

1 **CO<sub>2</sub> enhances the ability of *Candida albicans* to form biofilms, overcome**  
2 **nutritional immunity and resist antifungal treatment**

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11 **Working title** – CO<sub>2</sub> enhances *Candida albicans* biofilm formation

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1 **Abstract**

2 *C. albicans* is the predominant fungal pathogen of humans and frequently colonises  
3 medical devices, such as voice prosthesis, as a biofilm. It is a dimorphic yeast that  
4 can switch between yeast and hyphal forms in response to environmental cues, a  
5 property that is essential during biofilm establishment and maturation. One such cue  
6 is the elevation of CO<sub>2</sub> levels, as observed in exhaled breath.. However, despite the  
7 clear medical relevance, the effects of CO<sub>2</sub> on *C. albicans* biofilm growth has not  
8 been investigated to date. Here, we show that physiologically relevant CO<sub>2</sub> elevation  
9 enhances each stage of the *C. albicans* biofilm forming process;from attachment  
10 through to maturation and dispersion. . The effects of CO<sub>2</sub> are mediated via the  
11 Ras/cAMP/PKA signalling pathway and the central biofilm regulators Efg1, Brg1,  
12 Bcr1 and Ndt80. Biofilms grown under elevated CO<sub>2</sub> conditions also exhibit  
13 increased azole resistance, tolerance to nutritional immunity and enhanced glucose  
14 uptake to support their rapid growth. These findings suggest that *C. albicans* has  
15 evolved to utilise the CO<sub>2</sub> signal to promote biofilm formation within the host. We  
16 investigate the possibility of targeting CO<sub>2</sub> activated processes and propose 2-  
17 Deoxyglucose as a drug that may be repurposed to prevent *C. albicans* biofilm  
18 formation on medical airway management implants. We thus characterise the  
19 mechanisms by which CO<sub>2</sub> promotes *C. albicans* biofilm formation and suggest new  
20 approaches for future preventative strategies.

21

## 1 **Introduction**

2 *C. albicans* is a commensal yeast located on the mucosal surfaces of the oral cavity,  
3 gastrointestinal and genitourinary tracts of most healthy individuals <sup>12</sup>. Despite being  
4 a commensal organism, it is also an opportunistic pathogen <sup>13</sup>; in fact, it is the most  
5 widespread of all the human fungal pathogens <sup>4</sup> and is the fourth most common  
6 cause of hospital-acquired infections in the USA <sup>1</sup>. Infection with *C. albicans* is a  
7 particular problem among immunocompromised individuals or persons with  
8 implanted medical devices such as catheters or voice prostheses <sup>56</sup> upon which the  
9 yeast grows as a biofilm <sup>7</sup>.

10

11 Biofilms are structured communities of microorganisms attached to a surface. The  
12 cells are often encased within an extracellular matrix (ECM) which is commonly  
13 comprised of DNA <sup>89</sup>, lipids <sup>8</sup>, proteins <sup>810</sup> and polysaccharides <sup>8</sup>. *C. albicans* is able  
14 to form biofilms on both abiotic and biotic surfaces and biofilm-associated cells are  
15 considerably more resistant to traditional antifungals when compared to planktonic  
16 cells <sup>11</sup>. The reasons for this increased resistance are complex but include; the  
17 presence of an ECM which can act as a barrier to prevent antimicrobial agents  
18 reaching the cells <sup>1213</sup>, the presence of metabolically dormant persister cells inherent  
19 to biofilms <sup>14</sup>, and the upregulation of drug efflux pumps <sup>15</sup>. A significant percentage  
20 of human microbial infections arise from or are mediated via the formation of a  
21 biofilm <sup>161718</sup>, and this, combined the limited treatment options available, means the  
22 ability of *C. albicans* to grow as a biofilm is of particular medical interest.

23

24 *C. albicans* is a dimorphic fungus, it has the ability to undergo a morphogenic switch  
25 from a yeast, to pseudohyphal or hyphal forms in response to environmental cues.

1 The virulence of *C. albicans* is closely linked with the capacity to switch between  
2 these forms; hyphal *C. albicans* cells are frequently located at sites of tissue  
3 invasion, and cells which are unable to readily form hyphae exhibit reduced virulence  
4 <sup>1</sup>. The yeast-to-hyphal switch is also critical to biofilm formation as hyphal cells  
5 express a number of specific cell surface adhesins that enable cell-cell and cell-  
6 surface attachment <sup>19</sup>. These adhesins, such as the agglutinin-like sequence (Als)  
7 proteins, possess a folded N-terminal domain required for protein-ligand interaction  
8 and a C-terminal peptide which covalently bonds to glycosylphosphatidylinositol  
9 (GPI) to anchor the adhesin in the fungal cell wall <sup>20</sup>. The Als proteins also contain an  
10 amyloid forming region (AFR) in the N-terminal domain <sup>21</sup> which interacts with AFRs  
11 of other Als proteins. This results in the formation of large molecular weight clusters  
12 of Als proteins on the fungal cell wall called nanodomains which can bind multivalent  
13 ligands with high avidity <sup>22</sup>. These nanodomains form in response to sheer forces  
14 applied to the adhesin molecules which cause the AFR to unfold and facilitate Als  
15 molecule aggregation <sup>23,24</sup>. Nanodomains therefore strengthen adhesion and support  
16 the structure of mature biofilms <sup>25</sup>.

17

18 *C. albicans* biofilm formation is a complex process involving tightly regulated,  
19 interwoven signalling pathways centrally controlled by a set of nine transcription  
20 factors; Bcr1, Brg1, Efg1, Flo8, Gal4, Ndt80, Rob1, Rfx2 and Tec1. These nine  
21 essential regulators function at different stages throughout *C. albicans* biofilm  
22 formation <sup>26</sup> and coordinate the expression of over 1000 target genes upregulated  
23 during biofilm formation <sup>27</sup>. *C. albicans* biofilm formation can be divided into distinct  
24 stages that are governed by programmes of gene expression; attachment, initiation,  
25 maturation and dispersion. The attachment stage involves the initial attachment of *C.*

1 *albicans* cells, primarily in the yeast-form<sup>28</sup>, to a surface<sup>29</sup>. Both nonspecific factors,  
2 such as cell surface hydrophobicity and electrostatic forces, and specific factors,  
3 such as adhesins on the yeast cell surface binding to precise ligands on the  
4 substratum to be colonised, are responsible for the preliminary attachment<sup>29</sup>.  
5 Approximately 3-6 hours after the initial attachment, pseudohyphal and hyphal cells  
6 start forming from the proliferating yeast-form cells<sup>3</sup>. This initiation step is  
7 characterised by the appearance of extracellular material. The maturation phase of  
8 biofilm growth lasts between 24-48 hours<sup>29</sup>. Colonies of *C. albicans* continue to grow  
9 and secrete ECM, increasing the amount of material encasing the biofilm<sup>28</sup>. The final  
10 stage of biofilm development is the dispersal stage during which yeast-form cells bud  
11 off from hyphal cells within a mature biofilm and disperse in order to establish  
12 additional biofilms elsewhere<sup>2830</sup>. The yeast-form cells emerging from mature  
13 biofilms have distinct characteristics compared to typical planktonic yeast cells; with  
14 enhanced adherence, an increased propensity to filament, and increased biofilm  
15 forming capability<sup>31</sup>.

16  
17 Elevated CO<sub>2</sub> levels, as found in a number of physiologically relevant scenarios such  
18 as in exhaled breath or hypercapnia, have been shown to promote the yeast-to-  
19 hyphal switch in *C. albicans*. CO<sub>2</sub> is converted to bicarbonate ions HCO<sub>3</sub><sup>-</sup> by the  
20 enzyme carbonic anhydrase which in turn activate the adenylate cyclase Cyr1,  
21 resulting in increased cAMP levels and the PKA dependent activation of hyphal  
22 specific genes<sup>32</sup>. The yeast-to-hyphal switch is critical to the biofilm maturation  
23 process of *C. albicans*<sup>33</sup>, as well as being important to its virulence<sup>1</sup>. The effect of  
24 CO<sub>2</sub> may be particularly important within the context of biofilm development on voice  
25 prostheses (VPs) since these devices are situated in the throat of patients where

1 they are consistently exposed to high CO<sub>2</sub> (5%) levels during exhalation. If CO<sub>2</sub> does  
2 play a role in *C. albicans* biofilm maturation it could offer a possible explanation as to  
3 why *C. albicans* is found in such high frequencies on failed VPs. In addition, CO<sub>2</sub>  
4 content within the blood is also elevated (46mmHg and 40mmHg for venous and  
5 arterial blood respectively versus 0.3mmHg found in atmospheric air)<sup>34,35</sup>, and it has  
6 been estimated that as many as 80% of all microbial infections directly or indirectly  
7 involve pathogenic biofilms<sup>36</sup>. Thus, the work presented here could be more widely  
8 applicable to bloodstream infections and biofilm formation within the body.

9  
10 Here we demonstrate that physiologically relevant increases in CO<sub>2</sub> accelerate *C.*  
11 *albicans* biofilm formation on silicone surfaces. Transcriptome analysis reveals that  
12 several core biofilm regulatory pathways, including those governed by Efg1, Bcr1,  
13 Brg1, and Ndt80, are upregulated. We also demonstrate that a high CO<sub>2</sub>  
14 environment results in increased resistance of biofilms to azole antifungals,  
15 enhanced dispersal of cells from mature biofilms and an increase in capacity for  
16 glucose uptake. Moreover, a transcription factor knockout (TFKO) library screen  
17 demonstrated transcription factors involved in the acquisition of iron, such as the  
18 HAP transcription factors Hap43, Hap2, Hap3 and Hap5, to be important for *C.*  
19 *albicans* biofilm formation on silicone surfaces in atmospheric CO<sub>2</sub> conditions.  
20 However, high CO<sub>2</sub> was able to overcome the requirement for HAP transcription  
21 factor activity and enable *C. albicans* biofilms to forage for essential metabolites to  
22 support growth. Overall, we propose that *C. albicans* has adapted to utilise the high  
23 CO<sub>2</sub> environment found in the host to promote its ability to colonise and to compete  
24 for nutrition. Our analysis reveals new approaches that can be taken to prevent *C.*

- 1 *albicans* biofilm formation in high CO<sub>2</sub> environments that pave the way for new
- 2 therapeutic approaches to treat these highly drug resistant structures.
- 3

1 **Materials and Methods**

2 ***Candida* strains and growth media**

3 *Candida* strains (Supplementary Table S1) were routinely grown at 30°C in yeast  
4 peptone dextrose (YPD) media (2% peptone (BD Bacto), 2% D-glucose (Fisher  
5 Scientific), 1% yeast extract (BD Bacto)). For biofilm growth assays, *Candida*  
6 biofilms were grown at 37°C in RPMI-1640 media (Sigma-Aldrich, R8755)  
7 supplemented with 80µg/ml uridine (Sigma-Aldrich, U3750) if required.

8

9 ***In vitro* biofilm growth assays**

10 *C. albicans* biofilms were grown on a PDMS silicone elastomer (Provincial Rubber,  
11 S1). The silicone was cut into 1cm<sup>2</sup> squares and placed in clips in a modified 24-well  
12 plate lid (Academic Centre for Dentistry Amsterdam, AAA-model) so they could be  
13 suspended in media within a 24-well plate. Silicone squares were incubated in 1ml  
14 50% Donor Bovine Serum (DBS) (Gibco, 16030074) for 30 minutes at 30°C, then  
15 washed twice with 1ml PBS to remove excess DBS. 1ml of *C. albicans* were added  
16 to each well of a 24 well plate following resuspension in PBS at an OD<sub>600</sub> of 1.0 and  
17 the lid with the silicone squares attached was placed on top so the silicone squares  
18 protrude into the cell suspension. Plates were then incubated at 37°C (in either  
19 0.03% CO<sub>2</sub> or 5% CO<sub>2</sub>) without shaking for 90 min to allow cell attachment to the  
20 silicone. After the attachment phase, the silicone squares were washed twice with  
21 1ml PBS to remove any unattached cells and transferred to 1ml RPMI-1640 media  
22 (Sigma-Aldrich, R8755). They were then incubated at 37°C (in either 0.03% CO<sub>2</sub> or  
23 5% CO<sub>2</sub>) without shaking for 48h to allow biofilm maturation.

24

25



## 1 **Biofilm quantification via XTT assay**

2 Biofilm growth was quantified using an XTT assay <sup>37</sup>. Biofilms were washed twice  
3 with 1ml PBS to remove any planktonic cells before proceeding to quantification.  
4 After washing, the biofilms were transferred to a new pre-sterilised 24-well plate  
5 (Greiner Bio-one, CELLSTAR, 662160) containing 30µg/ml XTT labelling reagent  
6 (Roche, 11465015001) and incubated at 37°C for 4h. After incubation, the biofilms  
7 were removed from the 24-well plate and the absorbance of the remaining XTT  
8 labelling reagent was measured at 492nm using a BMG LABTECH FLUOstar  
9 Omega plate reader machine.

10

## 11 ***C. albicans* transcription factor knockout (TFKO) screen**

12 Biofilms using mutants from a *C. albicans* TFKO library <sup>38</sup> were seeded and grown  
13 for 48h on a PDMS silicone elastomer (Provincial Rubber, S1) as described above.  
14 Biofilm growth was quantified using the XTT assay. Experiments were performed in  
15 biological triplicate.

16

## 17 **Iron starvation of *C. albicans* biofilms**

18 Biofilms were set up as described previously except they were incubated at 37°C in  
19 either 0.03% CO<sub>2</sub> or 5% CO<sub>2</sub> for 48h in RPMI-1640 containing varying  
20 concentrations of the Fe<sup>2+</sup> chelator 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-  
21 disulfonic acid monosodium salt hydrate (Ferrozine – Sigma-Aldrich, 160601) or the  
22 Fe<sup>3+</sup> chelator Deferasirox (Cambridge Bioscience, CAY16753-5). Ferrozine was  
23 made as a 100mM stock solution in sterile MQ H<sub>2</sub>O and diluted in RPMI-1640 to final  
24 concentrations of 250-500µM. Deferasirox was made as a 20mg/ml stock solution in

1 DMSO and diluted in RPMI-1640 to final concentrations of 70-210µg/ml. Relevant  
2 solvent controls were included. Final biofilms were quantified using an XTT assay.

