Receptor-kinase EGFR-MAPK adaptor proteins mediate the epithelial response to *Candida albicans via* the cytolytic peptide toxin, candidalysin

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Nicole O. Ponde[1,2], Léa Lortal[1], Antzela Tsavou[1], Olivia W. Hepworth[1], Don N. Wickramasinghe[1], Jemima Ho[1], Jonathan P. Richardson[1], David L. Moyes[1], Sarah L. Gaffen[2,*], and Julian R. Naglik[1,*]

From the [1]Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral and Craniofacial Sciences, King's College London, London, United Kingdom; [2]Division of Rheumatology and Clinical Immunology, University of Pittsburgh, Pittsburgh Pennsylvania, USA

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***Candida albicans* (*C. albicans*) is a dimorphic commensal human fungal pathogen that can cause severe oropharyngeal candidiasis (oral thrush) in susceptible hosts. During invasive infection, *C. albicans* hyphae invade oral epithelial cells (OECs) and secrete candidalysin, a pore-forming cytolytic peptide that is required for *C. albicans* pathogenesis at mucosal surfaces. Candidalysin is produced in the hyphal invasion pocket and triggers cell damage responses in OECs. Candidalysin also activates multiple MAPK-based signaling events that collectively drive the production of downstream inflammatory mediators that coordinate downstream innate and adaptive immune responses. The activities of candidalysin are dependent on signaling through the epidermal growth factor receptor (EGFR). Here, we interrogated known EGFR-MAPK signaling intermediates for their roles mediating the OEC response to *C. albicans* infection. Using RNA silencing and pharmacological inhibition, we identified five key adaptors, including growth factor receptor-bound protein 2 (Grb2), Grb2-associated binding protein 1 (Gab1), Src homology and collagen (Shc), SH2-containing protein tyrosine phosphatase-2 (Shp2), and casitas B-lineage lymphoma (c-Cbl). We determined that all of these signaling effectors were inducibly phosphorylated in response to *C. albicans*. These phosphorylation events occurred in a candidalysin-dependent manner and additionally required EGFR phosphorylation, matrix metalloproteinases (MMPs), and cellular calcium flux to activate a complete OEC response to fungal infection. Of these, Gab1, Grb2, and Shp2 were the dominant drivers of ERK1/2 activation and the subsequent production of downstream innate-acting cytokines. Together, these results identify the key adaptor proteins that drive the EGFR signaling mechanisms that underlie oral epithelial responses to *C. albicans*.**

Fungal infections are highly underappreciated contributors of morbidity and mortality in humans, but to date, there are no vaccines to any pathogenic fungi (1). *Candida* species are a dominant cause of both superficial and life-threatening fungal infections in humans, with *Candida albicans* being the most prevalent. Though normally a benign member of the oral, vaginal, and gut commensal microbiota, disturbances caused by environmental cues or a compromised immune system result in the overgrowth of *C. albicans*, leading to superficial infections of the mucous membranes, in particularly the oral mucosa, that can significantly lower quality of life and affect millions of individuals annually (2, 3).

An essential feature of *C. albicans* pathogenesis is the formation of hyphae, which cause physical damage to the mucosal epithelium, thereby inducing expression and secretion of innate immune factors such as antimicrobial peptides, alarmins, and proinflammatory cytokines and chemokines (4). During infection, *C. albicans* hyphae secrete candidalysin (Clys) , a cytolytic peptide that is critical for pathogenesis at mucosal surfaces (5). Clys is encoded by *ECE1*, which is highly expressed in hyphae, though not required for filamentation. Clys is generated by processing of its parent protein Ece1p by the kexin proteases (6, 7). Clys secretion triggers epithelial cell damage and activation of a mitogen-activated protein kinase (MAPK)–based signaling pathway that leads to secretion of inflammatory mediators and cell survival signals (8–11). The epidermal growth factor receptor (EGFR) is key for mediating the host immune response against several microbial pathogens (12), including *C. albicans* (13–17). However, the downstream signals that orchestrate epithelial responses to this fungus are still incompletely understood.

