Candidalysin delivery to the invasion pocket is critical for host epithelial damage induced by *Candida albicans*

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Abstract
The human pathogenic fungus *Candida albicans* is a frequent cause of mucosal infections. Although the ability to transition from the yeast to the hypha morphology is essential for virulence, hypha formation and host cell invasion per se are not sufficient for the induction of epithelial damage. Rather, the hypha-associated peptide toxin, candidalysin, a product of the Ece1 polyprotein, is the critical damaging factor. While synthetic, exogenously added candidalysin is sufficient to damage epithelial cells, the level of damage does not reach the same level as invading *C. albicans* hyphae. Therefore, we hypothesized that a combination of fungal attributes is required to deliver candidalysin to the invasion pocket to enable the full damaging potential of *C. albicans* during infection. Utilising a panel of *C. albicans* mutants with known virulence defects, we demonstrate that the full damage potential of *C. albicans* requires the coordinated delivery of candidalysin to the invasion pocket. This process requires appropriate epithelial adhesion, hyphal extension and invasion, high levels of *ECE1* transcription, proper Ece1 processing and secretion of candidalysin. To confirm candidalysin delivery, we generated camelid VHHs (nanobodies) specific for candidalysin and demonstrate localization and accumulation of the toxin only in *C. albicans*-induced invasion pockets. In summary, a defined combination of virulence attributes and cellular processes is critical for delivering candidalysin to the invasion pocket to enable the full damage potential of *C. albicans* during mucosal infection.

Take Aways
- Candidalysin is a peptide toxin secreted by *C. albicans* causing epithelial damage.
- Candidalysin delivery to host cell membranes requires specific fungal attributes.
- Candidalysin accumulates in invasion pockets created by invasive hyphae.
- Camelid nanobodies enabled visualisation of candidalysin in the invasion pocket.

KEYWORDS
*Candida albicans*, candidalysin, hyphal formation, pathogenicity mechanisms, virulence
Candida albicans is a commensal fungus in the majority of the human population, but also frequently causes mucosal infections and, in severe cases, life-threatening systemic infections (Brown et al., 2012). The ability of C. albicans to cause such diseases is mediated by a wide range of virulence attributes. This includes the morphological transition between the yeast and the filamentous form, and the production of the peptide toxin candidalysin (Mayer et al., 2013; Moyes et al., 2016; Wilson, Naglik, & Hube, 2016).

Although yeast cells are required for dissemination via the bloodstream during systemic infections, the hyphal form is the more invasive morphology (Jacobsen et al., 2012; Kornitzer, 2019). In fact, hypha formation is accompanied by the deployment of several additional virulence attributes, including adhesins, invasins, metal acquisition factors, hydrolytic and detoxifying enzymes and thigmotropism (Brunke, Mogavero, Kasper, & Hube, 2016; Mayer et al., 2013; Wachtler, Wilson, Haedicke, Dalle, & Hube, 2011). Of the multiple genes identified as essential for mediating the full virulence potential of C. albicans, many are required for or associated with filamentation, demonstrating the critical role of hypha formation in C. albicans pathogenesis (Mayer et al., 2013).

During mucosal infection, the yeast-to-hypha transition is initiated by contact to epithelial cells (Dalle et al., 2010; Phan, Belanger, & Filler, 2000; Sudbery, 2011; Wachtler et al., 2011; Zakikhany et al., 2008). The initial step of filamentation, described as germ tube formation, is associated with increased adhesion to epithelia, mediated by hypha-associated adhesins such as Als3 (Hoyer, Payne, Bell, Myers, & Scherer, 1998). Extending hyphae grow along host cell surfaces and change their directional growth by contact sensing, also termed thigmotropism (Brand et al., 2007; Gow, 1997). This feature is required for the invasive properties of hyphae, since mutants lacking proteins associated with thigmotropism, such as Bud2, show reduced invasive growth (Brand et al., 2008). Invasion of epithelial cells occurs via “induced endocytosis” and “active penetration” depending on the epithelial cell type and both routes require hypha formation (Mayer et al., 2013; Zakikhany et al., 2007). Induced endocytosis is a host-driven process triggered by hypha-associated invasins, in particular Als3, which acts as both an adhesin and invasin (Phan et al., 2000; Wachtler et al., 2011). Active penetration is a fungal-driven process mediated by the physical forces and properties of elongating hyphae (Dalle et al., 2010; Zakikhany et al., 2007). Both induced endocytosis and active penetration cause invagination of epithelial cells and the formation of an “invasion pocket” in which the hypha is tightly surrounded by the host membrane (Wachtler et al., 2012; Zakikhany et al., 2007). Since the majority of proteins secreted by hyphae are transported to the hyphal tip via a complex secretion machinery (the Spitzenkörper) (Weiner et al., 2019), proteins or peptides secreted by hyphae in this scenario are expected to be secreted into the invasion pocket.

Mutants incapable of producing hyphae, such as a mutant lacking two key transcriptional regulators of the yeast-to-hypha transition—Cph1 and Efg1—have poor adherence kinetics and are non-invasive (Lo et al., 1997; Ramage, VandeWalle, Lopez-Ribot, & Wickes, 2002; Wachtler et al., 2011). Mutants that are unable to extend their growth at the hyphal tip, like a mutant lacking the protein Eed1 (Martin et al., 2011; Zakikhany et al., 2007), are only poorly able to invade host tissues. The eed1ΔΔ mutant produces short hyphae, expresses the full set of hypha-associated genes (HAGs) at early time points, and invades superficially, but then fails to maintain hyphal elongation (Martin et al., 2011; Polke et al., 2017; Zakikhany et al., 2007). Instead, eed1ΔΔ cells switch back to yeast growth and proliferate as yeast cells, a portion of them remaining inside the invaded cell (Zakikhany et al., 2007). Another C. albicans mutant lacking Hgc1, a G1 cyclin-related protein and regulator of morphogenesis, forms very short germ tubes but is unable to maintain filamentation (Zheng, Wang, & Wang, 2004). Although hgc1ΔΔ still expresses HAGs (Zheng et al., 2004), it has a reduced potential to invade epithelial cells (Wachtler et al., 2011). A systematic study of 26 C. albicans mutants showed that all mutants with reduced potential to adhere to and/or invade into epithelial cells, including mutants with dysfunctional contact sensing, are also unable to fully induce epithelial damage (Wachtler et al., 2011).

