Oestrogen promotes innate immune evasion of *Candida albicans* through inactivation of the alternative complement system

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Abstract

Gender is a risk factor for several infections that, for many pathogens, has been linked to sex hormones impacting host immunity and directly affecting microbial virulence. *Candida albicans* is a commensal of the urogenital tract and the predominant cause of vulvovaginal candidiasis (VVC). Factors that increase circulatory oestrogen levels like pregnancy, the use of oral contraceptives, and hormone replacement therapy predispose women to VVC, but the reasons for this are largely unknown. Here, we investigate how adaptation of *C. albicans* to oestrogen impacts the fungal host-pathogen. Physiologically relevant concentrations of oestrogen promoted fungal virulence by enabling *C. albicans* to avoid the actions of the innate immune system. Oestrogen-induced innate immune evasion was mediated via inhibition of opsonophagocytosis through enhanced acquisition of Factor H on the fungal cell surface and was dependent on the *C. albicans* moonlighting protein Gpd2. Oestrogen dependent derepression of *GPD2* was mediated via a non-canonical signalling pathway involving Ebp1 and Bcr1. Therefore, we propose that, in addition to affecting the antifungal potential of vaginal epithelial cells, elevated oestrogen levels predispose women to VVC by directly enhancing fungal pathogenicity. The discovery of this new hormone sensing pathway might pave the way in explaining gender biases associated with fungal infections and may provide an alternative approach to improving women's health.

Key words: *Candida albicans*, innate immune evasion, Factor H, hormone sensing, Gpd2

Introduction

Microbial infections exhibiting gender bias are common. This bias may predispose one sex to infection over the other, or result in one sex exhibiting severer infection outcomes [1-3]. Sex hormones like oestrogen, testosterone and progesterone regulate many functions of the immune system, and generally males are more prone to infection than females, as overall immune responses are lower in the male population [4-6]. Besides the impact of sex hormones on immune cell function, sex hormones also have a direct effect on microbial pathogenicity increasing microbial persistence, metabolism and virulence gene expression [7, 8].

The opportunistic fungal pathogen *Candida albicans* is the predominant fungal coloniser of the female reproductive tract and the major cause of genital thrush (vulvovaginal candidiasis, VVC). Approximately 75% of the female population will encounter at least one episode of VVC in their life-time, while up to 15% experience recurrent infection (RVVC) defined as four or more episodes in a twelve-month period [9]. Although not life threatening, mucosal infections are expensive to treat, impact quality of life, and increase population morbidity.

One of the major risk factors associated with the development of VVC is elevated levels of oestrogen which occur as a result of pregnancy, the use of high oestrogen-containing oral contraceptives and hormone replacement therapy [10, 11]. Therefore, oestrogen plays a key role in predisposing women to VVC, but the precise mechanism(s) for this are unknown. Oestrogen promotes glycogen production at the vaginal mucosa which might provide a nutrient rich environment to promote the expansion of *C. albicans* [10]. In mouse models of VVC, were pseudoestus is induced to maintain fungal colonisation, vaginal epithelial cells have a diminished ability to control the growth of *C. albicans* [12]. In addition, oestrogen decreases the infiltration of phagocytes and suppresses cell mediated immunity [13]. However, in rats, preincubation of *C. albicans* with oestrogen prior to vaginal infection enhances fungal survival [14], suggesting that in addition to affecting host immunity, oestrogen

77 may directly affect the virulence of *C. albicans*. In agreement with this idea, oestrogen has
78 been shown to promote hyphal morphogenesis of *C. albicans* [15], a key virulence factor of
79 the fungus. Furthermore, an oestrogen binding protein (Ebp1) has been identified in *C.*
80 *albicans* [16], although the importance of this protein in VVC is not known. Therefore, how *C.*
81 *albicans* adapts to oestrogen is still unclear.
82
83 The *C. albicans* cell wall is a multi-layered structure consisting of an inner layer of chitin and
84 beta-glucan, and an outer layer of heavily glycosylated proteins [17]. The fungal cell wall is a
85 highly dynamic structure providing rigidity, strength and protection from the environment. In
86 addition, many components of the cell wall act as pathogen associated molecular patterns
87 (PAMPs) and are recognised by the innate immune system [17, 18]. Recently, remodelling of
88 the cell wall in response to adaptation to host environments has been shown to regulate the
89 host-pathogen interaction [19-30]. In addition to direct recognition of cell wall PAMPs
90 mediating phagocytosis, *C. albicans* also activates the alternative complement system,
91 resulting in the deposition of complement proteins (i.e. C3) on the fungal cell surface, resulting
92 in opsonophagocytosis [31]. However, *C. albicans* can evade opsonophagocytosis through
93 the binding of complement regulatory proteins to its cell surface that inactivate the complement
94 cascade [32]. Here we show that *C. albicans* does adapt to oestrogen, and that this adaptation
95 perturbs the host-pathogen interaction, inhibiting phagocytosis of the fungal pathogen.
96 Avoidance of the innate immune system was mediated via the fungal cell surface protein,
97 Gpd2, recruiting the human complement regulatory protein, Factor H, to the fungal cell surface
98 inactivating the alternative complement system.

99

100 ## Results

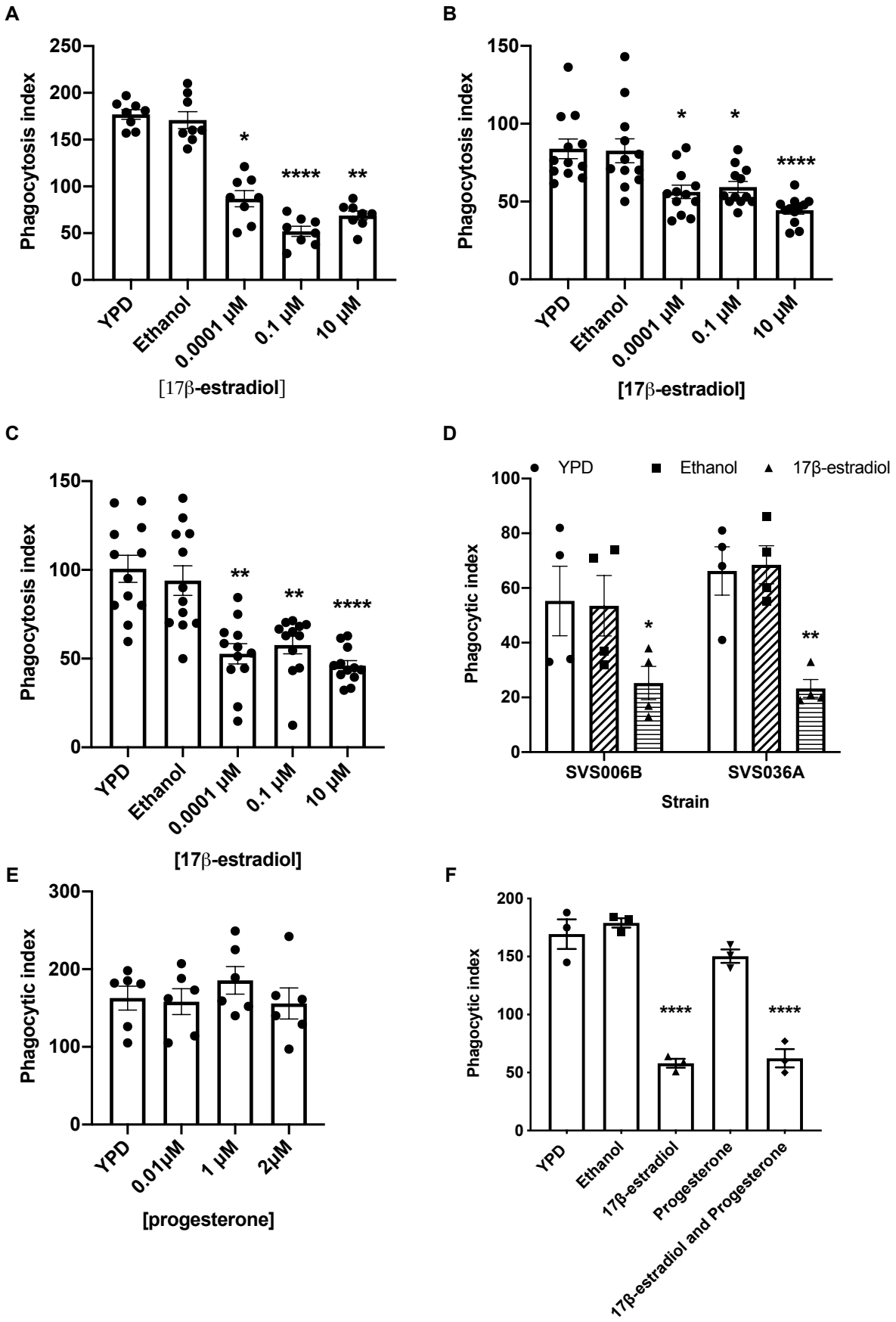
101 **Adaptation of *C. albicans* to oestrogen promotes immune evasion**

102 Pregnant women and women taking high oestrogen-containing oral contraceptives are more
103 prone to vulvovaginal candidiasis (VVC) [33], while VVC occurs less frequently in

104 postmenopausal women, indicating that oestrogen may play a role in promoting the virulence
105 of *C. albicans*. There are four main forms of oestrogen, estrone (E1), 17β-estradiol (E2), estriol
106 (E3) and 17α-ethynylestradiol (EE2). Estrone is the weakest form of oestrogen produced by
107 the ovaries and adipose tissue, and is only found in menopausal women, while 17β-estradiol
108 is the strongest form of oestrogen produced by the ovaries and has been associated with
109 many gynaecological disorders. Estriol is a by-product from the metabolism of estradiol, and
110 as such is found in high concentrations during pregnancy. Finally, 17α-ethynylestradiol is a
111 synthetic oestrogen used in oral contraceptive pills. To ascertain whether adaptation of *C.*
112 *albicans* to oestrogen affects the host-pathogen interaction, *C. albicans* cells were grown in
113 the presence of physiological (0.0001 μM) and super-physiological (0.1 μM, 10 μM)
114 concentrations of 17β-estradiol, estriol, or 17α-ethynylestradiol, and phagocytosis rates
115 quantified. Physiological and super-physiological concentrations of all three forms of
116 oestrogen significantly inhibited macrophage and neutrophil phagocytosis of *C. albicans,*
117 resulting in a 50% drop in phagocytosis compared to the ethanol vehicle control (Fig 1A-C, S1
118 Fig A, B). The reduced rates of *C. albicans* phagocytosis were independent of any impact of
119 oestrogen on fungal growth (S1 Fig C) or morphology (S1 Fig D). Given that all tested forms
120 of oestrogen elicited similar results, all subsequent experiments were performed only with
121 17β-estradiol.
122
123 To determine whether the observed inhibition of phagocytosis is truly mediated by adaptation
124 of *C. albicans* to oestrogen, and not a result of residual oestrogen coating the surface of *C.*
125 *albicans* inhibiting phagocyte function, macrophages were pre-treated with oestrogen prior to
126 the addition of *C. albicans* or latex beads. Macrophages pre-incubated with oestrogen
127 phagocytosed *C. albicans* at rates comparable to non-treated cells (S1 Fig E), suggesting that
128 under the tested conditions oestrogen does not directly impact the ability of macrophages to
129 phagocytose target particles. Pre-incubating inert particles like latex beads with oestrogen had
130 no effect on the ability of macrophages to phagocytose the particles (S1 Fig F), suggesting
131 that any residual oestrogen on the surface of particles does not interfere with the ability of

macrophages to phagocytose them. In agreement with this, incubation of *C. albicans* with oestrogen on ice, which inhibits fungal growth and metabolism, did not affect phagocytosis rates (S1 Fig G). Furthermore, re-inoculation of oestrogen-adapted *C. albicans* cells into fresh YPD media restored *C. albicans* phagocytosis rates (S1 Fig H). Therefore, in response to oestrogen, *C. albicans* undergoes some form of adaptation that alters the host-pathogen interaction.

