1/Arn1 transporter [169, 170]. The reductive pathway is utilised to take up iron from transferrin, ferritin, and free iron from the environment [72]. This pathway consists of several ferric reductases, multicopper oxidases and iron permeases, which are localised inside the plasma membrane [171, 172]. The hyphal protein Als3 has been identified as the receptor for binding host ferritin [173]. In order to utilise haem-derived iron from haemoglobin, haemolysis needs to be induced by the expression of specific haemolysins. One of these as yet unidentified haemolysins is hypothesised to be a secreted mannoprotein [174]. After the release of haemoglobin from the erythrocyte, uptake is mediated by the haem-receptor gene family members *RBT5*, *RBT51*, *CSA1*, *CSA2*, and *PGA7* [175]. Once inside the cell, further degradation is carried out by the haem oxygenase encoded by *HXM1*, resulting in release of iron from the molecule [176, 177]. A siderophore-like mechanism has been described for zinc acquisition. Extracellular zinc is bound by the secreted protein Pra1 (pH regulated antigen 1), which then re-associates with the cell surface via the Pra1 receptor Zrt1, which also serves as a zinc transporter [178, 179].

1.3.5 Stress response

Inside the human body, *C. albicans* is constantly exposed to a variety of environmental and immune-derived stresses. Therefore, the ability to cope with these stresses presents a prerequisite for life inside the human host – during both commensalism and pathogenicity. Mitogen-activated protein kinase (MAPK) pathways play a crucial role in sensing environmental and stress signals, and activation leads to a phosphorylation cascade, eventually resulting in the activation of specific transcription factors [180]. Three MAPK pathways are known to get activated in *C. albicans*, each being characterised by a specific kinase, namely Mkc1, Hog1 and Cek1. The Mkc1 (MAP Kinase from *C. albicans*) pathway is involved in cell wall salvage and maintaining cellular integrity and is phosphorylated upon oxidative and osmotic stress conditions [180, 181]. The Hog1 (High Osmolarity Glycerol response) pathway is considered to be the core response to many types of stresses, including oxidative, osmotic, heavy metal, cell wall and thermal stresses, and also plays a role in cell wall formation and morphogenesis [180]. Activation of Hog1 induces an accumulation of glycerol within the cell, which acts protectively during osmotic stress by counteracting the loss of water connected to the high osmotic gradient building up between the inside and outside of the fungal cell [180]. The Cek1 (*Candida* ERK-like kinase) pathway is involved in filamentation, mating, and possibly also the adaptation to thermal stress [180, 182]. This pathway is regulated by growth signals and quorum sensing. As would be expected of factors exhibiting this kind of importance, the loss of either Mkc1, Hog1 or Cek1 results in attenuated virulence in the mouse model [183–185].

When phagocytosed by an immune cell of the human host, *C. albicans* is exposed to both reactive oxygen species (ROS) and reactive nitrogen species (RNS). Enzymes detoxifying these molecules are pivotal in response to these stresses. ROS, such as hydroxyl radicals and peroxide, can be rendered inactive by the enzymes catalase (Cta1) and superoxide dismutases (Sod1-5), while the flavohaemoglobin enzyme Yhb1 may be utilised to detoxify RNS [161, 186–191].

A great flexibility in terms of pH tolerance is also of importance for the colonisation of the human host, as pH values within the human body vary from neutral (~pH 7.4) in the blood and tissues, acidic in the vaginal lumen (~pH 4) and range from highly acidic to alkaline (pH 2-8) inside the digestive system [192]. The environmental pH influences processes such as protein stability, enzyme function, nutrient uptake and the availability of micronutrients [192, 193]. Therefore, the sensing of the environmental pH and an appropriate response are of importance. An alkaline pH is sensed via the Rim101 signal transduction pathway [192]. The membrane receptors Dfg16 and Rim21 activate a signalling cascade upon sensing of an alkaline pH, which will result in the activation of Rim101 through C-terminal proteolytic cleavage [192]. Rim101 will then enter the nucleus and induce pH-appropriate transcriptional responses [192]. As mentioned earlier, *C. albicans* is also able to actively alkalinise the immediate environment via the excretion of

ammonia resulting from intracellular cleavage of amino acids and other alternative carbon sources. This is exported by the transporter Ato (ammonia transport outward) [164].
Heat shock proteins (Hsps) act as chaperones, preventing proteins from misfolding or aggregation during thermal and oxidative stress [194, 195]. They also play a role under non-stress conditions, as Hsps also mediate the correct folding of newly synthesised polypeptides [196]. Further roles include the assembly and disassembly of proteins in oligomeric structures, the transport of client proteins across the plasma membrane and the labelling of proteins via ubiquitylation for proteasome-mediated degradation. Until now, six major and six small heat shock proteins have been identified in *C. albicans* [72]. Hsps of importance are Hsp90, which is activated via the Hog1, Cek1 and Mkc1 pathways and is involved in temperature-dependent morphological changes [197, 198], and the small heat shock protein Hsp21, which is involved in stress adaptation and virulence and activated via the Cek1 pathway [182].

1.4 Host response

Upon first contact with *C. albicans*, the host's innate immune system is rapidly activated [60]. Immune cells involved in this primary response are particularly phagocytes, such as macrophages and neutrophils, and also epithelial cells of the mucosal surfaces. This primary response is characterised by direct antifungal activities, such as phagocytosis and the release of antimicrobial peptides [199]. This is followed by the more specific, albeit much slower, immune response of the adaptive immune system, which is mediated by T lymphocytes (cellular immunity) and B lymphocytes (humoral immunity) [60]. The generation of mature antigen-specific T and B lymphocytes may take as long as several days after first contact with a pathogen [200].

1.4.1 Innate immunity

The first step to combat an intruding pathogen is recognition. Therefore, epithelial cells of mucosal surfaces, which are the first cells to come into contact with *C. albicans*, express pattern recognition receptors (PRRs). Additionally, PRRs are also expressed by polymorphonuclear leukocytes (PMNs), dendritic cells, monocytes, macrophages, B cells and T cells. These receptors interact with specific pathogen associated molecular patterns (PAMPs) on the fungal cells, leading to recognition of the fungus. PAMPs specific for *C. albicans* are e.g., cell wall components (ß-glucan and mannan) and nucleic acids [7]. PRRs are divided into the three major groups of toll-like receptors (TLRs), C-type lectin receptors (CLRs), and nod-like receptors (NLRs) [201, 202]. TLRs involved in the recognition of *C. albicans* are TLR2 (recognising the phospholipomannan component of the cell wall), TLR4 (recognising short linear *O*-linked

mannans), and TLR9 (recognising fungal DNA) [203]. The binding of a PAMP by a PRR leads to the activation of a signal transduction pathway, finally activating a downstream adaptor molecule (mainly MyD88; myeloid differentiation primary response gene 88) and stimulating the release of pro-inflammatory cytokines [204]. CLRs, including the mannose receptor (MR), dectin-1, dectin-2, DC-SIGN, and Mincle, recognise *C. albicans* via binding of specific fungal polysaccharide structures [205]. Dectin-1 does have an extracellular carbohydrate recognition domain (CRD), which binds ß-glucan [206]. Dectin-2 can recognise high mannose structures on hyphae as well as α-mannan on both yeast and hyphae [207–209]. DC-SIGN is mainly expressed on dendritic cells and the binding of *N*-linked mannan to this receptor triggers the uptake of *C. albicans* into the cell [56, 209, 210]. MBL (mannose-binding lectin), in contrast to the other receptors, is a soluble and circulating CLR. Binding of this receptor to carbohydrates on microbial cell walls results in the activation of the complement system and subsequent opsonisation and uptake of the *Candida* cell [211]. The family of NLRs are intracellular receptors containing leucine-rich repeats and a nucleotide-binding domain [212]. The NLR NLRP3 forms an inflammasome complex with ASC (apoptosis-associated, speck-like protein) and the pro-enzyme pro-caspase-1 [45]. Once this complex gets activated, pro-caspase-1 is processed into an active caspase-1, which after being released results in the processing of pro-IL-1ß and pro-IL-18 and the secretion of the active cytokines IL-1ß and IL-18 [213]. As the first line of defence, epithelial cells are able to secrete antimicrobial peptides (AMPs) upon recognition of microbial pathogens via their PRR receptors. AMPs are small soluble molecules consisting of 10-50 amino acids and function by either killing or inhibiting the growth of the pathogen [214, 215]. AMPs known to be of importance for the interaction between *C. albicans* and human cells are the cathelicidin LL-37, histatins, and defensins [214]. The ß-1,3-exoglucanase Xog1 of the *C. albicans* cell wall serves as a receptor for LL-37 [216]. Binding of this AMP to Xog1 leads to an increased activity of this enzyme, thus hindering *C. albicans* in its ability to adhere to the host cell [216, 217]. Histatin 5 uses the cell envelope proteins Ssa1 and Ssa2 as receptors and enters the fungal cell via the transporters Dur3 and Dur31 [218–220]. Intracellular accumulation of this AMP, as well as the induction of reactive oxygen species (ROS) formation and an efflux of ions and ATP, contribute to the killing of the fungal cell [221, 222]. The group of human ß-defensins (hBD1- hBD3) permeabilise the membrane of intruding pathogens [223].

Phagocytic cells are key players of the innate immune system and these cells are recruited to the site of infection by pro-inflammatory cytokines released upon activation of the mucosal PRRs [224–226]. Neutrophils can directly kill *C. albicans* through phagocytosis and degranulation [227]. After uptake into these cells, *C. albicans* is exposed to ROS and an array of granule products, e.g. lactoferrin, myeloperoxidase, defensins and azurophil [228, 229]. Degranulation, accompanied by the release of antimicrobial peptides, and the formation of neutrophil extracellular traps (NETs) allows neutrophils to also kill pathogens extracellularly. Such NETs

are composed of a web of chromatin fibres containing serine proteases and antimicrobial proteins [227]. The role of mononuclear phagocytes during an infection is not known in detail so far, it is however clear that the secretion of pro-inflammatory cytokines by monocytes in the blood and macrophages in the tissues plays a role in recruitment and activation of PMNs [60]. Dendritic cells (DCs) represent the bridge between the innate and adaptive arms of the immune system. These cells recognise *Candida* via the C-type lectin receptors MR and DC-SIGN, which is followed by internalisation of the fungus and subsequent traffic to a local lymph node [115]. Processed *Candida*-specific antigens are eventually presented to T cells, initiating the response of the adaptive immune system [115, 230]. Besides cell-mediated antifungal responses, soluble factors also play a role in clearing fungal infections. The complement system, an amplifying cascade of protein cleavages consisting of over 60 small proteins and protein fragments, labels cells for phagocytosis via opsonisation [231–233]. The complement factor C3b is deposited on the *C. albicans* cell surface, making these cells recognisable for the complement receptor 3 on the surface of phagocytic cells, which will engulf, endocytose and finally kill the fungal cell [234].

1.4.2 Adaptive immunity

The adaptive immune response against *Candida* is mediated by T lymphocytes and B cells. The presentation of *Candida*-specific antigens by DCs triggers the priming of T cells into a specific subset. Depending on different factors such as molecular structures and morphology of the fungus, naïve $CD4^+$ Th precursor cells differentiate into one of three lineages of effector T helper cells: Th1, Th2, and Th17 cells [235]. Each subset is characterised by a different set of cytokines being secreted [236]. A Th1 response is associated with the secretion of inflammatory interferon-γ (IFN-γ), leading to the activation of neutrophils and subsequent phagocytosis and killing of the pathogen [60, 237, 238]. Therefore, the Th1 response primarily mediates the control of disseminated and established infections. Th2 cells on the other hand produce IL-4, IL-5, and IL-13 and this immune response is involved in promoting allergy and protecting against helminths, while it is considered to be non-protective against disseminated infections [236, 239, 240]. Th17 cells assist in controlling initial growth of *Candida* and preventing invasion of the tissue and are mainly involved in mucosal host defences [236, 241]. Th17 cells are specifically directed to mucosal areas, where they release the cytokines characteristic for a Th17 response, namely IL-17A, IL-17F, and IL-22, thereby inducing the recruitment of neutrophils to the site of infection and driving the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides by epithelial cells [8, 242]. The important role of the Th17 subset is highlighted by the fact, that excessive growth of *Candida* on the skin and mucosa of patients with hyper-IgE syndrome (HIES) or CMC results from the inability to induce a Th17 response. As a

consequence, the deficiency of IL-17 will lead to an insufficient recruitment of neutrophils from the bloodstream [243].

1.4.3 *Candida* immune escape strategies

Invasion of a host cell by *C. albicans* is followed by a constant battle between the host's immune responses and mechanisms evolved by *C. albicans* to escape these attacks. One of these mechanisms is to hide from recognition by the host via shielding of important PAMPs on the fungal cell surface. Detection by the PRR dectin-1 for example is avoided by shielding the receptor's ligand ß-glucan with the outer mannoprotein layer of the cell wall [244]. Antifungal drugs (e.g. echinocandins) or activity of host enzymes leading to the disturbance of the fungal cell wall structure therefore result in a better recognition of the pathogen by phagocytic cells [245–247]. White budding yeast cells release a chemoattractant for neutrophils. By phenotypically switching from a white to a mating-competent, oblong and opaque phenotype, the fungal cell makes itself invisible for neutrophils [248]. Indeed, when confronted with both white and opaque cells, neutrophils selectively engulf the white cells, while on encounter of opaque cells no phagocytosis can be observed. A further immune evasion mechanism exhibited by *C. albicans* is the inactivation of the host's complement system. This is accomplished either via the degradation of specific complement factors or the expression of complement inhibitors. It could be shown *in vitro*, that C3b, C4b and C5 are cleaved by secreted aspartic proteases [249]. The surface protein Pra1 (pH regulated protein 1), present in both yeast and hyphae, can also get secreted, resulting in the binding of human immune regulators and plasma proteins like factor H and the C4b-binding protein [250–252]. Even after phagocytosis by host immune cells, *C. albicans* is able to escape. Hyphal growth inside the immune cell after activation of the hyphal regulator Efg1 via the cAMP/PKA pathway will result in the piercing and eventually killing of the immune cell [253, 254]. Furthermore, *C. albicans* is able to intervene into the intracellular membrane trafficking, thereby inhibiting the fusion of lysosomes with the pathogen-containing phagosome [255, 256]. An array of fungal enzymes (e.g., a catalase and a surface superoxide dismutase) additionally counteract the oxidative stress induced by ROS production in phagocytes [199, 257]. The antifungal activities executed by the secretion of AMPs is overcome via secretion of peptide effectors, AMP efflux pumps, and regulation of signalling pathways [258]. The salivary AMP histatin 5 for example can either be degraded by the proteases Sap9 and Sap10, or get excluded from the cytoplasm via the polyamine transporter Flu1, a member of the *MDR* family [27, 259, 260]. In addition, the plasma membrane-bound signalling mucin Msb2 can shed its extracellular glycodomain Msb2*, which is able to counteract the antifungal activity of the cathelicidin LL-37, histatin 5 and human ß-defensin 1 [258, 261, 262]. Last but not least, *C. albicans* is able to

actively modulate the host's T cell response, by avoiding TLR4 recognition and thus inhibiting a pro-inflammatory Th1 response [263]. The favoured TLR2 activation instead leads to a non-protective Th2 response and to the induction of anti-inflammatory cytokines [264–266]. Moreover, the release of tryptophan metabolism products of *C. albicans* hyphal cells results in the down-modulation of the important Th17 response [267].

1.4.4 Discrimination of commensal and pathogenic *C. albicans*

The fact that *C. albicans* is part of the normal microbiota of most healthy humans demands the ability of the host's immune system to discriminate between harmless colonising cells and invading pathogenic cells, as both an overreaction of the immune system against the constantly present colonising yeast cells as well as a too weak response against invading hyphal cells may result in a fatal outcome. One essential step that was observed to be different between yeast and hyphae is the activation of the inflammasome, which is activated by PAMPs that vary between the different morphologies [238]. The important PAMP ß-glucan for example is thought to be expressed only on the bud scars of yeasts, but on the whole surface of hyphal cells, resulting in a different cytokine stimulation [244]. Furthermore, mannan fibrils present on hyphae are shorter and less abundant compared to the ones found on yeast cells. As the outer mannan layer serves to shield the cells from recognition by PRRs, hyphae are an easier target for recognition due to the loss of shielding of the underlying ß-glucan [268]. Additionally, it has been suggested that the biochemical structure of the mannan varies between yeast and hyphae, resulting in a differential recognition by dectin-2 [269]. Recognition of these PAMPs will lead to the activation of the inflammasome and subsequent processing and secretion of IL-1ß and IL-18, thereby inducing a Th17 or Th1 response, respectively. IL-1ß production is only induced by hyphae, while yeast cells or *C. albicans* mutants locked in the yeast form are unable to trigger activation of the inflammasome and thus the secretion of IL-1ß [111, 268]. Upon recognition of any morphological form of the fungus, three different intracellular signalling pathways get activated: the three main MAPK pathways p38, JNK and ERK1/2, the phosphatidylinositide-3-kinase (PI3K) pathway and the nuclear factor-kappa-enhancer of B cell function (NF-κB) pathway [226, 270–272]. The host defence mechanism of epithelial cells is triggered by a variety of danger signals, including the yeast-to-hypha transition and an increase in fungal burden. Moyes and colleagues were able to distinguish two distinct phases of signal pathway activation in response to *C. albicans*. The first phase is induced independent of the fungal morphology. The activation of NF-κB and PI3K signalling along with an initial, transient activation of the three MAPK pathways p38, JNK and ERK1/2 will lead to the activation of the transcription factor c-Jun via JNK and ERK1/2. This first phase displays an early response and can be observed as early as 5 min post-infection. If no

further signal is received, however, the signalling fades and disappears within 1h. In the presence of hyphae and in case the fungal burden exceeds a specific threshold, a second, stronger activation of the MAPK pathways is triggered, resulting in a p38-driven activation of the transcription factor c-Fos and ERK1/2-mediated phosphorylation of the MAPK phosphatase MKP1. c-Fos activation will lead to an up-regulated production of cytokines, chemokines and other inflammatory mediators, while the phosphatase MKP1 serves as a negative feedback regulator by deactivating both JNK and p38, thus inhibiting further MAPK signalling. Neither yeast nor hyphal cells below a certain threshold do trigger this second phase of MAPK signalling, allowing the immune system to remain quiescent and protecting against an immune overreaction [97].

1.5 Pore forming proteins

Pore-forming proteins (PFPs) are soluble proteins that generate pores inside membranes [273]. These are found in organisms from all kingdoms, including non-pathogenic bacteria, but even vertebrates [274]. In vertebrates, PFPs are utilised to kill bacteria (e.g. the complement membrane attack complex, MAC), to kill infected and malignant cells (e.g. perforin) or to permeabilise mitochondria, resulting in the triggering of apoptosis (e.g. members of the Bak family) [274]. PFPs are, however, also common in human pathogens, the best characterised being those of bacterial origin. A major class of PFPs are indeed pore-forming toxins (PFTs) of proteinaceous origin, which present the largest class of bacterial toxins [275]. The advantages of such protein toxins are obvious, as they may help in direct killing of the host cell or other bacteria, escaping from phagosomal immune cells and gaining access to intracellularly stored nutrients [276]. Depending on the structure of the peptide and the type of pore formed, PFTs can be divided into two different groups. While α-PFTs are generally rich in α-helices and pore-formation is induced via insertion of the α-helical domains into a membrane, ß-PFTs contain mainly ß-sheets and create pores by building ß-barrels inside the membrane [273, 277, 278]. In both cases, these proteins are generally produced in a soluble monomeric form that can assemble into oligomeric complexes [275]. Therefore, the toxin needs to undergo a conformational change that generates hydrophobic patches, as this is a prerequisite for allowing insertion into a lipid bilayer [274]. The process of oligomerisation is promoted by an increased toxin concentration and thus a higher chance of monomer-monomer-interaction on the host cell surface, due to the presence of specific PFT receptors [274]. Many of the receptors that have been identified so far are either lipid raft components or are associated with such rafts, although there are also non-lipid receptors, e.g. the GPI-anchored protein CD59, the integrin CD11b/CD18, certain claudins, and the metalloprotease ADAM10 [279–283]. ß-PFTs also strongly interact with sugar moieties protruding from the target

membrane, such as glycans that are covalently attached to membrane-associated proteins [275]. The general mechanism of pore formation is depicted in Figure 4.

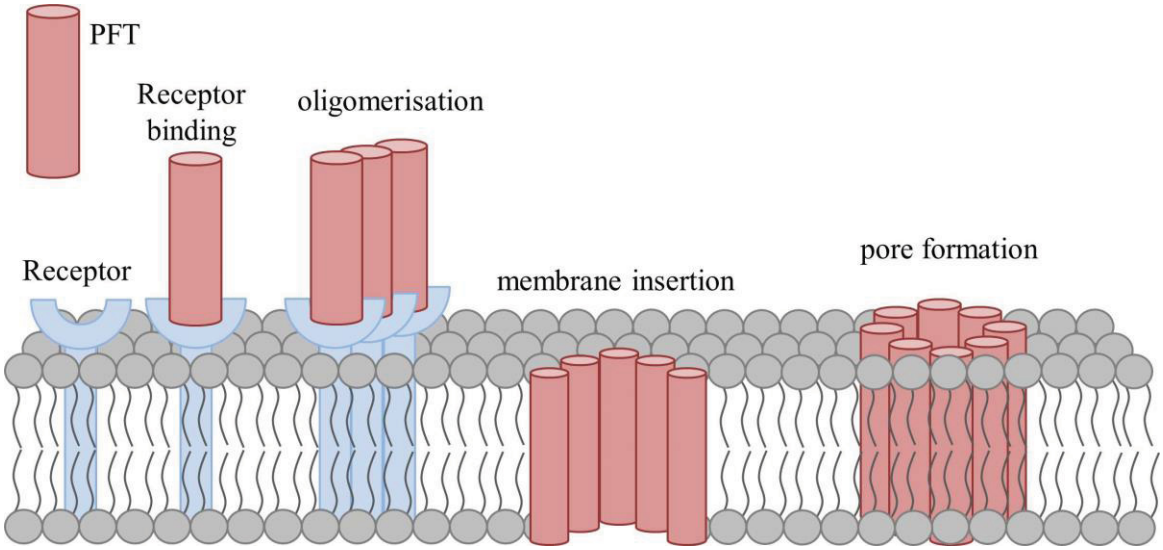


Figure 4: Mechanism of pore formation. Soluble pore-forming toxins (PFTs) bind to receptors and/or lipid components of the membrane. The concentration of PFTs on the cell surface will induce oligomerisation of the toxin monomers, resulting in membrane insertion and pore formation.

Different models exist to describe how peptide monomers insert into a membrane and eventually form a pore (see
Figure 5). One of these models is the so-called barrel-stave model, which has been originally established for the peptide alamecithin [284]. In this model, it is hypothesised that the hydrophilic faces of the monomers come together to form a hydrophilic pore. The more monomers aggregate, the larger the pore will become in size. The toroidal model on the other hand is based on the assumption that the hydrophilic parts of the peptides remain in contact with the hydrophilic head groups of the membrane lipids [285]. Aggregation of several peptides will therefore result in a bending of the associated lipids, leading to the formation of a pore that is lined by both peptides and head groups of the lipids [286]. Finally, the carpet model predicts a rather loose arrangement of peptides on the surface of the lipid bilayer, which after reaching a sufficient concentration will eventually result in a disruption of the bilayer in a detergent-like manner, due to the amphipathic nature of the peptides [287–289]. The type of pore formed predicted by the carpet model is in accordance to the toroidal model.

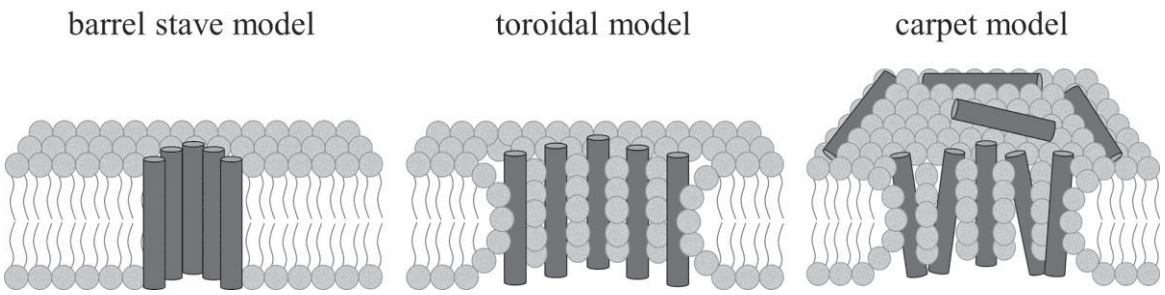


Figure 5: Models of pore formation. Different models have been proposed to explain membrane permeabilisation. In the barrel stave model, the hydrophilic peptide regions form the inside of the pore, while the hydrophobic regions interact with the lipid core of the membrane bilayer. In the toroidal model, bending of the lipid bilayer is induced, resulting in a pore lined by both the peptides and the polar lipid head groups. In the carpet model, peptides accumulate on the membrane surface, eventually disrupting the bilayer in a detergent-like manner.

The formation of a proper pore inside the membrane is followed by changes of the ion concentration inside the host cell's cytosol, which is mostly characterised by a massive influx of calcium into the cell, as well as an efflux of potassium ions and ATP [274, 280]. As a potent second messenger, calcium can activate various signalling cascades, e.g. the release of even more calcium from intracellular stores and the activation of proteolytic cascades [274]. The strong decrease of the intracellular potassium level on the other hand causes the host cell to enter a quiescence-like state, during which activation of autophagy, the formation of lipid droplets and a reduced anabolic activity can be observed [290, 291]. Furthermore, PFTs trigger the activation of phosphorylation cascades and activate all three MAP kinases, i.e. p38, JNK and ERK [290, 292]. Various toxins have also been reported to activate the inflammasome, resulting in the downstream activation of caspase-1 and cleavage of IL-1ß [275]. The decreased levels of both potassium and ATP finally result in a deregulation of mitochondrial activity and death of the cell [293, 294].
Despite the ubiquitous existence of PFTs among human pathogens, no PFTs have been described for human fungal pathogens until now.

1.6 Ece1 (Extent of cell elongation 1)

The *C. albicans* protein Ece1 is one of the most abundantly expressed proteins during hypha formation, being up-regulated to an at least 22-fold level in a *C. albicans* wild type strain when grown under hypha-inducing conditions [173] (Figure 6 B). Furthermore, expression is notably rapid, with the first transcripts being detectable as early as 30 min after stimulation of hyphal growth [295]. Although the first characterisation of this protein dates back to 1993, the function of Ece1 so far remained unknown [295]. Birse *et al*. were able to show that *ECE1* expression is

associated with cell morphology, but that the gene product is not necessary for hypha formation *per se*, as an *ECE1* null mutant was still capable of forming regular hyphae [295]. Despite its unknown function inside the cell, *ECE1* is regularly used as a marker for hyphal growth, due to the striking correlation between hypha formation and expression and the high expression levels that can be observed.

Ece1 is a unique protein of 271 amino acids (28.9 kDa), with the primary structure showing an unusual composition. The protein consists of a signal peptide and eight tandem repeats of a degenerate 34-amino acid sequence, which are separated by lysine-arginine (KR) residues [295] (Figure 6 A). Bader *et al*. could show, that these KR motifs present cleavage sites for the proprotein-converting protease Kex2, which is localised in the late trans-Golgi network [296]. Processing by Kex2 usually hints to an integration of the protein into the plasma membrane or cell wall, or to a subsequent secretion. Complete cleavage of Ece1 by Kex2 was hypothesised to result in a signal peptide and eight other peptides, with seven of these peptides ending in KR [296] (Figure 6 C). However, it could be shown by liquid chromatography/mass spectrometry (LC/MS) performed in our laboratory at a later time point during the study, that the final peptides secreted by the fungus under hypha-inducing conditions slightly vary from the proposed peptides. This may be due to further processing by different proteases after the initial cleavage by Kex2. The fact that a *kex1*Δ/Δ mutant does indeed secrete peptides of the proposed amino acid sequence, does assign this intracellular protease a role in further processing.

In silico analysis showed that the polypeptide precursor structure of Ece1 resembles that of the repellent protein Rep1 of the pathogenic plant fungus *Ustilago maydis*, the causative organism for corn smut disease [297]. *Um*Rep1 consists of twelve tandem repeats of a 37-amino acid sequence and is known to be processed by *Um*Kex2, resulting in small peptides which are integrated into the cell wall [297].

Under laboratory conditions, expression of *ECE1* is dependent on the transcription factors *EFG1* and *BCR1* [298–300]. The dependency on the major regulator for biofilm formation, *BCR1*, led to the hypothesis that Ece1 may play a role in biofilm formation [299]. Nobile *et al*. were able to show that while Ece1 is not required for biofilm formation *in vitro*, overexpression of the Bcr1 target *ECE1* does partially rescue biofilm formation in a *bcr1*Δ/Δ mutant, ascribing Ece1 a possible role in adhesion.

A screen of > 120 mutants, performed in the laboratory of Dr Julian Naglik at King's College London (UK), identified the *ece1*Δ/Δ mutant as the only mutant that despite being capable of forming normal hyphae was unable to induce the epithelial danger response pathways, characterised by the activation of MAPK-p38/c-Fos [301]. *ECE1* therefore may represent a candidate gene for the epithelial mechanism of discrimination between commensal and pathogenic *C. albicans* cells.

