

C. albicans incorporates methaemoglobin into surface attached and aggregate biofilms that exhibit increased virulence and antifungal resistance

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Abstract

Candida albicans is a leading cause of invasive fungal infections, yet how host-derived factors shape pathogenic biofilm states remains poorly understood. Here we show that oxidised haemoglobin (methaemoglobin; MetHb) which is readily formed upon red blood cell lysis, promotes the formation of *C. albicans* biofilms. MetHb also induces the rapid agglutination of *C. albicans* cells into haemoglobin aggregates, that display hallmark biofilm properties including antifungal tolerance, and enhanced virulence. The interaction between *C. albicans* and MetHb is enhanced by hyphal growth and requires the adhesins Als1 and Als3. Temporal transcriptomic analysis of *C. albicans*-MetHb aggregate formation revealed a rapid activation of adhesion and haem-iron acquisition pathways, followed by metabolic remodelling and enrichment of hypoxia-associated genes suggesting the establishment of localised microenvironments that favour fermentative metabolism. Consistent with *C. albicans*-MetHb aggregates acting as aggregate biofilms, they exhibited increased tolerance to antifungals and increased virulence in a *Galleria mellonella* infection model. Agent-based modelling of *C. albicans*-MetHb interactions supports a mechanism by which hypha-mediated adhesion drives aggregation and biofilm formation as observed experimentally, providing a valuable tool for future investigation. Together, these findings identify Methaemoglobin as a host-derived driver of rapid *C. albicans* community assembly. This raises the possibility that high MetHb levels increase the chance that *C. albicans* drug-tolerant, hypervirulent biofilm or aggregate biofilms become established *in vivo*. As MetHb levels are found to be elevated during infection, sepsis, surgery or medical device insertion, our novel findings raise the possibility that *C. albicans*-MetHb agglutination may play a significant, and yet underappreciated, role in multiple pathologies.

Introduction

Candida albicans is an opportunistic fungal pathogen capable of causing both superficial and invasive disease. Invasive candidiasis and *Candida* bloodstream infections collectively affect more than 1.56 million individuals globally each year, with mortality rates exceeding 63%, accounting for approximately 39% of all deaths attributable to fungal infections (1). Due to limited options for antifungal therapies, the high prevalence and mortality rates led to the World Health Organisation declaring *C. albicans* a critical priority (2).

Key to *C. albicans* virulence is its ability to transition from a yeast to hyphal form under key physiological stresses. While growth in the yeast form is typically associated with commensalism, the hyphal form of *C. albicans* is linked to tissue invasion, immune modulation and biofilm formation. Biofilm formation is the colonisation of surfaces or substrates by microorganisms, followed by the production and/or encapsulation within an extracellular matrix. Colonised surfaces can be either biotic such as organs and tissues, or abiotic such as medical tubing (3, 4). Cells grown in the biofilm form are strongly linked to chronic infection, persistence and resistance to antimicrobials.

C. albicans interacts with a broad range of human host proteins. For adhesion and invasion, *C. albicans* targets cell surface proteins such as E-cadherin for binding (5). In the extracellular matrix, proteins such as fibronectin and fibrinogen also aid in adhesion and colonisation. These matrix proteins are particularly relevant to biofilm formation on indwelling medical devices, where the absorption of these proteins onto device surfaces, termed conditioning, generates a more biocompatible substrate for *C. albicans* colonisation (6). Beyond adhesion, host proteins such as transferrin and ferritin are exploited for their nutritional provisions. As free iron is tightly regulated in sequestering systems such as these, *C. albicans* has evolved mechanisms to bind to these proteins and extract iron directly (7, 8). In the context of immunity, serum proteins albumin and factor H are also utilised by *C. albicans*. Albumin interacts with the secreted *C. albicans* toxin candidalysin to neutralise it, reducing immune activation to facilitate colonisation, whereas factor H is bound at the cell surface to mask key pathogen associated molecular patterns and reduce immune recognition (9, 10).

Haemoglobin (Hb) is the most abundant iron sequestering protein in humans, and functions to bind oxygen within red blood cells for transport. It is a 64.5kDa tetramer formed of four globin subunits, The most common form, HbA is composed of 2 α and 2 β -globin chains, each containing a single haem molecule with a central Fe atom. The redox state of this Fe atom is critical to haem's oxygen binding capacity and is therefore tightly regulated, with approximately 99% of Hb maintained in the ferrous state, termed oxyhaemoglobin (oxyHb). Oxidation of Fe(II) to Fe(III) forms methaemoglobin (MetHb) which is incapable of coordinating oxygen (11). Oxidation of oxyHb to MetHb occurs spontaneously at a low level under normal conditions where O₂ is reduced to superoxide. Any circulating reactive oxygen species can also convert oxyHb to MetHb (12). To maintain the functional, oxyHb state, MetHb is continuously reduced to oxyHb, intracellularly by the NADH-cytochrome-b₅ reductase system (13).

If Hb is released from the red blood cells, such as following haemolysis, it is autooxidised to MetHb. Extracellular Hb causes a local inflammatory response via toll-like receptor 4, initiating NF- κ B signalling. Innate response cells activated via this pathway release pro-inflammatory cytokines that promote an acute phase response in the liver, including the production of haptoglobin and hemopexin. Haptoglobin and hemopexin are specialised proteins that bind to Hb and haem respectively for degradation in macrophages or the liver (14). Such elevations are characteristic of systemic infection and septic shock, where significant inflammatory mediated haemolysis can occur (15).

The interactions between Hb and *C. albicans* have been investigated to a limited extent. When grown in Hb solutions, it was observed that *C. albicans* was bound to amorphous aggregates of protein, and that this occurred in both the yeast and hyphal form (16, 17). Hb was also demonstrated to induce the yeast-hyphal transition via the core morphogenesis regulator Efg1. More recently, hemin was shown to drive biofilm formation in *C. albicans* but in a mechanism independent of iron acquisition (18). Iron dependent biofilm enhancement via Hb has also been observed in Gram positive bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (19, 20).

We aimed to further characterise the interactions between Hb and *C. albicans*, particularly within the context of biofilm formation. We demonstrate that the previously observed amorphous protein aggregates binding *C. albicans* are specifically composed of MetHb, which is actively incorporated into both surface-attached, and aggregate biofilms. Here it acts as a structural component constituting a substantial proportion of the observed biomass where it helps to build biofilms rapidly and independently of its potential as a source of iron. MetHb inclusion into aggregate biofilms promoted significant antifungal tolerance to both amphotericin B and anidulafungin, likely through physical exclusion of these compounds and a transcriptional shift towards fermentative metabolism programs and persistence. Aggregate biofilms assembled rapidly in the presence of both MetHb released from whole blood lysis by *C. albicans*, or in purified MetHb solutions. We also identified Als1 and Als3 as essential adhesins required for MetHb-*C. albicans* aggregate biofilm formation. Interaction of *C. albicans* with MetHb also increased virulence in a *Galleria mellonella* infection model. Together our findings suggest that *C. albicans* can actively liberate and make use of MetHb to build biofilm structures on surfaces or in solution. This raises the possibility that MetHb binding plays a previously underappreciated role in the host interaction strategy employed by this successful opportunistic pathogen.

Materials and methods

Maintenance of strains and cultures

The complete details of all strains are summarised in the Supplementary material (Table S1). *C. albicans* isolates were maintained on yeast extract peptone dextrose (YPD) (1% yeast extract (BD Bacto), 2% peptone (BD Bacto), 2% D-glucose (Fisher)) agar (Oxoid) at 30°C. Prior to use, strains were grown overnight at 30°C, 180 RPM in YPD. For all assays, *C. albicans* was grown in Roswell Park Memorial Institute (RPMI) 1640 media (Oxoid) at 37°C unless specified otherwise.

Preparations of haemoglobin and blood solutions.

MetHb solutions were prepared using lyophilised human haemoglobin by Sigma-Aldrich (H7379) who state that these preparations are “primarily methaemoglobin” under non-reducing conditions. For experiments requiring oxyHb we used a lyophilised, ferrous stabilised haemoglobin preparation also by Sigma-Aldrich (H0267). Each preparation was dissolved in Mill-Q water or RPMI-1640 media and made fresh daily. The oxidation state of Hb solutions was confirmed using a BMG Labtech FLUOstar Omega plate reader to measure the absorbance spectra from 500-700nm. MetHb was confirmed by a peak at 630nm and oxyHb by dual peaks at 540 and 578nm. Solutions of 5% blood were prepared in RPMI-1640 from whole sheep blood (TCS bioscience).

Haemolysis

A haemolysis protocol was adapted from Mogavero *et al.* (21). Erythrocytes were isolated from whole sheep blood in Alsevers (TCS biosciences) by centrifugation at 2000 RCF for 10 minutes and the supernatant, and central layer of leukocytes aspirated. The erythrocytes were then resuspended in PBS. This process was then repeated, and the cells resuspended in RPMI-1640. Erythrocytes were counted using a haemocytometer and mixed in a 1:1 ratio with 5×10^7 CFU/mL *C. albicans* cells in a final volume of 10 mL RPMI-1640 media in a 50mL centrifuge tube. The tube was incubated horizontally at 37°C, 5% CO₂, 50 RPM for 24 hours. The samples were then centrifuged at 3200 RCF for 5 minutes and the supernatant containing Hb collected for use in complex formation assays. For cell free haemolysis, isolated erythrocytes were resuspended in Milli-Q water and vortexed for 30 seconds. Cell debris was then removed by vortexing at 3200 RCF for 1 minute.

Microtiter biofilm assay

For biofilm growth in microtiter plates, we used untreated flat-bottom 96-well plates (Starlab) with a working volume of 100 µL. Each well was pre-treated with 50% donor bovine serum (Thermofisher)(DBS) (50% PBS) for 30 minutes at 30°C without shaking. Excess DBS was then aspirated. *C. albicans* cells, previously grown overnight in YPD, were washed in PBS and diluted to an optical density (OD) of OD = 0.2 in PBS and incubated in each well for 90 minutes at 37°C, 5% CO₂, without shaking. Excess PBS containing planktonic cells was then aspirated leaving only the adhered population. RPMI-1640 media was then applied by gently pipetting the media down the side of each well and the plate incubated for a further 24 hours at 37°C, 5% CO₂, without shaking. The excess media was then aspirated and the mature biofilms washed with PBS.