3

#### 4 **Preparation of PDMS-coated microscope slides**

5 To prepare PDMS for coating microscope slides 16g ( $6.16 \times 10^{-4}$  mol) silanol-  
6 terminated PDMS (cSt 1000,  $M_w$  26000, from Fluorochem Ltd.) and 0.26g ( $12.48 \times$   
7  $10^{-4}$  mol, 1:4 stoichiometric ratio) cross-linking agent tetraethyl orthosilicate (TEOS –  
8 Sigma-Aldrich, 131903) were mixed at 3500rpm for 1 min using a DAC 150FV2-K  
9 speedmixer. At this point, 720µl tin(II) ethylhexanoate (Sigma-Aldrich, S3252) made  
10 up at a concentration of 0.6M in toluene was added as a catalyst and the mix spun  
11 for a further 60 secs at 3500rpm. The elastomer mixture was then doctor bladed onto  
12 microscope slides using an automatic precision film applicator MTCX4 (Mtv-  
13 Messtechnik – blade width = 70mm, thickness adjustability 0-3000µm). The doctor  
14 blade height was set 10µm higher than the thickness of the microscope slide. The  
15 elastomer mix was poured over the top of the slide (with a bias towards the side of  
16 the microscope slide closest to the doctor blade), and then the doctor blade is moved  
17 at a constant speed over the substrate. The microscope slide was then air cured for  
18 2h before being heat cured for 18h in a 70°C oven.

19

#### 20 **Preparation of *C. albicans* biofilms on silicone coated slides for microscopy**

21 Biofilms were grown directly on microscope slides that had been pre-coated with a  
22 PDMS silicone polymer (see above) for confocal imaging. Biofilms were grown with a  
23 prefabricated well. PDMS-coated microscope slides were incubated with 400µl 50%  
24 Donor Bovine Serum (DBS) (Gibco, 16030074) in the wells for 30 min at 30°C and  
25 washed twice with 400µl PBS. *C. albicans* overnight cultures were grown in YPD at

1 30°C and washed in PBS as described previously. 400µl of the OD<sub>600</sub> 1.0 standard  
2 cell suspension was added to the wells and incubated at 37°C (in either 0.03% CO<sub>2</sub>  
3 or 5% CO<sub>2</sub>) without shaking for 90 min to allow cell attachment to the silicone  
4 surface of the microscope slide. After the attachment phase, the microscope slides  
5 were washed twice with 400µl PBS to remove any unattached cells and then  
6 incubated at 37°C with 400µl RPMI-1640 media in the wells for 6h, 24h or 48h (in  
7 either 0.03% CO<sub>2</sub> or 5% CO<sub>2</sub>). Biofilms were washed twice with 400µl PBS and then  
8 incubated in the dark for 45 min at 30°C in 400µl PBS containing 50µg/ml ConA-  
9 FITC (Sigma-Aldrich, C7642) and 20µM FUN-1 (Invitrogen Molecular Probes,  
10 F7030). After incubation with the dyes, the stained biofilms were washed again with  
11 400µl PBS to remove any residual dye. The well was removed and 2 drops of  
12 ProLong<sup>TM</sup> Diamond Antifade Mountant (Invitrogen, P36965) was added to each  
13 stained biofilm. A cover slip was placed on top and the microscope slides were  
14 incubated in the dark at room temperature overnight to allow the mountant to cure.

15

### 16 **Confocal scanning laser microscopy (CSLM) of *Candida albicans* biofilms on** 17 **silicone slides**

18 Stained biofilms grown on silicone coated slides were imaged using a Zeiss  
19 LSM880/Elyra/Axio Observer.Z1 Confocal Microscope (Carl Zeiss Inc.) using the  
20 488nm argon and the 561nm DP55 lasers. Images of the green (ConA-FITC) and  
21 the red (FUN-1) were taken simultaneously using a multitrack mode. Z-stacks were  
22 taken using the inbuilt 'optimal' settings to determine the optimal intervals (typically  
23 1.5-2.0µm slices) based upon sample thickness and magnification. The 20x and oil-  
24 immersion 40x objective lenses were used throughout. The image acquisition  
25 software used was ZENBlack and the image processing software was ZENBlue.

## 1 **RNA isolation from *C. albicans* biofilms**

2 Total RNA was extracted in biological triplicate per condition (0.03% and 5% CO<sub>2</sub>)  
3 using the E.Z.N.A.<sup>TM</sup> Yeast RNA Kit (Omega Bio-Tek, R6870-01) as per the  
4 manufacturer's instructions with a few modifications. Specifically, *C. albicans* CAI-4  
5 biofilms were seeded and grown in 0.03% and 5% CO<sub>2</sub> as described for *in vitro*  
6 biofilm growth assays. Mature biofilms were washed twice with 1ml ice-cold PBS to  
7 remove any planktonic cells. Biofilm cells were harvested by transferring silicone  
8 squares upon which the biofilms were growing into 5ml cold SE buffer/2-  
9 mercaptoethanol (provided in the E.Z.N.A.<sup>TM</sup> Yeast RNA Kit) and vortexing at  
10 2500rpm for 1 min. The resulting biofilm cell suspension was pelleted by  
11 centrifugation at 4000rpm for 10 min at 4°C. The supernatant was discarded and the  
12 cells re-suspended in fresh 1ml cold SE buffer/2-mercaptoethanol, this cell  
13 suspension was transferred to a 2ml Eppendorf tube. The cell suspension was  
14 centrifuged again for 10 min at 4°C, the supernatant discarded and the pellet re-  
15 suspended in 480µl fresh SE buffer/2-mercaptoethanol. The biofilm cell suspension  
16 was incubated with 80µl lyticase stock solution (5000units/ml in SE buffer) at 30°C  
17 for 90 min. The resulting spheroplasts were pelleted by centrifugation at 2900rpm for  
18 10 min at 4°C and the supernatant aspirated and discarded. The spheroplasts were  
19 gently re-suspended in 350µl YRL buffer/2-mercaptoethanol (provided in the  
20 E.Z.N.A.<sup>TM</sup> Yeast RNA Kit). The rest of the RNA extraction proceeded as per the  
21 manufacturer's instructions including the optional DNase digestion step. RNA was  
22 eluted in 30µl DEPC water, the concentration and purity established using a  
23 NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and stored at -  
24 80°C.

25

## 1 **Library Preparation and RNA Sequencing**

2 RNA samples were sent to the Centre for Genome Enabled Biology and Medicine  
3 (Aberdeen, UK) for library preparation and sequencing. Before library preparation,  
4 the quality and quantification of RNA samples were evaluated with TapeStation  
5 (Agilent) and Qubit (Thermal Fisher). Samples with a minimum RIN of 8.0 proceeded  
6 to library preparation. The input of RNA was based on the specifically measured  
7 RNA concentration by Qubit. The mRNA-Seq libraries were prepared using  
8 TruSeq™ Stranded mRNA Sample Preparation Kit (Illumina) according to the  
9 manufacturer's instructions. Briefly, Poly-A RNA were purified from 500ng of total  
10 RNA with 1ul (1:100) ERCC spike (Thermal Fisher) as an internal control using RNA  
11 purification oligo(dT) beads, fragmented and retrotranscribed using random primers.  
12 Complementary-DNAs were end-repaired, and 3-adenylated, indexed adapters were  
13 then ligated. 15 cycles of PCR amplification were performed, and the PCR products  
14 were cleaned up with AMPure beads (Beckman Coulter). Libraries were validated for  
15 quality on Agilent DNA1000 Kit and quantified with the qPCR NGS Library  
16 Quantification kit (Roche). The final libraries were equimolar pooled and sequenced  
17 using the High Output 1X75 kit on the Illumina NextSeq500 platform producing 75bp  
18 single-end reads. For each library a depth of 50-70M reads was generated.

19

## 20 **Analysis of RNA-Seq data**

21 Analysis of RNA-Seq data was performed using the Galaxy web platform <sup>39</sup>. The  
22 quality of the RNA sequencing reads was checked using FastQC v0.11.5 <sup>40</sup> with  
23 default settings. Low quality ends (Phred score < 20) and any adaptor sequences  
24 were trimmed using TrimGalore! v0.4.3 <sup>41</sup>. Reads which became shorter than 40bp  
25 after trimming were removed from further analysis. After trimming, 97.7% of initial

1 reads remained and the quality was checked again using FastQC v0.11.5<sup>40</sup>. There  
2 were no Poly-A reads (more than 90% of the bases equal A), ambiguous reads  
3 (containing N) or low quality reads (more than 50% of the bases with a Phred score  
4 < 25). After processing, the mean Phred score per read was 35. Processed reads  
5 were aligned with the reference *C. albicans* genome SC5314 version A21-s02-m09-  
6 r10 using HISAT2 v2.1.0<sup>42</sup> with single-end reads and reverse strand settings (rest of  
7 the settings were default). After alignment, the number of mapped reads which  
8 overlapped CDS features in the genome (using the *C.*  
9 *albicans*\_SC5314\_version\_A21-s02-m09-r10\_features.gtf annotation file<sup>43</sup>) were  
10 determined using htseq-count v0.9.1<sup>44</sup> with default settings. Reads aligning to  
11 multiple positions or overlapping more than one gene were discarded, counting only  
12 reads mapping unambiguously to a single gene. Differential gene expression  
13 analysis between conditions (0.03% and 5% CO<sub>2</sub>) was performed using DESeq2  
14 v1.18.1<sup>45</sup> with default settings.

15

## 16 **Gene Set Enrichment Analysis of transcription profiles**

17 Downstream analysis of RNA Sequencing data was performed using the PreRanked  
18 tool of Gene Set Enrichment Analysis (GSEA; Broad Institute)<sup>46</sup> which compares a  
19 pre-ranked significantly differentially expressed gene list to a functional gene set  
20 database. False discovery rate (FDR) q-values were calculated based upon 1000  
21 permutations. The gene set database used was assembled by Sellam, A. *et al.* as  
22 described in<sup>47</sup>, which is based upon experimental analyses from published studies,  
23 Gene Ontology (GO) term categories curated by the *Candida* Genome Database<sup>48</sup>  
24 and protein-protein interaction information derived from *Saccharomyces cerevisiae*  
25 data curated by the *Saccharomyces* Genome Database<sup>49</sup>. Gene set networks were

1 generated in Cytoscape 3.7.1 (Available at: <https://cytoscape.org/>)<sup>50</sup> using the  
2 EnrichmentMap plug-in (Available at:  
3 <http://apps.cytoscape.org/apps/enrichmentmap>). Gene expression heat maps based  
4 on GO term categories were created using the Pheatmap package in R Studio.

5

## 6 **Antifungal treatment of *C. albicans* biofilms**

7 Biofilms were set up as described previously and grown in RPMI-1640 for 24h at  
8 37°C. The biofilms were then transferred to fresh RPMI-1640 media containing a  
9 select antifungal. Three antifungals were tested; Fluconazole, Miconazole and  
10 Nystatin. Fluconazole (Santa Cruz Biotechnology, sc-205698) was made as a  
11 50mg/ml stock solution in ethanol and diluted in RPMI-1640 final concentrations  
12 ranging from 8-256µg/ml. Miconazole (Santa Cruz Biotechnology, sc-205753) was  
13 made as a 50mg/ml stock solution in DMSO and also diluted in RPMI-1640 to final  
14 concentrations ranging from 8-256µg/ml. Nystatin (Santa Cruz Biotechnology, sc-  
15 212431) was made as a 5mg/ml stock solution in DMSO and diluted in RPMI-1640 to  
16 final concentrations ranging from 1-8µg/ml. Drug vehicle controls (0.5% ethanol for  
17 Fluconazole, 0.5% DMSO for Miconazole and Nystatin) were used in all cases. The  
18 biofilms matured in the RPMI-1640 media containing the select antifungal for a  
19 further 24h at 37°C in both 0.03% and 5% CO<sub>2</sub> before proceeding to quantification  
20 via the XTT assay. Experiments were performed in biological and technical triplicate.

21

## 22 ***C. albicans* attachment assay**

23 CAI-4 cells were seeded onto silicone-coated microscope slide for 90 min as  
24 described previously for growing biofilms for confocal microscopy, except here an  
25 OD<sub>600</sub> of 0.1 was used instead of 1.0 and the silicone-coated microscope slides were

1 not pre-incubated with DBS. The slide surface was washed twice with 400µl PBS to  
2 remove any unattached cells. Images were taken using a Leica DMR fitted with a  
3 Leica DFC9000 GT camera using a 20x objective lens and brightfield settings. The  
4 image acquisition software used was the Leica Application Suite X package. Using  
5 identical microscope settings throughout, five images were taken of each of three  
6 biological replicates in both 0.03% and 5% CO<sub>2</sub> and the cells per image counted  
7 using the 'Cell Counter' function in ImageJ.

8

### 9 **2-deoxyglucose (2-DG) treatment of *C. albicans* biofilms**

10 Biofilms were set up as described previously except they were incubated at 37°C in  
11 either 0.03% CO<sub>2</sub> or 5% CO<sub>2</sub> for 48h in RPMI-1640 containing varying  
12 concentrations of 2-DG. 2-DG (Sigma-Aldrich, D6134) was made up as a 20% stock  
13 solution in sterile MQ H<sub>2</sub>O and diluted in RPMI-1640 to final concentrations of 0.25-  
14 1%. Biofilms were quantified using XTT assays and images were also taken.  
15 Experiments were performed in biological and technical triplicate.