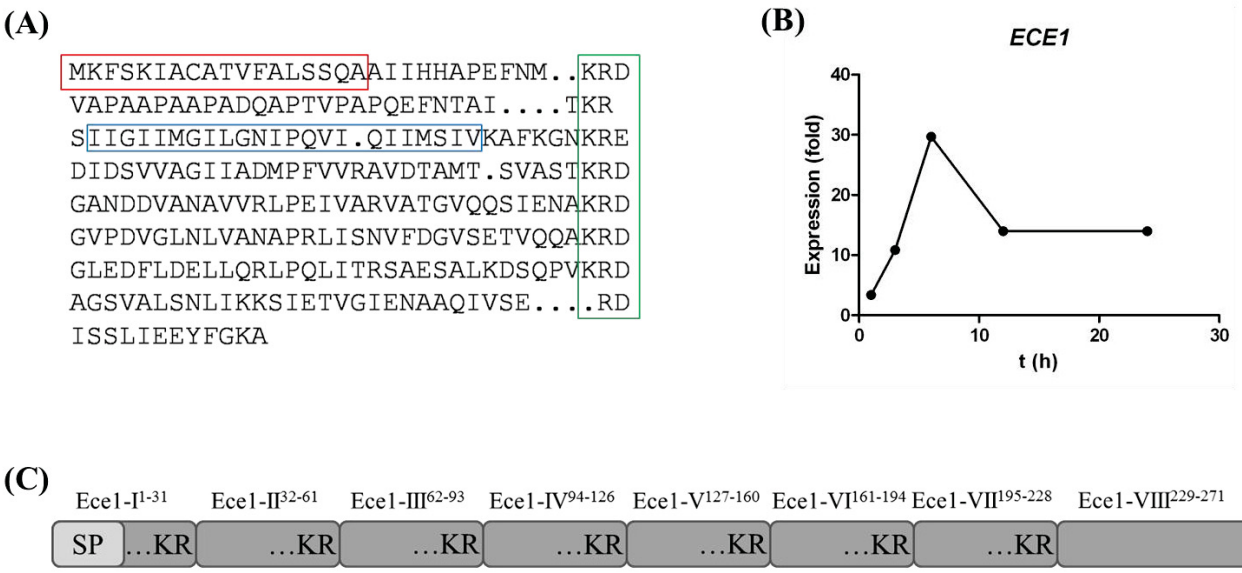


Figure 6: Properties of the hypha-associated protein Ece1. (A) Ece1 consists of 271 amino acids, containing a signal peptide (red box), a hypothesised transmembrane region (blue box) and eight tandem repeats separated by lysine-argine (KR)-motifs (green box). Modified from Birse *et al.* 1993 [295]. (B) Expression of *ECE1* is highly up-regulated during infection of epithelial cells. Expression levels increase up to 30-fold within a few hours and remain up-regulated during the course of infection. Data generated by K. Zakikhany (2007). (C) Complete cleavage of Ece1 is proposed to result in a signal peptide and eight short peptides ranging in size from 1.5 – 4.5 kDa.

1.7 Aims of this study

Given the major health and economic problems associated with *C. albicans* infections, especially regarding the ever rising number of immunocompromised patients in succession to modern medical therapies, the identification of possible virulence factors as well as the establishment of effective antifungal treatments is of utmost importance.

The strong correlation between Ece1 expression and hypha formation and the identification of this protein as a possible activator of epithelial damage response pathways, hints at Ece1 being a virulence factor involved in epithelial damage. Thus, the aim of this study was the elucidation of the so far unknown function of this hypha-associated protein.

In silico analyses, using BLAST and the protein structure prediction programme PHYRE^2, were performed to identify potential homologies to other known protein sequences, thereby gaining further insight into a probable function of Ece1.

Using a set of existing *ece1*Δ/Δ mutants, the role of the Ece1 protein during virulent behaviours, such as adhesion to, invasion into and damage of host epithelial cells was analysed. Considering the fact that Ece1 is most probably processed by the protease Kex2 in the late trans-Golgi network

and secreted in the form of short peptides, recombinantly expressed Ece1 digested with recombinant Kex2, as well as synthetically produced peptides corresponding to the proposed peptide sequences have been tested for their damage potential on different epithelial lineages. Furthermore, mycological researchers have long been aware of *C. albicans* producing a haemolytic factor. The exact identity of this haemolytic factor, however, remained unknown so far. Hypothesising that Ece1 may be involved in the lysis of erythrocytes, a theory which is supported by the finding that *ECE1* expression is induced in the presence of haemoglobin, the *ece1*Δ/Δ mutant strains as well as the Ece1-derived peptides were also examined for their ability to lyse red blood cells.

Based on the findings of the above experiments, a possible mode of action for Ece1 as a virulence factor was to be determined. This included the identification of a possible interaction site on the surface of the human host cell.

With Ece1 seemingly being important for *C. albicans*-induced host cell damage, the protein represents a promising target for the development of new anti-candidal therapeutics. Therefore, antibodies targeting either the full length Ece1 protein or a single peptide within the protein sequence have been produced and tested for their ability to abolish Ece1-induced cell damage on red blood cells.

2 Material and Methods

2.1 Materials

2.1.1 Strains

Candida albicans, *Candida glabrata*, *Saccharomyces cerevisiae*, *Pichia pastoris* and *Escherichia coli* strains used in this study are listed in Table 1.

Table 1: Yeast and bacterial strains used in the study.

Strain	Genotype	Reference
C. albicans		
SC5314	isogenic wild type	[302]
BWP17/CIp30	isogenic wild type	[303]
*ece1*Δ/Δ	*ura3*::*λimm434/ura3*::*λimm434* *arg4*::*hisG/arg4*::*hisG his1*::*hisG/his1*::*hisG* *ece1*::*HIS1*/*ece1*::*ARG4* *RPS10/rps10*::CIp10-*URA3*	[301]
*ece1*Δ/*ECE1*	*ura3*::*λimm434/ura3*::*λimm434* *arg4*::*hisG/arg4*::*hisG his1*::*hisG/his1*::*hisG* *ece1*::*HIS1/ece1*::*ARG4* *RPS10/rps10*::CIp10-*URA3-ECE1*	[301]
*ece1*Δ/*ECE1*[ΔECE1-III$^{62\text{-}93}$] clones1-3, 3-1, 3-3	*ura3*::*λimm434/ura3*::*λimm434* *arg4*::*hisG/arg4*::*hisG his1*::*hisG/his1*::*hisG* *ece1*::*HIS1/ece1*::*ARG4* *RPS10/rps10*::CIp10-*URA3-ECE1*$^{\Delta 184\text{-}279}$	[301]
*ece1*Δ/*ECE1*[ΔECE1-VII$^{195\text{-}228}$] clones K3, K6, K9, K12	*ura3*::*λimm434/ura3*::*λimm434* *arg4*::*hisG/arg4*::*hisG his1*::*hisG/his1*::*hisG* *ece1*::*HIS1/ece1*::*ARG4* *RPS10/rps10*::CIp10-*URA3-ECE1*$^{\Delta 583\text{-}684}$	A. Franke
*kex2*Δ/Δ	Δkex2::hisG / Δkex2::hisG	[304]
*eed1*Δ/Δ	Δipf946::HIS1/ Δipf946::ARG4+CIp10	[127]
*cph1*Δ/*efg1*Δ	Δcph1::hisG / cph1::hisG Δefg1::hisG / Δefg1::hisG-URA3-hisG	[305]

C. glabrata		
ATCC2001	clinical isolate	[306, 307]
S. cerevisiae		
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	[308]
P. pastoris		
ScKex2	Expression of ScKex2	[309]
E. coli		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	[310]
BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	[311]

2.1.2 Cell lines

The cell line TR-146 was used as an *in vitro* model to study the interaction between *C. albicans* and oral epithelial cells. This cell line originally derived from a squamous cell carcinoma of buccal epithelial cells and was obtained from Cancer Research Technology, London. The cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS. After thawing, the cells were maintained in a humidified incubator at 37°C in presence of 5% CO_2, routinely split after confluency was reached, and used in experiments for 15 passages.

The vaginal epithelial cell line A-431 was commercially obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and used as an *in vitro* model of vaginal *C. albicans* infection. The A-431 cell line is derived from an epidermoid carcinoma of an 85-year old female patient. This cell line was cultured in RPMI 1640 medium with the addition of 10% heat-inactivated FBS. In accordance with the cultivation of the TR-146 cells, the vaginal cell line was also maintained at 37°C in 5% CO_2 and used for 15 passages after initial thawing.

The Caco-2 gastrointestinal epithelial cell line originally derived from a human colorectal adenocarcinoma of a 72-year old male patient. These cells were maintained in DMEM culture

medium supplemented with 10% heat-inactivated FBS and non-essential amino acids. Cultivation was also carried out at 37°C in a humidified incubator in 5% CO_2.

For experiments carried out in 24-well plates (e.g. adhesion and invasion assays), $1x10^5$ cells of the respective cell line were seeded on a 12 mm glass coverslip placed inside each well and kept in the incubator for up to 48h until infection with *C. albicans*. For assays carried out in 96-well plates (e.g. damage assays), $2x10^4$ cells of the respective cell line were seeded per well, and the cells were incubated for 24-48h prior to infection.

2.1.3 Peptides

The eight peptides hypothesised to result from complete cleavage of Ece1 at the KR sites (Ece1-I-Ece1-VIII), as well as the peptides that have been verified by LC-MS/MS to be secreted by *C. albicans in vivo*, have been synthesised by the company ProteoGenix (Schiltigheim/France). The peptides were delivered in a lyophilised form and 1.5 mM aliquots of each peptide were prepared in DPBS and stored at -20°C. The synthetic peptides used during the course of this study are specified in Table 2.

Table 2: Amino acid sequences and molecular weights of the synthetic peptides used in this study.

Peptide	Sequence	MW (Da)
Ece1-I$^{19-31}$	AIIHHAPEFNMKR	1564
Ece1-II$^{32-61}$	DVAPAAPAAPADQAPTVPAPQEFNTAITKR	3016
Ece1-III$^{62-93}$	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR	3466
Ece1-IV$^{94-126}$	EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR	3466
Ece1-V$^{127-160}$	DGANDDVANAVVRLPEIVARVATGVQQSIENAKR	3577
Ece1-VI$^{161-194}$	DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR	3580
Ece1-VII$^{195-228}$	DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR	3882
Ece1-VIII$^{229-271}$	DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGKA	4567
Ece1-III$^{62-93R\rightarrow A}$	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKA	3381
Ece1-III$^{62-93KR\rightarrow AA}$	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNAA	3324
Ece1-III$^{62-92}$	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK_	3310
Ece1-III$^{62-92(K\rightarrow A)}$	SIIGIIMGILGNIPQVIQIIMSIVKAFKGNA	3253
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2848

MW = molecular weight in Dalton; underlined amino acids: modified C-termini

2.1.4 Media and chemicals

Unless otherwise stated, all chemicals were obtained from Carl Roth (Karlsruhe), Sigma (Munich), Merck (Darmstadt) or Roche (Mannheim). Cell culture media were purchased from Gibco.

Table 3: Media used in the study.

Medium	Composition	Application
YPD	1% yeast extract, 1% peptone, 2% glucose	*Candida* spp. growth
RPMI 1640	commercially obtained from Gibco	Hyphal induction
LB	1% tryptone, 0.5% yeast extract, 1% NaCl	*E. coli* growth
SOC	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose	*E. coli* transformation
BMG/BMM	100 mM potassium phosphate buffer (pH 6); 1.34% YNB with ammonium sulphate; $4x10^{-5}$% biotin; 1% glycerol (BMG) or 0.5% methanol (BMM)	*P. pastoris* growth

To obtain solid media, all media were supplemented with 2% agar (Roth) or 2% pure agar (Oxoid, defined media).

Table 4: Buffers used in the study.

Buffer	Composition	Application
PBS II	140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH 7.4	all experiments
PBSKMT	1xPBS II, 3 mM KCl, 2.5 mM $MgCl_2$, 0.1% TritonX-100	protein isolation
Binding buffer	20 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole, pH7.5	protein purification
Elution buffer	20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, pH7.5	protein purification
DPBS	commercially obtained from Gibco	blood experiments

HBSS	commercially obtained from PAA	blood experiments
TBS	50 mM Tris-HCl, 150 mM NaCl; pH 7.6	Western Blot, PIP strips
Stacking gel buffer	1 M Tris-HCl; pH 6.8	SDS-PAGE
Separating gel buffer	1 M Tris-HCl; pH 8.8	SDS-PAGE
4xLaemmli	240 mM Tris-HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 28% (v/v) H_2O	SDS-PAGE
Tris glycine buffer	25 mM Tris, 200 mM glycine, 0.1% SDS	SDS-PAGE

Table 5: Chemicals used in the study.

Chemical	Company	Application
Human serum albumin	Sigma	blood experiments
Methyl-ß-cyclodextrin	Carbosynth (UK)	blood experiments
Bovine serum albumin (BSA)	Serva	Western Blot, PIP strips
IPTG	Peqlab	rEce1 expression in *E. coli*

Table 6: Antibodies used in the study.

Antibody	Target	Produced in
Ece1-III polyclonal (Eurogentec)	Ece1-III$^{62-93}$	rabbit
anti-rabbit-HRP (Dako)	rabbit IgG	swine

2.2 Methods

2.2.1 Cultivation and storage of microorganisms

For long term storage, all microorganisms were kept at -80°C in their preferred growth media supplemented with 50% (v/v) glycerol. The glycerol stocks were used to inoculate fresh plates every 2-3 weeks. For short time storage, all yeast strains (*C. albicans*, *C. glabrata*, *S. cerevisiae*, *P. pastoris*) were maintained on YPD plates at 4°C. For use in experiments, cells from a single colony were incubated overnight (~16h) in liquid YPD in a shaking incubator at 30°C (*C. albicans*, *S. cerevisiae*) or 37°C (*C. glabrata*). Cells were harvested by centrifugation and, after counting with the help of a Neubauer counting chamber (Neubauer improved, Roth), diluted to the desired cell concentration.

P. pastoris, used for the overexpression of the recombinant protease Kex2 from *S. cerevisiae*, was precultured in BMG medium overnight. The following day, the preculture was used for inoculation of a larger volume of BMG. After 24h of further incubation, the cells were harvested by centrifugation and transferred into BMM medium to induce expression of ScKex2. All cultures were incubated in a shaking incubator at 30°C, using large flasks to allow adequate aeration of the cultures.

E. coli strains were routinely grown in Luria-Bertani (LB) broth at 37°C. For strains carrying a plasmid-conferred ampicillin resistance, the media was supplemented with 50 µg/ml ampicillin.

2.2.2 Growth kinetics of *C. albicans* strains

The effect of complete deletion of the *ECE1* gene or partial deletion of sequences coding for a single peptide on the growth of the respective strains was analysed by growth assays. Therefore, cells grown overnight were diluted into YPD medium to an optical density OD_{600} = 0.2 and transferred to a 96-well plate. Growth was continuously monitored by measuring the optical density every 30 min in an Infinite M200 plate reader [Tecan, Austria]. Measurements were carried out automatically by use of the following programme: constant incubation at 37°C, interrupted by 30 s shaking intervals (orbital, 6 mm amplitude) and subsequent OD measurement at 600 nm every 30 min. The cells were incubated for a total of 100 kinetic cycles (50h).

The influence of the synthetic Ece1-derived peptides and melittin on the growth of different *C. albicans* mutant strains and further yeast species was determined by the addition of the respective peptides to the growth medium.

2.2.3 Adhesion assay

To determine the ability of different *C. albicans* strains to adhere to epithelial cells, oral TR-146 epithelial cells were grown on 12 mm coverslips and inoculated with $1x10^5$ *C. albicans* cells for 1h in DMEM without FBS. After the incubation period, non-adherent cells were removed via extensive rinsing with PBS, followed by fixation of the cells with 4% paraformaldehyde. The adherent *C. albicans* cells were stained using 10µg/ml Calcofluor White. Next, the coverslips were mounted on glass slides and adherence evaluated via fluorescence microscopy. For this purpose, the number of adherent cells in 100 randomly chosen squares à 200 μm^2 was assessed and the percentage of adherent cells calculated based on the total area of the coverslip and total amount of seeded cells. The adherence of each *C. albicans* strain was tested in four technical replicates and three biological replicates.

2.2.4 Invasion assay

The number of invading *C. albicans* cells was determined based on a protocol from Park *et al* [113]. Oral epithelial TR-146 cells were grown for 2 days on 12 mm glass coverslips in DMEM with 10% FBS. The resulting epithelial monolayers were infected with $5x10^4$ stationary *Candida* cells for either 3h or 6h in DMEM cell culture medium without FBS. After the incubation period, non-adherent *Candida* cells were removed via extensive rinsing with PBS and the cells were fixated using 4% paraformaldehyde. To distinguish invasive from non-invasive parts of the *Candida* hyphal cells by fluorescence microscopy, a differential staining was carried out. For this purpose, the non-invasive parts of the hyphae were stained first by incubating the cells with a primary anti-*Candida* antibody (from rabbit), followed by incubation with a secondary goat anti-rabbit, Alexa488-coupled antibody. This was followed by a permeabilisation step, in which 0.5% Triton X-100 was used to lyse the membranes of the epithelial cells. Both intracellular and extracellular parts of the invasive *Candida* hyphae were subsequently stained with 10 µg/ml Calcofluor White solution. Next, the coverslips were mounted on glass slides and invasiveness evaluated via fluorescence microscopy. The percentage of invasive *Candida* cells was determined by dividing the number of [partly] invasive cells by the total number of adherent cells. At least 100 hyphae were counted for each *C. albicans* strain. In addition to determining the percentage of invasive cells, the lengths of *Candida* hyphae were measured using the Leica Application Software. Invasion and hyphal length after 3h and 6h were tested in quadruple for each strain and in three independent experiments.

2.2.5 Damage assay

Epithelial cell damage caused by different *C. albicans* strains and Ece1-derived peptides during their interaction with epithelial monolayers was measured by assessing the release of lactate dehydrogenase (LDH) into the surrounding medium. Epithelial cells were grown to confluency in a 96 well microtitre plate and infected with $2x10^4$ *C. albicans* cells and/or varying concentrations of Ece1-derived peptides in cell culture medium without FBS. Uninfected epithelial cells in cell culture medium were used to assess the spontaneous LDH-release of the cells during the incubation period. A positive control containing the highest possible LDH content was obtained by lysing the appropriate monolayers using 0.1% Triton X-100 following the incubation period. After 24h at 37°C, LDH activity was determined using the Cytotoxicity Detection Kit from Roche Applied Science according to the manufacturer's instructions. Briefly, a catalyst solution containing diaphorase and NAD^+, and a dye solution containing iodotetrazolium (INT) and sodium lactate were added to the sample supernatants. LDH-catalysed conversion of lactate to pyruvate results in the reduction of NAD^+ to $NADPH/H^+$. H/H^+ from the $NADPH/H^+$ are then transferred to the tetrazolium salt INT, which is catalysed by diaphorase and results in the reduction of the tetrazolium salt to the water-soluble dye formazan. Absorption of this dye can be measured spectrophotometrically at 490 nm. The percentage of lysis of epithelial cells was calculated relatively to the 100% lysis control, while the concentration of released LDH (in ng/ml) was determined using an LDH standard curve ranging from 3.8 ng/ml to 500 ng/ml. All experiments were performed at least in triplicate for each condition and repeated at least three times.

2.2.6 Haemolysis experiments

2.2.6.1 Human erythrocyte preparation

Whole human blood donated by healthy volunteers within the HKI was collected in 9 ml EDTA K3 tubes [Sarstedt], containing 1.6 mg Ethylene Diamine Tetra Acetic Acid (EDTA) to prevent the blood from coagulating.

Depending on the type of experiment, the preparation of erythrocytes was carried out using two different protocols. Blood that was to be tested for lysis by synthetic peptides was first centrifuged at 2000 x g for 5 min at room temperature (RT). Afterwards, the serum was removed and replaced by the same volume of sterile Dulbecco's PBS (DPBS [Gibco]) to wash the remaining serum from the cells. Following a second centrifugation step at 2000 x g, DPBS at a slightly smaller volume than the original volume of serum was added, to obtain the erythrocytes at an as high

concentration as possible. A sample of this blood preparation was analysed in the Auto Hematology Analyzer BC-5300 Vet [Mindray] to obtain the red blood cell count. For use in the experiments, the red blood cell concentration was adjusted to $5x10^8$ cells/ml.
Aliquots of whole blood, which were to be used for peptide lysis experiments and prepared by the described protocol, were stored refrigerated for up to 3 days.
A different type of blood preparation was applied when *C. albicans* strains were tested for their ability to lyse human red blood cells. For this purpose, whole blood was first centrifuged at 2000 x g for 10 min at RT, followed by the removal of the plasma layer as well as the top layer of the red blood cells, in order to eliminate as many white blood cells as possible from the sample. The remaining cell pellet was then resuspended to the starting volume in DPBS containing 2% heat-inactivated FBS [Gibco]. Following another centrifugation step at 2000 x g for 10 min, the supernatant and top layer of red blood cells was again removed and the remaining cells resuspended to starting volume, this time in DPBS without FBS. After a last round of centrifugation (2000 x g, 10 min) and removal of the top layer, the remaining cell pellet was resuspended in clear RPMI 1640 medium [Gibco] to starting volume.
During blood preparation, 500 µl samples of both whole blood ("pre-wash") and washed blood cells ("post-wash") were kept and used for a differential count with the Auto Hematology Analyzer to verify successful diminishment of white blood cells and to determine the red blood cell concentration. For use in the experiments, the red blood cell concentration was adjusted to $5x10^8$ cells/ml.

To investigate the influence of the cholesterol content of the erythrocyte membrane on lysis by Ece1, the membranes were depleted of cholesterol using the compound methyl-ß-cyclodextrin. To obtain cholesterol-depleted erythrocytes, the cells were incubated in DPBS containing 5 mM methyl-ß-cyclodextrin for 30 min at 37°C and under light shaking (70 rpm). Afterwards, the erythrocytes were washed several times with DPBS, and finally diluted to a concentration of $5x10^8$ cells/ml for use in experiments. As a control, a second aliquot of erythrocytes was prepared identically, but incubation was carried out in DPBS without added methyl-ß-cyclodextrin.

For the preparation of human serum to be used in experiments, whole blood was collected in serum syringes [Sarstedt] containing silicate beads coated with a clotting activator. After blood collection, the content of the syringe was transferred into a 50 ml test tube and left undisturbed until clotting of the blood could be observed (approximately 30 min). The tube was then centrifuged at 4000 x g for 10 min in order to remove the clotted blood components. The serum was aliquoted and stored at -20°C until needed.

2.2.6.2 Erythrocyte lysis by synthetic Ece1-peptides

To test the eight synthetic peptides corresponding to the peptides resulting from complete cleavage of the Ece1 protein for their ability to lyse red blood cells, these peptides were incubated in blood prepared after the protocol described above. For a standard experiment, 9 µM of each peptide were mixed with DPBS to a final volume of 130 µl. After the addition of 20 µl red blood cell suspension at a concentration of $5x10^8$ cells/ml, the cells were incubated for 1h at 37°C. Afterwards, the cells were centrifuged at 2000 x g for 3 min and 100 µl of the supernatant were transferred to a 96 well plate. Evaluation was carried out by measuring the absorbance of the released haemoglobin at 541 nm in a microplate reader.
To test the influence of different factors like e.g. pH value, ion concentrations, or the presence of specific substances on the efficiency of peptide lysis, the assay was adapted respectively.

2.2.6.3 Erythrocyte lysis by *C. albicans* strains

To investigate the effect of Ece1 on erythrocyte lysis, different *C. albicans* wild type strains and mutants were incubated in the presence of red blood cells under hypha-inducing conditions. An 8 ml YPD overnight culture of each strain was harvested and after washing and counting, the cells were adjusted to a concentration of $1x10^8$/ml in clear RPMI. 500 µl of this cell suspension were mixed with 100 µl of an erythrocyte suspension at $5x10^8$ cells/ml (preparation see part 2.2.6.1), giving an MOI of 1. The samples were then incubated at 37°C and 50 rpm. After 16h, 24h and 48h, samples were taken out, centrifuged and 100 µl of the supernatant were transferred onto a 96-well plate. The amount of released haemoglobin in each sample was determined by measuring the supernatants spectrophotometrically at 541 nm in a microplate reader.

2.2.7 Lipid Binding Assay

To test the ability of the synthetic peptides Ece1-III$^{62-93}$, Ece1-III$^{62-93R\rightarrow A}$, and Ece1-III$^{62-93KR\rightarrow AA}$ to bind to different phospholipids, PIP Strips™ [Echelon Biosciences, Salt Lake City/USA] were incubated with 10 µg of the respective synthetic peptide in Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) for 16h at 4°C. Afterwards, the strips were incubated with a polyclonal rabbit anti-Ece1-III antibody [Eurogentec, Seraing/Belgium] as primary antibody and an HRP-conjugated anti-rabbit antibody [Dako, Glostrup/Denmark] as secondary antibody. The signals were visualised by chemiluminescence using ECL Western Blotting substrate [Pierce, Rockford/USA].

2.2.8 *E. coli* transformation

In order to recombinantly express a His-tagged Ece1 protein, the plasmid p*ECE1* (see Figure 7) was first transformed into competent cells of the strain *E. coli* DH5α. This strain is characterised by the property to methylate inserted plasmids, thereby protecting the plasmid from degradation by *E. coli* DNAses. Afterwards, the plasmid was isolated and transformed into the desired overexpression strain *E. coli* BL21(DE3). This strain carries the T7 RNA polymerase gene and *lacI*^{q} on the λ prophage DE3 and T7 promoter driven expression on transformed plasmids can be induced with IPTG.

For transformation, approximately 50 ng of plasmid DNA were added to frozen competent cells and the mixture was incubated on ice for 20 min. Cells were then heat-shocked by transfer to 42°C for 60 s, followed by a further 1 min incubation on ice. After the addition of 900 µl of pre-warmed SOC-medium, the cells were incubated for 45 min at 37°C in a shaking incubator. Different dilutions of the transformation mixture were then plated onto selective LB-plates, containing 50 µg/ml ampicillin, and plates were incubated at 37°C overnight.

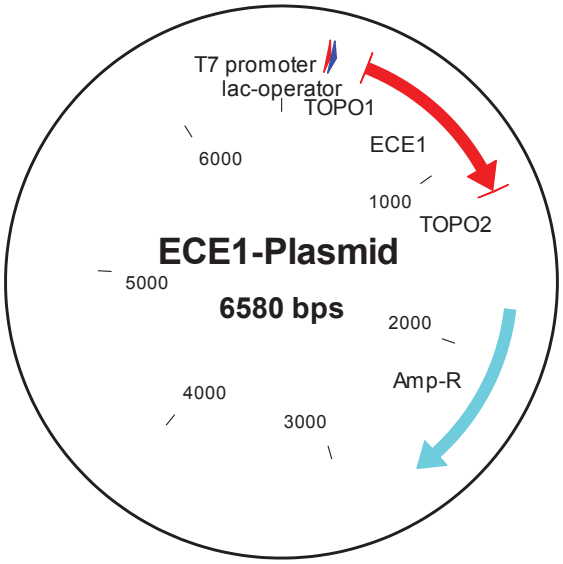


Figure 7: Expression vector used for overexpression of recombinant Ece1 in *E. coli* BL21(DE3). The plasmid consists of the commercial vector pET100/DTOPO (Invitrogen) with the *ECE1* sequence including an N-terminal His-Tag inserted after the T7 promoter. This vector was created by O. Bader (2008).

2.2.9 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using a no-column plasmid preparation protocol. *E. coli* cells were harvested from an overnight LB-culture by centrifugation (13000 rpm, 1 min) and resuspended in 300 µl of resuspension buffer P1 (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8) by vortexing. After the addition of 300 µl of lysis buffer P2 (200 mM NaOH, 1% SDS, pH 12.5), samples were carefully mixed and incubated at RT for 5 min. Finally, 300 µl of neutralisation buffer P3 (3M KOAc, pH 5.5) were added and the samples centrifuged for 5 min at 13000 rpm. The resulting supernatant was transferred into a new test tube and mixed with half the volume of isopropanol in order to precipitate the plasmid DNA. Following another centrifugation step (10 min at 13000 rpm), the supernatant was discarded and the pellet washed with 500 µl of cold 70% ethanol. After air-drying, the pellet was dissolved in 30 µl ddH_2O and stored at -20°C.

2.2.10 Overexpression of recombinant Ece1 (rEce1)

For overexpression of rEce1, 100 ml of LB medium containing 50 µg/ml ampicillin were inoculated with 1 ml of an overnight culture of *E. coli* BL21(DE3) carrying the *ECE1*-plasmid. The culture was incubated at 37°C and 180 rpm and after an OD_{600} of 0.4 was reached, protein expression was induced by the addition of IPTG to a final concentration in the culture of 1 mM. After incubation for a further 4h, cells were harvested by centrifugation and stored at -80°C.

2.2.11 Protein isolation from *E. coli*

To isolate *E. coli* proteins, frozen cell pellets were thawed on ice and resuspended in two pellet volumes of cold PBSKMT buffer containing a protease inhibitor cocktail [Roche]. After the addition of one pellet volume glass beads, cells were lysed for 2x30 s at 5500 rpm with a 30 s break in a Precellys 24 homogeniser [Peqlab], followed by a centrifugation step at 13,000 rpm for 10 min at 4°C. The protein-containing supernatant was transferred into a fresh 1.5 ml eppendorf reaction tube.

2.2.12 Purification of recombinant Ece1

The His-tagged rEce1 protein was purified by metal chelate affinity chromatography using High Density Zinc Agarose His-Columns [Jena Bioscience]. After initial equilibration of the column with binding buffer, protein-containing *E. coli* cell lysate was applied and kept on the column for at least 15 min to facilitate protein binding and yield. The column was then washed several times with binding buffer and the desired protein eluted by application of elution buffer.

2.2.13 Overexpression of recombinant *S. cerevisiae* Kex2 (ScKex2)

ScKex2 was overexpressed using a *P. pastoris* strain. *P. pastoris* is a methylotrophic yeast species and therefore capable of metabolising methanol. The oxidation of methanol to formaldehyde is the first step in the metabolism of methanol and is catalysed by the enzyme alcohol oxidase (*AOX*). Due to a poor O_2 affinity, large amounts of this enzyme are produced, making the *AOX* promoter an ideal promoter for the overexpression of recombinant proteins. The cells of the overexpression strain were first grown in Buffered Minimal Glycerol (BMG) medium for two days, before protein expression was induced by transferring the cells into Buffered Minimal Methanol (BMM) medium. The *P. pastoris* strain was constructed to secrete heterologous proteins into the medium, and therefore ScKex2 was harvested by centrifugation and disposal of the cell pellet.

2.2.14 Purification of recombinant ScKex2

As mentioned above, ScKex2 was secreted into the medium during overexpression. Due to very low levels of endogenous proteins being secreted by *P. pastoris*, a secreted heterologous protein represents the majority of the total protein concentration in the medium. Using Amicon Ultra concentrator columns [Merck Millipore] with a molecular-weight cut-off of 30 kDa, possible endogenous proteins were removed and the spent medium changed against PBS buffer.

2.2.15 Determination of protein concentration

Protein concentrations inside cell lysates and purified samples were determined using a Protein Assay Kit [Pierce]. A BSA standard, ranging from 0 (blank) to 2 mg/ml was prepared following the manufacturer's instructions. 25 µl of each standard concentration and each sample were pipetted in a 96-well plate and mixed with 200 µl of a 50:1 dilution of solutions A and B. After

30 min of incubation at 37°C, absorbance was measured at 562 nm using a microplate reader. The kit is based on the Biuret reaction and Cu^{2+} present in solution B is getting reduced to Cu^{+} by the proteins present in the sample. The subsequent chelation of one cuprous ion by two molecules of bicinchoninic acid (BCA), contained in solution A, will lead to the formation of a purple-coloured reaction product which exhibits a strong absorption at 562 nm.

