

## **The globular C1q receptor is required for epidermal growth factor receptor signaling during *Candida albicans* infection**

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## ABSTRACT

During oropharyngeal candidiasis, *Candida albicans* activates the epidermal growth factor receptor (EGFR), which induces oral epithelial cells to both endocytose the fungus and synthesize proinflammatory mediators that orchestrate the host immune response. To elucidate the signaling pathways that are stimulated when *C. albicans* interacts with EGFR, we analyzed the proteins that associate with EGFR when *C. albicans* infects human oral epithelial cells. We identified 1214 proteins that were associated with EGFR in *C. albicans*-infected cells. We investigated the function of seven of these proteins that either showed increased association with EGFR in response to *C. albicans* or that mediated the interaction of other microbial pathogens with epithelial cells. Among these proteins, EGFR was found to associate with WW domain-binding protein 2, toll-interacting protein, interferon-induced transmembrane protein 3, and the globular C1q receptor (gC1qR) in viable epithelial cells. Each of these proteins was required for maximal endocytosis of *C. albicans* and they all regulated fungal-induced production of IL-1 $\beta$  and/or IL-8, either positively or negatively. gC1qR functioned as a key co-receptor with EGFR. Interacting with the *C. albicans* Als3 invasin, gC1qR was required for the fungus to stimulate both EGFR and the ephrin type-A receptor 2. The combination of gC1qR and EGFR was necessary for maximal endocytosis of *C. albicans* and secretion of IL-1 $\beta$ , IL-8, and GM-CSF. Thus, this work provides an atlas of proteins that associate with EGFR and identifies several that play a central role in the response of human oral epithelial cells to *C. albicans* infection.

## IMPORTANCE

Oral epithelial cells play a key role in the pathogenesis of oropharyngeal candidiasis. In addition to being target host cells for *C. albicans* adherence and invasion, they secrete proinflammatory cytokines and chemokines that recruit T cells and activated phagocytes to foci of infection. It is known that *C. albicans* activates EGFR on oral epithelial cells, which induces these cells to

endocytose the organism and stimulates them to secrete proinflammatory mediators. To elucidate the EGFR signaling pathways that govern these responses, we analyzed the epithelial cell proteins that associate with EGFR in *C. albicans*-infected epithelial cells. We identified four proteins that physically associate with EGFR and that regulate different aspects of the epithelial response to *C. albicans*. One of these is gC1qR, which is required for *C. albicans* to activate EGFR, induce endocytosis, and stimulate the secretion of proinflammatory mediators, indicating that gC1qR functions as a key co-receptor with EGFR.

## **KEYWORDS**

*Candida albicans*, oral epithelial cells, endocytosis, epidermal growth factor receptor, host defense

## 1 INTRODUCTION

2 *Candida albicans* grows as a harmless commensal in the oral cavity of at least 50% of  
3 normal adults (1). However, when either local or systemic immune defenses are weakened, *C.*  
4 *albicans* can proliferate and cause oropharyngeal candidiasis (2). This disease causes  
5 substantial morbidity in patients with HIV/AIDS, dentures, organ transplantation, cancer,  
6 diabetes, and xerostomia (3-6). Although most patients with their first episode of oropharyngeal  
7 candidiasis readily respond to treatment with antifungal agents such as fluconazole, patients  
8 with recurrent disease are at risk for developing infection with an azole-resistant strain (7, 8).

9 During oropharyngeal candidiasis, *C. albicans* invades the epithelial cell lining of the  
10 oropharynx, stimulating a strong proinflammatory host response that is driven by IL-17 and  
11 IL-22 signaling that is initiated from innate lymphocytes and subsequently from adaptive  
12 immune response (9, 10). When *C. albicans* adheres to an epithelial cell, it interacts with and  
13 activates multiple epithelial cell receptors, including the ephrin type-A receptor 2 (EphA2), E-  
14 cadherin, the platelet-derived growth factor receptor BB, HER2, and the epidermal growth factor  
15 receptor (EGFR) (11-13). Among these receptors, EGFR plays a central role in triggering the  
16 epithelial cell endocytosis of *C. albicans* and stimulating a proinflammatory response. EGFR  
17 interacts with the ephrin type-A receptor 2 (EphA2), HER2, E-cadherin, src family kinases, and  
18 the aryl hydrocarbon receptor, triggering changes in the actin cytoskeleton that lead to the  
19 endocytosis of the fungus (11, 13, 14). EGFR also interacts with EphA2 to stimulate epithelial  
20 cells to secrete proinflammatory mediators, including defensins, IL-1 $\alpha$ , IL-1 $\beta$ , CXCL8/IL-8, and  
21 CCL20 (11, 15, 16). The defensins have direct candidacidal activity, while the cytokines and  
22 chemokines recruit phagocytes to the focus of infection and enhance their capacity to kill the  
23 invading fungus.

24 Because of the key role of EGFR in mediating the oral epithelial cell response to *C.*  
25 *albicans*, we sought to obtain a more comprehensive view of the spectrum of host cell proteins  
26 that interact with this receptor. Using a proteomics approach, we found that EGFR associates

27 with 1214 epithelial cell proteins in *C. albicans*-infected cells. Of these proteins, 13 had  
28 increased association with EGFR in the *C. albicans*-infected cells relative to uninfected cells. In  
29 intact epithelial cells, four proteins associated with EGFR in the vicinity of *C. albicans* hyphae:  
30 WW domain-binding protein 2 (WBP2) which governs EGFR expression and signaling in cancer  
31 cells, toll-interacting protein (TOLLIP) which is a negative regulator of toll-like receptor  
32 signaling, interferon-induced transmembrane protein 3 (IFITM3) which is an antiviral protein,  
33 and the globular C1q receptor (gC1qR) which is a multifunctional protein that interacts with a  
34 variety serum components. We interrogated these in the context of *C. albicans* infection of  
35 human oral epithelial cells, and found that each of these proteins is required for maximal  
36 endocytosis of *C. albicans*. Moreover, they all regulate the production of innate cytokines such  
37 as IL-1 $\beta$  and/or IL-8, either positively or negatively. Additionally, gC1qR functions as a key co-  
38 receptor that is required for *C. albicans* to stimulate EGFR and to induce endocytosis and an  
39 epithelial cell proinflammatory response.

40

## 41 RESULTS

42 **EGFR associates with multiple proteins that mediate endocytosis and govern**  
43 **actin dynamics.** To identify epithelial cell proteins that associate with EGFR, we infected the  
44 OKF6/TERT-2 oral epithelial cell line (17) with *C. albicans* yeast for 90 min, cross-linked the  
45 proteins with formaldehyde and then performed immunoprecipitation of whole cell lysates with  
46 an anti-EGFR antibody. The proteins that were associated with EGFR were then identified by  
47 LC-MS/MS. As controls, we processed in parallel uninfected epithelial cells as well as epithelial  
48 cells incubated with epidermal growth factor (EGF) for 5 min. In total, 1214 proteins were  
49 associated with EGFR in all three samples of epithelial cells infected with *C. albicans* (Table S1)  
50 and 1278 were associated with EGFR in both samples of epithelial cells that had been  
51 incubated with EGF (Table S2). The majority of these proteins were constitutively associated  
52 with EGFR. However, 13 proteins showed at least a 2-fold increase in association with EGFR in

53 all three samples of *C. albicans*-infected cells relative to uninfected cells (Table 1) and 37 had  
54 increased association with EGFR in the EGF-exposed cells (Table S3). Among the proteins that  
55 had increased association with EGFR in response to *C. albicans* infection, four associated with  
56 EGFR only in cells exposed to *C. albicans*, but not EGF. Among these were: WW domain-  
57 binding protein 2 (WBP2), guanylate binding protein family member 6 (GPB6), subunit J of  
58 eukaryotic translation initiation factor 3, and calcineurin subunit B type 1 (Table 1). Only three  
59 proteins were found to associate with EGFR in cells exposed to EGF but not *C. albicans*. One  
60 was EGF itself and the others were galectin-9B and ERBB receptor feedback inhibitor 1 (Table  
61 S3). Collectively, these results suggest that while the profiles of proteins that associate with  
62 EGFR in response to *C. albicans* and EGF are similar, some proteins are uniquely recruited in  
63 response to *C. albicans*.

64 Analysis of proteins constitutively associated with EGFR provided insight into the  
65 mechanisms by which EGFR induces epithelial cells to endocytose *C. albicans*. We found that  
66 EGFR associated with EphA2, E-cadherin, and HER2 (Table 2), consistent with our previous  
67 findings that EGFR functions in the same pathways as these three receptors (11, 13, 18). The  
68 src family kinases are known to activate EGFR (14), and we found that two members of this  
69 family, Lyn and Yes associate with this receptor (Table 2). Receptors are sometimes located in  
70 specific microdomains on the plasma membrane. It was notable that EGFR was associated with  
71 caveolin-1, caveolin-2, flotillin-1, flotillin-2, arf6, and RhoA (Table 2), which are components of  
72 lipid rafts (19, 20).

73 The endocytosis of *C. albicans* is also mediated in part by the clathrin and cortactin  
74 internalization pathway (21). Consistent with this mechanism, the clathrin heavy and light chains  
75 and cortactin were found to associate with EGFR (Table 2). Also associated with EGFR was the  
76 phosphatidylinositol-binding clathrin assembly protein, an adapter protein that is required for  
77 proper clathrin function (20). Activation of the clathrin pathway induces rearrangement of actin  
78 filaments, leading to the formation of pseudopods that engulf the fungus, leading to the

79 formation of an endocytic vacuole (22). We found that EGFR associated with all seven  
80 members of the actin-related protein 2/3 (arp2/3) complex, including actin-related proteins 2 and  
81 3, and actin-related protein complex subunits 1B, 2, 3, 4, 5 (Table 2). These proteins play a key  
82 role in organizing actin filaments during clathrin-mediated endocytosis and the formation of  
83 pseudopods (23, 24).

84 EGFR was also associated with numerous actin binding proteins, including  $\alpha$ -actinin,  
85 calponin 3, cofilin-1, drebrin, and fascin (Table 2). The association of EGFR with filamin A and  
86 B, which link actin to membrane glycoproteins (25) suggests that these both may connect EGFR  
87 with actin and its associated proteins. Actin dynamics are known to be regulated by small  
88 GTPases (26), and we found that EGFR was associated with RhoA, RhoB, RhoC, RhoG,  
89 RAC1, RAC2, Ran, and CDC42 (Table 2). EGFR associated with additional small GTPases,  
90 including HRas, KRas, NRas, R-Ras, R-Ras2 and 28 different Rabs (Table 2). Most small  
91 GTPases require guanine nucleotide-exchange factors (GEFs) for activation (27), and EGFR  
92 associated with guanine nucleotide exchange factor 16 (neuroblastoma, ephexin 4) and  
93 regulator of chromosome condensation 1 (RCC1) (Table 2). Collectively, these results indicate  
94 that EGFR has extensive interactions with the actin cytoskeleton and its regulatory proteins that  
95 likely induce the endocytosis of *C. albicans* hyphae and secretion of proinflammatory mediators  
96 when EGFR is activated.