16

### 17 ***C. albicans* biofilm dispersion assay**

18 Biofilms were set up as described previously and grown in RPMI-1640 for 48h at  
19 37°C. The spent media, containing dispersed cells, was sonicated at amplitude 4µm  
20 for 10 secs to separate clumps of hyphal cells (previous work in our lab has  
21 demonstrated these sonication settings do not affect viability). After sonication, the  
22 dispersed cells were diluted 1 in 10 and 200µl of this suspension was plated on YPD  
23 agar plates in triplicate. YPD plates were incubated for 48h at 37°C to allow colonies  
24 to form, at which point the number of colonies were manually counted using a Stuart



- 1 Scientific Colony Counter. Experiments were performed in biological and technical
- 2 triplicate.
- 3

## 1 **Results**

### 2 **CO<sub>2</sub> enhances *C. albicans* biofilm formation**

3 *C. albicans* biofilms were seeded and grown on silicone in CO<sub>2</sub> levels found in  
4 exhaled air (5%) and atmospheric air (0.03%) for 24 and 48 hours. Biofilms were  
5 quantified using the XTT assay which acts as a readout of cell number<sup>37</sup>. After 24  
6 hours of growth, both CAI4pSM2 and SN250 *C. albicans* strains exhibited a  
7 significantly higher cell number within biofilms grown in a 5% CO<sub>2</sub>. However, after 48  
8 hours, cell numbers within biofilms grown in both CO<sub>2</sub> conditions appeared equal  
9 (Figure 1A). Interestingly, although cell number appeared equivalent at 48h, the  
10 resultant biofilm mass appeared noticeably larger in biofilms grown under elevated  
11 CO<sub>2</sub> conditions (Figure 1B).

12

### 13 **Analysis of the effects of CO<sub>2</sub> on phases of *C. albicans* biofilm formation**

14 We sought to determine which phases of biofilm growth were influenced by CO<sub>2</sub>  
15 elevation. To investigate the attachment phase *C. albicans* cells were seeded for 90  
16 min onto silicone-coated microscope slides under 0.03% CO<sub>2</sub> or 5% CO<sub>2</sub> levels. We  
17 observed that exposure to elevated CO<sub>2</sub> led to a significant increase in the number  
18 of cells that attached to silicone (mean of 2108 cells in 0.03% vs. a mean of 7033  
19 cells in 5% CO<sub>2</sub>) (Figure 2A). Cells also appeared to attach as larger aggregations in  
20 5% CO<sub>2</sub> when compared to those in 0.03% CO<sub>2</sub> (Supplementary Figures S1A and B)  
21 indicating that both cell-substrate and cell-cell attachments may be enhanced.

22

23 Confocal scanning laser microscopy (CSLM) was used to investigate the effects of  
24 CO<sub>2</sub> on biofilm growth during maturation. *C. albicans* biofilms were seeded on  
25 silicone-coated microscope slides and images were taken at 6h, 24h and 48h of

1 growth under either 0.03% or 5% CO<sub>2</sub> growth conditions (Figure 2B). Biofilm images  
2 are displayed as maximum intensity ortho-projections of Z-stacks to give a view of  
3 the overall structures of the biofilms. After 6h growth in 0.03% CO<sub>2</sub>, the majority of  
4 cells were found in the yeast form with some visibly initiating hyphae. In comparison,  
5 biofilms grown in 5% CO<sub>2</sub> appeared to consist of a high proportion of hyphal cells,  
6 were visibly denser and had begun to exhibit an ordered structure in the Z-plane  
7 (Figure 2B). After 24h growth in 0.03% CO<sub>2</sub>, biofilms were progressing through the  
8 maturation stage with the appearance numerous hyphal cells. However, the 5% CO<sub>2</sub>  
9 biofilms displayed a fully mature biofilm organisation displaying hyphal cells  
10 organised in a brush-like structure above a basal layer of yeast cells (Figure 2B). At  
11 48h, biofilms grown in both 0.03% and 5% CO<sub>2</sub> appeared as dense mature  
12 structures, however, biofilms grown under elevated CO<sub>2</sub> appeared larger (Figure 2B)  
13 as had been observed macroscopically (Figure 1B).

14

15 Dispersion is the final stage of biofilm formation, we therefore investigated whether  
16 CO<sub>2</sub> elevation resulted in increased levels of cell shedding. We routinely observed  
17 that spent RPMI-1640 media isolated after biofilm growth in 5% CO<sub>2</sub> contained more  
18 cells than that of taken from biofilms grown in 0.03% CO<sub>2</sub> (Supplementary Figure  
19 S2A). We quantified this by seeding and growing *C. albicans* biofilms for 48h in  
20 0.03% and 5% CO<sub>2</sub> and conducting colony forming unit (CFU) assays using the  
21 spent RPMI-1640 media (Figure 2C and Supplementary Figure S2B). Our results  
22 showed an approximate four-fold increase in cell number released from mature  
23 biofilms when grown under elevated CO<sub>2</sub> conditions, consistent with an increase in  
24 dispersal. Overall, these data demonstrate that the elevation of CO<sub>2</sub> enhances each

1 stage of the *C. albicans* biofilm forming process, from attachment through maturation  
2 to dispersion.

3

#### 4 **Identification of the regulatory mechanisms that govern CO<sub>2</sub> acceleration of *C.*** 5 ***albicans* biofilm formation**

6 In planktonic *C. albicans* cells CO<sub>2</sub> is converted to bicarbonate ions (HCO<sub>3</sub><sup>-</sup>) which  
7 stimulates the adenylate cyclase Cyr1 (Cdc35), an increase in cAMP and activation  
8 of PKA<sup>32</sup>. We investigated whether CO<sub>2</sub> elevation may drive biofilm formation and  
9 maturation via a similar Ras/cAMP/PKA mechanism. We conducted biofilm growth  
10 assays using *C. albicans* mutants lacking key components of the pathway; *ras1Δ/Δ*,  
11 *cdc35Δ/Δ*, *CDC35<sup>ΔRA</sup>* (adenylate cyclase missing the Ras1 interacting domain),  
12 *tpk1Δ/Δ* (missing a catalytic subunit isoform of PKA), and *tpk2Δ/Δ* (missing the other  
13 catalytic subunit isoform of PKA). Biofilm formation was quantified after 48h of  
14 growth and compared to an isogenic wild type control. The *ras1Δ/Δ* mutant displayed  
15 significantly attenuated biofilm growth in 0.03% CO<sub>2</sub> but this was rescued to wild  
16 type levels in 5% CO<sub>2</sub> (Figure 3A), indicating that Ras1 function is dispensable for  
17 biofilm formation under conditions of elevated CO<sub>2</sub>. The *cdc35Δ/Δ* and *CDC35<sup>ΔRA</sup>*  
18 mutants both exhibited significantly reduced biofilm growth when grown under either  
19 atmospheric or elevated CO<sub>2</sub> conditions (Figure 3A). Conversely, both *tpk1Δ/Δ* and  
20 *tpk2Δ/Δ* mutants exhibited biofilm growth equivalent to the wild type under both CO<sub>2</sub>  
21 conditions (Figure 3A). Taken together, this data implies the CO<sub>2</sub>-mediated effect on  
22 *C. albicans* biofilm growth is reliant on Cyr1 but can bypass a requirement for Ras1  
23 and that the PKA isoforms Tpk1 and Tpk2 are functionally redundant with respect to  
24 cAMP activation of the biofilm programme. Our findings are consistent with the

1 adenylate cyclase Cyr1 as a key CO<sub>2</sub> sensor in the enhanced biofilm growth  
2 observed under elevated CO<sub>2</sub> conditions.

3

4 To investigate the molecular basis of the activation of *C. albicans* biofilm formation in  
5 5% CO<sub>2</sub>, we conducted a screen of an available transcription factor knockout (TFKO)  
6 library<sup>38</sup> containing 147 mutants each lacking a non-essential transcription factor.  
7 This screen identified 122 deletions that had no effect upon biofilm formation in  
8 either CO<sub>2</sub> condition (Supplementary Figure S3) and 22 transcription factors which  
9 attenuated biofilm formation (Supplementary Table S2). Six TFKO mutants (*tup1Δ/Δ*,  
10 *sef1Δ/Δ*, *swi4Δ/Δ*, *pho4Δ/Δ*, *bcr1Δ/Δ* and *efg1Δ/Δ*) had diminished biofilm growth in  
11 both 0.03% and 5% CO<sub>2</sub>, 12 had reduced biofilm formation only in 0.03% CO<sub>2</sub>  
12 (*hap2Δ/Δ*, *rbf1Δ/Δ*, *rob1Δ/Δ*, *fgr15Δ/Δ*, *dal81Δ/Δ*, *mig1Δ/Δ*, *brg1Δ/Δ*,  
13 *C4\_00260WΔ/Δ*, *zcf27Δ/Δ*, *C1\_13880CΔ/Δ*, *crz1Δ/Δ*, and *hap43Δ/Δ*), and 4 TFKOs  
14 had reduced biofilm formation only in 5% CO<sub>2</sub> (*leu3Δ/Δ*, *mbp1Δ/Δ*, *bas1Δ/Δ*, and  
15 *try6Δ/Δ*) (Supplementary Table S2 and Supplementary Figure S3). Intriguingly, 3  
16 additional mutants, *zcf17Δ/Δ*, *zcf30Δ/Δ* and *mac1Δ/Δ*, displayed increased biofilm  
17 growth in 0.03% CO<sub>2</sub> but no significant difference in 5% CO<sub>2</sub>.

18

19 We performed gene ontology enrichment analysis on the genes encoding the 25  
20 transcription factors whose loss impacted upon biofilm growth to group them  
21 according to biological processes. This revealed that 7 of these transcription factors  
22 – Brg1, Bcr1, Efg1, Rob1, Dal81, Leu3, and Try6 – were already known to be  
23 involved in the regulation of single-species biofilm formation within *C. albicans* (Table  
24 S2). Interestingly, out of these, only the *efg1Δ/Δ* and *bcr1Δ/Δ* mutant exhibited  
25 attenuated biofilm growth in both 0.03% and 5% CO<sub>2</sub>. The *brg1Δ/Δ*, *dal81Δ/Δ*, and

1 *rob1Δ/Δ* mutants had significantly reduced biofilm growth in 0.03% CO<sub>2</sub> but this was  
2 rescued in the 5% CO<sub>2</sub> environment (Supplementary Table S2), possibly indicating  
3 redundancy of these TFs when cells are exposed to high CO<sub>2</sub>. This important finding  
4 suggests that CO<sub>2</sub> elevation can bypass the requirement for some of the key  
5 transcriptional regulators of biofilm formation (Figure 3B). The *leu3Δ/Δ* and *try6Δ/Δ*  
6 mutants had significantly reduced biofilm growth in 5% CO<sub>2</sub> but no significant  
7 difference in 0.03% CO<sub>2</sub>. Eight of the TFs identified – Brg1, Crz1, Efg1, Mac1, Mig1,  
8 Rob1, Zcf27, Zcf30 – are associated with the positive regulation of filamentous  
9 growth and Tup1 is involved in the negative regulation of filamentous growth. *C.*  
10 *albicans* biofilm formation is strongly linked with the yeast-to-hyphal switch, with  
11 mutants unable to perform this switch having previously been shown to be deficient  
12 in biofilm growth<sup>33</sup>.

13

14 The TFKO screen also revealed that mutants lacking transcription factors associated  
15 with metal ion homeostasis, specifically iron homeostasis, had altered biofilm-  
16 formation in 0.03% and/or 5% CO<sub>2</sub> (Figure 4A and Supplementary Table S2).  
17 Mutants lacking genes expressing components of the HAP complex; *hap2Δ/Δ*,  
18 *hap3Δ/Δ*, *hap5Δ/Δ*, and *hap43Δ/Δ*, exhibited significantly reduced biofilm growth  
19 after 48h compared to wild type in 0.03% CO<sub>2</sub>. However, their biofilm growth was  
20 significantly higher in 5% CO<sub>2</sub>, reaching wild type levels in the cases of the *hap3Δ/Δ*,  
21 *hap5Δ/Δ*, and *hap43Δ/Δ* mutants (Figure 4A). This is an important observation as it  
22 indicates that an increase in CO<sub>2</sub> concentration is sufficient to compensate for the  
23 loss of these transcription factors. The HAP complex in *C. albicans* is responsible for  
24 the regulation of iron homeostasis<sup>51</sup>. Sef1 acts downstream of this complex and the  
25 *sef1Δ/Δ* mutant displayed significantly reduced biofilm growth in both 0.03% and 5%

1 CO<sub>2</sub> after 48h growth (Figure 4A). These data suggest that the elevation of CO<sub>2</sub> can  
2 bypass the requirement for HAP complex activity in biofilm formation in a Sef1  
3 dependent manner.

4

5 To further determine whether elevated CO<sub>2</sub> enhanced *C. albicans* ability to  
6 appropriate iron from the environment biofilms were grown in the presence of the  
7 Fe<sup>2+</sup> chelator 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid  
8 monosodium salt hydrate (Ferrozine). Iron chelation was observed to have a marked  
9 effect on biofilm growth at and above 350µM Ferrozine (Figure 4B). As had been  
10 observed with TFKO strains of the HAP complex, the elevation of CO<sub>2</sub> counteracted  
11 the effects of iron limitation (Figure 4B) providing further evidence of a role for CO<sub>2</sub> in  
12 enhancing iron uptake or utilisation capability. This tolerance to iron starvation of *C.*  
13 *albicans* biofilms grown in 5% CO<sub>2</sub> was also exhibited by *tpk1Δ/Δ* and *tpk2Δ/Δ*  
14 mutants (Supplementary Figure S4), indicating these PKA isoforms are functionally  
15 redundant with respect to iron homeostatic pathways. Clinical strains of *C. albicans*  
16 isolated from failed voice prostheses displayed the same phenotype with biofilm  
17 formation rescued in 5% CO<sub>2</sub> when grown in the presence of 500µM Ferrozine  
18 (Figure 4C). A transcriptomic analysis comparing biofilms grown in 0.03% and 5%  
19 CO<sub>2</sub> (described below) revealed that several genes related to iron acquisition were  
20 upregulated in 5% CO<sub>2</sub> biofilms, providing an explanation for the tolerance to iron  
21 sequestration in high CO<sub>2</sub> (Figure 4). For example, *FTR2*, which encodes the high  
22 affinity iron permease Ftr2, had increased expression. In addition, *CFL4* which  
23 encodes a putative ferric reductase, is regulated by Sef1 and induced in low-iron  
24 conditions<sup>52</sup> was also upregulated in high CO<sub>2</sub> biofilms after 48h. Finally, the *CSA2*  
25 and *RBT5* genes, which encode proteins involved in the acquisition of iron from

1 haem groups, were also upregulated. Csa2 and Rbt5 have both previously been  
2 found to be required for normal biofilm formation in RPMI-1640 media <sup>5354</sup>.