2.2.16 ScKex2-mediated digestion of rEce1

Recombinant ScKex2 obtained from overexpression in *P. pastoris* was utilised for *in vitro* digestion of recombinantly expressed Ece1 to verify processing of Ece1 by this protease. For digestion experiments, 100 µg of rEce1 were mixed with 2 µg of ScKex2. On account of the Kex2-activity being dependent on calcium, $CaCl_2$ was added to a final concentration of 10 mM in the test reaction. Incubation of the digestion samples was carried out at 37°C for either 1 min, 5 min, 10 min or 20 min. Afterwards, samples were directly put on ice and a small volume of each sample was used for analysis via SDS gel electrophoresis. The remaining volume was stored at -80°C until needed for experiments.

2.2.17 Erythrocyte lysis by rEce1 digestion products

Digestion products obtained from ScKex2-mediated digestion of rEce1 were tested for their lytic properties via utilisation in an erythrocyte lysis assay. Considering that digestion might have been incomplete due to a reduced efficiency, digestion products were added to the erythrocytes in a final concentration of 0.3 mg/ml (10 µM), 0.6 mg/ml (20 µM) and 1.2 mg/ml (40 µM), corresponding to a concentration of fully processed Ece1-III of 10 µM, assuming that digestion efficiency was 100%, 50%, or 25%, respectively. The samples were incubated for 1h at 37°C and haemoglobin release from erythrocytes was determined by a spectrophotometrical measurement at 541 nm.

2.2.18 SDS-PAGE

Proteins were analysed by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein gels containing 12% acrylamide were prepared according to Laemmli and run in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell [Bio-Rad]. Prior to electrophoresis, the protein samples were mixed with 1x Laemmli buffer (+ ß-mercaptoethanol) and denatured at 95°C for 5 min. In addition to the samples, 5 µl of Page Ruler™ Prestained

Protein ladder [Fermentas] were applied to every gel as a molecular weight marker. The gels were run in Tris-glycine buffer for ~2h at 120V. Following separation of the proteins via SDS-PAGE, the gels were either stained with Coomassie or used for subsequent Western Blotting.

2.2.19 Coomassie staining

After a quick rinse in ddH_2O, SDS gels were transferred into Coomassie Brilliant Blue R-250 staining solution [Bio-Rad] for 2h. Background staining was removed by thorough washing in a destaining solution containing H_2O, methanol and acetic acid in a ratio of 50:40:10 (v/v/v).

2.2.20 Western Blot

Using a PerfectBlue semi-dry blot system [Peqlab], proteins were transferred from SDS gels onto nitrocellulose membranes [Millipore]. After blotting of the proteins, the membrane was blocked by incubation with 5% milk powder in TBST for 1h at RT. Afterwards the membrane was probed with a primary antibody in 2% milk powder in TBST, either overnight at 4°C or for 2h at RT. Following three washes with TBST, a secondary horseradish-peroxidase-conjugated antibody (swine anti-rabbit-IgG-HRP; 1:1700; [Dako/Denmark]) in 2% milk powder in TBST was added and incubated for 2h at RT. Detection of the signals was performed using ECL Western blotting substrate [Pierce/USA] according to the manufacturer's instructions.

2.2.21 Induction of hyphal growth

To induce the switch from growth as a budding yeast to the filamentous hyphal growth form in a liquid culture, RPMI medium was inoculated with $5x10^5$ *C. albicans* cells per ml and incubated for 16-24h at 37°C in a shaking incubator. For the induction of hyphal growth on plastic, $5x10^4$ *C. albicans* cells per well were seeded into a 12-well plate and incubated for up to 16h at 37°C in a humidified incubator.

2.2.22 Statistical analyses

Mean values and standard deviations were calculated from repeated independent experiments using the programme GraphPad Prism 5. For calculation of significance, the one-way Anova test was used and *p*-values < 0.05 were considered as significant differences. If not otherwise mentioned, at least three independent experiments were performed.

2.2.23 Databases and *in silico* analyses

Candida gene and protein sequences were obtained from the *Candida* Genome Database (CGD). This database was also used for sequence comparisons and homology analyses using the BLAST function. For the prediction of 3D peptide structures, the PHYRE^2 Protein Fold Recognition Server was used. Sequence alignments were performed with the ESPript 3 Multiple Sequence Alignment tool. Molecular weights of peptides and proteins were calculated using the ExPASy Compute pI/Mw tool. For the prediction of transmembrane regions within the Ece1 protein, the HMMTOP prediction programme was used, while information about the physiochemical properties of peptides was obtained from PepCalc.com.

3 Results

3.1 *In silico* analyses

Although the hypha-associated gene *ECE1* had been identified in 1993, the function of the protein remained unknown until today. The strong up-regulation during hypha formation, however, suggests that this protein may play an important role in the infection process. To gain further insight into the function of Ece1, *in silico* analyses were performed to learn more about evolutionary conservation within the *Candida* genus, to obtain a possible 3D structure of the protein and to find similar proteins present in other species.

3.1.1 *ECE1* conservation

The search for orthologous genes in other *Candida* species using the *Candida* Genome Database showed that orthologues to Ece1 of *C. albicans* SC5314 can be found in the *C. albicans* strain *WO-1*, as well as in *C. dubliniensis* (Cd36_43260) and in *C. tropicalis* (CTRG_00476) (Figure 8), two other members of the CTG-clade of *Candida* species. These species translate the codon CTG as serine instead of leucine under laboratory growth conditions and most of the pathogenic *Candida* species belong to this group. Therefore, Ece1 orthologues can only be found among the pathogenic species of the *Candida* genus.

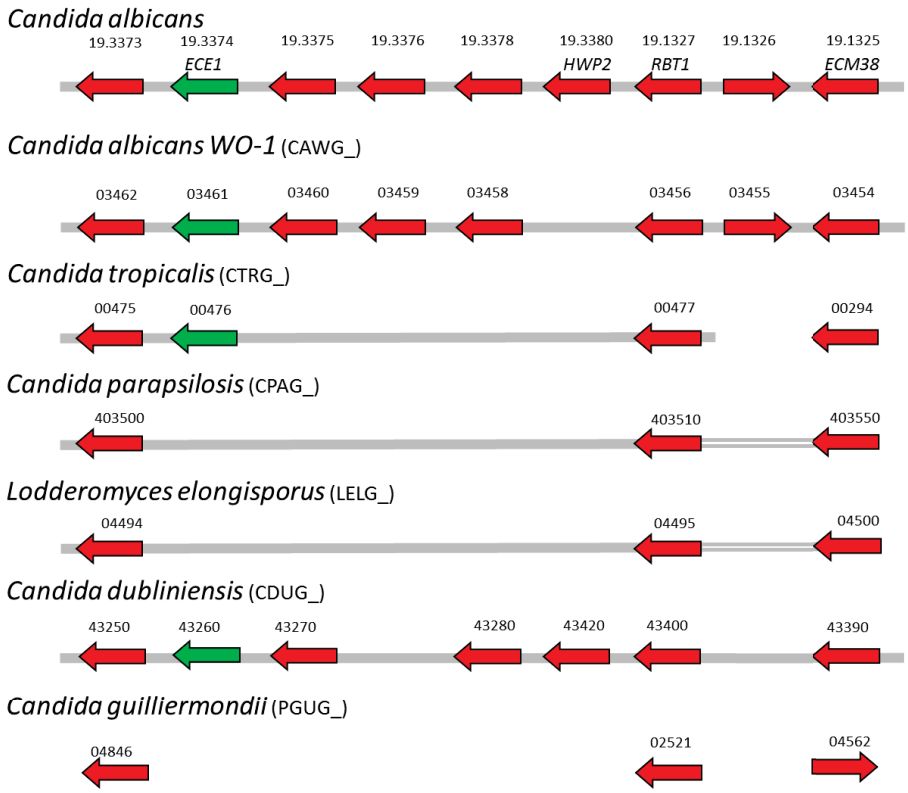
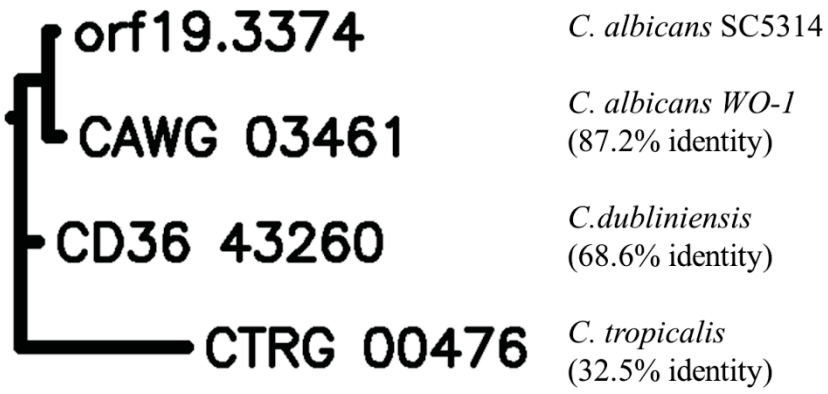


Figure 8: Orthologues of *C. albicans ECE1* within the CTG clade. Apart from *C. albicans*, orthologues for *ECE1* can only be found in *C. dubliniensis* and *C. tropicalis*.

3.1.1.1 Similarity between Ece1 orthologues from different *Candida* species

Alignment of the Ece1 orthologues from different species showed that the identity between *C. albicans* Ece1 (orf 19.3374) and the *C. dubliniensis* orthologue (CD36_43260) is 68.6%, while the *C. tropicalis* orthologue CTRG_00476 still only shows an identity of 32.5% to the *C. albicans* gene (Figure 9 A, B). These differences are also reflected in the length of the amino acid sequences of the proteins. While the Ece1 protein of *C. albicans* SC5314 is composed of 271 amino acids, the amino acid sequence of the Ece1 orthologue from *C. dubliniensis* (268 amino acids) is almost identical in length, while a greater variation does exist in the lengths of the *C. albicans* and *C. tropicalis* (282 amino acids) orthologues. Interestingly, the lysine-arginine motifs, which have been shown to play a role in Kex2-mediated processing of the *C. albicans* protein, seem to be highly conserved within all four different orthologues (see Figure 9 B).

(A)



(B)

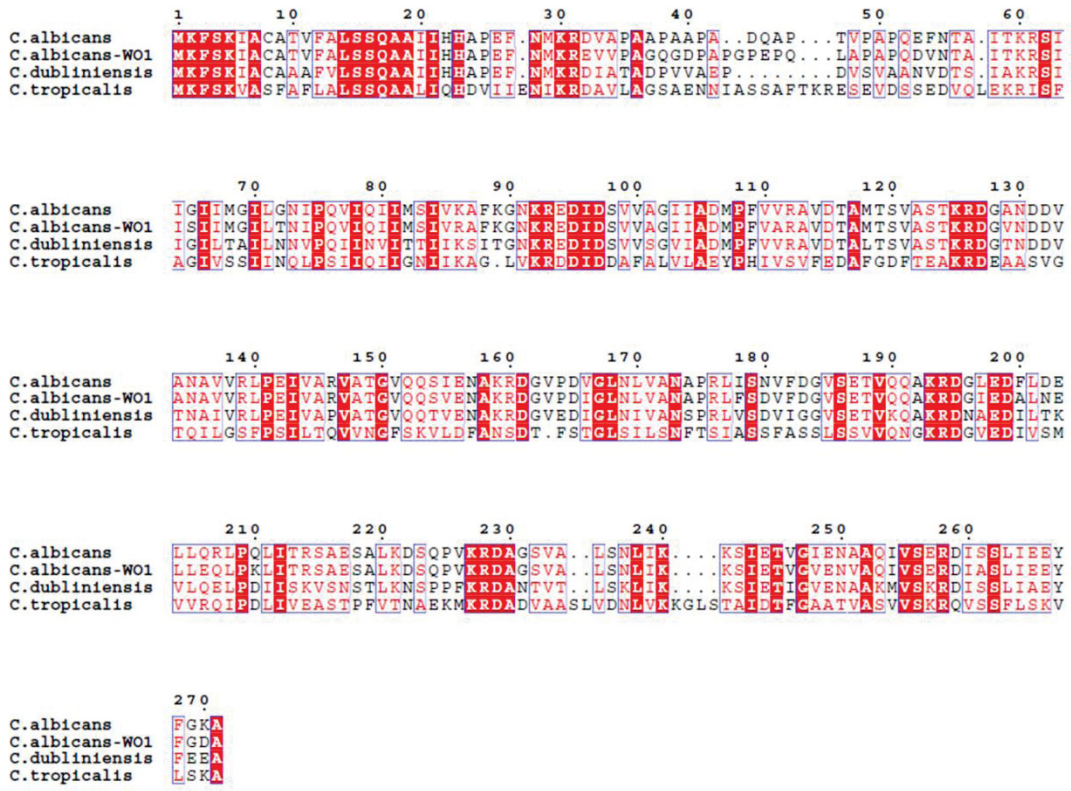


Figure 9: Phylogenetic relationship and identity between the different Ece1 orthologues. (A) Identity between Ece1 orthologues from different *Candida* species. (B) Alignment of protein sequences of Ece1 orthologues.

3.1.2 Predicted structure of Ece1

Using the HMMTOP server for the prediction of transmembrane helices, it was found that only a short part of the amino acid sequence is predicted to adopt an α-helical conformation. According to the *in silico* data, the helix is composed of the amino acids 63 to 85 (Figure 10 A). This is in accordance to the predicted transmembrane domain for this protein and noticeably correlates to peptide Ece1-III$^{62-93}$, comprising the amino acids 62 to 93. Analysing peptide Ece1-III$^{62-93}$ for a predicted 3D structure using the PHYRE^2 protein fold recognition server, supported this prediction, although confidence in the model is low (24.8%) (Figure 10 B). While the corresponding peptide from *C. dubliniensis* is also predicted to form a transmembrane helix, no α-helical structure is formed by the matching peptide from *C. tropicalis*.

(A)

```
seq  MKFSKIACAT VFALSSQAAI IHHAPEFNMK RDVAPAAPAA PADQAPTVPA    50
pred OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOOOO OOOOOOOooo

seq  PQEFNTAITK RSIIGIIMGI LGNIPQVIQI IMSIVKAFKG NKREDIDSVV   100
pred oooooooooo ooHHHHHHHH HHHHHHHHHH HHHHHiiiii iiiiiiiiii

seq  AGIIADMPFV VRAVDTAMTS VASTKRDGAN DDVANAVVRL PEIVARVATG   150
pred IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

seq  VQQSIENAKR DGVPDVGLNL VANAPRLISN VFDGVSETVQ QAKRDGLEDF   200
pred IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

seq  LDELLQRLPQ LITRSAESAL KDSQPVKRDA GSVALSNLIK KSIETVGIEN   250
pred IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

seq  AAQIVSERDI SSLIEEYFGK A  271
pred IIIIIIIIII IIIIIIIIII I
```

(B)

Figure 10: *In silico* prediction for the structure of Ece1. (A) Only a small part of the protein (amino acids 63-85) is predicted to be localised in the membrane. (O/o: extracellular localisation, I/i: intracellular localisation, H: part of α-helix) (B) The predicted 3D-structure of peptide Ece1-III$^{62-93}$.

3.1.3 Similarity between Ece1-III and the peptide melittin

Due to the striking structure of peptide Ece1-III$^{62-93}$ in comparison to the other seven peptides, this peptide was analysed in more detail. It was found that this peptide is structurally similar to the peptide melittin, which is the active component of the venom of the European Honey Bee (*Apis mellifera*) and represents more than 50% of the venom's dry weight [312]. Both peptides are of approximately the same length (26 amino acids for melittin versus 32 amino acids for Ece1-III$^{62-93}$) and are of amphipathic nature. They possess a hydrophilic N- and C-terminus, while the middle parts of both peptides are highly hydrophobic, resulting in a poor solubility in water.

These hydrophobic peptide regions are predicted to insert into a membrane as an α-helix (Figure 11, A, B). Melittin is a well-known pore-forming toxin and has been studied in great detail. This peptide effectively binds to erythrocytes and induces the release of haemoglobin.

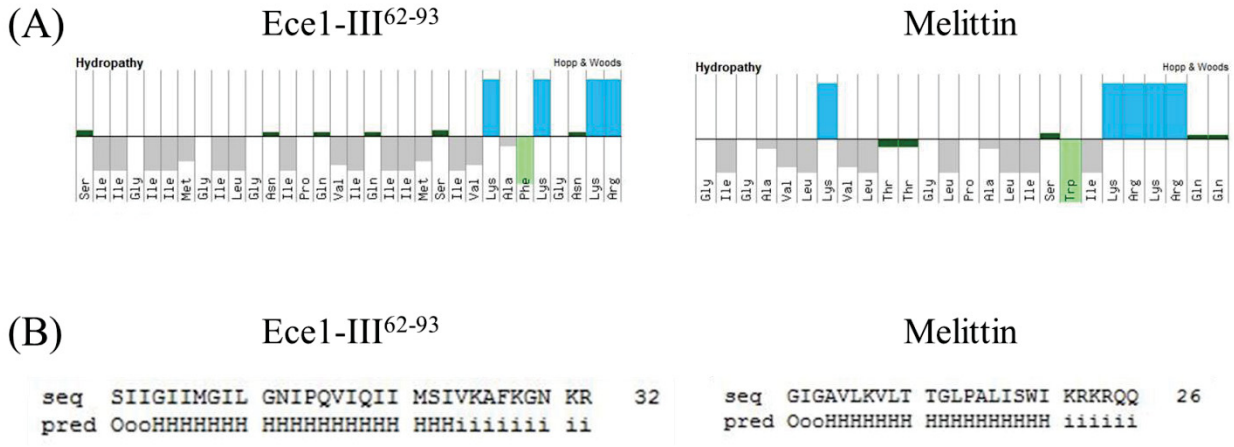


Figure 11: Structural similarities between melittin from bee venom and the *C. albicans* peptide Ece1-III$^{62-93}$. (A) Both peptides contain a hydrophilic N- and C-terminus, while the middle part consists mainly of hydrophobic amino acids. Top: hydrophilic, bottom: hydrophobic. Light green: aromatic, cyan blue: basic, grey: aliphatic, dark green: polar (B) The hydrophobic parts of the peptides are predicted to form a transmembrane α-helix (H), while the N- and C-termini are predicted to be localised extracellularly (O/o) and intracellularly (i), respectively.

3.2 Properties of the *ece1*Δ/Δ mutant versus the wild type

In order to establish an infection, it is important for *C. albicans* to be able to adhere to and invade into the host cells. Both processes are highly dependent on hypha formation, due to the expression of adhesins, invasins and hydrolytic enzymes associated with this morphological form. A mutant being defective in any of these processes could hint to the deleted gene being involved in the exertion of any of these virulence traits. Therefore, the *ece1*Δ/Δ mutant was tested in comparison to its parental wild type for its ability to form hyphae and to adhere to and invade into epithelial cells.

3.2.1 Impact of *ECE1* deletion on *C. albicans* growth

To initially characterise the *ece1*Δ/Δ mutant, growth was assessed by incubating the mutant as well as two wild type strains and the revertant strain in YPD medium for 50h. The resulting growth curve demonstrated that no differences in growth exist between the mutant and parental strain. All tested strains exhibited a similar growth trend, with a short lag phase followed by a

rapid log phase and all strains reached their growth plateau at the same time with an OD_{600} of approximately 1.2 (Figure 12).

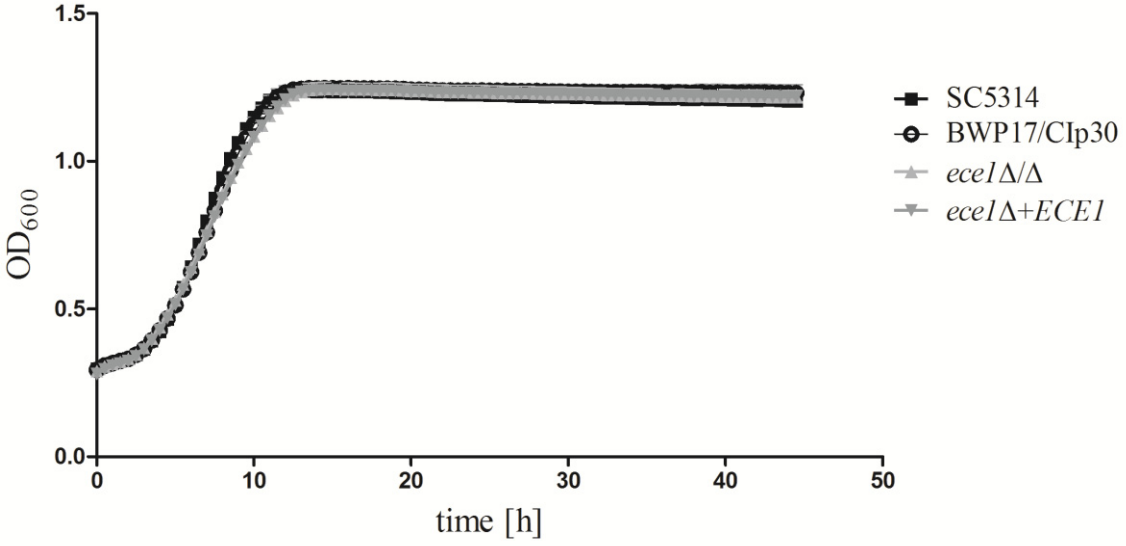


Figure 12: Impact of *ECE1* deletion on *C. albicans* growth. No differences in growth can be observed between the *ece1*Δ/Δ mutant, revertant strain (*ece1*Δ+*ECE1*) and the parental wild type BWP17/CIp30.

3.2.2 Hypha formation of the *ece1*Δ/Δ

The ability of the *ece1*Δ/Δ mutant to form proper hyphae was tested either by the incubation under hypha inducing conditions on plastic and in RPMI medium at 37°C, or via the infection of epithelial monolayers for 6h and subsequent differential staining and fluorescence microscopy.
When grown on plastic, hypha formation of the parental wild type BWP17/CIp30, *ece1*Δ/Δ and the revertant strain *ece1*Δ+*ECE1* were examined after 3h, 5h and 24h with an inverse microscope. At all time points, the hyphal growth that could be observed was similar between the three strains (Figure 13A). The mutant also formed hyphae similar to the wild type during an infection of oral epithelial cells. Differential staining performed after the incubation period also illustrated, that the mutant is capable of invading the host cells (Figure 13B).

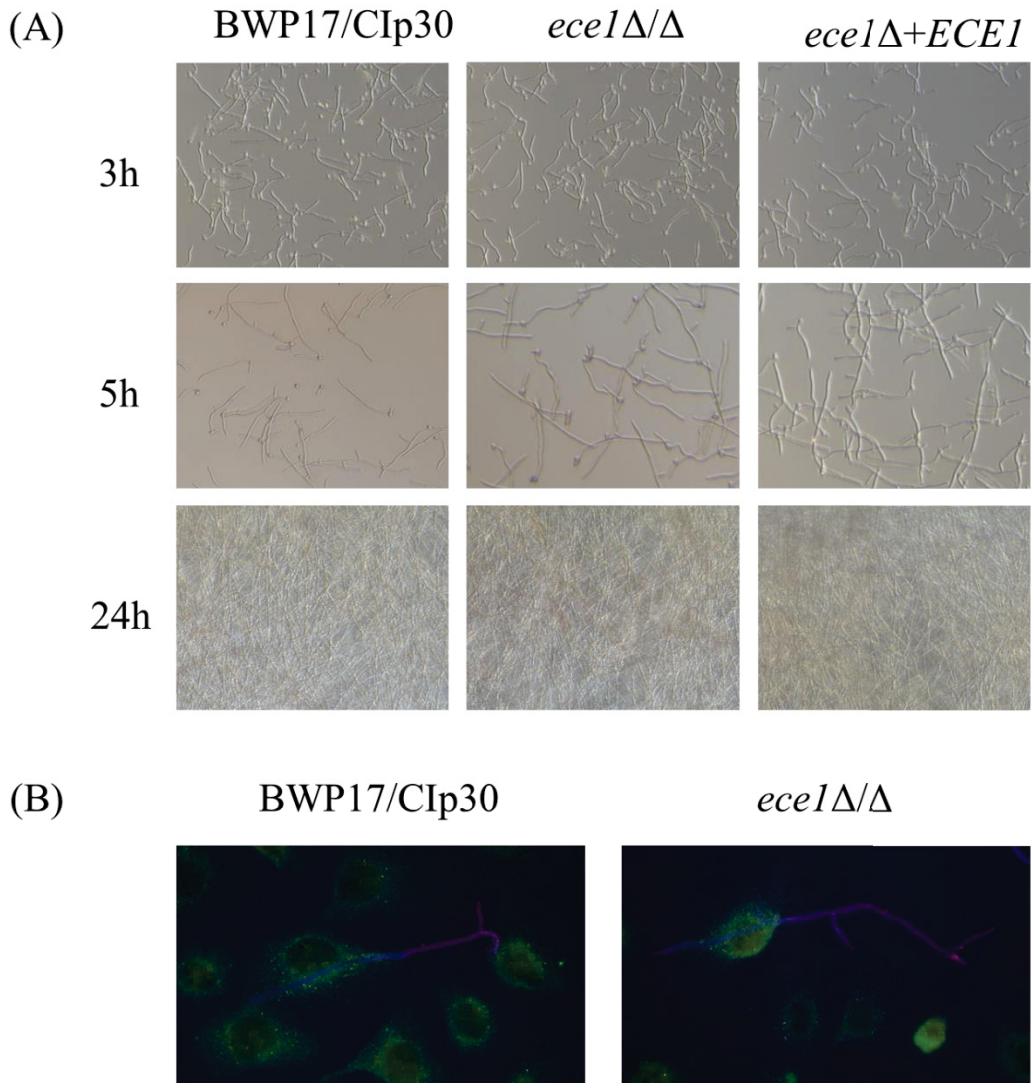


Figure 13: Hyphal growth of the *ece1*Δ/Δ in comparison to the parental wild type BWP17/CIp30 on plastic and during infection of an epithelial monolayer. (A) Cells were grown under hypha-inducing conditions in RPMI medium and hyphal growth assessed after 3h, 5h and 24h. (B) When infecting a monolayer, *ece1*Δ/Δ mutant cells formed hyphae to the same extent as the parental wild type and invasion into epithelial cells could be observed. (Blue: invasive parts of the hyphae; purple: extracellular parts of the hyphae; green: epithelial cells)

3.2.3 Adhesion and invasion properties of the *ece1*Δ/Δ mutant

To examine the adhesion properties of the *ece1*Δ/Δ strain, the mutant as well as the parental wild type BWP17/CIp30 and the revertant strain *ece1*Δ+*ECE1* were co-incubated with the oral epithelial cell line TR-146 for 1h. After the incubation period, the adhesion rates were calculated using fluorescence microscopy. Although it was found that adhesion rates of the mutant and revertant strain were slightly lower compared to that of the wild type, the differences were not

significant. Approximately 23% of all wild type cells adhered to the epithelial cells, compared to 20% and 17% of *ece1*Δ/Δ and *ece1*Δ+*ECE1* cells, respectively (Figure 14A).

The ability of the same strains to invade oral epithelial cells was determined by co-incubation with TR-146 cells for 3h and 6h. In addition to the determination of invasion rates, the lengths of the invasive hyphae were measured and compared. After 3h of co-incubation, 61% of all wild type cells had invaded. Again, both, the mutant and the revertant strain behaved similarly, exhibiting invasion rates of 64% (*ece1*Δ/Δ) and 58% (*ece1*Δ+*ECE1*). Furthermore, no differences in the lengths of the invasive hyphae could be detected. The average lengths of the hyphae ranged between 26-28 µm for all strains. After an incubation period of 6h on TR-146 epithelial cells, an invasion rate of 92% could be observed for the wild type, while 90% of *ece1*Δ/Δ cells and 87% of *ece1*Δ+*ECE1* cells were invasive. The average lengths of the hyphae of invasive wild type and mutant hyphae were almost identical (89 µm for wild type versus 90 µm for *ece1*Δ/Δ), while the hyphae of the revertant strain were slightly, but not significantly shorter (80 µm). Therefore, *ECE1* is dispensable for the formation of proper hyphae, adhesion to and invasion into host cells. The invasion rates and hyphal lengths of all strains are depicted in Figure 14 B and C.

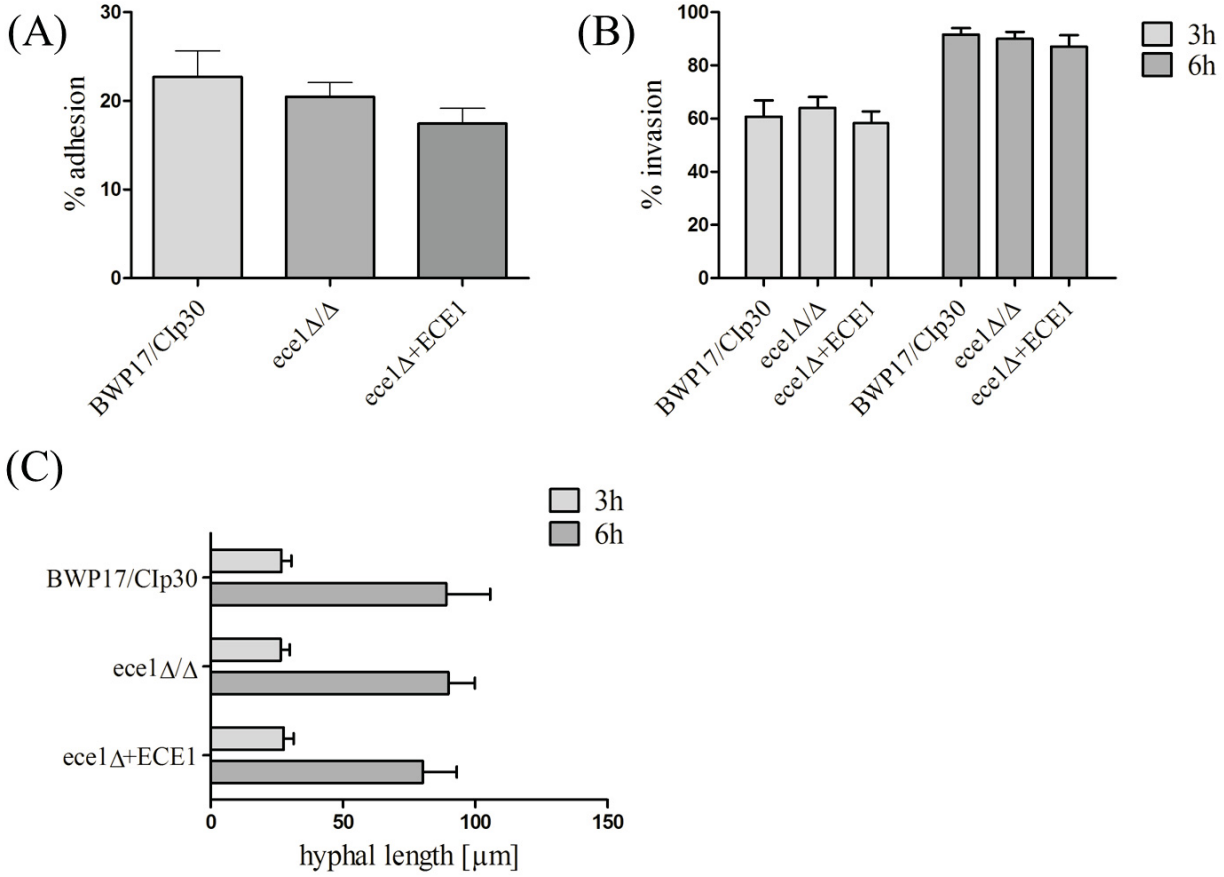


Figure 14: Adhesion, invasion and filament formation of BWP17/CIp30, *ece1*Δ/Δ and *ece1*Δ+*ECE1* on oral epithelial cells. (A) Adhesion of *C. albicans* to TR-146 epithelial cells after 1h of co-incubation. (B) Invasion of *C. albicans* into TR-146 epithelial cells after 3h and 6h. (C) Lengths of invasive hyphae during co-incubation with TR-146 epithelial cells after 3h and 6h.