Finally, we tested several vaginal *C. albicans* isolates and observed the all isolates displayed reduced phagocytosis after adaptation to oestrogen, confirming that oestrogen-induced immune evasion is a general trait of clinically relevant *C. albicans* (Fig 1D). Taking this data together we conclude that the reduction in *C. albicans* phagocytosis is due to the fungus reversibly adapting to the oestrogen, and that this adaptation interferes with the host mechanism of fungal clearance.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Phagocytosis index
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YPD
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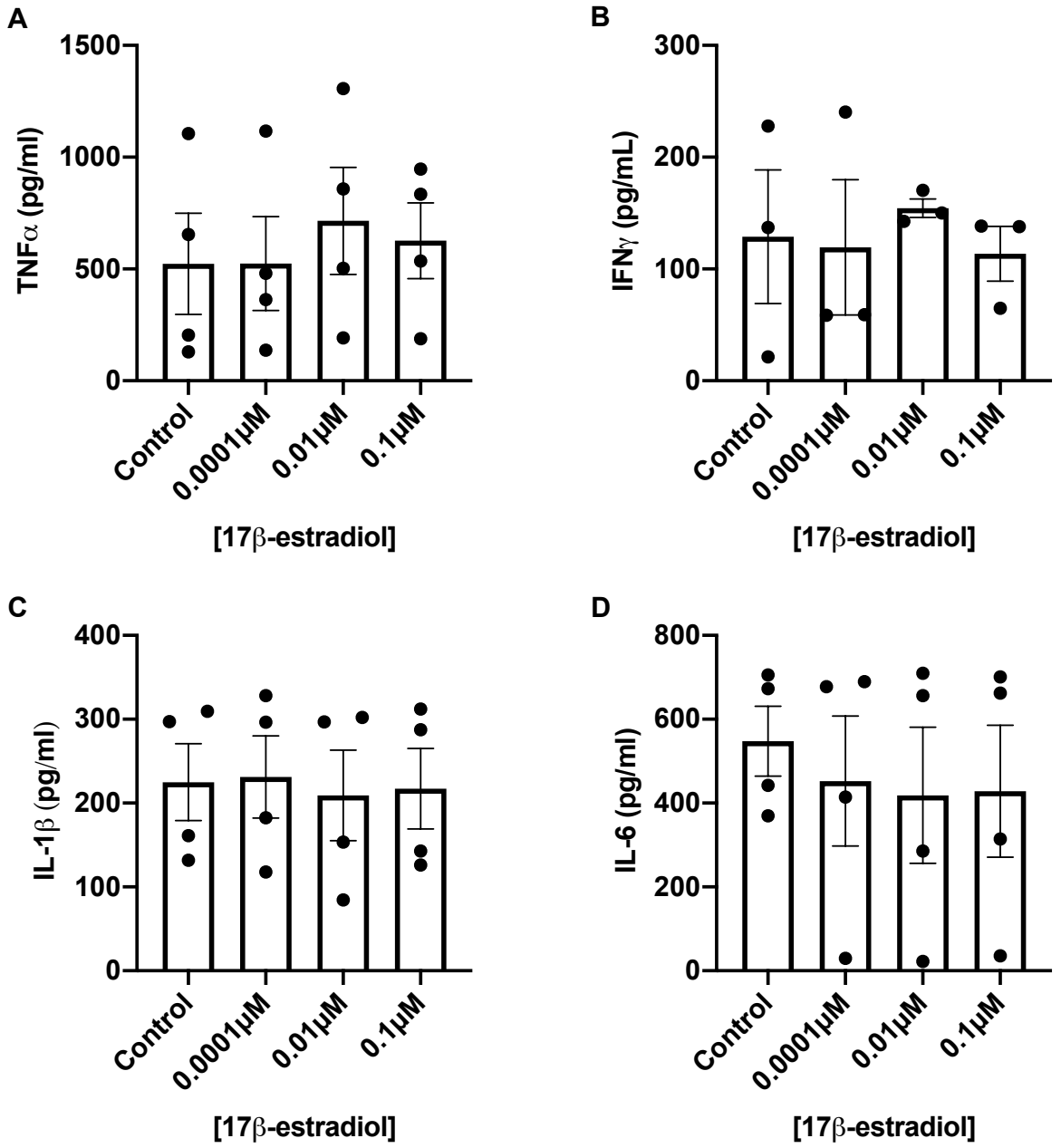
146

147 **Fig 1. Oestrogen promotes innate immune evasion of *C. albicans*** *C. albicans* cells were
148 grown in YPD with or without oestrogen supplementation. Cells were harvested, washed in
149 PBS and co-incubated with (**A**) J774A.1 cells (**B**) human macrophages (**C**), human
150 neutrophils. (**D**) J774A.1 phagocytosis rates of *C. albicans* clinical isolates after exposure to
151 10 μM 17β-estradiol. **(E)** J774A.1 phagocytosis rates of *C. albicans* clinical isolates after
152 exposure to progesterone **(F)** J774A.1 phagocytosis rates of *C. albicans* clinical isolates after
153 exposure to progesterone and oestrogen. Cells were fixed with 4% PFA, imaged by
154 microscopy, scored using ImageJ software and phagocytic index determined. All data
155 represent the mean ± SEM from at least three independent experiments. (*$p < 0.05$, **$p <$
156 0.01, ****$p < 0.0001$).
157
158 **Hormone induced innate immune evasion is specific to Oestrogen**
159 Progesterone been shown to affect phagocytosis rates [34], and is structurally quite similar to
160 oestrogen. Therefore, we hypothesised that *C. albicans* may adapt to progesterone in a similar
161 way to oestrogen to evade the innate immune system. *C. albicans* cells pre-exposed to
162 physiological and super-physiological concentrations of progesterone exhibited comparable
163 phagocytosis rates as *C. albicans* cells grown in YPD (Fig 1E), while *C. albicans* treated with
164 both oestrogen and progesterone still exhibited innate immune evasion (Fig 1F). Therefore,
165 the promotion of innate immune evasion appears to be a specific and dominant attribute of
166 oestrogen.
167
168 **Altered phagocytosis rates does not affect pro-inflammatory cytokine production.**
169 Previous studies have shown that oestrogen can interfere with NF-κB signalling to attenuate
170 inflammatory responses [35, 36]. Therefore, we investigated whether adaptation of *C. albicans*
171 to oestrogen dampens the pro-inflammatory innate immune response. Human peripheral
172 blood monocytes (PBMCs) were exposed to oestrogen-adapted *C. albicans* cells and
173 secretion of TNFα, IFN-γ, IL-6 and IL-1β was evaluated. Adaptation to oestrogen did not affect
174 the cytokine response (Fig 2). Therefore, although significantly fewer oestrogen-adapted cells

175 are phagocytosed by the innate immune system, the extracellular *C. albicans* cells still
176 stimulate significant cytokine production, likely through non-phagocytic receptors like TLR4.
177



178

179 **Fig 2. Adaptation of *C. albicans* to oestrogen does not affect cytokine secretion.** *C.*
180 *albicans* cells were grown in YPD with or without 10 μM 17β-estradiol for 4 h. Cells were
181 washed, fixed with 4% PFA and co-incubated with PBMCs for 24h. Secretion of (**A**) TNFα, (**B**)
182 IFNγ (**C**) IL-1β and (**D**) IL-16 by PBMCs was quantified by ELISA. Data represent the mean ±
183 SEM from four independent experiments using different donors.

184

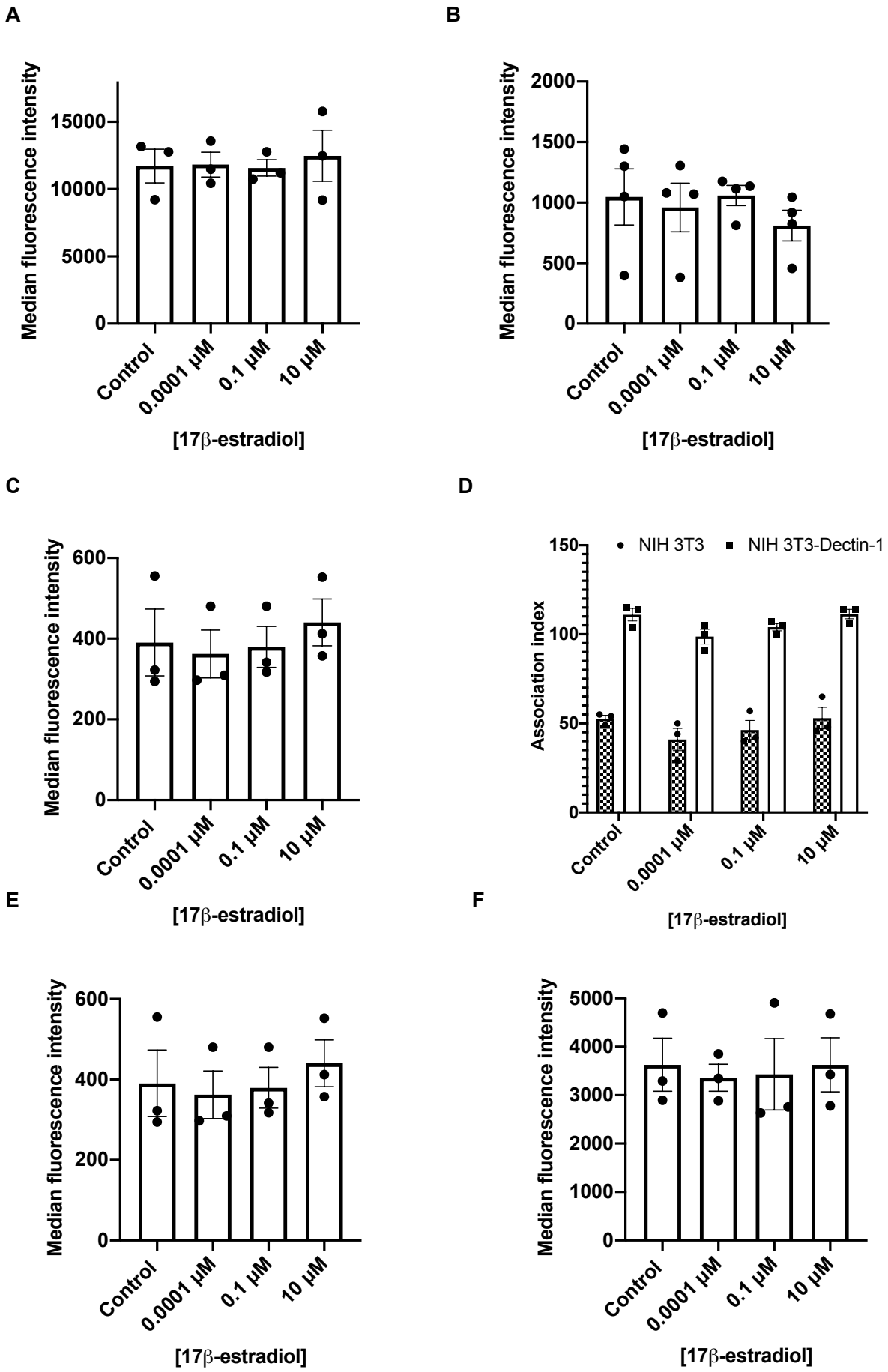
185 **Oestrogen dependent innate immune evasion is not dependent on fungal carbohydrate**
186 **cell wall remodelling.**

187 Recently, modulation of the *C. albicans* cell wall carbohydrates has been shown to influence
188 the host-pathogen interaction [19-30]. Therefore, the ability of oestrogen to promote
189 remodelling of the fungal cell wall was quantified by flow cytometry. However, initial studies
190 confirmed that adaptation of *C. albicans* to oestrogen does not result in increased
191 incorporation of mannan, β-glucan or chitin into the cell wall (Fig 3A-C).

192

193 Dectin-1 is a phagocytic receptor important for controlling fungal infections through the
194 recognition of surface exposed beta-glucan [37]. Host environmental cues that reduce *C.*
195 *albicans* phagocytosis do so through masking surface exposed beta-glucan inhibiting Dectin-
196 1 dependent recognition [21-23, 30]. To ascertain whether the observed reduction in
197 phagocytosis was dependent on Dectin-1, the attachment of oestrogen-adapted *C. albicans*
198 cells to Dectin-1 expressing fibroblasts was quantified. Dectin-1 recognised and bound
199 oestrogen-adapted cells at rates comparable to control cells, suggesting that the altered
200 phagocytosis rates are not due to reduced Dectin-1 recognition (Fig 3D). In agreement with
201 this, adaptation of *C. albicans* to oestrogen did not affect the surface exposure of either β-
202 glucan or chitin (Fig 3E, F). Taken together, these data indicate that oestrogen does not affect
203 the overall structure of the *C. albicans* cell wall, and that the oestrogen-dependent reduction
204 in phagocytosis is not dependent on fungal carbohydrate cell wall remodelling.