3

#### 4 **Transcriptome analysis of *C. albicans* biofilms grown in high and low CO<sub>2</sub>**

5 To further investigate the effect of high CO<sub>2</sub> on *C. albicans* biofilm growth, we  
6 performed an RNA Sequencing analysis of *C. albicans* biofilms grown in 0.03% and  
7 5% CO<sub>2</sub>. Growth in 5% CO<sub>2</sub> led to a global response resulting in the significant  
8 differential expression (false-discovery-rate adjusted p-value ( $q$ )  $\leq$  0.05) of 2875  
9 genes, with roughly equal numbers of genes up- and downregulated (1441 up and  
10 1434 down) (Supplementary Figure S5A). 80 genes were strongly (log<sub>2</sub> fold change  
11  $>2$ ) upregulated and 25 were strongly (log<sub>2</sub> fold change  $<-2$ ) downregulated. To  
12 investigate the cellular pathways which these differentially expressed genes are  
13 within we conducted Gene Set Enrichment Analysis (GSEA; Broad Institute) <sup>46</sup>.  
14 GSEA revealed that biofilm formation pathways controlled by four of the nine 'core'  
15 biofilm regulator transcription factors <sup>2755</sup> were upregulated in 5% CO<sub>2</sub> biofilms at 48h  
16 (Figure 5A). Genes downstream of Brg1, Efg1, Ndt80 and Bcr1 are enriched in the  
17 upregulated genes at the top of the ranked list of differentially expressed genes  
18 (NES = +2.79, +2.66, +2.56, and +2.60 respectively) (Figure 5A), indicating high CO<sub>2</sub>  
19 drives expression of genes previously described as important in the biofilm-forming  
20 ability of *C. albicans*.

21

22 As GSEA can show enrichment profiles exhibiting correlations with several  
23 overlapping gene sets, we visualised networks of similar gene sets using the  
24 Cytoscape 3.7.1 EnrichmentMap plug-in <sup>50</sup>. Upregulated gene sets included those  
25 encoding membrane transporters, ribosome biogenesis, peroxisomal  $\beta$ -oxidation and



1 white-opaque switching (Figure 5B). Pathways involved in cytoskeleton organisation,  
2 cell-cycle progression and membrane biosynthesis were downregulated in high CO<sub>2</sub>  
3 conditions (Figure 5B), indicating cells within biofilms in high CO<sub>2</sub> may have stopped  
4 dividing at the 48h time point assessed, consistent with such biofilms having moved  
5 more rapidly through the maturation process.

6  
7 GSEA suggested that cell adhesion processes were upregulated in high CO<sub>2</sub>  
8 biofilms (NES = +2.13) (Figures 6A). GO Slim process analysis also highlighted  
9 many genes associated with cell adhesion were upregulated in *C. albicans* biofilms  
10 grown in 5% CO<sub>2</sub> after 48h (Figure 6B). The second highest upregulated gene in 5%  
11 CO<sub>2</sub> biofilms compared to 0.03% CO<sub>2</sub> biofilms was *ALS1* with a 3.77 log<sub>2</sub> fold  
12 change (Figure 6B). The Als1 cell surface adhesin has previously been shown to  
13 have important roles in biofilm formation<sup>56</sup>, and its expression is controlled by the  
14 biofilm transcription regulation network composed of Brg1, Rob1, Tec1, Ndt80, Bcr1  
15 and Efg1<sup>27</sup>. Other genes encoding cell surface adhesins such as *ALS4* and *ALS2*  
16 were also upregulated in 5% CO<sub>2</sub> biofilms after 48h (Figure 6B).

17  
18 GSEA has also identified that genes involved in membrane transporter activity are  
19 enriched in the upregulated genes at the top of the ranked list of differentially  
20 expressed genes (NES = +2.22) (Figure 6A). Specifically, genes encoding amino  
21 acid transporters were enriched in the significantly upregulated genes of 5% CO<sub>2</sub>  
22 biofilms (NES = +2.62) (Figure 6B). Likewise, significantly differentially expressed  
23 genes possessing the GO term 'amino acid transport' were primarily upregulated  
24 (Figure 6B). The most highly upregulated of these was *GAP2* which encodes a  
25 general amino acid permease<sup>57</sup>. *GAP2* was in fact the highest upregulated gene of

1 the entire RNA-Seq data set with a 4.60 log<sub>2</sub> fold change. The basic amino acid  
2 permease genes *CAN1*, *CAN2*, and *CAN3* were also upregulated (Figure 6B).

3  
4 Genes associated with carbohydrate transmembrane transport were also enriched in  
5 the significantly upregulated genes of 5% CO<sub>2</sub> biofilms as revealed by GSEA (NES =  
6 +1.88) (Figure 6B). Amongst these were 12 genes encoding putative major facilitator  
7 superfamily (MFS) glucose transmembrane transporters present in the significantly  
8 differentially expressed gene list and these are almost all upregulated. The only  
9 exceptions are *HGT9* and *HGT18* with log<sub>2</sub> fold changes of -1.24 and -1.70  
10 respectively (Figure 6B).

11  
12 Genes previously identified to be involved in hyphal formation in response to foetal  
13 bovine serum (FBS) exposure or 37°C were enriched in the downregulated genes  
14 (NES = -3.41). Likewise, the majority of genes under the GO term 'hyphal growth' in  
15 the significantly differentially expressed gene list were downregulated in 5% CO<sub>2</sub>  
16 biofilms at 48h growth (Supplementary Figure S5B). Significantly differentially  
17 expressed genes associated with the cytoskeleton, as identified by GO term  
18 analysis, were enriched in the downregulated genes (NES = -2.69) (Supplementary  
19 Figure S5C). Cytoskeleton reorganisation is important for the growth of *C. albicans*  
20 hyphal cells<sup>58</sup> as well as cell division<sup>5960</sup>, indicating cell growth is lower in 48h old *C.*  
21 *albicans* biofilms grown in 5% CO<sub>2</sub> than in those grown in 0.03% CO<sub>2</sub>. Consistent  
22 with this, genes involved in the transition through the G1/S checkpoint were also  
23 enriched in the significantly downregulated genes (NES = -2.83) (Supplementary  
24 Figure S5C).

1 Initially, these data appear to be contradictory to the previous data highlighting the  
2 increased biofilm formation of *C. albicans* under high CO<sub>2</sub> conditions. However, due  
3 to the increased biofilm formation in a 5% CO<sub>2</sub> environment, *C. albicans* biofilms  
4 reach full maturity much quicker in high CO<sub>2</sub>, as observed via confocal microscopy  
5 (Figure 2B). Thus, by 48h *C. albicans* biofilms grown in high CO<sub>2</sub> have been fully  
6 mature for several hours, and hence would contain fewer dividing cells or cells  
7 extending hyphae in comparison to low CO<sub>2</sub> biofilms.

8

### 9 **CO<sub>2</sub> elevation enhances azole resistance in *C. albicans* biofilms**

10 In addition to the observed increase in expression of genes that drive biofilm  
11 formation we observed an elevation in certain stress response pathways in *C.*  
12 *albicans* biofilms grown in 5% CO<sub>2</sub>. Gene sets involved in the response of *C.*  
13 *albicans* to antifungals such as Ketoconazole<sup>61</sup> (Figure 7A) were upregulated as well  
14 as several drug transporters (Figure 7B), indicating that elevation may lead to  
15 increased drug resistance. Upregulated genes included the *MDR1* gene (2.56 log<sub>2</sub>  
16 fold change) which encodes the multidrug resistance pump Mdr1 and is associated  
17 with resistance to several antifungals such as azoles<sup>62</sup>. To test the significance of  
18 this *C. albicans* biofilms were seeded and grown for 24h in 0.03% and 5% CO<sub>2</sub>  
19 before the addition of antifungals, after which they were grown for an additional 24h  
20 in both conditions to observe the effect of drug application. Antifungal concentrations  
21 were selected based upon previously reported MIC values for these antifungals  
22 against *C. albicans* biofilms<sup>28</sup>. Overall, Fluconazole and Miconazole treatment led to  
23 a significant reduction in biofilm growth in 0.03% CO<sub>2</sub> (Figures 7C and  
24 Supplementary Figure S6). Treatment with Fluconazole and Miconazole also  
25 significantly reduced biofilm formation in 5% CO<sub>2</sub>, however, their effects were

1 markedly reduced (Figures 7C and Supplementary Figure S6). This suggests an  
2 increased resistance of biofilms grown in 5% CO<sub>2</sub> to azole treatment. Interestingly,  
3 Nystatin was equally as effective against biofilms in either CO<sub>2</sub> environment (Figure  
4 7D).

5

## 6 **Precision approaches to overcome CO<sub>2</sub> acceleration of *C. albicans* biofilm** 7 **formation**

8 Our data indicate that elevation of CO<sub>2</sub> leads to an increase in the ability to scavenge  
9 for iron and glucose, both essential for biofilm formation and growth. We therefore  
10 wished to test whether these represented potential targets to combat *C. albicans*  
11 growth in high CO<sub>2</sub> environments such as the airway. An Fe<sup>3+</sup> chelator called  
12 Deferasirox, which is approved for treating patients with iron overload, has recently  
13 been shown to reduce infection levels in a murine oropharyngeal candidiasis model  
14 <sup>63</sup>. With this in mind, we repeated our previous iron starvation biofilm growth assay  
15 (Figure 4B) using Deferasirox in place of Ferrozine. We observed that Deferasirox  
16 treatment completely eradicates *C. albicans* biofilm growth in 0.03% CO<sub>2</sub> but has  
17 very little effect on biofilm growth in 5% CO<sub>2</sub> (Figure 8A). Thus adding further  
18 evidence that exposure to high levels of CO<sub>2</sub> can enable *C. albicans* biofilms to  
19 overcome the effects of iron starvation. Deferasirox does not therefore appear to be  
20 an effective treatment against *C. albicans* biofilms in high CO<sub>2</sub> such as in the context  
21 of voice prostheses colonisation.

22

23 *C. albicans* biofilms grown in 5% CO<sub>2</sub> exhibited the upregulation of genes encoding  
24 glucose transporters. Accordingly, we contemplated whether treatment of *C. albicans*  
25 biofilms with the glucose analogue 2-deoxyglucose (a glycolytic inhibitor) may

1 decrease biofilm growth in high CO<sub>2</sub> environments. 2-deoxyglucose (2-DG) has  
2 previously made it to Stage II clinical trials as an anti-prostate cancer treatment and  
3 is considered safe for use in humans<sup>64</sup>. Thus, it could be a potential therapeutic  
4 option to combat *C. albicans* biofilm formation on medical devices, specifically on  
5 voice prostheses. *C. albicans* biofilm formation was significantly reduced in the  
6 presence of 2-DG regardless of CO<sub>2</sub> environment (Figure 8B and Supplementary  
7 Figure S7). Interestingly, the biofilm reductions in all 2-DG concentrations were  
8 similar for biofilms in both low and high CO<sub>2</sub>, despite the fact several glucose  
9 transporters were upregulated in 5% CO<sub>2</sub> biofilms (Figure 6B). The reduction in  
10 biofilm growth upon 2-DG treatment was also quite apparent macroscopically (Figure  
11 8C).

12

## 1 **Discussion**

2 Our data demonstrate for the first time how a physiologically relevant elevation of  
3 CO<sub>2</sub> accelerates biofilm formation in *C. albicans* by activating the cAMP/PKA  
4 pathway (Figure 9). Although CO<sub>2</sub> elevation is dependent on Cyr1 it appears to  
5 bypass a requirement for Ras1. CO<sub>2</sub> elevation enhances each stage of the *C.*  
6 *albicans* biofilm forming process, from attachment through maturation to dispersion.  
7 The observed increase in cell attachment is accompanied by an increase in the  
8 abundance of mRNA transcripts for *ALS1*, *ALS2* and *ALS4* which encode adhesins  
9 of the agglutinin-like sequence family that function in the cell-surface and cell-cell  
10 attachment of *C. albicans*<sup>19</sup>. Our observed increase in dispersion also correlates  
11 with an upregulation of the known regulator of this stage of biofilm growth, *NRG1*<sup>65</sup>.  
12 Our observations have important clinical implication in scenarios where prosthetic  
13 devices are placed in areas of elevated CO<sub>2</sub>, for example voice prostheses or  
14 tracheostomy tubing. We would anticipate that high CO<sub>2</sub> may increase the probability  
15 of *C. albicans* colonisation and dissemination.

16  
17 Of the original six 'core' biofilm regulators (Efg1, Bcr1, Brg1, Rob1, Ndt80 and Tec1)  
18 only four (Efg1, Bcr1, Brg1, and Rob1) were identified as required for normal biofilm  
19 growth in our TFKO screen. The original TFKO screen which identified these  
20 regulators was carried out using a polystyrene surface<sup>27</sup> whereas we used silicone.  
21 This may suggest that attachment to and biofilm growth on silicone requires a more  
22 limited set of core transcription factors reflecting the importance of surface upon  
23 biofilm formation as has been observed previously<sup>55</sup>. In support of the environment  
24 being critical to biofilm establishment, CO<sub>2</sub> elevation was able to compensate for the  
25 loss of the core biofilm regulators Brg1 and Rob1. Intriguingly, of the four biofilm

1 regulators whose gene sets were predicted to be upregulated in 5% CO<sub>2</sub> biofilms,  
2 Efg1, Bcr1 and Brg1 were identified in our TFKO screen whereas Ndt80 was not.  
3 Moreover, only *efg1Δ/Δ* and *bcr1Δ/Δ* had reduced biofilm growth in both 0.03% and  
4 5% CO<sub>2</sub>. This disparity may be explained by the degree of overlap in downstream  
5 target genes between the core regulators of biofilm formation <sup>27</sup> which implies  
6 significant potential for functional redundancy.