97 **The EGFR-associated proteins WBP2, TOLLIP, IFITM3, and gC1qR govern the**  
98 **epithelial cell response to *C. albicans*.** Among the proteins that were predicted by the  
99 proteomics data to have increased association with EGFR in response to *C. albicans*, we  
100 selected six for additional study: WBP2, guanylate binding protein 6 (GBP6), TOLLIP, early  
101 endosome antigen 1 (EEA1), desmoglein-3 (DSG3), and IFITM3. These proteins were chosen  
102 because of their potential roles in governing the endocytosis of *C. albicans* and the secretion of  
103 proinflammatory mediators. We also investigated the globular C1q receptor (gC1qR), which was  
104 constitutively associated with EGFR. Our rationale was that gC1qR is known to function as a

105 receptor for *L. monocytogenes*, and there are significant similarities between the mechanisms of  
106 host cell invasion by this bacterium and *C. albicans* (18, 21, 28-30).

107 To verify that the selected proteins were associated with EGFR in intact oral epithelial  
108 cells and to determine their subcellular location with respect to *C. albicans* cells, we used a  
109 proximity ligation assay (16). This assay forms a fluorescent spot where two proteins are  
110 located within 40 nm of one another (31). All seven proteins were associated with EGFR in both  
111 infected and uninfected epithelial cells, but only WBP2, TOLLIP, IFITM3, and gC1qR  
112 accumulated with EGFR in the vicinity of *C. albicans* hyphae (Fig. 1 and Fig. S1). The  
113 accumulation of WBP2, TOLLIP, IFITM3, and gC1qR around *C. albicans* suggested that these  
114 proteins might govern the epithelial cell response to the fungus.

115 To investigate this hypothesis, we used siRNA to knock down the levels of each these  
116 proteins in oral epithelial cells. We then measured *C. albicans* adherence to epithelial cells and  
117 subsequent endocytosis using a standard differential fluorescence assay (18, 22). We also  
118 analyzed the secretion of IL-1 $\beta$  and IL-8 by ELISA. As a control, we used siRNA to knock down  
119 EGFR in the epithelial cells. Consistent with previous results (13, 16), knockdown of EGFR  
120 modestly reduced the number of cell-associated *C. albicans* cells and decreased the number of  
121 endocytosed organisms by almost 50% (Fig. 2A). Reduction of EGFR also inhibited *C. albicans*-  
122 induced secretion of IL-1 $\beta$  and IL-8.

123 WBP2 is a multifunctional protein that has mainly been studied in the context of breast  
124 cancer. Functioning as an adapter protein and transcriptional co-activator, WBP2 is required for  
125 maximal EGFR expression and for the normal activity of the PI3K/Akt signaling pathway (32).  
126 WBP2 also links JNK to Wnt signaling (32). Both PI3k/Akt and JNK have been shown to govern  
127 the response of oral epithelial cells to *C. albicans* (33, 34). As shown, siRNA knockdown of  
128 WBP2 reduced total EGFR levels in oral epithelial cells, leading to a decrease in *C. albicans*  
129 endocytosis and a reduction in IL-1 $\beta$  and IL-8 production (Fig. 2B). Thus, knockdown of WBP2



130 largely phenocopies the knockdown of EGFR, and is required for normal cellular EGFR levels in  
131 oral epithelial cells.

132 TOLLIP is a membrane-associated endocytic adapter protein that is a negative regulator  
133 of the innate immune response. TOLLIP inhibits signaling by STAT1, toll-like receptor, and IL-1 $\beta$   
134 (35-37), but was not previously known to associate with EGFR. Knockdown of TOLLIP inhibited  
135 secretion of IL-8 but had no impact on the adherence or endocytosis of *C. albicans* or the  
136 secretion of IL-1 $\beta$  (Fig. 2C). These results suggest that TOLLIP is a positive regulator of  
137 epithelial cell IL-8 secretion in response to *C. albicans* infection.

138 IFITM3 plays a key role in the host defense against viral infections by binding to virus  
139 particles and shuttling them to lysosomes for degradation (38). By a similar process, IFITM3  
140 also enhances the degradation of activated EGFR (38). Knockdown of IFITM3 in oral epithelial  
141 cells had paradoxical effects. Although loss of IFITM3 inhibited the adherence and endocytosis  
142 of *C. albicans*, it stimulated *C. albicans*-induced secretion of IL-1 $\beta$  and IL-8 (Fig. 2D), thereby  
143 dissociating the process of endocytosis from cytokine production.

144 gC1qR (HABP1/p32) is present in the mitochondrial matrix where is involved in oxidative  
145 phosphorylation (39). Although gC1qR lacks a transmembrane sequence, it is also expressed  
146 on the cell surface where it functions as a receptor for C1q, high-molecular weight kininogen,  
147 factor XII, vitronectin, and hyaluronic acid (40-43). gC1qR It also acts as a host cell receptor for  
148 multiple pathogens including *L. monocytogenes*, *Staphylococcus aureus*, and *Plasmodium*  
149 *falciparum* (28, 44, 45). In carcinoma cells, inhibition of gC1qR with a monoclonal antibody is  
150 known to reduce EGFR phosphorylation and block stimulation of migration and lamellipodia  
151 formation in response to EGF (46). Knockdown of gC1qR inhibited the endocytosis of *C.*  
152 *albicans* but stimulated the secretion of IL-1 $\beta$  and IL-8 (Fig. 2E), suggesting that gC1qR may  
153 play a key role in the activation of EGFR by *C. albicans*.

154 **Surface-exposed gC1qR is required for EGFR-mediated endocytosis of *C.***

155 ***albicans*.** Based on the above results, we focused on gC1qR for in-depth study. Because

156 gC1qR is expressed both on the cell surface and intracellularly, the gC1qR siRNA likely reduced  
157 the levels of both cell surface and intracellular gC1qR. To test whether the inhibition of just  
158 surface-exposed gC1qR altered the epithelial cell response to *C. albicans*, we evaluated two  
159 different anti-gC1qR monoclonal antibodies, 60.11 and 74.5.2 that bind to different domains of  
160 gC1qR (47). Both antibodies decreased the endocytosis of *C. albicans* (Fig. 3A), and antibody  
161 74.5.2 slightly reduced the number of cell-associated organisms (Fig. S2A). Also, treating the  
162 epithelial cells with both monoclonal antibodies together did not result in a further inhibition of  
163 endocytosis. Thus, surface-exposed gC1qR is required for maximal endocytosis of *C. albicans*  
164 by oral epithelial cells.

165 To investigate the functional relationship between gC1qR and EGFR, we treated the oral  
166 epithelial cells with an anti-gC1qR monoclonal antibody, the specific EGFR inhibitor gefitinib, or  
167 the antibody and gefitinib in combination. Both the anti-gC1qR antibody and gefitinib inhibited  
168 the endocytosis of *C. albicans* similarly, and combining the anti-gC1qR antibody with gefitinib  
169 did not decrease endocytosis further (Fig. 3B). None of the treatments altered the number of  
170 cell-associated organisms (Fig. S2B). These results suggest that gC1qR may function in the  
171 same pathway as EGFR to mediate the endocytosis of *C. albicans*.

172 We further explored the relationship between gC1qR and EGFR in the endocytosis of *C.*  
173 *albicans* using a heterologous expression approach. We obtained two NIH/3T3 mouse  
174 fibroblastoid cell lines, a wild-type cell line and one that had been transfected with human EGFR  
175 and HER2 (48). Each of these cell lines was then transfected with either GFP as a control or  
176 human gC1qR. When wild-type NIH/3T3 cells were transfected with gC1qR, they endocytosed a  
177 similar number of *C. albicans* cells as the control cells (Fig. 3C), indicating that gC1qR was  
178 unable to induce endocytosis in the absence of EGFR. As we found previously (13), NIH/3T3  
179 cells that expressed human EGFR and HER2 endocytosed more *C. albicans* cells than the wild-  
180 type cells. When the EGFR-expressing cells were transfected with gC1qR, they endocytosed  
181 even more organisms. This increase in endocytosis was due to the presence of surface

182 expressed gC1qR because treating these cells with an anti-gC1qR antibody reduced  
183 endocytosis to basal levels (Fig. 3D). There was an increase in the number of *C. albicans* cells  
184 that were associated with NIH/3T3 cells that expressed EGFR relative to cells that did not (Fig.  
185 S2C). However, expression of gC1qR had no significant effect on the number of cell-associated  
186 organisms (Fig. S2C, D). Collectively, these data indicate that gC1qR is a key cofactor that  
187 enhances EGFR-mediated endocytosis of *C. albicans*.

188 **Surface-expressed gC1qR is required for *C. albicans*-induced stimulation of**  
189 **epithelial cell production of proinflammatory mediators.** To investigate the role of surface-  
190 expressed gC1qR in the oral epithelial cell inflammatory response to *C. albicans*, we analyzed  
191 the effects of an anti-gC1qR antibody on the secretion of IL-1 $\beta$  and IL-8. In contrast to  
192 knockdown of gC1qR with siRNA, inhibiting gC1qR with the monoclonal antibody significantly  
193 reduced the secretion of both mediators, indicating that surface expressed gC1qR is required  
194 for *C. albicans* to stimulate their production (Fig. 4A and B).

195 Next, we analyzed the functional relationship between gC1qR and EGFR in the epithelial  
196 cell proinflammatory response. We used a cytometric bead array to measure the levels of IL-1 $\beta$ ,  
197 IL-8, IL-1 $\alpha$ , and GM-CSF that were secreted in response to *C. albicans*. Inhibition of surface-  
198 expressed gC1qR significantly reduced the production of all four inflammatory mediators (Fig  
199 4C-F), although the absolute levels of IL-1 $\beta$  and IL-8 were somewhat different when measured  
200 by the cytometric bead array instead of the ELISA. Inhibition of EGFR with gefitinib also  
201 significantly decreased the levels of IL-1 $\beta$ , IL-8, and GM-CSF, but had no effect on IL-1 $\alpha$  levels,  
202 as reported previously (15, 16). The finding that inhibition of gC1qR reduced IL-1 $\alpha$  production  
203 whereas inhibition of EGFR had no effect suggests that gC1qR is required for the production of  
204 IL- $\alpha$  by interacting with a receptor other than EGFR.

205 When the epithelial cells were incubated with the anti-gC1qR antibody and gefitinib in  
206 combination, the production of IL-1 $\beta$  and GM-CSF was not inhibited more than in cells  
207 incubated with the anti-gC1qR antibody alone (Figs. 4C and F). However, dual inhibition of

208 gC1qR and EGFR resulted in a modest further reduction in IL-8 production (Fig. 4F).

209 Collectively, these results indicate that gC1qR and EGFR function in the same pathway to

210 induce the production of IL-1 $\beta$ , IL-8, and GM-CSF in response to *C. albicans*.

211 **gC1qR is necessary for intact *C. albicans* to interact with EGFR.** Our finding that

212 gC1qR functions in the same pathway as EGFR to mediate epithelial cell endocytosis and

213 stimulation prompted us to investigate whether gC1qR was necessary for *C. albicans* to activate

214 EGFR. We found that treatment of oral epithelial cells with an anti-gC1qR antibody decreased

215 *C. albicans*-induced phosphorylation of EGFR by approximately 58% (Figs. 5A and B). As

216 expected, treatment of the infected cells with the EGFR kinase inhibitor, gefitinib reduced EGFR

217 phosphorylation to below basal levels. Notably, inhibition of gC1qR did not block EGFR

218 phosphorylation in response to either EGF or candidalysin, a pore-forming toxin released by *C.*

219 *albicans* (15, 49) (Fig. S3). Thus, the effects of inhibiting surface-expressed gC1qR were

220 specific to intact *C. albicans*.