205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Control
0.0001 μM
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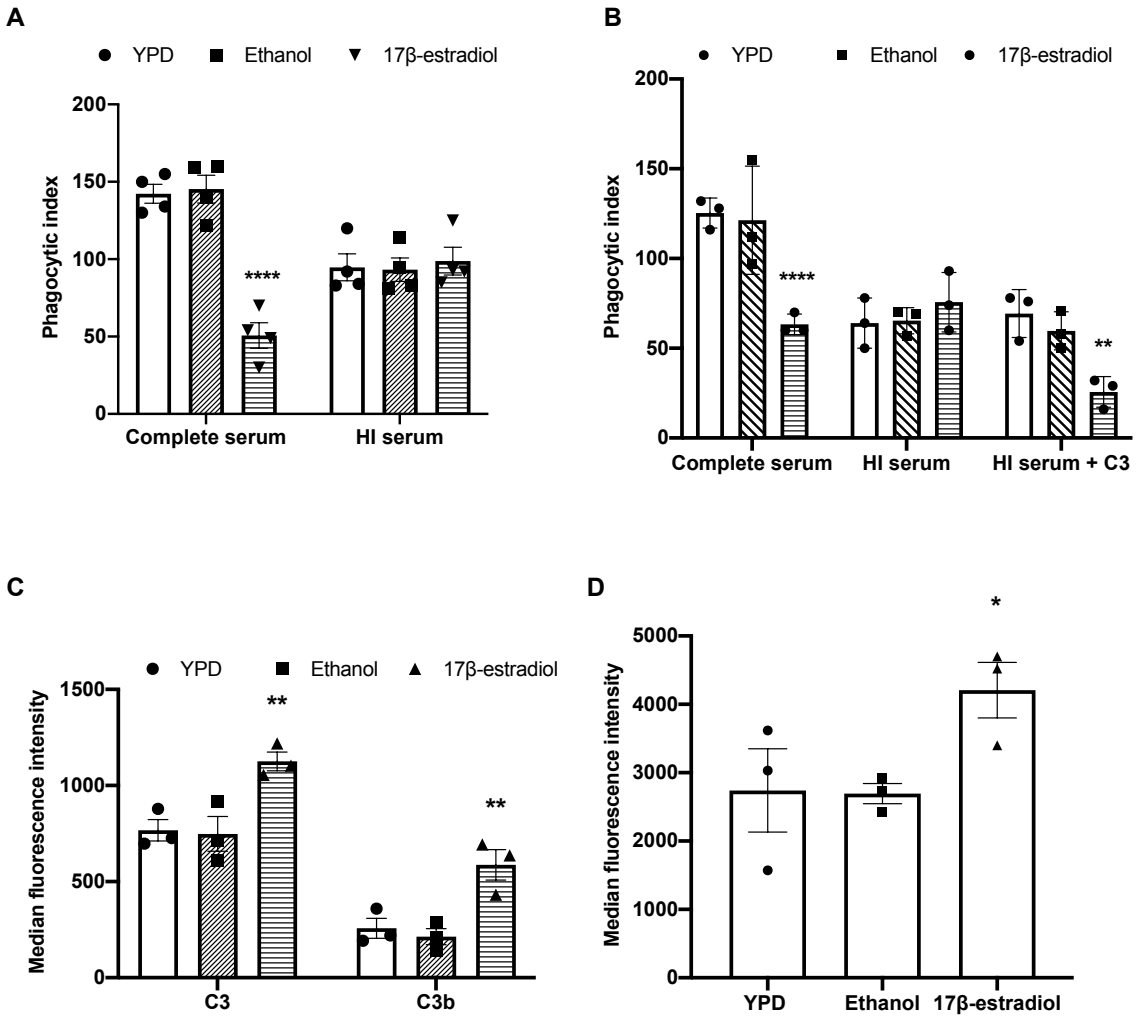
10 μM
[17β-estradiol]

206

207 **Fig 3. Reduced phagocytosis rates are not due to gross changes in cell wall**
208 **carbohydrates.** *C. albicans* cells were grown in YPD with or without 10 μM 17β-estradiol.
209 Cells were harvested, washed in PBS, fixed with 4% PFA and stained for (**A**) total mannan
210 (**B**) total glucan (**C**) total chitin. (**D**) To assess the role of Dectin-1 in *C. albicans* phagocytosis,
211 Dectin-1 was expressed in NIH 3T3 fibroblasts. *C. albicans* cells were stained for (**E**) exposed
212 β1,3-glucan and (**F**) exposed chitin levels. Staining was quantified by flow cytometry and
213 median fluorescence intensities (MFI) determined. All data represent the mean ± SEM from
214 at least three independent experiments.
215
216 **Oestrogen-induced innate immune evasion is mediated via inhibition of**
217 **opsonophagocytosis.**
218 In addition to direct detection of cell wall PAMPs leading to phagocytosis, *C. albicans* can be
219 opsonised. *C. albicans* activates the alternative complement cascade resulting in the
220 deposition of complement (C3) on its surface inducing opsonophagocytosis via the
221 complement receptors CR1 and CR3. To confirm whether oestrogen-induce innate immune
222 evasion was mediated via inhibition of complement, we assessed phagocytosis rates in heat-
223 inactivated serum, where the major complement proteins (i.e. C3) are denatured. Overall, the
224 phagocytosis rates of *C. albicans* in heat-inactivated serum were lower than in live serum (Fig
225 4A), confirming the importance of opsonophagocytosis in the recognition of *C. albicans*.
226 However, adaptation of *C. albicans* to oestrogen did not result in further evasion of
227 phagocytosis in heat-inactivated serum (Fig 4A), suggesting that pre-exposure of *C. albicans*
228 to oestrogen impacts complement activation. Supplementation of heat-inactivated serum with
229 purified C3 restored oestrogen-induced immune evasion (Fig 4B), confirming that oestrogen-
230 induced immune evasion likely occurs through the avoidance of opsonophagocytosis.
231



232

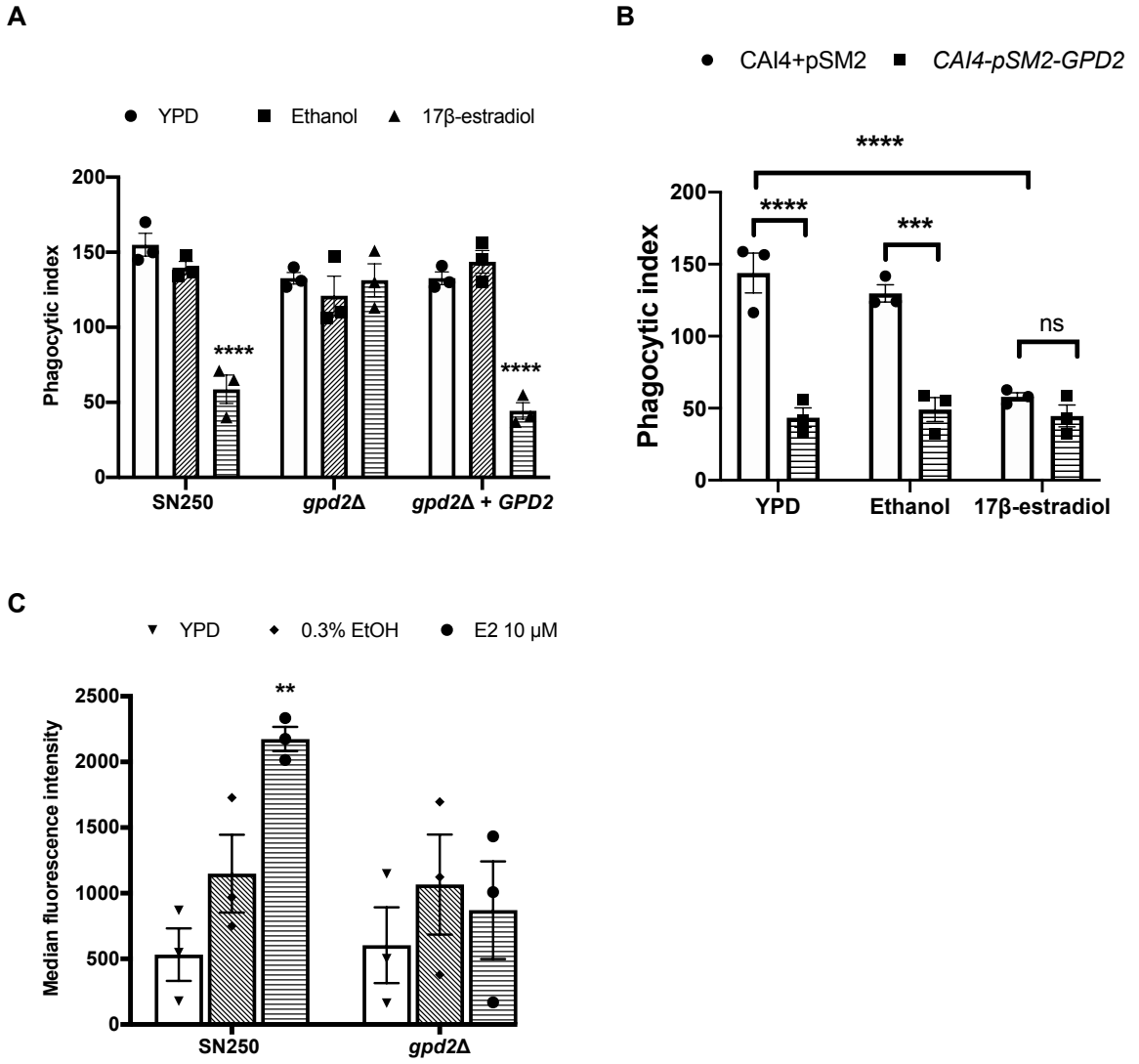
233 **Fig 4. Oestrogen promotes innate immune evasion of *C. albicans* through the inhibition**
234 **of opsonophagocytosis. A)** J774A.1 macrophages were maintained in either complete
235 serum or heat-inactivated (HI) serum and infected with *C. albicans* pre-exposed to YPD, 0.3%
236 ethanol or 10 μM 17β-estradiol at a MOI of 5. **B)** J774A.1 macrophages were maintained in
237 either complete serum, heat-inactivated (HI) serum, or heat-inactivated serum supplemented
238 with purified C3 and infected with *C. albicans* pre-exposed to YPD, 0.3% ethanol or 10 μM
239 17β-estradiol at a MOI of 5. **C)** *C. albicans* was grown in YPD with or without 10 μM 17β-
240 estradiol, incubated in human serum for 20 minutes, fixed with PFA and C3 and C3b binding
241 quantified by FACS. **(D)**. *C. albicans* was grown in YPD with or without 10 μM 17β-estradiol,
242 incubated in human serum for 20 minutes, fixed with PFA and Factor H binding quantified by

243 FACS. Data represent the mean ± SEM from at least three independent experiments. (*p <
244 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
245
246 Next, we tested whether adaptation of *C. albicans* to oestrogen affected the deposition of C3
247 and C3b on the fungal cell surface. Oestrogen-adapted cells exhibited enhanced C3 and C3b
248 deposition compared to non-adapted cells (Fig 4C), suggesting that adaptation to oestrogen
249 promotes C3 and C3b binding on the *C. albicans* cell surface. Increased deposition of
250 complement is normally associated with increased phagocytosis. However, processing of C3
251 can be inhibited by the recruitment of host regulatory proteins like Factor H, and several
252 pathogens are known to avoid opsonophagocytosis through enhanced recruitment of Factor
253 H [38, 39]. Therefore, the ability of oestrogen adapted *C. albicans* cells to bind Factor H was
254 quantified. Oestrogen-adapted cells bound significantly more Factor H, compared to the
255 solvent control (Fig 4D), suggesting that oestrogen-adapted *C. albicans* evade the innate
256 immune system through enhanced Factor H recruitment and decreased opsonophagocytosis.
257

258 **Oestrogen-induced innate immune evasion is mediated through Gpd2**

259 *GPD2* is predicted to encode a cytosolic protein with glycerol-3-phosphate dehydrogenase
260 activity [40]. However, more recently, Gpd2 has been shown to localise to the fungal cell
261 surface in response to serum [41], and has been classed as a moonlighting protein (a protein
262 that has a biological function not predicted by its amino acid sequence). The moonlighting
263 activities of Gpd2 include binding Factor H, Factor H like protein-1 (FHL-1) and plasminogen
264 [40], key components of the alternative complement system. Binding of these host
265 complement regulatory proteins to microbial surfaces has been shown to promote immune
266 evasion via inhibition of complement-mediated phagocytosis [42]. Thus, we hypothesised that
267 adaptation of *C. albicans* to oestrogen promotes binding of these complement regulatory
268 proteins to Gpd2 thereby inhibiting opsonophagocytosis. To test this hypothesis, *GPD2* was
269 deleted in *C. albicans* and phagocytosis rates in the presence and absence of oestrogen

270 determined. Deletion of *GPD2* prevented oestrogen-dependent inhibition of macrophage
271 phagocytosis rates (Fig 5A), confirming that *GPD2* is required for oestrogen dependent innate
272 immune evasion.
273