While hypha formation and maintenance are critical for invasion, the key virulence attribute responsible for inducing epithelial damage is candidalysin (Moyes et al., 2016). This peptide toxin, a product of the larger Ece1 (extent of cell elongation, [Birse, Irwin, Fonzi, & Sypherd, 1993]) polyprotein, is exclusively secreted by C. albicans hyphae, and is responsible for pore-induced damage of different epithelial cell types, including oral, vaginal, and intestinal cells (Allert et al., 2018; Moyes et al., 2016; Richardson et al., 2017, 2018). Processing of Ece1 requires the sequential activities of the Golgi-located proteases Kex2 and Kex1 (Moyes et al., 2016; Richardson et al., 2018). C. albicans mutants lacking Ece1 or the candidalysin-coding sequence only, are unable to damage epithelial cells, despite normal hypha formation. Incomplete processing of candidalysin also prevents epithelial damage (Richardson et al., 2018). Importantly, these studies showed that it is candidalysin’s activity that damages host cells rather than C. albicans hypha formation and host cell invasion per se, which finally decoupled epithelial damage from hypha formation (Wilson et al., 2016). However, while synthetic, exogenously added candidalysin is sufficient to lyse epithelial cells (Moyes et al., 2016), the degree of damage does not reach the same level as C. albicans hyphae invading epithelial cells, unless extremely high concentrations of the peptide are applied (Allert et al., 2018; Moyes et al., 2016). Therefore, we hypothesized that a combination of fungal attributes and cellular processes is required for the full damage potential of C. albicans during epithelial infection.

In this study, we utilised a selected panel of C. albicans mutants with known virulence defects to demonstrate that the full damage observed with wild-type C. albicans cells requires a defined combination of virulence attributes to deliver candidalysin to the invasion pocket during mucosal infections. This process requires appropriate adhesion, hyphal extension and invasion, high levels of ECE1 transcription, proper Ece1 processing and, finally, secretion of candidalysin. Candidalysin delivery to the invasion pocket...
pocket was confirmed using camelid V₅Hs (nanobodies) specific for candidalysin.

2 | RESULTS

2.1 | Diverse C. albicans mutants show reduced oral epithelial damage

To dissect which virulence attributes of C. albicans are required for full epithelial damage (relative to the wild-type type) in combination with the production of mature candidalysin, we analysed a panel of mutants with known defects in hypha formation (cph1ΔΔ/efg1ΔΔ, hgc1ΔΔ), hypha extension (eed1ΔΔ), thigmotropism (bud2ΔΔ), adhesion (als3ΔΔ), Ece1 processing (kex2ΔΔ, kex1ΔΔ) and lacking Ece1 (ece1ΔΔΔ) (Table 1). All mutants grew normally in their yeast morphology and thus have no general growth defect. However, some mutants show multiple other defects, for example, the kex2ΔΔ mutant is not only unable to process the Ece1 polypeptide (Richardson et al., 2018) but also multiple other pro-proteins with Lys-Arg processing sites (e.g., secreted aspartic proteases [Newport & Agabian, 1997; Naglik, Challacombe, & Hube, 2003]) and is known to have defects in hypha formation (Newport & Agabian, 1997, Newport et al., 2003). Damage caused to TR146 oral epithelial cells (OECs) is summarised in Figure 1a. All mutants showed either an intermediate (hgc1ΔΔ, bud2ΔΔ) or a (very) strongly reduced damage potential (cph1ΔΔΔ/efg1ΔΔ, eed1ΔΔΔ, als3ΔΔΔ, kex1ΔΔΔ, kex2ΔΔΔ and ece1ΔΔΔ), indicating that hypha formation and extension, thigmotropism, adhesion, Ece1 production and processing are all critical to enable full epithelial damage by C. albicans.

2.2 | High ECE1 transcription rates are necessary but not sufficient to predict damage

Hypha formation is strongly linked to the transcription of ECE1 (Martin et al., 2013). Therefore, the inability of yeast-locked mutants (such as cph1ΔΔΔ/efg1ΔΔΔ) to cause full epithelial damage may be solely linked to the silenced transcription of ECE1. We thus quantified ECE1 transcription (in the absence of epithelial cells but in strong hyphae-inducing conditions) in the selected mutants (Figure 1b). Almost all mutants with strongly reduced damage potential also showed no or severely/highly impaired ECE1 transcription (cph1ΔΔΔ/efg1ΔΔΔ, eed1ΔΔΔ, kex2ΔΔΔ and ece1ΔΔΔ). Thus, strongly reduced ECE1 transcription levels strongly correlate with reduced damage potential. Notably, other mutants with a strong/intermediate reduction in their damage capacity (hgc1ΔΔΔ, bud2ΔΔΔ, als3ΔΔΔ and kex1ΔΔΔ) had ECE1 transcript levels similar to the isogenic wild type, or only mildly reduced.

2.3 | Reduced adhesion, invasion and hypha length are confirmed phenotypes predictive for reduced damage potential

We next investigated which virulence parameters were required to facilitate candidalysin-induced epithelial damage by quantifying the ability of each mutant to adhere, invade and produce hyphae (Figure 2). Only the two mutants lacking the transcription factors Cph1/efg1 or the adhesin Als3 showed adhesion defects (Figure 2a). The same mutants also showed significantly reduced invasion potential, in line with (Wachtler et al., 2011), where adhesion was shown to be a prerequisite for invasion. All other mutants invaded at similar rates, or only mildly reduced compared with the wild type (Figure 2b), confirming that invasion per se does not necessarily cause epithelial damage (Moyes et al., 2016). We thus hypothesized that hypha extension, hyphal length and delivery of candidalysin are all essential attributes required for invasive hyphae to cause damage. As expected, a significant reduction in the ability to produce full length hyphae (relative to wild-type cells), at 3 hr post infection (p.i.), was observed for mutants lacking Cph1/efg1, Hgc1, or Kex2 (Figure 2c). The mutant lacking Eed1 showed a mild reduction in hyphal length. In fact, this mutant is known to switch to yeast growth after approximately 3 hr (Zakikhany et al., 2007).

These data suggest that the ability to produce full length hyphae in combination with high ECE1 transcription is critical for the full potential to damage epithelial cells. This explains why the hgc1ΔΔ mutant can cause intermediate epithelial damage despite not producing full length hyphae, as it also transcribes ECE1 at levels similar to the isogenic wild type.