3.2.4 Epithelial damage caused by *C. albicans* on different cell lines

Data produced in Dr Julian Naglik's laboratory had demonstrated that the *ece1*Δ/Δ mutant does not activate the epithelial damage response pathway [301]. Given this finding, and the structural properties of Ece1, a possible role for Ece1 in epithelial damage was assessed. A cytotoxicity assay was performed, in which different epithelial cell lines (oral, vaginal and gastrointestinal) were co-incubated with *C. albicans* cells for 24h, followed by the measurement of released lactate dehydrogenase (LDH) as a marker for cell damage. It was found that on all three epithelial cell lines, the *ece1*Δ/Δ strain did not cause damage and in all cases damage was significantly reduced in comparison to the wild type and revertant strain (Figure 15).

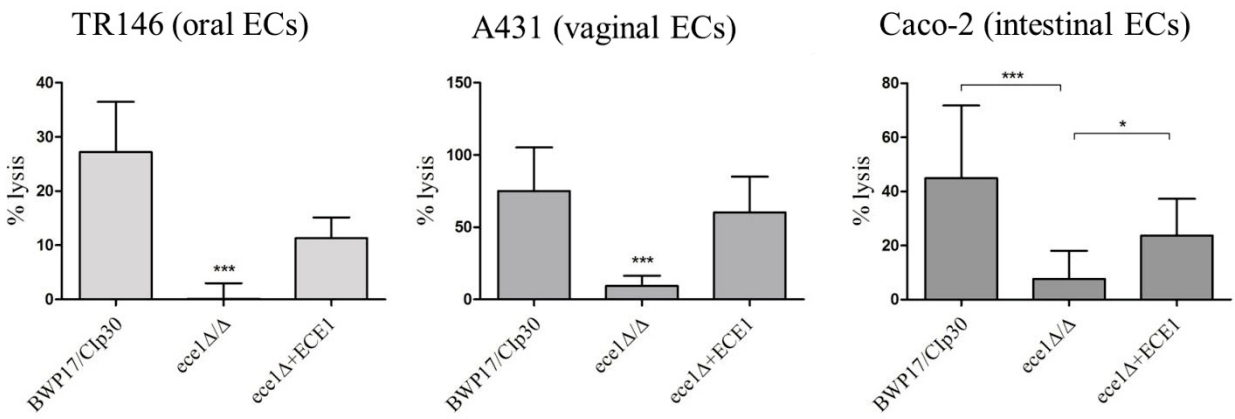


Figure 15: Epithelial damage caused by *C. albicans* strains on different epithelial cell lines. The *ece1*Δ/Δ mutant shows a highly reduced cytotoxicity level on all tested epithelial cell types and damage is significantly reduced in comparison to the wild type and revertant strain.

3.3 Synthetic peptides

As the full length Ece1 protein is predicted to be processed into eight short peptides, which may each exert a different function, these peptides were produced synthetically to test their effect on different cell types, such as epithelial cells and erythrocytes. Data obtained from co-workers and other collaboration partners during the course of the study showed that the peptide profile secreted *in vivo* may vary slightly from the hypothesised peptides. In particular, it was found that Ece1-III$^{62-93}$ was likely to be secreted *in vivo* without the C-terminal arginine. Therefore, two versions of this peptide (Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$) were tested simultaneously in most experiments.

3.3.1 Effect of synthetic peptides on epithelial damage

To assess the effect that the single peptides, predicted to be present after cleavage by the protease Kex2, exert on epithelial cells, all peptides were incubated individually with oral epithelial cells for 24h at a concentration of 10 µM. The result of this experiment showed that the epithelial cells were strongly damaged by the peptide Ece1-III$^{62-93}$ with an approximate lysis rate of 32% of the epithelial cells after 24h of co-incubation, while none of the other peptides caused any damage. Interestingly, when in addition to 10 µM of peptide the epithelial cells were infected with *C. albicans*, an obvious increase of damage could be observed compared to infection with the peptides or *Candida* strains alone. When epithelial cells were infected with a combination of synthetic peptide and the *C. albicans* wild type strain BWP17/CIp30, damage was measurable for all of the eight peptides. However, damage only significantly increased, compared to BWP17/CIp30 alone, for the peptides Ece1-III, Ece1-IV, Ece1-V and Ece1-VIII. In combination with wild type *Candida* cells, the damage observed to be caused by these peptides was higher than lysis caused by the *C. albicans* wild type alone. Remarkably, the highest increase of damage potential occurred in the combination of peptide Ece1-VIII with the wild type. While the peptide alone does not cause damage, a combinatorial infection with *C. albicans* resulted in an even higher lysis than that observed for the highly lytic peptide Ece1-III in combination with *C. albicans*. When infecting epithelial cells with a combination of 10 µM peptide and the *ece1*Δ/Δ strain, an increase of damage in comparison to the peptide alone could only be observed for peptides Ece1-III, Ece1-IV and Ece1-VIII. While none of these increases was significant, the relative increase of damage caused by *ece1*Δ/Δ in combination with peptide Ece1-VIII is greater than that caused by the mutant strain in combination with peptide Ece1-III. Nevertheless, it is noticeable, that the *ece1*Δ/Δ mutant, which alone does not cause any damage on epithelial cells, showed a lysis rate of 40% in combination with peptide Ece1-III, which is slightly higher than observed for peptide Ece1-III alone (Figure 16). Due to the striking cytolytic properties of Ece1-III$^{62-93}$, all further experiments were mainly focussed on this peptide.

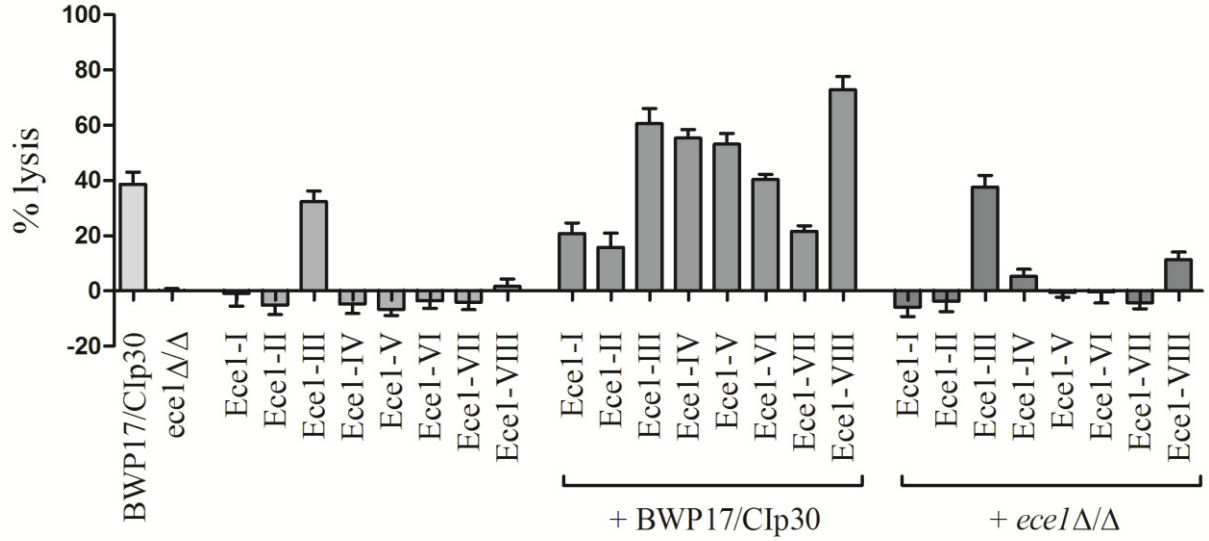


Figure 16: Epithelial damage caused by synthetic Ece1-peptides alone or in combination with *C. albicans* cells. Of all eight peptides, only Ece1-III causes damage to epithelial cells. When added in combination with the wild type BWP17/CIp30, an increase of damage can be observed for all peptides, while combination with the *ece1*Δ/Δ mutant results in an increased damage of only peptides Ece1-III, Ece1-IV and Ece1-VIII.

Lysis by peptide Ece1-III$^{62-93}$ could also be observed to occur rather rapidly in comparison to damage by *C. albicans* hyphae. Significant damage of various epithelial cell lines could already be detected after only 3h of co-incubation of the epithelial cells with the peptide. As no further damage could be detected after co-incubation with oral and vaginal epithelial cells for 6h, the cytotoxicity reached after 3h or possibly even reached earlier corresponds to the highest possible damage potential of Ece1-III$^{62-93}$ for these cell lines. Damage exerted by Ece1-III$^{62-93}$ on Caco-2 cells after 3h on the other hand was noticeably lower compared to the other epithelial cell lines and a further increase in damage could be observed after 6h (Figure 17). Epithelial damage caused by the *C. albicans* cells was undetectable at 3h post-infection, and only rose above background levels at 6h post-infection for BWP17/CIp30-infected Caco-2 cells and *ece1*Δ+*ECE1*-infected A-431 epithelia.

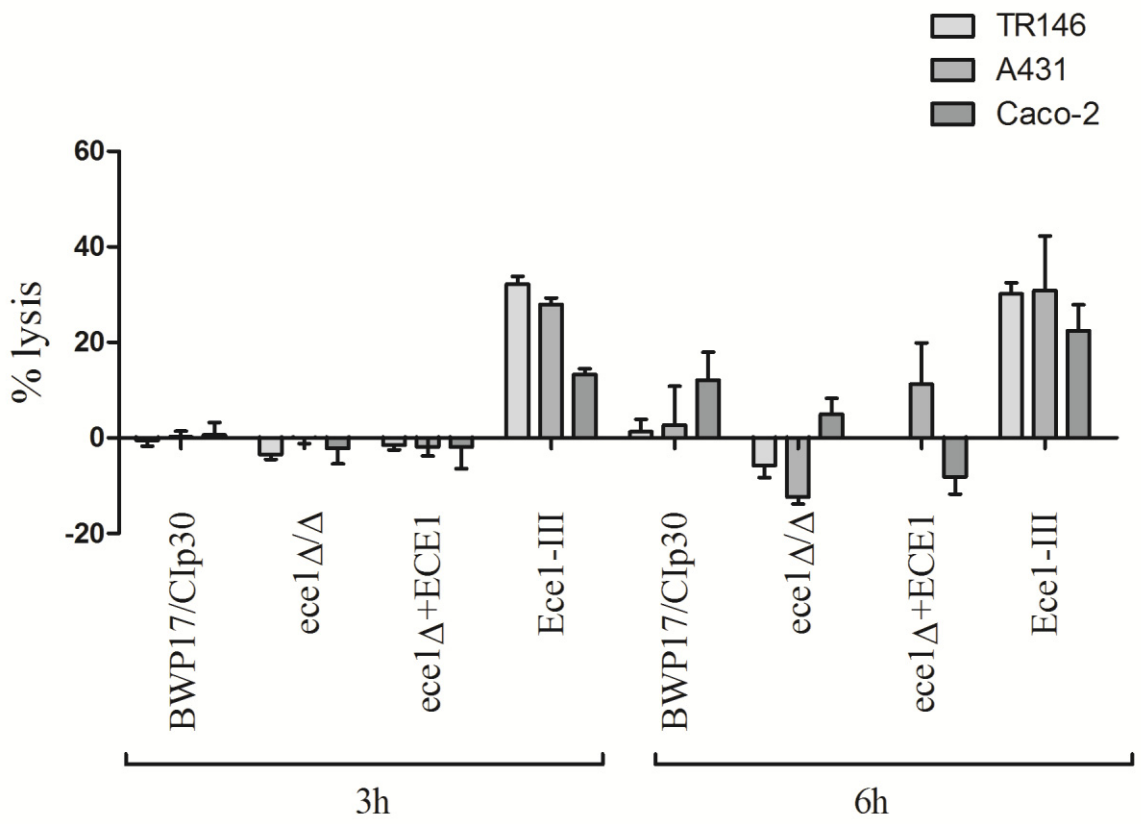


Figure 17: Damage of different epithelial cell lines after 3h and 6h. Peptide Ece1-III$^{62-93}$ rapidly induces lysis of epithelial cells, with 15-30% (depending on cell line) of the host cells being damaged after only 3h. In contrast, initial damage by *C. albicans* strains can first be observed after 6h.

By infecting epithelial cells with different concentrations (5-20 µM) of peptide Ece1-III$^{62-93}$ and peptide Ece1-III lacking the C-terminal arginine (Ece1-III$^{62-92}$), it could be shown that the damaging effect of both peptides is concentration dependent. While at a concentration of 5 µM Ece1-III$^{62-93}$ causes 18% of the epithelial cells to lyse after 24h of co-incubation, damage significantly increases at higher peptide concentrations, reaching the highest observed lysis rate of epithelial cells of 51% when added in a concentration of 20 µM. Peptide Ece1-III$^{62-92}$ elicited a less lytic effect on epithelial cells than Ece1-III$^{62-93}$. While only little damage (~ 6%) can be observed at a peptide concentration of 5 µM, the cytotoxicity continuously rises with an increasing concentration of added peptide. However, a maximum lysis rate of only 24% can be observed after the addition of 20 µM of peptide Ece1-III$^{62-92}$. As a means of comparison and due to the structural similarity to peptide Ece1-III, epithelial cells were also exposed to the same concentrations of the potent cytolytic toxin melittin. The obtained results demonstrated that melittin exerts a much stronger lytic effect on the epithelial cells than Ece1-III$^{62-93}$. When present

in a concentration of at least 10 µM, 100% of the epithelial cells could be observed to be lysed by this peptide (Figure 18).

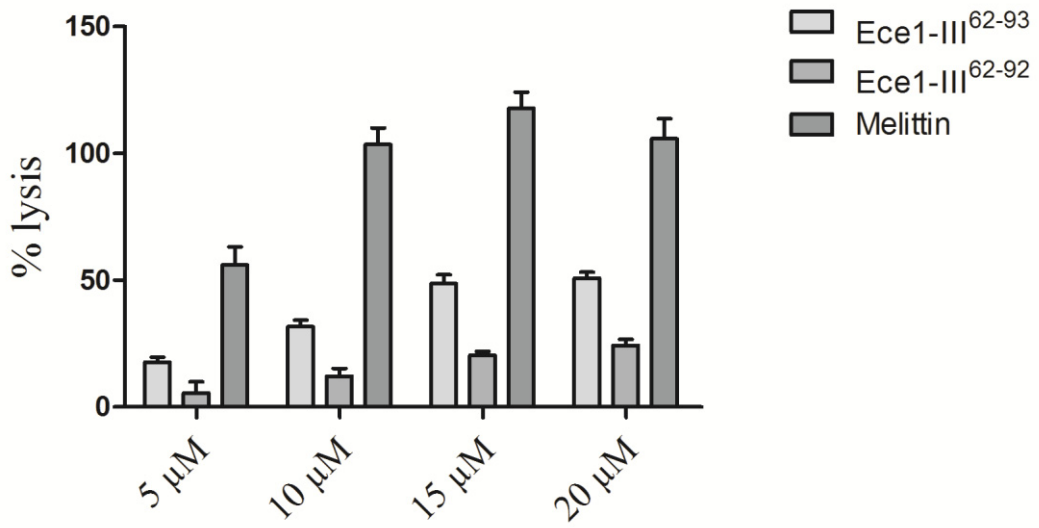


Figure 18: Concentration-dependency of Ece1-III-induced epithelial lysis. Co-incubation of epithelial cells with different concentrations of Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ demonstrated that lysis by these peptides is concentration-dependent. Melittin, used as a positive control peptide of known toxicity, exerted a very high lysis even at low concentrations.

As already shown in Figure 16, a combinatorial infection of epithelial cells with synthetic Ece1-III$^{62-93}$ together with viable *C. albicans* cells, results in an increased damage potential compared to an infection with either peptide or strain alone. Interestingly, when comparing the effects of the strains BWP17/CIp30, *ece1*Δ/Δ and *ece1*Δ+*ECE1* on the damage potential of Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$, it was found that in combination with the strains BWP17/CIp30 and *ece1*Δ+*ECE1*, peptide Ece1-III$^{62-92}$ resulted in a higher damage of epithelial cells, even though this peptide alone has a less lytic effect than Ece1-III$^{62-93}$. This effect could not be observed when Ece1-III$^{62-92}$ was added to the epithelial cells in combination with the *ece1*Δ/Δ mutant strain. While damage in comparison to the peptide alone measurably increased (24% versus 16%), it was significantly lower than damage caused by the combination of peptide Ece1-III$^{62-93}$ with the *ece1*Δ/Δ strain (41%) (Figure 19).

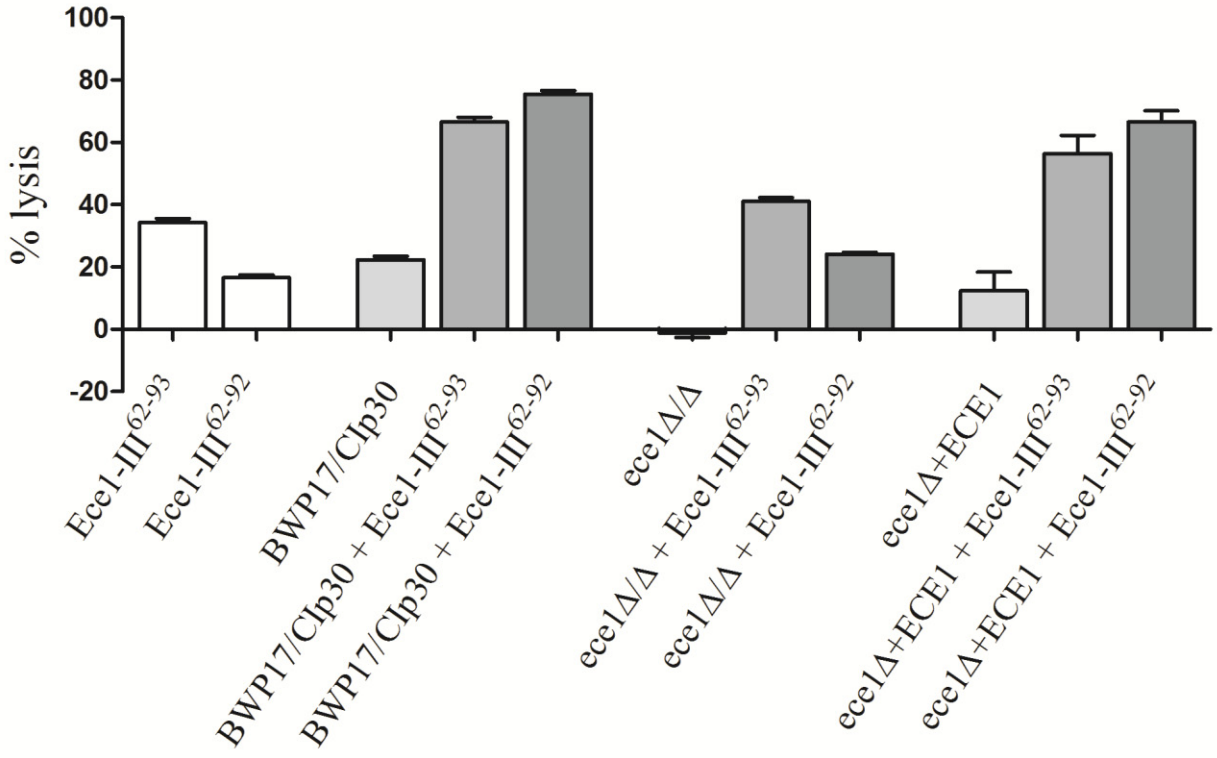


Figure 19: Combinatorial infection of epithelial cells with different versions of Ece1-III. The damage caused by both Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ alone is increased when epithelial cells are infected in combination with *C. albicans* strains. While damage by Ece1-III$^{62-92}$ alone is lower compared to Ece1-III$^{62-93}$, combination with either BWP17/CIp30 or *ece1*Δ+*ECE1* results in a higher lysis rate than the respective combinations with Ece1-III$^{62-93}$.

3.3.1.1 Importance of hypha formation for the cytotoxicity of synthetic peptides

To investigate the role that hypha formation may play in enhancing the cytolytic effect of the synthetic peptides alone, oral epithelial cells were incubated with a combination of peptides and non-hypha-forming yeast species or *C. albicans* mutant strains. The budding yeasts *C. glabrata* (strain ATCC2001) and *S. cerevisiae* (strain BY4741) were chosen for this experiment, as well as the two *C. albicans* mutant strains *eed1*Δ/Δ and *cph1*Δ/*efg1*Δ. The protein Eed1 is involved in filamentation and epithelial escape and while a strain lacking the *EED1* gene is capable of initially inducing hyphal growth, it is unable to maintain this growth form and eventually switches back to yeast cell growth. The *eed1*Δ/Δ mutant, therefore, is able to invade epithelial cells, but will revert back to yeast growth inside the host cell. The *cph1*Δ/*efg1*Δ mutant on the other hand is completely yeast-locked, as the lack of the two important hypha-inducing transcription factors Cph1 and Efg1 precludes the formation of hyphae. In addition to these viable yeast strains and

mutants, heat-killed yeast cells of the *C. albicans* wild type BWP17/CIp30 and the *C. glabrata* and *S. cerevisiae* wild types ATCC2001 and BY4741 were tested for their ability to increase the damage potential of the synthetic peptides. Similar to the experiment described in part 3.3.1, all strains were incubated in combination with 10 µM of peptide Ece1-III$^{62\text{-}93}$ or Ece1-III$^{62\text{-}92}$ for 24h at 37°C.

It was found that all tested non-hypha forming strains showed a significantly higher damage on epithelial cells when added in combination with either of the synthetic peptides. However, the observed damage was never higher than that of the equivalent peptide concentration alone, ascribing this increase in cytotoxicity to the peptide alone. The only exception, however, was the *C. glabrata* strain ATCC2001. While this strain alone causes practically no damage on oral epithelial cells, a combinatorial infection with the *C. glabrata* strain and synthetic peptide resulted in a highly increased damage potential and the observed damage was considerably higher than the sum of the damage potentials of the strain and the peptide alone. This effect could be noted with either peptide Ece1 III$^{62\ 93}$ or Ece1-III$^{62\text{-}92}$ (Figure 20).

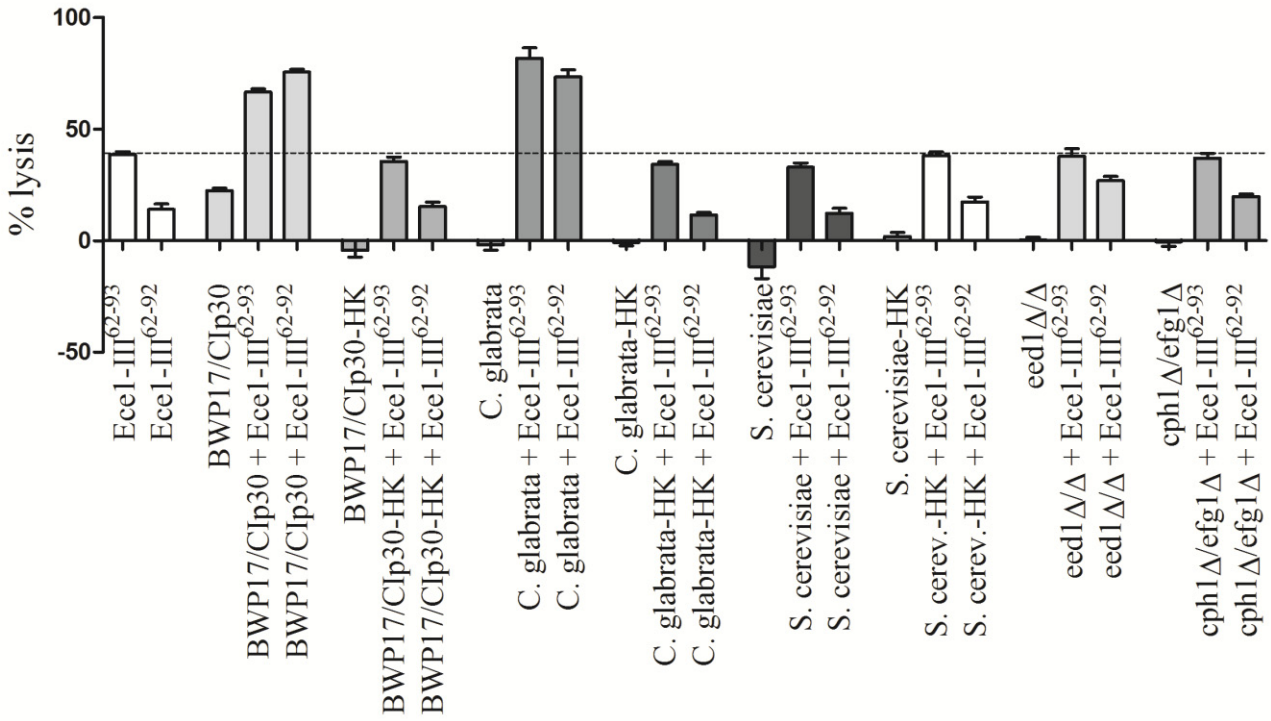


Figure 20: Cytotoxicity of synthetic peptides added in combination with different yeast strains. *C. albicans* mutant strains locked in the yeast-form, as well as non-hyphae forming yeast strains and heat-killed (HK) *C. albicans* strains do not increase the damage of synthetic peptides during a combinatorial infection. The only exception within the tested strains was the *C. glabrata* wild type ATCC2001.

3.3.2 Erythrocyte damage induced by synthetic Ece1 peptides

As melittin has long been known to effectively bind to erythrocytes and induce the release of haemoglobin, the question arose whether any of the synthetic Ece1-peptides may exhibit a haemolytic effect too. Therefore, a mixture of the peptides predicted to result from Kex2 cleavage (Ece1-I – Ece1-VIII) was added to human erythrocytes in different concentrations and incubated for 1h at 37°C. Haemoglobin release occurring during the incubation was measured spectrophotometrically at 541 nm, corresponding to the absorption peak of this molecule. The peptide mixture was added to the blood cells in a final concentration ranging from 3-15 µM with all peptides in the mixture being present at the same molar ratio. The result of this experiment showed that erythrocyte lysis by this peptide mixture is very efficient and red blood cells are completely lysed after the addition of peptides in the concentration of 9-15 µM. At a peptide concentration of 6 µM, erythrocyte lysis is still very effective (90%), while only after the addition of the lowest tested peptide concentration of 3 µM a significantly decreased lysis of erythrocytes (14%) could be observed (Figure 21A).

Similarly to the experiments carried out using oral epithelial cells, the eight peptides were tested for their individual effects on erythrocytes by adding the single peptides to the red blood cells in a concentration of 9 µM each. As can be seen in Figure 21B, a highly lytic effect could only be observed for peptide Ece1-III$^{62-93}$, which caused an erythrocyte lysis of almost 90%. All other peptides were completely unharmful to the erythrocytes. Red blood cell lysis by peptide Ece1-III$^{62-93}$ was also confirmed microscopically. After the 1h incubation period, erythrocytes in the sample containing the peptide were visually depleted, while in the control sample the cells were still intact (Figure 21 C).

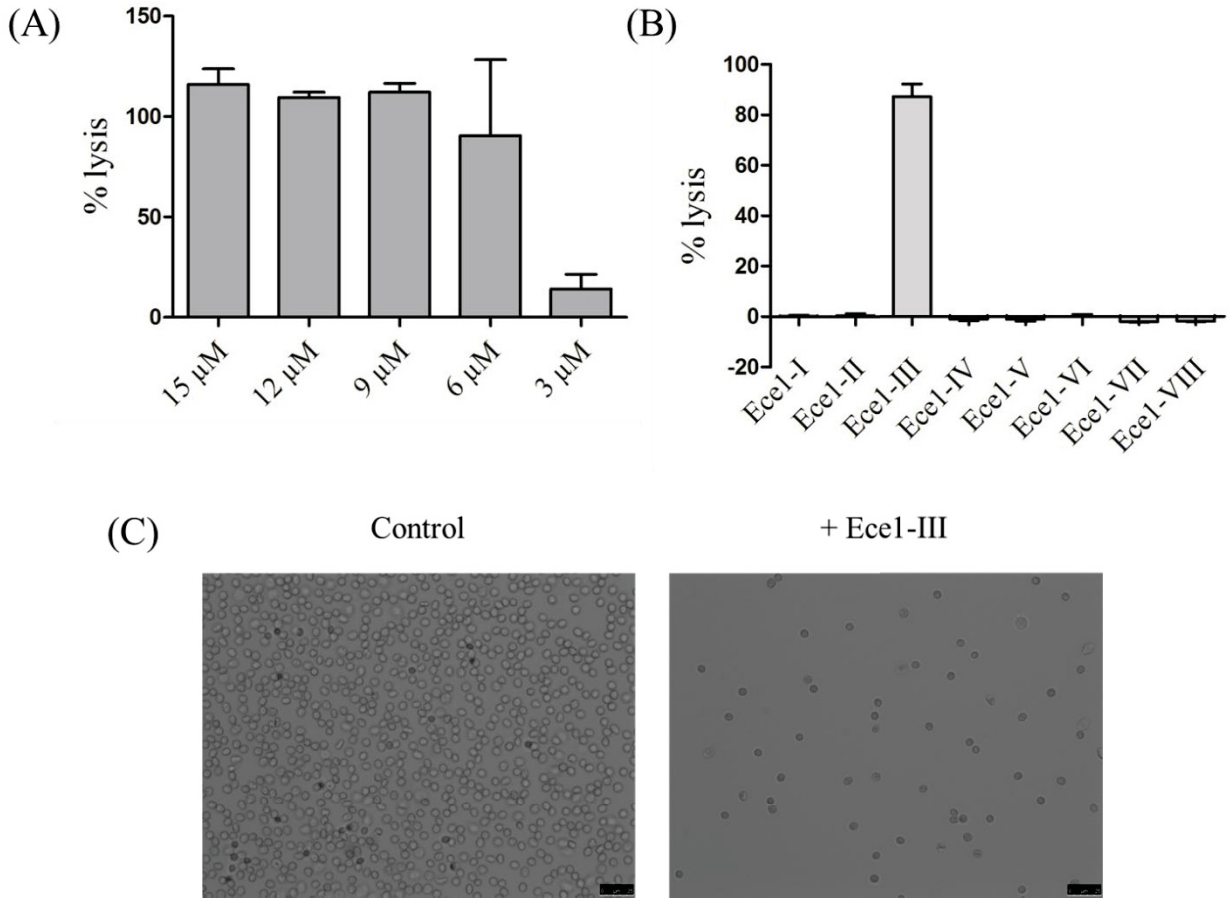


Figure 21: Erythrocyte lysis by synthetic Ece1-peptides. (A) Erythrocytes are efficiently lysed by a mixture of the eight synthetic peptides Ece1-I – Ece1-VIII. Only at a concentration 3 µM lysis of red blood cells is significantly reduced. (B) Of the eight Ece1-peptides, only Ece1-III causes lysis of erythrocytes, while the other peptides do not damage. (C) After 1h incubation with Ece1-III, erythrocytes are visibly depleted in comparison to the control (incubation in PBS only).