274

275 **Fig 5. Inhibition of opsonophagocytosis is dependent on Gpd2. A)** J77A.1 macrophages
276 were infected with *C. albicans* strains grown in YPD with or without 10 μM 17β-estradiol and
277 phagocytosis rates quantified. **B)** J774A.1 macrophages were infected with CAI4-pSM2 and
278 CAI4-pSM2-GPD2 (*GPD2* over-expression strain) grown in YPD with or without 10 μM 17β-
279 estradiol and phagocytosis rates quantified. **C)** *C. albicans* strains were grown in YPD with or
280 without 10 μM 17β-estradiol, incubated in human serum for 20 minutes, fixed with PFA and

281 Factor H binding quantified by FACS. All data represent the mean ± SEM from at least three
282 independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001).
283
284 To determine whether increased expression of *GPD2* is sufficient to promote innate immune
285 evasion, we over-expressed *GPD2* in *C. albicans*. Over-expression of *GPD2* (2.75-fold
286 increased mRNA expression compared to wild type cells) resulted in reduced phagocytosis
287 rates irrespective of oestrogen treatment (Fig 5B), thereby confirming that enhanced
288 expression of *GPD2* is sufficient to promote *C. albicans* innate immune evasion. To determine
289 whether the expression levels of *GPD2* correlate with Factor H binding, we quantified Factor
290 H binding in the *gpd2*Δ mutant. As predicted, the *gpd2*Δ mutant did not bind more Factor H
291 than wild type cells in the presence of oestrogen (Fig 5C). Therefore, the expression of Gpd2
292 plays a key role in regulating the host-pathogen interaction.
293
294 **Oestrogen elicits a mild transcriptional response in *C. albicans.***
295 To elucidate how quickly *C. albicans* adapts to oestrogen the fungus was grown in the
296 presence of 10 μM 17β-estradiol for varying lengths of time before macrophage phagocytosis
297 rates were quantified. This time course analysis confirmed that phagocytosis rates gradually
298 reduced after 60 minutes of growth in the presence of oestrogen (S2 Fig A), suggesting that
299 *C. albicans* undergoes some form of transcriptional or translational response to oestrogen. To
300 identify how oestrogen affects the global transcriptional profile of *C. albicans*, RNA-Seq was
301 performed on *C. albicans* cells that had been adapted to 10 μM 17β-estradiol for 4 hours.
302 Despite having a strong impact on the host-pathogen interaction, oestrogen only had a mild
303 effect on the transcriptome of *C. albicans*, (S2 Fig B, Table 1) with 5 genes being significantly
304 up-regulated and 23 genes being significantly down-regulated in the presence of oestrogen
305 (fold change > 2, FDR < 0.01). Genes that were upregulated were largely involved in cellular
306 redox or efflux, suggesting that oestrogen imposes oxidative stress on *C. albicans*. The most
307 significantly down regulated genes were *FRE7*, *SAP2*, *HWP1* and *ECE1*. *FRE7* is a cupric

308 reductase that has previously been confirmed to be repressed by oestrogen [43]. *ECE1* and
309 *HWP1* are strongly associated with hyphal formation, suggesting that in YPD oestrogen
310 represses hyphal formation. As phagocytosis occurs through interactions with the fungal cell
311 surface we further analysed the expression of cell surface proteins in response to oestrogen
312 stimulation to see if other surface proteins contributed to the observed innate immune evasion.
313 In addition to *GPD2*, transcripts corresponding to *CDR1*, *EBP1, SOD6, ALS2, UTR2* and
314 *FGR23* were moderately up regulated in the presence of oestrogen (Table 2). Oestrogen is a
315 substrate of Cdr1, and it is thought that *CDR1* is upregulated in the presence of oestrogen to
316 increase the efflux of oestrogen thereby reducing toxicity [43]. To determine whether Cdr1 is
317 involved in oestrogen-dependent innate immune evasion, phagocytosis rates of the *cdr1*Δ and
318 *cdr1*Δ/*cdr2*Δ mutants were quantified. Deletion of *CDR1* alone or in combination with *CDR2*
319 did not affect oestrogen dependent inhibition of *C. albicans* phagocytosis (S2 Fig C).
320
321 **Table 1 RNA Seq analysis**

Gene name	Fold change	FDR p-value	Function
AOX2	4.25	5.04E-11	Alternative oxidase
orf19.3120	3.16	2.36E-07	PDR-subfamily ABC transporter
orf19.355	2.46	0.03	Predicted oxidoreductase activity
orf19.3337	2.18	4.62E-03	Protein of unknown function
GST1	2.17	0.04	Putative glutathione S-transferase
GAP2	-2.02	1.12E-03	Broad specificity amino acid permease
PST1	-2.03	4.12E-08	Flavodoxin-like protein
FOX2	-2.03	6.50E-11	3-hydroxyacyl-CoA epimerase
orf19.94	-2.07	2.09E-06	Protein of unknown function
MLS1	-2.11	8.93E-08	Malate synthase
ICL1	-2.15	1.71E-03	Isocitrate lyase
orf19.7310	-2.15	1.53E-10	Uncharacterized
CAT1	-2.33	4.45E-10	Catalase
ASR1	-2.33	4.69E-11	Heat shock protein
orf19.6688	-2.37	6.53E-03	Protein of unknown function
orf19.2515	-2.4	3.86E-10	ZZ-type zinc finger protein
IHD1	-2.4	1.72E-12	GPI-anchored protein
FDH1	-2.43	3.22E-09	Formate dehydrogenase
HSP30	-2.55	0.04	Heat shock protein
orf19.7029	-2.64	7.66E-03	Putative guanine deaminase
HPD1	-2.69	0.03	3-hydroxypropionate dehydrogenase

GIT1	-2.73	1.85E-06	Glycerophosphoinositol permease
HMX1	-2.91	0	Heme oxygenase
FRE30	-3.17	3.71E-03	Similarity to ferric reductases
FRE7	-3.23	2.25E-03	Copper-regulated cupric reductase
SAP2	-3.49	1.20E-03	Secreted aspartyl proteinase
HWP1	-3.72	0.03	Host transglutaminase substrate
ECE1	-6.66	1.34E-03	Candidalysin cytolytic peptide toxin

322

323 **Table 2 Analysis of gene expression for cell surface proteins**

Gene name	Fold change	p-value	Function
EBP1	1.86	7.85E-09	NADPH oxidoreductase
CDR1	1.65	0.0000396	Multidrug transporter of ABC superfamily
SOD6	1.56	0.000749	Superoxide dismutase
ALS2	1.31	0.01	ALS family protein
UTR2	1.27	0.02	Cell wall glycosidase
FGR23	1.26	0.03	Protein of unknown function
GPD2	1.21	0.06	Glycerol 3-P dehydrogenase
ECM331	-1.21	0.03	GPI-anchored protein
HSP21	-1.22	0.07	Small heat shock protein
PGA4	-1.23	0.03	beta-1,3-glucanosyltransferase
CSA1	-1.25	0.03	Surface antigen
SAP10	-1.27	0.03	Secreted aspartyl protease
PLB4.5	-1.27	0.02	Phospholipase B
PGA23	-1.31	0.02	GPI-anchored protein of unknown function
PGA10	-1.32	0.01	GPI anchored membrane protein
RBT4	-1.32	0.01	Pry family protein
HSP104	-1.35	0.00253	Heat-shock protein
PHR1	-1.36	0.03	Cell surface glycosidase
IFF4	-1.37	0.02	Adhesin-like cell surface protein
CSP37	-1.37	0.00229	Hyphal cell wall protein
GPH1	-1.43	0.000767	Putative glycogen phosphorylase
XOG1	-1.46	0.000325	Exo-1,3-beta-glucanase
HSP70	-1.47	0.000249	Putative Hsp70 chaperone
CSH1	-1.55	0.0000623	Aldo-keto reductase
MET6	-1.62	3.51E-08	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
PGA26	-1.65	0.07	GPI-anchored adhesin-like protein
PHO100	-1.77	0.05	Putative inducible acid phosphatase
DDR48	-1.77	0.000000264	Immunogenic stress-associated protein
IFF9	-1.89	0.02	Predicted GPI-linked cell-wall protein

IHD1	-2.4	5.55E-16	GPI-anchored protein
PGA15	-2.43	0.06	Putative GPI-anchored protein
PGA46	-2.44	0.06	Putative GPI-anchored protein
HWP1	-3.72	0.000928	Host transglutaminase substrate

324

325 **Ebp1 is a negative regulator of innate immune evasion.**

326 The most significantly differentially regulated cell surface gene was oestrogen binding protein
327 1 (Ebp1). Ebp1 is homologous to old yellow enzyme 2 (*OYE2*) of *S. cerevisiae* and is an
328 NADPH oxidoreductase [16]. Oestrogen inhibits the NADPH oxidoreductase activity of
329 recombinant Ebp1, yet the function of this enzyme in the *C. albicans* oestrogen response is
330 unknown. To determine whether Ebp1 plays a role in oestrogen induced innate immune
331 evasion, *EBP1* was deleted in *C. albicans* and phagocytosis rates of the mutant in the
332 presence and absence of oestrogen quantified. Deletion of *EBP1* resulted in consistently low
333 phagocytosis even in the absence of hormone stimulation (Fig 6A). To determine whether this
334 enhanced innate immune evasion was linked to *GPD2*, the expression of *GPD2* in the *ebp1*Δ
335 mutant was quantified by RT-PCR. Deletion of *EBP1* resulted in 2.74-fold higher expression
336 of *GPD2* (comparable levels of *GPD2* expression to the over-expression strain) compared to
337 wild type cells (Fig 6B), suggesting Ebp1 is a negative regulator of *GPD2* expression and
338 therefore innate immune evasion.

339

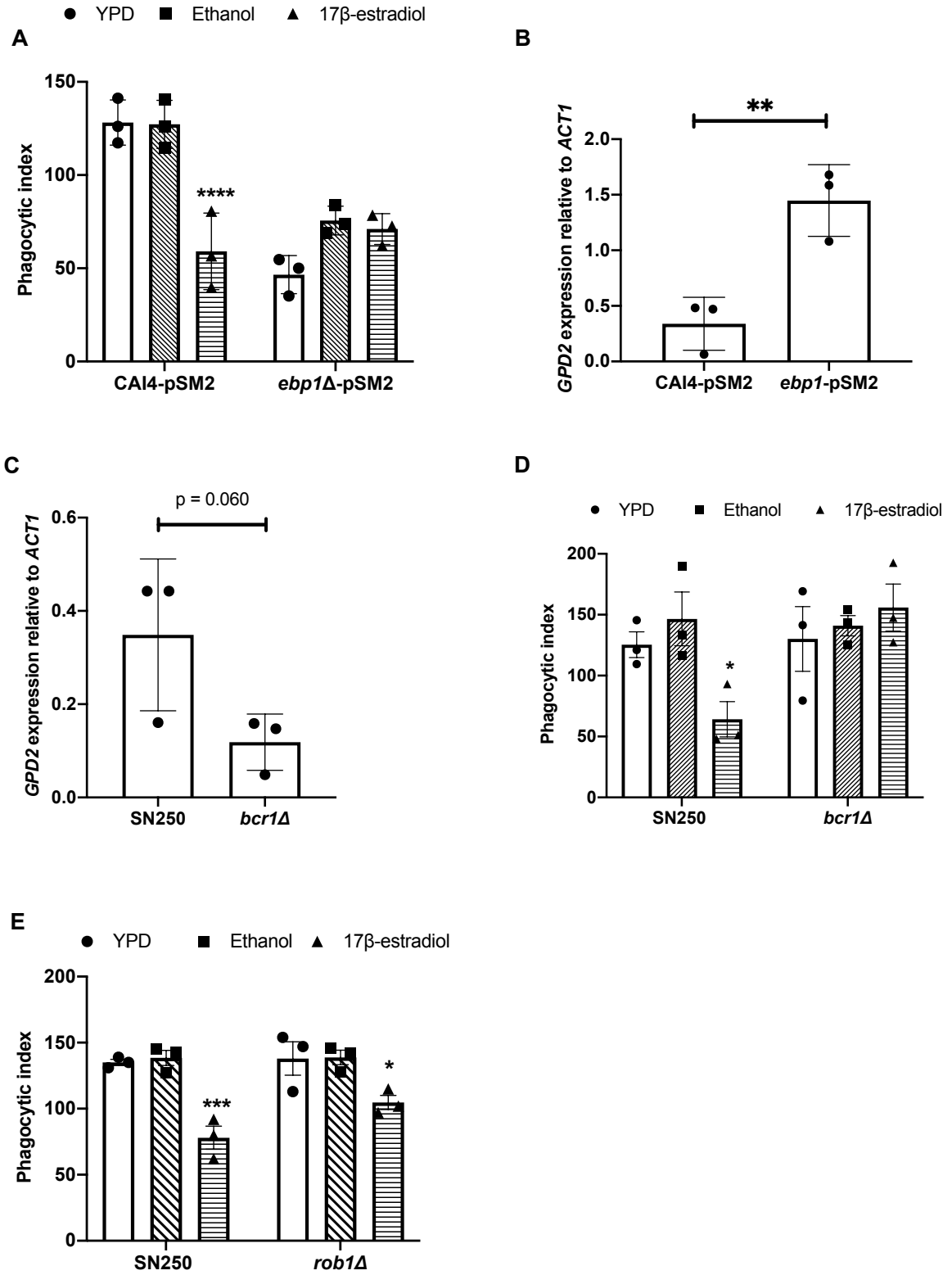


Fig 6. Adaptation of *C. albicans* to oestrogen is mediated via Ebp1 and Bcr1 A) The *ebp1*Δ mutant and the parental control strain were grown with or without 10 μM 17β-estradiol for 4 hrs and then exposed to J774A.1 macrophages for 45 minutes and phagocytosis rates