2.4 | Constitutive transcription of ECE1 in a yeast-locked mutant does not rescue its damaging phenotype

Given that hgc1ΔΔ caused an intermediate level of epithelial damage despite expressing ECE1 at high levels, we next determined whether filamentation and ECE1 transcription are linked to enable epithelial cell damage. To achieve this, we generated a yeast-locked strain that constitutively transcribes ECE1: cph1ΔΔΔ/efg1ΔΔΔ + pENO1-ECE1 (Westman et al., 2018). Hypha-producing control strains were transformed with the same construct, obtaining BW17/Cp30 + pENO1-ECE1 (contains 1 copy of ECE1 driven by its original promoter, and 1 copy driven by the enolase promoter) and ece1ΔΔ + pENO1-ECE1 (contains only 1 copy of ECE1 driven by the enolase promoter). As shown in Figure 1a, both BW17/Cp30 + pENO1-ECE1 and ece1ΔΔ + pENO1-ECE1 were able to damage at levels similar to the isogenic wild type. However, the cph1ΔΔΔ/efg1ΔΔΔ + pENO1-ECE1 mutant was unable to cause damage. RT-qPCR analysis showed that the enolase promoter was able to induce transcription of ECE1 mRNA to levels only mildly reduced compared to the isogenic wild type (Figure 1b) in both cph1ΔΔΔ/efg1ΔΔΔ + pENO1-ECE1 and ece1ΔΔ + pENO1-ECE1. BW17/Cp30 + pENO1-ECE1 showed ECE1 transcript levels similar to the BW17/Cp30 isogenic wild type.

Since the forced expression of ECE1 in the yeast-locked strain cph1ΔΔΔ/efg1ΔΔΔ was insufficient to enable this mutant to induce damage but was sufficient to enable damage in the hypha-producing strain ece1ΔΔ + pENO1-ECE1, we conclude that filamentation in combination with ECE1 expression is required for inducing damage.
TABLE 1  Strains used in this study

| Strain name       | Internal name | Relevant genotype                                      | Relevant published or expected phenotype                                      | Reference                  |
|-------------------|---------------|--------------------------------------------------------|--------------------------------------------------------------------------------|----------------------------|
| BWP17/CIP30       | M1477         | ura3::::im434/ura3::::im434                             | Isogenic wild type.                                                           | Zakikhany et al. (2007)    |
|                   |               | iro1::::im434/iro1::::im434                             |                                                                            |                            |
|                   |               | his1::::hisG/his1::::hisG                                |                                                                            |                            |
|                   |               | arg4::::hisG/arg4::::hisG                               |                                                                            |                            |
|                   |               | RPS1/ps1::::UR43-HA3                                     |                                                                            |                            |
|                   |               |                                                         |                                                                            |                            |
|                   |               |                                                         |                                                                            |                            |
| cph1ΔΔ/efg1ΔΔ      | M2065         | cph1::::hisG/cph1::::hisG                               | Yeast-locked. No expression of HAGs.                                           | Wartenberg et al. (2014)   |
|                   |               | efg1::::hisG/efg1::::hisG                               |                                                                            |                            |
|                   |               | RPS1/ps1::::UR43-HA3                                     |                                                                            |                            |
| hgc1ΔΔ             | M1309         | hgc1::::HIS1/hgc1::::ARG4                               | Forms germ tubes but is unable to properly filament. Still expresses HAGs.     | Zheng et al. (2004)        |
| eed1ΔΔ             | M1263         | eed1::::HIS1/eed1::::ARG4                               | Switches back to yeast growth after initial germ tube formation. Starts invasion, but remains trapped, no interepithelial dissemination. | Zakikhany et al. (2007)    |
| bud2ΔΔ             | M1412         | bud2::::ARG4/URA3-bud2::::bud2::::HIS1                  | Aberrant thigmotropic growth. Reduced invasion.                                | Hausauer, Gerami-Nejad, Kisler-Anderson, and Gale (2005) |
| als3ΔΔ             | M1284         | als3::::ARG4/als3::::HIS1/als3::::im434/als3::::im434   | Reduced adhesion to ECs.                                                      | Nobile et al. (2006)       |
|                   |               | (URA3-::iro1)                                           |                                                                            |                            |
| kex2ΔΔ             | M215          | kex2::::hisG/kex2::::hisG-URA3-::riesG                  | Inability to form hyphae. Inability to process Ece1.                          | Newport and Agabian (1997) |
| kex1ΔΔ             | M2248         | kex1::::HIS1/kex1::::ARG4                               | Incomplete processing of candidalysin, reduced damage on ECs.                 | Moyes et al. (2016)        |
| ece1ΔΔ             | M2057         | ece1::::ARG4/ene1::::HIS1                               | No damage on ECs.                                                            | Moyes et al. (2016)        |
|                   |               | RPS1/ps1::::UR43                                       |                                                                            |                            |
| cph1ΔΔ/efg1ΔΔ + pENO1-ECE1 | M2360     | cph1::::hisG/cph1::::hisG                               | Yeast-locked. Forced transcription of ECE1.                                    | Westman, Moran, Mogavero, Hube, and Grinstein (2018) |
|                   |               | efg1::::hisG/efg1::::hisG                               |                                                                            |                            |
|                   |               | RPS1/ps1::::UR43                                       |                                                                            |                            |
|                   |               | caSAT1-peno1-ECE1                                      |                                                                            |                            |
|                   |               | ECE1                                                   |                                                                            |                            |
| BWP17/CIP30 + pENO1-ECE1 | M2682     | caSAT1-peno1-ECE1                                      | Wild type expressing ECE1 from constitutive promoter, but in original ECE1 locus. Control strain. Also transcribes ECE1 from native promoter (second allele). | This study                 |
| ece1ΔΔ + pENO1-ECE1 | M2725         | caSAT1-peno1-ECE1                                       | Only transcribing ECE1 from constitutive promoter, but in original ECE1 locus. Second allele is disrupted. | This study                 |

Abbreviations: ECs, epithelial cells; HAGs, hypha-specific genes.

