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The SPS amino acid sensor mediates nutrient acquisition and immune evasion in *Candida albicans*

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Summary

Candida albicans is well adapted to its host and is able to sense and respond to the nutrients available within. We have shown that *C. albicans* avidly utilizes amino acids as a carbon source, which allows this opportunistic pathogen to neutralize acidic environments, including the macrophage phagosome. The transcription factor Stp2 is a key regulator of this phenomenon, and we sought to understand the mechanism of activation of Stp2, focusing on the SPS sensor system previously characterized for its role in nitrogen acquisition. We generated deletion mutants of the three components, *SSY1*, *PTR3* and *SSY5* and demonstrated that these strains utilize amino acids poorly as carbon source, cannot neutralize the medium in response to these nutrients, and have reduced ammonia release. Exogenous amino acids rapidly induce proteolytic processing of Stp2 and nuclear translocation in an SPS-dependent manner. A truncated version of Stp2, lacking the amino terminal nuclear exclusion domain, could suppress the growth and pH neutralization defects of the SPS mutants. We showed that the SPS system is required for normal resistance of *C. albicans* to macrophages and that mutants defective in this system reside in more acidic phagosomes compared with wild type cells; however, a more equivocal contribution was observed in the murine model of disseminated candidiasis. Taken together, these results indicate that the SPS system is activated under carbon starvation conditions resembling host environments, regulating Stp2 functions necessary for amino acid catabolism and normal interactions with innate immune cells.

Introduction

The opportunistic fungal pathogen *Candida albicans* is a polymorphic yeast intimately associated with the human host. It is able to colonize diverse niches in the body including mucosal surfaces and the intestinal lumen, without causing any clinical symptoms (McManus and Coleman, 2014; Pérez and Johnson, 2013). However, several predisposing factors make the host susceptible to *Candida* infections, such as immunosuppression, disruption of anatomical barriers and disturbance of the normal microbiota (Brown *et al*., 2012; Fidel, 2006; Perlroth *et al*., 2007; Pfaller and Diekema, 2007).

The ability to utilize the nutrients available in the host to generate energy and biomass, so as to resist the host immune defenses, is an important criterion for any pathogen. *C. albicans* is

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metabolically flexible and capable of adapting to microenvironments in different anatomical sites (Wilson *et al.*, 2009). In some of these environments, sugars are limiting, but organic acids such as lactate are available at relatively high concentrations, one example being the environment within the gut or the vaginal cavity (Brown *et al.*, 2014). Other nutrient sources available in the host are proteins and peptides. To make use of these, *C. albicans* has an expanded family of secreted aspartic proteases and oligopeptide transporters that have a role in the acquisition of nutrients during colonization and infection (Hube *et al.*, 1994; Naglik *et al.*, 2003; Reuβ and Morschhäuser, 2006). In addition, several amino acid auxotrophs retain full virulence, indicating that a ready supply of these nutrients are found in the host (Kingsbury and McCusker, 2010; Noble and Johnson, 2005).

Professional phagocytes are one of the first lines of defense against *C. albicans* and employ diverse killing mechanisms to damage ingested fungal cells (Collette and Lorenz, 2011; Jiménez-López and Lorenz, 2013; Miramón *et al.*, 2013), one of which is nutrient (specifically carbon) deprivation. Transcriptional and genetic data indicate that *C. albicans* adapts to this environment by upregulating metabolic pathways for alternative (non-sugar) carbon sources, such as the glyoxylate cycle, gluconeogenesis and fatty acid β-oxidation, as well as transport systems for oligopeptides, amino acids and carboxylic acids. Further, a number of these processes are required for virulence in cell culture and/or whole animal models (Barelle *et al.*, 2006; Fradin *et al.*, 2003; Fradin *et al.*, 2005; Lorenz *et al.*, 2004; Rubin-Bejerano *et al.*, 2003).

C. albicans uses amino acids as a sole source of carbon with much greater avidity than other model fungi (Vylkova *et al.*, 2011), and they appear to be a significant nutrient source for phagocytosed cells. *In vitro*, growth in glucose-poor, amino acid-rich conditions induces a dramatic increase in the media pH, which we have demonstrated is due to the excretion of amino acid-derived ammonia (Vylkova *et al.*, 2011). Following phagocytosis, this adaptation allows *C. albicans* to interfere with the normal maturation and acidification of the phagosome, thereby promoting fungal survival (Danhof and Lorenz, 2015; Vylkova and Lorenz, 2014). Indeed, we have shown that cells lacking Stp2, a transcription factor (TF) known to regulate amino acid permease expression, fail to neutralize *in vitro* environments, occupy a more acidic phagolysosome and are impaired in multiple aspects of the *Candida*-macrophage interaction and in virulence *in vivo* (Vylkova and Lorenz, 2014; Vylkova *et al.*, 2011).

Regulation of carbon utilization has been extensively studied in organisms such as *Saccharomyces cerevisiae* and *Aspergillus nidulans* (Turcotte *et al.*, 2010). Glucose repression of genes required for utilization of non-fermentable carbon sources is mediated by Mig1 (CreA in filamentous fungi). When this repression is lifted, other TFs activate specific uptake and catabolic pathways to capitalize on the available nutrients. This structure is broadly conserved in *C. albicans* (Corvey *et al.*, 2005; Zaragoza *et al.*, 2000), although there has been some reassignment of TFs function (Ramírez and Lorenz, 2009) and intriguing adaptations in post-translational regulation (Sandai *et al.*, 2012).

There is essentially no information on the regulatory networks that control utilization of amino acids as a carbon source, as model fungi have limited ability to do so. In contrast,

more is known about their use as a source of nitrogen or for direct protein synthesis, which is regulated in part by Stp1 and Stp2, paralogous C2H2-type Zinc finger TFs. In *S. cerevisiae*, these TFs are activated by a proteolytic event regulated by the SPS system, comprised of Ssy1, Ptr3 and Ssy5 (Forsberg and Ljungdahl, 2001; Forsberg *et al.*, 2001; Klasson *et al.*, 1999). Ssy1 is an amino acid transporter homolog that senses amino acids in the extracellular environment, although it does not have transport activity. Ptr3 is a peripherally membrane-associated protein (Liu *et al.*, 2008) that mediates the interaction of casein kinase I (CKI) with the last component of the system, Ssy5, a chymotrypsin-like serine endoprotease. This endoprotease becomes hyperphosphorylated in an inhibitory domain, leading to a proteolytic event that releases a catalytically active fragment (Abdel-Sater *et al.*, 2004; Abdel-Sater *et al.*, 2011; Omnus *et al.*, 2011; Pfirrmann *et al.*, 2010). In turn, Ssy5 processes Stp1 and Stp2 by removing a nuclear exclusion domain at the amino termini, thereby allowing translocation into the nucleus where they drive the expression of genes involved in amino acid utilization (Forsberg *et al.*, 2001). Close homologs of these proteins exist in *C. albicans* where, in response to nitrogen starvation, they control the expression of two different set of genes: Stp1 induces the expression of genes involved in protein degradation and peptide transport, whereas Stp2 regulates the expression of genes involved in amino acid utilization (Martínez and Ljungdahl, 2005).