221 Activation of EGFR is also required for *C. albicans* to induce sustained phosphorylation

222 of the EphA2 receptor tyrosine kinase (11, 16). We determined that inhibition of gC1qR

223 decreased *C. albicans*-induced EphA2 phosphorylation (Figs. 5A and B). These findings are

224 consistent with the model that gC1qR is necessary for *C. albicans* to activate EGFR and its

225 downstream signaling pathways in oral epithelial cells.

226 Previously, we found that EGFR associated, either directly or indirectly, with *C. albicans*

227 hyphae (13). To determine if gC1qR plays a role in this association, we infected oral epithelial

228 cells with *C. albicans* in the presence of either an anti-gC1qR antibody or an anti-EGFR

229 antibody. After 90 min, we lysed the epithelial cells with a detergent, collected the *C. albicans*

230 hyphae, and rinsed them extensively to remove unbound proteins. Using high molar urea, we

231 eluted the epithelial cell proteins that remained associated with the organisms and analyzed

232 them by immunoblotting. In control cells, both gC1qR and EGFR were associated with the *C.*

233 *albicans* hyphae (Figs 5C and D). When the cells were incubated with the anti-gC1qR antibody,

234 there was a significant reduction in the amounts of gC1qR and EGFR that were associated with  
235 *C. albicans*. When the cells were incubated with an anti-EGFR antibody, the amount of fungal-  
236 associated gC1qR and EGFR was also significantly reduced. Collectively, these data suggest  
237 that gC1qR and EGFR have a reciprocal relationship such that each protein is necessary for the  
238 other to maximally associate with *C. albicans*.

239 ***C. albicans* Als3 but not candidalysin is required for increased association of**  
240 **gC1qR with EGFR.** Both the Als3 invasin and the candidalysin pore forming toxin are required  
241 for *C. albicans* to maximally activate EGFR in oral epithelial cells (15, 16). We investigated  
242 whether these factors were required gC1qR to associate with EGFR around *C. albicans* cells in  
243 intact oral epithelial cells. Using the proximity ligation assay, we determined that when oral  
244 epithelial cells were infected with an *als3* $\Delta/\Delta$  deletion mutant, there was no accumulation of the  
245 gC1qR-EGFR containing complex around the hyphae (Fig. 5E). By contrast, when the epithelial  
246 cells were infected with the wild-type strain, an *als1* $\Delta/\Delta$  deletion mutant, or a candidalysin-  
247 deficient *ece1* $\Delta/\Delta$  deletion mutant, this complex formed around the hyphae. Thus, Als3, but not  
248 Als1 or candidalysin, is necessary for gC1qR to associate with EGFR around *C. albicans*  
249 hyphae.

250 **Inhibition of gC1qR does not alter *C. albicans* virulence or activation of EGFR in**  
251 **mice.** Next, we investigated the role of gC1qR in the pathogenesis of oropharyngeal candidiasis  
252 in mice. To verify that monoclonal antibodies raised against human gC1qR could inhibit mouse  
253 gC1qR, we tested the capacity of these antibodies to inhibit the endocytosis of *C. albicans* by  
254 primary mouse epithelial cells. We found monoclonal antibody 74.5.2, which is directed against  
255 the binding site for high-molecular weight kininogen (47) significantly reduced *C. albicans*  
256 endocytosis (Fig. 6A). It also decreased the number of cell-associated organisms (Fig. S4). By  
257 contrast, monoclonal antibody 60.11, which recognizes the binding site for C1q (47), had no  
258 detectable effect on either interaction.

259           Based on these results, we treated immunocompetent mice with antibody 74.5.2 and  
260 orally infected them with wild-type *C. albicans*. Control mice were treated with either mouse IgG  
261 or gefitinib. We found that after 1 day of infection, mice treated with the anti-gC1qR antibody  
262 had the same oral fungal burden as mice that received the control IgG (Fig. 6B). The level of  
263 myeloperoxidase (MPO), a measure of phagocyte accumulation (50, 51), in the oral tissues was  
264 also not changed by administration of the anti-gC1qR antibody (Fig. 6C). As expected (16),  
265 treatment with gefitinib significantly reduced both the oral fungal burden and oral MPO levels in  
266 the mice. These results suggest the gC1qR is dispensable for mediating the epithelial cell  
267 response to *C. albicans* in mice.

268           To investigate these results further, we analyzed the capacity of the anti-gC1qR antibody  
269 74.5.2 to inhibit *C. albicans*-induced phosphorylation of EGFR in mouse epithelial cells. We  
270 determined that the antibody had no effect, although EGFR phosphorylation was inhibited by  
271 gefitinib (Fig. 6D). Thus in mice, gC1qR is likely dispensable for *C. albicans*-induced EGFR  
272 activation, unlike in humans.

273

## 274 **DISCUSSION**

275           In this work, we determined that EGFR interacts with numerous proteins that function in  
276 a multitude of different signaling pathways, consistent with the concept that this receptor is a  
277 key regulator of epithelial cell physiology. The proteomics data indicate that EGFR is part of a  
278 multi-protein complex that contains E-cadherin, EphA2, and HERS. This result provides an  
279 explanation for our previous findings that these three receptors function with EGFR in the same  
280 pathway to induce the epithelial cell response to *C. albicans* (11, 13, 18).

281           Previously, we had determined that *C. albicans* activates the aryl hydrocarbon receptor,  
282 leading to the de-repression of src family kinases that phosphorylate EGFR (14). However, the  
283 member(s) of the src family responsible for this phosphorylation remained unknown. The current

284 finding indicate that Lyn and Yes associate with EGFR suggests that these two members of the  
285 src family kinases phosphorylate EGFR in response to *C. albicans* infection.

286 EGFR mediates the endocytosis of *C. albicans* by activating the cortactin-clathrin  
287 pathway, leading to the formation of pseudopods that engulf the organism and pull it into the  
288 epithelial cell (21, 22). The proteomics data were consistent with this mechanism and suggest  
289 that the arp2/3 complex mediates the rearrangement of actin filaments that induce pseudopod  
290 formation. Although the arp2/3 complex can be activated by the Wiskott-Aldrich syndrome  
291 protein (WASP) or vasodilator-stimulated phosphoprotein (VASP) (52-54), neither protein was  
292 consistently associated with EGFR in cells infected with *C. albicans*. Thus, it remains to be  
293 determined how EGFR activates this complex

294 We also established roles for four key proteins that interact with EGFR and induce the  
295 epithelial cell response to *C. albicans*, none of which had been previously implicated in the  
296 response to fungal pathogens. Three of these proteins, WBP2, IFITM3, and gC1qR, were  
297 required for maximal epithelial cell endocytosis of the fungus, suggesting that they function  
298 along with EGFR to orchestrate this process.

299 Perhaps not surprisingly, siRNA knockdown of these proteins had pleiotropic effects on  
300 the production of IL-1 $\beta$  and IL-8 by the infected oral epithelial cells, as these are regulated by a  
301 myriad of inflammatory and infectious signals. Knockdown of WBP2 led to reduced cellular  
302 levels of EGFR and thus resulted in decreased production of IL-1 $\beta$  and IL-8, similar to siRNA  
303 knockdown of EGFR itself. This result is consistent with a report that WBP2 is required for  
304 normal EGFR expression (32). Knockdown of TOLLIP inhibited the production of IL-8 but had  
305 no effect on IL-1 $\beta$  secretion. Although TOLLIP is generally considered to be a negative regulator  
306 of the host inflammatory response (35-37), our results suggest that it has the capacity to be a  
307 positive regulator of IL-8 production in epithelial cells infected with *C. albicans*. While siRNA  
308 knockdown of IFITM3 inhibited *C. albicans* endocytosis, it stimulated the production of both IL-  
309 1 $\beta$  and IL-8. IFITM3 has been found to enhance the degradation of activated EGFR in

310 pulmonary epithelial cells (38). Although knockdown of IFITM3 in oral epithelial cells had no  
311 detectable effect on total cellular EGFR levels, our results indicate that IFITM3 is a positive  
312 regulator of epithelial cell endocytosis of *C. albicans* but a negative regulator of cytokine  
313 production.

314 Our in-depth analysis of gC1qR enabled us to develop a model for how this protein  
315 interacts with EGFR and is required for EGFR signaling in response to *C. albicans* infection  
316 (Fig. 7). We propose that gC1qR and EGFR form part of a complex that interacts either directly  
317 or indirectly with the *C. albicans* Als3 invasin. According to this model, gC1qR is required for  
318 Als3 to maximally interact with and activate EGFR. In turn, EGFR is required for *C. albicans* to  
319 interact optimally with gC1qR. This arrangement is deduced from the proteomics analysis,  
320 which showed that gC1qR was associated with EGFR, and from pull-down assays, which  
321 indicated that blocking gC1qR inhibited the physical association of EGFR with *C. albicans* and  
322 that blocking EGFR in turn reduced the association of gC1qR with the organism. Although the  
323 proteomics analysis suggested that gC1qR associated with EGFR constitutively, the proximity  
324 ligation assay showed that the gC1qR-EGFR complex was enriched in regions surrounding *C.*  
325 *albicans* hyphae that expressed Als3. Previously, we found that Als3 is required for *C. albicans*  
326 to induce maximal EGFR activation (13). Accordingly, these results suggest that gC1qR is a key  
327 co-factor that is required for Als3 to activate EGFR (Fig. 7).

328 Although EGF also activates EGFR, we found that it does so independently of gC1qR.  
329 These findings differ from Kim et al. (46), who reported that inhibition of gC1qR in the A549 lung  
330 cancer cell line blocked activation of EGFR induced by EGF. Possible reasons for these  
331 discrepant results include differences in the cell lines and the anti-gC1qR antibodies that we  
332 used as compared those used by the other investigators. We also found that blocking gC1qR  
333 did not prevent activation of EGFR by candidalysin. Collectively, these data suggest that soluble  
334 stimuli activate EGFR in oral epithelial cells independently of gC1qR whereas *C. albicans*  
335 hyphae that express Als3 activate EGFR by a mechanism that requires gC1qR.



336           When investigating the role of gC1qR in mediating the production of IL-1 $\beta$  and IL-8 in  
337 response to *C. albicans* infection, we observed that treatment with the anti-gC1qR antibody  
338 inhibited the production of these inflammatory mediators whereas siRNA knockdown of gC1qR  
339 had the opposite effect. The likely explanation for these results is that inhibiting just extracellular  
340 gC1qR has a different effect than decreasing the levels of intracellular gC1qR. Our experiments  
341 with the anti-gC1qR antibody indicate that it inhibits the production of proinflammatory mediators  
342 by blocking *C. albicans*-induced activation of EGFR and EphA2. These latter two receptors play  
343 key roles in stimulating the production of cytokines and chemokines in response to *C. albicans*  
344 (15, 16). Why reducing the levels of intracellular gC1qR stimulates the production of  
345 proinflammatory mediators in oral epithelial cells is unknown. However, knockdown of gC1qR in  
346 macrophages is known to reduce the cytoplasmic levels of tumor necrosis factor  $\alpha$  inducible  
347 protein 3 (TNFAIP3, A20), a suppressor of NF- $\kappa$ B activation, thereby stimulating the production  
348 of proinflammatory mediators (55). We speculate that a similar process may occur when gC1qR  
349 is knocked down in oral epithelial cells.