During the course of the study, mass spectrometry results suggested that Ece1-III$^{62-93}$ was further cleaved by Kex1, removing the C-terminal arginine residue, resulting in secretion of the peptide Ece1-III$^{62-92}$. Therefore, this alternative peptide was included into all further experiments using red blood cells. In a preliminary experiment, the lytic effects of both peptide Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ were tested for their concentration dependency. It was found for both peptides, that erythrocyte lysis increased in a concentration-dependent manner. Lysis exhibited by peptide Ece1-III$^{62-92}$, however, was reduced compared to Ece1-III$^{62-93}$. Considerable red blood cell damage (35%) through peptide Ece1-III$^{62-93}$ could already be observed at a peptide concentration of 6 µM, with damage increasing proportionally up to 96% at a peptide concentration of 15 µM. In comparison, only 7% of erythrocytes were lysed by Ece1-III$^{62-92}$ in a concentration of 6 µM, and at the highest tested concentration of 15 µM a total of approximately 30% of erythrocytes had been damaged (Figure 22).

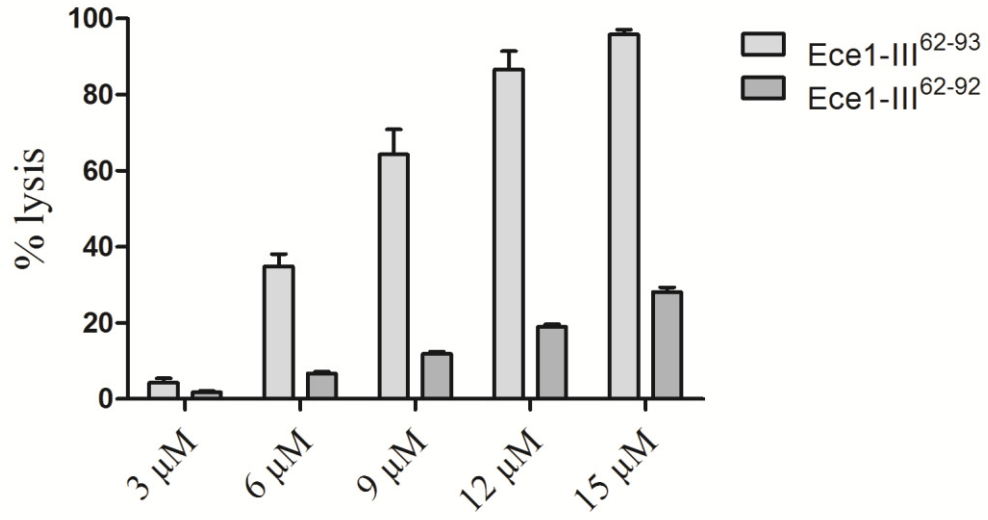


Figure 22: Erythrocyte damage caused by two versions of Ece1-III at different concentrations. Erythrocyte damage by both peptides increases concentration-dependently. While lysis by Ece1-III$^{62-93}$ is very efficient (96% at 15 µM), damage by Ece1-III$^{62-92}$ is considerably lower (30% at 15 µM).

To analyse how rapid lysis of erythrocytes occurs, a time course experiment was carried out, surveying haemoglobin release at different time points ranging from 2 min to 180 min. Due to the short incubation times, the 2 min, 5 min and 10 min samples were incubated at room temperature, while all other samples were incubated at 37°C. The result clearly shows that lysis by peptide Ece1-III$^{62-93}$ occurs almost instantly after the addition to the red blood cells. After only 2 min at room temperature, almost 20% of all erythrocytes are already lysed. Lysis by this peptide continuously increases with longer incubation periods and reaches its maximum after 45 min, when 98% of the erythrocytes are lysed. On the other hand, lysis by peptide Ece1-III$^{62-92}$ is not only lower, but also much slower. The first measurable lysis (~7%) was observed after 15 min. During the course of 180 min, the lysis rate of this peptide slowly but steadily increases, reaching its highest lysis rate of 33% at the latest measured time point of 180 min (Figure 23).

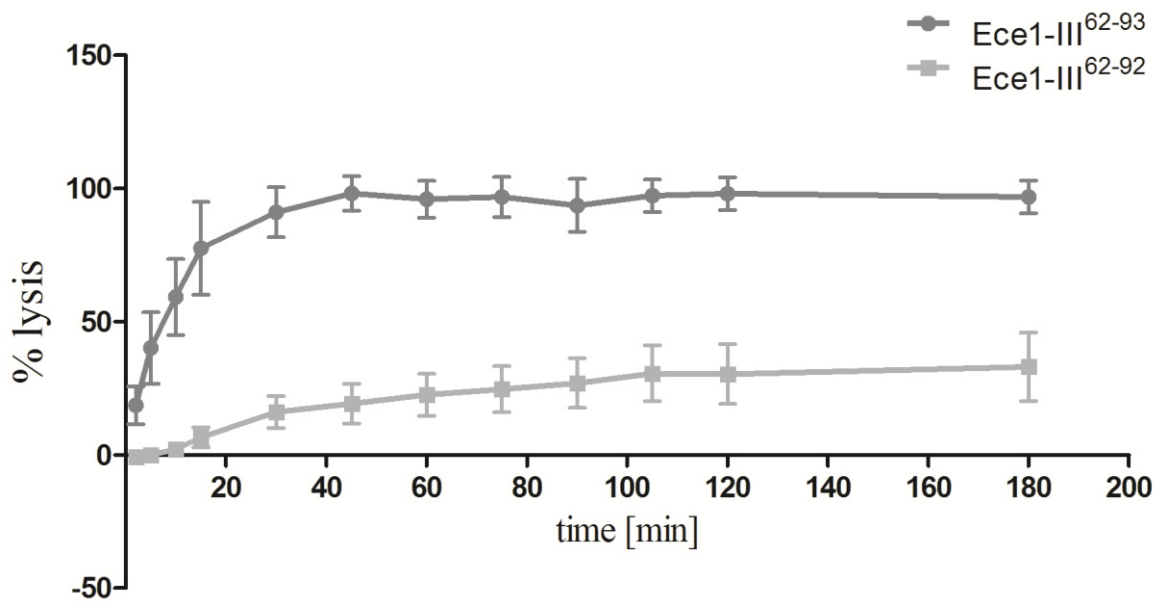


Figure 23: Time course of erythrocyte lysis by Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$. While lysis by Ece1-III$^{62-93}$ is induced instantly and reaches its maximum of 98% after only 45 min, erythrocyte damage by Ece1-III$^{62-92}$ is induced much slower and maximal damage does not exceed 33%.

As many peptide toxins are only active at very specific pH values, lysis efficiency of Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ was tested at different pH values. Due to the sensitivity of red blood cells to extremes of pH, peptide lysis was only tested in samples ranging in pH from 5-8. It was found that neither peptide was influenced in its lytic activity by any of the tested pH values. Both peptide Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ reached their maximum lysis rates of approximately 100% and 30%, respectively, which could already be observed in earlier experiments (Figure 24). The very slight decrease of damage by Ece1-III$^{62-93}$ at pH 7 was ascribed to normal experimental variations, as this condition corresponds most closely to the natural environment of erythrocytes at a pH of ~7.4 in the blood.

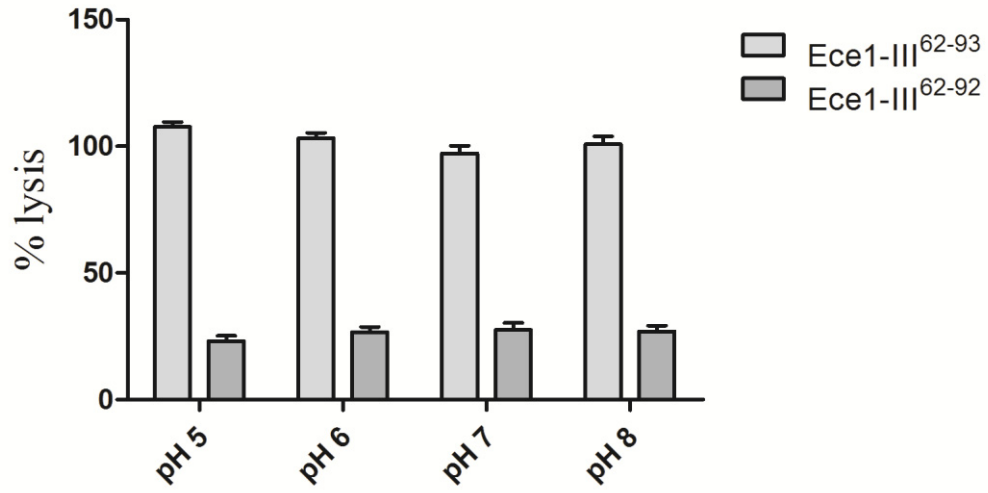


Figure 24: Influence of the pH on erythrocyte lysis by Ece1-III. Damage of erythrocytes by Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ is not influenced by the pH value in the range from pH 5 to pH 8.

3.3.2.1 Effect of human serum and serum albumin on Ece1-III-induced erythrocyte lysis

During a *C. albicans* bloodstream infection, the Ece1-peptides will be secreted into whole blood. While erythrocytes represent the most abundant blood cell type, a total of ~55% of whole blood is made up of plasma. To examine whether any of its components may influence red blood cell lysis by Ece1, human blood serum (plasma devoid of the clotting factor fibrinogen) was prepared and lysis efficiency of the peptides analysed in the presence of different serum concentrations. Indeed it was found that human serum exhibits a highly inhibitory effect on red blood cell lysis by Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$. While incubation of the erythrocytes with peptide Ece1-III$^{62-93}$ alone resulted in the lysis of 92% of all red blood cells after 1h, the presence of human serum in a dilution of 1:100 caused a strong decrease of the lysis rate, with only 23% of the erythrocytes being lysed after the incubation period. When added in lower concentrations, i.e. in a 1:200 and 1:500 dilution, human serum still negatively affected the cell lysis, but to a lower extent compared to the higher serum concentration. When added in a 1:200 dilution, 36% of the erythrocytes were found to be lysed, while a 1:500 diluted serum resulted in a lysis rate of 53%. Erythrocyte lysis by peptide Ece1-III$^{62-92}$, which already causes much less lysis compared to Ece1-III$^{62-93}$, was completely abolished by the addition of human serum in a 1:100 and 1:200 dilution. Even when added in a 1:500 dilution, only 3% lysis of erythrocytes by this peptide could be detected (Figure 25A).

Human blood serum contains 91% of water and 7% of proteins [313, 314]. Of these proteins the major component (60%) is albumin [314]. To test whether this serum component may be responsible for the inhibitory effect exhibited by the serum, human serum albumin in different concentrations was added to the samples. For Ece1-III$^{62-93}$, no inhibitory effect on lysis was

observed for any of the tested albumin concentrations. The lysis rate of Ece1-III$^{62-92}$ on the other hand continuously decreased with increasing concentrations of serum albumin present. While the peptide alone caused lysis of 37% of the erythrocytes, only 17% of erythrocytes were lysed in the presence of the highest tested albumin concentration of 2 mg/ml. However, while noticeable, this decrease in lysis was not significant (Figure 25B).

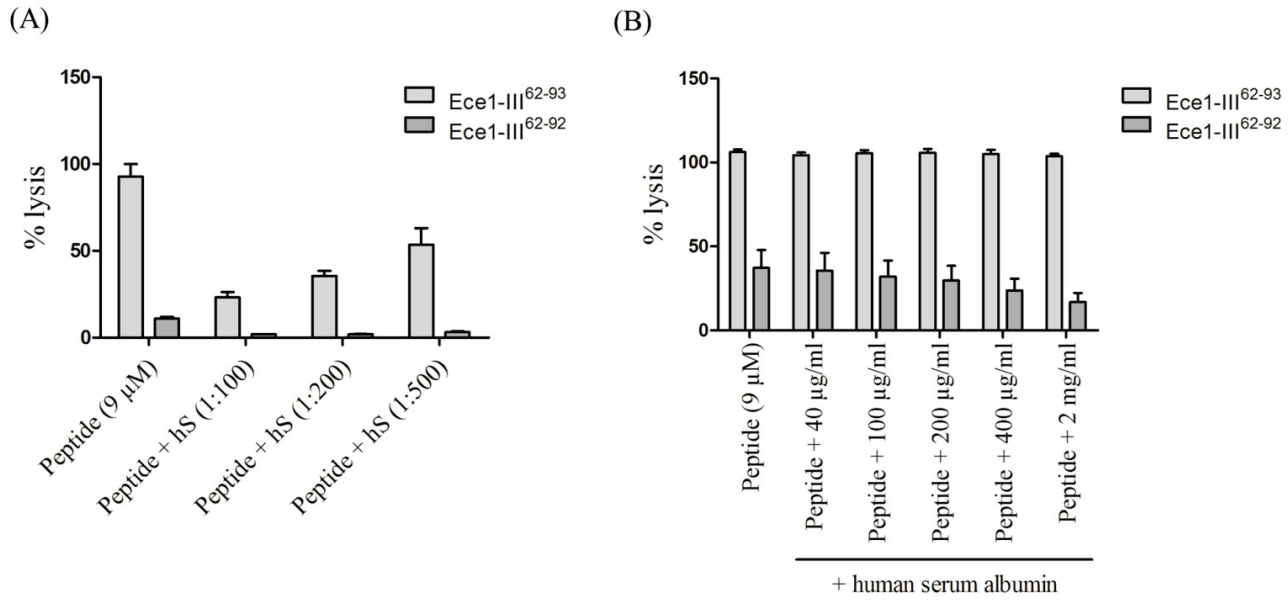


Figure 25: Influence of human serum and human serum albumin on erythrocyte lysis. (A) The addition of human serum (hS) effectively inhibits lysis of red blood cells by Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$. (B) Addition of human serum albumin did not decrease Ece1-III induced erythrocyte lysis, demonstrating that this serum component is not responsible for the inhibitory effect of serum.

3.3.2.1 Influence of ion-chelation on Ece1-III-induced erythrocyte lysis

Hypothesising that in accordance to many other lytic peptides the damage exerted by the Ece1-peptides may also be ion-dependent, the majority of divalent ions present in the test samples was chelated using the potent chelating agent EDTA. The lack of free ions in the surrounding buffer may result in a reduced flow of ions inside the red blood cell, thereby preventing damage caused to the cell by dramatically changing intracellular ion concentrations and the connected triggering of damaging pathways. The addition of 20 mM of EDTA to the test samples only caused a slight decrease of lysis, while the presence of EDTA concentrations of 50 mM and higher resulted in a significantly reduced lysis rate for both peptides. Erythrocyte lysis by both peptides was observed to be halved in the presence of 50 mM in comparison to the damage caused by the respective peptide alone. At concentrations of 100 mM and 200 mM of EDTA, damage exerted by

Ece1-III$^{62-93}$ was only minimal (11% and 3%, respectively), while damage by Ece1-III$^{62-92}$ was completely abolished in the presence of 200 mM of EDTA (Figure 26A).

To assess whether the observed effect of EDTA was caused by ion chelation or by a direct inhibition of the peptides, an excess amount of magnesium ions (as $MgCl_2$) was added in addition to the EDTA, hypothesising that lysis inhibition by EDTA-induced ion-chelation should be restored. An inhibiting EDTA concentration of 100 mM was chosen, to obtain a significantly reduced rate of lysis. To restore the damaging capacity of the peptides, the samples were supplemented with either 50 mM or 100 mM of $MgCl_2$. The lysis rate of 14% of Ece1-III$^{62-93}$ in the presence of 100 mM could be restored to 34% by the addition of 50 mM $MgCl_2$. Addition of 100 mM $MgCl_2$ resulted in lysis significantly increasing to 65%. Similar results could be observed for peptide Ece1-III$^{62-92}$. The decreased lysis of 3% due to the inhibitory effect of EDTA could be gradually restored by the addition of increasing concentrations of $MgCl_2$. Supplementation with 100 mM of $MgCl_2$ even resulted in a higher lysis (19%) than the original lysis observed for the peptide alone (14%) (Figure 26B).

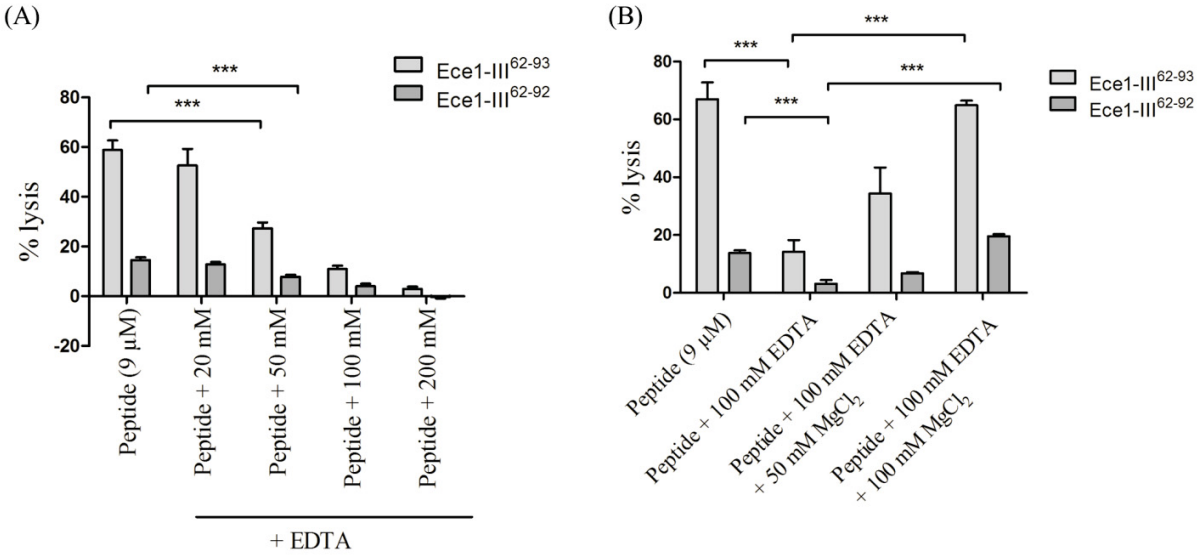


Figure 26: Influence of EDTA on erythrocyte lysis. (A) Lack of free ions caused by addition of the chelating agent EDTA inhibits the damaging effect of Ece1-III. (B) Damage by Ece1-III can be restored by the addition of an excess amount of magnesium ions in the form of $MgCl_2$. These results point to the damage-mechanism of Ece1-III being ion-dependent.

3.3.2.3 Impact of membrane cholesterol-content on Ece1-III-mediated erythrocyte lysis

To examine whether cholesterol may play a modulatory role on the haemolytic activity of Ece1-III, cholesterol was specifically depleted from the erythrocytes using the compound methyl-ß-cyclodextrin (MßCD). Cyclodextrins are cyclic oligosaccharides, containing a hydrophobic cavity which enables this molecule to bind various hydrophobic compounds. Exposure of cells to a high concentration of MßCD (5-10 mM) for a prolonged time (> 2h) results in the removal of 80-90% of total cellular cholesterol [315, 316]. In this study, cholesterol-depletion was carried out by incubation of erythrocytes in 5 mM of MßCD for 30 min at 37°C. Afterwards, these erythrocytes were used in haemolysis experiments comparing the Ece1-III-mediated lysis rates for both depleted and non-treated erythrocytes. To eliminate the possibility of MßCD exhibiting a damaging effect on the erythrocytes, a negative control without peptide was included and subtracted from the measured values. So far, only preliminary experiments were carried out using the peptide Ece1-III$^{62-93}$ and it should be noted that the experiment has only been performed twice and in duplicate.

The results obtained so far, however, indicate that cholesterol may exert a protective effect on the erythrocyte membrane, as MßCD-treated red blood cells are more easily lysed by Ece1-III$^{62-93}$. Particularly at low peptide concentrations, e.g. 1.5 µM or 0.75 µM, the observed lysis rate of cholesterol-depleted erythrocytes is significantly higher compared to that of untreated erythrocytes (Figure 27).

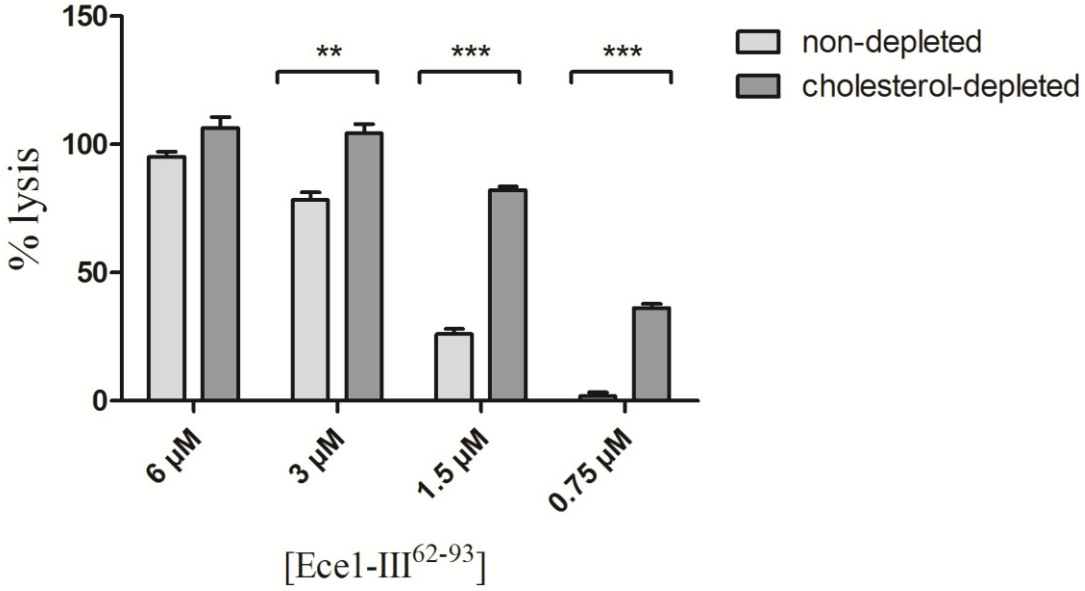


Figure 27: Impact of membrane cholesterol-content on Ece1-III-mediated erythrocyte lysis. Reduction of the cholesterol inside the erythrocyte membrane results in a higher lysis rate by Ece1-III$^{62-93}$. Particularly at low peptide concentrations, cholesterol-depleted erythrocytes are more efficiently lysed than red blood cells of the untreated control.

3.3.3 Yeast growth in the presence of synthetic Ece1 peptides

Discovering the highly lytic effect that peptide Ece1-III$^{62-93}$ exerts on both epithelial cells and erythrocytes, the question arose whether high concentrations of the peptide in the surrounding medium could also exhibit an inhibitory effect on the growth of *C. albicans* itself or other yeast species, such as *C. glabrata* and *S. cerevisiae*. To analyse the influence of peptides Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ on the growth of these yeast species, growth experiments were carried out in the presence of these peptides. The two *C. albicans* wild type strains SC5314 and BWP17/CIp30, the *ece1*Δ/Δ and revertant strain, the *ece1*Δ+*ECE1*[ΔEce1-III] strains (see part 3.4) and a wild type strain of both *C. glabrata* (ATCC2001) and *S. cerevisiae* (BY4741) were grown in the presence of 20 µM of either peptide Ece1-III$^{62-93}$ or Ece1-III$^{62-92}$. As a means of comparison, growth of these strains was also tested in the presence of the same concentration of melittin. After 50h of incubation at 37°C, however, no differences in growth could be detected for any of the strains in the presence of either Ece1-III$^{62-93}$, Ece1-III$^{62-92}$ or melittin. All strains showed a growth comparable to that in medium without added peptide and reached the same final OD, which was OD_{600} of 1.2 for all tested *C. albicans* strains, OD_{600} of 1.4 for *C. glabrata* and OD_{600} of 1.1 for *S. cerevisiae* (Figure 28).

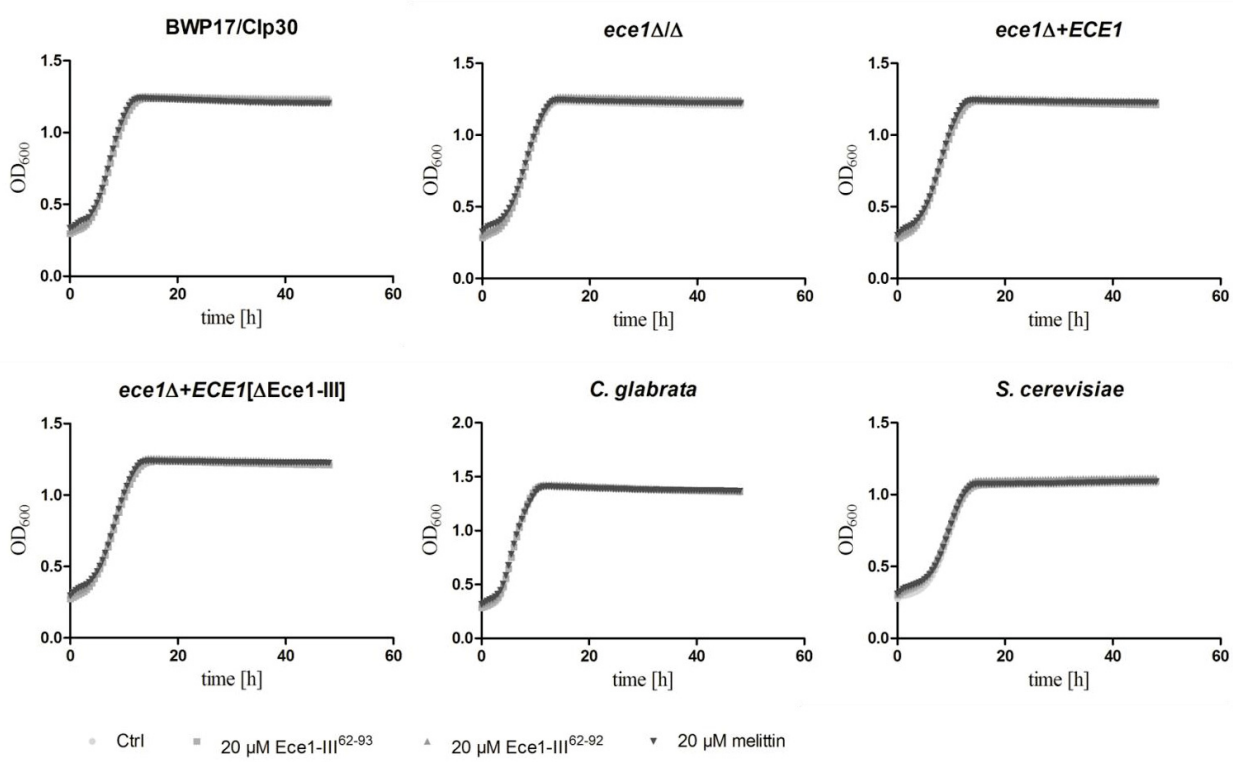


Figure 28: Impact of peptides on the growth of different yeast species. The presence of 20 µM of either Ece1-III$^{62-93}$, Ece1-III$^{62-92}$ or melittin did not influence the growth of the tested *C. albicans* strains, *C. glabrata* and *S. cerevisiae* compared to growth in YPD only (Ctrl).

Furthermore, all *C. albicans* strains were subjected to growth in the presence of a higher concentration of the three peptides, i.e. 70 µM. Even at this high peptide concentration in the surrounding medium, growth characteristics in the presence of Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ were observed to be similar to the control, with all strains reaching the same final OD. On the contrary, growth in the presence of 70 µM of melittin resulted in a slight initial drop of OD, which was most prominent for the *C. albicans* wild type BWP17/CIp30. No further influences of melittin on *C. albicans* growth could be observed however (Figure 29).