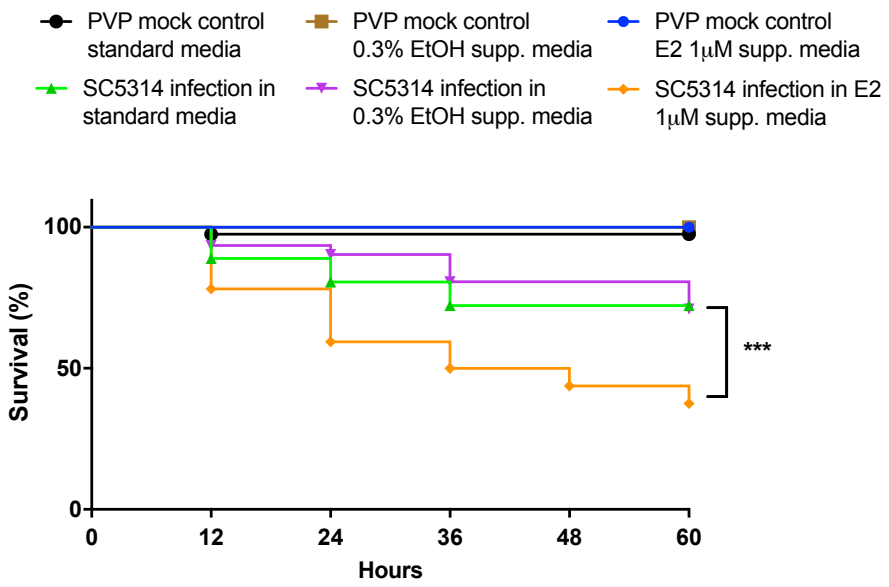
344 quantified. **B)** The *ebp1*Δ mutant and the parental control strain were grown in YPD to mid-log
345 phase, total RNA extracted and *GPD2* gene expression quantified by RT-PCR relative to
346 *ACT1*. **C)** The *bcr1*Δ mutant and the parental control strain were grown in YPD to mid-log
347 phase, total RNA extracted and *GPD2* gene expression quantified by RT-PCR relative to
348 *ACT1*. **D)** The *bcr1*Δ mutant and parental control strain were grown with or without 10 μM 17β-
349 estradiol for 4 hrs and then exposed to J774A.1 macrophages for 45 minutes and
350 phagocytosis rates quantified. **E)** The *rob1*Δ mutant and the parental control strain were grown
351 in YPD with or without 10 μM 17β-estradiol for 4 h and then co-incubated with J774A.1
352 macrophages for 45 min and phagocytosis rates quantified. All data represent the mean and
353 SEM from three biological replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).
354

355 **Oestrogen dependent innate immune evasion requires a threshold level of *GPD2***
356 **expression.**

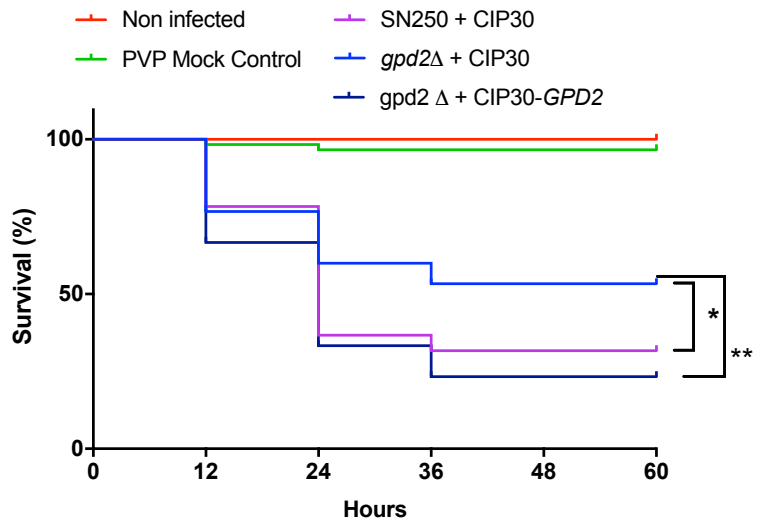
357 To identify transcription factors involved in the expression of *GPD2,* we analysed the promoter
358 region of *GPD2*. The Pathoyeastract program [44] identified 17 transcription factors
359 associated with the promoter region of *GPD2.* Of these 17 transcription factors, 10 have been
360 deleted in *C. albicans* and are available in the transcription factor knockout library [45]. To
361 investigate the role of these 10 transcription factors in the regulation of *GPD2*, the expression
362 of *GPD2* was quantified by qPCR. Of the 10 transcription factor mutants tested, deletion of
363 *BCR1* and *ROB1* had a significant impact on the expression of *GPD2* (Fig 6C, S3 Fig),
364 reducing the expression of *GPD2* to 25% of the wildtype control. To assess whether the
365 reduced expression levels of *GPD2* in the *bcr1*Δ and *rob1*Δ mutants are sufficient to perturb
366 the response to oestrogen, the phagocytosis rates of the *bcr1*Δ and *rob1*Δ mutants in the
367 presence or absence of oestrogen were quantified. Deletion of *BCR1* resulted in a loss of
368 oestrogen-mediated innate immune evasion (Fig 6D), while deletion of *ROB1* partially blocked
369 innate immune evasion (Fig 6E), confirming that a threshold level of *GPD2* expression is
370 required for innate immune evasion.

371 **Oestrogen-induced immune evasion plays a key role in *C. albicans* pathogenicity.**
372 Having established that adaptation of *C. albicans* to oestrogen inhibits phagocytosis, we
373 explored whether this phenomenon could influence virulence *in vivo* using a zebrafish larval
374 model for disseminated disease. Previously, it was shown that exposing zebrafish larvae (3
375 hours post fertilisation) to media containing 1 μM oestrogen results in an *in vivo* oestrogen
376 concentration of 0.0057 μM, equivalent to physiological levels during pregnancy in humans
377 [46, 47]. Taking advantage of this observation, zebrafish larvae were infected with *C. albicans*
378 SC5314 and maintained in E3 media supplemented with 1 μM oestrogen and survival rate
379 monitored for up to 5 days post fertilisation. Compared to zebrafish incubated in E3 media
380 alone, or E3 media supplemented with ethanol, supplementation of E3 media with 1 μM
381 oestrogen enhanced the virulence of *C. albicans,* resulting in an 63% reduction in zebrafish
382 survival 60 hrs post infection (Fig 7A).
383
384 To investigate whether Gpd2 is required for virulence in this model, zebrafish were infected
385 with the parental control strain, the *gpd2*Δ mutant, or reconstituted control strains of *C.*
386 *albicans*. Deletion of *GPD2* led to attenuation of *C. albicans* virulence, which was restored to
387 parental control levels via complementation with a single copy of *GPD2* (Fig 7B). To assess
388 whether increased expression of Gpd2 is sufficient to drive fungal virulence, *GPD2* was over
389 expressed in the CAI4 background. Infection of zebrafish larvae with *C. albicans* over-
390 expressing *GPD2* enhanced *C. albicans* virulence in the absence of oestrogen compared to
391 the respective control strain (Fig 7C). Therefore, Gpd2 plays a key role in promoting *C.*
392 *albicans* virulence *in vivo*.
393

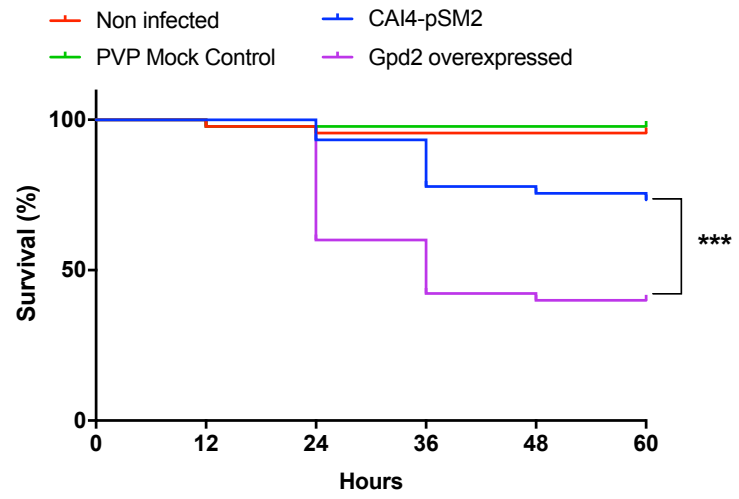
A


PVP mock control standard media
PVP mock control 0.3% EtOH supp. media
PVP mock control E2 1μM supp. media
SC5314 infection in standard media
SC5314 infection in 0.3% EtOH supp. media
SC5314 infection in E2 1μM supp. media
Survival (%)
100
50
0
0
12
24
36
48
60
Hours
***

B


Non infected
PVP Mock Control
SN250 + CIP30
gpd2Δ + CIP30
gpd2 Δ + CIP30-GPD2
Survival (%)
100
50
0
0
12
24
36
48
60
Hours
*
**

C


Non infected
PVP Mock Control
CAI4-pSM2
Gpd2 overexpressed
Survival (%)
100
50
0
0
12
24
36
48
60
Hours
***

394

395 **Fig 7**. **Oestrogen promotes the virulence of *C. albicans* in a zebrafish larval infection**
396 **model. A)** *C. albicans* (SC5314) were microinjected into the hindbrain ventricle of zebrafish
397 larvae in the Prim25 stage. Infected larvae were maintained in E3 media, or E3 media
398 supplemented with 0.3% ethanol, or 1 μM 17β-estradiol and larval survival monitored every
399 24 h until day 5 post fertilisation. **B**) SN250-CIP30, *gpd2Δ-CIP30* and *gpd2Δ-CIP30-GPD2*
400 were microinjected into the hindbrain ventricle of zebrafish larvae in the Prim25 stage dpf.
401 Larvae were maintained in E3 media and larval survival monitored every 24 h until day 5 post
402 fertilisation. **C)** CAI4-pSM2 or CAI4-pSM2-GPD2 were microinjected into the hindbrain
403 ventricle of zebrafish larvae in the Prim25 stage dpf. Larvae were maintained in E3 media and
404 larval survival monitored every 24 h until day 5 post fertilisation. The survival curves represent
405 data pooled from three independent experiments. Statistically significant differences were
406 determined by Log-rank (Mantel-Cox) test. (*$p < 0.05$, **$p < 0.01$; ***$p < 0.001$).
407

408 ## Discussion

409 Vulvovaginal candidiasis (VVC) is a mucosal infection affecting 75% of the female population
410 of reproductive age [11]. Oestrogen is known to govern susceptibility to VVC infections, with
411 women with low circulatory oestrogen levels (i.e. postmenopausal women) having a low risk
412 of developing VVC, and women with high oestrogen levels (i.e. during pregnancy, or women
413 taking high oestrogen containing oral contracepts) having a high risk of VVC. Elevated
414 oestrogen levels increase glycogen at the vaginal mucosa, reduce leukocyte infiltration, and
415 reduce antifungal activity of epithelial cells, promoting infection [10, 12, 13]. However, here we
416 show that, in addition to these effects on the host, oestrogen promotes adaptation responses
417 in *C. albicans* which induce evasion of the innate immune system through Gpd2-dependent
418 inhibition of complement mediated opsonophagocytosis. Unlike the induction of fungal
419 morphogenesis, which appears to be limited to 17β-estradiol [43], all tested forms of oestrogen
420 promoted innate immune evasion, suggesting that *C. albicans* has evolved at least two
421 signalling pathways that are responsive to oestrogen.