350           Inhibition of EGFR in mice with oropharyngeal candidiasis reduces oral fungal burden  
351 and inflammation (16). Thus, we were surprised to determine that treating mice with the anti-  
352 gC1qR antibody had no effect on either of these parameters. Subsequently, we found that this  
353 antibody did not block *C. albicans*-induced phosphorylation of EGFR in primary oral epithelial  
354 cells. The probable explanation for these results is that gC1qR is dispensable for the fungus to  
355 activate EGFR in mouse epithelial cells. A less likely possibility is that the anti-gC1qR antibody  
356 used in the mouse studies did not block the association of gC1qR with EGFR in mouse cells,  
357 even though it did so in human cells.

358           gC1qR is exploited by multiple microbial pathogens. gC1qR mediates the adherence of  
359 *Plasmodium falciparum* and *Staphylococcus aureus* to vascular endothelial cells and the  
360 adherence of *Bacillus cereus* spores to epithelial cells (44, 45, 56, 57). It is also an epithelial cell  
361 receptor for the *L. monocytogenes* internalin B (InlB) invasin and mediates the endocytosis of

362 the organism (28). Because gC1qR lacks a transmembrane sequence, it is thought to induce  
363 endocytosis by signaling via another cell surface receptor. In the case of *L. monocytogenes*, the  
364 second receptor appears to be Met, the hepatocyte growth factor receptor (58). In some cell  
365 lines, gC1qR and Met have been found to function cooperatively to mediate the endocytosis of  
366 this bacterium (59). Although the primary amino acid sequence of the *C. albicans* Als3 invasin  
367 shares no homology with InIB, we demonstrate that Als3 also interacts with gC1qR. Instead of  
368 Met, EGFR is the second receptor that associates with gC1qR and transduces the epithelial cell  
369 response *C. albicans*. Not only is gC1qR required for EGFR-mediated endocytosis of the  
370 fungus, but it is also necessary for the maximal epithelial cell inflammatory response to this  
371 organism. We have identified additional host cell receptors for *C. albicans* including E-cadherin,  
372 HER2, the platelet-derived growth factor BB, the aryl hydrocarbon receptor, and gp96 (12-14,  
373 18, 60). Whether gC1qR also interacts with these receptors remains to be determined.

374

## 375 **MATERIALS AND METHODS**

376 **Epithelial cells and fungal strains.** The OKF6/TERT-2 oral epithelial cell line was  
377 provided by J. Rheinwald (Dana-Farber/Harvard Cancer Center, Boston, MA) and cultured as  
378 outlined previously (14, 17). Primary oral mucosal epithelial cells from BALB/c mice were  
379 obtained from Cell Biologics Inc. and grown following the manufacturer's instructions. NIH/3T3  
380 cells that expressed human EGFR and HER2 were provided by Nadege Gaborit (Institut de  
381 Recherche en Cancérologie de Montpellier, France) and grown as described (48). The *C.*  
382 *albicans* wild-type strain SC5314 and the *als1* $\Delta/\Delta$ , *als3* $\Delta/\Delta$ , and *ece1* $\Delta/\Delta$  mutants (16) were  
383 grown in yeast extract, peptone, dextrose (YPD) broth in a shaking incubator at 30°C for 18 h,  
384 after which the cells were pelleted by centrifugation and washed twice with PBS. Yeast cells  
385 were suspended in PBS, diluted and counted with a hemacytometer.

386 **Immunoprecipitation.** OKF6/TERT-2 oral epithelial cells were grown to confluency in  
387 75 cm<sup>2</sup> tissue culture flasks and the culture medium was changed to KSF medium without

388 supplements (Thermo Fisher Scientific; # 17005042) the night before the experiment. The next  
389 morning, cells were incubated for 90 min with supplement-free KSF medium containing  $10^8$  C.  
390 *albicans* yeast cells or for 5 min with 50 ng/ml EGF. Cell incubated with fresh supplement-free  
391 KSF alone were processed in parallel as a negative control. After incubation, the medium was  
392 aspirated and the cells were washed once with ice cold PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (PBS<sup>++</sup>). The  
393 proteins were cross-linked by incubation with 1% paraformaldehyde for 10 min. After rinsing the  
394 cells twice with ice cold PBS<sup>++</sup>, they were detached from the culture flasks with a cell scraper  
395 and collected by centrifugation. The cell pellet was lysed with 5.8% octyl- $\beta$ -D-glucopyranoside  
396 (VWR; # 97061-760) with sonication. The lysate was clarified by centrifugation and then  
397 precleared with protein A/G magnetic beads (Thermo Fisher Scientific, # PI88802,) for 30 min at  
398 4°C. The precleared cell lysates were incubated with an anti-EGFR antibody (Santa Cruz  
399 Biotechnology; # SC-101, clone R-1), for 1 h at 4°C, and precipitated with protein A/G magnetic  
400 beads for 2 h at 4°C. After washing the beads 3 times with 1.5% octyl- $\beta$  –D-glucopyranoside, the  
401 proteins were eluted with 8M Urea, stored at -80°C.

402 **Mass Spectrometry.** The protein samples were treated with Tris (2-carboxyethyl)  
403 phosphine hydrochloride (Thermo Fisher Scientific; # 20491) and 2-chloroacetamide (cat#  
404 154955) for reduction and alkylation, respectively. Tris buffer (0.1 M, pH 8.5) was added to each  
405 sample decrease the urea concentration to final 2 M. After dilution, the samples were digested  
406 with lysyl endopeptidase (Fujifilm Wako Chemicals; # 125-05061) and trypsin (Thermo Fisher  
407 Scientific; # 90058) for 20 h at 37°C. After quenching the reaction by adjusting the pH to 3, the  
408 digested samples were desalted using C18 columns (Thermo Fisher Scientific; # 89870) and  
409 then dried by vacuum centrifuge.

410 For the LC-MS/MS analysis, the samples were dissolved in 0.2% formic acid (FA)  
411 solution. The analysis was performed using an EASY-nLC 1000 (Thermo Fisher Scientific  
412 connected to a Q Exactive Orbitrap Mass Spectrometers (Thermo Fisher Scientific). Solvent A  
413 consisted of 99.9% H<sub>2</sub>O and 0.1% FA, and solvent B consisted of 19.9% H<sub>2</sub>O, 80% acetonitrile,

414 and 0.1% FA. Each sample was loaded onto an Easy Spray Column (25 cm x 75  $\mu$ m, 2  $\mu$ m  
415 C18, ES802, Thermo Fisher Scientific) and separated over 90 min at a flow rate of 0.5  $\mu$ L/min  
416 with the following gradient: 2-35% B (75 min), 35-85% B (5 min), and 85% B (10 min). The full  
417 MS scan was acquired at 70,000 resolution with a scan range of 350-2000 m/z, the automatic  
418 gain control target was  $1 \times 10^6$ , and the maximum injection time was 100 ms. The dd-MS2 scan  
419 was acquired at 17,500 resolution with a scan range of 200-2000 m/z, the automatic gain  
420 control target was  $5 \times 10^4$ , the maximum injection time was 64 ms, and the isolation window was  
421 2.0 m/z. The proteomic data processing was performed using Proteome Discoverer 1.4 (Thermo  
422 Fisher Scientific) and the Sequest HT Search Engine. The search allowed for a precursor mass  
423 tolerance of 10 ppm, a minimum peptide length of 6, and a minimum peptide sequence number  
424 of 1.

425 **Proximity ligation assay.** To determine if the selected proteins associated with EGFR  
426 in intact epithelial cells, OKF6/TERT-2 cells were grown to confluency on fibronectin coated  
427 coverslips. The cells were infected with  $3 \times 10^5$  *C. albicans* yeast in supplement-free KSF  
428 medium. After 90 min, the medium was aspirated and the cells were fixed with 4%  
429 paraformaldehyde for 10 min. The coverslips were washed 3 times with PBS, after which the  
430 epithelial cells were permeabilized with 0.1% triton X-100 in PBS for 20 min. The association  
431 between EGFR and WBP2, GBP6, TOLLIP, EEA1, DSG3, IFITM3, or gC1qR, was detected  
432 using the Duolink in Situ Red Starter Kit Mouse/Rabbit (Sigma-Aldrich; #DUO92101-1kit)  
433 according to the manufacturer's instruction. The antibodies used were rabbit anti-EGFR  
434 (Genetex; # GTX121919, clone N1-2), mouse anti-EGFR (Santa Cruz Biotechnology # SC-101,  
435 clone R-1), anti-WBP2 (Santa Cruz Biotechnology; # SC-514247, clone D-12), anti-GBP6  
436 (Sigma-Aldrich; # HPA027744), anti-TOLLIP (Proteintech # 117315-1), anti-EEA1 (Santa Cruz  
437 Biotechnology; # SC-365652 clone E-8), anti-DSG3 (Santa Cruz Biotechnology; # SC-53487,  
438 clone 5g11), anti-IFITM3 (Proteintech; # 11714-1), and gC1qR (Santa Cruz Biotechnology; #

439 SC-23885, clone 74.5.2). The cells were imaged by confocal microscopy and z stacks of the  
440 images were constructed using the Leica Application Suite X software (Leica).

441 **siRNA.** To knock down the levels of selected proteins, the OKF6/TERT-2 cells were  
442 grown in 6-well tissue culture plates to 50-80% confluency overnight. The next morning, the  
443 cells were transfected with EGFR (Santa Cruz Biotechnology; # SC-29301), WBP2 (Santa Cruz  
444 Biotechnology; # SC93955), TOLLIP (Dharmacon; # L-016930-00-0005), IFITM3 (Santa Cruz  
445 Biotechnology; # SC-97053), gC1qR (Santa Cruz Biotechnology; # SC-42880), or control  
446 (Qiagen; # 1027281) siRNA using Lipofectamine RNAiMAX (Invitrogen; #13778150) following  
447 the manufacturer's instructions. After 24 h post-transfection, the cells were seeded onto  
448 fibronectin coated coverslips and incubated for another 24 h before use in the experiments. The  
449 extent of protein knockdown was determined by immunoblotting and total loading was detected  
450 by probing the blots with an anti-GAPDH antibody (Cell Signaling; #5174, clone D16H11).

451 **Epithelial cell endocytosis and cell-association.** Our standard differential  
452 fluorescence assay was used to measure the number of *C. albicans* cells that were  
453 endocytosed by and cell-associated with the OKF6/TERT-2 epithelial cells, primary mouse  
454 epithelial cells, and NIH/3T3 cells (13, 18, 22). The host cells were infected with  $10^5$  *C. albicans*  
455 yeast cells. To ensure similar levels of endocytosis among the different host cells, the incubation  
456 time was 2.5 h for OKF6/TERT-2 cells, 2 h for the mouse oral epithelial cells and 1.5 h for the  
457 NIH/3T3 cells. In the siRNA experiments, the transfected epithelial cells were seeded onto  
458 fibronectin-coated glass coverslips 24 h before infection. For experiments using gefitinib (1  $\mu$ M;  
459 Selleck Chem, Inc; # S1025) or the anti-gC1qR antibodies (10  $\mu$ g/ml), the inhibitor or antibodies  
460 were added to the host cells 1 h prior to infection and they remained in the medium for the  
461 duration of the experiment. All experiments performed in triplicate at least three times.