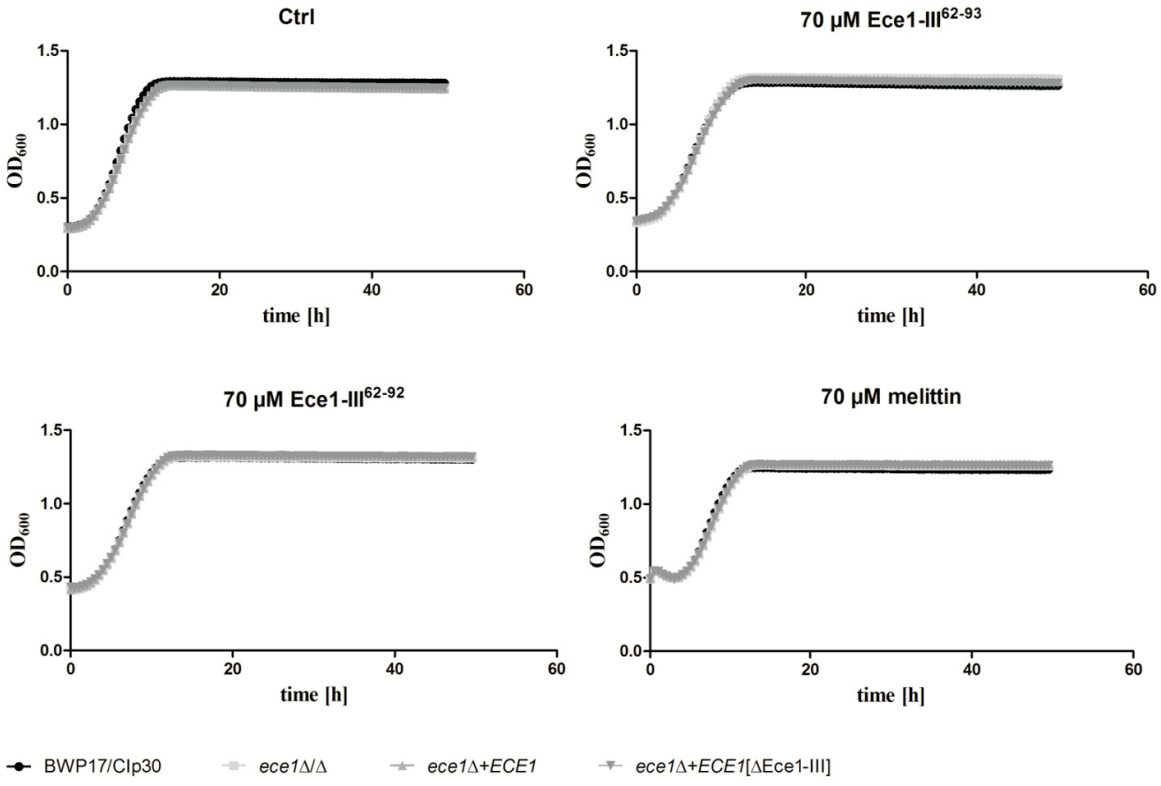


Figure 29: Impact of higher peptide concentration on the growth of different *C. albicans* strains. While the presence of 70 µM of Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ did not influence the growth of the tested *C. albicans* strains, this concentration of melittin caused a prolonged lag phase, which could be observed for all tested strains.

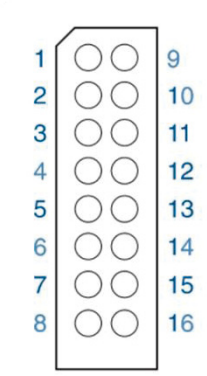
3.3.4 Interaction of Ece1-III with the host cell membrane

The previously obtained results pointed in the direction of Ece1-III acting as a pore-forming peptide. Trying to identify possible binding partners acting as a receptor in the host cell membrane, a lipid binding assay was performed by overlaying the synthetic peptide onto PIP Strips™ Membranes. These membranes contain 15 spots containing 100 picomoles of different membrane lipids (Figure 30A). Incubation of synthetic Ece1-III with the PIP Strips™

Membranes and subsequent antibody detection was performed to identify membrane lipids that may allow localisation of Ece1-III to the membrane via direct binding. Hypothesising that the C-terminal positively charged lysine-arginine-motif of Ece1-III$^{62-93}$ may be involved in mediating binding to the negatively charged head groups of the membrane lipids, synthetic Ece1-III peptides with a slight variation of this motif were tested for their binding capacity as well. These included peptide Ece1-III$^{62-93R\rightarrow A}$ and Ece1-III$^{62-93KR\rightarrow AA}$, in which one or both amino acids of the C-terminus were substituted with the nonpolar amino acid alanine.

Antibody detection revealed that Ece1-III$^{62-93}$ did readily bind to several of the present phospholipids. The strongest binding could be observed between this peptide and the phospholipids phosphatidylinositol-4-phosphate, phosphatidic acid and phosphatidylserine. Binding to phosphatidylinositol, phosphatidylinositol-3-phosphate, phosphatidylinositol-6-phosphate and phosphatidylinositol-3,5-bisphosphate was weaker, but still noticeable. Exchange of the positively charged amino acid arginine at the C-terminus of peptide Ece1-III$^{62-93}$ with alanine resulted in a remarkable loss of binding capacity to the tested phospholipids. Ece1-III$^{62-93R\rightarrow A}$ only bound to one of the spotted lipids, phosphatidic acid. Curiously, this strong reduction in binding capacity was not observed when both positively charged amino acids of the peptide's C-terminus (lysine and arginine) were substituted with alanine. The binding pattern that could be observed after incubation of the PIP Strips™ Membranes with Ece1-III$^{62-93KR\rightarrow AA}$ was similar to that of Ece1-III with the intact C-terminus, albeit binding to the respective phospholipids was perceivably weaker for most lipids. Aberrant to peptide Ece1-III$^{62-93}$ however, the peptide with the completely nonpolar C-terminus did bind most strongly to the phospholipid phosphatidylinositol-3-phosphate. The results of the lipid binding assay for all three tested peptides are depicted in Figure 30B.

(A)



Spot #	PIP Strips * and PIP MicroStrips †
1	Lysophosphatidic acid
2	Lysophosphatidylcholine
3	Phosphatidylinositol (PtdIns)
4	PtdIns(3)P
5	PtdIns(4)P
6	PtdIns(5)P
7	Phosphatidylethanolamine
8	Phosphatidylcholine
9	Sphingosine 1-phosphate
10	PtdIns(3,4)P_2
11	PtdIns(3,5)P_2
12	PtdIns(4,5)P_2
13	PtdIns(3,4,5)P_3
14	Phosphatidic acid
15	Phosphatidylserine
16	Blank

(B)

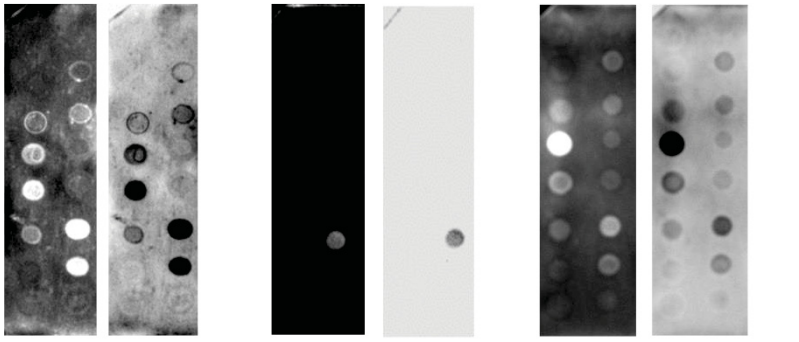


Figure 30: Identification of possible Ece1-III binding partners within the cell membrane via lipid binding assay. (A) Schematic of a PIP Strips™ Membrane and table of the phospholipids spotted on each strip at a concentration of 100 picomoles (images taken from Thermofisher). (B) Binding of the Ece1-III peptides with varying C-terminal endings to the different phospholipids.

3.4 Single peptide knockout-mutants

Seeing that of the eight Ece1-peptides predicted to result from complete cleavage of the protein only Ece1-III demonstrated a damaging effect on host cells, a mutant strain was created in our laboratory, in which the *ECE1* allele, including promoter and coding sequence, but specifically lacking the Ece1-III-encoding DNA was reinserted in the mutant lacking *ECE1* (resulting in the strain *ece1*Δ+*ECE1*[ΔEce1-III]). Hypothesising that the other seven peptides are negligible for the effects carried out by Ece1, a mutant deficient in only this one peptide should behave similarly to the *ece1*Δ/Δ null mutant. Three different clones of this mutant were tested in parallel for their ability to form hyphae and to cause damage of oral epithelial cells.

The *ece1*Δ+*ECE1*[ΔEce1-III] strains were incubated in RPMI medium at 37°C to induce the yeast-to-hypha transition. The formation of hyphae was assessed after 3h, 5h and 24h, respectively. As shown in Figure 31, the cells of all tested clones showed the development of

germ tubes and short hyphae after 3h of incubation. Continued hyphal growth could be observed after 5h, while after 24h of incubation, the *Candida* cells had formed a dense layer of long, interwoven hyphae. Therefore, hypha formation of the *ece1*Δ+*ECE1*[ΔEce1-III] strains is in accordance to that of the *ece1*Δ/Δ null mutant, which shows a similar hyphal growth at the respective time points (compare Figure 13).

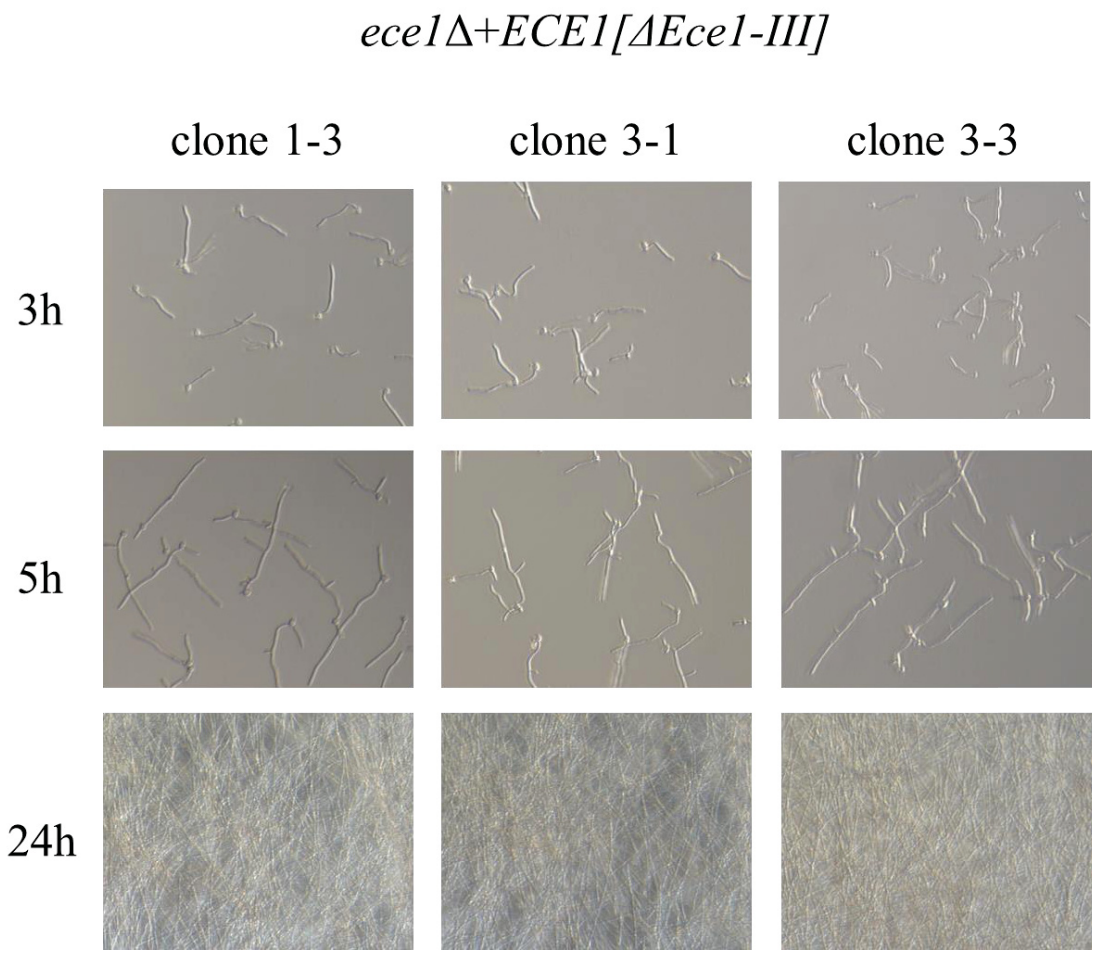


Figure 31: Hyphal growth of the *ece1*Δ+*ECE1*[ΔEce1-III] mutants on plastic. Cells were grown under hypha-inducing conditions in RPMI medium and hyphal growth was assessed after 3h, 5h and 24h. All three clones showed normal hypha-forming abilities.

The damaging capacity of the *ece1*Δ+*ECE1*[ΔEce1-III] strains was tested via a cytotoxicity assay on oral epithelial cells in comparison to the parental wild type strain BWP17/CIp30, the *ece1*Δ/Δ mutant and the *ece1*Δ+*ECE1* strain. After 24h of co-incubation with the epithelial cells, damage was determined via measurement of LDH release. The results showed that all three strains deficient in Ece1-III behaved similarly to the *ece1*Δ/Δ mutant, i.e. no damage was caused by these strains. However, four clones of an Ece1-VII-deficient strain (*ece1*Δ+*ECE1*[ΔEce1-VII]) that had

been constructed and included into this experiment as a control, did not exert any damage on the oral epithelial cells either. As expected, the highest damage was caused by the wild type (~20% lysis), while the revertant strain *ece1*Δ+*ECE1* caused an intermediate damage of approximately 5% (Figure 32).

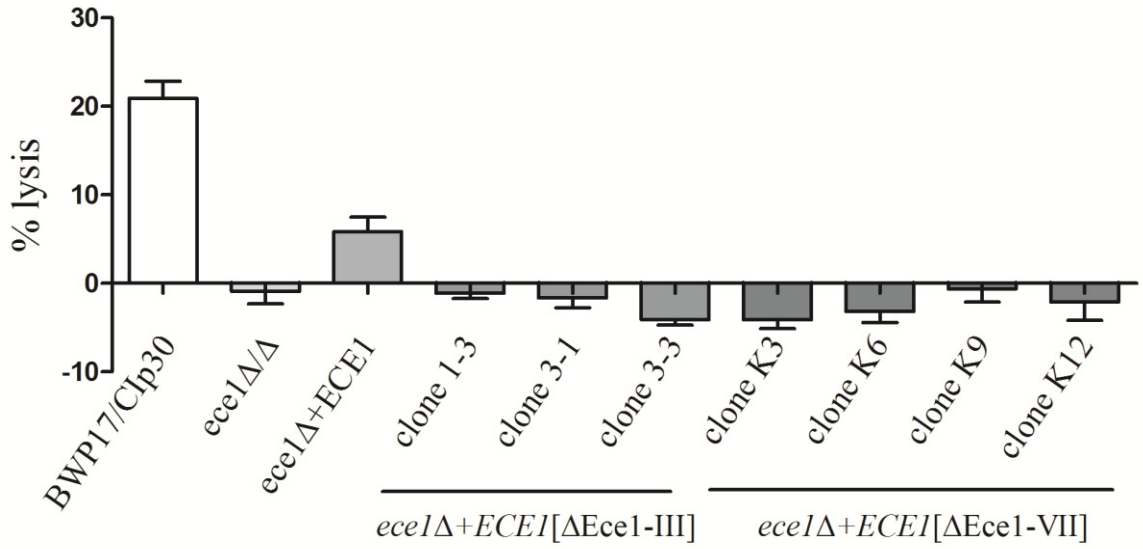


Figure 32: Impact of single peptide deletion on damage of epithelial cells. The three clones of the *ece1*Δ+*ECE1*[ΔEce1-III] strain, which are only deficient in the Ece1-III encoding gene sequence, behave similarly to the *ece1*Δ/Δ mutant and do not cause damage of oral epithelial cells. The four clones of the *ece1*Δ+*ECE1*[ΔEce1-VII] strain however did not damage the epithelial cells either.

3.5 Lysis of erythrocytes by *C. albicans*

C. albicans wild type and mutant strains were tested for their ability to lyse red blood cells by co-incubation for different periods of time (16h, 24h and 48h) and subsequent determination of erythrocyte lysis determined by measuring absorbance of released haemoglobin at 541 nm. The tested strains included two *C. albicans* wild types (SC5314 and BWP17/CIp30), the *ece1*Δ/Δ, *ece1*Δ+*ECE1*, all three clones of the mutant lacking Ece1-III$^{62-93}$, the clones lacking Ece1-VII, *kex2*Δ/Δ, *eed1*Δ/Δ and *cph1*Δ/*efg1*Δ. Co-incubation was carried out under hypha-inducing conditions, i.e. at 37°C and in RPMI 1640 medium.

After 16h of co-incubation, 24% and 30% of the erythrocytes were lysed by the wild type strains SC5314 and BWP17/CIp30, respectively, while a lysis rate of 18% could be observed for the revertant strain *ece1*Δ+*ECE1*. For all of these three strains, damage gradually increased with an elongated co-incubation time. While both wild types had caused approximately 80% lysis after 48h, lysis by the revertant strain was overall less and reached a maximum of 53% after 48h of

incubation. No damage was exerted by the *ece1*Δ/Δ and all three clones of the *ece1*Δ+*ECE1*[ΔEce1-III] mutant. Likewise, no damage of erythrocytes could be observed after co-incubation with the strain lacking the gene encoding for the Kex2 protease (*kex2*Δ/Δ) and with the strains *eed1*Δ/Δ and *cph1*Δ/*efg1*Δ, which are characterised by switching back to the yeast form after initial hypha formation or by being locked in the yeast form, respectively. While the synthetic peptide Ece1-VII did not show a lytic effect on red blood cells, the loss of the gene sequence encoding this single peptide resulted in an abolished damage effect of the respective strains on erythrocytes. After a co-incubation for 48h, only a very small percentage of blood cells (4-5%) had been lysed by the clones *ece1*Δ+*ECE1*[ΔEce1-VII] K6 and *ece1*Δ+*ECE1*[ΔEce1-VII] K12 (Figure 33). This result may hint to a more prominent function of Ece1-VII *in vivo*, but further experiments would be needed to confirm this role.

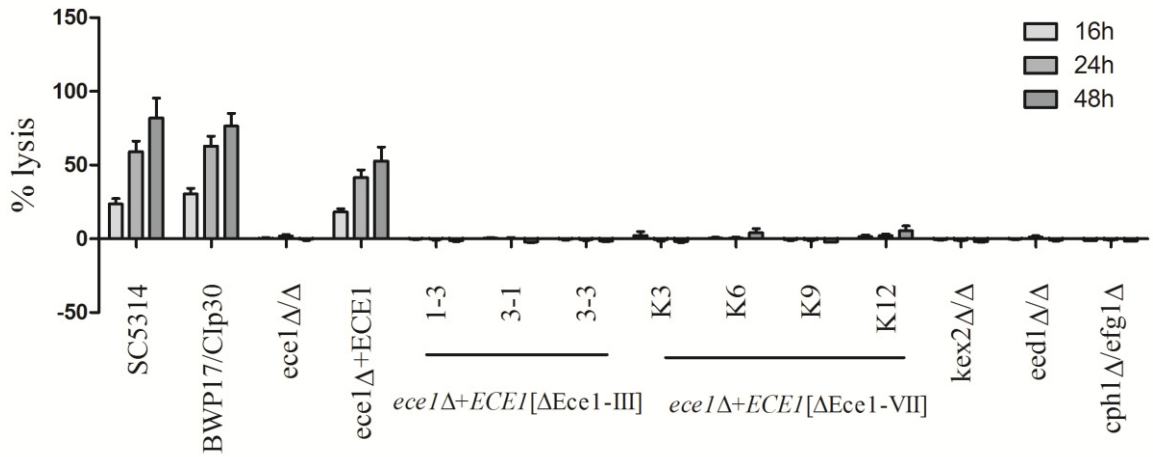


Figure 33: Erythrocyte lysis by different *C. albicans* wild type and mutant strains. While both tested wild types (SC5314 and BWP17/CIp30) and the *ece1*Δ+*ECE1* strain are able to lyse red blood cell, no haemoglobin release could be observed after incubation with the *ece1*Δ/Δ, the *ece1*Δ+*ECE1*[ΔEce1-III] strains, most *ece1*Δ+*ECE1*[ΔEce1-VII] strains, *kex2*Δ/Δ, *eed1*Δ/Δ and *cph1*Δ/*efg1*Δ. Very low lysis was caused by the two *ece1*Δ+*ECE1*[ΔEce1-VII] clones K6 and K12 after 48h.

3.6 ScKex2-mediated *in vitro* digestion of rEce1

In order to determine the importance of processing of the full length Ece1 protein into single peptides, a His-tagged version of the full length protein was recombinantly expressed in *E. coli* BL21(DE3), followed by isolation and purification of the protein utilising the His-tag. In parallel, the protease Kex2, which had already been shown to process Ece1 *in vitro*, was overexpressed

using a *P. pastoris* strain. Subsequently, digestion of the produced recombinant Ece1 (rEce1) with the recombinant Kex2 (ScKex2) and comparison of damage efficiency between the full length rEce1 and the digest products was carried out in order to identify a possible increase of cytotoxicity coming along with the processing into single peptides.

3.6.1 Protein overexpression

Both rEce1 and ScKex2 were successfully overexpressed using the described expression systems. After purification and concentration of the proteins from *E. coli* cell lysates and *P. pastoris* culture supernatants, respectively, protein solutions containing > 2 mg/ml protein could be obtained (Figure 34).

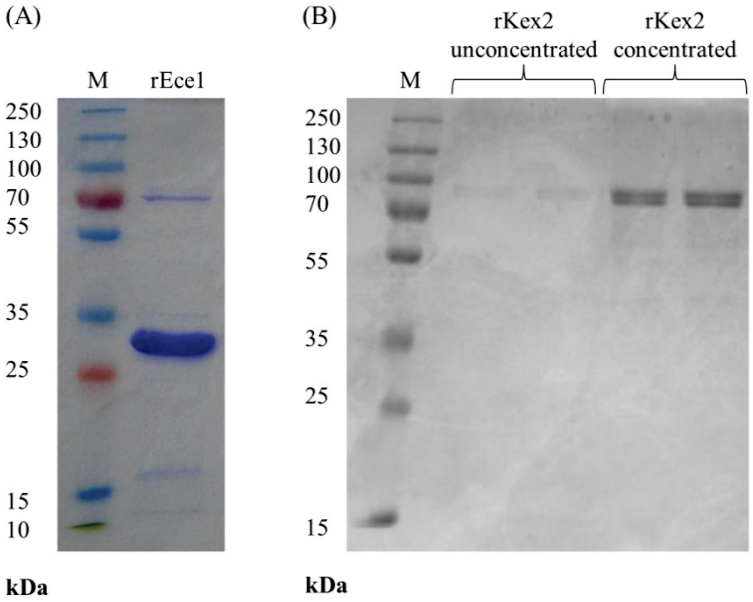


Figure 34: Overexpression of recombinant Ece1 (rEce1) and recombinant Kex2 (ScKex2). (A) SDS-PAGE of purified and concentrated rEce1. The His-tagged Ece1 protein has a molecular weight of approximately 30 kDa. (B) SDS-PAGE of overexpressed ScKex2. While the protein concentration was very low in the supernatant (unconcentrated), it was increased by using concentrator columns (concentrated). The ScKex2 protein has a molecular weight of approximately 90 kDa.

3.6.2 *In vitro* digestion of rEce1

To digest full length rEce1, the protein was co-incubated with ScKex2 in the presence of $CaCl_2$ for either 1 min, 5 min, 10 min or 20 min at 37°C. Afterwards, the samples were directly put on ice until loading on an SDS-gel for examination of a successful digest. It was found, that digestion had occurred rapidly, as the previously strong protein band of rEce1 was noticeably reduced in the digestion samples, while an increase of lower bands, corresponding to smaller protein products, was observed. After 20 min of co-incubation, digestion had progressed to a point where no individual proteins could be detected. The lowest detectable band after the shortest incubation times was below 10 kDa, but did not appear to correspond to the molecular weight of single Ece1-peptides, as it resolved at a higher molecular weight than synthetic Ece1-III. This was identical for all samples incubated between 1 to 10 min. Blotting of the protein gel revealed, that a great proportion of rEce1 had seemingly formed dimers or trimers, as strong bands were detectable at 60 kDa and 90 kDa, corresponding to multiples of the molecular weight of the full length Ece1 protein. Therefore, only a small fraction of the present protein seems to have been digested by ScKex2, explaining the very weak band of the lower weight proteins. Blotting however also revealed very weak bands, which in comparison to the synthetic Ece1-III correlate to fully processed rEce1 (Figure 35).

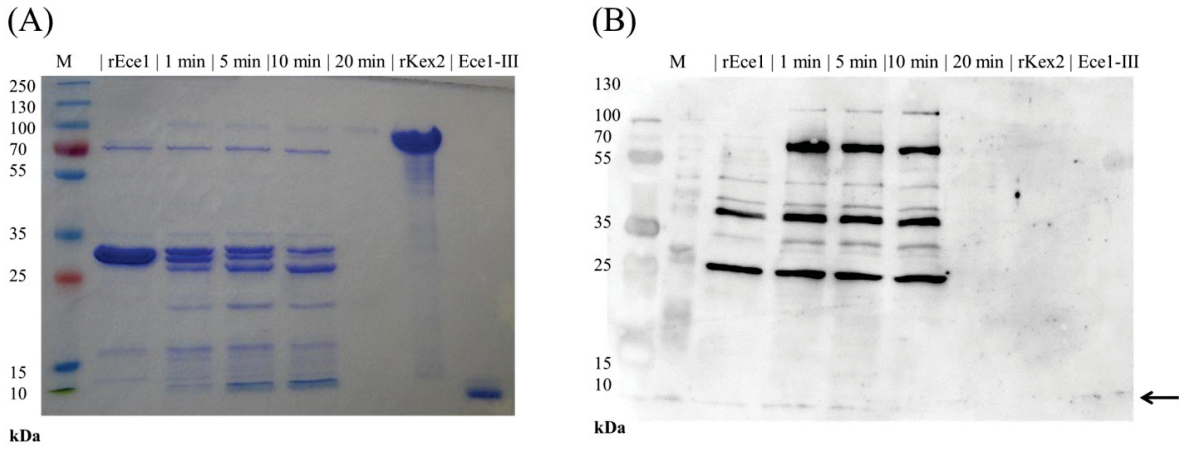


Figure 35: *In vitro* digestion of rEce1. (A) SDS-PAGE of rEce1, ScKex2 and the digestion products after 1 min, 5 min, 10 min and 20 min of co-incubation. Synthetic Ece1-III was added as a control. (B) Western Blot of the protein gel using a polyclonal antibody against Ece1-III. While strong detection can be observed for the full length rEce1 protein and multiples thereof (30 kDa, 60 kDa, 90 kDa), detection of lower weight peptides, including synthetic Ece1-III, is only weak. Arrow: peptides with molecular weights corresponding to fully processed rEce1.

3.6.3 Erythrocyte lysis by rEce1 digestion products

Digestion products resulting from ScKex2-mediated cleavage of rEce1 were tested for their ability to lyse erythrocytes. Different concentrations (0.3 mg/ml, 0.6 mg/ml, 1.2 mg/ml) of the digestion products were added to the erythrocyte samples, corresponding to 10 µM of free Ece1-III, assuming digestion efficiencies of 100%, 50%, or 25%, respectively. Both unprocessed rEce1 and ScKex2, in concentrations correlating to their presence in the digestion samples, were used as controls.

Haemoglobin release observed after the incubation period revealed that no lysis occurred following incubation with ScKex2. While no erythrocyte lysis could be observed after incubation with 10 µM and 20 µM of unprocessed rEce1 either, the addition of 40 µM of undigested rEce1 resulted in 13% erythrocyte lysis. No erythrocyte lysis, however, could be detected after the incubation with rEce1 digestion products. Only the addition of 40 µM of digested rEce1, corresponding to 10 µM of free Ece1-III at an assumed digestion efficiency of 25%, caused minimal lysis (2%) of the red blood cells. In comparison, synthetic Ece1-III$^{62-93}$ at a concentration of 10 µM led to complete lysis of the erythrocytes, while the same concentration of Ece1-III$^{62-92}$ resulted in 22% haemolysis.

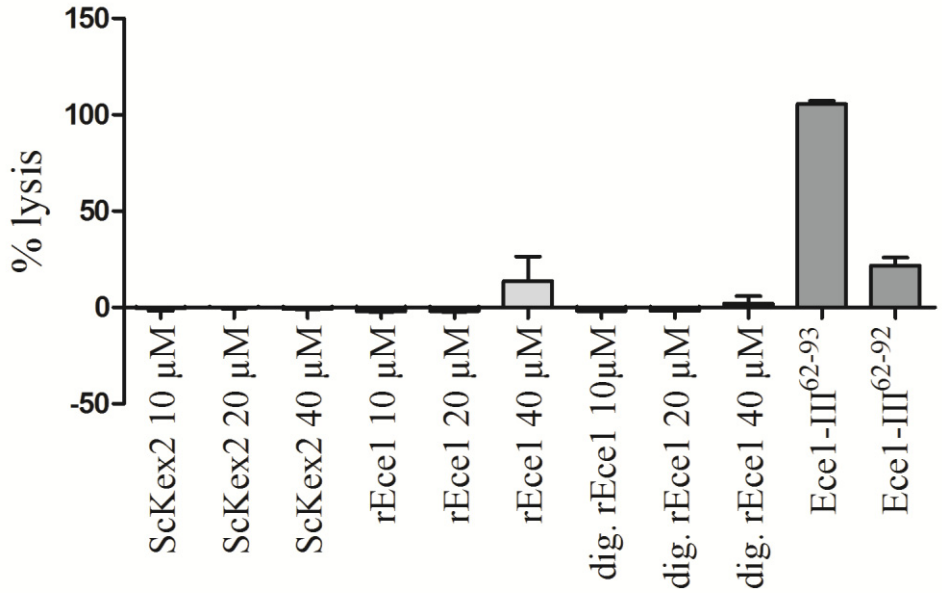


Figure 36: Erythrocyte lysis by rEce1 digestion products. While moderate lysis (13%) of red blood cells is caused by 40 µM of undigested rEce1, the highest concentration of digested rEce1 only caused lysis of 2% of the erythrocytes. In comparison, synthetic Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ cause lysis of 100% and 22% of erythrocytes, respectively.

4 Discussion

C. albicans is the cause for a variety of infections in humans, ranging from harmless, albeit unpleasant, superficial infections of the skin and mucosa to life-threatening systemic infections. Especially in light of the constantly increasing number of immunocompromised persons due to HIV infection, anti-cancer chemotherapy and organ transplantation, a high percentage of persons are at risk of developing a *Candida* infection. Therefore, the identification of new virulence factors and the development of novel antifungal drugs is an important step in preventing and treating infections by this fungal pathogen.

Yeast-to-hypha transition represents an important virulence mechanism of *C. albicans* during infection of its human host [74]. Hyphae are responsible for excessive tissue invasion and damage and hypha formation is also accompanied by the expression of various hypha-associated proteins, which contribute to the three main steps during an infection: adhesion to, invasion into and damage of the host cell [15, 72, 76]. These proteins include, for example, adhesins and invasins of the Als family as well as secreted aspartic proteases. However, with over 70% of the *C. albicans* ORFs still being uncharacterised, a high number of possibly important virulence factors remain to be discovered.

The aim of this work was to identify the function of the hypha-associated protein Ece1, as previously performed experiments hinted at this protein playing an important role in inducing epithelial damage. Even though Ece1 has already been characterised over two decades ago and is regularly used as a marker for hyphal growth, the exact function of this protein during a fungal infection remained to be elucidated. Ideally, this work would result in the identification of Ece1 function and provide a possible mode of action.