Quantification of total biomass by crystal violet staining

Biofilms were covered with 0.1% crystal violet (Sigma-Aldrich) solution for 15 minutes at room temperature. Excess crystal violet was then removed, and the biofilm washed 4 times by running distilled water down the edge of the plate well. Biofilms were then left at room temperature until dry. For quantification, the biofilms were destained with 30% acetic acid for 15 minutes at room temperature. The acetic acid was then transferred to a fresh plate, and absorbance read at 550 nm using a BMG LABTECH FLUOstar Omega plate reader.

Quantification of metabolic activity by XTT assay.

30 µg/mL XTT reagent (Roche) was added to mature, washed biofilms and incubated at 37°C for 4 hours. The microtiter plates were then centrifuged at 3200 RCF for 5 minutes. The supernatant was then transferred to a clean plate and the absorbance at 492 nm measured using a BMG LABTECH FLUOstar Omega plate reader.

Immunostaining

For immunostaining of *C. albicans*-MetHb complexes, samples were initially washed twice in PBS and fixed in 100%, 4°C methanol on ice for 15 minutes. Samples were then washed twice in PBS and blocked in 1% bovine serum albumin (BSA) solution for 1 hour at room temperature. Samples were then resuspended in 1:1000 anti-haemoglobin-FITC (Thermofisher #A80-134F) and incubated at room temperature for 1 hour. Samples were then washed twice in PBS and mounted on coverslips with 100 µg/mL CFW.

Growth and staining of biofilms for confocal microscopy.

Biofilms for CLSM were grown as described above in untreated 8-well chamber slides (Ibidi) with a working volume of 300 µL. After 24 hours of growth, biofilms were fixed in 4% paraformaldehyde (Thermofisher) for 30 minutes at room temperature, washed 3 times in PBS and counterstained with 30 µg/mL of calcofluor white (CFW) (Sigma-Aldrich) and/or immunostained with anti-haemoglobin-FITC. For identification of the extracellular matrix biofilms were stained with FilmTracer™ SYPRO® Ruby for (Thermofisher) for 30 minutes. MetHb emitted a strong fluorescence when excited at 561nm and detected by using emission at 580-700nm. Imaging of biofilms and *C. albicans* complexes was completed using a Zeiss LSM880/Elyra/Axio Observer.Z1 confocal microscope with the ZenBlack acquisition software. The 405 nm diode, 488 nm argon and 561 nm DP55 laser were used for blue (CFW), green (anti-Hb-FITC) and red (MetHb) respectively. Images were taken using a multi-track mechanism with Airyscan detector. To capture z-stacks the “optimal” setting in ZenBlack was utilised to take slices between 1.5–1.8µm. All images were processed using standard airyscan processing. Z-stacks were further processed into maximum intensity projections.

***C. albicans*- Hb complex formation assay**

Formation of *C. albicans*-Hb complexes in solution was performed by inoculating OD = 1.0 *C. albicans* cells into RPMI-1640 media + 0.3% MetHb and incubating at 37°C, 180 RPM for 4 hours unless otherwise specified. Experiments using oxyHb were limited to 2 hours as the product would begin to oxidise to MetHb beyond this point, as confirmed by spectrophotometry (Figure S1). To isolate complexes for downstream analysis, solutions were centrifuged at 100 RCF for 30 seconds and resuspended in PBS. To quantify mean *C. albicans*-Hb aggregate/complex size, images were captured using a Denovix CellDrop™ FI automatic cell counter. *C. albicans*-Hb complexes were formed and isolated as described above and images were captured using the 4X objective lens with brightfield illumination. Batch size analysis was performed using thresholding and the analyse particles functionality of ImageJ.

To image the interactions between separate *C. albicans*-MetHb complexes we strain expressing a GFP tagged mitochondria (mtGFP) in the SN250 background (22). SN250-MetHb and SN250-mtGFP-MetHb complexes were initially formed independently for 1 hour at 37°C in RPMI-1640 + 0.3% MetHb. The two cultures were then combined in a 1:1 ratio and incubated for a further 3 hours. The resulting complexes were then counterstained with 100 µg/mL calcofluor white and imaged by CLSM as described above.

SDS-PAGE and Western Blotting

Isolation of proteins from aggregate biofilms was performed by hot alkali extraction. Briefly aggregate biofilms were resuspended in lysis buffer (0.069 M SDS, 0.1 M NaOH, 0.05 M EDTA, 0.028 β -mercaptoethanol) and heated at 90°C for 10 minutes. Acetic acid was then added to a final concentration of 0.15% and heated for a further 10 minutes at 90°C. Cell free controls were dissolved directly into lysis buffer at the appropriate concentration. Proteins were then separated by SDS-gel electrophoresis using 4–12% Bis-Tris NuPAGE (Thermo) gels and either stained with Coomassie brilliant blue or transferred to polyvinyl difluoride membranes. For western blotting, the membranes were blocked in 5% BSA and probed with anti-haemoglobin-FITC using a 1:1000 dilution and visualised using 488nm illumination.

1. *C. albicans* adhesin KO library screen

C. albicans single gene deletion mutants from the Shaprio et al. (11) adhesion knockout library were inoculated into RPMI-1640 + 0.3% MetHb and incubated at 37°C, 180 RPM for 1.5 hours. The suspension was then centrifuged at 100 RCF for 30 seconds to separate out the complexes from the planktonic cells. The absorbance at 600nm of the remaining supernatant was then measured using a BMG LABTECH FLUOstar Omega plate reader. An agglutination score was then calculated using the reciprocal of the raw absorbance measurement. Here a lower score reflects less aggregation/MetHb binding and therefore a smaller reduction of the turbidity of the solution when briefly centrifuged. To calculate percentage binding of specific mutants to MetHb, complexes were imaged by CLSM and the number of cells bound to MetHb per frame counted. For images containing large multicellular complexes where counting was not possible, a percentage binding of 99 was recorded.

Antifungal tolerance assay

24-well microtiter plates in RPMI-1640 media. 1 mL of RPMI-1640 $\pm$ 0.3% MetHb was inoculated with OD = 1.0 *C. albicans* and incubated for 4 hours at 37°C, 180 RPM. Following incubation, the resulting complexes were centrifuged at 3200 RCF for 5 minutes and washed in PBS. The plate was then centrifuged again, and the complexes resuspended in 1 mL RPMI-1640 media containing increasing concentrations of 0–20 μ g/mL of amphotericin B, or 0–10 μ g/mL of anidulafungin. The complexes were then incubated for 24 hours at 37°C, 180 RPM. As the *C. albicans*-MetHb complexes could not be reliably disrupted or diluted without affecting cell viability, we could not calculate viable CFUs by traditional methods. Instead, the suspension was washed once in PBS then resuspended in 200 μ L of fresh PBS and the whole volume spread on YPD agar plates. The plates were incubated for 48 hours at 30°C. Plates were then scanned and the percentage coverage of each plate calculated using thresholding in ImageJ. The resulting percentage area was then normalised against the percentage area of the untreated plates to calculate a survival index at each concentration.

RNA isolation from *C. albicans* aggregates

Total RNA extraction was performed using the RiboPure™ Yeast RNA purification kit by Invitrogen™ with some additional pre-treatments. Specifically, *C. albicans* was grown in RPMI-140 ± 0.3% MetHb for 30 or 90 minutes. The resulting solution was then washed in PBS to remove excess protein and sonicated on ice for 30 seconds (20% amplitude, 5 second pulses). The suspensions were then washed again in PBS and resuspended in PBS containing 2.5 mg/mL zymolyase and 2 mg/mL proteinase K and incubated for 30 minutes at 37°C. Following incubation, the solutions were resuspended in 48µL, 10% SDS, 480µL lysis buffer, and 480µL phenol:chloroform:IAA and the RNA extraction performed as per the RiboPure™ manual. RNA was eluted in 100uL DEPC water and quantified with a NanoDrop ND-1000 to check A_{260}/A_{280} and A_{260}/A_{230} for protein and solvent contamination respectively.

RNA sequencing and analysis

Library preparation and sequencing was performed by Novogene Limited (Cambridge, UK). Messenger RNA was purified from total RNA extracts using poly-T oligo attached magnetic beads. cDNA was then synthesised using random hexamer primers followed by the second strand cDNA synthesis. Novogene then performed A-tailing, adapter ligation, size selection, amplification, and purification to generate the library. Quality control was performed by real-time PCR and bioanalyzer.

RNAseq analysis was completed using the Galaxy platform (12). Firstly the RNA reads were checked for quality using FastQC v0.12.1 (13). Average sequence quality (Phred score) was 39 Adaptor ends were trimmed with TrimGalore! v0.6.7 (14). The remaining reads were re-checked by FastQC with Phred score maintained at 39. Processed reads were then aligned against SC5314 Assembly 22 s08-m01-r01 using Bowtie2 v2.5.3 (15). Based on the associated .gtf annotation file, aligned, annotated reads were counted using FeatureCounts v2.0.8 (16). Differential expression between RPMI-1640 and 0.3% MetHb treated aggregates was determined using DESeq2 v2.11.40.8. Based on the resulting principal component plot, one MetHb treated replicate was discarded, and the DESeq2 analysis repeated. Analysis of the significantly differentially expressed genes (adjusted $p < 0.05$) was then performed using the online bioinformatics resource STRINGdb, which analyses protein-protein interactions within a gene-set to identify processes that are significantly enriched (17). Processes with a false discovery rate > 0.05 were classified as enriched and were merged in STRINGdb with a similarity score of 0.1. The relationship/overlap between processes was determined using REVIGO. (18) This data was then used to create perfuse-force directed ball and stick plots in Cytoscape v3.9.1 (10) where distance between nodes corresponds to similarity in process, size of node corresponds to enrichment, and colour corresponds to whether a process is up or down regulated. For processes of interest, annotated heat maps were manually constructed to show individual gene expression profiles.

Gene set enrichment analysis (GSEA) was performed using the GSEA PreRanked tool (19) to compare our list of differentially expressed genes to a large functional gene set database assembled by Sellam et al. (2014) (20) encompassing experimental analyses, GO terms from the Candida Genome Database (21), and relevant protein-protein interactions from the Saccharomyces Genome Database (22). Raw,

processed data and metadata are available at the NCBI Gene Expression Omnibus accession number GSE249653.

In vivo *G. mellonella* infection assay

In vivo infection assays were conducted using *Galleria mellonella* larvae (Live Foods Direct, Sheffield, UK) Larvae were stored at 15°C on receipt to prevent pupation. Prior to assays, larvae allowed to warm at room temperature for at least 30 minutes. $N = 40$ Larvae per condition were inoculated with 10 µL containing 2×10^5 CFU/mL *C. albicans* cells in PBS ± 0.3% MetHb. Post inoculation, the larvae were incubated at 37°C for 7 days and mortality assessed daily. Death was recorded if no motility was visible on contact with a pair of forceps.