We have shown in *C. albicans* that Stp2 is required for the utilization and catabolism of amino acids as a carbon source and the resulting extrusion of ammonia (Vylkova *et al.*, 2011), but the origin of these amino acids (from host or fungal pools) and the mechanism of activation of Stp2 under carbon starvation conditions has not been clear. To address these questions, we asked whether the SPS system also responds to carbon starvation and whether it, similar to Stp2, modulates the macrophage-fungal interaction. We demonstrate here that *C. albicans* requires a functional SPS system to grow on amino acids as a carbon source and to manipulate the extracellular pH. The SPS system regulates the proteolytic cleavage of a negative regulatory domain of Stp2 and expression of a truncated version of Stp2 lacking this domain suppresses the defects of mutants lacking a functional SPS system. The SPS system plays a major role in the interaction of *C. albicans* with macrophages, but it has a more modest role in the murine model of disseminated candidiasis. Our data suggest that the SPS system is a key player in the sensing of amino acids in the environment, regardless of eventual catabolic outcomes and that proper SPS function is important in facilitating fungal survival during phagocytosis.

Results

The SPS system mediates amino acid utilization and pH manipulation

In order to investigate the role of the SPS system in amino acid utilization, we generated deletion strains lacking *SSY1*, *PTR3* or *SSY5*, the three components of this system, in the SC5314 background as well as complemented strains. We first tested their ability to grow on amino acids as the sole carbon source in glucose-depleted conditions. As shown in Fig. 1A, the *ssy1*Δ/Δ, *ptr3*Δ/Δ and *ssy5*Δ/Δ strains grew poorly on YNB supplemented with 1% casamino acids; growth was similar to the *stp2*Δ/Δ strain. The ability to utilize this carbon source was restored to wild type levels upon reintegration of one allele of the respective

gene. Similar to the *stp2*Δ/Δ strain, the SPS mutants were also unable to neutralize the initial acidic pH of the medium (Fig. 1B). The *ssy1*Δ/Δ, *ptr3*Δ/Δ and *ssy5*Δ/Δ strains behaved identically in growth and pH assays therefore we focused on *ssy1*Δ/Δ in further experiments, which lacks the transmembrane sensor protein.

To exclude the possibility that the inability to manipulate the pH of the medium is due to a general fitness defect, we supplemented the medium with glycerol, a carbon source that does not interfere with the ability to neutralize the pH in response to amino acids (Vylkova *et al.*, 2011). As shown in Fig. S1A, glycerol greatly improves the growth of the three SPS mutant strains, reaching higher culture densities compared with medium without glycerol (Fig. S1A). Because the wild type and reconstituted strains are able to utilize both glycerol and amino acids, their culture densities remain higher than the SPS mutants. Despite the improved ability to grow in medium supplemented with glycerol, the SPS mutants were unable to neutralize the initial acidic pH (Fig. S1B), indicating that a functional SPS system is required to manipulate the external pH in response to amino acids.

Cells growing in amino acid-rich media excrete ammonia derived from the amino and side chain amines and this drives the neutralization of the environment. Standard yeast media contains ammonium sulfate, and we have established that this can complicate assessment of ammonia generation (Danhof and Lorenz, 2015). Thus, we replaced the ammonium sulfate with allantoin. This nitrogen source supports optimal growth and pH changes, which are again impaired in the SPS mutants (Figs. 1A and B and data not shown). This confirms that the neutralization of the environmental pH is independent of the nitrogen source available in the medium. To demonstrate that ammonia is being excreted during growth on amino acids, we determined the release of ammonia by SC5314, *ssy1*Δ/Δ and reconstituted + *SSY1* strains growing on solid YNBA medium (YNB-Allantoin) supplemented with casamino acids. As shown in Fig. 2C, SC5314 cells continuously released ammonia over the course of the experiment (72 h). In contrast, the *ssy1*Δ/Δ strain failed to release any detectable ammonia, which correlates with its inability to neutralize the environmental pH. As discussed later, a truncated allele of *STP2* (*STP2**) that mimics the proteolytically processed form suppresses all phenotypes of the *ssy1*Δ/Δ strain.

The SPS system is responsible for Stp2 processing

The expression of genes necessary to utilize amino acids is controlled by the TF Stp2 (Martínez and Ljungdahl, 2005), which translocates to the nucleus because of a proteolytic cleavage that occurs in response to extracellular amino acids. We sought to determine the activation of Stp2 in wild type cells as well as in cells with a defective SPS system in the context of utilization of amino acids as carbon source. We generated a tagged version of Stp2 fused to mCherry at the carboxyl terminus to allow the identification of the protein by Western blotting and fluorescence microscopy.

To demonstrate activation of Stp2, cells were grown on glucose and amino acids. As shown previously in carbon replete conditions (Martínez and Ljungdahl, 2005), Stp2 is converted to a lower molecular weight form (from 94 kDa to ~82 kDa). This processing is extremely rapid when grown on amino acids, with a portion of Stp2 seen in the processed form within 2 min and the entire sample within 5 min, while Stp2 remained unprocessed even after 1 h

incubation on glucose (Figs. 3A and S2A). Processing of Stp2 requires the SPS system, as *ssy1*Δ/Δ cells express only the full-length form, regardless of the carbon source (Fig. 3B). In agreement with Martínez and Ljungdahl (2005), Stp2 is processed only by a subset of amino acids (Fig. S2B), showing a certain degree of specificity.

Nuclear localization of Stp2 is SPS-dependent

We next investigated the subcellular localization of Stp2 upon amino acid sensing. When wild type cells expressing Stp2-mCherry were grown on glucose, diffuse red fluorescence was detected in the cytoplasm with no apparent nuclear localization (Fig. 4A). In contrast, upon incubation with amino acids, red fluorescence co-localized with the nucleic acid-staining dye Hoechst 33342 (Fig. 4B), revealing translocation of the Stp2-mCherry into the nucleus. This nuclear localization was not observed in the *ssy1*Δ/Δ cells after incubation with glucose or amino acids (Fig. 4C and D), demonstrating that the SPS system is needed for processing and nuclear translocation of Stp2.

Stp2 lacking the N-terminal domain is able to restore amino acid utilization

Once we demonstrated that the SPS system is required for activation of Stp2, and thus, for amino acid utilization and pH manipulation, we investigated whether the expression of a constitutively active form of Stp2 is able to suppress the defects observed in the SPS mutants. For this purpose, we created an *STP2* allele lacking the first 100 residues containing the nuclear exclusion domain (Andreasson and Ljungdahl, 2004) and placed it under the control of the *ACT1* promoter, similar to a strategy used previously (Martínez and Ljungdahl, 2005). The truncated *STP2** allele induces the expression of genes involved in amino acid utilization in the absence of a functional SPS system (Martínez and Ljungdahl, 2005). As shown in Fig. 5A, expression of Stp2* is sufficient to suppress the growth defect observed in the three *ssy1*Δ/Δ, *ptr3*Δ/Δ and *ssy5*Δ/Δ mutant strains when growing on amino acids as carbon source. Moreover, the truncated *STP2** allele also recovers the ability to neutralize the acidic pH of the medium (Fig. 5B). The truncated *STP2** allele restores growth, the ability to neutralize the pH and ammonia production to the *ssy1*Δ/Δ mutant in YNBA (Fig. 2A–C).