422

423 Complement evasion is a successful mechanism employed by viruses, bacteria, parasites and
424 fungi to escape the innate immune system [48]. Evasion of the complement system can be
425 mediated through the secretion of degradative proteins, or by recruitment of host regulatory
426 proteins [49]. One of the most common methods pathogens use to evade complement is
427 through enhanced recruitment of Factor H, a key regulatory protein in the complement system,
428 to the microbial surface. Bound Factor H prevents further activation of the alternative
429 complement system through both the destabilisation of the C3 convertase and enhancement
430 of Factor I mediated degradation of C3b to iC3b, reducing opsonisation and inhibiting the
431 formation of the membrane attack complex [49]. *C. albicans* has been shown by others to
432 recruit Factor H to its surface through the expression of moonlighting proteins. Moonlighting
433 proteins are proteins that perform a biological function not predicted from the amino acid
434 sequence. So far, four moonlighting proteins (Phr1, Gpd2, Hgt1 and Gpm1) have been
435 identified in *C. albicans* [40, 50-52]. Although Gpd2 is predicted to be a cytoplasmic protein
436 involved in cellular redox, Gpd2 has also been identified in cell wall proteomic studies [41],
437 suggesting that Gpd2 functions in the cell wall. Interestingly, Gpd2 was only identified in the
438 cell wall proteome of *C. albicans* grown in complete serum, and not heat-inactivated serum
439 where complement proteins have been inactivated [41]. Therefore, deposition of complement
440 on the fungal cell surface may drive localisation of Gpd2 to the cell surface.

441

442 Purified Gpd2 binds all three complement regulatory proteins [40], although the biological
443 significance of these interactions in infections is not known. Factor H and FHL1 bound to Gpd2
444 remain active, cleaving C3b and thereby inhibiting the alternative complement system [40]. In
445 addition, Gpd2 can also bind plasminogen, which is then processed into plasmin, contributing
446 to inactivation of the alternative complement system [40]. FACS analysis of the deposition of
447 complement proteins on the surface of *C. albicans* in response to oestrogen suggested that
448 oestrogen-adapted cells bound more Factor H, indicative of oestrogen promoting complement
449 evasion. However, oestrogen-adapted *C. albicans* also bound more C3 and C3b, which is

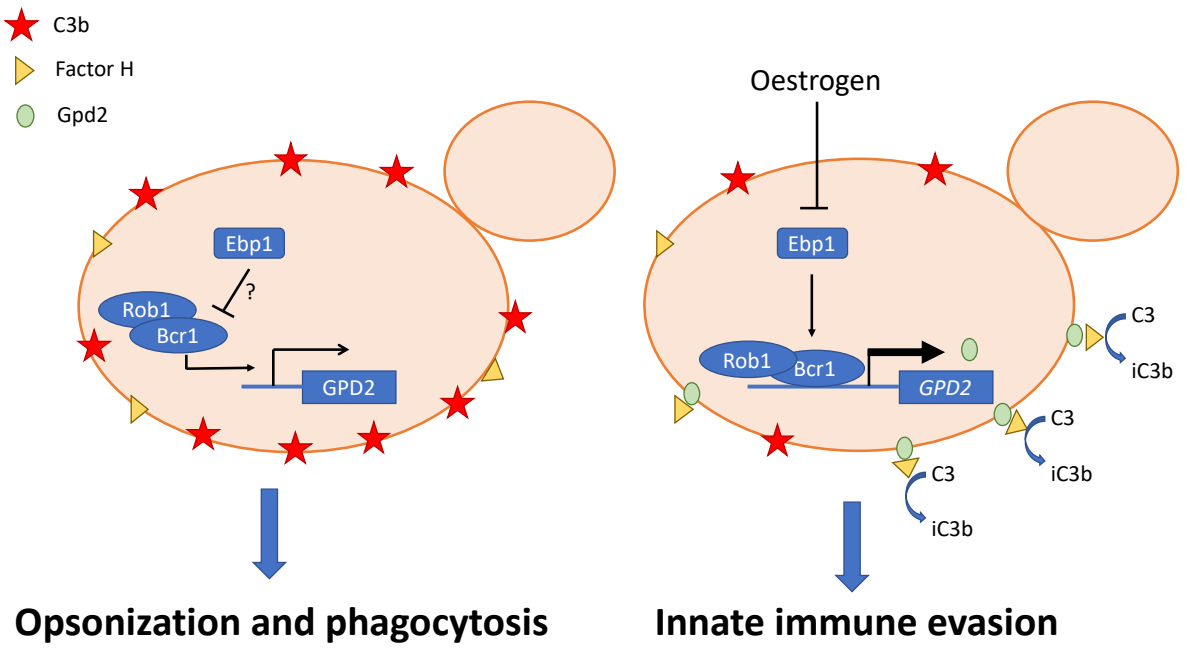
450 surprising as binding of Factor H should prevent C3 deposition. Factor H is a glycoprotein that
451 is made up of 20 complement control proteins (CCP). Microbes bind Factor H at various
452 positions, with *Neisseria* species binding Factor H at CCP6-8 [53]. However, multiple microbes
453 bind Factor H at CCP20, and this has been termed the Common Microbial Binding site [54].
454 Binding of Factor H to some of these microbial cell surface proteins at CCP20 results in
455 increased affinity of Factor H for C3b [54]. The enhanced affinity of Factor H for C3b might
456 explain why we observe elevated binding of both C3b and Factor H, and would suggest that
457 Gpd2 binds to CCP20. The formation of this stable tri-partite complex (microbial protein,
458 Factor H, C3b) results in enhanced activity of Factor H and therefore rapid inactivation of the
459 alternative complement cascade [54], which would explain why we observe reduced levels of
460 phagocytosis.
461
462 Innate immune evasion was only observed after *C. albicans* had been grown in the presence
463 of oestrogen for 60 minutes, suggesting that the response required either transcriptional or
464 post-transcriptional regulation. However, in line with previous studies [43], global
465 transcriptional analysis confirmed that only a small proportion of *C. albicans* genes were
466 differentially regulated by oestrogen. In comparison to the work of Cheng *et al.,* where most
467 of the differentially regulated genes are involved in fungal morphogenesis [43], our analysis
468 mainly identified genes involved in metabolism and oxidation-reduction processes. This lack
469 of overlap is likely reflective of the growth conditions used, as Cheng *et al.*, used RPMI where
470 oestrogen promotes hyphal formation [13, 43], while under our experimental conditions (YPD)
471 oestrogen did not promote hyphal formation. Although innate immune evasion was dependent
472 on the presence of Gpd2, *GPD2* mRNA levels were only moderately increased in response to
473 oestrogen, suggesting that other factors (i.e. post-translational modifications, protein
474 localisation) may be involved. Given that Gpd2 has only been identified as a cell wall protein
475 under specific conditions [41], it might be that in response to oestrogen a higher level of Gpd2
476 is recruited to the cell wall, enabling the fungus to bind more Factor H. In *S. cerevisiae*, the
477 activity of both Gpd1 and Gpd2 is regulated via post-translational modification [55]. Snf1-

478 dependent phosphorylation of *Sc*Gpd1 (orthologue of Ca*GPD2*) results in decreased enzyme
479 activity [55]. Although *Ca*Gpd2 has several predicted phosphorylation sites, these are located
480 in different regions of the protein compared to *Sc*Gpd1, and therefore it is possible that
481 phosphorylation/dephosphorylation at any of these sites in *Ca*Gpd2 could affect the
482 localisation, or potential to bind Factor H.

483

484 Ebp1, despite showing no homology to mammalian hormone binding proteins, has been
485 described as an oestrogen binding protein. Ebp1 is homologous to old yellow enzyme 2
486 (*OYE2*) of *S. cerevisiae*, an NADPH oxidoreductase. Biochemical characterisation of
487 recombinant Ebp1 confirms that Ebp1 is an NADPH oxidoreductase, and that oestrogen
488 inhibits this enzymatic activity [16, 56]. The importance of Ebp1 enzymatic activity in *C.*
489 *albicans* pathogenicity is not known, but here we demonstrate that Ebp1 may serve as a
490 negative regulator of innate immune evasion through the regulation of *GPD2*. Interaction of
491 oestrogen with Ebp1, through an as yet unidentified pathway likely involving Bcr1, results in
492 elevated expression of *GPD2* and, potentially in association with other post-translational
493 modifications, promotes innate immune evasion and pathogenicity (Fig 8).

494



495

496 **Fig 8 Proposed mechanism of oestrogen-induced innate immune evasion.** Under
497 standard laboratory conditions the C3 is deposited on the surface of *C. albicans* resulting in
498 effective phagocytosis. However, in the presence of oestrogen the NADPH activity of Ebp1 is
499 reduced resulting in derepression (through an as yet to be identified signalling pathway) and
500 surface localisation of Gpd2. Gpd2 recruits Factor H to the fungal cell surface, preventing the
501 formation of the C3 convertase and disposition of C3 on the fungal cell surface. Reduced
502 opsonisation results in innate immune evasion and insufficient clearance of *C. albicans*,
503 promoting fungal pathogenicity.
504
505 In recent years zebrafish larvae have become an excellent model for studying pathogenicity
506 mechanisms. Zebrafish have a complement system that is structurally and functionally similar
507 to the mammalian complement system [57], and an orthologue of Factor H has been identified
508 and cloned [58]. The presence of oestrogen in the zebrafish larval model of disseminated *C.*
509 *albicans* infection, confirmed that oestrogen promotes the virulence of *C. albicans*, suggesting
510 that oestrogen dependent inactivation of the alternative complement system promotes the
511 virulence of *C. albicans in vivo*. In support of this, deletion of *GPD2* attenuated fungal
512 virulence, while over expression of *GPD2* enhanced virulence in the zebrafish model of
513 infection, confirming that Gpd2 is an important fungal virulence factor. Given the strong
514 association of elevated oestrogen levels with the development of VVC, it is important to
515 understand the role of Gpd2 in promoting innate immune evasion, and may lead to the
516 development of alternative treatment options for VVC and RVVC.
517
518

519 Materials and Methods

520 Ethics

521 Protocols for human blood collection and isolation of neutrophils and peripheral blood
522 mononuclear cells (PBMCs) were approved by ethical review board of the School of

523 Biosciences at the University of Birmingham. Blood was collected anonymously and on
524 voluntary basis after getting written informed consent.
525 Zebrafish care and experiment protocols were performed in accordance with the Home Office
526 project license 40/3681 and personal license IE905E215 as per Animal Scientific Procedures
527 Act 1986.
528

529 **Strains and media**

530 Unless indicated otherwise, all media and chemicals were purchased from Sigma-Aldrich UK.
531 All strains used in the study are listed in S1 Table. *Candida* strains were routinely maintained
532 on YPD agar (1% yeast extract, 1% peptone, 2% glucose and 2% agar). For broth cultures all
533 strains were cultured in YPD (1% yeast extract, 1% bacto-peptone, 2% glucose) buffered to
534 pH6 with 3.57% HEPES. Oestrogen was diluted in 10% ethanol to a stock concentration of
535 100 μg/ml and diluted into YPD at the required concentrations, maintaining the final ethanol
536 concentration at 0.3%.
537

538 **Phagocytosis experiments**

539 Phagocytosis assay was performed as previously described [59]. Briefly, overnight cultures of
540 *C. albicans* were sub-cultured 1:100 in fresh YPD media, media supplemented with 0.3%
541 ethanol, or media supplemented with oestrogen (0.0001 μM, 0.01 μM or 10 μM) and incubated
542 at 37ºC, 200 rpm for 4 h. *C. albicans* cells were washed three times in PBS and 1 x10^5 J774A.1
543 macrophages (Sigma, UK) were infected with 5 x 10^5 yeast cells (multiplicity of infection [MOI]
544 of 5) for 45 min at 37°C, 5% CO_2. Cells were washed with PBS to remove non-phagocytosed
545 yeast cells, and phagocytosis stopped by fixing with 4% paraformaldehyde (PFA) for 20
546 minutes. Samples were stained for 30 minutes with 50 μg/ml ConA-FITC to stain non-
547 phagocytosed fungal cells, washed and imaged. Phagocytosis events were scored from
548 multiple fields of view using imageJ. When required, J774A.1 cells were maintained in DMEM
549 supplemented with heat inactivated serum and the assay was complemented with 1 μg/mL