Statistical analyses

Statistical analyses were performed using GraphPad PRISM v11.0.0. Bar plots were analysed by One-way ANOVA with Tukey's multiple comparisons or Dunnett's test and group bar plots were analysed by Two-way ANOVA with Šidák's multiple comparisons test. *Galleria mellonella* survival curves were analysed by log-rank test. In all plots: (*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$, (****) = $p < 0.0001$, (ns) = $p > 0.05$. Error bars denote standard deviation.

Agent based model for *C. albicans*-MetHb biofilm formation

Modelling of *C. albicans*-MetHb biofilm formation was based on the lattice-free agent-based model as described in Binder (2025)(23). The binding and aggregation of *C. albicans* with MetHb and subsequent growth of hyphae was simulated within a cube of side length $2X$, centred at the origin. Simulations were initiated with a distribution of m MetHb agent-clusters on the plane $z = -X$. A population of n yeast-form *C. albicans* were distributed uniformly at random throughout the three-dimensional domain and allowed to undergo random walks with coefficient of diffusion $D = 1$. *C. albicans* and MetHb agents were idealised as spheres of unit radius and were considered adhered upon contact when the distance between their centres were less than 3 units apart with probability $0 \leq B \leq 1$. Upon successful adhesion the yeast-form agents cease their random walk and initiate hyphal growth, idealised as a third species of unit-length spherical hyphal agents, which is an extension to the base model of Binder (2025)(23) Hyphal agents are initially generated adjacent to the yeast-agent with subsequent agents giving rise to new hyphal agents with the probability $H = 10^{-3}$ at each iteration. Natural variability in filament length was modelled based on a normal distribution $\mathcal{N}(55, 6^2)$ and location was determined using the directional vector

$$v(\rho, \theta, \phi) = (\rho \sin\phi \cos\theta, \rho \sin\phi \sin\theta, \rho \cos\phi)$$

To the position vector of the previous agent in the filament. Here ρ is the length of $\mathbf{v}$, $\theta \in [0, 2\pi]$ is the polar angle between $\mathbf{v}$ and the x -axis, and $\phi \in [0, \pi]$ is the azimuthal angle between $\mathbf{v}$ and the z -axis. For the first hyphal agent in a filament, the values of θ and ϕ are chosen at random. For

subsequent hyphal agents θ and ϕ are chosen as the sum of previous θ and a random amount of up to $\pm 15^\circ$. The length of the directional vector is chosen at random from the interval $2 \leq \rho \leq 3$. Additionally, yeast-agents were allowed to adhere to hyphal agents in three-dimensional space with probability $0 \leq Y \leq 1$.

The intensity of segregation of the resulting aggregate biofilms was calculated according to Binder *et al.* (24) where the index $I = 1$ refers to a highly segregated distribution, and $I = 0$ refers to a completely uniform distribution. The rate of aggregate formation was ascertained from the number of iterations for which the model requires before completion, where all agents have been bound.

Results

Methaemoglobin enhances biofilm formation.

C. albicans biofilms showed a significant increase in biomass when grown in the presence of MetHb ((as confirmed by spectroscopy (Figure S1)) when compared to the untreated control. The increase in biomass required live *C. albicans* as no equivalent increase was observed when MetHb was incubated with ethanol killed cells (Fig. 1a). Elevated *C. albicans* biofilm biomass was not observed when supplemented with an equivalent concentration of bovine serum albumin (BSA), a globular protein of similar molecular weight to Hb (Hb = 64.5 kDa, BSA = 66.5 kDa)(Fig. 1b). A similar MetHb associated increase in *C. albicans* biofilm biomass was also observed in clinical isolates (Fig. 1c). However, despite an increase in biomass the metabolic activity of MetHb supplemented *C. albicans* did not increase suggesting a non-cell or quiescent cell basis for this finding (Fig. 1d). To investigate this, we examined the nature of *C. albicans* biofilms grown in the presence of MetHb by confocal microscopy. By differentially staining for *C. albicans* cells and the protein content of the ECM we observed that MetHb grown biofilms exhibited a more extensive matrix with protein distributed throughout as opposed to being concentrated on the apical surface as observed in the untreated control (Fig. 1e). To determine whether iron acquisition from MetHb supported the observed increase in *C. albicans* biofilm biomass, we assessed the effects of deletions for known iron homeostasis (*hap3Δ/Δ*, *hap5Δ/Δ*, *hap43Δ/Δ*, *sef1Δ/Δ*, *zcf20Δ/Δ*) and haemoglobin uptake (*rht5Δ/Δ*, *pga7Δ/Δ*) genes. All deletion strains showed a comparable enhancement of biofilm biomass in the presence of MetHb in line with that observed in the wild-type strain (Fig. 1f), suggesting that iron acquisition from MetHb is not the primary driver of this phenotype.

C. albicans can liberate MetHb from red blood cells to form aggregate biofilms in solution

We hypothesised that MetHb may be directly incorporated into *C. albicans* biofilms and that this was the principle cause of the observed increase in biomass. To investigate whether *C. albicans* could directly bind to MetHb we turned to solution-based aggregation assays to allow for a more accurate quantification of the process. On preparation of MetHb solutions, we observed that small aggregates of protein were formed independently of the presence of *C. albicans* cells. MetHb aggregation was significantly enhanced when *C. albicans* cells were introduced. Lambda scanning of the resulting MetHb aggregates revealed they were auto-fluorescent under 561nm excitation (Figure S1) providing a

convenient method for their microscopic identification. Co-incubation of *C. albicans* with MetHb at 30°C and 37°C demonstrated binding of *C. albicans* to MetHb aggregates in both the yeast and hyphal forms respectively (Fig. 2A). Immunostaining and SDS-PAGE of *C. albicans*-MetHb aggregates confirmed the presence of MetHb subunits (Fig. 3a)(Figure S2). Importantly, complex formation between *C. albicans* and MetHb was not observed when stabilised in its oxyHb form (Fig. 2b). The ability of *C. albicans* to utilise MetHb liberated from red blood cells, as opposed to from a purified commercial product, was tested using media supplemented with sheep's blood. *C. albicans*-MetHb aggregate formation was evident in both RPMI-1640 solutions supplemented with either whole blood, or the supernatant of a lysed blood solution and significantly elevated compared to the untreated controls (Fig. 2c). Fluorescence confocal imaging of these aggregates revealed they were phenotypically identical to aggregate biofilms formed in the purified MetHb solutions, with auto-fluorescent inclusions, interspersed with *C. albicans* hyphae (Fig. 2e). We also aimed to confirm whether *C. albicans* mediated haemolysis was adequate to enable MetHb release and aggregate formation. To test this, we pre-treated red blood cells with *C. albicans* before centrifugation to remove all cells and debris and then re-inoculated the supernatant with fresh *C. albicans* cells. We observed that the MetHb released as a result of *C. albicans* haemolysis was sufficient to induce aggregate formation in a manner comparable to those produced when incubated with purified MetHb (Fig. 2e).

Under physiological conditions, free MetHb is bound by haptoglobin (Hpt) and trafficked for degradation (14). To investigate whether Hpt binding could limit *C. albicans*-MetHb aggregate formation, we inoculated *C. albicans* into MetHb alone, and into a 1:1 mixture of MetHb and Hpt (1mg/mL). Although the presence of Hpt did not abolish the aggregation phenotype, the resulting complex area was significantly reduced, suggesting that Hpt competes with *C. albicans* for MetHb binding and that this limits aggregation (Fig. 2f-g).

Morphological transition and cell-cell interactions facilitate *C. albicans*-MetHb aggregation.

Hyphal growth and hyphae-hyphae interactions are critical for *C. albicans* biofilm formation (25). To address if this is also the case for *C. albicans*-MetHb aggregation, we investigated aggregate formation using the hyphal-defective mutant, *efg1*Δ/Δ. Imaging of the resulting aggregates showed limited interaction between the *efg1*Δ/Δ cells and MetHb aggregates, with no significant increase in aggregation complex area observed. These data suggest that morphological transition and/or hyphal expressed genes may be of importance for the formation of *C. albicans*-MetHb complexes (Fig. 3a-e). We further hypothesised that hyphal growth was required to knit smaller MetHb containing aggregates together leading to exponential expansion of complex size. To assess this, we formed *C. albicans*-MetHb complexes using either a wild-type or a GFP-tagged strain independently. After 1 hour, the reactions were combined and incubated a further 3 hours before resultant complexes were stained with calcoflour white and observed using confocal microscopy. Large *C. albicans*-MetHb complexes were observed with distinct areas of GFP signal indicating that cell-based fusion of smaller complexes is indeed an explanation for rapid aggregation (Fig. 3f).

The adhesins Als1 and Als3 facilitate *C. albicans*-MetHb complex formation

With the aim of characterising the molecular interactions underpinning *C. albicans*-MetHb complex formation, we utilised a knockout library (26) containing strains deleted for 12 key *C. albicans* adhesin genes alone or in combination. Initially we assessed mutants with single deletions (Δ/Δ) of either *ALS1*, *ALS3*, *ALS5*, *ALS7*, *ALS9*, *EAP1*, *HWP1*, *HWP2*, *RBR3*, *RBT1*, *HYR1*, or *IFF4*. Only *als1* Δ/Δ and *als3* Δ/Δ strains exhibited significantly reduced agglutination scores compared to the library wild type (fRS29xfRS32)(Fig. 4a). The products of incubation of MetHb with *als1* Δ/Δ , *als3* Δ/Δ and *als1* Δ/Δ /*als3* Δ/Δ strains were then imaged and the percentage of MetHb-bound *C. albicans* cells calculated. Per-cell binding of both the *als1* Δ/Δ and *als3* Δ/Δ strains to MetHb aggregates was not significantly different to the wild-type, however a decrease in binding in the *als1* Δ/Δ /*als3* Δ/Δ strain was observed (Fig. 4b). Batch imaging of the resulting complexes was also performed to calculate mean complex area, which revealed that MetHb aggregation enhanced complex formation in the *als1* Δ/Δ strain but a loss of this phenotype in the *als3* Δ/Δ strain (Fig. 4c). Confocal imaging of the formed complexes confirmed that the loss of *ALS3* led to a significant reduction in *C. albicans*-MetHb aggregates size and that this was reduced by the additional loss of *ALS1* (Fig. 4d). Whole well scans of macroscopic *C. albicans*-Hb aggregate formation in wild type, *als1* Δ/Δ , *als3* Δ/Δ and *als1* Δ/Δ /*als3* Δ/Δ experiments are included in the supplementary material (Figure S3).