2.5  |  Secretion of candidalysin does not always predict damage

Next, we considered the importance of candidalysin secretion, as producing high levels of ECE1 transcripts does not necessarily mean that the transcripts are efficiently translated, or that the translated toxin is properly secreted. To test this, mutant strains were cultured in strong hypha-inducing conditions and their supernatants analysed by LC-MS/MS. Detailed results are available in Table S1, and a summary of the results is shown in Table 2. Candidalysin secretion was measured as “Peptide Spectrum Matches” (PSMs), which allows quantitative analysis of peptide abundances. Candidalysin was not secreted by cph1ΔΔ/efg1ΔΔ, kex2ΔΔ or ece1ΔΔ and was negligible/low in eed1ΔΔ, bud2ΔΔ and kex1ΔΔ. Of these, only bud2ΔΔ and kex1ΔΔ expressed moderate levels of ECE1 transcripts (Figure 1b). For kex1ΔΔ, it is known that the toxin’s precursor is produced, and the low levels of candidalysin in the supernatant are due to the fact that the precursor is not processed into the final toxin (Richardson et al., 2018). Mutant bud2ΔΔ probably either poorly translates ECE1 transcripts or is impaired in efficient Ece1 processing or candidalysin secretion. The reduced secretion of candidalysin explains why bud2ΔΔ induces moderate levels of damage.
All mutants with reduced PSM counts for candidalysin were also severely impaired in their ability to damage epithelial cells. Only als3ΔΔ secreted sufficient levels of candidalysin; however, since this mutant cannot efficiently adhere or invade epithelial cells, candidalysin is not delivered to the invasion pocket and hence als3ΔΔ does not damage. This further supports the view that efficient adhesion and invasion are pre-requisites to enable full damage-inducing capacity of C. albicans. This was further confirmed with the yeast-locked mutant cph1ΔΔ/efg1ΔΔ + pENO1-ECE1, which expressed substantial amounts of ECE1 transcripts (Figure 1b) but was unable to adhere, invade (Figure 2) or damage epithelial cells (Figure 1a).

2.6 | Secreted candidalysin localises and accumulates in the invasion pocket

Our data suggest that while candidalysin is the fungal factor that induces damage, the toxin must be secreted by hyphae in the invasion pocket to optimise damage induced by C. albicans. The secretion of candidalysin (or any protein or peptide) is expected to primarily occur at the tip of the invading hypha (Brand et al., 2008; Crampin et al., 2005; Jones & Sudbery, 2010). To visualise candidalysin in situ, we generated Llama-derived (camelid) anti-candidalysin nanobodies. After phage-display selection, two VHH clones with binding affinity towards synthetic candidalysin were isolated, named CAL1-F1 and CAL1-H1. Clone CAL1-F1 gave brighter signals in immunofluorescence infection experiments and was used to visualise candidalysin during epithelial infection by C. albicans. Indirect immunofluorescence demonstrated that candidalysin localises and accumulates at the invasion site and throughout the invasion pocket (Figure 3a), confirming that candidalysin is delivered to the invasion pocket.

We next investigated candidalysin localization with the panel of mutants and quantified the percentage of invasive/non-invasive hyphae that showed positive/negative candidalysin staining. For the BWP17/Clp30 wild type, the majority of invasive hyphae were candidalysin-positive, and the majority of non-invasive hyphae were candidalysin-negative (Figure 3b). This holds true for most
mutants that invaded to any extent, except ece1ΔΔ, which does not produce candidalysin, kex1ΔΔ, and the ece1Δ + pENO1-ECE1. Figure 3c shows a representative micrograph for each mutant stained with CAL1-F1. A visual evaluation of candidalysin fluorescence allowed us to arbitrarily assign the mutants into five categories: “++++” (BWP17/C1p30 wild type and BWP17/C1p30 + pENO1-ECE1); “+++” (als3ΔΔ and kex1ΔΔ); “++” (hgc1ΔΔ and bud2ΔΔΔ); “+/−” (eed1ΔΔ and ece1Δ + pENO1-ECE1); and “−” (cph1ΔΔ/efg1ΔΔ, kex2ΔΔ, ece1ΔΔ and cph1ΔΔ/efg1ΔΔ + pENO1-ECE1). When comparing the damage data (Figure 1a) with subjective immunofluorescence intensity evaluation from Figure 3c, there is a correlation between the intensity of the candidalysin fluorescence signal around the hypha and damage induction. One exception is mutant kex1ΔΔ, which does not secrete candidalysin but does secrete the precursor (Ece1-III, only differing from candidalysin by the additional Arg residue at the C terminus [Moyes et al., 2016, Richardson et al., 2018]).

![Figure 2](image_url)

**FIGURE 2** Reduced adhesion, invasion and hypha length are predictive of reduced damage potential. Data presented as % relative to the BWP17/C1p30 isogenic wild type (dotted line) ± standard deviation (n ≥ 3 biological replicates). (a) Adhesion at 1 hr post infection (p.i.), recorded as % adherent cells compared to initial inoculum (mean adhesion of the BWP17/C1p30 isogenic wild type = 35.6%). (b) Invasion at 3 hr p.i., recorded as % of hyphae that invaded into the TR146 OECs, (mean invasion of the BWP17/C1p30 isogenic wild type = 34.6%). (c) Hypha length at 3 hr p.i. (mean hypha length of BWP17/C1p30 isogenic wild type = 30.2 μm). Statistical significance was calculated using a one-way ANOVA post hoc Dunnett’s multiple comparisons test (vs BWP17/C1p30) on log-transformed data. **p ≤ .01; ***p ≤ .001; ****p ≤ .0001; no stars, not significant. Colours indicate differences vs BWP17/C1p30 isogenic wild type: green—no difference (<20%); light green—mild difference (20–40%); yellow—intermediate difference (40–60%); orange—high difference (60–80%); red—very high difference (>80%).
TABLE 2 LC-MS/MS analysis of secreted candidalysin and its precursor

| Strain name                        | PSMs for candidalysin* | PSMs for Ece1-III |
|------------------------------------|------------------------|------------------|
| BWP17/Clp30                        | 633                    | 16               |
| cph1ΔΔ/efg1ΔΔ                      | 0                      | 0                |
| hgc1ΔΔ                             | 71                     | 0                |
| eed1ΔΔ                             | 1                      | 0                |
| bud2ΔΔ                             | 11                     | 0                |
| als3ΔΔ                             | 238                    | 1                |
| kex1ΔΔ                             | 0                      | 0                |
| ece1ΔΔ                             | 14                     | 65               |
| cph1ΔΔ/efg1ΔΔ + pENO1-ECE1         | 637                    | 0                |
| BWP17/Clp30 + pENO1-ECE1           | 405                    | 0                |
| ece1Δ + pENO1-ECE1                 | 61                     | 0                |

Abbreviation: PSM, peptide spectrum match.
*PSMs are normalised to 100 ml hyphal supernatant; shown is the average of PSMs obtained in two independent sample preparations and subsequent LC-MS/MS runs. Full details of LC-MS/MS datasets are provided in Table S1.

indicates that, not surprisingly, the anti-candidalysin nanobody also recognises Ece1-III. Another exception is als3ΔΔ, which is defective in adhesion and invasion compared with the wild type, but when this mutant does manage to invade, it secretes and accumulates reasonable amounts of candidalysin in the invasion pocket. Also, the ece1Δ + pENO1-ECE1 mutant induces levels of damage comparable to the wild type at 24 hr (Figure 1a), but far less extracellular candidalysin can be detected at 4 hr (Figure 3b,c). Possibly, the kinetics of translation of ECE1 transcripts produced by the enolase promoter are slower than the native ECE1 promoter, hence extracellular candidalysin levels accumulate at a slower rate in the invasion pocket.