We also tagged the truncated *STP2** allele expressed in *ssy1*Δ/Δ with mCherry. As shown in Fig. 3B, the truncated Stp2* expressed by glucose-grown or amino acids-grown *ssy1*Δ/Δ cells has a similar mobility pattern as the processed Stp2 in amino acids-grown wild type cells. The Stp2*-mCherry fusion is fully functional, because its expression suppresses the growth defect on amino acids and pH neutralization ability of *ssy1*Δ/Δ (Fig. 3C and D). In addition, the truncated Stp2* is constitutively localized to the nucleus under both glucose and amino acids when expressed in *ssy1*Δ/Δ (Fig. S3).

Candida albicans requires a functional SPS system for normal interaction with macrophages

Our group has shown that *C. albicans* lacking a functional Stp2 cannot efficiently escape from macrophage killing (Vylkova and Lorenz, 2014). We investigated whether the absence of a functional SPS system has an impact on the normal interaction of *C. albicans* with these phagocytes. First, we tested whether *ssy1*Δ/Δ cells are more susceptible to macrophage

killing using an accepted end-point dilution assay (Miramón *et al.*, 2014; Rocha *et al.*, 2001). During coincubation with RAW264.7 murine macrophages, the *ssy1*Δ/Δ strain was significantly less robust than wild-type strains (Fig. 6A), suggesting increased susceptibility. The resistance to macrophage killing was restored to wild-type levels in the reconstituted strain, and partially restored in strains expressing the truncated *STP2** allele.

We next determined the capacity of these strains to damage phagocytes by means of a cytotoxicity assay that detects release of the cytosolic enzyme lactate dehydrogenase (LDH) into the media as a proxy for macrophage membrane damage. As shown in Fig. 6B, *ssy1*Δ/Δ cells induced less cytotoxicity at two different multiplicities of infection (2:1 and 1:1), and the phenotype was at least partially restored in both the reconstituted strain as well as in the strain expressing the truncated *STP2** allele. Thus, a functional SPS system is necessary for normal resistance towards macrophage attack as well as for normal killing of phagocytes.

Because the SPS mutants cannot neutralize acidic environments, we tested whether the increased susceptibility of these mutants is due to their inability to block acidification of the phagosome. To this end, LysoTracker Red LTR, Invitrogen-loaded RAW264.7 macrophages were infected with SC5314, *ssy1*Δ/Δ and *ssy1*Δ/Δ + *SSY1* cells expressing a Pma1-GFP fusion (Pma1 localizes to the plasma membrane). Using the microscope software Slidebook 6.0, we measured the fluorescence signal of GFP and LTR along a line drawn through the fungal cell (Fig. 7A). Plotting the GFP signal outlines the fungal cell shape, whereas the LTR signal delineates acidic sites. We quantified the LTR signal in the immediate vicinity of the ingested fungal cells (20 pixels, or 2 μm, from the maximum GFP peak, on both sides) and determined the total area under the curve as an estimation of the degree of acidification of the phagosomal compartment (Fig. 7B). Similar to the *stp2*Δ/Δ mutant, cells of the *ssy1*Δ/Δ mutant reside in a more acidic organelle than wild type or the reconstituted strain, as indicated by the intensity of LTR staining in the lumen of the vesicle. These data demonstrate that *C. albicans* requires the SPS system to avoid phagosomal acidification, which suggests that there is a source of amino acids in the lumen of this organelle.

The SPS system has limited effect on virulence in the murine model of disseminated candidiasis

Previous work revealed that a *C. albicans* mutant lacking Stp2 (*stp2*Δ/Δ) has a moderate but significant attenuation in virulence in the murine model of disseminated infection. We tested the three SPS mutant strains in this animal model to investigate the impact of amino acid sensing in virulence. The animals infected with the wild type strain (SC5314) succumbed within 6 days post infection whereas the survival curves for animals infected with the SPS mutants was slightly shifted toward longer survival, although it was not significantly different from the wild type strain (Fig. S4), suggesting that the SPS system has a limited role in the murine model of hematogenously disseminated candidiasis.

Discussion

The fungal regulatory networks that govern the utilization of disparate compounds including sugars, alcohols, carboxylic acids and fatty acids have been extensively studied in several model systems in which there is a conserved framework that involves glucose repression/

derepression and subsequent activation of catabolic pathways for specific alternate nutrients. The same cannot be said for the utilization of amino acids as a source of carbon for the simple reason that this is disfavored in many fungi. *C. albicans*, in contrast, eagerly catabolizes amino acids, which appear to be an important and available nutrient *in vivo*. We have previously shown that amino acid metabolism has been adapted in this species to promote survival during contact with phagocytes and virulence and that Stp2, a TF with a known role in amino acid metabolism, compromises interactions with the host (Martínez and Ljungdahl, 2005; Vylkova and Lorenz, 2014; Vylkova *et al.*, 2011). Thus, it is important to understand the regulatory mechanisms that underlie these adaptations.

In yeast, utilization of exogenous amino acids for synthesis of proteins or other nitrogenous compounds requires the SPS system, a three-protein complex (Ssy1, Ptr3 and Ssy5) that senses extracellular amino acids and results in the proteolytic processing and activation of Stp2 (Forsberg and Ljungdahl, 2001; Forsberg *et al.*, 2001; Klasson *et al.*, 1999). Available evidence suggests this works similarly in *C. albicans* (Martínez and Ljungdahl, 2004; Martínez and Ljungdahl, 2005), but whether this was important in the context of glucose repression/derepression was unknown. In this study, we have shown that the SPS system is also required for the ability to utilize amino acids as a carbon source with deletion of any single component (*SSY1*, *PTR3* or *SSY5*) conferring identical phenotypes. Further, although wild type and complemented strains rapidly raise extracellular pH, the mutants cannot do so. This is independent of the growth defect, because addition of glycerol restores growth without ameliorating the inability to manipulate the pH, which results from a failure to excrete ammonia. The defects of SPS mutants in amino acid utilization, pH modulation and ammonia release occur regardless of the nitrogen source and in several different media (Figs 1 and 2, and data not shown).

We next explored the contribution of amino acid sensing during the interaction of *C. albicans* with macrophages. Our group has shown that *stp2*Δ/Δ strains are more susceptible to macrophage killing and upon phagocytosis, they reside in an acidified phagosome in contrast to wild-type cells (Vylkova and Lorenz, 2014). Consistent with this, the *ssy1*Δ/Δ cells exhibited increased susceptibility to RAW264.7 murine macrophages. In addition, this mutant germinates less readily in macrophages and is less cytotoxic to the phagocytes, indicating that these effects require a functional amino acid sensing mechanism. Indeed, *ssy1*Δ/Δ cells reside in a more acidic compartment inside the macrophage, demonstrating that the SPS system is required for normal inhibition of the phagosomal acidification.