Agent-based modelling of *C. albicans*-MetHb biofilm formation supports the importance of hyphal growth in aggregation

To understand the kinetics of aggregation and biofilm formation we developed a computational, agent-based model, based on the work of Binder *et al.* 2025 (23). The model was able to reproduce simulated surface attached biofilms that were phenotypically similar to those produced *in vitro* (Fig. 5a). This simulation was then re-run to investigate the importance of hyphal-based adhesion to the process. In one condition the model allowed only for MetHb to interact with *C. albicans* in its budding yeast form, while in the second hyphae – hyphae and hyphae-MetHb adhesions were also permitted, simulating the role of the influence of the hyphae expressed adhesin, Als3. The models were then realised 10 times and the number of iterations to model completion recorded. Model completion was achieved in significantly less iterations when permitting hyphal adhesion suggesting these adhesive properties accelerate aggregate formation (Fig. 5b). However, the distribution index of the model formed when permitting hyphal adhesions was lower than that of the yeast adhesion only control, indicating hyphal adhesions may lead to less dense, or more evenly distributed structures (Fig. 5c).

Temporal transcriptomic analysis of the *C. albicans*-MetHb aggregation process

To investigate the effects of *C. albicans*-MetHb aggregate formation in more detail we performed RNA sequencing at 30 and 90 minutes post-incubation, corresponding to initial and complete complex formation. Based on an FDR adjusted p-value of < 0.05 , at 30 minutes, 2607 genes were significantly upregulated and 2633 were downregulated. At 90 minutes 1790 genes were significantly upregulated

and 1778 were downregulated (Table S2). Analysis was performed using STRING-DB to identify upregulated and downregulated processes at each time point.

After 30 minutes several processes were significantly upregulated, including cell adhesion, iron ion transport, mannose biosynthesis as well as ribosome biosynthesis and translation. Mitochondrial organisation/respiration, as well as sugar and fatty acid metabolism were downregulated. Both protein folding and copper ion transport were both positively and negatively enriched showing upregulation and downregulation of relevant genes. At 90 minutes an opposing profile was observed, with adhesion, translation and ribosome assembly each negatively enriched. Positive enrichment was instead seen for trehalose and alcohol biosynthesis. Interestingly there was positive enrichment for DNA repair, cell cycle checks and regulation of chromatin organisation suggesting DNA stress (Fig. 6a). These findings show a rapid and dynamic transcription response to the detection and aggregation of MetHb by *C. albicans* (Fig. 6b). The initial response to MetHb presence may be one of iron scavenging, as at 30 minutes, the haem binding component of the haem-iron uptake system, *CSA2*, was the most upregulated annotated gene ($\log_2FC = 6.17$). The downstream elements of the CFEM system, *PGA7*, *PGA10* were then upregulated in the $t = 90$ results, and *CSA2* expression was reduced suggesting haem had been bound and was in the process of being chaperoned for degradation. A range of ferric reductases, multicopper oxidases and iron permeases were also upregulated at $t = 30$ supporting iron redox and uptake. Both *ALS1* and *ALS3* were significantly upregulated at $t = 30$ with a decrease in expression seen at $t = 90$, consistent with our observation that aggregation is largely complete by 90 minutes in our *in vitro* system. *ALS4* and *ALS5* were also upregulated at $t = 30$ alongside other adhesins such as *EAP1* and *RBT1*.

Trehalose biosynthesis and gluconeogenesis were other processes that were activated between $t = 30$ and $t = 90$. At $t = 30$ the trehalase *NTH1* was upregulated with downregulation of the trehalose synthases *TPS1* and *TPS2*. At $t = 90$ regulation of these genes was inverted suggesting a switch from carbohydrate utilisation to carbohydrate storage. Genes involved in glycolysis such as *ADH*, *ADH2* and *ENO1* were generally downregulated at $t = 30$ with a switch to upregulation at $t = 90$. This shift to glycolysis, carbohydrate storage and fermentation could be indicative of a survival/quiescence phenotype. Many DNA damage repair systems including *RAD1*, *RAD2*, *RAD53*, and *RAD9* were variably expressed at $t = 30$ but predominantly upregulated at $t = 90$ implying DNA damage in response to exposure to MetHb. Further examination of the superoxide dismutase (SOD) systems showed downregulation of *SOD1*, *SOD2* and *SOD5* with upregulation of *SOD3* and *SOD4* suggesting the observed DNA damage may involve ROS accumulation.

The profile of biofilm and filamentation regulation was variable within the *C. albicans*-MetHb aggregation process. Of the 'Filamentous growth' gene ontology term (GO:0030447) 407 genes were significantly differentially expressed at $t = 30$ 230 of which were upregulated. At $t = 90$ 281 genes of the same GO term were differentially expressed, 167 of which were upregulated. Of the 9 key biofilm formation regulators, 5 were significantly upregulated at $t = 30$. (*BRG1*, *ROB1*, *GAL4*, *RFX2*, *FLO8*). By $t = 90$ only *ROB1*, *GAL4* and *FLO8* remained upregulated. (Fig. 6b)

Hypoxia-associated transcriptional programs are enriched in *C. albicans*-MetHb complexes.

Following our transcriptomic analysis with String-DB, we conducted a secondary analysis using gene set enrichment analysis (GSEA) (27). In *C. albicans*-MetHb complexes, (t = 90) GSEA revealed significant enrichment of hypoxia response pathways compared to the control. Notably pathways regulated by the hypoxia response repressor Sko1, and the hypoxia induced metabolic regulators Tye7 and Gal4 were enriched. Genes upregulated in a *sko1* Δ/Δ strain were positively enriched (NES + 3.249), while downregulated genes were negatively enriched (NES - 3.203), consistent with repression of Sko1. Genes downregulated in a *tye7* Δ/Δ strain showed positive enrichment (NES + 3.422), indicating induction of Tye7 and its targets. A positive enrichment of ChIP-chip targets of Gal4 (NES + 3.052) also indicated activation of Gal4. During *C. albicans*-MetHb complex formation. Repression of Sko1 and activation of Tye7 and Gal4 suggest that *C. albicans* in MetHb complexes are under hypoxic stress and have transitioned from aerobic, mitochondrial respiration to Tye7 and Gal4 mediated fermentative processes such as glycolysis. Equivalent hypoxia responses were not observed in GSEA analysis performed on the t = 30 dataset (Fig. 6c).

***C. albicans* -MetHb aggregates exhibit hallmarks of aggregate biofilms including antifungal tolerance and increased virulence.**

Biofilm formation is closely associated with tolerance to antimicrobial therapies. To assess if the formation of *C. albicans*-MetHb complexes could facilitate tolerance to antimicrobials we evaluated the efficacy of two antifungals, the polyene amphotericin B, and the echinocandin, anidulafungin. *C. albicans* complexes were formed in the presence or absence of MetHb and then collected, washed, and challenged with increasing concentrations of the relevant antifungal at 37°C with shaking for 24 hours. As the resulting complexes could not be evenly diluted for viability assessment by traditional microbiology methods, the complete solution of complexes was washed to remove residual antifungals and spread on YPD agar. These plates were then imaged and the percentage coverage of the plate used to calculate a survival index relative to the untreated control. When plotted as a dose response curve, complexes formed in the presence of MetHb contained significantly more viable cells to those in RPMI-1640 alone for both amphotericin B and anidulafungin (Fig. 7a-c). To test if this survival was due to a hereditary or non-hereditary mechanism we re-isolated colonies grown following amphotericin B exposure and performed a traditional microtiter broth dilution MIC assay. Testing of all the colonies resulted in the restoration of anti-fungal sensitivity with IC₅₀ values for amphotericin B within the expected susceptibility range for *C. albicans* (31)(Fig. 7d).

To assess the *in vivo* virulence potential of *C. albicans*-MetHb complexes a *G. mellonella* model was used. We inoculated n = 40 larvae with *C. albicans* in the presence or absence of 0.3% MetHb. We observed a significant increase in mortality when inoculating with *C. albicans* in the presence of MetHb compared to the *C. albicans* control. This enhanced virulence in the presence of MetHb was lost when inoculating with the *als1* Δ/Δ , *als3* Δ/Δ or *als1* $\Delta/\Delta/*als3* Δ/Δ strains. Inoculation with MetHb alone had no impact on larval survival (Fig. 7e).$

Discussion

This study describes oxidised haemoglobin, MetHb as a driver of biofilm formation, promoting both surface-attached biofilms and the rapid assembly of non-adhered aggregates that exhibit biofilm properties in solution. These findings elaborate upon prior observations that *C. albicans* binds to amorphous aggregates in haemoglobin preparations and that haemoglobin promotes hyphal transition (32, 33), by specifying the required haem oxidation state, and the essential adhesins required for this interaction. Additionally, we explored the elicited transcriptional response, *in vivo* virulence and an observed non-hereditary phenotypic tolerance to antifungals.

Complex formation of *C. albicans* in haemoglobin solutions was exclusively observed in MetHb solutions and not with oxyHb, indicating that the oxidation state of haem iron is critical for this interaction. Our screen of adhesin knockout mutants identified Als1 and Als3 as the primary mediators of agglutination and complex expansion in the presence of MetHb. Individual deletion of *ALS1* or *ALS3* did not significantly decrease per-cell binding of MetHb, whereas the double deletion *als1Δ/Δ als3Δ/Δ* showed a statistically relevant reduction, implying a partial functional redundancy in initial adhesion. Loss of *ALS3* alone was sufficient to prohibit macroscopic complex formation, consistent with its role in cell-cell adhesion and biofilm formation (34). The observation of limited association between *C. albicans* and aggregated MetHb in all conditions implies that physical or electrostatic forces may contribute to initial contact, with Als1/Als3 mediated adhesion driving the subsequent complex expansion.

Hyphal morphogenesis was shown to be essential for the formation of macroscopic complexes. The hyphal impaired mutant *efg1Δ/Δ* formed limited association and imaging of complexes formed with differentially labelled wild-type strains confirmed that hyphal growth physically mediates the fusion of independently formed complexes. Again, this is consistent with the essential nature of filamentous growth in accelerating biofilm formation and development. However, as Efg1 regulates both hyphal transition, and the expression of hyphal associated genes, including both Als1 and Als3, the precise contribution of hyphal structure, vs hyphal gene expression cannot be fully confirmed (35). This could be addressed by utilising a mutant of the downstream gene *HGC1* as this is required specifically for cell polarisation and elongation/filamentation without broadly limiting the expression of hypha-associated genes.