In conclusion, epithelial damage during C. albicans infection is the result of a combination of virulence attributes that work in concert to deliver candidalysin to the invasion pocket (Table 3).

3 | DISCUSSION

Mucosal C. albicans infections are associated with several virulence attributes, are initiated by hypha formation and culminate in host cell damage induced by the peptide toxin candidalysin (Moyes et al., 2016), which subsequently activates protective innate immune responses (Naglik, Gaffen, & Hube, 2019; Naglik, Konig, Hube, & Gaffen, 2017; Verma et al., 2017). Hyphal invasion was long thought to be sufficient to cause damage, but the discovery of candidalysin made it possible to decouple hypha formation from damage (Wilson et al., 2016). Likewise, ECE1 transcription does not necessarily correlate with the ability of clinical C. albicans strains to induce epithelial damage (Schonherr et al., 2017). Interestingly, while synthetic candidalysin can damage host cells by direct application, its damage potential does not reach the level of damage induced by invading wild-type C. albicans cells (Allert et al., 2018). Also, the damage induced by synthetic, exogenously added candidalysin is higher in the presence of an ece1ΔΔ mutant than in its absence (Allert et al., 2018), indicating that fungal hyphae facilitate candidalysin toxicity. This led us to hypothesize that several virulence attributes work in concert to facilitate C. albicans-induced damage through candidalysin activity. Utilising a panel of C. albicans mutants and a camelid VHH (nanobody) specific for candidalysin, this study demonstrates that candidalysin needs to be delivered to the invasion pocket by filamenting cells to enable the full damage potential of C. albicans during mucosal infection.

C. albicans infection of epithelial cells requires multiple steps, including hypha formation, adhesion, induced endocytosis, hyphal growth into an invasion pocket and candidalysin secretion (Mayer et al., 2013; Moyes et al., 2016). However, it was unclear whether hyphae are required to deliver candidalysin and whether the toxin mediated damage from the epithelial surface or from within the invasion pocket. Therefore, the contribution of each virulence attribute was assessed for its importance to enable C. albicans to induce epithelial damage via candidalysin activity. As expected, a yeast-locked C. albicans mutant (cph1ΔΔ/efg1ΔΔ) was unable to adhere, invade, express ECE1 or secrete candidalysin, and was thus unable to damage epithelial cells. To demonstrate that the absence of damage was not due to the lack of ECE1 expression, we generated cph1ΔΔ/efg1ΔΔ + pENO1-ECE1, where ECE1 is expressed under the control of the constitutive ENO1 (encoding enolase 1) promoter. This yeast-locked strain expressed substantial levels of ECE1 transcripts, but was unable to cause epithelial damage. This provided strong evidence that damage induction requires candidalysin to be delivered by hyphae. This is supported by the phenotypes of the hgc1ΔΔ and eed1ΔΔ mutants. Mutant hgc1ΔΔ forms short germ tubes but can nevertheless adhere, invade and express moderate levels of ECE1 transcripts, but notably is unable to secrete high amounts of candidalysin and hence induces limited damage. Mutant eed1ΔΔ initially produces normal hyphae and thus adheres to and invades normally (Zakikhany et al., 2007). However, after ≈3 hr eed1Δ reverts to yeast phase growth, shutting down the transcription of HAGs (Polke et al., 2017), including ECE1, and candidalysin secretion, and is unable to induce damage. This confirms not only that candidalysin needs to be delivered by hyphae but also transient ECE1 transcription is not sufficient to cause damage, additionally suggesting that hyphal maintenance is critical for sustained damage during infection. This could also explain why some clinical C. albicans strains poorly induce epithelial damage, as they are impaired in hypha formation (Schonherr et al., 2017).

Given that candidalysin delivery by hyphae appears critical for damage, we investigated the role of hyphal adhesion and invasion (pocket formation) in enabling candidalysin-induced damage by assessing the als3ΔΔ mutant. In agreement with previous studies, mutant als3ΔΔ produces normal hyphae and strongly expresses ECE1 (Cleary et al., 2011), but very poorly adheres to and invades into epithelial cells. Notably, als3ΔΔ is also very poor at inducing epithelial

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damage, indicating that hyphae need to adhere and subsequently invade epithelial cells to enable candidalysin’s cytolytic effects on host membranes. The data support our previous studies (Murciano et al., 2012; Wachtler et al., 2011) and indicate that candidalysin needs to be delivered to the invasion pocket in order to accumulate at sufficient concentrations to induce epithelial cell damage. Furthermore, our data are supported by a recent study showing that Als3 promotes the targeting of candidalysin to host cells by inducing the formation of an endocytic vacuole (the “invasion pocket”) in which candidalysin accumulates (Swidergall et al., 2021).

FIGURE 3  Secreted candidalysin localises and accumulates in the invasion pocket. Candidalysin is stained after infection (4 hr) of TR146 oral epithelial cells. After fixation, extracellular, non-invasive fungal components were stained with Concanavalin A-AlexaFluor 647 (magenta), permeabilized, and candidalysin was then stained with the anti-candidalysin V_h CAL-F1 (green). (a) Detail about *C. albicans* BWP17/Clp30 isogenic wild type; (b) Quantification of candidalysin positive/negative staining around invasive/non-invasive hyphae; (c) Representative micrographs of the selected *C. albicans* mutants. Scale bar = 10 μm.
Once the invasion pocket is formed, hyphae need to grow in it via a process that may be linked to thigmotropism (contact sensing). To assess the importance of thigmotropism in candidalysin-induced epithelial damage we used \textit{bud2ΔΔ}, which is required for directional growth of hyphae (Brand et al., 2008). Notably, we found that \textit{bud2ΔΔ} had no defect in hypha formation, adhesion, or invasion of epithelial cells, indicating that thigmotropism may not be critical for hyphal growth into the pocket. However, while \textit{bud2ΔΔ} expressed only mildly reduced levels of \textit{ECE1} transcripts, it secreted candidalysin in very low amounts and thus was impaired in inducing damage. The
### Table 3: Visual summary of phenotypes