The function of the SPS sensor is mediated through Stp2, which is also required for utilization of amino acids and the associated pH changes (Vylkova *et al.*, 2011). We showed that Stp2 is cleaved in an SPS-dependent manner extremely rapidly, within minutes of a switch to media containing amino acids as the carbon source and that this promotes nuclear localization. Finally, expression of a constitutively nuclear isoform (Andreasson and Ljungdahl, 2004), created through an amino terminal truncation (Stp2*), suppresses all SPS phenotypes in vitro.

Overall, our data reveal that the SPS sensor in *C. albicans* has a similar architecture as in *S. cerevisiae* (Bernard and André, 2001; Didion *et al.*, 1998; Forsberg and Ljungdahl, 2001;

Forsberg *et al.*, 2001; Iraqui *et al.*, 1999; Klasson *et al.*, 1999; Poulsen *et al.*, 2005), which had been suggested by earlier characterization of Ssy1 (Csy1) in *C. albicans* by Ljungdahl and colleagues (Brega *et al.*, 2004; Martínez and Ljungdahl, 2004; Martínez and Ljungdahl, 2005). Importantly, however, the SPS complex had not been investigated for a role in responses to carbon starvation, and our data indicate that this system is critical for amino acid sensing under these conditions as well. This has important implications, as increasing evidence has shown that peptides and protein may be important sources of carbon during interaction with the host (Barelle *et al.*, 2006; Fernández-Arenas *et al.*, 2007; Fradin *et al.*, 2003; Fradin *et al.*, 2005; Lorenz *et al.*, 2004; Rubin-Bejerano *et al.*, 2003). This adaptation has potentially selected for specialization of SPS targets: in *C. albicans*, Stp2 controls the expression of genes involved in amino acids utilization such as amino acid permeases, whereas its paralogue Stp1 controls the expression of genes involved in protein degradation (Martínez and Ljungdahl, 2005; Ramachandra *et al.*, 2014). This is in contrast to *S. cerevisiae*, where these TFs have redundant roles (Eckert-Boulet *et al.*, 2004; Forsberg and Ljungdahl, 2001; Forsberg *et al.*, 2001).

Because the *STP2** allele suppressed the SPS mutants, we took a step further and explored whether constitutive expression of three known or suspected Stp2 targets, *GAP2* (general amino acid permease), *CAN2* (basic amino acid permease) and *DIP5* (dicarboxylic amino acid permease) (Martínez and Ljungdahl, 2005; Vylkova *et al.*, 2011) could suppress the SPS growth phenotypes on amino acids and found that they could not (data not shown). This suggests that targets of Stp2 extend beyond the permeases, potentially to catabolic enzymes or other factors that are also necessary for growth under these conditions. Indeed, we have recently described a large family of putative ammonia/acetate transporters ATOs, some of which are Stp2-regulated, that mediate neutralization. Strikingly, over-expression of individual *ATO* genes in an *stp2*Δ/Δ strain confers significant growth defects thereby reinforcing the idea that efficient utilization of amino acids and active manipulation of pH requires coordinate regulation of multiple processes (Danhof and Lorenz, 2015).

Our previous work left unanswered questions of whether the source of the amino acids in the phagolysosome is from the host or the pathogen. Conceivably, protein recycling in the fungus may contribute to release peptides and amino acids to support growth. Indeed, as part of the response to phagocytes, *C. albicans* upregulates the expression of vacuolar proteases (Fradin *et al.*, 2005; Lorenz *et al.*, 2004). However, the survival of a mutant defective in autophagy, *atg9*Δ/Δ, is not affected following macrophage phagocytosis (Palmer *et al.*, 2007), suggesting that protein recycling plays only a minor role in the nutrient acquisition during the interaction with phagocytes. In stark contrast, we have shown that the ability to properly sense extracellular amino acids is crucial for the normal resistance of *C. albicans* towards macrophages. This strongly suggests that the amino acids originate from the host. These may come from the hydrolysis of phagosomal proteins and peptides given the expression of multiple secreted proteases in phagocytosed cells (Fradin *et al.*, 2005; Lorenz *et al.*, 2004). Indeed, the only assay in which the constitutively active *STP2** allele does not fully suppress *ssy1*Δ/Δ phenotypes is in the macrophage, which may indicate a role for Stp1 in proteolysis and import of luminal proteins. Alternatively, others have shown that *C. albicans* actively modifies the composition of the vesicles through its trafficking inside the

cell (Bain *et al.*, 2014; Fernández-Arenas *et al.*, 2009; Okai *et al.*, 2015), which may result in the fungal cell inhabiting a compartment distinct from a classical phagolysosome.

Inhibition of the phagosomal acidification is a strategy that fungal pathogens have exploited extensively. *Histoplasma capsulatum* has been appreciated to neutralize the phagolysosome for many years (Eissenberg *et al.*, 1993), while we and others have shown that *C. albicans* also occupies a neutral phagolysosome (Bain *et al.*, 2014; Fernández-Arenas *et al.*, 2009; Vylkova and Lorenz, 2014) and have proposed a metabolism-driven mechanism. The distantly related pathogenic yeast *Candida glabrata* also inhibits phagosomal acidification in a process that depends on Golgi-localized α-mannosyl transferases (Kasper *et al.*, 2014). Recently, *Cryptococcus neoformans* was shown to efficiently block phagosomal acidification when phagocytosed by macrophages (Smith *et al.*, 2015). Further characterization of the phagosomal composition upon phagocytosis of strains defective in neutralization will shed light on the mechanisms by which *C. albicans* and other fungal pathogens are able to hijack and alter phagosomal maturation.

In contrast to findings with the *stp2*Δ/Δ mutant, in which virulence in a mouse model is attenuated (Vylkova and Lorenz, 2014), the *ssy1*Δ/Δ, *ssy5*Δ/Δ and *ptr3*Δ/Δ strains have only a small shift towards delayed mortality in the animals, a difference that was not statistically significant. There may be an SPS-independent pathway that could activate Stp2 *in vivo* or other SPS-independent functions of Stp2. Alternatively, SPS sensing may be less important in the intravenous injection model, which replicates only the late stages of the human disease, and may have a more striking role in other aspects of the host-pathogen interaction, such as during adhesion to epithelia and tissue invasion. In fact, genes important for amino acid transport and ammonia extrusion (*ATO*) are upregulated when *C. albicans* interacts with oral epithelial cells (Wächtler *et al.*, 2011). Therefore, there may be a more prominent role for the SPS system in biofilm-related models, such as in the oral cavity (Dongari-Bagtzoglou *et al.*, 2009; Solis and Filler, 2012), the urogenital tract (Harriott *et al.*, 2010) or on abiotic surfaces (Andes *et al.*, 2004; Nett *et al.*, 2010; Ricicova *et al.*, 2010; Wang and Fries, 2011). Amino acids (or proteins) are also likely a key nutrient in the gut and may be important for colonization in this native niche (Koh, 2013; Koh *et al.*, 2008; Samonis *et al.*, 1990; Wiesner *et al.*, 2001). Thus, further study is needed to define the nutritional parameters of infection in diverse host niches.