462 **Lentivirus construction and production and host cell transduction.** The transfer  
463 vectors (pLenti-EF1A-EGFP-Blast or pLenti-EF1A-hC1QBP[NM\_001212.4]-Blast) were  
464 constructed by cloning eGFP or hC1QBP[NM\_001212.4] into pLenti-Cas9-Blast (Addgene; #

465 52962) at the BamHI and XbaI sites. The virus was produced by transfecting HEK293T cells  
466 with plasmid psPAX2 (Addgene; # 12260), plasmid pCMV-VSVG (Addgene; # 8454), and  
467 transfer vector (pLenti-EF1A-EGFP-Blast or pLenti-EF1A-hC1QBP[NM\_001212.4]-Blast ) using  
468 the X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich; # 6365787001) according to the  
469 manufacturer's instructions. The supernatant containing the virus was collected at 60 h post-  
470 transfection, passed through a 0.45µm PVDF filter and stored at 4°C (short-term) or -80°C (long-  
471 term).

472 For transduction, the NIH/3T3 mouse fibroblast cells (untransformed control cells or the  
473 human EGFR/hHER2 transformed cell line (48)) were seeded into a 6-well plate in DMEM+10%  
474 bovine calf serum. The cells were transduced with lentivirus in the presence of 0.5µg/ml  
475 polybrene (Santa Cruz Biotechnology; #SC134220). The plates were centrifuged at 1000g for  
476 30 min and then incubated at 37°C in 5%CO<sub>2</sub> overnight. The next morning, the cells were  
477 transferred to 10 cm diameter tissue culture dishes. Two days post transduction, 10 µg/ml of  
478 blasticidin (Gibco; # A1113903) was added to the medium to select for transduced cells and  
479 selection was maintained for 7 days. The successful transduction of eGFP was determined by  
480 fluorescent microscopy and transduction of hC1QBP (gC1qR) was verified via immunoblotting  
481 with an anti-gC1qR antibody (clone 74.5.2) and an anti-EGFR antibody (Cell Signaling  
482 Technology; # 4267, clone 38B1).

483 **Receptor phosphorylation.** Analysis of the phosphorylation of EGFR and EphA2 was  
484 performed as previously described (11, 16). Briefly, OKF6/TERT-2 cells were seeded onto 24-  
485 well tissue culture plates and incubated overnight in supplement-free KSF medium. The next  
486 morning, the cells were treated with gefitinib, an anti-gC1qR antibody (clone 74.5.2) or control  
487 mouse IgG (R&D systems; #MAB002) for 1 h. The cells were then stimulated with 10<sup>6</sup> C.  
488 *albicans* yeast cells for 90 min, 40 µM candidalysin (Biomatik) for 5 min, or 1 ng/ml EGF for 5  
489 min in the presence of the inhibitor or antibody. Next, the cells were lysed with 100µl 2X SDS  
490 loading buffer in the present of phosphatase inhibitor cocktail (Sigma-Aldrich), protease inhibitor

491 cocktail (Sigma-Aldrich), and PMSF (Sigma-Aldrich). After the samples were denatured at 90°C  
492 for 2 min, they were clarified by centrifugation. The proteins were separated by SDS-PAGE and  
493 transferred to PVDF membranes. Phosphorylated EGFR (Tyr 1068) was detected with a  
494 phosphospecific antibody (Cell Signaling Technology; # 2234) and enhanced  
495 chemiluminescence. Total EGFR was detected with an anti-EGFR antibody Cell Signaling  
496 Technology; # 4267). Phosphorylation of EphA2 (Ser 897) was detected with a phosphospecific  
497 anti-EphA2 antibody (Cell Signaling Technology; #6347, clone D9A1) and total EphA2 was  
498 detected with an anti-EphA2 antibody (Cell Signaling Technology; # 6997, clone D4A2). The  
499 phosphorylation of EGFR in mouse oral epithelial cells was determined similarly except that  
500 total EGFR was detected with anti-EGFR antibody from Santa Cruz Biotechnology (#  
501 SC373746, clone A-10). All experiments were repeated at least three times.

502 ***C. albicans* association with epithelial cell gC1qR and EGFR.** The capacity of anti-  
503 gC1qR and anti-EGFR antibodies to block the association of *C. albicans* with gC1qR and EGFR  
504 was determined using our previously described method (61). Confluent OKF6/TERT-2 epithelial  
505 cells in a 6-well tissue culture plate were switched to supplement-free KSF medium the night  
506 before the experiment. The next morning, the epithelial cells were incubated with 10 µg/ml of  
507 control mouse IgG, an anti-gC1qR antibody (clone 74.5.2), or an anti-EGFR antibody  
508 (cetuximab; Lilly) for 1 h, and then infected with  $6 \times 10^6$  *C. albicans* blastospores. After 90 min of  
509 infection, the cells were detached with a cell scraper and collected by centrifugation. The  
510 epithelial cells were lysed by incubation with 5.8% n-octyl-β glucopyranoside in PBS<sup>++</sup> in the  
511 present of protease inhibitors and PMSF on ice for 1 h. The samples were centrifuged at 5,000  
512 g for 1 min and the supernatants containing the total cell lysates were collected. The pellets,  
513 which contained the intact *C. albicans* cells and the epithelial cells proteins that were associated  
514 with them, were washed three times with cold 1.5% n-octyl-β D glucopyranoside in PBS<sup>++</sup>  
515 containing protease inhibitors. The proteins that remained associated with the *C. albicans* cells  
516 were eluted by incubation with 6M urea for 30 min on ice. After pelleting the organisms by

517 centrifugation, the eluted proteins in the supernatants were separated by SDS PAGE. The  
518 presence of gC1qR and EGFR in the eluates was determined by immunoblotting with an anti  
519 gC1qR antibody (Abcam; # ab24733, clone 60.11) and an anti EGFR antibody (clone 38B1).  
520 The experiment was repeated four times.

521 **Cytokine and chemokine measurement.** To measure the production of IL-1 $\beta$  and IL-8  
522 by oral epithelial cells, the OKF6/TERT-2 cells were grown in 48-well tissue culture plates  
523 overnight in supplement-free KSF and then infected with  $6.25 \times 10^5$  *C. albicans* yeast cells for 8  
524 h. The conditioned medium was collected and clarified by centrifugation. The levels of IL-1 $\beta$  and  
525 IL-8 in the conditioned medium were measured by ELISA using the human IL-1 $\beta$  DuoSet (R&D  
526 Systems; # DY20105) and the human IL-8 set (BD Biosciences; # BD555244).

527 The production of IL-1 $\beta$ , IL-8, IL-1 $\alpha$ , and GM-CSF by the epithelial cells was measured  
528 similarly except that the epithelial cells were grown in 24-well tissue culture plates and infected  
529 with  $1.5 \times 10^6$  *C. albicans* yeast cells. The levels of the inflammatory mediators were measured  
530 by Luminex Multiplex (R&D systems; # LXSAHM-06). The experiments were repeated three  
531 times in duplicate.

532 **Mouse experiments.** Our standard immunocompetent mouse model of oropharyngeal  
533 candidiasis was used to assess the effects of an anti-gC1qR antibody and gefitinib the infection  
534 (11, 16, 62). Immunocompetent Balb/c were administered 100  $\mu$ g of the anti-gC1qR antibody  
535 (clone 74.5.2) by intraperitoneal injection on day -1 relative to infection. Control mice were  
536 injected with a similar amount of mouse IgG. Gefitinib was administered by adding the drug to  
537 powdered chow to a final concentration of 200 parts per million, starting at day -2 relative to  
538 infection and continuing throughout the experiment. On the day of the infection, the mice were  
539 sedated and a calcium alginate swab saturated with  $2 \times 10^7$  *C. albicans* yeast cells was placed  
540 sublingually for 75 min. After 1 day of infection, the mice were sacrificed and the tongues were  
541 excised, weighed, and homogenized. The oral fungal burden was determined by quantitative  
542 culture of an aliquot of the homogenates and the level of MPO in the homogenates was



543 determined by commercial ELISA (Hycult Biotech; # HK210-02). The experiment was repeated  
544 twice using 4-5 mice per condition and the results were combined.

545 **Statistical analysis.** The *in vitro* data were analyzed using one-way analysis of variance  
546 with Dunnett's test for multiple comparisons. The mouse data were analyzed by the Kruskal-  
547 Wallis test with Dunn's test for multiple comparisons. *P* values < 0.05 were considered to be  
548 significant.

549

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**Table 1.** List of proteins that had increased association with EGFR in response to infection with *C. albicans*. Shown are individual results from 3 biological replicates for epithelial cells infected with *C. albicans* and 2 biological replicates from epithelial cells incubated with EGF. Values are the ratio of the amount of protein detected in cells exposed to *C. albicans* or EGF relative to unstimulated control cells. Inf indicates proteins that were undetectable in unstimulated control cells.

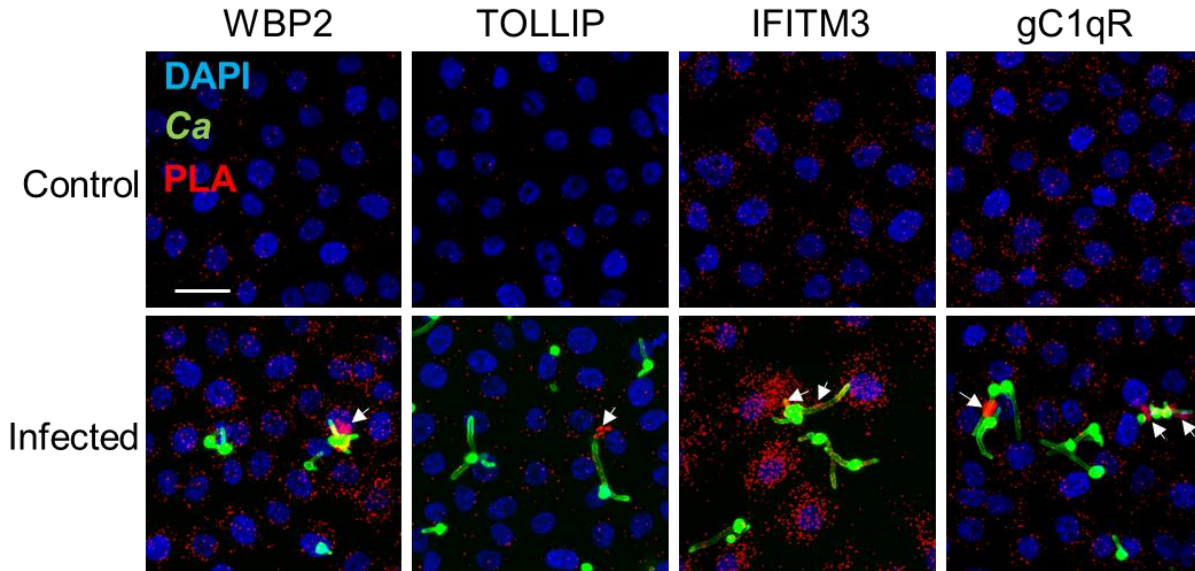
Accession Number	Gene	Protein	<i>C. albicans</i> -1	<i>C. albicans</i> -2	<i>C. albicans</i> -3	EGF-1	EGF-2
K7EIJ0	WBP2	WW domain-binding protein 2	Inf	Inf	Inf	-	-
B4DRS8	GBP6	cDNA FLJ54753, highly similar to guanylate binding protein family, member 6	Inf	Inf	Inf	-	-
B4DUI3	EIF3J	Eukaryotic translation initiation factor 3 subunit J	2.63	Inf	Inf	-	-
F6U1T9	PPP3R1	Calcineurin subunit B type 1	Inf	2.15	Inf	0.00	-
Q9H0E2	TOLLIP	Toll-interacting protein	Inf	Inf	Inf	Inf	-
P35354	PTGS2	Prostaglandin G/H synthase 2	3.29	Inf	Inf	-	Inf
Q15075	EEA1	Early endosome antigen 1	15.80	12.72	3.11	6.38	1.83
P32926	DSG3	Desmoglein-3	3.30	5.99	2.08	0.61	0.76
Q01628	IFITM3	Interferon-induced transmembrane protein 3	3.17	3.18	2.68	0.00	0.91
Q14165	MLEC	Malectin	2.10	2.46	2.57	1.45	1.61
P02787	TF	Serotransferrin	4.06	2.05	2.24	2.30	1.32
P21281	ATP6V1B2	V-type proton ATPase subunit B, brain isoform	4.10	7.36	Inf	4.03	Inf
P36543	ATP6V1E1	V-type proton ATPase subunit E 1	2.47	Inf	2.26	Inf	1.94

**Table 2.** List of proteins that had constitutive association with EGFR in response to infection with *C. albicans*. Shown are individual results from 3 biological replicates for epithelial cells infected with *C. albicans* and 2 biological replicates from epithelial cells incubated with EGF. Values are the ratio of the amount of protein detected in cells exposed to *C. albicans* or EGF relative to unstimulated control cells. Inf indicates proteins that were undetectable in unstimulated control cells.