4.1 Involvement of Ece1 in host cell damage

Upon recognition of *C. albicans* yeast or hyphal cells by the host's immune system, activation of the three main MAPK pathways p38, JNK and ERK1/2, the PI3K and the NF-κB pathway is initiated [226, 270–272]. While this initial activation is independent of the morphological form of *C. albicans*, only hyphal cells do trigger a second, much stronger response of these pathways, eventually resulting in the activation of the transcription factor c-Fos and ERK1/2-mediated phosphorylation of the MAPK phosphatase MKP1 [97]. Activation of c-Fos is characterised by an up-regulated production of cytokines, chemokines and other inflammatory mediators, while the phosphatase MKP1 functions as a negative feedback regulator by contributing to the inhibition of MAPK signalling via deactivation of JNK and p38 [97]. As previous work had showed, the

*ece1*Δ/Δ mutant is unique in its ability to form proper hyphae, but not activating the epithelial danger response pathway, therefore ascribing an important function to the Ece1 protein in the discrimination between *C. albicans* colonisation and invasion [301]. Furthermore, *in silico* analysis revealed that orthologues of the *ECE1* gene can only be found in two other members within the CTG clade of *Candida* species, i.e. in *C. dubliniensis* and *C. tropicalis*. Both species are medically relevant, with *C. tropicalis* presenting a quickly emerging non-*albicans Candida* species, which has recently been identified as the most common species associated with candidaemia in Asia [317, 318]. While *C. dubliniensis* is primarily associated with OPC, it is the causative agent of 2-5% of all IC cases, with a crude mortality rate similar to *C. albicans* [66, 68, 319–321]. Finding that *ECE1* orthologues are not present in the less pathogenic *Candida* species demonstrates that this gene may be an important determinant of pathogenicity by influencing the severity of an infection.

4.1.1 *ece1*Δ/Δ mutant versus wild type

The examination of adhesion and invasion properties performed in this study revealed that no differences in these attributes do exist between the *ece1*Δ/Δ mutant strain and the parental wild type BWP17/CIp30. This shows that although the expression of Ece1 is tightly correlated to the formation of hyphae, the protein is not important for the hypha formation *per se* and neither does it function as an adhesin or invasin, as attachment to and invasion into the host cell are not impaired in the deletion strain. On the contrary, the ability to damage host epithelial cells is completely abolished in the *ece1*Δ/Δ mutant, emphasising the contribution of this protein to *C. albicans*-induced tissue damage. Considering this distinctive phenotype of the mutant, the question arises how this strain is able to invade epithelial cells without damaging them during the process. As invasion so far has only been proven by differential staining and subsequent fluorescent microscopy, it is possible that instead of actually piercing the host cell membrane, *ece1*Δ/Δ induces an invagination of the membrane, thereby creating a membrane pocket, which tightly surrounds the elongating hyphal cell. Given the fact that the single Ece1 peptides are secreted after the processing step, such a membrane pocket would also prevent diffusion of these secreted peptides into the surrounding environment and instead allow the accumulation of the peptides, resulting in an increased peptide concentration in close proximity to the host cell membrane, thereby leading to an enhanced effectiveness.

4.1.2 Ece1 is cleaved into single peptides of which Ece1-III is responsible for Ece1-mediated epithelial damage

Bader *et al.* identified Ece1 as a substrate of the proprotein-converting protease Kex2 by performing *in vitro* digestion experiments [296]. This protease is localised in the late trans-Golgi network, indicating that *in vivo* processing of Ece1 occurs intracellularly. The resulting cleavage products, i.e. the individual Ece1 peptides, therefore represent the functionally active form of Ece1 and have been hypothesised to end up secreted into the surrounding environment, given the localisation of the Kex2 protease within the secretory pathway. Indeed, LC-MS/MS analysis, performed at a later time point during this study at our institute, identified Ece1-derived peptides in culture supernatants of a *C. albicans* wild type strain grown under hypha-inducing conditions [301].

Assuming these individual peptides to be responsible for the Ece1-induced damage during infection, synthetically produced peptides were obtained and analysed for their cytolytic properties on both epithelial cells and red blood cells.

Infection of oral epithelial cells with the individual synthetic peptides clearly demonstrated that peptide Ece1-III is responsible for the damaging effect of the Ece1 protein. While co-incubation of the epithelial cells with 10 µM of this peptide resulted in a lysis rate of over 30% after 24h, the remaining peptides could not be observed to exert an effect on the host cells. This result is in accordance to *in silico* analyses predicting only peptide Ece1-III to adopt an α-helical conformation and to localise inside plasma membranes. When added to epithelial cells in combination with a *C. albicans* wild type strain, however, all peptides caused noticeable lysis of the epithelial cells, often exceeding the lysis rate of the wild type alone. On the contrary, such an increase of peptide-induced lysis could not be observed when epithelial cells were co-incubated with a combination of synthetic peptide and the *ece1*Δ/Δ mutant strain. This may suggest that peptides Ece1-I, Ece1-II and Ece1-IV - Ece1-VIII are involved in Ece1-mediated damage, but that the presence of the other peptides, particularly Ece1-III, is needed for these peptides to actively contribute to the damaging effect. While secretion of all Ece1-peptides is given in the presence of a *C. albicans* wild type strain, but not in the presence of the *ece1*Δ/Δ mutant strain, only the combinatorial infection of synthetic peptides with the wild type strain caused a drastic increase of the peptides' damage potential. Elucidation of the exact function of these other peptides therefore is of great interest and remains the work of further research. Combinatorial infection of epithelial cells with the *ece1*Δ/Δ mutant and Ece1-III resulted in a noticeably increased damage compared to infection with the *ece1*Δ/Δ strain alone and caused a slight increase in damage compared to peptide Ece1-III alone (37% versus 32%). This might be due to the hypha formation of the *C. albicans* mutant enhancing the activity of the peptide, possibly by facilitating membrane interaction through the formation of a membrane pocket by the invading hyphae.

Interestingly, it was shown that while Ece1-III$^{62-92}$, which lacks the C-terminal arginine, does cause an overall much lower lysis than Ece1-III$^{62-93}$, combinatorial infection of this peptide with either the *C. albicans* wild type or *ece1*Δ+*ECE1* strain resulted in a higher lysis of epithelial cells (75% and 67%, respectively) compared to an equivalent infection using Ece1-III$^{62-93}$ (67% and 56%, respectively). The finding that less lysis is caused by Ece1-III$^{62-92}$ than Ece1-III$^{62-93}$ in case of a combinatorial infection with the *ece1*Δ/Δ mutant strain might be a hint that full activity of this peptide requires the presence of other Ece1 peptides. This demonstrates that while this peptide alone may be less effective in causing damage on host cells than the peptide with the C-terminal lysine-arginine-motif, lysis by Ece1-III$^{62-92}$ might be superior under certain conditions. This may include an environment more closely resembling *in vivo* conditions, i.e. in the presence of a *C. albicans* strain expressing all Ece1 peptides.

4.1.3 Ece1-III induced damage is rapid and concentration-dependent

Examining how fast lysis by synthetic Ece1-III occurs in comparison to *C. albicans*-induced damage, a *C. albicans* wild type strain, the *ece1*Δ/Δ and reconstituted *ece1*Δ+*ECE1* strain as well as 10 µM of synthetic peptide were co-incubated with different epithelial cell lines for 3h and 6h, respectively. The result showed that lysis by synthetic Ece1-III is rapid, with 13 – 32% of the epithelial cells being lysed within three hours, depending on the cell line. While lysis of both oral and vaginal epithelial cells seems to be initiated immediately, lysis of gastrointestinal epithelial cells was observed to happen more slowly, with a total of 22% of the host cells being lysed after 6h, compared to an averaged 30% of oral and vaginal cells being lysed in the same time frame. On the contrary, first damage induced by the *C. albicans* wild type and reconstituted strain was detectable after 6h. However, considering that first *ECE1* transcripts are found 30 min after the stimulation of hyphal growth, and the production of active peptides includes further steps such as translation, processing and secretion, damage being detectable within 6h also appears rather rapid. The fact that Ece1 is expressed and secreted in such a fast manner underscores the relevance of this protein in infection-associated epithelial damage during a *C. albicans* infection.

Incubation of epithelial cells with different concentrations of synthetic Ece1-III also showed that damage is induced concentration-dependently. Peptide-induced lysis proportionally increased for both Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ with rising concentrations. Using melittin as a positive control demonstrated that Ece1-III is less cytotoxic than the bee-venom derived toxin, as melittin caused a remarkably higher lysis of epithelial cells than either of the Ece1-III variations. This may be due to the molecular mechanism underlying the lytic activity of the peptides. It is, for example, possible that melittin is better adapted to the lipid composition present in the plasma membrane of human epithelial cells and might preferentially bind to lipids that are ubiquitous. Moreover, it

might be of importance that Ece1-III is produced by an opportunistic pathogen and therefore its evolutionary purpose may originally lie in accessing nutrients or competing against other microorganisms inside the fungus' environmental niche, while bee venom is actively produced to ward off enemies, therefore requiring a stronger toxicity.

It could be observed that at a concentration of 20 µM, melittin did cause less lysis than at a concentration of 15 µM. This may indicate that peptide saturation is eventually reached and that a higher peptide concentration will result in an oversaturation favouring peptide-peptide interaction instead of peptide-membrane interaction. Such an effect could not be observed for either peptide Ece1-III$^{62-93}$ or Ece1-III$^{62-92}$ in the respective concentrations, demonstrating that even at high micromolar concentrations peptide efficiency is not impaired.

4.1.4 Influence of hypha formation and cell viability on peptide-induced epithelial damage

As has been shown in previous experiments, *C. albicans* hyphae contribute to an increase of peptide-induced epithelial damage, even if the hypha-forming strain itself is not causing damage, as in the case of the *ece1*Δ/Δ mutant strain. By using *C. albicans* mutants that are locked in the yeast form (*cph1*Δ/*efg1*Δ) or unable to maintain hyphal growth (*eed1*Δ/Δ) this could be confirmed. Combinatorial infection of epithelial cells with neither of these strains together with Ece1-III did result in an elevated lysis rate compared to infection with only the peptide. The same result was found for the budding yeast *S. cerevisiae*, which is also non-pathogenic. *C. glabrata*, a yeast species which is evolutionary more closely related to *S. cerevisiae* than *C. albicans*, could also be shown not to bring about damage of epithelial cells. Interestingly, when epithelial cells were co-incubated with a combination of *C. glabrata* and synthetic Ece1-III, lysis rates comparable to equivalent experiments using a *C. albicans* wild type strain were reached. Therefore, *C. glabrata* so far represents the only known non-hypha forming yeast enhancing Ece1-III-induced damage of epithelial cells. This indicates that also in case of *C. albicans* factors other than hypha formation alone might be involved in augmenting peptide-induced damage. Such factors may for example include hydrolytic enzymes that aid in the disintegration of epithelial cell-cell contacts, leading to the loss of epithelial integrity and possibly facilitating Ece1-III activity. As, in contrast to the rest of the *Candida* species, *C. glabrata* does not produce secreted proteases which are released into the extracellular space [322, 323], hydrolytic enzymes of a different kind can be expected to be involved in *C. glabrata*-mediated enhancement of Ece1-III activity, e.g. phospholipases or lipases. Furthermore, *C. glabrata*, among other non-*albicans Candida* species, has been shown to be able to degrade haemoglobin via the utilisation of haemolysins. In *C. glabrata*, the haemoglobin-like protein (HLP) gene has been associated with haemolytic activity exhibited by this fungus [324]. Having shown that the *C. albicans* peptide

Ece1-III itself is able to induce erythrocyte lysis (see part 4.1.5), the assumption of a *C. glabrata* haemolysin enhancing its activity may be plausible.

Performing combinatorial infections of a reconstituted human vaginal epithelium (RHVE) with both *C. albicans* and *C. glabrata*, Alves *et al*. were able to demonstrate that both colonisation and invasion of the epithelium by *C. glabrata* is significantly enhanced in the presence of a *C. albicans* strain [325]. Even though only half the inoculum of *C. albicans* cells was used for a co-infection as opposed to an infection with *C. albicans* alone, tissue damage was found to be higher during a co-infection of the epithelium with *C. glabrata* and the *C. albicans* strain ATCC 90028 than with the *C. albicans* strain alone [325]. Additionally, quantitative RT-PCR revealed that the relative expression of *EPA* genes, a major group of *C. glabrata* adhesins [326], is down-regulated or absent during a co-infection with *C. albicans*, suggesting that the increase of *C. glabrata* colonisation and invasion is mediated by *C. albicans*-associated factors. Indeed, expression of the *SAP4-10* genes as well as genes of the *ALS* family could be shown to be up-regulated in *C. albicans* during a co-infection [325]. As these results indicate that pathogenicity of *C. glabrata* is enhanced through the epithelial damage induced by *C. albicans*, studies on the relative expression of *ECE1* during a co-infection with *C. glabrata* may prove to generate interesting results. The increased damage potential of *C. glabrata* observed during co-incubation with Ece1-III indicates that a highly up-regulated expression of only *ECE1* may already be sufficient to enhance *C. glabrata* pathogenicity. *In vivo*, however, the expression of a variety of *C. albicans* virulence factors may contribute to enabling *C. glabrata* to better colonise and invade host cells. RT-PCR performed in our laboratory for example showed that expression of *ECE1* is strikingly correlating to the expression of different *SAP* proteases, showing that the expression of different virulence factors is precisely coordinated.

An indicator for the involvement of enzymatic factors in the augmentation of Ece1-III-induced damage is the finding that this effect is lost in heat-killed cells of both *C. albicans* and *C. glabrata*. Confirmation for this hypothesis could be obtained via simultaneous co-incubation of epithelial cells with both synthetic Ece1-III and various purified hydrolytic enzymes from both *C. albicans* and *C. glabrata*. An involvement of *C. albicans* Saps might furthermore be verified by co-incubation of synthetic Ece1-III and a *C. albicans* wild type strain in the presence of the Sap inhibitor pepstatin A.

4.1.5 Ece1-III induces erythrocyte damage

C. albicans has long been known to produce and secrete a haemolytic factor which is involved in iron acquisition from host erythrocytes [327, 328]. While Watanabe *et al.* were able to characterise this factor as a sugar moiety of a *C. albicans* mannoprotein, the exact identity of this protein and the molecular mechanism underlying erythrocyte lysis could not be determined [174]. They did, however, conclude that the activity of secreted aspartic proteases is not involved in erythrocyte lysis, as the addition of the potent aspartic protease inhibitor pepstatin A did not influence the haemolytic activity of *C. albicans*. Additionally, enzymatic activity could be ruled out as being responsible for erythrocyte lysis by incubation of the culture supernatant at 100°C. Heat-inactivation did not affect the haemolytic properties of *C. albicans*, providing further evidence that haemolysis is not mediated by proteolytic enzymes [174].

Given the structural similarity between the Ece1-derived peptide Ece1-III and the bee venom toxin melittin that has been found through *in silico* analysis, it was hypothesised that Ece1 may very well represent the *C. albicans* haemolytic factor, as melittin is a pore-forming toxin which efficiently integrates into and disrupts erythrocyte membranes [329–331]. The involvement of Ece1 in the lysis of erythrocytes is further supported by the finding that *ECE1* expression is induced in the presence of haemoglobin [332].

It could be shown in this study, that synthetic Ece1-III does indeed exert a potent cytolytic effect on human erythrocytes. Using different peptide concentrations and performing a time course experiment, it could be demonstrated that both Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ do rapidly and efficiently lyse red blood cells, with Ece1-III$^{62-93}$ being active at much lower concentrations than Ece1-III$^{62-92}$ though. The peptide featuring the C-terminal lysine-arginine-motif was also found to induce haemolysis more rapidly, with almost 100% of the erythrocytes already being lysed after 45 minutes of incubation. Ece1-III$^{62-92}$-induced lysis in contrast is still increasing after 180 min, indicating that a higher lysis rate may be reached at some point. Considering the fact that a concentrated erythrocyte suspension as well as micromolar concentrations of the synthetic peptides have been used for these experiments, it is rather unlikely that the haemolytic activity of Ece1-III does play a significant role *in vivo* in the bloodstream. This peptide may, however, be a possible candidate for the production of haemocidins in the vaginal niche through the lysis of haemoglobin present in the menstrual discharge. Haemocidins are a group of microbicidal peptides arising from haem-binding proteins such as haemoglobin [333]. *In vivo*, such peptides can be found in tick guts [334–336], in human post-partum uterine fluid [337] or in the menstrual discharge [338, 339]. In the latter, these peptides might strongly influence the composition of the vaginal microflora and therefore contribute to a *C. albicans* overgrowth, eventually resulting in vaginal candidiasis. Even though Bocheńska *et al*. could demonstrate that *C. albicans* secreted aspartic proteases are able to liberate bactericidal haemocidins from haemoglobin, with the

liberated peptides showing a strong killing activity against *Lactobacillus acidophilus* and, to a lower degree, against *E. coli* [340], the exact process underlying the generation of menstrual haemocidins remains to be elucidated. Hence, a role of Ece1-III in the production of these bactericidal peptides cannot be excluded.

4.1.6 Impact of the pH on Ece1-III-mediated erythrocyte lysis

As *C. albicans* encounters a variety of environmental niches when colonising or infecting its human host, an effective virulence factor needs to be active in a broad range of different pH values. In isotonic media a decrease of the pH over the range of 9.4-4.8 has been shown to result in an increased erythrocyte volume, a decrease in density and a more spherical shape of these blood cells [341]. Therefore, the haemolytic properties of Ece1-III have only been tested at a pH between 5-8, to prevent the occurrence of false-positive results arising from pH-induced impairment of erythrocyte viability. No differences in lysis efficiency could be observed at the tested pH values, demonstrating that at least in the range that has been tested the pH value does not influence the haemolytic activity of Ece1-III. This is not surprising, as many known toxins have been shown to be active among a wide range of pH values. The VacA toxin released from *Helicobacter pylori* for example is activated by a short exposure to acidic solutions (pH 1.5-5.5) and once activated remains stable at pH 1.5 and can even resist pepsin digestion at pH 2 at 37°C [342]. In the case of *H. pylori* VacA, a low pH also mediates the disassembly from an oligomeric structure into monomers, resulting in the exposure of hydrophobic patches that are crucial for insertion into a host membrane [343, 344]. This transition only takes place at a very specific pH around 5.2 [342, 344], illustrating how tightly toxin function can be dependent on an environmental cue. Given the fact that only a low range of pH values could be tested in this study due to the above mentioned viability issues of the erythrocytes, this may explain the differences that could be observed between Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$, as the optimal pH for a more efficient haemolysis by Ece1-III$^{62-92}$ might be outside the tested range. Hypothesising a role of Ece1-III in the generation of menstrual haemocidins, as discussed in part 4.1.5, full activity may very well be reached at more acidic pH values, such as pH 4, corresponding to the vaginal pH and to the finding that Sap-mediated hydrolysis of haemoglobin, resulting in the production of haemocidins, was also most effective at pH 4 [340].

4.1.7 Ece1-III induced erythrocyte lysis is inhibited by human serum

Haemolysis experiments carried out in the presence of human blood serum demonstrated that the serum exerts a highly inhibiting effect on Ece1-III-induced erythrocyte lysis. In the presence of 0.2% human serum, lysis by Ece1-III$^{62-93}$ was reduced by 50%, while at a concentration of 1%, lysis reduction reached 75%. Lysis induced by Ece1-III$^{62-92}$ could even be completely abolished by the addition of human serum in a concentration of 1% and 0.5%. Based on the fact that *C. albicans* must acquire the essential micronutrient iron from the host and assuming that Ece1-III is the main factor responsible for erythrocyte lysis, inactivation of this peptide by human serum will result in an iron deficiency and eventually lead to an impaired growth and ultimately death of the fungal cell, as the iron-binding haem cannot be released from the erythrocytes. This is in accordance with the results of earlier studies that demonstrated a growth inhibition of *C. albicans* in the presence of human serum [345–348]. Regarding the fact that serum makes up an approximate 55% of whole blood, Ece1-III function should be effectively inhibited *in vivo*, thereby preventing *C. albicans* iron sequestration from the host and inhibiting fungal growth. Human blood serum is composed of approximately 91% of water, 7% of proteins and 2% of electrolytes and low molecular substances. Albumins represent the majority of serum proteins, making up 60% of the total serum protein content [314]. Therefore, in the attempt to identify the Ece1-III-inhibiting serum component, serum albumin was chosen to be tested for its effect on Ece1-III-induced haemolysis. Different albumin concentrations were tested, ranging from 40 µg/ml to 2 mg/ml. While even at the highest concentrations albumin could not be observed to exert an inhibiting effect on red blood cell lysis by Ece1-III$^{62-93}$, a minor inhibition of Ece1-III$^{62-92}$-induced erythrocyte lysis could be detected. However, in comparison to the inhibitory effect of whole serum in which albumins are present in a much lower concentration, the effect of purified albumins is very low and it therefore appears unlikely that these proteins represent the serum component responsible for the inhibiting effect on Ece1-III. Previous studies demonstrated an involvement of serum transferrin in the inhibition of *C. albicans* growth in human serum [347, 349–351]. This iron-chelating protein makes up 4% of the serum proteins and is responsible for the binding of free serum iron and iron transport. “Nutritional immunity”, resulting from the binding of iron within proteins, effectively prevents microbial growth [166, 167]. Indeed, addition of free iron at a concentration exceeding transferrin saturation could be shown to diminish the inhibitory effect of human serum on *C. albicans* growth [349]. Usually, iron is bound to approximately 30% of the available iron-binding sites within the transferrin molecules. This means that residual binding sites are available to bind additional iron that may be released from erythrocytes by Ece1-III. Whether nutritional immunity is responsible for the inhibitory effect of human serum on Ece1-III-induced erythrocyte lysis may be verified by repeating the lysis experiment in the presence of human serum and simultaneously adding free

iron in a concentration leading to transferrin oversaturation. Additional iron resulting from erythrocyte lysis and subsequent breakdown of haem could then not be effectively sequestered by transferrin, thereby enabling growth of *C. albicans*.

4.1.8 An ion-dependent mechanism is involved in Ece1-III-induced haemolysis

Ece1-III-induced erythrocyte lysis could be shown to be significantly reduced in the presence of the potent chelating agent EDTA. This chelator is known to form stable complexes with divalent cations, e.g. calcium, magnesium or zinc ions. As removal of such ions from the surrounding medium was shown to impair erythrocyte lysis, it can be assumed that the molecular mechanism underlying Ece1-III-mediated haemolysis is ion-dependent. Divalent cations play an important role in various processes connected to microbial toxins. Heavy metal ions for example represent essential cofactors for the activity of metalloproteases, which often act as receptors for pore-forming toxins [352]. Ion-chelation would therefore impair receptor activity, resulting in a reduced toxin function. The strong effect that EDTA-mediated ion-chelation was shown to exert on Ece1-III activity suggests that the cytotoxic mechanism of this peptide may involve the activity of a metalloprotease. As the majority of known metalloproteases is zinc-dependent, it would be beneficial to repeat this experiment using a metalloprotease inhibitor such as *o*-Phenanthroline instead of EDTA, as this compound exhibits a considerably higher stability constant for zinc than for calcium [353]. A further possibility explaining the ion-dependency of Ece1-III-mediated cytotoxicity would be the initiation of cellular signalling pathways within the host cell in response to Ece1-III. Divalent cations are potent second messengers involved in the activation of various cellular processes. Particularly calcium has been shown to be responsible for many intracellular signalling pathways [354]. As Mg^{2+} is chelated as well as Ca^{2+} by EDTA, Ece1-III-induced signalling may, however, also be dependent on Mg^{2+}. Indeed, it was shown that the addition of an excess amount of Mg^{2+}-ions to cells pre-treated with EDTA restored the cytotoxicity of Ece1-III. In case of Ece1-III$^{62-92}$, the addition of 100 mM of $MgCl_2$ to the sample pre-treated with EDTA even resulted in a lysis rate higher than that of the peptide alone, supporting the hypothesis that Ece1-III may induce Mg^{2+}-concentration-dependent pathways. Due to the experimental setup, unfortunately no comparative experiment could be performed with calcium ions. The dependency of cytotoxic function on divalent cations has also been demonstrated for bacterial toxins, e.g. the *Bacillus thuringiensis* Cry1Aa and Cry1Ab toxins [352, 355]. Furthermore, Kirouac *et al.*showed that the inhibition of *B. thuringiensis* Cry1Aa by EDTA was reversed by addition of an excess amount of calcium, magnesium or barium [352], while the cytotoxicity of Cry1Ab was only restored with magnesium, but not calcium ions [355].

4.1.9 Role of membrane cholesterol for Ece1-III activity

Preliminary results obtained from haemolysis experiments using cholesterol-depleted erythrocytes indicated this membrane lipid to exhibit a protective effect against lysis by Ece1-III. Red blood cells that were pre-treated with the cholesterol-depleting compound methyl-ß-cyclodextrin clearly demonstrated a decreased resistance to Ece1-III-mediated damage. A similarly protective effect of cholesterol could be observed for the bee venom-derived toxin melittin [356, 357]. The haemolytic activity was shown to increase ~3-fold when cholesterol within the erythrocyte membranes was depleted by ~55% [358]. Furthermore, the antifungal agent syringomycin E had been demonstrated to exhibit an increased pore-forming activity on erythrocyte membranes when these were depleted of cholesterol [359]. Erythrocyte membranes contain high amounts (~45 mol%) of cholesterol [360], which is an essential component of eukaryotic membranes. This lipid is involved in membrane organisation and dynamics and has been suggested to play a role in the maintenance of lipid rafts [360–362]. As cholesterol is known to induce tight phospholipid acyl chain packing in cell membranes, depletion of this lipid may facilitate the insertion of peptide toxins into the then loosened structure of the membrane, thus enhancing toxin activity [360, 363]. In addition to cholesterol, bacterial lipopolysaccharides (LPS) could be shown to also exert a protective effect against melittin, possibly due to a similarly tight packing of the lipid acyl chains that can be observed for cholesterol [364]. Hypothesising that LPS might also reduce the activity of Ece1-III, gram-negative bacteria should prove more resistant against this fungal peptide toxin than gram-positive bacteria, given the fact that only those contain LPS in their outer membrane structure.

On the other hand, however, there are also toxins whose activity is dependent on the presence of cholesterol and in which case cholesterol depletion results in a decreased cytotoxicity. Such examples are the large clostridial cytotoxins toxin A and toxin B from *Clostridium difficile*. Giesemann *et al*. were able to show that treatment of membranes with MßCD resulted in a reduced pore formation, as was determined by a decreased efflux of the radioactive cation $^{86}Rb^{+}$, which was used to preload the cells prior to their exposure to the toxins [365].

Given the structural similarity between melittin and the *C. albicans* peptide Ece1-III, as well as the first results obtained from experiments utilising cholesterol-depleted erythrocytes, it is tempting to speculate that cholesterol exhibits the same effect on Ece1-III than it does on melittin. Further experiments are, however, needed to gain a more detailed knowledge about the importance of the membrane cholesterol content on Ece1-III activity. Moreover, it needs to be verified that the observed sensitivity of cholesterol-depleted erythrocytes to Ece1-III-mediated lysis is not caused by an overall alteration of the membrane composition induced by MßCD.

4.1.10 *ECE1* deletion results in the inability to lyse red blood cells

The importance of Ece1 during growth with erythrocytes as the only nutrient source was also demonstrated by erythrocyte lysis experiments using the *ece1*Δ/Δ mutant strain. While the null mutant with both *ECE1* alleles deleted was not able to release haemoglobin from the erythrocytes, efficient haemoglobin release by both tested wild type strains (SC5314 and BWP17/CIp30) was observed. Through reintegration of one of the *ECE1* alleles, haemoglobin release from the blood cells was restored. The finding that all *ece1*Δ+*ECE1*[ΔEce1-III] mutants, which are only deficient in the Ece1-III-encoding part of *ECE1*, exhibit the same phenotype as the *ece1*Δ/Δ null mutant, confirms the previous results of only Ece1-III being able to lyse erythrocytes. Besides *ece1*Δ/Δ, three other *C. albicans* mutants were tested for their ability to release haemoglobin from red blood cells, namely the *kex2*Δ/Δ, *eed1*Δ/Δ and *cph1*Δ/*efg1*Δ mutants. As expected, due to the involvement of Kex2 in Ece1 processing, the *kex2*Δ/Δ mutant did not induce haemoglobin release. As an active Kex2 protease, however, is important for a plethora of proteolytic processes in the fungal cell, deletion of the *KEX2* gene has a strong impact on *C. albicans* growth and a homozygous mutant is not capable of proper hypha formation, but instead shows a pleomorphic growth. Therefore, the inability of the *kex2*Δ/Δ mutant to release haemoglobin from erythrocytes cannot solely be ascribed to the importance of Kex2 activity for Ece1 function. Furthermore, neither the *eed1*Δ/Δ nor the *cph1*Δ/*efg1*Δ mutant was able to lyse erythrocytes. That can however be attributed to the fact that both mutants lack the ability to form hyphae. While *cph1*Δ/*efg1*Δ is completely locked in the yeast form, *eed1*Δ/Δ does induce an initial hypha formation but switches back to yeast growth eventually. Furthermore, Martin *et al*. showed that in the *eed1*Δ/Δ mutant expression of *ECE1*, among several other hypha-associated genes, was down-regulated during growth on plastic and infections of reconstituted human epithelia [366]. Down-regulation of *ECE1* expression may also be expected for the *cph1*Δ/*efg1*Δ mutant. Although Cph1 is not required for *ECE1* expression, deletion of *EFG1* results in an abrogation of *ECE1* expression [298, 367].

4.1.11 Cytolytic activity of peptides obtained through *in vitro* digestion of rEce1

A different approach to examine the cytolytic activity of single Ece1 peptides was taken by overexpressing both Ece1 and Kex2 and utilising the recombinant proteins for *in vitro* digestion and subsequent use of the digestion products in haemolysis assays. Both proteins could be successfully overexpressed and purified using the respective expression systems *E. coli* BL21(DE3) and *P. pastoris* ScKex2. Likewise, Kex2-mediated digestion of rEce1 was verified. Digestion efficiency, however, was observed to be very low, as a large amount of the full length

protein remained intact. This might be due to different reasons, including the finding that the recombinant Ece1 seems to be prone to the formation of multimers. In fact, protein bands correlating to multiples of the molecular weight of rEce1 were detected in rEce1 preparations in this study. Low digestion efficiency and the formation of multimers might also be caused by the His-tag attached to the N-terminus of the protein. The additional amino acids constituting the tag might negatively influence the activity of the Kex2 protease, resulting in an impaired or incomplete digestion of the substrate. Furthermore, the protein's His-tag may lead to a stronger interaction between several protein molecules, thereby facilitating the formation of rEce1-multimers. Additionally, it has to be considered that an *S. cerevisiae*-derived Kex2 protease was utilised for these experiments, instead of the orthologous *C. albicans* protease, as attemps to express this enzyme in *P. pastoris* failed [296]. Even though *Ca*Kex2 is a functional homologue of *Sc*Kex2 [304], processing of *C. albicans* proteins might be carried out more successfully in its native host.