Experimental procedures

Media, culture conditions and strains

Strains were routinely grown on YPD medium (1% Yeast extract, 2% Peptone and 2% Dextrose) at 30 °C in a roller incubator. Neutralization assays were performed in yeast nitrogen base (YNB) medium (0.17% YNB and 0.05% ammonium sulfate) or YNBA (0.17% yeast nitrogen base and 0.25% allantoin) supplemented with 1% casamino acids and 2% glycerol, when specified; pH was adjusted to 4.0 with HCl. When needed, agar was added at 2% final concentration.

Deletion of *SSY1*, *PTR3* and *SSY5* was performed using the *SAT1*-flipper method (Reuβ *et al.*, 2004), in the wild type strain SC5314 (Gillum *et al.*, 1984). In brief, fragments of ~350

bp upstream and downstream relative to the open reading frame of interest were polymerase chain reaction (PCR) amplified and cloned between the KpnI and XhoI (upstream fragment) or the SacII and SacI (downstream fragment) in pSFS2. The deletion cassette was PCR amplified using Phusion High-Fidelity DNA polymerase (NEB) and used for electroporation of the recipient *C. albicans* strain. Transformants were selected on YPD with nourseothricin (200 μg/mL; Werner Bioagents, Jena, Germany). Clones were screened by colony-PCR to confirm correct integration of the cassette. In order to excise the deletion cassette, positive clones were grown on YPM (1% yeast extract, 2% peptone and 2% maltose) overnight at 30°C in a roller incubator and spread on YPD. Correct excision of the *SAT1-FLP1* cassette was verified by PCR. A second round of deletion was carried out to generate the homozygous strains described in Table S1. Reintegration of one allele of the respective gene was performed using a CIp10-based plasmid (Murad *et al.*, 2000), where the *URA3* marker has been substituted with the *SAT1* gene (Jiménez-López *et al.*, 2013). open reading frames including the upstream (promoter) and downstream (terminator) intergenic regions were cloned in the MCS of CIp10-*SAT1*. The resulting plasmids were linearized with StuI and used for transformation of the homozygous strains. Correct integration was verified by PCR.

Deletion of the first 100 residues of Stp2 was performed using a pSFS2-based plasmid (Reuβ *et al.*, 2004), containing the *ACT1* promoter (1012 bp) cloned in the NotI site. A 369 bp fragment upstream *STP2* was PCR amplified and cloned between the KpnI and XhoI sites, and a 329 bp fragment (+321 to +629, as measured from the ATG codon) was amplified and cloned between the SacII and SacI of pSFS2-ACT1p; this fragment contains an initiation codon. The deletion cassette was PCR-amplified as described previously and used for electroporation of the SPS mutant strains. Correct integration and excision of the cassette was performed as described previously.

Tagging of one allele of *STP2* was performed using a newly generated pFA-mCherry-*SAT1* plasmid, using pFA-*MYC-HIS1* (Lavoie *et al.*, 2008) as backbone. First, the *SAT1* gene was PCR-amplified from pSFS2 and cloned between the AscI and PmeI sites, replacing the *HIS1* marker. Then, the mCherry gene (Keppler-Ross *et al.*, 2008) was PCR amplified and cloned between the XmaI and AscI sites. A 447 bp *STP2* fragment (+1306 to +1752, from ATG codon) was cloned in the XmaI site in frame with mCherry and 353 bp fragment downstream *STP2* was cloned in the PmeI site of pFA-mCherry-*SAT1*. The tagging cassette was PCR-amplified and used to transform the recipient strains. Correct integration was verified by PCR.

Pma1-GFP strains were generated by cloning the GFP sequence (Barelle *et al.*, 2004) in the ApaI site of pSFS2. A 366 bp fragment (+2320 to +2685) of *PMA1* was cloned in the KpnI site, in frame with the GFP sequence. The *PMA1* 3′ UTR fragment (431 bp) was cloned between SacII and SacI. The tagging cassette was PCR amplified and used to transform the recipient strains. Correct integration was verified by PCR.

Ammonia release assay

We determined ammonia released by *C. albicans* growing on casamino acids by means of citric acid traps, as described previously (Danhof and Lorenz, 2015; Vylkova *et al.*, 2011). Briefly, cells were grown overnight in YPD and diluted to an OD_{600} of 1. Cells (5 μL) were

spotted onto solid YNBA with casamino acids, pH 4. Citric acid traps (120 μL of 10% citric acid) were placed in microtube caps attached to the lid of the dish, directly underneath the spotted inoculum. The plates were incubated at 37°C, and 20 μL samples were drawn every 24 h over a period of 3 days. Ammonia concentration was determined using the Nessler's reagent. Experiments were performed in triplicate.

Western blot analysis

Strains were grown overnight on YPD at 30°C in a roller incubator. Cells were diluted and grown for a further 4 h at 30°C starting at an OD_{600} of 0.2 in YNB + 2% glucose. Cells were collected at 4°C and resuspended in prewarmed (37 °C) YNB + 1% casamino acids. To investigate Stp2 processing in response to specific amino acids, cells were incubated for 15 min in YNBA in presence of the respective amino acid (10 mM), pH 4.0, at 37°C. Cells were collected by centrifugation at 4 °C and washed with TEGN-TX100 (20 mM Tris-HCl pH 7.9, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl, 0.1% Triton X-100) lysis buffer with protease inhibitors (Complete ULTRA, Roche, 1 tablet per 10 mL). Cell pellets were resuspended in TEGN-TX100 with protease inhibitors and mechanically disrupted using acid-washed glass beads. Samples were subjected to six rounds of 1.5 min of bead beating in a vortex mixer and 1.5 min on ice. Protein extracts were clarified by centrifugation and protein quantified by the Pierce BCA protein assay (ThermoFisher Scientific). Protein samples (50 μg) were separated by SDS-PAGE in a 6% acrylamide gel, and transferred to a 0.2 μm nitrocellulose membrane (BioRad). Membranes were blocked overnight at 4°C with 5% milk powder in 1× TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Blots were probed with a primary rabbit anti-mCherry antibody (1:5000, Rockland) and a secondary goat anti-rabbit horseradish peroxi-dase (HRP)-conjugated antibody (1:3000, BioRad). Blots were developed using the Pierce ECL Western blotting substrate (ThermoFisher Scientific) and visualized in an ImageQuant LAS4000 mini apparatus (GE).

Visualization of Stp2 nuclear translocation

To visualize the cellular localization of Stp2-mCherry in response to amino acids, cells were exposed to glucose or amino acids as described previously. Cells were collected and fixed in 2.7% paraformaldehyde (pH 7.5) for 15 min at 37 °C, washed twice with 1× PBS and incubated in 500 μL 1× PBS with Hoechst 33342 (1 drop of NucBlue, Molecular Probes) overnight at room temperature. Cells were collected and resuspended in 20 μL 1× PBS and visualized by fluorescence microscopy. Images were captured and processed using Slidebook 6.0. Z-stacks were analyzed by deconvolution using the nearest-neighbor algorithm, and a projection was generated as the final image.