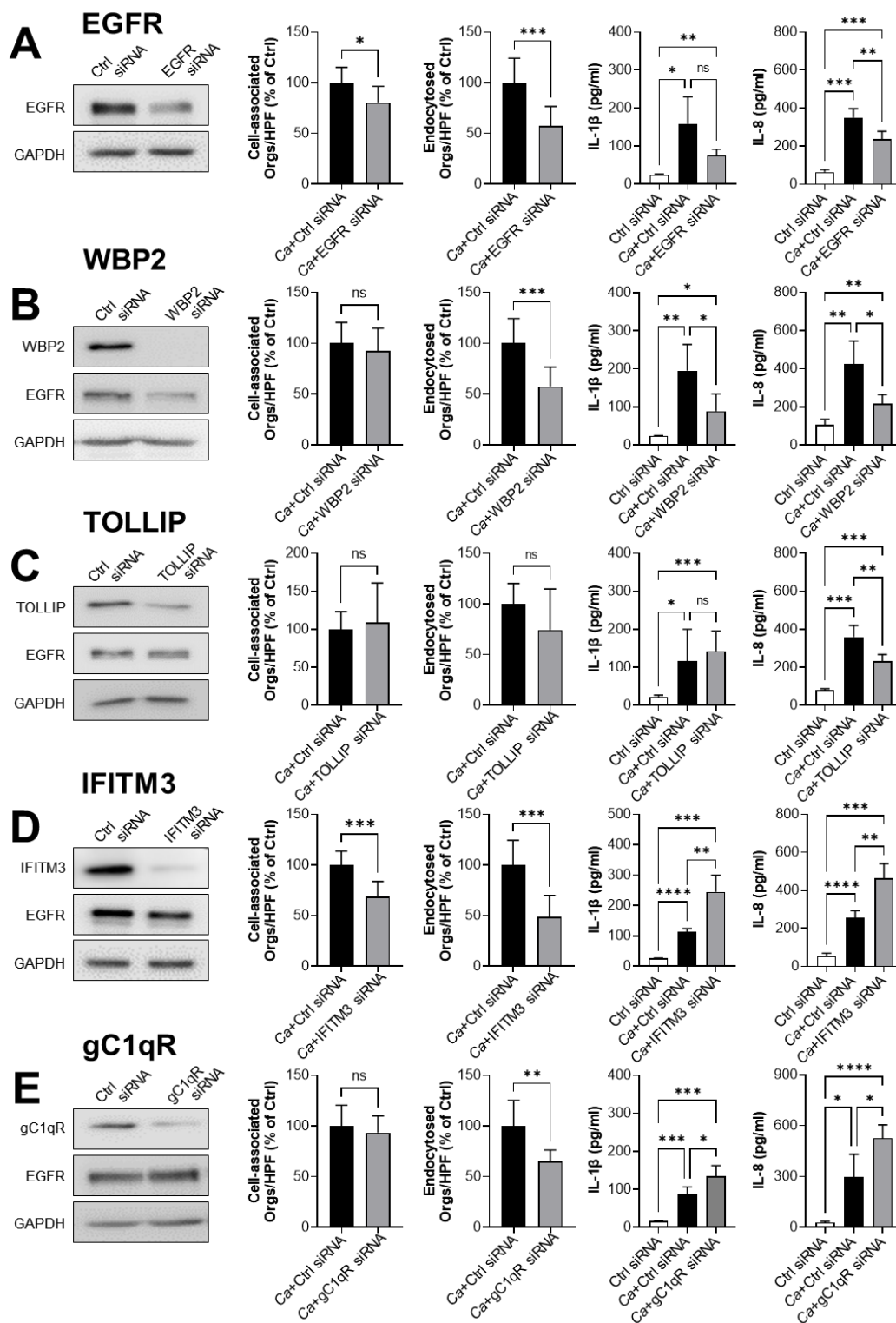
Function/location	Accession Number	Gene	Protein	<i>C. albicans</i> -1	<i>C. albicans</i> -2	<i>C. albicans</i> -3	EGF-1	EGF-2
Receptor	P29317	EPHA2	Ephrin type-A receptor 2	1.65	0.68	0.44	0.58	0.33
	D3XNU5	CDH1	E-cadherin 1	0.48	0.79	0.64	0.43	0.42
	P04626-4	ERBB2	Isoform 4 of ErbB-2 (HER2)	0.63	1.57	0.93	1.04	1.02
Src tyrosine kinase	P07948-2	LYN	Isoform 2 of Tyrosine-protein kinase Lyn	6.58	0.68	0.63	0.57	0.58
	B2RA70	YES1	Highly similar to Homo sapiens v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	4.23	0.65	0.53	0.75	0.46
Lipid rafts	Q03135	CAV1	Caveolin-1	1.62	1.22	0.87	1.19	0.83
	P51636	CAV2	Caveolin-2	Inf	Inf	0.76	Inf	0.46
	O75955	FLOT1	Flotillin-1	1.64	1.01	0.97	0.90	0.80
	Q6FG43	FLOT2	Flotillin-2	1.34	1.04	0.90	0.93	0.68
	P62330	ARF6	ADP-ribosylation factor 6	1.18	0.68	0.72	0.78	0.53
	P61586	RHOA	RhoA	1.90	0.96	0.66	0.89	0.77
Clathrin internalization pathway	Q00610-2	CLTC	Isoform 2 of Clathrin heavy chain 1	1.69	0.88	0.80	2.22	1.12
	P09496-2	CLTA	Isoform Non-brain of Clathrin light chain A	4.64	1.05	1.61	2.72	1.55
	P09497-2	CLTB	Isoform Non-brain of Clathrin light chain B	Inf	0.94	1.20	2.03	1.44
	Q14247	CTTN	Cortactin	3.56	1.31	0.90	0.39	0.39
	B5BU72	PICALM	Phosphatidylinositol-binding clathrin assembly protein isoform 2	1.61	0.75	1.12	2.18	1.44
Actin-related protein 2/3 complex	P61160	ACTR2	Actin-related protein 2	2.96	1.12	0.94	0.61	0.50
	P61158	ACTR3	Actin-related protein 3	1.93	1.17	0.55	0.43	0.49
	O15143	ARPC1B	Actin-related protein 2/3 complex subunit 1B	2.14	1.75	1.06	0.58	0.36

	O15144	ARPC2	Actin-related protein 2/3 complex subunit 2	4.10	1.09	1.31	0.75	0.74
	B2R4D5	ARPC3	Highly similar to Homo sapiens actin related protein 2/3 complex, subunit 3	2.45	1.73	0.92	0.39	0.48
	P59998	ARPC4	Actin-related protein 2/3 complex subunit 4	2.15	1.03	0.78	0.65	0.41
	B3KPC7	ARPC5	Actin-related protein 2/3 complex subunit 5	1.39	1.30	0.83	0.00	0.52
Actin binding protein	P12814	ACTN1	Alpha-actinin-1	1.44	0.69	0.91	2.21	0.54
	B4DP09	CNN3	Highly similar to Calponin-3	2.24	Inf	1.02	Und	0.79
	P23528	CFL1	Cofilin-1	1.72	0.70	0.58	1.08	0.88
	Q16643	DBN1	Drebrin	1.51	1.39	1.08	0.00	0.00
	P21333-2	FLNA	Isoform 2 of Filamin-A	3.46	1.02	1.02	0.61	0.55
	O75369-2	FLNB	Isoform 2 of Filamin-B	4.18	1.08	1.38	0.74	0.61
Regulator of actin dynamics	P62745	RHOB	RhoB	1.96	0.56	0.89	0.76	0.86
	Q5JR08	RHOC	RhoC	1.80	0.96	0.80	0.85	0.86
	P84095	RHOG	RhoG	1.30	0.78	5.60	0.74	0.40
	P63000	RAC1	Ras-related C3 botulinum toxin substrate 1	1.98	0.70	0.90	0.85	0.71
	P15153	RAC2	Ras-related C3 botulinum toxin substrate 2	1.87	0.74	1.30	0.73	0.83
	P62826	RAN	Ran	1.16	0.73	0.68	0.58	0.98
	P60953	CDC42	Cell division control protein 42	1.49	0.61	1.00	0.88	0.86
Small GTPase	P01112	HRAS	HRas	2.55	0.37	0.99	0.40	0.96
	P01116-2	KRAS	KRas	3.46	0.33	0.83	0.78	0.84
	P01111	NRAS	NRas	3.06	0.40	0.83	0.56	0.82
	P10301	RRAS	Ras-related protein R-Ras	2.36	0.98	1.00	0.34	1.01
	P62070	RRAS2	Ras-related protein R-Ras2	1.66	0.59	0.83	0.31	0.64
Guanine nucleotide exchange factor	Q86TW5	ARHGEF16	Rho guanine nucleotide exchange factor 16	2.96	0.99	1.10	1.17	1.04
	P18754	RCC1	Regulator of chromosome condensation	2.46	Inf	0.58	Inf	1.00