550 C3 (Sigma, C2910) or C3b (Sigma, 204860). All experiments were performed in technical
551 duplicates, with a minimum of three independent biological repeats. Data were analysed by
552 Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons test at 95%
553 confidence.
554
555 **Human macrophages and neutrophils**
556 PBMCs and neutrophils were isolated as previously described [20]. Neutrophils were seeded
557 at 2 x 10^5 cells/mL in 24-well plates in serum free RPMI supplemented with 100 mM L-
558 glutamine, incubated for 1 h at 37°C, 5% CO_2 and then co-incubated with 1 x 10^6 *C. albicans*
559 cells (MOI = 5) for 45 min at 37°C, 5% CO_2. Cells were immediately fixed with 4% PFA and
560 stained with 50 μg/ml ConA-FITC for 30 minutes to stain non-phagocytosed fungal cells,
561 washed and imaged. Phagocytosis events were scored from multiple fields of view using
562 imageJ. To assess primary macrophage phagocytosis rates PBMCs (0.5 x10^6) were seeded
563 into 24 well plates in differentiation media (RPMI 1640 supplemented with 100 mM L-
564 glutamine, 10% human AB serum, 100 mM Pen/Strep and 20 ng/ml M-CSF) for 7 days
565 replacing the media every 2-3 days, and then phagocytosis rates determined as described
566 above. To assess cytokine production 2.5 x10^4 PBMCs were stimulated with 5 x10^4 PFA fixed
567 *C. albicans* for 24 hours, supernatants collected and stored at -20°C and cytokine
568 concentrations quantified by ELISA. All experiments were performed in technical duplicates,
569 with a minimum of three independent biological repeats using different donors. Data were
570 analysed by Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons test at
571 95% confidence.
572
573 **Genetic manipulation of *C. albicans***
574 All primers used in genetic manipulation of *C. albicans* are listed in S2 Table. To reintroduce
575 *GPD2* into *C. albicans gpd2*Δ mutant, the *GPD2* locus was PCR-amplified from *C. albicans*
576 SC5314 genomic DNA using primers GPD2-SacI-F and GPD2-NotI-R. The PCR product was

577 cloned into CIp30 plasmid[60] restricted with *SacI* and *NotI* using T4 DNA ligase. The
578 generated CIp30-*GPD2* was linearized with *StuI* and transformed into *C. albicans gpd2*Δ
579 mutant by standard heat-shock transformation [61].
580 To generate a *C. albicans* strain that over expresses *GPD2*, the open reading frame of *GPD2*
581 was cloned into pSM2[62] using primers GPD2-OE-F and GPD2-OE-R under the control of
582 the *TEF2* promoter. The resulting plasmid was linearized with *PacI*, and integrated into CAI4
583 at the *URA3* locus by standard heat-shock transformation.
584 To generate *ebp1Δ*, 500 bp of the 5' and 3' UTR were amplified from genomic DNA using
585 primers EBP1-5F, EBP1-5R, and EBP1-3F and EBP1-3R. The resulting PCR products were
586 purified, digested with *EcoRV* and *SacI* or *HindIII* with *KpnI* and cloned into the mini URA
587 blaster cassette pDDB57 [63]. The resulting disruption cassette was digested with *EcoRV* and
588 *KpnI* and transformed in CAI4 by standard heat-shock transformation. Resulting colonies were
589 screened by PCR and positive colonies were plated onto YNB supplemented with 5-
590 fluoroorotic acid (5-FOA) and uridine to select for spontaneous homologous recombination
591 and loss of *URA3*. Resulting colonies were then re-transformed with the *EBP1* knockout
592 cassette, and loss of both alleles was confirmed by PCR, and lack of expression was
593 confirmed by qPCR. The URA baster cassette was recycled and *URA3* replaced at its native
594 locus by transforming the strain with pSM2.
595
596 **Immunofluorescent staining of *C. albicans* cell wall components**
597 *C. albicans* cells were stained as previously described [20]. Briefly, *C. albicans* cells from
598 overnight culture were sub-cultured in YPD broth with or without oestrogen supplementation
599 and incubated at 37°C, 200 rpm for 4 h. Cells were harvested by centrifugation, washed in
600 PBS and fixed with 4% PFA. To quantify total mannan, glucan and chitin levels in the cell wall,
601 cells were stained with 50 μg/ml TRITC-conjugated concanavalin A (Molecular Probes, Life
602 Technologies), 33.3 μg/ml Aniline Blue fluorochrome (Bioscience supplies) and 3 μg/ml
603 Calcofluor White for 30 minutes. To quantify surface exposure of β1,3-glucan and chitin, cells

604 were stained with 3 μg/ml Fc-Dectin-1 (a gift from G. Brown, University of Aberdeen) and 50
605 μg/ml TRITC-conjugated wheat germ agglutinin (Molecular Probes, Life Technologies). Cells
606 were analysed by flow cytometry and median fluorescence intensity (MFI) quantified. FACS
607 data were analysed by Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons
608 test at 95% confidence.
609
610 **RNA sequencing**
611 *C. albicans* cells were grown for 4 h in YPD broth with or without 10 μM 17β-estradiol at 37ºC,
612 200 rpm. Cells were harvested by centrifugation, washed three times in PBS, and snapped
613 frozen in liquid nitrogen. Total RNA was extracted as per manufacturer's instructions using the
614 RNeasy Plus mini kit (Qiagen). Total RNA was quantified using NanoDrop 8000
615 spectrophotometer (ND-8000-GL; Thermo Fisher). RNA samples were assessed for genomic
616 DNA contamination by PCR and agarose gel electrophoresis. Samples were then processed
617 as previously reported [59]. Sequencing reads are available at the Gene Expression Omnibus
618 (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) at the following accession number
619 GSE145240.
620
621 Reads were analysed following a previous published method[59] and using CLC Genomic
622 workbench 11.0.1 software (Qiagen). In summary, adapter and quality trimming was
623 performed before reads were mapped to *C. albicans* reference genome (Assembly 21, version
624 s02-m09-r10). Transcript Per Kilobase Million (TPM) were reported for each open reading
625 frame (ORFs). Statistical analysis was performed after addition to all values of the lowest TPM
626 measurement, then data were log10 transformed and differential expression between
627 condition were considered significant if the absolute value Fold Change >2 and FDR < 0.01.
628 CGD GO term finder [64] was used to perform Gene ontology (GO) analysis with P-values
629 corresponding to Bonferroni-corrected hypergeometric test P-values.
630

631 **RT-PCR**

632 *C. albicans* cells were grown for 4 h in YPD broth with or without 10 μM 17β-estradiol at 37ºC,
633 200 rpm. Cells were harvested by centrifugation, and snapped frozen in liquid nitrogen. RNA
634 was extracted using the RNeasy Plus mini kit (Qiagen) as per manufacturer's instructions.
635 RNA quality and quantity were checked by electrophoresis and spectroscopy. The qRT-PCR
636 was performed using the 2x qPCRBIO SyGreen mix kit (PCRbiosystem) according to
637 manufacturer's recommendations with 50 ng of total RNA (primers shown in S2 Table).
638 Relative quantification of gene expression was determined by the Delta Delta Ct method with
639 *ACT1* as an endogenous control. mRNA expression was performed in technical triplicate, and
640 data represent the mean and SEM from three independent biological repeats, and were
641 analysed using a paired T-test with 95% confidence.

642

643 **Complement binding**

644 *C. albicans* cells were grown for 4 h in YPD broth with or without 10 μM 17β-estradiol at 37ºC,
645 200 rpm. Cells were harvested, washed three times in PBS, fixed on ice for 30 min with 4%
646 PFA. About 2 x 10^6 yeast cells were labelled with 400 µL 10% normal human serum for 20
647 min at 37ºC, 200 rpm. Cells were washed thrice in PBS and incubated on ice with 100 µL of
648 either 10 µg/mL Anti-Factor H Goat pAb (Sigma, 341276-1ML) or 1 µg/mL Goat anti Chicken
649 IgY (H+L) diluted in 1% BSA/PBS. Cells were washed thrice in PBS and incubated in dark
650 with 100 µL of either Rabbit anti Goat IgG (H+L) Secondary Antibody, Alexa Fluor 488
651 (Invitrogen, A11078) or Goat anti Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 594,
652 (Invitrogen, A11042) diluted 1:200 in 1% BSA/PBS. Cells were analysed by flow cytometry
653 and median fluorescence intensity determined. FACS data were analysed by Kruskal-Wallis
654 test followed by a post-hoc Dunn's multiple comparisons test at 95% confidence.

655

656 **Zebrafish infection**

657 Wild type (AB) *Danio rerio* zebrafish used in the study were housed in a recirculating system
658 of gallon tanks at the University of Birmingham Zebrafish Facility. To obtain embryos, 4 male
659 and 5 female fish were transferred into a breeding tank and maintained at 28°C, 14 h light/10
660 h dark cycle. Embryos were collected the following day, sorted and incubated at 30°C for 24
661 h in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00003%
662 methylene blue, pH 7). Embryos were maintained at a density of 100 per 14.5 cm dish
663 containing 150 mL E3 media supplemented with 0.02 mg/mL Phenylthiourea.
664
665 Hind brain infections were performed as previously described [65]. Briefly, zebrafish at the
666 prim25 stage were manually dechorionated, and anesthetized in 160 µg/mL Tricaine.
667 Approximately 5 nL of injection buffer (10% PVP-40 in PBS, 0.05% phenol red) or *C. albicans*
668 suspension at 5×10^7 cells/mL in injection buffer was microinjected into the hindbrain ventricle
669 via the otic vesicle to achieve a dose of 20-50 yeast/larva. Within 1 h of infection, larvae were
670 screened by microscopy to remove fish noticeably traumatised from microinjection and to
671 ascertain correct injection site and inoculum size. At least 15 larvae per condition were
672 transferred into a 6-well plate, incubated at 28°C in E3 media either with or without 1 μM
673 oestrogen and observed for survival every 24 h until day 5 post fertilisation. Data from three
674 independent biological replicates were pooled together to determine percent survival. Data
675 were analysed by Log-rank Mantel-Cox test and Gehan-Breslow-Wilcoxon test (extra weight
676 for early time points).
677
678 **Statistics**
679 Unless indicated otherwise, data were analysed in Prism (version 8) using a Kruskal-Wallis
680 test followed with a post-hoc Dunn's multiple comparisons test at 95% confidence. Data
681 represent the mean +/- SEM from at least three independent biological experiments.
682

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690

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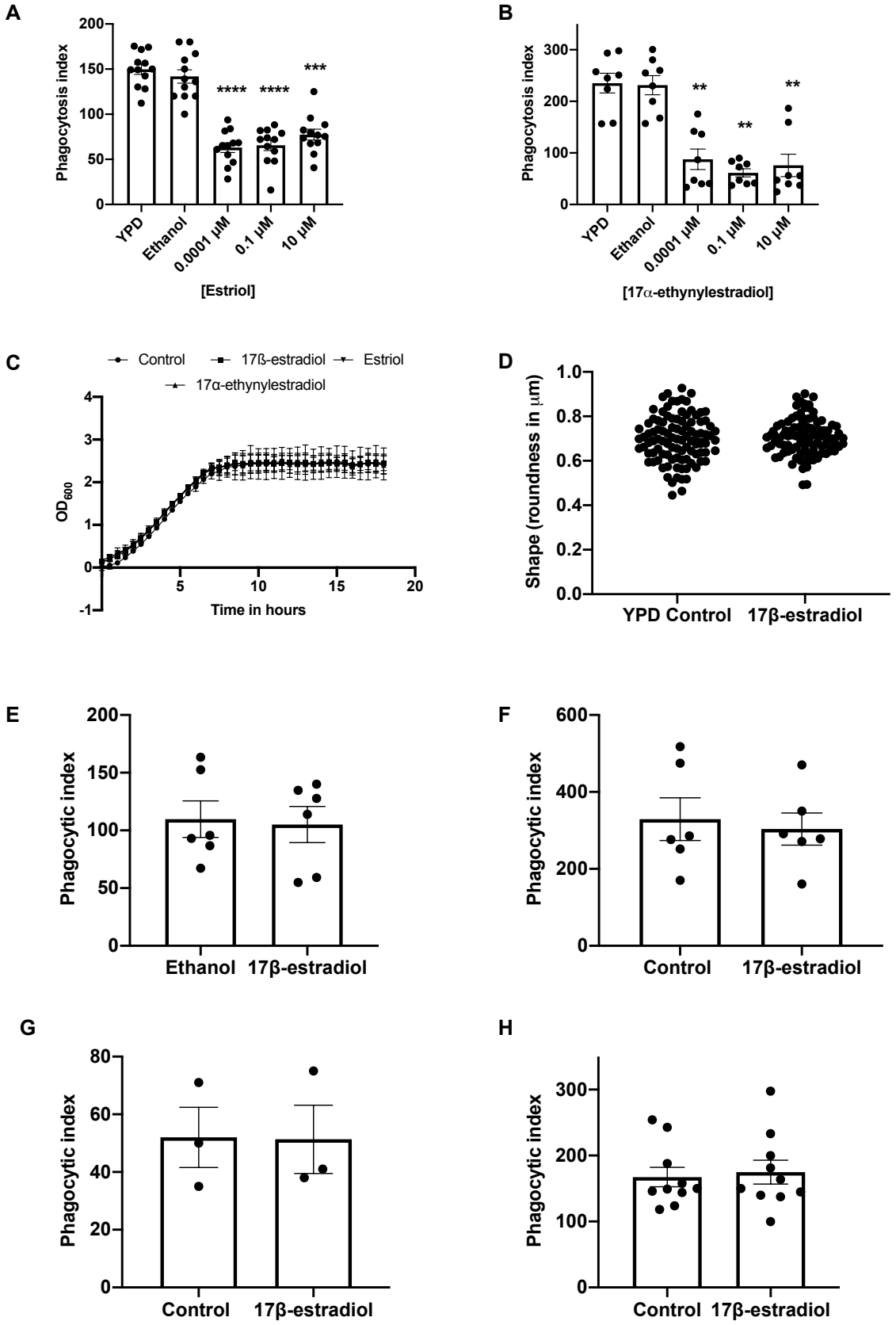
927 **Supporting information**