End point dilution assay

To determine the sensitivity of the fungal strains to macrophage killing, we performed the end point dilution assay (Rocha *et al.*, 2001). In brief, RAW264.7 macrophages in RPMI 1640 with phenol red supplemented with 10% fetal bovine serum (RPMI-10) were seeded in wells in a 96-well plate at a concentration of 2.5×10^5 cells mL^{-1}, 100 μL per well, incubated overnight at 37°C and 5% CO_2. Fungal cells were grown overnight in YPD at 30°C, collected and washed twice with 1× PBS, and resuspended at a concentration of 5×10^5 cells mL^{-1}. Prior to infection, the medium was replaced with 150 μL RPMI without

phenol red. The first well in each column was inoculated with 50 μL of the fungal suspension, and then serially diluted 1:4 six times. Media controls were prepared in the same manner, and incubated 15 h at 37°C and 5% CO_2. Quantification of fungal biomass (microcolonies developed at the bottom of the wells) was performed by means of the XTT assay as previously described (Miramón *et al.*, 2014). In brief, media was carefully removed from each well and 150 μL of XTT/Coenzyme Q0 were added in each well and incubated at 37°C for 60 min. An aliquot of 100 μL was removed from each well and transferred to a new plate. Absorbance was read at 451 nm in a microplate reader. Experiments were performed five independent times.

Lactate dehydrogenase release assay

Cytotoxicity of the fungal strains on macrophages was determined by detecting the release of LDH after coincubation, using the CytoTox96 Non-Radioactive Cytotoxicity assay (Promega) according to manufacturer's instructions (Vylkova and Lorenz, 2014). Briefly, RAW264.7 macrophages in RPMI-10 were seeded in a 96-well plate at a concentration of 2.5×10^5 cells mL^{-1}, 100 μL per well, incubated overnight at 37°C and 5% CO_2. Fungal suspensions were prepared as described previously, at 2×10^6 (for multiplicity of infection of 2) and 1×10^6 cells mL^{-1} (for MOI of 1). Prior to infection, the medium was replaced with 50 μL RPMI without phenol red. Macrophages were infected with 50 μL of the fungal suspensions and incubated for 15 h at 37°C and 5% CO_2.

LDH release was normalized to chemically lysed macrophages. Experiments were performed in triplicate.

Phagosome acidification assay

Phagosomal acidification was investigated using the acidotropic fluorescent probe LysoTracker® Red DND-99, which accumulates in acidic organelles. Macrophages were seeded in glass-bottom μ-Slides (Ibidi) at a concentration of 1.5×10^5 cells mL^{-1} and loaded for 2 h with 50 nM LTR in RPMI without phenol red. Medium was replaced prior to infection. Overnight cultures of Pma1-GFP *C. albicans* strains were regrown in YPD for 5 h at 30°C, cells were collected and washed in 1× PBS, and diluted to a concentration of 1.5×10^7 cells mL^{-1}. Macrophages were infected with 10 μL of the *C. albicans* suspensions and incubated for 1 h at 37°C, 5% CO_2. Cells were fixed in paraformaldehyde, washed with 1× PBS, and nuclei were stained with NucBlue for visualization purposes. Images were captured and analyzed using Slidebook 6.0. To quantify the accumulation of LTR in the phagosome, we obtained the signal intensity profiles of GFP and LTR along a line extending beyond the edges of the fungal cell. We quantified the area under the curve of the LTR signal for a region of 20 pixels (2 μm) from each side of the *C. albicans* cell, outlined by the GFP signal. Experiments were performed in triplicate; a minimum of 30 fungal cells were analyzed per strain in each experiment.

Murine model of disseminated candidiasis

The murine model of hematogenously disseminated candidiasis was performed as previously described (Vylkova and Lorenz, 2014). Female ICR mice (10 individuals per strain, housed in groups of five) were inoculated via the tail vein with 100 μL of fungal suspensions in 1×

PBS at a concentration of 5×10^6 cells mL^{-1}. Animals were monitored twice daily and humanely terminated when moribund. Protocols were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Statistical analyses

Statistics were performed in GraphPad Prism 6. Tukey's multiple comparisons test was used to analyze the ammonia release assay. Two-tailed paired Student's *T*-test was used to analyze the fungal survival data (end point dilution assay). Macrophage cytotoxicity data (LDH assay) and phagosomal acidification (LTR assay) were analyzed using Dunn's multiple comparisons test. Survival proportions were evaluated by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. P values≤0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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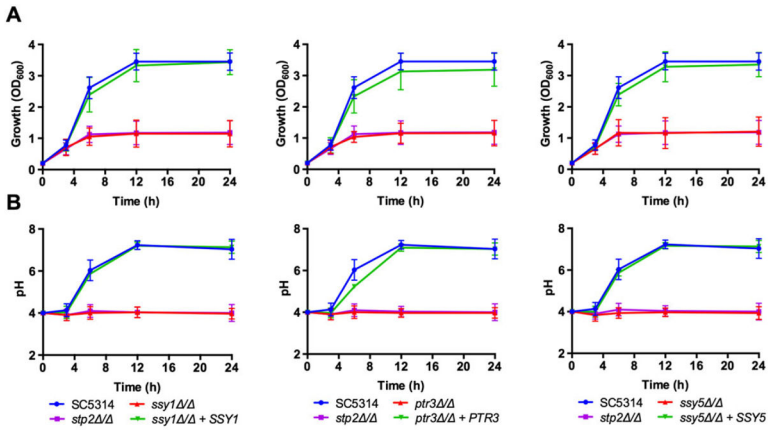

Fig. 1.

The SPS system is required for amino acid utilization and environmental pH manipulation. Wild type SC5314 *Candida albicans*, *ssy1*Δ/Δ (CaPM07), *ptr3*Δ/Δ (CaPM15) and *ssy5*Δ/Δ (CaPM23), and complemented strains were grown at 37°C on yeast nitrogen base supplemented with 1% casamino acids, with an initial pH of 4.0. The *stp2*Δ/Δ (SVC17) strain was included for comparison purposes.

A. Growth was determined by measuring the OD_{600} at indicated time points.

B. Aliquots of the cultures were used to measure the pH of the medium at the same time points. Graphs show mean and standard deviation from three independent replicates.

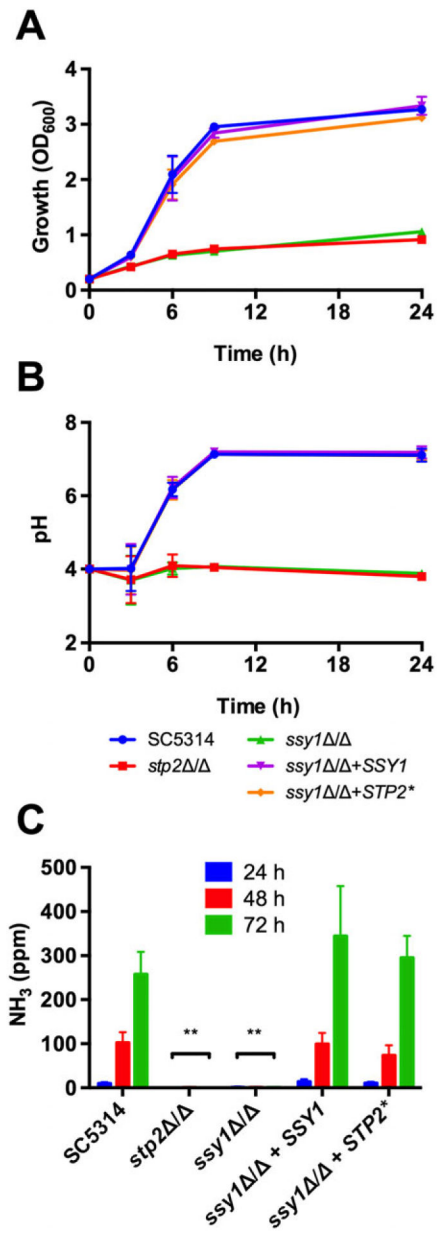

Fig. 2.