| Strain (mutant) name | Damage | ECE1 transcription | Adhesion | Invasion | Hypha length | CaL secretion | CaL visual quantification from stainings |
|----------------------|--------|--------------------|----------|----------|--------------|---------------|------------------------------------------|
| BWP17/Clp30          |        |                    |          |          |              |               |                                          |
| cph1ΔΔ/efg1ΔΔ        |        |                    |          |          |              |               |                                          |
| hgc1ΔΔ               |        |                    |          |          |              |               |                                          |
| eed1ΔΔ               |        |                    |          |          |              |               |                                          |
| bud2ΔΔ               |        |                    |          |          |              |               |                                          |
| als3ΔΔ               |        |                    |          |          |              |               |                                          |
| kex2ΔΔ               |        |                    |          |          |              |               |                                          |
| kex1ΔΔ               |        |                    |          |          |              |               |                                          |
| ece1ΔΔ               |        |                    |          |          |              |               |                                          |
| cph1ΔΔ/efg1ΔΔ + pENO1-ECE1 | | | | | | | |
| BWP17/Clp30 + pENO1-ECE1 | | | | | | | |
| ece1Δ + pENO1-ECE1 | | | | | | | |

**Color code for observed phenotypes:**

- **Damage / Adhesion / Invasion / Hypha length** (difference vs BWP17/Clp30 isogenic wild type)
  - no (<20%)
  - mild (20-40%)
  - intermediate (40-60%)
  - high (60-80%)
  - very high (>80%)

- **ECE1 transcription** (difference vs BWP17/Clp30 isogenic wild type)
  - no ($\Delta\Delta C_{\text{Gt}} \leq 1$)
  - mild ($1 < \Delta\Delta C_{\text{Gt}} \leq 2$)
  - intermediate ($2 < \Delta\Delta C_{\text{Gt}} \leq 4$)
  - high ($4 < \Delta\Delta C_{\text{Gt}} \leq 7$)
  - very high ($\Delta\Delta C_{\text{Gt}} > 7$)

- **CaL secretion (PSMs)**
  - very high (>200)
  - high (101-200)
  - medium (51-100)
  - low (21-50)
  - negligible (0-20)

- **CaL signal from stainings**
  - very bright (+++)
  - bright (++)
  - visible, not so bright (+)
  - barely visible (+/-)
  - not visible (-)

Abbreviations: CaL, candidalysin; PSM, peptide spectrum match.
reduced candidalysin secretion may derive from bud2ΔΔ also having suspected defects in the secretory pathway (Brand et al., 2008) and may, as a consequence, not efficiently transport candidalysin to the hyphal tip for delivery/secretion. Therefore, the intermediate damage induced by bud2ΔΔ may be due to impaired candidalysin secretion.

The data demonstrate that hyphae need to deliver candidalysin into the invasion pocket to induce epithelial damage. However, candidalysin delivery and secretion first requires the parent protein Ece1 to be sequentially processed by the Golgi-located serine proteases Kex2 and Kex1 (Moyes et al., 2016; Richardson et al., 2018). Kex2 is critical for processing several \textit{C. albicans} proteins in addition to Ece1 (Bader, Krauke, & Hube, 2008) and, therefore, kex2ΔΔ has a general fitness defect that manifests in lack of hypha formation (Newport & Agabian, 1997), ECE1 expression and candidalysin secretion, thus explaining its defective damage phenotype. After Kex2 generates peptides terminating in Lys-Arg amino acid residues, Kex1 removes the terminal Arg to form fully matured candidalysin terminating in Lys. The mature candidalysin is then thought to be packaged and transported to the hyphal tip for secretion. We found that, while kex1ΔΔ exhibits normal adhesion, invasion and hyphal phenotypes and expresses only mildly reduced levels of ECE1 transcripts, it poorly secretes candidalysin and hence induces low levels of damage. On the other hand, kex1ΔΔ produces substantial amounts of the candidalysin precursor terminating in Lys-Arg, as detected by LC-MS/MS (Tables 2 and S1) and by the α-candidalysin nanobody, which is as damaging as candidalysin (Moyes et al., 2016). Given that Kex1 is probably also involved in the processing of numerous other hitherto unknown and unrelated proteins (with possible roles in virulence), kex1ΔΔ likely has other defects in packing fungal peptides to the secretory pathway and may not efficiently transport the candidalysin precursor to the hyphal tip for secretion and delivery. These data indicate that correct Ece1 processing and packaging/transport of candidalysin to the hyphal tip for secretion is essential for induction of epithelial damage.

To confirm our supposition that candidalysin needs to be secreted into the invasion pocket to induce damage, we generated novel anti-candidalysin camelid \textit{V}_{\text{w}}\textit{Hs} (nanobodies) to detect candidalysin in situ. Indirect immunofluorescence demonstrated that candidalysin is secreted at the hyphal tip at the point of epithelial entry and subsequently throughout the invasion pocket as the hypha extends into it. The strength of immunofluorescence in situ directly correlated with the amount of candidalysin secreted in vitro by the \textit{C. albicans} mutant strains. Together, the data demonstrate that during \textit{C. albicans} mucosal infection, a combination of virulence attributes work in concert to deliver candidalysin to the invasion pocket via the hyphal tip. Sustained hypha formation and extension permit candidalysin to accumulate at concentrations sufficient to induce host cell damage and subsequent immunopathology. This is in agreement with the concept of “The cards of virulence, where each virulence factor is considered a card that contributes to the overall virulence phenotype” of a pathogenic microbe (Casadevall, 2006). Even a strong card like candidalysin alone is not sufficient for full virulence and to win the game—from the perspective of a pathogen.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and culture conditions

\textit{C. albicans} strains used in this study are described in Table 1. Strains were cultured in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose, with 1.5% [wt/vol] agar for solid medium) at 30°C, shaking at 180 rpm for liquid cultures. Hyphal growth was induced in RPMI 1640 medium (Life Technologies) for 3 hr at 37°C and 5% CO\textsubscript{2}. Cells from overnight cultures were washed twice with phosphate-buffered saline (PBS), and the concentration of cells was adjusted as indicated in each experiment.