Transcriptomic analysis revealed a biphasic response to MetHb. At 30 minutes, prior to macroscopic complex formation, haem-acquisition pathways were prominently upregulated, including the CFEM family haem-binding protein *CSA2*, alongside adhesins *ALS1*, *ALS3*, *ALS4*, *ALS5*, *EAP1* and *RBT1*. This early profile represents the initiation of complex formation through adhesion, and possibly haem-iron scavenging. At 90 minutes, when macroscopic complexes had formed, this expression profile was replaced by the activation of fermentative metabolism, trehalose biosynthesis and glycolytic enzymes, alongside repression of mitochondrial respiration. GSEA confirmed the enrichment of hypoxia-associated transcriptional programmes at this later time point, including repression of Sko1 and activation of Tye7 and Gal4, suggesting cells within mature complexes experience localised oxygen

limitation. Hypoxic microenvironments have been previously documented in fungal biofilms, and these findings imply that such niches may form in aggregate biofilms independently of a surface substrate (37).

Despite the prominent transcriptional upregulation of iron acquisition pathways, deletion of core iron homeostasis regulators (*SEF1*, *HAP3*, *HAP5*, *HAP43*, *ZCF20*) and the haem-receptor *RBT5* did not impair MetHb-enhanced biofilm formation. This dissociation between iron homeostasis and MetHb-induced biofilm formation may reflect functional redundancy within these pathways but also highlights our demonstration of MetHb protein as a structural component within the *C. albicans* biofilm. The initial formation of *C. albicans*-MetHb complexes may be independent of iron homeostasis, but then subsequently activate haem-iron scavenging from within the biofilm, with the MetHb inclusions acting as a local iron reservoir.

C. albicans-MetHb aggregates formed in solution displayed markedly increased tolerance to both amphotericin B and anidulafungin, with viability maintained above EUCAST clinical resistance breakpoints (31). Re-isolation and MIC testing of surviving colonies demonstrated this to be a non-hereditary phenotypic tolerance rather than an acquired resistance. One possibility is that the physical density of the formed complexes may impede the penetration of antifungals to the core of the complex allowing for the survival of unexposed subpopulations, in a manner similar to that seen with the extracellular matrix of classical, surface attached biofilms. Furthermore, the shift to slower, fermentative metabolism and the formation of hypoxic micro-niches may further reduce the susceptibility of antifungals that target metabolically active cells (38). The direct sequestration of antifungals by MetHb aggregates remains a possibility that cannot be ruled out without additional investigation, but it remains clear that *C. albicans*-MetHb aggregation may promote the presence of persister populations that would prove difficult to eradicate.

G. mellonella *in vivo* infection with *C. albicans*-MetHb complexes resulted in increased mortality compared to *C. albicans* alone and this phenotype was lost with the deletion of *ALS1* or *ALS3*. This suggests that MetHb induced virulence may be directly related to complex formation and expansion, however as Als1 and Als3 are both key adhesins for *C. albicans* virulence further investigation, preferably with a mammalian model and histological examination is required to confirm this (34, 39). Furthermore, while MetHb alone did not impact larval mortality, it is plausible that it may cause an inflammatory response that sensitises the larvae to fungal killing. In humans, free haemoglobin has been shown to activate innate immune responses via toll-like receptor 4. Whether analogous mechanisms exist in invertebrates is not clear (40).

The experimental system described here has some limitations that should be noted. Primarily the use of commercial, lyophilised haemoglobin, which is predominantly pre-oxidised to the ferric state. Additionally, a free haemoglobin concentration of 0.3% exceeds the typical range in healthy individuals but may fall in the range reported during severe haemolytic pathologies or local tissue concentrations, the latter of which are not well defined (41–43). Importantly, we demonstrated that *C. albicans*-mediated

haemolysis generates sufficient free haemoglobin from erythrocytes to support aggregate formation, and that complexes can form in both whole blood and haemolysed blood supernatants, confirming the physiological plausibility of this interaction. Competition with haptoglobin, the principal physiological scavenger of free haemoglobin, reduced but did not abolish complex formation, indicating that *C. albicans* can compete for MetHb binding under conditions where haptoglobin is overwhelmed, as often occurs during severe haemolysis or haemorrhage (44, 45).

The growth of biofilms as non-attached aggregates as opposed to surface attached structures has been well documented and reviewed, particularly with regard to Gram negative bacteria such as *Pseudomonas aeruginosa* (46). The models proposed mainly detail the secretion of a polysaccharides that are then bound by the pathogen in order to aggregate and form multicellular structures. Aggregate biofilm formation is not well documented in *C. albicans*. While *C. albicans* is known to release extracellular matrix components such as glucans and mannans, this typically occurs in later biofilm development stages following an established adhesion and proliferative phase (47). The release and formation of aggregated MetHb protein following haemolysis and the subsequent agglutination of *C. albicans* more closely resembles the *P. aeruginosa* model of secreted polysaccharides but instead the fungus is utilising a host-derived substance which is less commonly documented.

C. albicans has been described in clinical literature as associating with blood clots in the body. Pathologies such as *Candida* thrombophlebitis and *Candida* septic emboli describe the infection of blood clots in the body resulting in persistent/recurrent bloodstream infections with often poor prognoses (48, 49). It is implied in the literature that these clots are colonised by *C. albicans* but it may be the case that *C. albicans* mediated haemolysis may drive aggregate formation and a clotting-like response. These pathologies are often associated with the presence of vascular access devices such as central venous catheters with are a prominent risk factor for biofilm formation (50). One mechanism of aggregate biofilm formation details the dislodging or detaching of portions of surface attached biofilms, including the extracellular matrix (46). The detachment or 'metastasis' of MetHb enriched biofilms from medical devices to secondary locations in the body may be particularly problematic considering their high tolerance to antimicrobial therapies.

One interpretation of these findings is that *C. albicans*-MetHb aggregates and surface attached biofilm formation may serve as virulence mechanism. However, it could be the case that the interaction acts as a clotting mechanism to limit and immobilise a haemolytic pathogen. Further evidence to suggest a protective or restrictive role comes from the upregulation of hypoxia response programmes and apparent reduction DNA damage repair systems that may suggest the aggregate biofilm represents a stressful environment. Within the *C. albicans*-MetHb aggregates, it may be that haemocidins, small antimicrobial peptides formed from fragmented haemoglobin and myoglobin proteins are formed (51), this possibility requires further investigation. Long term viability assays examining *C. albicans* and other haemolytic pathogens grown in the presence of MetHb could provide further insight into its role as a component of the innate immune system. It is also plausible that *C. albicans* has may developed the ability to utilise MetHb a way of rapidly generating a biofilm structure that can persist in sites of infection

and resist phagocytic clearance. As a number of bacteria are able to adhere to *C. albicans* it may be the case that such structures also provide a refuge for passengers and that this contributes to persistence.

The role of MetHb in polymicrobial interactions requires further exploration. Biofilms in nature are known to be polymicrobial and the interplay between fungal and bacterial pathogens is a complex and developing field (52). *C. albicans* biofilms are often referred to as 'scaffolds' for bacterial colonisers due to their hyphal growth rapidly forming large biomasses (53). *C. albicans* aggregate and surface attached biofilms produce large masses that could potentially harbour a range of bacterial co-pathogens. *S. aureus* and *S. pneumoniae* biofilms are both enriched by haemoglobin and both pathogens have documented synergism with *C. albicans* (19, 20, 54, 55).

In conclusion, *C. albicans* surface attached and aggregate biofilm formation is enhanced by the presence of MetHb and represents a novel and previously undescribed growth state for this opportunistic fungal pathogen. The nature of this interaction and its clinical significance require further exploration, but it may play an important role in host-pathogen interaction, infection and response to treatment. More complex *in vivo* systems and histological analyses could provide better insight into the physiological plausibility of such aggregates and their role in *C. albicans* infection and immunity.

Declarations

Competing Interests

All authors declare no financial or non-financial competing interests.

Author Contribution

E.D.R devised and conducted experiments, collected and analysed data, wrote manuscript; E.P. and B.B. conducted computational modelling, collected and analysed data; J.B. conducted experiments, collected and analysed data; C.W.G. devised experiments, conceived project, obtained funding, supervised project and edited the manuscript.

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Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets generated and/or analysed during the current study are available in the NCBI Gene Ontology Omnibus accession number GSE249653.