Growth and pH manipulation correlate with ammonia release. Wild type *Candida albicans* SC5314, *ssy1*Δ/Δ (CaPM07), *ssy1*Δ/Δ + *SSY1* (CaPM25) and *ssy1*Δ/Δ + *STP2** (CaPM36) strains were grown at 37°C on yeast nitrogen base allantoin (allantoin present as nitrogen source) with 1% casamino acids at an initial pH of 4.0. The *stp2*Δ/Δ (SVC17) strain was included for comparison purposes.

A. Growth was determined by measuring the OD_{600} at indicated time points.

B. Aliquots of the cultures were used to measure the pH of the medium at the same time points.

C. Ammonia excreted as byproduct of amino acid catabolism was collected in an acid trap and quantified with the Nessler's reagent. Graphs show mean and standard deviation from three independent replicates.

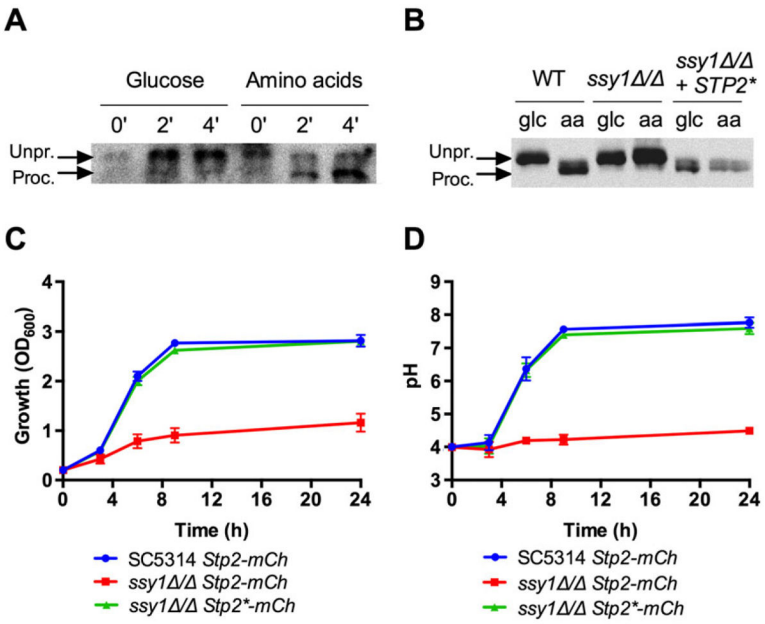

Fig. 3.

Processing of Stp2 in response to amino acids depends on the SPS system. A. Western blot analysis to detect Stp2 processing in response to amino acids. *Candida albicans* cells expressing a single *STP2* allele tagged with mCherry (CaPM41) were grown for 4 h in yeast nitrogen base supplemented with 2% glucose at 30°C, at an initial OD_{600} of 0.2. Cells were then exposed to yeast nitrogen base + 2% glucose or 1% casamino acids for the indicated time. Cell extracts were blotted and probed with an anti-mCherry antibody (see Materials and methods for details). The higher band corresponds to the unprocessed Stp2 ('Unpr.'), whereas the lower one represents the processed version ('Proc.'). B. Processing of Stp2 was not observed in *ssy1*Δ/Δ expressing *STP2-mCherry* (CaPM43) after exposure to glucose or amino acids for 1 h. As a control, an *ssy1*Δ/Δ strain expressing a truncated version *STP2*-mCherry* (CaPM45) lacking the N-terminal domain was included. Western blots were performed three independent times from biological replicates. Representative images are shown. (C) Growth and (D) pH was determined as stated in Fig. 1. Graphs show mean and standard deviation from three independent replicates.

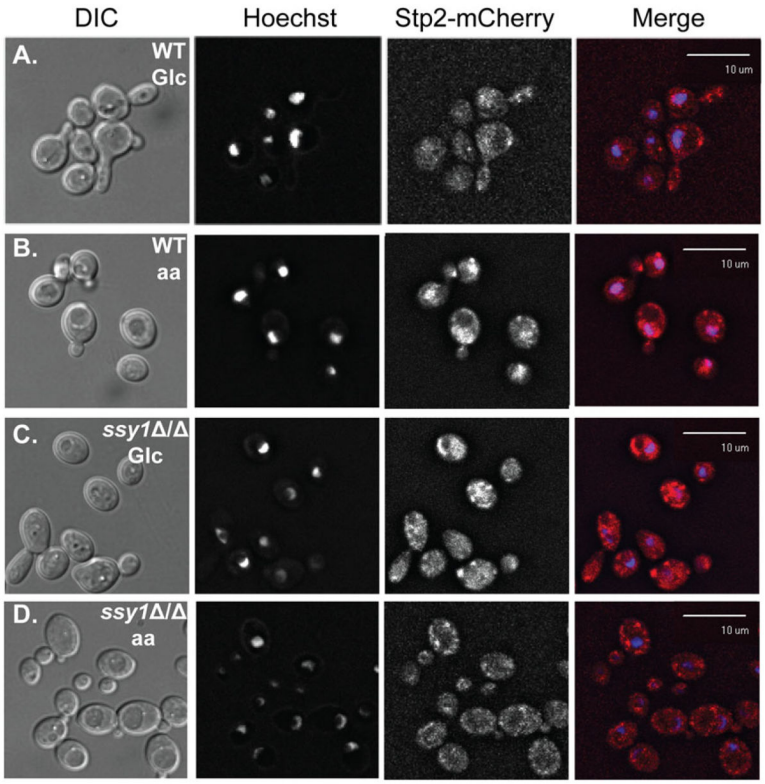

Fig. 4.

Nuclear translocation of Stp2 upon amino acid sensing relies on a functional SPS system. Wild type (CaPM41) and *ssy1*Δ/Δ cells expressing the *STP2-mCherry* fusion (CaPM43) were exposed to glucose or amino acids for 1 h at 37°C. Cells were fixed and stained with Hoechst 33342. Cells were visualized with fluorescence microscopy to detect DAPI and mCherry fluorescent signal in the corresponding channels. A) Wild type cells exposed to glucose. B) Wild type cells exposed to amino acids. C) *ssy1*Δ/Δ cells exposed to glucose or D) *ssy1*Δ/Δ cells exposed to amino acids. Experiments were performed three independent times. Representative images are shown.