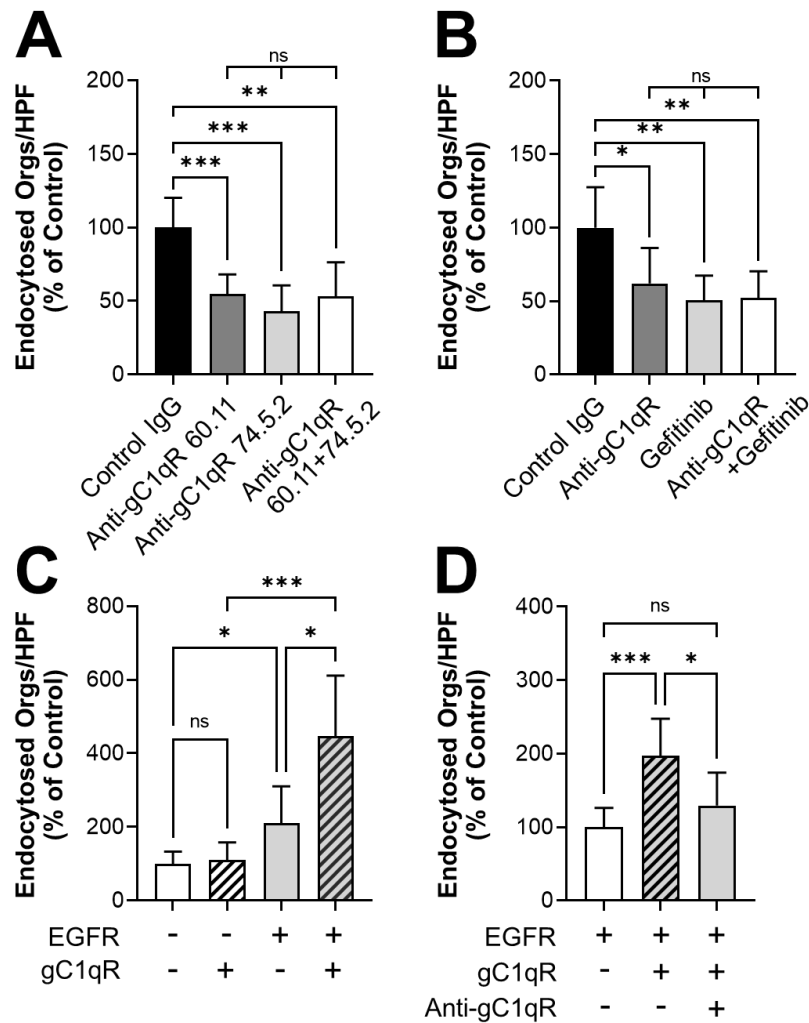


**Fig. 1** Proximity ligation assays showing the physical association of the epidermal growth factor receptor (EGFR) with WW domain-binding protein 2 (WBP2), toll-interacting protein (TOLLIP), interferon-induced transmembrane protein 3 (IFITM3), and the globular C1q receptor (gC1qR) in the OKF6/TERT-2 oral epithelial cell line. The epithelial cells were incubated with either medium alone (top) or infected with *C. albicans* (bottom) for 90 min. Red spots indicate the regions where the indicated proteins associate with EGFR. Arrows indicate the accumulation of the proteins around *C. albicans* hyphae. Results are representative of three independent experiments. Scale bar 25  $\mu$ m.

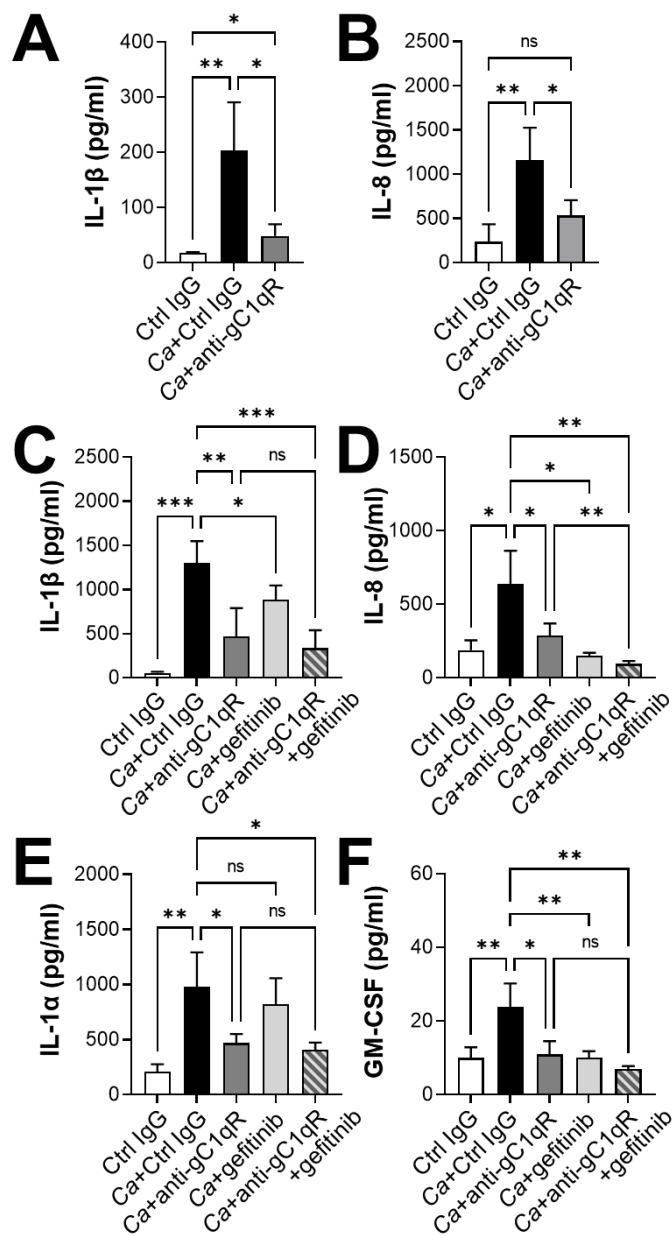


**Fig. 2** Functional analysis of proteins that interact with EGFR. Oral epithelial cells were transfected with EGFR (A), WBP2 (B), TOLLIP (C), IFITM3 (D), and gC1qR (E) siRNA. For each siRNA, the extent of protein knockdown and its effects on the number of cell-associated organisms, the number of endocytosed organisms, IL-1 $\beta$  secretion, and IL-8 secretion were determined. The graphs show the mean  $\pm$  SD of three independent experiments, each

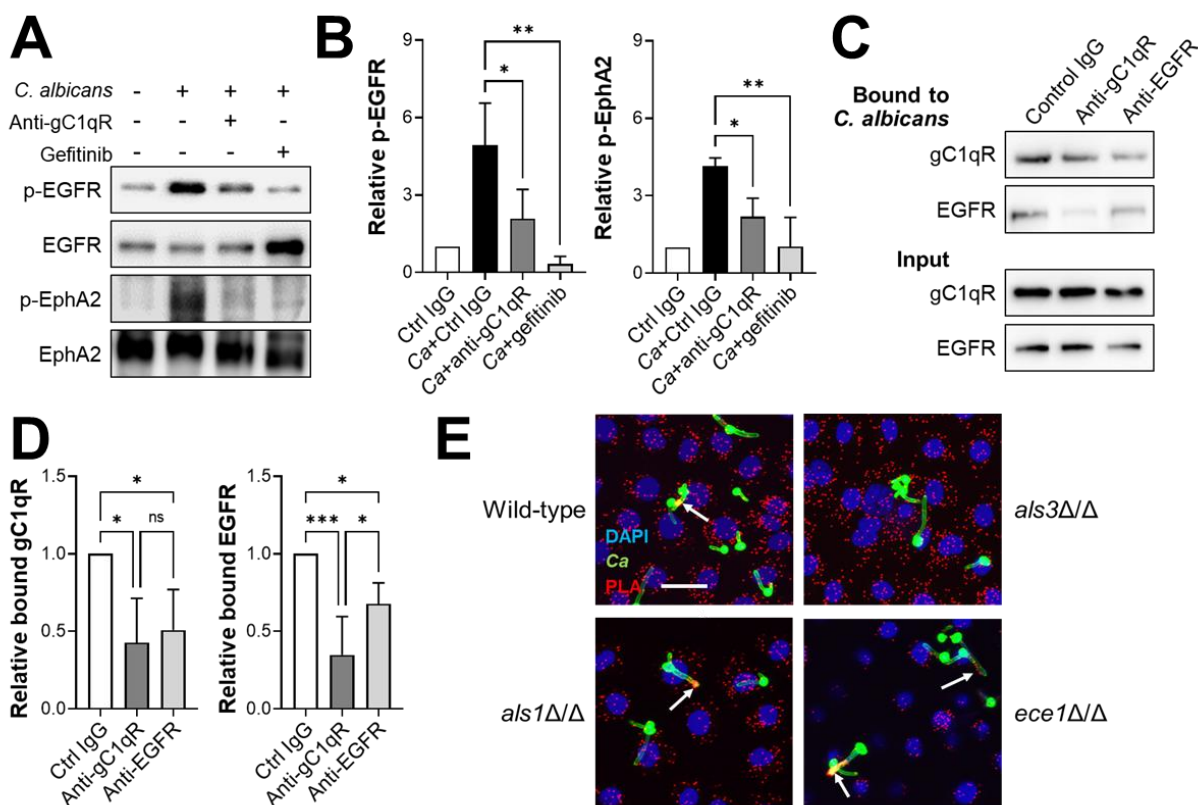
performed in triplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. Ca, *C. albicans*; Ctrl, control; NS, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .



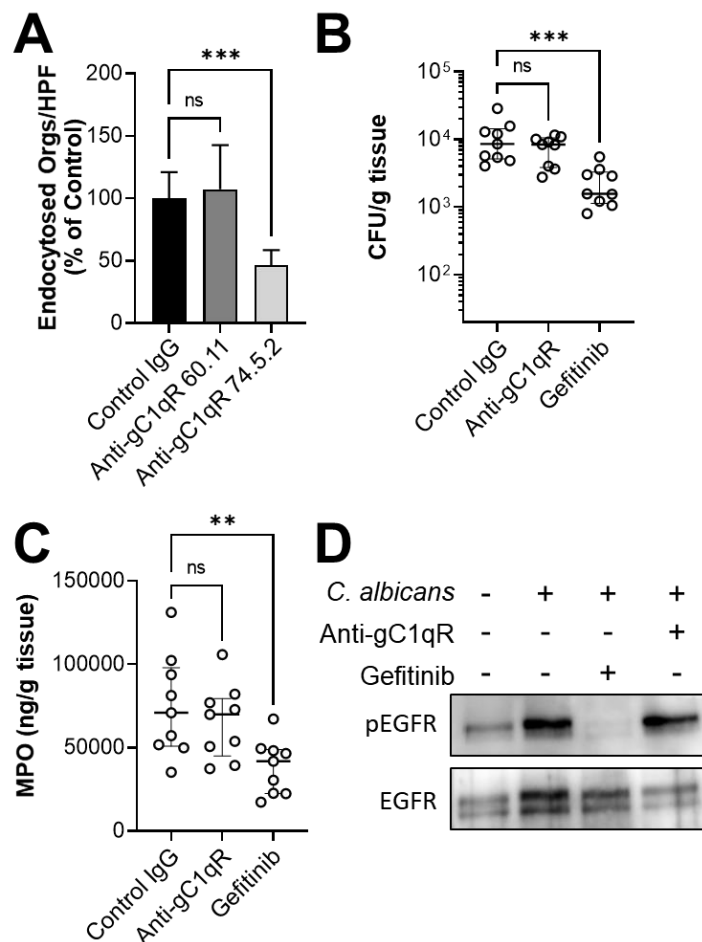
**Fig. 3** Surface-expressed gC1qR mediates the endocytosis of *C. albicans*. (A) Effects of two different anti-gC1qR monoclonal antibodies on the endocytosis of *C. albicans* by oral epithelial cells. (B) Effects of the anti-gC1qR antibody 74.5.2 and the EGFR kinase inhibitor, gefitinib on the endocytosis of *C. albicans* by oral epithelial cells. (C and D) Endocytosis of *C. albicans* by NIH/3T3 cells expressing human gC1qR and/or human EGFR. (C) Additive effects of EGFR and gC1qR on endocytosis. (D) Effects of inhibiting surface-expressed gC1qR with the anti-gC1qR antibody 74.5.2 on endocytosis. Results are the mean  $\pm$  SD of three independent experiments, each performed in triplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. ns, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