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Figures

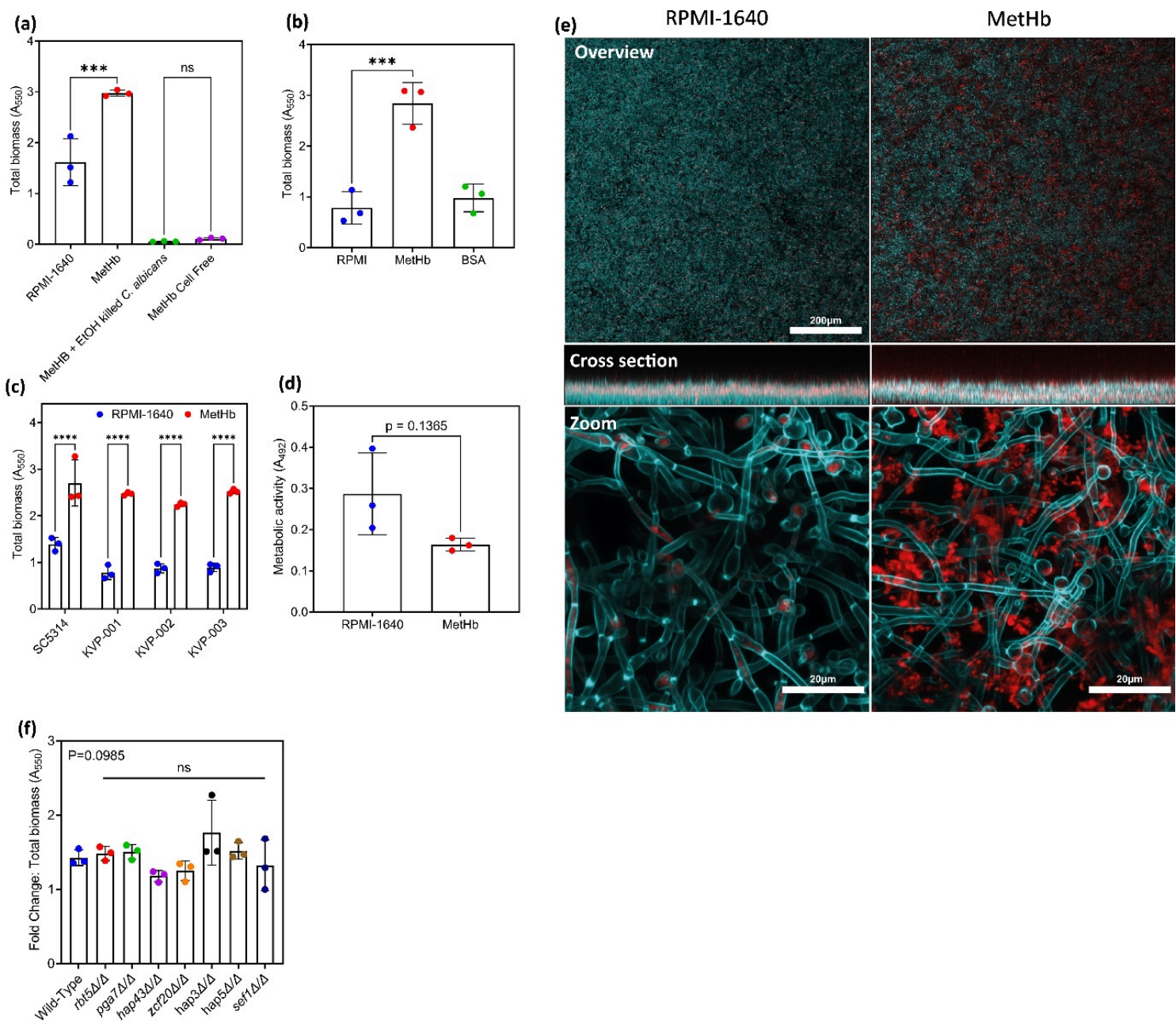


Figure 1

MetHb enhances biofilm biomass in an iron independent manner. *C. albicans* (SC5314) biofilms grown for 24 hours in the presence or absence of 0.3 % metHb. (a) Total biomass of biofilms as determined by crystal violet staining compared to an ethanol killed and cell free control. (b) Increase in biofilm biomass when treated with metHb compared to BSA. (c) Enhanced biofilm biomass is conserved across 3 clinical *C. albicans* isolates. (d) Metabolic activity of *C. albicans* biofilms grown in the presence of metHb. (e) Fluorescence confocal microscopy of *C. albicans* biofilms. Red = extracellular matrix (FilmTracer™ SYPRO™ Ruby) Cyan = *C. albicans* (CFW). (f) Fold change in total biomass of biofilms of iron-acquisition and homeostasis knockout mutants grown in the presence and absence of metHb. Statistical significance determined by way of One or Two-way ANOVA with Šidák's or Tukey test. Individual points represent biological replicates (n=3).

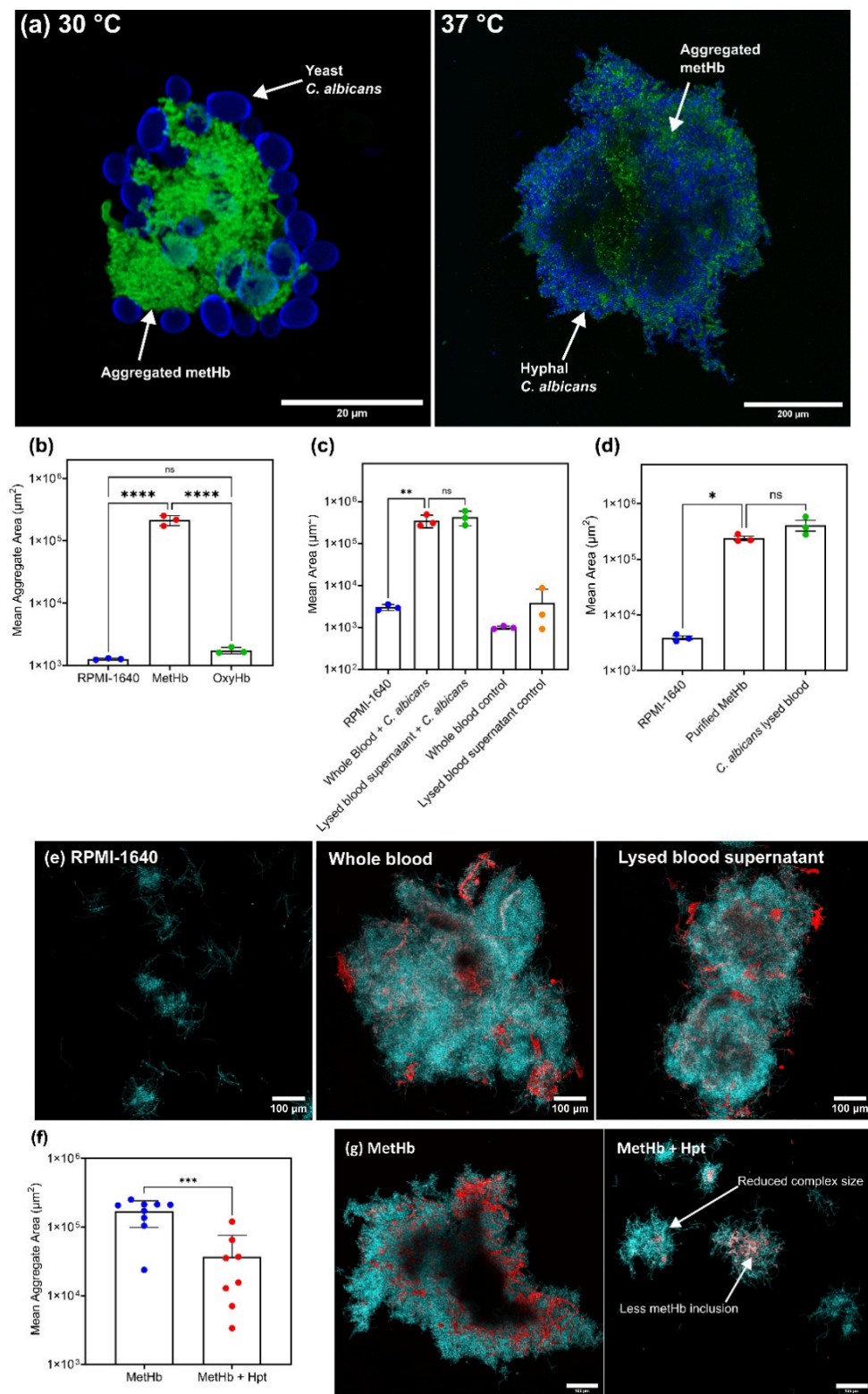


Figure 2

MetHb and blood facilitate aggregate biofilm formation. *C. albicans* cells were inoculated into RPMI-1640 media $\pm$ 0.3 % metHb/oxyHb/Haptoglobin or 5 % blood/lysed blood supernatant for 2-4 hours. Complexes were then isolated by centrifugation and imaged by light microscopy. Mean aggregate area was determined using ImageJ. (a) *C. albicans* metHb aggregate complexes (Blue = *C. albicans* (calcofluor white), Green = anti-haemoglobin-FITC)). (b) Growth of *C. albicans* in the presence of ferrous

stabilised haemoglobin (oxyHb) resulted in no aggregate formation following 2 hours of exposure at 37 °C. (c) MetHb complex formation when 5 % blood suspensions and the supernatant of 5 % blood manually lysed by osmotic stress were mixed with *C. albicans* for 4 hours. (d) Complex formation from blood lysed independently by *C. albicans*. (f) Mean aggregate area and (g) Representative CLSM images of *C. albicans*-metHb complexes formed in the presence or absence of hpt. Statistical analyses performed by way of One-way ANOVA with Tukey's test, or unpaired t-test. Data points represent biological replicates.