4.2 | Construction of pENO1-ECE1 mutants

Similar to (Westman et al., 2018), pENO1-ECE1 strains transcribing ECE1 under the control of the \textit{C. albicans} enolase promoter (pENO1) were generated from the parent strains BWP17/Cip30 (contains 2 copies of \textit{ECE1} at their original positions) and ece1Δ (heterozygous \textit{ece1} knockout strain, contains only 1 copy of \textit{ECE1} at its original position). A cassette containing the \textit{Candida}-adapted NAT1 (CanNAT1) marker and pENO1 was amplified from pNAT-ENO1 using primers ECE1\_ENO1\_PF (containing 80 nts of \textit{ECE1} gene-specific sequence starting from the start codon) and ECE1\_ENO1\_PR (containing 80 nts homologous to the \textit{ECE1} 5′ promoter region, starting 692 nts before the start codon, Table 4). The PCR product was then integrated before the \textit{ECE1} gene. Integration was confirmed with primers ENO1p-S (ENO1-specific) and ECE1-IR (internal to the \textit{ECE1} gene, Table 4). A PCR product of ~450 bp indicated a positive transformant.

4.3 | Mammalian cell culture

The human TR146 OEC line was chosen to evaluate the damage potential of the selected \textit{C. albicans} mutants, since it is a versatile cell line often used for damage screening purposes (Moyes et al., 2016). TR146 OECs were purchased from the European Collection of Authenticated Cell Cultures (Rupniak et al., 1985) and used throughout the study. Cells were cultured at \textit{37°C} and 5% CO\textsubscript{2} in Dulbecco’s modified Eagle medium/Nutrient Mixture F-12 (Life Technologies) supplemented with 10% (vol/vol) heat-inactivated (10 min at 56°C) fetal bovine serum (Life Technologies).

4.4 | Adhesion, invasion and hyphal length assay

Adhesion, invasion and hyphal length assays were performed similar to Allert et al., 2018. \textit{C. albicans} cells were added to confluent TR146 OECs in 24-well plates to a final concentration of \textbf{10}^5 cells/well. Adhesion of \textit{C. albicans} to OECs was determined 1 hr p.i. Non-attached \textit{C. albicans} cells were removed by washing 3× with PBS. Samples were fixed with Histofix (Roth) for 15 min at room temperature (RT) or overnight at 4°C and rinsed 3× with PBS. Adherent fungi
were stained with Calcofluor white (10 μg/ml in 0.1 M Tris–HCl (pH 9); Sigma-Aldrich) for 20 min at RT in the dark. Samples were then washed 3× with water, mounted on glass slides with ProLong Gold antifade mountant (ThermoFisher Scientific), and analysed by fluorescence microscopy. Pictures of each sample were taken until a total of at least 50 adherent C. albicans cells were counted. The total number of adherent cells on the entire coverslip was calculated based on the average of Candida cells counted in each picture within a defined area. This number was expressed as a percentage of adhered cells vs inoculated C. albicans cells. Invasion of C. albicans into TR146 cells was analysed by differential staining. After 3 hr of C. albicans infection, TR146 cells were washed 3× with PBS and fixed with Histofix. Extracellular, non-invasive fungal components were stained with Concanavalin A-AlexaFluor 647 (ThermoFisher Scientific) for 20 min, in the dark, on an orbital shaker (70 rpm). After the OECs were rinsed 1× with PBS, they were permeabilized with 0.1% saponin in PBS (0.1% PBSS) for 5 min at RT and washed again 1× with PBS. Samples were then blocked with 1% BSA in 0.05% PBSS for 1 hr at RT, shaking at 70 rpm and washed again 1× with PBS. Candidalysin staining was stained with a primary, a secondary, and a tertiary antibody. All antibodies were diluted in 0.5% BSA in 0.05% PBSS, and all incubations with antibodies were performed at RT for 1 hr, with shaking at 70 rpm, followed by 1× wash with PBS. The primary antibody used was the anti-candidalysin Vh CAL1-F1, at 1 μM concentration. The secondary antibody was a polyclonal rabbit-α-Vh IgG (1 mg/ml, QVQ B.V., 1:50). The tertiary antibody was a goat-α-rabbit IgG coupled with AlexaFluor 488 (Cat. no. A32731, 2 mg/ml, ThermoFisher, 1:400). Samples were then mounted with ProLong Gold antifade mountant (ThermoFisher Scientific) and visualised by fluorescence microscopy.

### 4.6 Immunofluorescence staining for visualisation of candidalysin

TR146 OECs grown to confluency in a 24-well plate were infected for 4 hr with C. albicans strains (initial inoculum 10^5/well). TR146 cells were washed 3× with PBS and fixed with Histofix. Extracellular, non-invasive fungal components were stained with Concanavalin A-AlexaFluor 647 (ThermoFisher Scientific) for 20 min, in the dark, on an orbital shaker (70 rpm). After the OECs were rinsed 1× with PBS, they were permeabilized with 0.1% saponin in PBS (0.1% PBSS) for 5 min at RT and washed again 1× with PBS. Samples were then blocked with 1% BSA in 0.05% PBSS for 1 hr at RT, shaking at 70 rpm and washed again 1× with PBS. Candidalysin staining was stained with a primary, a secondary, and a tertiary antibody. All antibodies were diluted in 0.5% BSA in 0.05% PBSS, and all incubations with antibodies were performed at RT for 1 hr, with shaking at 70 rpm, followed by 1× wash with PBS. The primary antibody used was the anti-candidalysin Vh CAL1-F1, at 1 μM concentration. The secondary antibody was a polyclonal rabbit-α-Vh IgG (1 mg/ml, QVQ B.V., 1:50). The tertiary antibody was a goat-α-rabbit IgG coupled with AlexaFluor 488 (Cat. no. A32731, 2 mg/ml, ThermoFisher, 1:400). Samples were then mounted with ProLong Gold antifade mountant (ThermoFisher Scientific) and visualised by fluorescence microscopy.

### 4.5 Anti-candidalysin llama VhHs (nanobodies)

Anti-Candidalysin Llama VhHs (i.e., variable domains of the heavy chain of the heavy-chain antibody, also termed nanobodies) were produced by QVQ B.V. (Utrecht, The Netherlands) using phage-display technology (Kuhn et al., 2016; Verheesen & Laeremans, 2012). For this purpose, two llamas were immunised with heat- or formaldehyde-inactivated C. albicans SC5314 yeast, germ tube and hypha cells, followed by VhH library construction in the phagemid vector pUR8100 for transformation into E. coli TG1. Phage-display selections were conducted on synthetic FLAG-tagged candidalysin peptides immobilised by affinity capture with an anti-FLAG M2 antibody (Sigma-Aldrich).