EGFR, also known as Her1 or ErbB1, belongs to a family of receptor tyrosine kinases (RTKs) that regulate cell survival, proliferation, apoptosis, differentiation, and development (18). Dysregulation of EGFR is also associated with tumorigenesis (19, 20). EGFR autophosphorylation is a proximal event that initiates downstream signaling, resulting in recruitment of various adaptors and ultimately activating downstream signaling cascades including MAPK, phosphatidylinositol 3-kinase/Akt/Protein Kinase B (PI3K/Akt), and signal transducers and activators of transcription (STATs). Multiple microbes are known to exploit EGFR during pathogenesis, using adaptor activation and phosphorylation to infiltrate host cells

* For correspondence: Sarah L. Gaffen, sarah.gaffen@pitt.edu; Julian R. Naglik, julian.naglik@kcl.ac.uk.

and evade the immune system (12, 21, 22). Additionally, a role for these adaptors in driving immunity to microbes has also emerged (23).

In the present study, we sought to elucidate the role of MAPK signaling and EGFR adaptors in the oral epithelial cell (OEC) response to *C. albicans*. We found that growth factor receptor–bound protein 2 (Grb2), Grb2-associated binding protein 1 (Gab1), Src homology and collagen (Shc), SH2 containing protein tyrosine phosphatase-2 (Shp2, also PTPN11), and casitas B-lineage lymphoma (c-Cbl) are all required to mediate an EGFR-dependent response to *C. albicans via* Clys. EGFR kinase inhibitors negatively impact this response, which also requires matrix metalloproteinases and cellular calcium flux. Accordingly, we illuminate the underlying mechanisms of how early EGFR-mediated epithelial responses are orchestrated by the novel fungal toxin Clys, with implications for defining the host response to the medically important fungus *C. albicans*.

Results

C. albicans *activates EGFR-associated adaptors through secreted Clys*

In establishment of oropharyngeal candidiasis (oral thrush), OECs respond to the pore-forming toxin (PFT) Clys secreted by *C. albicans* hyphae. OEC responses require EGFR signals, which leads to the release of antifungal proinflammatory mediators that ultimately coordinate an effective host response (4, 24). However, the underlying mechanisms of the early EGFR molecular events activated in response to *C. albicans* and Clys remain incompletely characterized. Accordingly, TR146 buccal epithelial cells were infected with a *C. albicans* (BWP17 + CIp30, parental strain) at a multiplicity of infection of 10 for 2 h, a time point known to be optimal for EGFR activation (13). Notably, all proteins known to interact with EGFR were inducibly phosphorylated following infection with *C. albicans* including Gab1 (Y659), Grb2 (p-Tyrosine), Shc (Y317), Shp2 (Y580), and c-Cbl (Y774) (Fig. 1*A*).

Since filamentation is required for *C. albicans*–induced epithelial responses (25), we asked whether Clys was required for adaptor activation. Mutant *C. albicans* strains lacking the Clys parent gene *ECE1* (*ece1*Δ/Δ) or with a deletion of the region that encodes Clys (*ece1*Δ/Δ+*ECE1*$_{\Delta 184-279}$) failed to activate Gab1, Shc, Shp2, and c-Cbl (Fig. 1*A*). However, both mutant strains lacking Clys were able to induce phosphorylation of Grb2, though to a lesser extent than the parental and revertant strains, suggesting Clys is not the sole driver of Grb2 phosphorylation. To determine whether Clys alone could activate this panel of adaptors, TR146 cells were stimulated with Clys over a time course of 2 h. We treated cells with a strongly lytic concentration (70 μM) that triggers membrane damage, calcium flux, and cytokine release, an intermediate sublytic concentration (15 μM) that activates epithelial signal transduction through MAPK, p38/MKP-1, and c-Fos and the production of immune regulatory cytokines, and a non-lytic concentration (3 μM) that induces low level secretion of selected cytokines (Moyes *et al.*, 2016). Phosphorylation of Gab1 at Y659 was detectable within 30 min of exposure to all concentrations of Clys, with phosphorylation levels increased strongly in response to higher concentrations of Clys (70 or 15 μM) at 2 h post treatment (Fig. 1*B*). Lower concentrations of Clys that do not cause cell lysis (3 μM) also induced pGab1 Y659 at 2 h post treatment. In contrast, treatment with 70 or 15 μM Clys induced Grb2 phosphorylation within 30 min, which was sustained until 2 h. Increased phosphorylation levels were only observed in cells stimulated with 3 μM Clys following 2 h of treatment. Phosphorylation of Shc Y317 was observed within 30 min following treatment with all concentrations of Clys, with a dose-dependent response observed at 30 min and 2 h post stimulation. Together, these data suggest that there is differential temporal organization of adaptors in response to *C. albicans* and Clys, which appears to be an early, coordinated event.