7

8 Many of the genes significantly upregulated in biofilms grown in high CO<sub>2</sub> after 48h  
9 were also found to be upregulated in other biofilm gene expression studies <sup>66,67</sup>. For  
10 instance, Nett, J. *et al.* observed an increase in the expression of adherence genes  
11 in an *in vivo* venous catheter biofilm model. Like us, this venous catheter biofilm  
12 study saw an increase in the transcript abundance of *ALS1* and *ALS2* however, this  
13 was only observed at earlier time point biofilms <sup>66</sup>. This was also the case in a  
14 temporal gene expression analysis using *in vitro* denture and catheter models <sup>67</sup>.  
15 Interestingly, some pathways such as hexose transport, amino acid uptake, and  
16 stress responses that our differential gene expression analysis predicted to be  
17 upregulated in mature biofilms grown in high CO<sub>2</sub> were concluded to be upregulated  
18 only in early phase biofilms (12h) in this denture and catheter study <sup>67</sup>. The authors  
19 concluded the result of the induction of these pathways is the increase of intracellular  
20 pools of pyruvate, pentoses and amino acids, preparing for the large increase in  
21 biomass that occurs later in biofilm development <sup>67</sup>. We hypothesise that high CO<sub>2</sub>  
22 may stimulate these pathways and maintain their activity even in mature biofilms,  
23 thus supporting the increased biomass and maturation rate of biofilms observed  
24 when grown in high CO<sub>2</sub>.

25

1 We observed an increase in azole antifungal resistance within *C. albicans* biofilms  
2 grown in 5% CO<sub>2</sub>. This, at least partly, could be explained by the increase in  
3 expression of drug transporter genes such as *MDR1* which have previously been  
4 implicated in azole resistance <sup>62,68</sup>. However, *mdr1Δ/Δ*, as well as *cdr1Δ/Δ* and  
5 *cdr2Δ/Δ*, mutants only exhibit reduced azole resistance in planktonic culture and  
6 early stage (6h) biofilms, while levels of resistance are maintained in mature biofilms  
7 <sup>62</sup>. Therefore, we propose that it is more likely the increased azole resistance  
8 phenotype of 5% CO<sub>2</sub> biofilms displayed is contributed to via another mechanism,  
9 possibly increased ECM deposition. β-1,3-glucan, a major component of biofilm  
10 ECM, can bind to azole antifungals and sequester them to prevent passage to the  
11 cells <sup>69</sup>. We have observed that after 48h, 5% CO<sub>2</sub> biofilms, while containing similar  
12 cell numbers to 0.03% CO<sub>2</sub> biofilms, often appear larger to the eye with a more  
13 bulbous appearance. This could suggest more ECM material being produced in high  
14 CO<sub>2</sub> environments, contributing to the increased azole resistance. Furthermore,  
15 Miconazole treatment has been shown to generate superoxide radicals within *C.*  
16 *albicans* biofilms and leads to the increased expression of *SOD5* and *SOD6* (encode  
17 superoxide dismutase enzymes) in an attempt to protect against the toxic  
18 superoxides. A *sod4Δ/Δsod5Δ/Δsod6Δ/Δ* triple mutant is hypersensitive to  
19 Miconazole treatment when growing as a biofilm <sup>70</sup>. Our transcriptome analysis  
20 revealed *SOD6* and *SOD4* are upregulated in 5% CO<sub>2</sub> biofilms, providing a potential  
21 further mechanism for increased Miconazole resistance.

22

23 Our study identified that transcription factors involved in iron homeostasis are  
24 important for *C. albicans* biofilm growth. Principal among these were the Hap  
25 transcription factors which come together to form the HAP complex, a CCAAT box-



1 binding transcriptional regulator, under iron-limiting conditions. Genetic studies have  
2 revealed a requirement of *HAP2*, *HAP3*, *HAP5* and *HAP43* for growth in low-iron  
3 media<sup>3871</sup>. Thus, the biofilm formation defect exhibited by the *hap2Δ/Δ*, *hap3Δ/Δ*,  
4 *hap5Δ/Δ*, and *hap43Δ/Δ* mutants in 0.03% CO<sub>2</sub> could be explained by this growth  
5 deficiency since RPMI-1640 media has a low iron content. Nevertheless, this makes  
6 it even more intriguing that simply an increase in ambient CO<sub>2</sub> levels was able to  
7 significantly increase the biofilm growth of these mutants.

8

9 The HAP complex represses a GATA-type transcription factor called Sfu1. Sfu1 is  
10 responsible for repressing iron-uptake genes along with *SEF1* under iron-replete  
11 conditions. Sef1 activates iron-uptake genes as well as *HAP43*, *HAP2* and *HAP3*<sup>52</sup>,  
12 in this way the HAP complex is able to induce iron-uptake pathways while repressing  
13 iron-utilisation genes<sup>5152</sup>. Deletion of Sef1 results in the aberrant downregulation of  
14 all the major iron-uptake pathways of *C. albicans* in low iron conditions<sup>52</sup>. Due to the  
15 fact the *sef1Δ/Δ* mutant had defective biofilm growth in both CO<sub>2</sub> conditions, we  
16 hypothesise that a high CO<sub>2</sub> environment may influence Sef1 directly, causing the  
17 HAP complex to become at least partially redundant under these conditions. It  
18 should be noted that we did not observe a significant effect on *SEF1* mRNA levels  
19 within 5% CO<sub>2</sub> biofilms after 48h. Thus, if it is influencing Sef1 activity, CO<sub>2</sub> may be  
20 acting at either the protein level or post-translational level as Sef1 is subject to a  
21 post-translational control loop consisting of Sfu1 and the cyclin-dependent kinase  
22 Ssn3<sup>72</sup>. However Ssn3 may not play a role as it has recently been found to be  
23 dephosphorylated, and thus inactive, in 5% CO<sub>2</sub><sup>73</sup>. Our current model suggests that  
24 under elevated CO<sub>2</sub> PKA can phosphorylate Sef1 and possibly also inhibit Sfu1  
25 (Figure 9). This is currently under investigation and may possibly explain the

1 tolerance to iron sequestration exhibited by *C. albicans* biofilms grown in high CO<sub>2</sub>.  
2 We believe any potential phosphorylation of Sef1 by PKA to be Tpk1/Tpk2 redundant  
3 since the *tpk1Δ/Δ* and the *tpk2Δ/Δ* mutants both exhibited a significant increase in  
4 biofilm growth in 5% CO<sub>2</sub> when in the presence of 500μM Ferrozine compared to  
5 0.03% CO<sub>2</sub>. This is supported by a Tpk1/Tpk2 phosphoproteomic study which  
6 predicted Sef1 to be a potential PKA target that can be phosphorylated by both Tpk1  
7 and Tpk2<sup>74</sup>.

8

9 The increased azole resistance of *C. albicans* biofilms in 5% CO<sub>2</sub> along with the  
10 observed iron starvation tolerance when treated with the Fe<sup>3+</sup> chelator Deferasirox  
11 has important implications for the development of potential biofilm treatment  
12 strategies. At a clinical level this also indicates the location of a *C. albicans* biofilm  
13 within the body should be taken into consideration when deciding upon the most  
14 effective treatment. Encouragingly, 2-deoxyglucose (2-DG) was able to attenuate *C.*  
15 *albicans* biofilm growth in both 5% and atmospheric (0.03%) CO<sub>2</sub> environments. 2-  
16 DG has exhibited antimicrobial effects against fungal moulds<sup>75</sup> and bacterial biofilms  
17<sup>76</sup>. This, together with its action against *C. albicans* biofilms presented here,  
18 highlights the potential for 2-DG to be used an anti-biofilm therapeutic. It may be  
19 particularly useful for medical devices such as voice prostheses which are situated in  
20 CO<sub>2</sub>-rich environments in the body and are often colonised by a mixture of bacterial  
21 and fungal species<sup>77787980</sup>. It's important to note however that 2-DG was unable to  
22 eradicate *C. albicans* biofilm growth completely. It may be that 2-DG treatment is  
23 beneficial in combination with other compounds, such as iron chelators or traditional  
24 antifungals; this possibility is yet to be explored.

25

1 In conclusion, our data demonstrates that elevated levels of CO<sub>2</sub> act as an important  
2 driver of *C. albicans* biofilm formation, growth and maturation. These CO<sub>2</sub>-mediated  
3 effects are likely to have important medical ramifications, particularly in the context of  
4 prosthetics and airway management devices, but also for host infections in CO<sub>2</sub>-rich  
5 environments in the body.

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6

1 **Author Contributions**

2 The study was conceived by C.W. Gourlay and F.A. Mühlischlegel. Experimental  
3 procedures and data analyses were conducted by D.R. Pentland. The manuscript  
4 was written and edited by D.R. Pentland, C.W. Gourlay and F.A. Mühlischlegel.

5

1 **Competing Interests**

2 The authors declare no competing or conflicting interests.

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1 **Figure Legends**

2 **Figure 1: The effect of high CO<sub>2</sub> (5%) on *C. albicans* biofilm formation. (A)**

3 Biofilms were seeded and grown for 24h or 48h in 0.03% or 5% CO<sub>2</sub>, the resulting  
4 biofilms were quantified using the XTT assay with absorbance at 492nm as a  
5 readout. The graph represents three biological replicates each containing technical  
6 triplicates, error bars denote Standard Deviation. Paired two-tail t-tests were carried  
7 out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = not significant. **(B)** Representative images  
8 of *C. albicans* (SN250 strain) biofilms grown in 0.03% and 5% CO<sub>2</sub> for 48h.

9

10 **Figure 2: The effects of CO<sub>2</sub> on *C. albicans* biofilm growth (A) Attachment: *C.***

11 *albicans* CAI-4 cells were seeded onto silicone-coated microscope slides under  
12 0.03% or 5% CO<sub>2</sub> and images taken at 20x objective magnification. Cells per image  
13 were counted and the mean calculated across three biological replicates (five  
14 images per replicate). A paired two-tail t-test carried out: \*\*p<0.01. Error bars denote  
15 Standard Deviation. **(B) Maturation**: Biofilms were seeded on silicone-coated  
16 microscope slide and grown for 6h, 24h and 48h. Biofilms were stained with ConA-  
17 FITC (green) and FUN-1 (red). Z-stack Images were taken using 20x (6h) and 40x  
18 (24 and 48h) magnifications. Experiments were repeated in triplicate and  
19 representative maximum intensity images are presented as well as a z stack profiles.

20 **(C) Dispersion**: Spent media was collected from biofilms grown for 48h in 0.03% or  
21 5% CO<sub>2</sub> and diluted 1:10 before being plated to assess the number of colonies.  
22 Three biological replicates each containing technical triplicates were conducted,  
23 error bars denote Standard Deviation. A paired two-tail t-test was carried out:  
24 \*p<0.05.

1

2 **Figure 3: Biofilm growth assays of *C. albicans* Ras1-Cyr1-PKA pathway and**  
3 **central biofilm regulator null mutants.** Cells were seeded and grown as biofilms  
4 for 48h before XTT quantification. Control wells with no cells were included. **(A)**  
5 Graph represents three biological replicates each containing technical triplicates,  
6 error bars denote Standard Deviation. **(B)** Graph represents three biological  
7 replicates, error bars denote Standard Deviation. Two-way ANOVAs followed by  
8 Tukey tests for multiple comparisons were carried out: \* $p < 0.05$ , \*\* $p < 0.01$ ,  
9 \*\*\* $p < 0.001$ , n.s. = not significant. Stars directly above the bars indicate a significant  
10 difference to the wild type in the same CO<sub>2</sub> environment.

11

12 **Figure 4: The effect of high (5%) CO<sub>2</sub> on iron homeostasis in *C. albicans***  
13 **biofilms.** Biofilms were seeded and grown for 48h before XTT quantification. Control  
14 wells with no cells were set up as media controls. **(A)** Biofilm growth assay using  
15 TFKO mutants lacking iron homeostatic transcription factors. **(B)** Iron starvation  
16 biofilm growth assay of in the presence of the Fe<sup>2+</sup> chelator Ferrozine. **(C)** Iron  
17 starvation biofilm growth assay of clinical isolates in the presence of Ferrozine.  
18 Graph represents three biological replicates each containing technical triplicates,  
19 error bars denote Standard Deviation. Two-way ANOVAs followed by Tukey tests for  
20 multiple comparisons were carried out: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , n.s. = not  
21 significant.

22

23 **Figure 5: Global gene expression changes in 5% CO<sub>2</sub> vs. 0.03% CO<sub>2</sub> *C.***  
24 ***albicans* biofilms (A)** GSEA enrichment plots of central biofilm regulator gene sets

1 with altered expression levels as assessed by RNA Sequencing, four of the nine  
2 identified core regulators of biofilm formation (Brg1, Efg1, Ndt80, and Bcr1<sup>27</sup>) were  
3 identified as having positive GSEA scores. Vertical black lines represent individual  
4 genes in the significantly differentially expressed ranked gene list from upregulated  
5 (left) to downregulated (right). The enrichment score increases if there are lots of  
6 genes towards the beginning of the ranked list (upregulated). NES = normalised  
7 enrichment score, positive NES indicates enrichment in the upregulated group of  
8 genes. **(B)** Gene set cluster map showing the most upregulated and downregulated  
9 gene sets as determined by GSEA along with their cellular functions. Each circle is a  
10 gene set and the lines between them depict how much they overlap, thicker line =  
11 more genes in common.

12

13 **Figure 6: Adhesion and transport processes are upregulated in 5% CO<sub>2</sub> C.**  
14 ***albicans* biofilms (A)** GSEA enrichment plot of the BIOLOGICAL ADHESION\_BIO  
15 and TRANSPORTER ACTIVITY\_MOL, AMINO ACID TRANSPORT\_BIO and  
16 CARBOHYDRATE TRANSPORTER ACTIVITY\_MOL gene sets containing genes  
17 under the GO terms 'transporter activity' and 'amino acid transport' NES =  
18 normalised enrichment score, positive NES indicates enrichment in the upregulated  
19 group of genes. **(B)** Heat map of significantly differentially expressed genes  
20 associated with cell adhesion, amino acid transport and glucose transport as  
21 identified by GO Slim process analysis. The colours saturate at log<sub>2</sub> fold change of 2  
22 and -2.