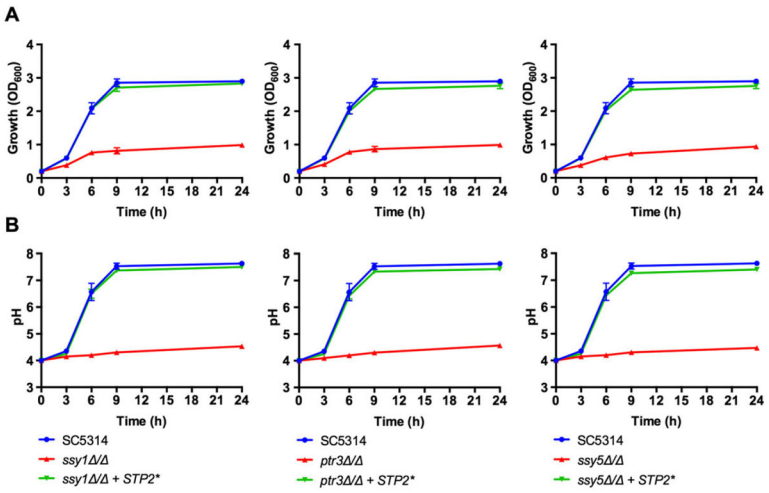

Fig. 5.

Expression of a truncated *STP2* allele lacking the N-terminal domain restores growth and neutralization ability in the SPS mutants. A truncated allele of *STP2* lacking the first 100 residues (Stp2*), corresponding to the N-terminal nuclear exclusion domain, was expressed in the *Candida albicans ssy1*Δ/Δ (CaPM36), *ptr3*Δ/Δ (CaPM38) and *ssy5*Δ/Δ (CaPM40) strains. Cultures were grown at 37°C on yeast nitrogen base supplemented with 1% casamino acids, with an initial pH of 4.0.

A. Growth was determined by measuring the OD_{600} at indicated time points.

B. An aliquot of the cultures was used to measure the pH of the medium at the same time points. Graphs show mean and standard deviation from three independent replicates.

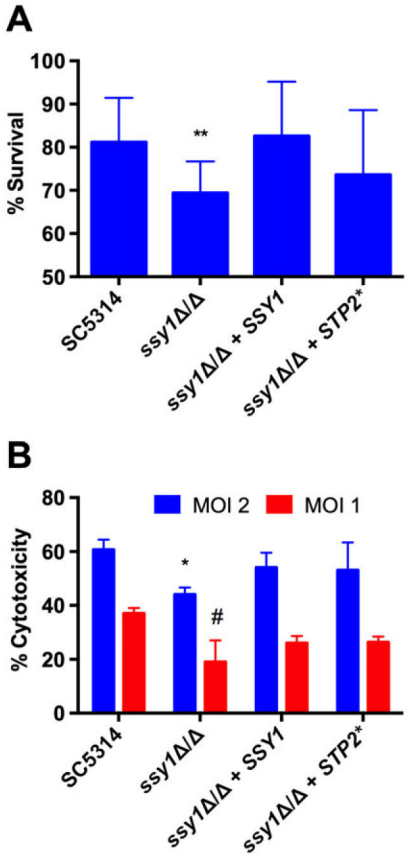

Fig. 6.

The SPS system is required for normal resistance towards macrophage attack and cytotoxicity. RAW264.7 murine macrophages were infected with wild type (SC5314), *ssy1*Δ/Δ (CaPM07), reconstituted (CaPM25) and *STP2**-expressing (CaPM36) cells.

A. An end point dilution assay coupled with an XTT metabolic assay was performed to determine the sensitivity of these strains towards macrophage killing. Bars show mean and standard deviation of five independent experiments. $^{**}P < 0.01$.

B. Macrophage cytotoxicity was determined by measuring the release of lactate dehydrogenase after coincubation of phagocytes with fungal cells for 15 h at two different multiplicities of infection (MOI). Bars show mean and standard deviation of three independent experiments. *P < 0.05 (compared with WT at MOI 2); $^{\#}P < 0.05$ (compared with WT at MOI 1).

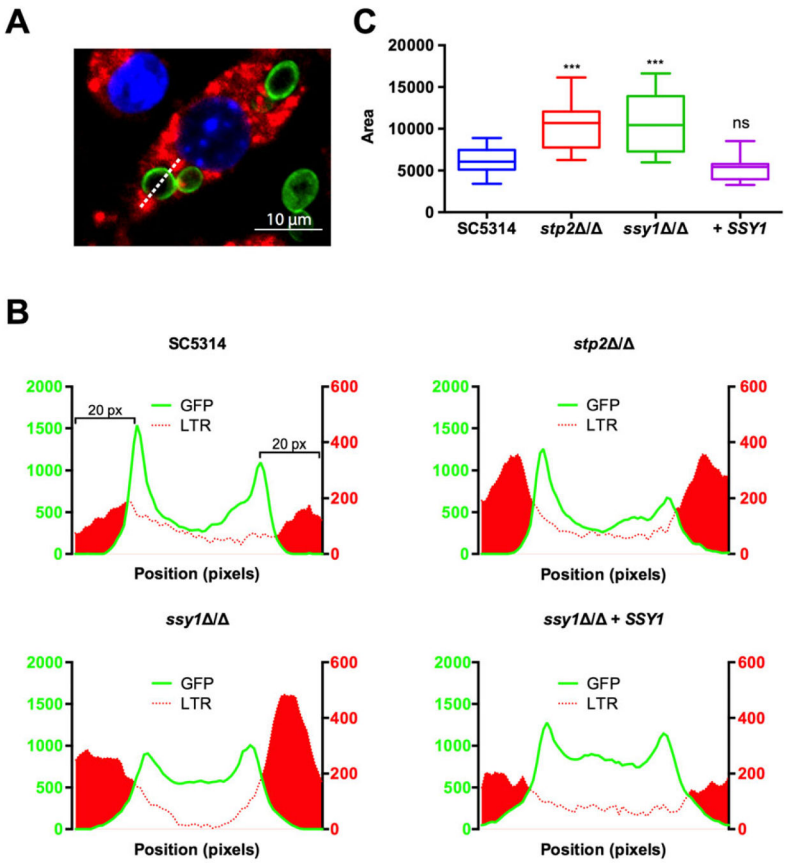

Fig. 7.

The SPS system is required for neutralization of the phagosome. LysoTracker Red (LTR)-loaded RAW264.7 macrophages were infected with SC5314 (CaPM57), *stp2*Δ/Δ (CaPM61), *ssy1*Δ/Δ (CaPM62) and *ssy1*Δ/Δ + *SSY1* (CaPM66) each expressing a C-terminal Pma1-GFP fusion.

A. Quantification of the LTR signal was performed using Slidebook 6.0. GFP and LTR signals were measured by drawing a line traversing the fungal cell (dashed line).

B. Representative profiles from each strain. GFP signal is plotted on the left axis, LTR signal plotted on the right axis. Area under the curve (filled in red) was calculated in Prism (GraphPad Software).

C. Graph shows box and whiskers (minimum to maximum values), representing the area under the curve from the LTR intensity. A minimum of 30 cells were analyzed per strain. The experiment was performed in triplicate.