Unexpectedly, the kinetics of Shp2 phosphorylation in response to Clys differed from Gab1, Grb2, and Shc, as phosphorylation of Shp2 Y580 was seen only after 2 h of treatment with 70 or 15 μM Clys, with negligible changes in phosphorylation observed with 3 μM (Fig. 1*B*). These data suggest either that Shp2 may play a role in the response against Clys when a significant amount of epithelial damage has been induced or slower kinetics of Shp2 activity in comparison to the other adaptors. Treatment of TR146 cells with 70 or 15 μM Clys triggered strong c-Cbl pY774 activation that was sustained at 30 min and 2 h post stimulation (Fig. 1*B*). Only very modest changes were observed with 3 μM Clys. Collectively, these data show that Clys can induce phosphorylation of adaptors in TR146 epithelial cells in a time- and dose-dependent manner, with distinct activation profiles for adaptors in response to lytic *versus* sublytic concentrations of Clys.

Gab1, Grb2, Shc, Shp2, and c-Cbl activation requires EGFR activation, matrix metalloproteinases, and calcium flux

While EGFR and the Ephrin family member, EphA2, play critical roles in mediating the innate epithelial response to Clys and *C. albicans* (13, 14), it is unknown whether the activation of corresponding adaptors in response to hyphae relies exclusively on EGFR. To determine whether EGFR drives Clys-induced adaptor activation, we analyzed the effects of the EGFR-specific kinase inhibitors on the activation of Gab1, Grb2, Shc, Shp2, and c-Cbl. Treatment of TR146 cells with EGFR inhibitors gefitinib (used at a final concentration of 1 μM) or PD153035 (0.5 μM) reduced *C. albicans*–induced phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl at 2 h post infection (Fig. 2*A*). Similarly, inhibition of EGFR either abolished or reduced phosphorylation of all adaptors in response to Clys (70 or 15 μM), indicating that EGFR activation is indeed required for Clys-induced adaptor activity (Fig. 2*B*).

Matrix metalloproteinases (MMPs) are zinc-dependent metalloendopeptidases that cleave and release EGF family proligands, activating EGFR signaling (13). Pretreatment of TR146 OECs with the pan-MMP inhibitor Marimastat (10 or 1 μm) suppressed phosphorylation of Gab1, Grb2, Shc, Shp2,