23

1 **Figure 7: Antifungal sensitivity of *C. albicans* biofilms grown in high (5%) CO<sub>2</sub>.**  
2 **(A)** GSEA enrichment plot of the KETOCONAZOLE\_UP gene set containing genes  
3 upregulated in *C. albicans* cells grown in the presence of Ketoconazole<sup>61</sup>. NES =  
4 normalised enrichment score, positive NES indicates enrichment in the upregulated  
5 group of genes. **(B)** Heat map of genes associated with drug transport, including the  
6 multidrug efflux pump gene *MDR1*. **(C)** Biofilm growth assay of CAI4pSM2 in the  
7 presence of Fluconazole. **(D)** Biofilm growth assay of CAI4pSM2 in the presence of  
8 Nystatin. The relative XTT activity is presented with the 0.03% CO<sub>2</sub> biofilms being  
9 normalised to the 0.03% CO<sub>2</sub> untreated control and the 5% CO<sub>2</sub> biofilms being  
10 normalised to the 5% CO<sub>2</sub> untreated control. Two-way ANOVAs followed by Tukey  
11 tests for multiple comparisons were carried out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. =  
12 not significant. Stars directly above the bars indicate a significant difference to  
13 untreated in the same CO<sub>2</sub> environment.

14

15 **Figure 8: Efficacy of potential treatments to combat *C. albicans* biofilms grown**  
16 **in high (5%) CO<sub>2</sub>.** Biofilms were seeded and grown for 48h before XTT  
17 quantification. Control wells with no cells were set up as media controls to monitor  
18 for contamination. **(A)** Biofilm growth assay of SN250 in the presence of the Fe<sup>3+</sup>  
19 chelator Deferasirox. Graph represents two biological replicates each containing  
20 technical triplicates, error bars denote Standard Deviation. **(B)** Biofilm growth assay  
21 of SN250 in the presence of the glycolytic inhibitor 2-DG. Graph represents three  
22 biological replicates each containing technical triplicates, error bars denote Standard  
23 Deviation. Two-way ANOVAs followed by Tukey tests for multiple comparisons were  
24 carried out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = not significant. Stars directly above  
25 the bars indicate a significant difference to the untreated SN250 in the same CO<sub>2</sub>

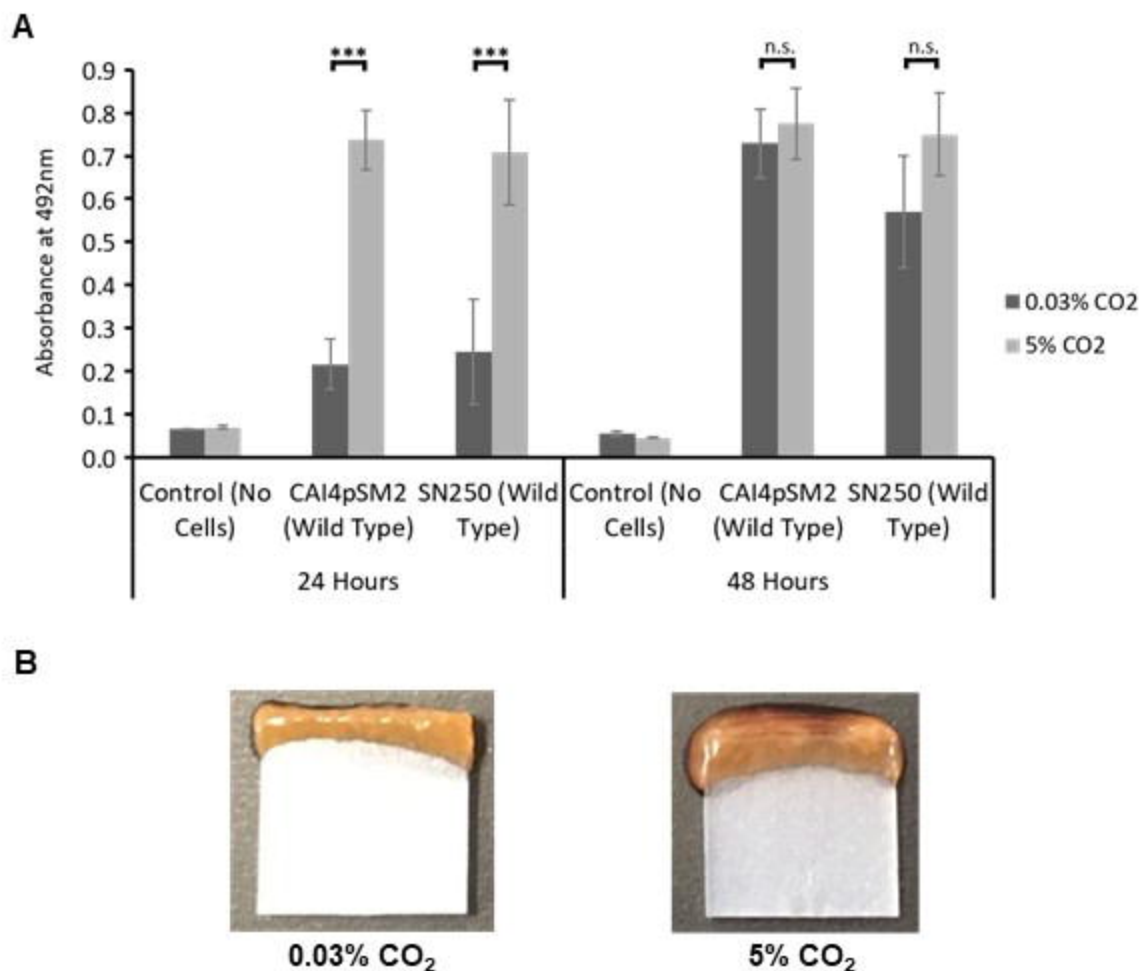


1 environment. **(C)** Representative images of SN250 biofilms grown in 5% CO<sub>2</sub> ±2-DG  
2 for 48h.

3

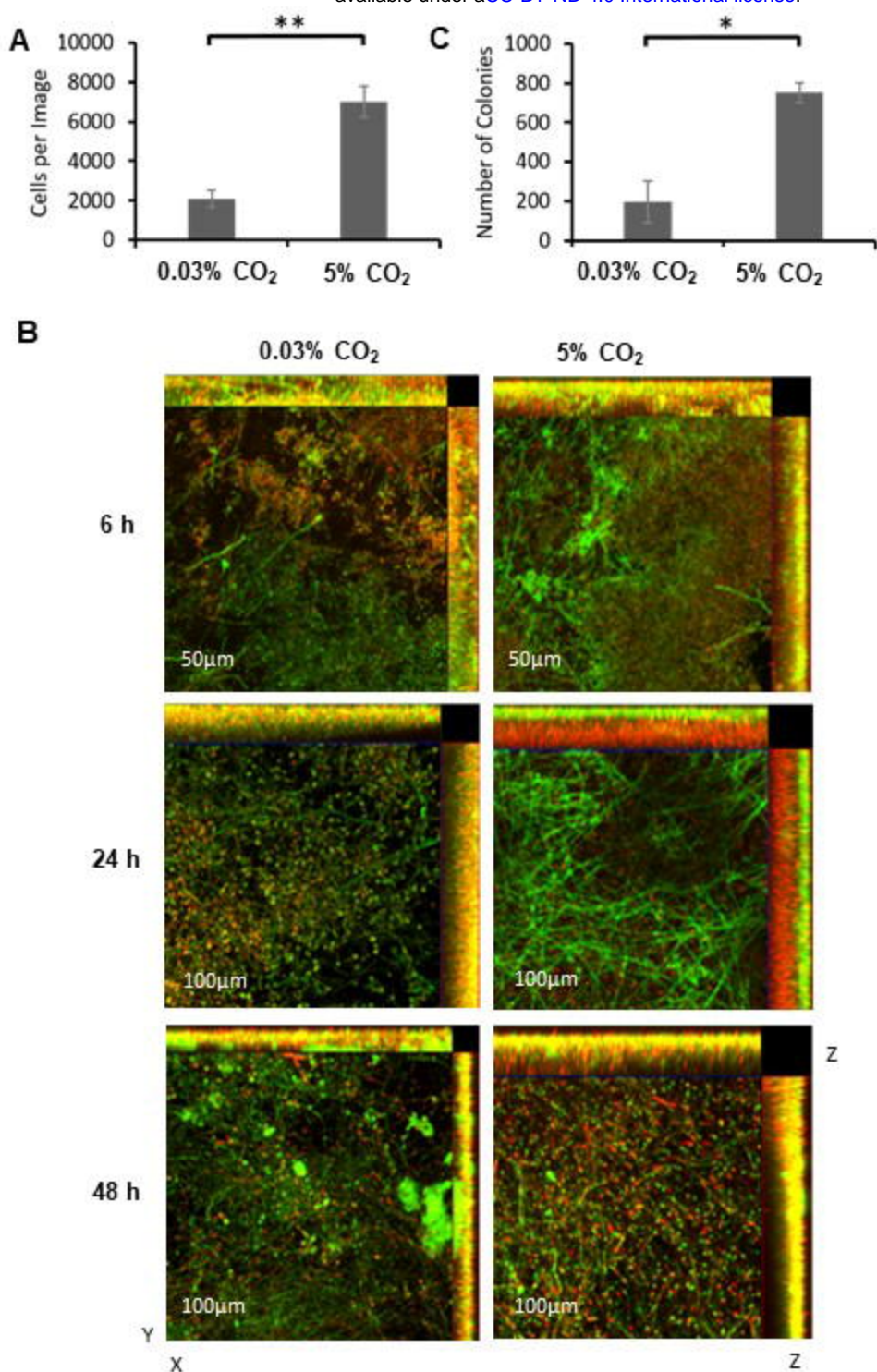
4 **Figure 9: Predicted model of the interplay between CO<sub>2</sub> signalling and iron**  
5 **homeostasis in *C. albicans* biofilms on silicone surfaces.** We hypothesise that  
6 the CO<sub>2</sub>-mediated activation of PKA via Cyr1 will result in increased activity of Sef1  
7 (via its phosphorylation and possibly the inhibition of Sfu1), thus increasing the  
8 expression of iron-uptake genes. PKA activation leads to Efg1 activation promoting  
9 the biofilm process.

**Figure 1**



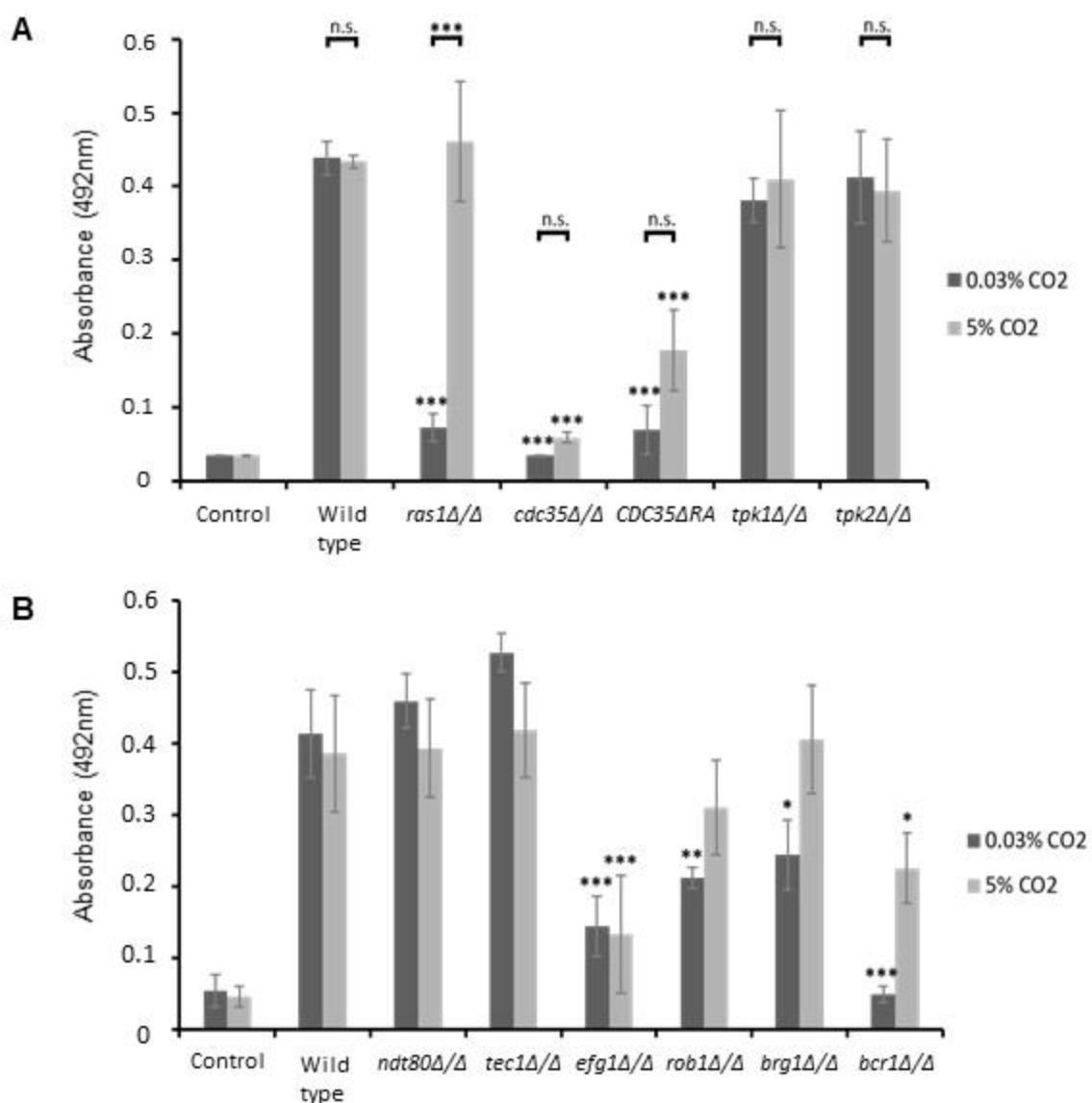
**Figure 1: The effect of high CO<sub>2</sub> (5%) on *C. albicans* biofilm formation. (A)** Biofilms were seeded and grown for 24h or 48h in 0.03% or 5% CO<sub>2</sub>, the resulting biofilms were quantified using the XTT assay with absorbance at 492nm as a readout. The graph represents three biological replicates each containing technical triplicates, error bars denote Standard Deviation. Paired two-tail t-tests were carried out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = not significant. **(B)** Representative images of *C. albicans* (SN250 strain) biofilms grown in 0.03% and 5% CO<sub>2</sub> for 48h.