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929 **S1 Fig Oestrogen inhibits *C. albicans* phagocytosis, but does not affect fungal growth**
930 **or morphology. A)** J774A.1 phagocytosis rates of *C. albicans* (SC5314) grown in the
931 presence of estriol. **B)** J774A.1 phagocytosis rates of *C. albicans* grown in the presence of
932 17α-ethynylestradiol. **C)** *C. albicans* cells were grown in 96-welled plate in YPD broth
933 supplemented with 10 μM 17β-estradiol, 10 μM 17α-ethynylestradiol or 10 μM estriol. Optical
934 densities (OD) of the cultures were recorded every 30 min for 18 h. **D)** *C. albicans* was grown
935 in YPD broth supplemented with 17β-estradiol 10 μM for 4 h. Cells were washed with PBS,
936 stained with concanavalin A, imaged by microscopy and analysed for shape. **E)** J774A.1
937 macrophages preincubated with 10 μM 17β-estradiol were infected with *C. albicans* cells
938 grown in YPD and phagocytosis rates quantified. **F)** Beads were incubated at room
939 temperature in PBS with or without 10 μM 17β-estradiol. Beads were washed and co-
940 incubated with J774A.1 macrophages and phagocytosis rates quantified. **G)** *C. albicans* cells
941 were incubated in PBS at 4°C for 4 h with or without 10 μM 17β-estradiol. Cells were washed
942 and co-incubated with J774A.1 macrophages and phagocytosis rates quantified **H)** *C. albicans*
943 cells previously grown in YPD with or without 10 μM 17β-estradiol were harvested, washed
944 and re-incubated in fresh YPD for 24 h. Cells were co-incubated with J774A.1 macrophages
945 and phagocytosis rates quantified. All data represent the mean ± SEM from at least three
946 independent experiments. (**$p<0.01$, ***$p<0.001$, ****$p<0.0001$).
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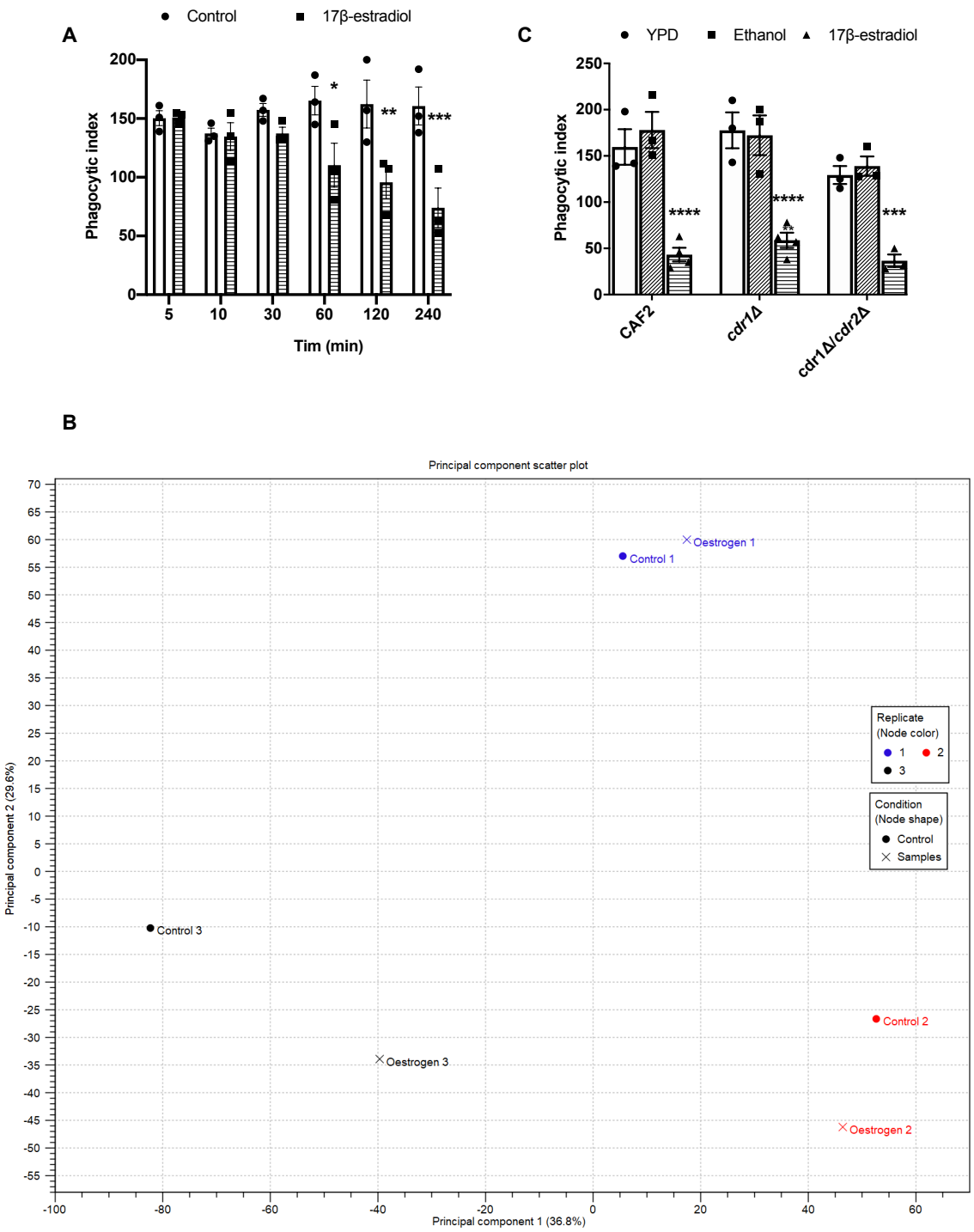

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950 **S2 Fig Oestrogen induces a mild transcriptional response in *C. albicans* A)** *C. albicans*

951 (SC5314) was grown with or without 10 μM 17β-estradiol for the indicated time and then

952 exposed to J774A.1 macrophages for 45 minutes and phagocytosis rates quantified. **B)** PCA

953 of RNA Seq data. **C)** The *cdr1*Δ, *cdr1*Δ/*cdr2*Δ mutants and the parental control strain were

954 grown with or without 10 μM 17β-estradiol for 4 hr, and then exposed to J774A.1 macrophages
955 for 45 minutes and phagocytosis rates quantified. All data represent the mean ± SEM from at
956 least three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).
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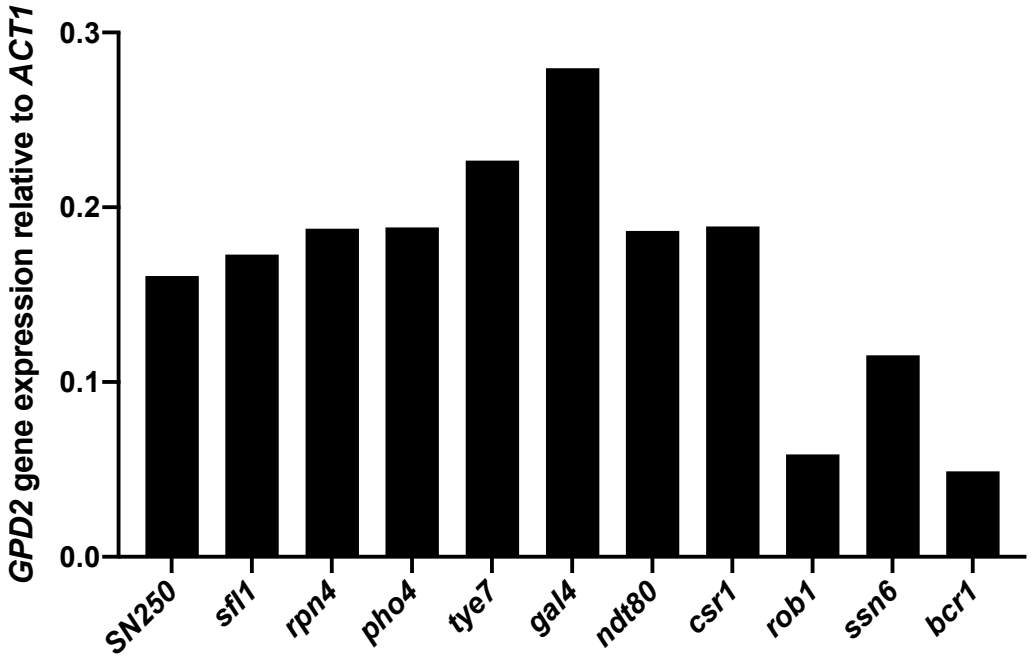

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960 **S3 Fig Bcr1 and Rob1 regulate the expression of *GPD2.*** Transcription factor mutants and
961 the parental control strain were grown in YPD to mid-log phase, total RNA extracted and *GPD2*
962 gene expression quantified by RT-PCR relative to *ACT1*.
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975 **S1 Table *Candida* strains used in this study**

Strain	Genotype	Source/reference
SC5314	Wild type	[66]
SVS006B	Clinical isolate	Prof Ramage, Glasgow University
SVS062A	Clinical isolate	Prof Ramage, Glasgow University
SN152	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ:: λimm434 IRO1/iro1Δ:: λimm434*	[67]
SN250	*his1Δ/his1Δ, leu2Δ::C. dubliniensis HIS1 /leu2Δ::C. maltosa LEU2-arg4Δ/arg4Δ, URA3/ura3Δ::imm434-IRO1/iro1Δ::imm434*	[67]
SN250-CIP30	As SN250 but *RPS1/rps1::*CIP30	This study
*rob1*Δ	As SN152 but *rob1Δ::C. dubliniensisHIS1/rob1Δ::C. maltose LEU2*	[67]
*bcr1*Δ	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::λimm434 IRO1/iro1Δ:: λimm434 bcr1::LEU/bcr1::HIS1*	[67]
*gpd2*Δ	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2*	[67]
*gpd2*Δ–CIP30	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2, RPS1/rps1::CIP30*	This study
*gpd2*Δ-CIP30-*GPD2*	As SN152 but *gpd2Δ::C. dubliniensisHIS1/gpd2Δ::C. maltose LEU2RPS1/rps1::CIP30-GPD2*	This study
CAI4	*ura3::imm434/ura3::imm434 iro1/iro1::imm434*	[68]
CAI4-pSM2	*ura3::imm434/ura3::imm434 iro1/iro1::imm434::*pSM2	[69]

CAI4-pSM2-*GPD2*	*ura3::imm434/ura3::imm434 iro1/iro1::imm434::*pSM2-pTef2-*GPD2*	This study
*ebp1*Δ	As CAI4 but *ebp1::*dlp200/*ebp1::*dlp200	This study
*ebp1*Δ-pSM2	As CAI4 but *ebp1::*dlp200/*ebp1*::dlp200-pSM2	This study
CAF2-1	*URA3/ura3:: λimm434*	[68]
*cdr1*Δ	As CAF2-1, but *crd1::hisG/cdr1:hisG-URA3-HisG*	[70]
*cdr1/2*Δ	As CAF2-1, but *crd1::hisg/cdr1:hisG, cdr2::hisG-URA3-hisG/cdr2::hisG*	[70]

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978 **S2 Table Primers used in this study**

Primer	**Sequence**
GPD2-SacI-F	CTCCGAGCTCGGTGATGGTGATGGTGATGG
GPD2-NotI-R	GGAGAGCGGCCGCTGGTAAATTGGACAACGAGTGG
EBP1-5F:	CTCCGATATCATCGCATGAG
EBP1-5R:	GGAGAGGAGCTCctgatgatatgataatttgc
EBP1-3F:	GGAGAGAAGCTTGGGAATGAAGTTCTATTAGC
EBP1-3R:	GGAGAGGGTACCgttacatctactactacagg
RT-ACT1-F	CCTACGTGTACTTGTGCAAGGCAA
RT-ACT1-R	TAGTTGTGTGCACTGAGCGTCGAA
RT-GDP2-F	GCCAACGAAGTTGCCAAAGGT
RT-GPD2-R	AGGCACCAGCAATAGAGGCA

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