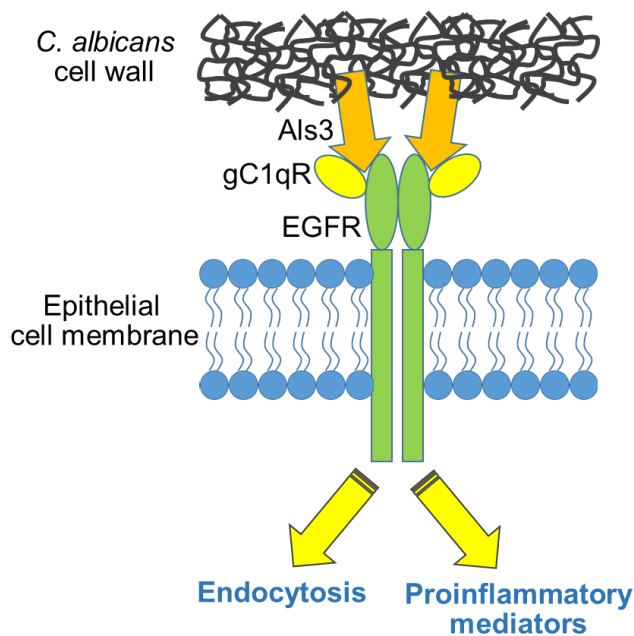
**Fig. 4** gC1qR is required for production of proinflammatory mediators by oral epithelial cells in response to *C. albicans* infection. (A-F) Oral epithelial cells were infected with *C. albicans* in the presence of an anti-gC1qR antibody 74.5.2 or gefitinib for 8 h, after which the concentration of the indicated inflammatory mediators in the medium was analyzed by ELISA (A and B) or Luminex cytometric bead array (C-F). Results are the mean  $\pm$  SD of three independent experiments, each performed in duplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. Ca, *C. albicans*; Ctrl, control; ns, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



**Fig. 5** Interactions of gC1qR and EGFR with *C. albicans*. (A and B) Effects of the anti-gC1qR antibody 74.5.2 and gefitinib on the phosphorylation of EGFR and EphA2 in response to 90-min infection with *C. albicans*. (A) Representative immunoblots. (B) Densitometric analysis of three immunoblots, such as the ones shown in (A). (C and D) Effects of anti-gC1qR and anti-EGFR antibodies on binding of gC1qR and EGFR to *C. albicans* hyphae. (C) Representative immunoblots. (D) Densitometric analysis of four immunoblots, such as the ones shown in (C). Results are the mean  $\pm$  SD of 3-4 independent experiments. (E) Proximity ligation assay showing the association of gC1qR with EGFR around hyphae of *C. albicans* wild-type, *als1Δ/Δ* and *ece1Δ/Δ* strains, but not the *als3Δ/Δ* mutant. Scale bar 25  $\mu$ m. The numerical data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. Ca, *C. albicans*; Ctrl, control; ns, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

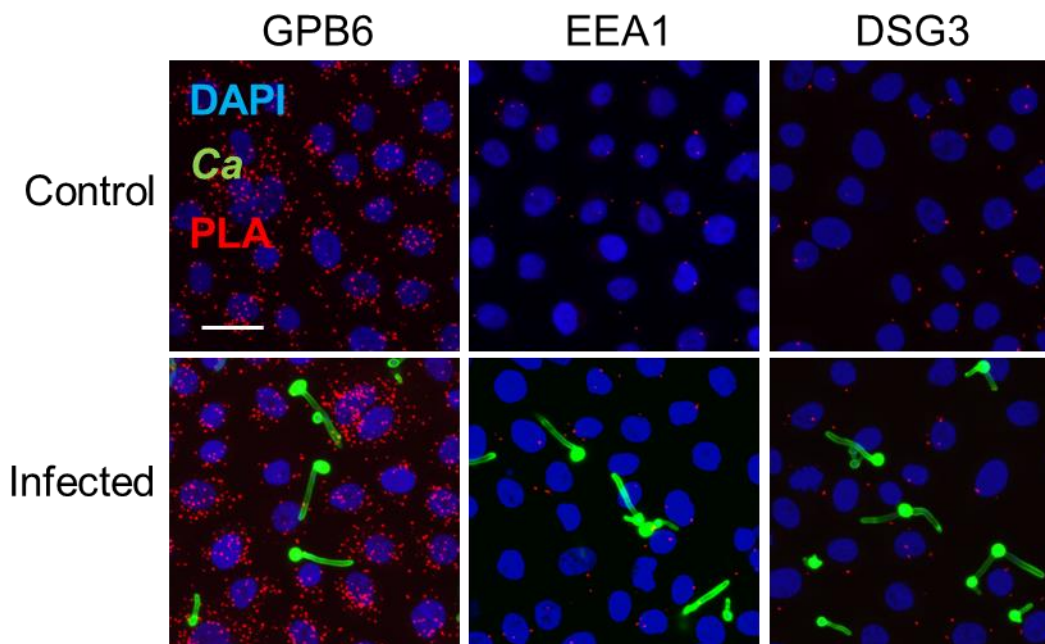


**Fig. 6.** Inhibition of gC1qR has no significant effect on the outcome of oropharyngeal candidiasis. (A) Effects of the indicated anti-gC1qR antibodies on the endocytosis of *C. albicans* by primary mouse oral epithelial cells. Results are the mean ± SD of three independent experiments, each performed in triplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. (B and C) Effects of the anti-gC1qR antibody 74.5.2 and gefitinib on the outcome of oropharyngeal candidiasis after 1 d of infection. Oral fungal burden (B). Oral myeloperoxidase (MPO) content (C). Results are the median ± interquartile range of two independent experiments, each with 4-5 mice per group. The data were analyzed using the Kruskal-Wallis test. (D). Immunoblot showing that the anti-gC1qR antibody 74.5.2 does not block *C. albicans*-induced phosphorylation of EGFR in primary mouse oral epithelial cells. Results are representative of three independent experiments. ns, not significant; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

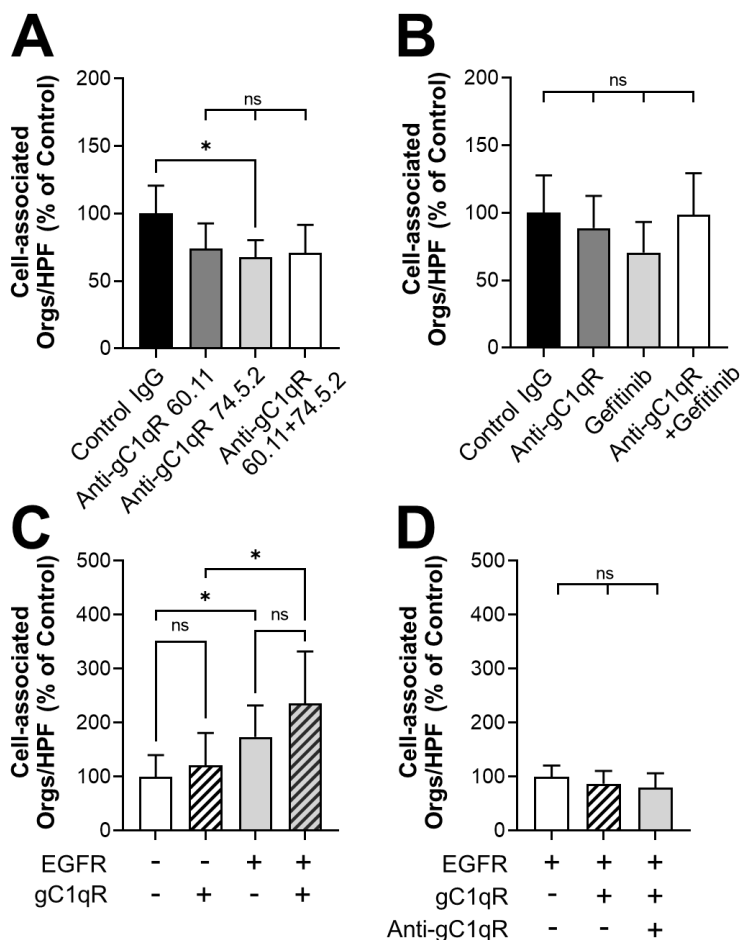


**Fig. 7** Diagram of the functional interaction of *C. albicans* Als3 with gC1qR and EGFR in human oral epithelial cells. Als3 interacts either directly or indirectly with both gC1qR and EGFR, leading to the activation of EGFR and subsequent induction of endocytosis and secretion of proinflammatory mediators.

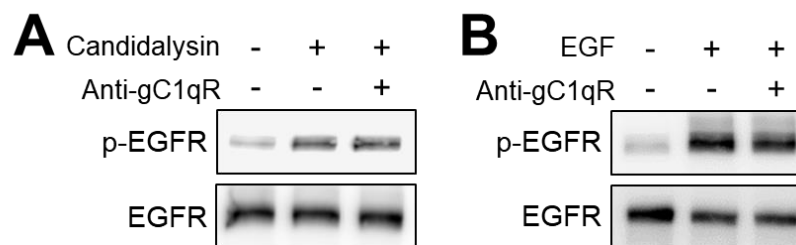




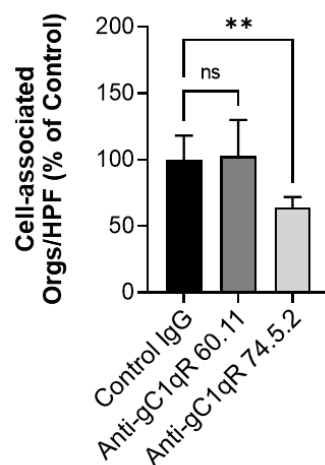
**Fig. S1** Proximity ligation assays to assess the physical association of the epidermal growth factor receptor (EGFR) with guanylate binding protein 6 (GBP6), early endosome antigen 1 (EEA1), and desmoglein-3 (DSG3) in the OKF6/TERT-2 oral epithelial cell line. The epithelial cells were incubated in either medium alone (top) or infected with *C. albicans* (bottom) for 90 min. Red spots indicate the regions where the indicated proteins associate with EGFR. Results are representative of three independent experiments. Scale bar 25  $\mu$ m.



**Fig. S2** Surface-expressed gC1qR has minimal effects on the number of *C. albicans* cells that are associated with oral epithelial cells. (A) Effects of two different anti-gC1qR monoclonal antibodies on the number of cell-associated *C. albicans* cells. (B) Effects of the anti-gC1qR antibody 74.5.2 and the EGFR kinase inhibitor, gefitinib on the number of cell-associated *C. albicans* cells. (C and D) Number of *C. albicans* cells that are cell-associated with NIH/3T3 cells expressing human gC1qR and/or human EGFR. (C) Effects of EGFR and gC1qR expression on cell-association. (D) Effects of inhibiting surface-expressed gC1qR with the anti-gC1qR antibody 74.5.2. Results are the mean  $\pm$  SD of three independent experiments, each performed in triplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. ns, not significant; \* $P < 0.05$ .



**Fig. S3** Inhibition of gC1qR does not block EGFR phosphorylation in response to 40  $\mu$ M candidalysin or 1 ng/ml epidermal growth factor (EGF). Representative immunoblots from three independent experiments.



**Fig. S4** Effects of the indicated anti-gC1qR antibodies on the number of *C. albicans* cells that were associated with primary mouse oral epithelial cells. Results are the mean  $\pm$  SD of three independent experiments, each performed in triplicate. The data were analyzed using one-way analysis of variance with Dunnett's test for multiple comparisons. ns, not significant; \*\* $P < 0.01$ .