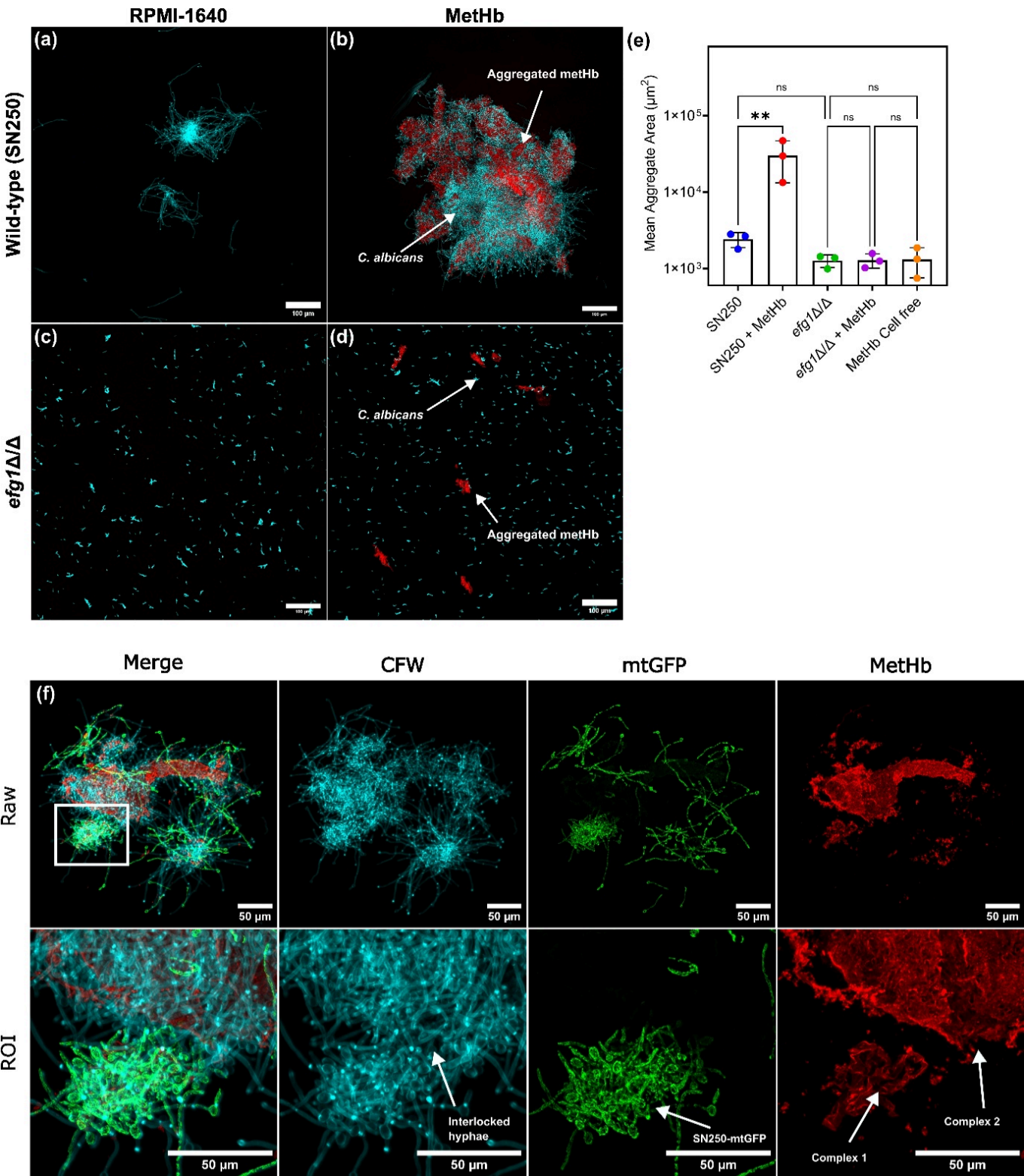


Figure 3

Hyphal growth and hyphal expressed genes drive *C. albicans*-MetHb complex expansion. Hyphal growth and hyphal expressed genes drive *C. albicans*-MetHb complex expansion. (a-d) Representative CLSM images of *C. albicans* wild-type (SN250) or *efg1* Δ/Δ strains grown in the presence or absence of metHb for 4 hours. (e) Quantification of resulting aggregates formed by wild-type or *efg1* Δ/Δ . Statistical analysis performed by way of one-way ANOVA with Tukey's test. Data points represent biological replicates (n=3). (f) Fusion of smaller aggregates through hyphal growth. Independent aggregates of wild-type or GFP-tagged were grown for 1 hour and the reactions combined for a further 3 hours. All cells were counterstained with CFW. Independent metHb subsections of larger aggregates can be identified by GFP expression which have been connected through hyphal growth.

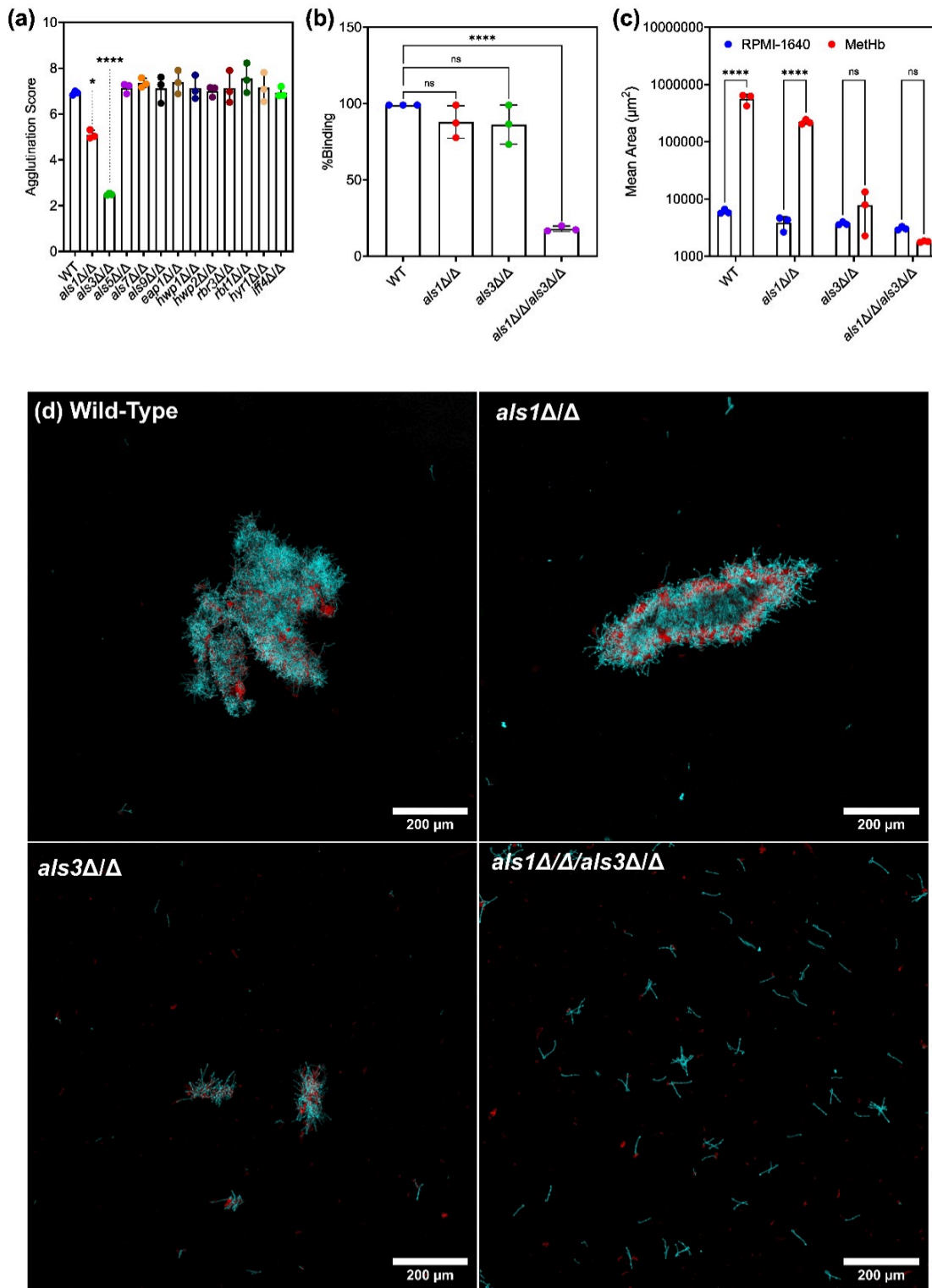


Figure 4

Adhesins Als1 and Als3 facilitate meHb complex formation. Strains were inoculated into RPMI-1640 ± 0.3 % methHb for 4 hours. Agglutination was then quantified by spectroscopy and *C. albicans*-methHb binding quantified imaged by CLSM. cyan = calcofluor white, red = methHb. (a) Screen of *C. albicans* adhesin knockout library by methHb agglutination. (b) Percentage binding of ALS1, ALS3, and ALS1/ALS3 mutant cells to methHb aggregates. (c) Mean aggregate area of complexes formed using ALS1, ALS3,

and ALS1/ALS3 mutants. Statistical analyses performed by way of one or two-way ANOVA with Tukey or Šidák's test. Data points depict biological replicates (n=3). (d) Representative images of complexes formed from adhesion deletion strains.

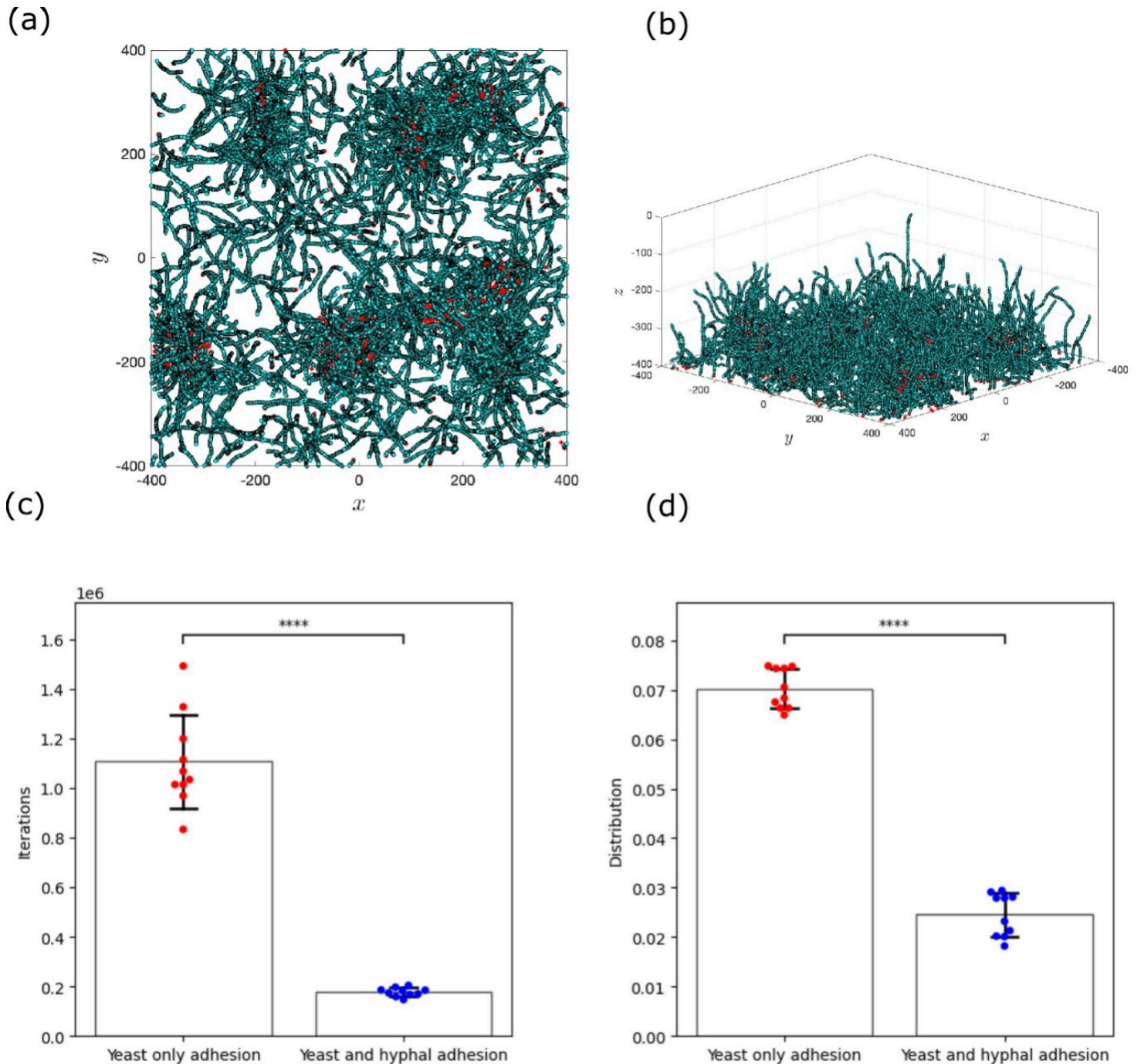


Figure 5

Agent-based modelling of *C. albicans*-MetHb interaction in surface attached biofilm formation. Agent-based model of filamenting *C. albicans* was able to simulate structures comparable in morphology to those observed in vitro. Cyan = simulated *C. albicans*, Red = Simulated MetHb. (a) Top-down view of model simulation. (b) 3D view of resulting structure. (c) Number of iterations required to reach model completion (binding of all agents) when controlling for yeast only or hyphal-based adhesion. (d)

Distribution index score for model when controlled for yeast only or hyphal-based adhesion. An index score of 1 refers to a high degree of segregation whereas 0 implies a perfectly uniform distribution. Each data point represents an independent realisation of the model. Statistical analyses performed by way of unpaired t-test.