Figure 2



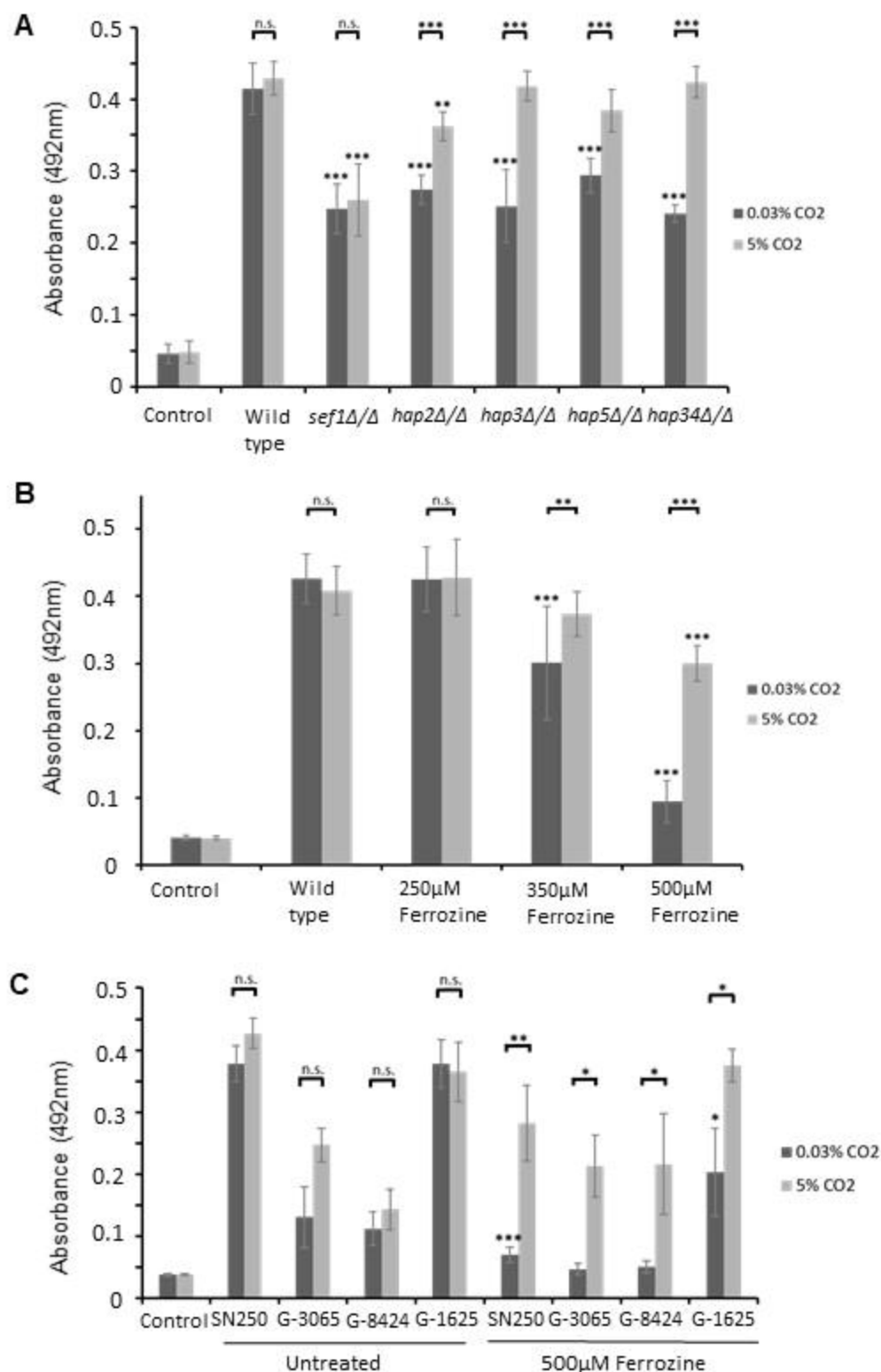
**Figure 2: The effects of CO<sub>2</sub> on *C. albicans* biofilm growth** (A) Attachment: *C. albicans* CAI-4 cells were seeded onto silicone-coated microscope slides under 0.03% or 5% CO<sub>2</sub> and images taken at 20x objective magnification. Cells per image were counted and the mean calculated across three biological replicates (five images per replicate). A paired two-tail t-test carried out: \*\*p<0.01. Error bars denote Standard Deviation. (B) Maturation: Biofilms were seeded on silicone-coated microscope slide and grown for 6h, 24h and 48h. Biofilms were stained with ConA-FITC (green) and FUN-1 (red). Z-stack Images were taken using 20x (6h) and 40x (24 and 48h) magnifications. Experiments were repeated in triplicate and representative maximum intensity images are presented as well as a z stack profiles. (C) Dispersion: Spent media was collected from biofilms grown for 48h in 0.03% or 5% CO<sub>2</sub> and diluted 1:10 before being plated to assess the number of colonies. Three biological replicates each containing technical triplicates were conducted, error bars denote Standard Deviation. A paired two-tail t-test was carried out: \*p<0.05.

Figure 3



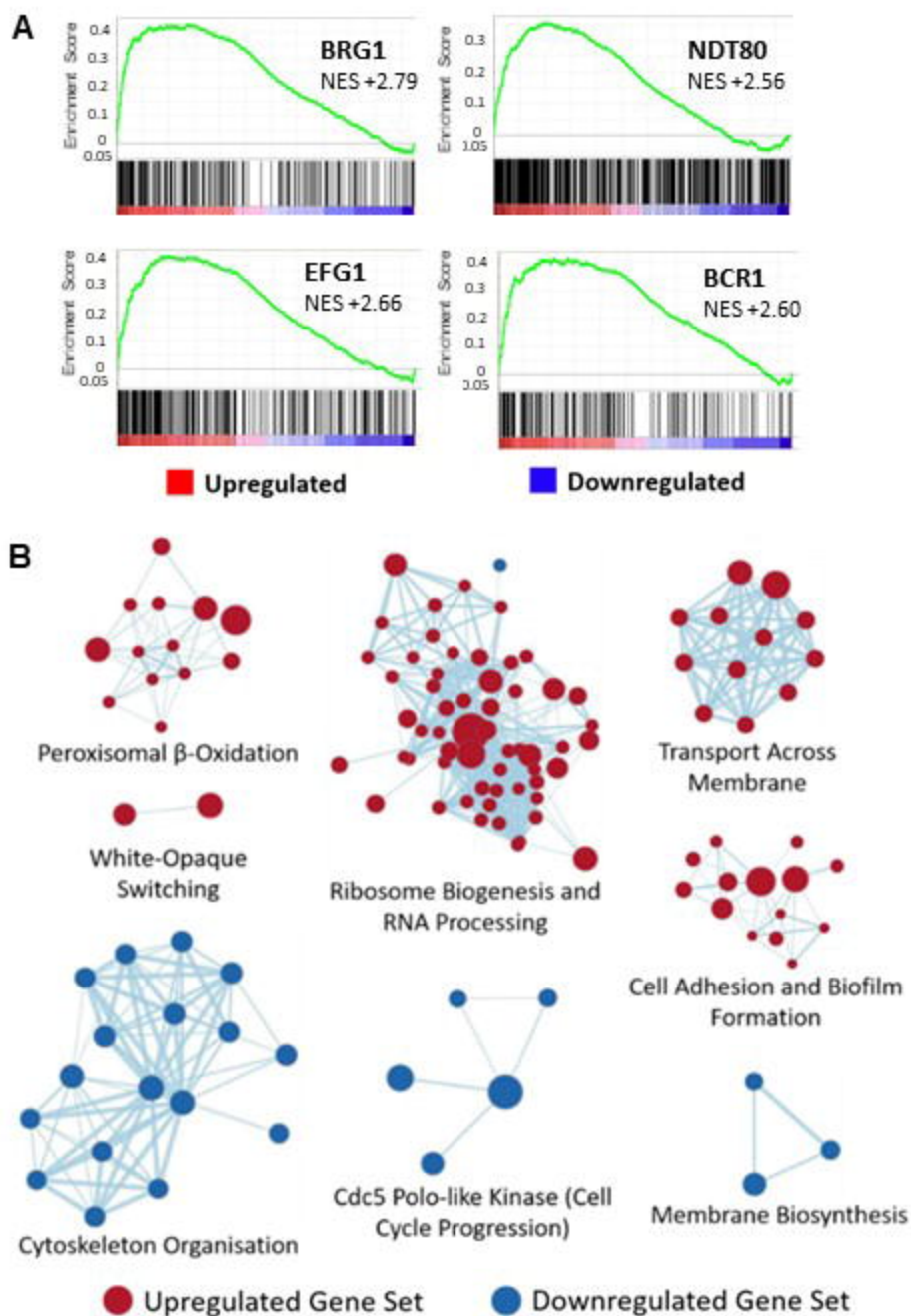
**Figure 3: Biofilm growth assays of *C. albicans* Ras1-Cyr1-PKA pathway and central biofilm regulator null mutants.** Cells were seeded and grown as biofilms for 48h before XTT quantification. Control wells with no cells were included. **(A)** Graph represents three biological replicates each containing technical triplicates, error bars denote Standard Deviation. **(B)** Graph represents three biological replicates, error bars denote Standard Deviation. Two-way ANOVAs followed by Tukey tests for multiple comparisons were carried out: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , n.s. = not significant. Stars directly above the bars indicate a significant difference to the wild type in the same CO<sub>2</sub> environment.

**Figure 4**



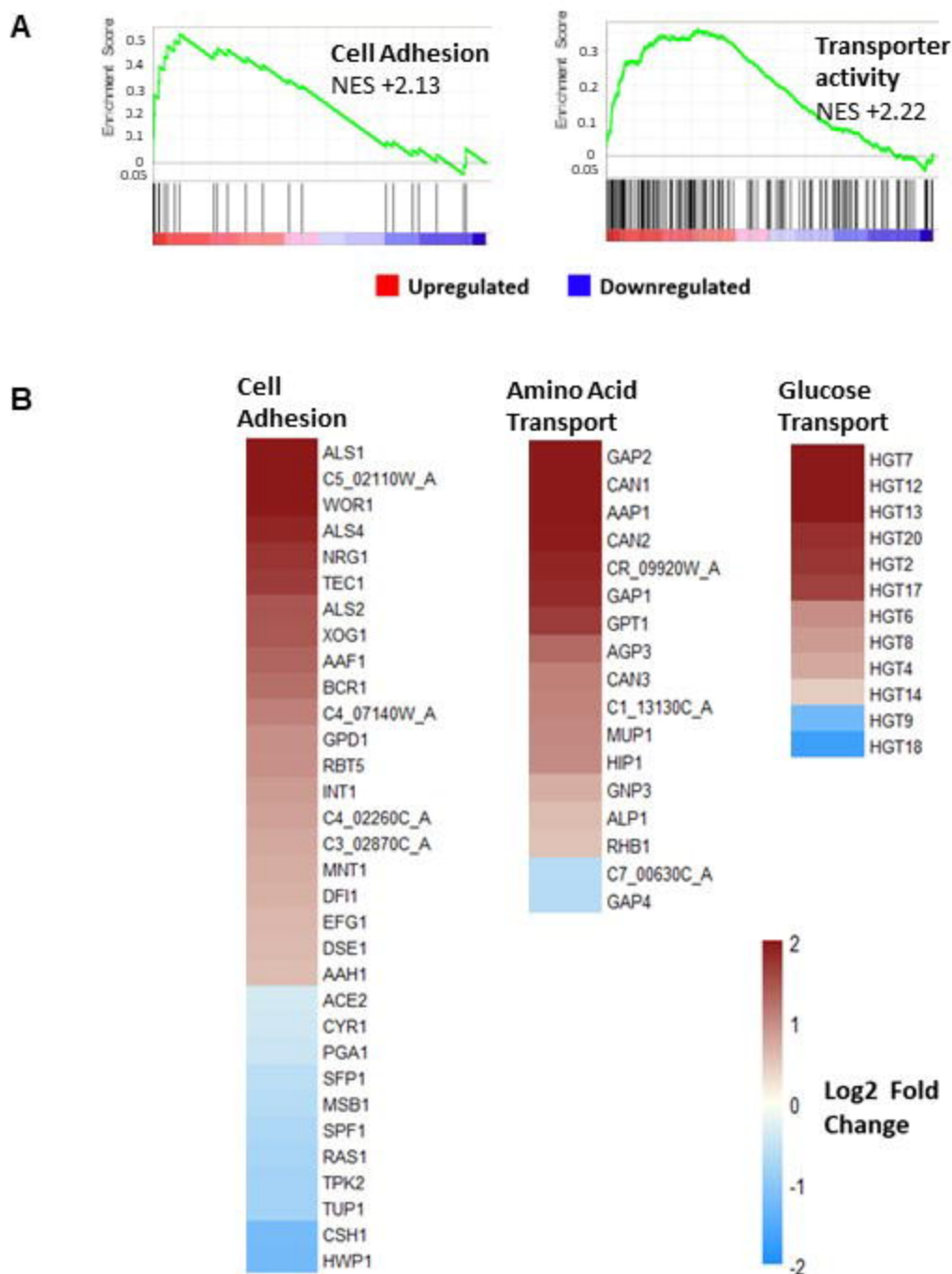
**Figure 4: The effect of high (5%) CO<sub>2</sub> on iron homeostasis in *C. albicans* biofilms.** Biofilms were seeded and grown for 48h before XTT quantification. Control wells with no cells were set up as media controls. **(A)** Biofilm growth assay using TFKO mutants lacking iron homeostatic transcription factors. **(B)** Iron starvation biofilm growth assay of in the presence of the Fe<sup>2+</sup> chelator Ferrozine. **(C)** Iron starvation biofilm growth assay of clinical isolates in the presence of Ferrozine. Graph represents three biological replicates each containing technical triplicates, error bars denote Standard Deviation. Two-way ANOVAs followed by Tukey tests for multiple comparisons were carried out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = not significant.