SDS-PAGE of the rEce1 digestion products clearly showed proteins of low molecular weight (~10-20 kDa) to result from Kex2 cleavage. These products, however, were not detected in Western Blots. Blotting was performed using a polyclonal anti-Ece1-III antibody, meaning that polypeptides present after Kex2 processing are only detectable as long as Ece1-III is part of these polypeptides. The fact that none of the protein bands in this size range was detectable with the antibody may indicate that the release of Ece1-III may happen early during the cleavage process. Peptides with a molecular weight below 10 kDa on the contrary were detected by Western Blotting and their location on the membrane in comparison to synthetic Ece1-III suggests these peptides were the result of complete Kex2 cleavage, i.e. to be present as single Ece1 peptides with a molecular weight of approximately 1.5-4.5 kDa.

Haemolysis experiments performed with peptides derived from *in vitro* digestion of rEce1 unfortunately remained inconclusive. Only a low erythrocyte lysis rate (13%) was observed after the addition of 40 µM of rEce1, while lysis induced by 40 µM of digested rEce1 was only minimal (2%). The reason why erythrocyte lysis could be detected to be carried out by the full length rEce1 protein, which in principle should not act cytolytically, may be based on spontaneous disaggregation of the polypeptide within the sample. Proteolytically digested rEce1 should then however be expected to induce at least as much lysis as the full length protein in the same concentration. The determination of the exact concentration of digested protein, however, is difficult, as protein assays do not provide information about the molecular weight of the proteins present inside the sample. Moreover, the Kex2 protease is still present in the digested samples and may exert an unknown influence on the activity of the peptides. More experiments and modified methods for *in vitro* purification of Ece1 peptides therefore are needed to further examine the haemolytic activity of peptides derived from recombinantly expressed Ece1.

4.1.12 Single peptide knockout-mutants

Given that of the eight synthetic Ece1-peptides only Ece1-III was found to exert damage on both epithelial cells and red blood cells, a knockout mutant had been constructed which, in contrast to the *ece1*Δ/Δ strain, is not deficient in the complete *ECE1* encoding sequence, but only lacks the part of the gene sequence coding for Ece1-III. Three different clones of this strain were used in this study to examine whether this partial deletion would sufficiently abolish Ece1-induced damage. Analysis of the cytotoxicity exerted by these *ece1*Δ+*ECE1*[ΔEce1-III] strains was performed by monitoring damage of oral epithelial cells and through quantifying erythrocyte lysis. Both assays revealed that the cytotoxicity exerted by all three clones was similar to that of the *ece1*Δ/Δ null mutant. To exclude the possibility that this impaired cytotoxicity might be due to morphological restrictions, the ability of the clones to form proper hyphae was verified via hypha induction experiments. Expression studies performed in our laboratory furthermore confirmed that the altered Ece1 protein is actually expressed and that the expression rate is comparable to that observed for the reconstituted strain *ece1*Δ+*ECE1*. Therefore, the loss of the ability of the *ece1*Δ+*ECE1*[ΔEce1-III] strain to damage host cells is likely to arise from the lack of peptide Ece1-III. Nevertheless, a single peptide knockout mutant deficient in Ece1-VII instead of Ece1-III, which had been constructed as a means of comparison, exhibited an identical phenotype, i.e. no damage of host cells was caused by this strain, even though hypha formation and expression of the altered Ece1 protein were not impaired. This demonstrates that although the peptides besides Ece1-III may not be directly involved in the induction of host cell damage, a completely intact Ece1 protein is crucial for full activity. Considering that the protein needs to be proteolytically cleaved, alteration of the primary amino acid structure via the deletion of single peptides might result in a faulty cleavage process. Furthermore, the other peptides may exert a chaperoning function by shielding the cytotoxic peptide Ece1-III until after secretion and lack of these chaperones might result in the degradation of Ece1-III to protect the own cell. Ece1-III has so far been shown to not act cytolytically on *C. albicans* or other yeast species, as demonstrated by growth experiments in the presence of 70 µM of the peptide, a concentration which is rather unlikely to be reached under *in vivo* conditions. However, as experiments have only been carried out with exogenously added peptide, the effect of free intracellular Ece1-III on *C. albicans* viability is not known. Given the different composition of fungal versus mammalian and bacterial plasma membranes, particularly characterised by the presence of the lipid ergosterol instead of cholesterol, a protective mechanism of *C. albicans* against its own lytic peptide may be assumed, but remains to be elucidated.

4.2 Interaction of Ece1-III with the host cell membrane

In order for a peptide to elicit damage on host cells, the first step is interaction with the host's cell membrane. In the case of all bacterial PFTs, this is achieved via binding to specific receptors, e.g. lipids, proteins, or sugars [275]. Interaction with these host membrane components will result in an increased concentration on the host cell surface and facilitate peptide oligomerisation and pore formation. Examination of Ece1-III interaction with various phospholipids revealed that this peptide strongly binds to several lipids that can be found as part of the host cell's membrane, i.e. phosphatidylinositol-4-phosphate, phosphatidic acid and phosphatidylserine. As a distinctive binding between Ece1-III$^{62-93}$ and phosphatidylserine was observed in three replicate experiments, the focus was directed to the interaction with this specific membrane lipid. This phospholipid is typically localised in the inner leaflet of a bilayer membrane and while it sporadically gets externalised to the outer leaflet, it usually is rapidly relocated to the inner leaflet by flippases [368]. Different studies, however, have demonstrated that rapid externalisation of phosphatidylserine, independent of apoptotic processes, can be induced via interaction of different types of bacteria with the host plasma membrane. Murata-Kamiya *et al.* were able to show, that interaction of the oncoprotein CagA of *H. pylori* with phosphatidylserine does play a key role in mediating delivery, localisation and the pathophysiological action of CagA [369]. They could observe that externalised phosphatidylserine remained at the position of bacterial attachment, suggesting that bacterial contact may result in an inhibition of the host flippase. Accordingly, shielding of the surface-exposed phosphatidylserine by annexin V or an anti-phosphatidylserine antibody successfully impaired CagA delivery into the host cell [369]. An earlier study also observed phosphatidylserine externalisation on the host cell's surface during a *Chlamydia* infection [370]. A similar mechanism of active phosphatidylserine externalisation may therefore be hypothesised for Ece1-III of *C. albicans*. Indeed, initial biophysical experiments using artificial membranes of different lipid composition revealed that membranes containing phosphatidylserine are faster and more effectively lysed by Ece1-III than membranes lacking this phospholipid (unpublished data, personal communication with T. Gutsmann). Another membrane lipid that has been suggested to influence the activity of Ece1-III is cholesterol (see part 4.1.9). However, while the interaction between Ece1-III and phosphatidylserine seems to induce the binding to the host cell membrane, the amount of cholesterol present inside the membrane rather affects the efficiency of membrane insertion, due to the impact of this lipid on membrane fluidity.
While binding to phosphatidylserine seems to be involved in establishing an initial cell-cell contact, it may be assumed that proper binding and induction of monomer oligomerisation is mediated via interaction with a receptor molecule, such as a lipid raft component, a GPI-anchored protein or a metalloprotease present in the host cell membrane. Experiments using oral epithelial cells and erythrocytes have demonstrated that micromolar concentrations of Ece1-III are needed

in vitro to obtain efficient lysis of those cells. Considering that invading *C. albicans* hyphae form a membrane pocket, which may prevent excessive diffusion of the secreted peptides, such concentrations may indeed be accomplished *in vivo*. For the alpha-haemolysin (Hla) of *S. aureus*, a well-known PFT with haemolytic properties, it has been shown that toxin concentrations exceeding 1 µM are needed to initiate significant binding and haemolysis of human erythrocytes [371]. Nevertheless, a high-affinity receptor-mediated host cell binding would be beneficial at low peptide concentrations. The dependency of Ece1-III activity on the presence of divalent cations, which could be shown via chelation experiments using EDTA (see part 4.1.8), might hint at a metalloprotease as a proteinaceous receptor. A metalloprotease often found to act as a receptor for bacterial toxins such as *S. aureus* Hla is a disintegrin and metalloprotease 10 (ADAM10) [283]. This enzyme contains an extracellular zinc-dependent metalloprotease domain, which catalyses the cleavage of a variety of host proteins [372]. E-cadherin, a protein present in the adherens junctions of epithelial cells and conveying cell-cell contact, represents a substrate of ADAM10 [372]. *S. aureus* Hla has been shown to enhance this ADAM10-mediated cleavage of E-cadherin, thereby inducing the loss of epithelial integrity and promoting bacterial virulence [373–375]. However, not all PFTs do interact with specific receptors. The bee venom-derived peptide melittin, for example, is proposed to interact with membrane lipids rather than a specific protein receptor, since there are $1.8x10^7$ binding sites for this peptide per erythrocyte [330]. Verification of receptor-mediated Ece1-III-binding and the identification of the specific membrane component acting as receptor therefore should be the aim of future research.

4.3 Ece1-III is a pore forming toxin

Several findings about peptide Ece1-III led to the conclusion that this peptide does act as a pore-forming toxin. This includes the short length and amphipathic nature of the peptide as well as the *in silico* prediction of the peptide to form an α-helical structure and to localise inside the membrane. Furthermore, binding of Ece1-III to several phospholipids that are present in host cell membranes could be demonstrated in this study. The cytotoxic effect of most pore-forming proteins is induced via changes of the ion concentration within the host cell. Once the membrane barrier is disrupted by a pore, the ions can flow freely, creating an intracellular imbalance eventually leading to the death of the cell. By using the potent ion chelator EDTA, the damaging mechanism of Ece1-III was shown to be ion-dependent too, further supporting the view that this peptide indeed induces pores in host cell membranes. Biophysical experiments using artificial membranes, carried out by our collaborating partner T. Gutsmann and his colleagues, also demonstrated that Ece1-III shows characteristics of a pore-forming toxin.

Summarising all of the above results, a model of Ece1-III-induced pore formation was established (Figure 37). Whether the type of pore formed corresponds more closely to the toroidal or barrel stave model cannot be predicted at this time point. Given the structural similarity to melittin, lysis might occur analogous for Ece1-III. The honeybee toxin is proposed to induce lysis by a colloid osmotic mechanism, resulting in the formation of membrane lesions [330]. This lysis mechanism is characterised by an equilibration of ions through the pore inside the host cell, followed by the influx of water and subsequent cell swelling and finally cell lysis. While the elucidation of the exact molecular mechanism of Ece1-III-induced lysis remains the work of future research, enough data have been gathered to this point to demonstrate that Ece1-III is in fact the first known peptide toxin of a human fungal pathogen. Due to its toxin nature, this peptide is now referred to as "Candidalysin" [301].

Considering that *C. albicans* mainly exists as a harmless coloniser of human mucosal surfaces, the main function of this pore-forming toxin may have been the exploitation of nutrients in its natural environment. While *in vitro* the expression of *ECE1* is invariably connected to the formation of hyphae, an attribute highly associated to fungal virulence, a different picture may arise under *in vivo* conditions. It has, for example, been demonstrated that inside the murine intestinal tract high levels of Ece1 are also expressed by *C. albicans* yeast cells during commensal colonisation [300]. Additionally, as has been shown by Moyes *et al.*, colonising *C. albicans* cells can also exist in the hyphal form without triggering a pro-inflammatory response pathway, when the number of cells does not exceed a certain threshold [226]. Therefore, it can be assumed that the original function of Candidalysin may have served the task of nutrient acquisition and competition with other colonising microorganisms in the commensal stage, while in the case of *C. albicans* overgrowth or impairment of the host's immune system the expression of this peptide toxin inevitably results in the damage of host tissue.

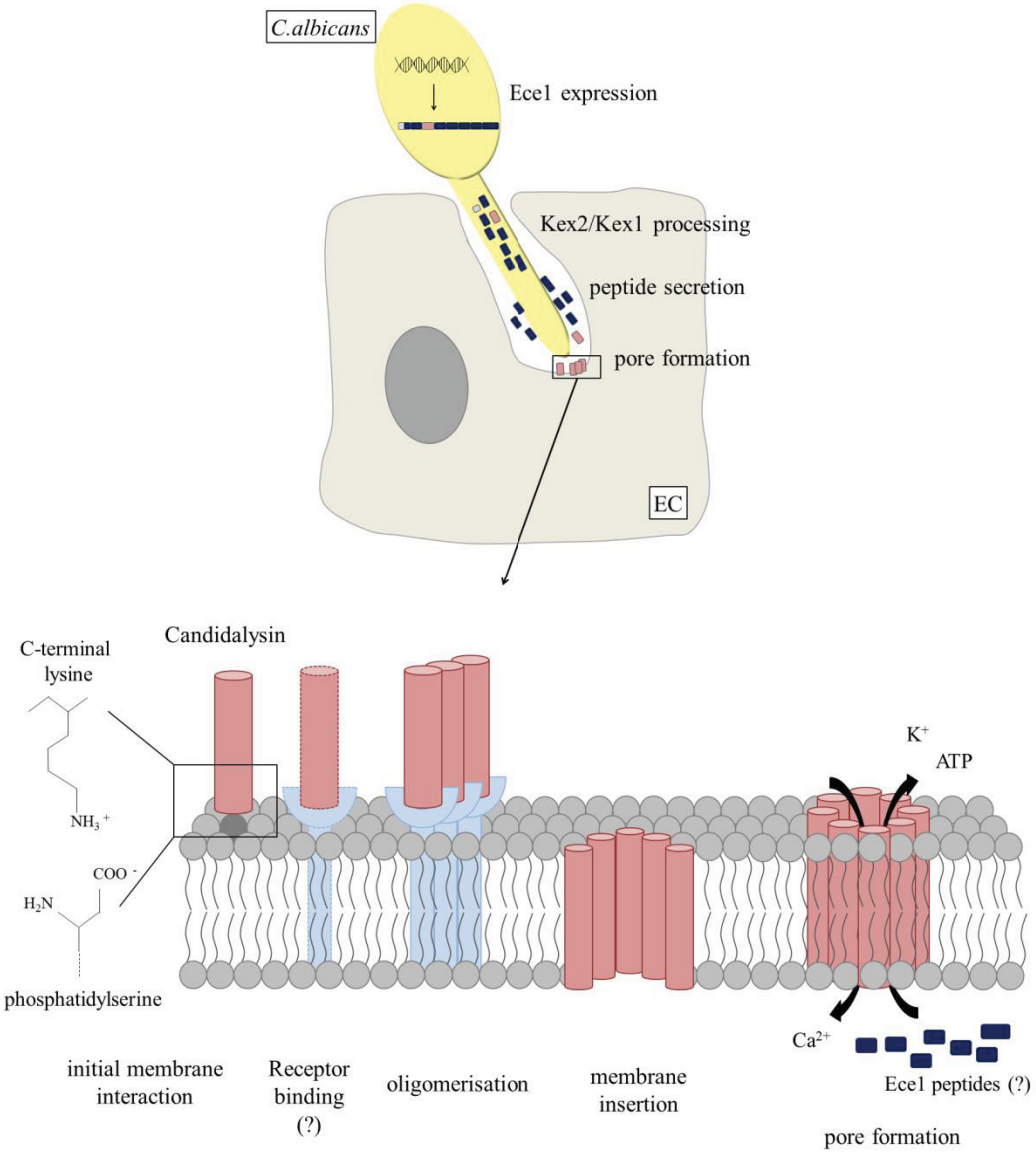


Figure 37: Model of Candidalysin-induced pore-formation. After expression of the Ece1 polypeptide precursor, the protein is hypothesised to be successively processed by the proteases Kex2 and Kex1. The resulting peptides will then get secreted and while the Candidalysin monomers interact with the host membrane, finally inserting and forming pores, the remaining peptides may influence pore formation, enter the host cell through the Candidalysin pore and exert an as yet unknown function inside the host cell or may have other unknown functions.

4.4 Ece1-III$^{62-93}$ versus Ece1-III$^{62-92}$

Ece1-III with a C-terminal lysine-arginine motif (Ece1-III$^{62-93}$) was predicted to result from processing by the protease Kex2. At a later time point of the study, however, data obtained from liquid chromatography/mass spectrometry indicated, that the peptide may rather be secreted without the C-terminal arginine. Repetition of the experiments including this slightly different version of the peptide showed that, while this peptide is still causing noticeable damage on both epithelial cells and erythrocytes, damage is strongly reduced in comparison to the peptide featuring the intact KR-motif. Utilising different mutant strains deficient in one of various proteases, a member of our laboratory later identified the protease Kex1 to be responsible for the final digestion leading from Ece1-III$^{62-93}$ to Ece1-III$^{62-92}$, as a *kex1*Δ/Δ secreted the expected peptides ending with a KR-motif [301]. Given the fact that Kex1 is an intracellular protease, it can be concluded that this final processing step has to occur intracellularly. Previous hypotheses of *C. albicans* detoxifying its own peptide in response to the extracellular concentration exceeding a certain threshold level did therefore proof wrong and the question arose, why this fungal pathogen would attenuate the effectiveness of such a potent toxin as Ece1-III$^{62-93}$ by a further processing step. Growth experiments in the presence of very high concentrations (70 µM) of both Ece1-III$^{62-93}$ and Ece1-III$^{62-92}$ showed, that even at such high concentrations, which are rather unlikely to occur *in vivo*, growth of all tested *C. albicans* strains was not influenced, while host cells are already strongly damaged at peptide concentrations around 10 µM. However, while *C. albicans* may have originally secreted the more toxic version of Ece1-III, different evolutionary developments could have contributed to the additional processing step, resulting in a less potent toxin. It needs to be kept in mind, that *C. albicans* is an opportunistic human pathogen, which is mostly found as a harmless coloniser of mucosal surfaces. In these natural habitats, *C. albicans* interacts with a plethora of other microorganisms, and while many of these interactions may be antagonistic, several beneficial interactions between *C. albicans* and various bacterial strains are known today. Different Streptococci species, e.g. *Streptococcus gordonii*, are regularly co-isolated with *C. albicans* [376, 377]. While the fungus is able to attach to the bacterial cells, thereby avoiding the competition for adhesion sites, and can use the *S. gordonii* metabolic waste product lactate as a carbon source for growth, the streptococci benefit from a reduction of the oxygen tension to a lower and more favourable level [376, 378, 379]. Other examples include the formation of polymicrobial biofilms, which results in an increased tolerance against antimicrobial agents and resistance to host clearance mechanisms. Such beneficial interactions with other microbial species might have eventually led to the change in toxin secretion, as Ece1-III$^{62-93}$ might as rapidly lyse bacterial cells as it does its human host cells. Indeed, co-incubation of the Ece1-III peptide with different lactobacilli and *E. coli* strains were observed to have an inhibitory or lethal effect on several bacterial species (unpublished data,

personal communication with T. Förster). However, further experiments including a variety of other bacterial strains need to be performed to obtain a clearer picture about the influence of *C. albicans* peptide Ece1-III on bacterial growth. Moreover, expression studies on *KEX1* might prove useful to show whether this protease gets down-regulated during mucosal infections. This would allow the fungus to secrete the more toxic peptide product after a shift from commensalism to pathogenicity.

4.5 Ece1 as a potential anticandidal drug target

The results obtained in this study clearly demonstrated that the hypha-associated protein Ece1 is essential for *C. albicans*-induced damage of different epithelial cell types and erythrocytes, as damage was completely abolished by deletion of the *ECE1* gene. Considering these data, Ece1 presents an attractive possible target for the development of novel anticandidal drugs. As the damaging effect of this protein is exerted through the pore-forming, toxin-like nature of peptide Ece1-III, an antibody directed against this part of the protein may help preventing epithelial damage during *C. albicans* infections. As experiments showed, the *ece1*Δ+*ECE1*[ΔEce1-III] strains, which are only deficient in this one peptide but still express all other Ece1-peptides, behaved similar to the *ece1*Δ/Δ null mutant, demonstrating that the role of these other peptides can possibly be neglected when considering Ece1 as a potential drug target. Preliminary experiments were carried out in this study, examining different antibodies directed against Ece1-III for an abolishing effect against peptide-mediated damage. Unfortunately, no protecting effect on erythrocytes was observed with the antibodies used, indicating that an antibody to be used for the treatment of infections most probably needs to be highly specific. As human serum was shown to be an efficient inhibitor of damage induced by Ece1-III, identification of the active serum component, however, might be a first step in finding an effective antagonist.

In addition to an Ece1-directed antibody, attenuation of Ece1-III-induced damage could be accomplished via inhibition of receptor binding on the host cell membrane. This might be realised by designing small drug molecules targeting a site of the PFT that is known to be involved in membrane binding. For PFTs of the aerolysin family, synthetic GPI molecules and GPI analogues for example have been proposed to inhibit pore assembly by impeding attachment of the PFT to the plasma membrane [380]. In case of Ece1-III, targeting the positively charged C-terminus of the peptide could prove as effective, because changes of the C-terminal amino acids significantly influence the membrane binding capacity of this peptide, as has been shown in this study. A different approach to prevent PFT-receptor binding could be to target the receptor rather than the PFT itself, as has been shown for *S. aureus* α-haemolysin (Hla) by targeting its receptor ADAM10 with a metalloprotease inhibitor [374]. While phosphatidylserine was already identified

as a binding partner of Ece1-III within the cell membrane, a potential protein receptor remains to be discovered though.

However, even with a potent antibody against Ece1, the problem of a timely diagnosis and initiation of treatment would still remain. Expression studies showed that expression of *ECE1* is rapidly and highly up-regulated, once hypha formation is induced. Furthermore, results obtained in this study revealed that epithelial damage, as detected with the protocols used in this study, occurs only several hours after the addition of Ece1-III. Therefore, a successful anticandidal therapy targeting Ece1 would have to be initiated at an early time point of the infection, in order to directly inactivate Ece1-III after the processing of the full length Ece1 protein and secretion of the single peptides.

Regarding the high cytolytic effect of Candidalysin on different human cell lines, this toxin might also be considered for application in anti-cancer therapy, once the molecular mechanism is completely elucidated. Binding of the PFT to specific receptors up-regulated in cancer cells and subsequent pore formation would result in a suicide response and thereby killing of the malignant cells. Such a strategy has already been successfully applied to target tumours expressing claudin 3, claudin 4 and claudin 6 [381, 382].

4.6 Conclusions and outlook

The data gathered in this study identify peptide Ece1-III of *C. albicans* as the first known peptide toxin of a human fungal pathogen and demonstrate the importance of this peptide for the mediation of infection-associated epithelial damage. This peptide, which is now referred to as Candidalysin, was shown to effectively lyse epithelial cells as well as human red blood cells and the physiological properties elucidated in this work led to the proposition of a possible mode of action for this toxin. This model hypothesises that Candidalysin monomers attach to the host membrane via binding of specific phospholipids, such as phosphatidylserine. An increased peptide concentration on the membrane surface will result in the aggregation of monomers and insertion into the host membrane as a pore-like structure. Subsequent changes in the intracellular ion composition and the ion-dependent activation of signalling pathways will eventually result in the death of the host cell. Elucidation of the exact molecular mode of action remains the work of future studies and includes the identification of a possible receptor molecule within the host membrane, which may facilitate membrane binding and oligomerisation. Moreover, the role of the remaining seven Ece1 peptides should be more closely examined, as several experiments indicated that while these peptides do not play a role in mediating direct damage, the presence of these peptides contributes to an overall more effective lysis process.

Elucidating the function of this protein, which has so far been unknown, underlines the importance of further characterisation of the many unidentified ORFs of the *C. albicans* genome, as this may result in the identification of essential virulence factors. More detailed knowledge of the molecular mechanisms underlying infection-associated damage will lead to a better understanding of the host-pathogen interaction during *C. albicans* infections and may eventually contribute to the development of new therapeutic antifungal options.

5 List of Abbreviations

BSA	Bovine Serum albumin
CIp30	Candida Integration plasmid 30
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EC	epithelial cell
ECL	enhanced chemiluminescence
FBS	Foetal Bovine Serum
HIV	human immunodeficiency virus
IPTG	Isopropyl-ß-D-thiogalactopyranosid
(k)Da	(kilo)Dalton
KOAc	potassium acetate
LC-MS/MS	Liquid Chromatography-mass spectrometry
LDH	lactate dehydrogenase
LB	Luria Bertani
M	Molar
MOI	Multiplicity of Infection
nm	nanometer
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PFP/PFT	pore forming protein/pore forming toxin
PRR	pattern recognition receptor
ROS	reactive oxygen species
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
SDS	sodium dodecyl sulphate
SOC	Super Optimal Broth with Catabolite Repression
TBS	Tris Buffered Saline
v/v	volume per volume
w/v	weight per volume
YNB	yeast nitrogen base
YPD	Yeast Peptone Dextrose

6 References

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7 Appendix

Publications

Höfs S, Mogavero S and Hube B (2016). Interaction of *Candida albicans* with host cells: virulence factors, host defense, escape strategies, and the microbiota. *J Microbiol* 54 (3), 149-169

Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Wernecke J, **Höfs S**, … Hube B and Naglik JR (2016). Candidalysin – a fungal peptide toxin critical for mucosal infection. *Nature* 532 (7597), 64-68

Posters and talks

Posters

18th Congress of the International Society for Human and Animal Mycology (2012), Berlin | Uncovering the role of Ece1 - a *Candida albicans* hypha-expressed gene essential for epithelial damage.

64. Jahrestagung der DGHM (2012), Hamburg | Characterisation of the first *Candida albicans* toxin: Ece1

Fifth FEBS Advanced Lecture Course on Human Fungal Pathogens (2013), La Colle sur Loup, France | Molecular characterisation of the *Candida albicans* damage factor Ece1

Symposium of the International Leibniz Research School (2014), Jena | Ece1 - a *Candida albicans* toxin

19th Congress of the International Society for Human and Animal Mycology (2015), Melbourne, Australia | Ece1 – a *Candida albicans* pore-forming toxin

49. Wissenschaftliche Tagung der Deutschsprachigen Mykologischen Gesellschaft (2015), Jena | Ece1 - a *Candida albicans* pore-forming toxin

Talks

DGHM Statusworkshop, FG Eurkaryontische Krankheitserreger (2012), Berlin | The biological function of the *Candida albicans* hyphal protein Ece1 and its role in epithelial damage.

3rd Central European Summer Course on Mycology (2012), Szeged, Hungary | Dissecting the function of Ece1 - a *Candida albicans* epithelial damage factor.

Symposium of the International Leibniz Research School (2013), Jena | Molecular characterisation of the *Candida albicans* damage factor Ece1

47. Wissenschaftliche Tagung der Deutschsprachigen Mykologischen Gesellschaft (2013), Tübingen | Ece1 - a *Candida albicans* pore-forming toxin

4. Gemeinsamer Kongress von DGHM und VAAM (2014), Dresden | Ece1 - a *Candida albicans* pore-forming toxin

Additional courses

JSMC practical course "Properties of luciferases for *in vivo* imaging of infections" by Dr. Brock (Jena, Germany, December 2014)

JSMC practical course "Calcium signaling in microbial communication" by Prof. Oelmüller (Jena, Germany, January 2015)

ILRS workshop "Interaction of human dendritic cells with *Candida albicans*" by Prof. Skerka (Jena, Germany, August 2015)

Travel grants and awards

FEMS travel grant for attending the 3rd Central European Summer Course on Mycology, Szeged, Hungary (July 2012)

DGHM travel grant for attending the 64th Annual Meeting of the DGHM, Hamburg, Germany (October 2012)

FEMS travel grant for attending the Fifth FEBS Advanced Lecture Course on Human Fungal Pathogens, La Colle sur Loup, France (May 2013)

DGHM travel grant for attending the 4th Joint Meeting of DGHM and VAAM, Dresden, Germany (October 2014)

YISHAM Scholarship for attending the 19th Congress of the International Society for Human and Animal Mycology, Melbourne, Australia (May 2015)

International Leibniz Research School poster award – annual ILRS Symposium (May 2014)

Collaborative Research Center/Transregio FungiNet poster award at the annual DMykG meeting (September 2015)

Acknowledgements

First of all I would like to express my gratitude to Prof Bernhard Hube, who provided this wonderful project as well as excellent work conditions and lab facilities within the HKI. His constant support throughout my time at his department is greatly appreciated. I also want to thank the reviewers of this work.

Furthermore, I want to thank my supervisors, Dr Selene Mogavero and Dr Duncan Wilson, for their amazing input and help, as well as for proof-reading of this thesis and loads of helpful advice.

I want to thank all members of the MPM and MI for providing such a nice working atmosphere. This includes the whole Ece1-team & E-04 office, and our hard-working technicians: Nadja, Stephie, Daniela and Birgit. Lab work would have been much more time-consuming without you! I'm thankful to Carolyn for helping to generate the adhesion/invasion data during her bachelor thesis. I also want to thank Christine, Nicole, Tony, Marcel, Annemarie, Karo, Osama, Volha, Steffi, Bettina, Daniel, Alexa and Elli for being great lab partners and for the many cheerful scientific and non-scientific conversations. I want to thank Annika for putting so much work into the creation of the Ece1-VII deletion mutants. I'm thankful to Sascha for always being immediately available whenever modern technology chose to annoy me. In particular, I want to thank Franzi and Melanie, for not only being great colleagues but also wonderful friends and travel companions. Our collective movie nights and BBP classes were as much appreciated as your professional opinions on work-related topics. Last but not least I would like to thank François for his moral support and organisational advice concerning my thesis.

I also wish to thank Prof Ilse Jacobsen and Prof Oliver Kurzai for contributing to the blood experiments by providing instruments and drawing blood. A big thank you goes to all the people who supported the experiments by regularly donating blood.

I appreciate the support of all internal and external collaborators involved in the Ece1-project, including Ting Luo and Dr Thomas Krüger from the MAM department, Dr Julian Naglik's lab in London, Dr Oliver Bader from the University of Göttingen and the group of Prof Thomas Gutsmann in Borstel.

I especially want to thank my parents and my sister and brother for their love and constant encouragement. You are truly the best!

Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Mir ist die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt.

Personen, die mich bei der Auswahl und Auswertung des Materials sowie der Herstellung des Manuskripts unterstützt haben, sind in der Danksagung der Dissertation vermerkt.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch bei keiner anderen Hochschule als Dissertation eingereicht und auch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung verwendet.

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Sarah Höfs

Jena, 13. September 2016