After two sequential rounds of biopanning phage-display selection, 2 independent clones were identified by ELISA screening on affinity captured candidalysin. VhH clones CAL1-F1 and CAL1-H1 exhibited high apparent affinity towards synthetic candidalysin or its recombinant parent protein rEce1 in ELISAs.

### 4.7 Quantification of candidalysin staining

The same samples prepared for visualisation of candidalysin were used to qualitatively record the percentage of hyphae with any candidalysin staining, and whether these were invasive or non-invasive. At least 100 hyphae per strain were counted, in three independent experiments, except for the less adhesive mutants, where 100 cells were not always found in the same sample.

### 4.8 Damage assay

Damage of TR146 OECs was determined by quantification of lactate dehydrogenase activity in the supernatant using a Cytotoxicity Detection Kit (Roche) according to the manufacturer’s instructions. Results
are presented as % damage relative to the BWP17/Cip30 isogenic wild type, after subtraction of the vehicle only.

4.9 | Fungal RNA extraction

C. albicans strains were adjusted to $10^7$ cells/ml in 25 ml RPMI 1640 (hypha-inducing) or 5 ml YPD (yeast-maintaining). For hyphae samples, fungal suspensions were distributed in 150 cm$^2$ petri dishes and incubated at 37°C and 5% CO$_2$ for 3 hr. After incubation, medium and non-adherent Candida cells were discarded. Adherent Candida cells were rinsed once with ice-cold PBS, loosened with a cell scraper and collected. For yeast samples, fungal suspensions were cultured at 30°C for 3 hr in a shaking incubator (180 rpm). After incubation, cells were collected by centrifugation (4,000g for 2 min at 4°C) and resuspended in 10 ml ice-cold PBS. Hyphae and yeast samples were washed again with 1 ml ice-cold PBS and centrifuged (20,000g, 2 min, 4°C), the supernatant was discarded, and cell pellets were frozen in liquid nitrogen. Frozen Candida pellets were thawed in 600 μL RTL buffer (Qiagen) containing 1% β-mercaptoethanol, mixed with 300 μl acid-washed glass beads (0.5 mm Ø), and run through a Precellys homogeniser (Bertin instruments) twice at 5,500 beats/min for 15 s, with 20 s pause in between. Lysates were centrifuged for 2 min at 20,000g, 4°C, the supernatant was mixed with an equal volume of 70% ethanol (prepared in diethyl pyrocarbonate [DEPC]-treated water) and total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer’s instructions. RNA integrity and concentration were confirmed using a Bioanalyzer (Agilent).

4.10 | ECE1 transcription analysis

RNA (500 ng) was treated with DNase (Episcience), confirmed DNA free, and cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. cDNA samples were used for qPCR with EvaGreen mix (Bio&Sell). Primers (ACT1-F and ACT1-R for ACT1 and ECE1-F and ECE1-R for ECE1 [Table 4]) were used at a final concentration of 500 nM. qPCR amplifications were performed using a CFX96 thermocycler (Bio-Rad). ECE1 transcription was calculated using the threshold cycle (ΔΔCt) method, with ACT1 as the reference gene and C. albicans isogenic wild-type strain (yeast morphology) as the control sample. Data are presented as % relative to the BWP17/Cip30 isogenic wild-type strain.

4.11 | Candidalysin detection in hyphal supernatants by LC–MS/MS analysis

Analysis of hypha-secreted Ece1 peptides was optimised for the detection of candidalysin and performed as previously described (Moyes et al., 2016). The method is aimed at the detection of peptides with highly hydrophobic moieties such as candidalysin. Briefly, C. albicans strains were cultured for 18 hr under strong hypha-inducing conditions (yeast nitrogen base medium containing 2% sucrose, 75 mM MOPS [3-(N-morpholino)-2-hydroxypropanesulfonic acid] buffer, pH 7.2, 5 mM N-acetyl-β-glucosamine, 37°C). Secreted peptides were enriched from culture supernatants by solid-phase extraction using silica-based C4 followed by C18 columns, dried in a vacuum concentrator, dissolved in 0.2% formic acid in 71:27:2 (vol/vol/vol) acetonitrile (ACN)-H$_2$O-dimethyl sulfoxide (DMSO) and passed through a 10-kDa molecular-mass cutoff filter in order to remove intact proteins. LC–MS/MS analysis was carried out on an Ultimate 3,000 nano-LC coupled to a Q Exactive Plus mass spectrometer (Thermo). Peptide separation was performed on an Accucore C4 column (75 μm i.D. × 150 mm, 2.6 μm) with eluents (A) 0.2% HCOOH in 95:5 H$_2$O-DMSO and (B) 0.2% HCOOH in 85:10:5 ACN-H$_2$O-DMSO using the following gradient: 0–1.5 min at 60% B, 35–45 min at 96% B, and 45.1–60 min at 60% B. The top 10 precursor ions (full scan at m/z 300–1,600, R = 70 k [full width at half maximum, FWHM]) per scan cycle underwent HCD (high energy collisional dissociation) fragmentation at 30% normalised collision energy. Resulting MS2 spectra were monitored at R = 17.5 k (FWHM). Proteome Discoverer 2.4 (Thermo) and the Sequest HT algorithm were used to search against the protein database of C. albicans SC5314 (http://www.candidagenome.org). Mass spectra were searched for both unspecific cleavages (no enzyme) and tryptic peptides up to four missed cleavages. Precursor and fragment mass tolerances were 10 ppm and 0.02 Da, respectively. At least 2 unique peptides per protein, a false discovery rate of <1% and cross-correlation (Xcorr) validation (from 2.0 at z = 2 up to 3.0 at z = 6) were required for identification.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Selene Mogavero and Bernhard Hube conceived the experiments. Selene Mogavero performed most of the experiments. Selene Mogavero analysed the data with help from Sascha Brunke. Selene Mogavero wrote the manuscript with substantial input from Frank M. Sauer, Sascha Brunke, Julian R. Naglik and Bernhard Hube.
Frank M. Sauer and Edward Dolk developed the α-candidalysin nanobody. Thomas Krüger, Olaf Kniemeyer and Axel A. Brakhage produced LC-MS/MS data. Stefanie Allert, Daniela Schulz, Stephanie Wisgott, Nadja Jablonowski and Osama Elshafee provided technical assistance. All authors read and approved the manuscript.

SERVICES

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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