Figure 5



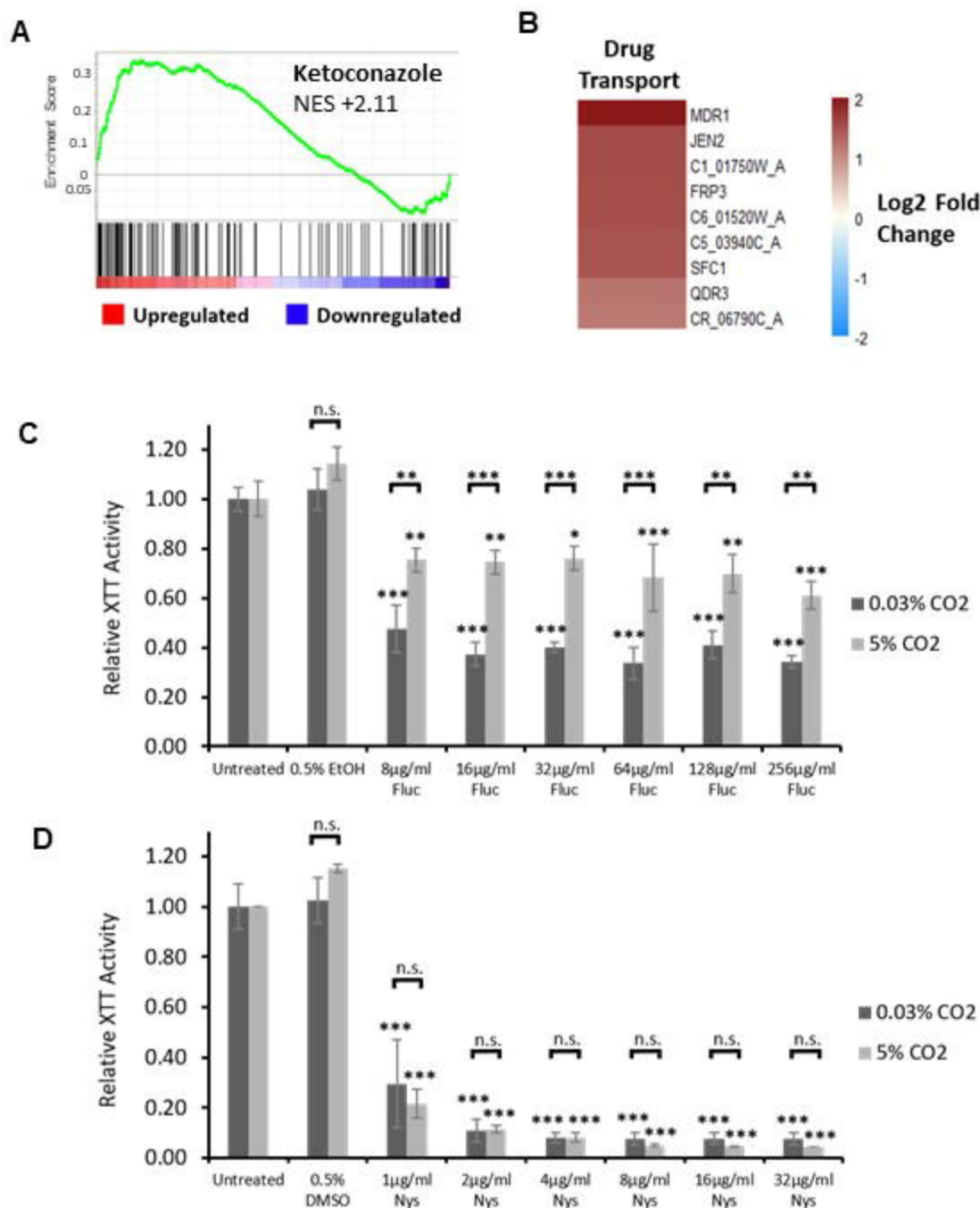
**Figure 5: Global gene expression changes in 5% CO<sub>2</sub> vs. 0.03% CO<sub>2</sub> *C. albicans* biofilms (A)** GSEA enrichment plots of central biofilm regulator gene sets with altered expression levels as assessed by RNA Sequencing, four of the nine identified core regulators of biofilm formation (Brg1, Efg1, Ndt80, and Bcr1 [27]) were identified as having positive GSEA scores. Vertical black lines represent individual genes in the significantly differentially expressed ranked gene list from upregulated (left) to downregulated (right). The enrichment score increases if there are lots of genes towards the beginning of the ranked list (upregulated). NES = normalised enrichment score, positive NES indicates enrichment in the upregulated group of genes. **(B)** Gene set cluster map showing the most upregulated and downregulated gene sets as determined by GSEA along with their cellular functions. Each circle is a gene set and the lines between them depict how much they overlap, thicker line = more genes in common.

**Figure 6**



**Figure 6: Adhesion and transport processes are upregulated in 5% CO<sub>2</sub> *C. albicans* biofilms (A)** GSEA enrichment plot of the BIOLOGICAL ADHESION\_BIO and TRANSPORTER ACTIVITY\_MOL, AMINO ACID TRANSPORT\_BIO and CARBOHYDRATE TRANSPORTER ACTIVITY\_MOL gene sets containing genes under the GO terms ‘transporter activity’ and ‘amino acid transport’ NES = normalised enrichment score, positive NES indicates enrichment in the upregulated group of genes. **(B)** Heat map of significantly differentially expressed genes associated with cell adhesion, amino acid transport and glucose transport as identified by GO Slim process analysis. The colours saturate at log<sub>2</sub> fold change of 2 and -2.

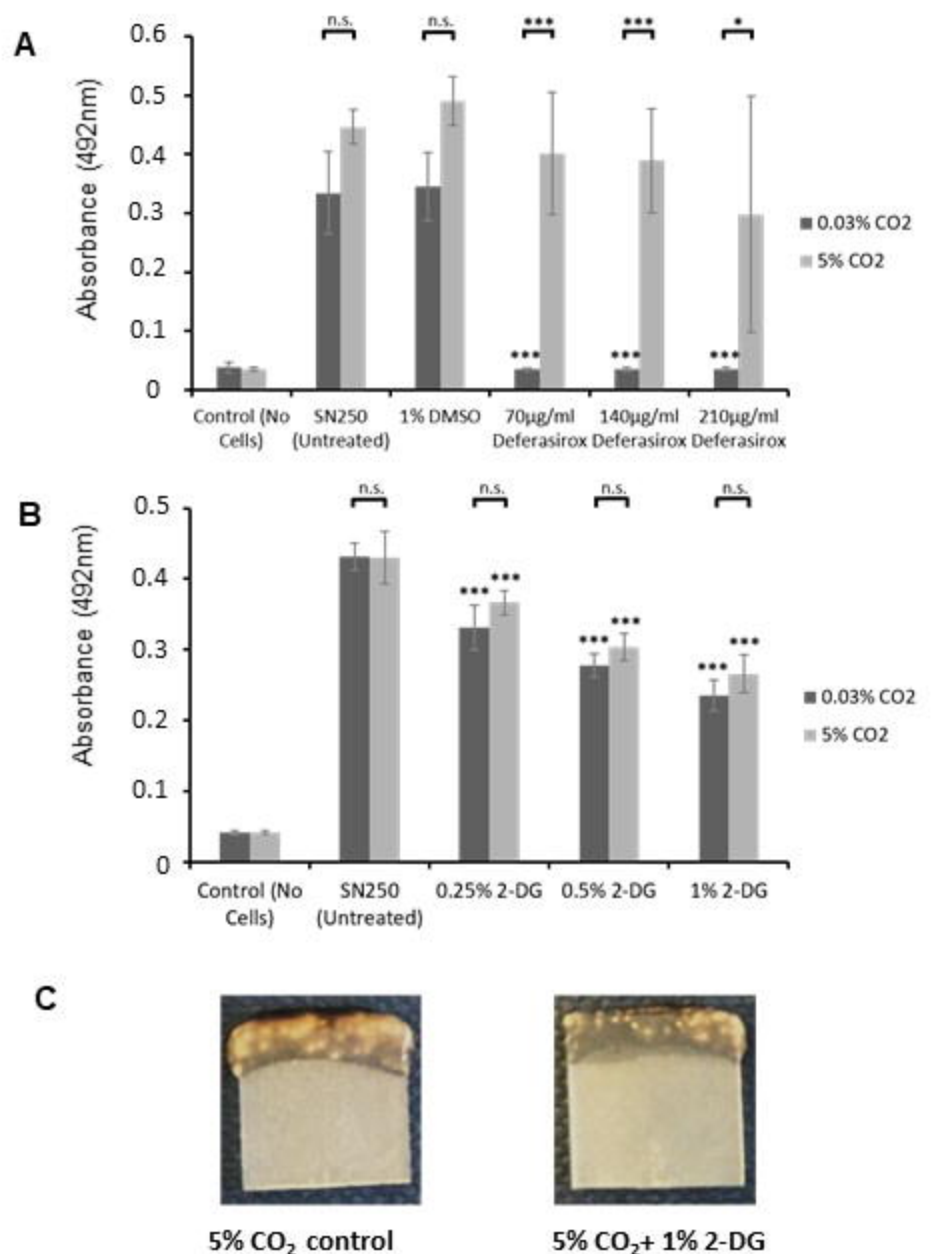
Figure 7



**Figure 7: Antifungal sensitivity of *C. albicans* biofilms grown in high (5%) CO<sub>2</sub>.** (A) GSEA enrichment plot of the KETOCONAZOLE\_UP gene set containing genes upregulated in *C. albicans* cells grown in the presence of Ketoconazole [62]. NES = normalised enrichment score, positive NES indicates enrichment in the upregulated group of genes. (B) Heat map of genes associated with drug transport, including the multidrug efflux pump gene *MDR1*. (C) Biofilm growth assay of CAI4pSM2 in the presence of Fluconazole. (D) Biofilm growth assay of CAI4pSM2 in the presence of Nystatin. The relative XTT activity is presented with the 0.03% CO<sub>2</sub> biofilms being normalised to the 0.03% CO<sub>2</sub> untreated control and the 5% CO<sub>2</sub> biofilms being normalised to the 5% CO<sub>2</sub> untreated control. Two-way ANOVAs followed by Tukey tests for multiple comparisons were carried out: \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, n.s. = not significant. Stars directly above the bars indicate a significant difference to untreated in the same CO<sub>2</sub> environment.

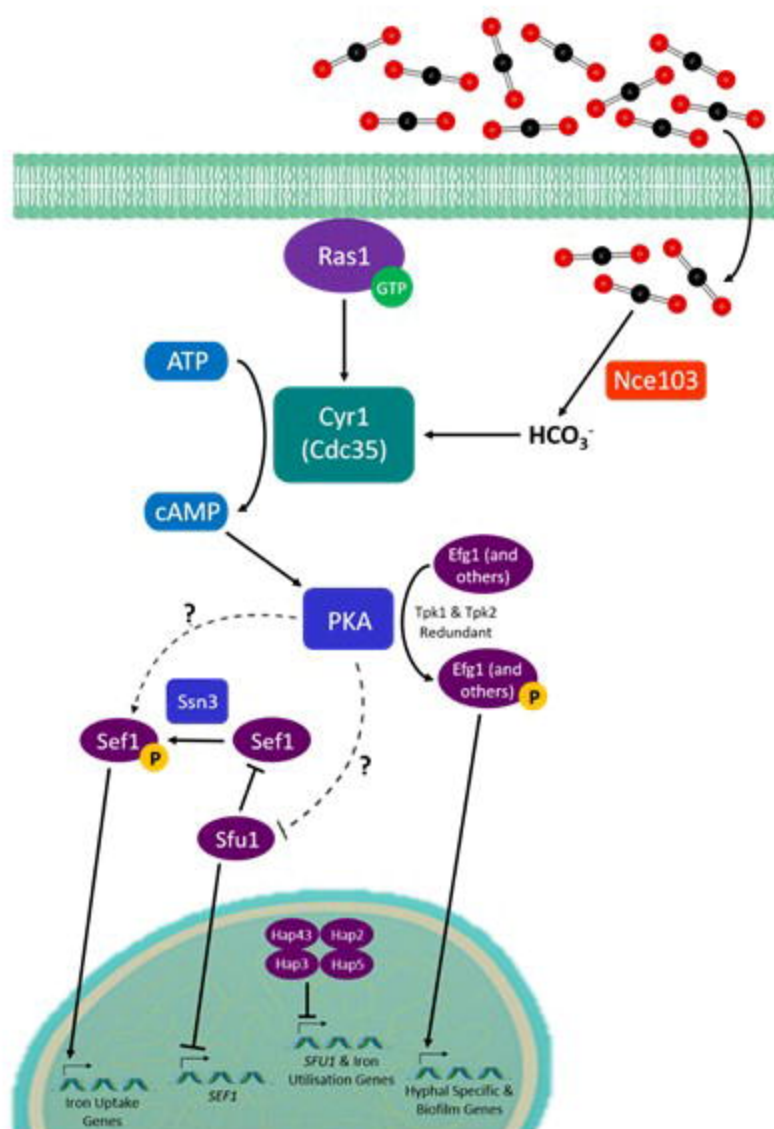


**Figure 8**



**Figure 8: Efficacy of potential treatments to combat *C. albicans* biofilms grown in high (5%) CO<sub>2</sub>.** Biofilms were seeded and grown for 48h before XTT quantification. Control wells with no cells were set up as media controls to monitor for contamination. **(A)** Biofilm growth assay of SN250 in the presence of the Fe<sup>3+</sup> chelator Deferasirox. Graph represents two biological replicates each containing technical triplicates, error bars denote Standard Deviation. **(B)** Biofilm growth assay of SN250 in the presence of the glycolytic inhibitor 2-DG. Graph represents three biological replicates each containing technical triplicates, error bars denote Standard Deviation. Two-way ANOVAs followed by Tukey tests for multiple comparisons were carried out: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n.s. = not significant. Stars directly above the bars indicate a significant difference to the untreated SN250 in the same CO<sub>2</sub> environment. **(C)** Representative images of SN250 biofilms grown in 5% CO<sub>2</sub> ±2-DG for 48h.

Figure 9



**Figure 9: Predicted model of the interplay between CO<sub>2</sub> signalling and iron homeostasis in *C. albicans* biofilms on silicone surfaces.** We hypothesise that the CO<sub>2</sub>-mediated activation of PKA via Cyr1 will result in increased activity of Sef1 (via its phosphorylation and possibly the inhibition of Sfu1), thus increasing the expression of iron-uptake genes. PKA activation leads to Efg1 activation promoting the biofilm process.