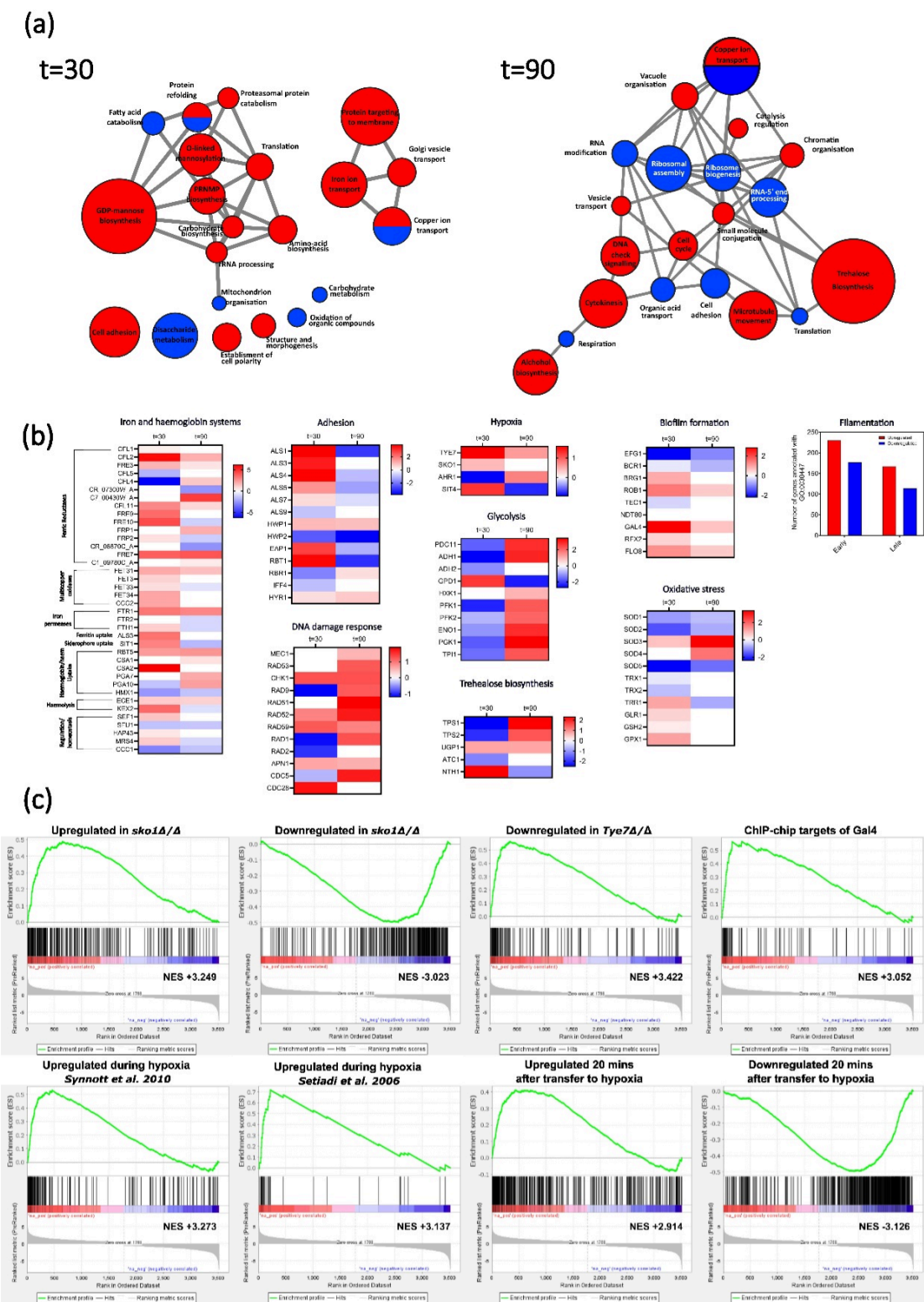


Figure 6

Transcriptomic analysis of metHb aggregate biofilms Differentially expressed genes were extracted using the DESeq2 statistical package. Significantly affected processes were identified by analysis of predicted protein-protein interactions using STRINGdb. (28) Enriched upregulated (red) and downregulated (blue) processes at (a) t=30 and (b) t=90 minutes. Processes were plotted as perfuse force directed ball and stick plots in Cytoscape (v3.9.1) with size of node representing enrichment score and distance between nodes demonstrating similarity in process as determined by analysis in REVIGO. (29) Red and blue nodes show processes that were significantly positively and negatively enriched. Heat maps showing difference in gene expression at t=30 and t=90 minutes. Gene sets were manually curated using gene ontology information from The Candida genome Database. (30) Genes annotated with “filamentous growth” were compared by count and graphed as a bar plot to visualise the difference in total genes.(c) GSEA plots show enrichment of gene sets in *C. albicans* after 90 minutes of exposure to metHb, compared to an RPMI-1640 control.

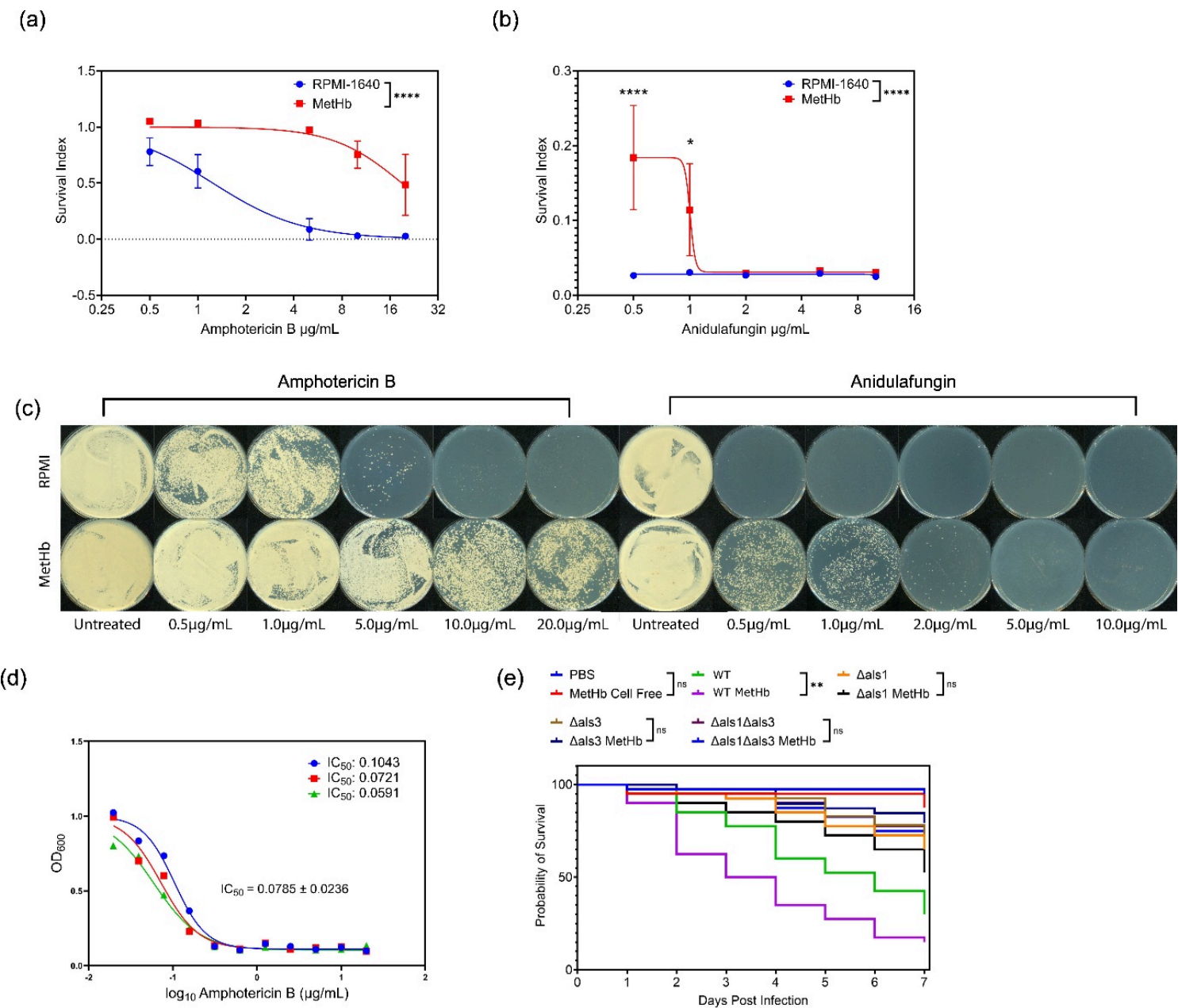


Figure 7

MetHb exposure increases antifungal tolerance and in vivo virulence. *C. albicans*-MetHb complexes were formed by incubation in RPMI-1640 + 0.3 % MetHb for 4 hours at 37°C. Complexes were then washed and incubated for a further 24 hours in RPMI-1640 + amphotericin or anidulafungin. The final complexes were then isolated by centrifugation, washed in PBS and spread on YPD agar plates. Colonies arising from the 20 µg/mL amphotericin B treatment were collected and used for further MIC testing. (a-b) Dose response relationship of *C. albicans* complexes grown in the presence and absence of metHb to amphotericin B and Anidulafungin respectively. Survival indexes calculated as a measure of percentage growth on agar plates. (c) Representative images of agar plates used to calculate survival index at each concentration. (d) IC₅₀ of recovered cells exhibit typical susceptibility to amphotericin B. *Galleria mellonella* larvae were inoculated with a planktonic suspension of *C. albicans* yeast cells ± 0.3 % metHb. (e) *G. mellonella* infection model. *G. mellonella* larvae were inoculated with wild-type or ALS1/ALS3 mutants and viability assessed for 7 days at 37 °C (n=40 larvae per treatment). Statistical analysis performed by way of log-rank test.

Supplementary Files

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