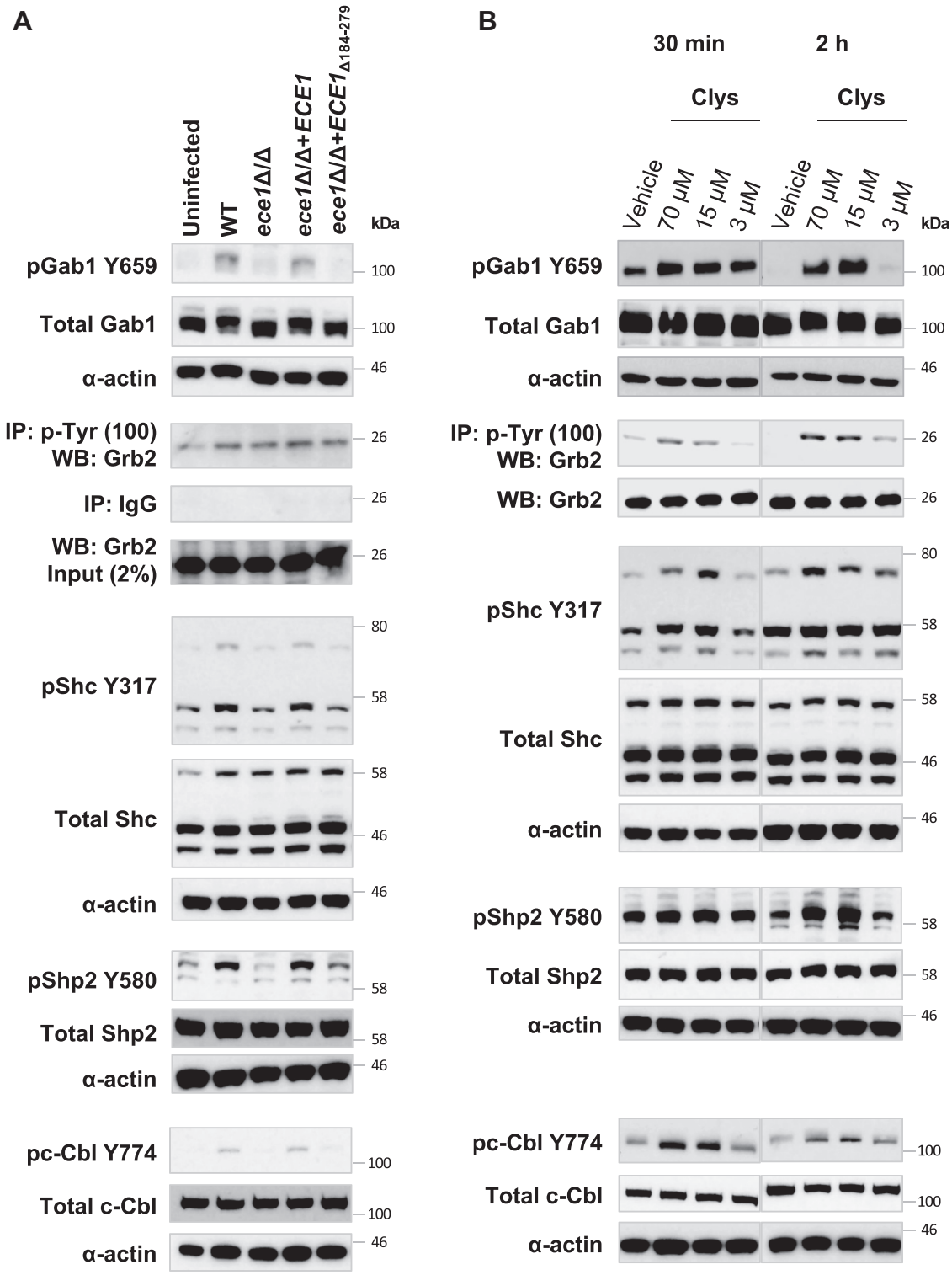

Figure 1. Candidalysin is necessary and sufficient to activate Gab1, Grb2, Shc, Shp2, and c-Cbl adaptors in human oral epithelial cells. *A*, candidalysin (Clys) activates Gab1, Grb2, Shc, Shp2, and c-Cbl adaptors in human TR146 oral epithelial cells (OECs). Immunoblot showing phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl in response to 2 h infection of TR146 OECs with the indicated strains of *C. albicans*. *B*, Clys is sufficient to activate Gab1, Grb2, Shc, Shp2, and c-Cbl. Immunoblot showing phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl following stimulation with Clys at 30 min and 2 h in OECs. Blots are representative of three independent experiments.

and c-Cbl upon Clys stimulation (Fig. 2*C*) and infection with *C. albicans* (Fig. S1). Consistent with this, a Clys-deficient *C. albicans* strain (*ece1*Δ/Δ+*ECE1*$_{\Delta 184-279}$), failed to phosphorylate Gab1, Shc, and c-Cbl, though induced very modest levels of phosphorylation for Grb2 and Shp2. Additionally, a blocking anti-EGFR mAb (Cetuximab, 10 μg/mL) reduced/abolished the phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl in response to Clys and *C. albicans* infection (Fig. 3),

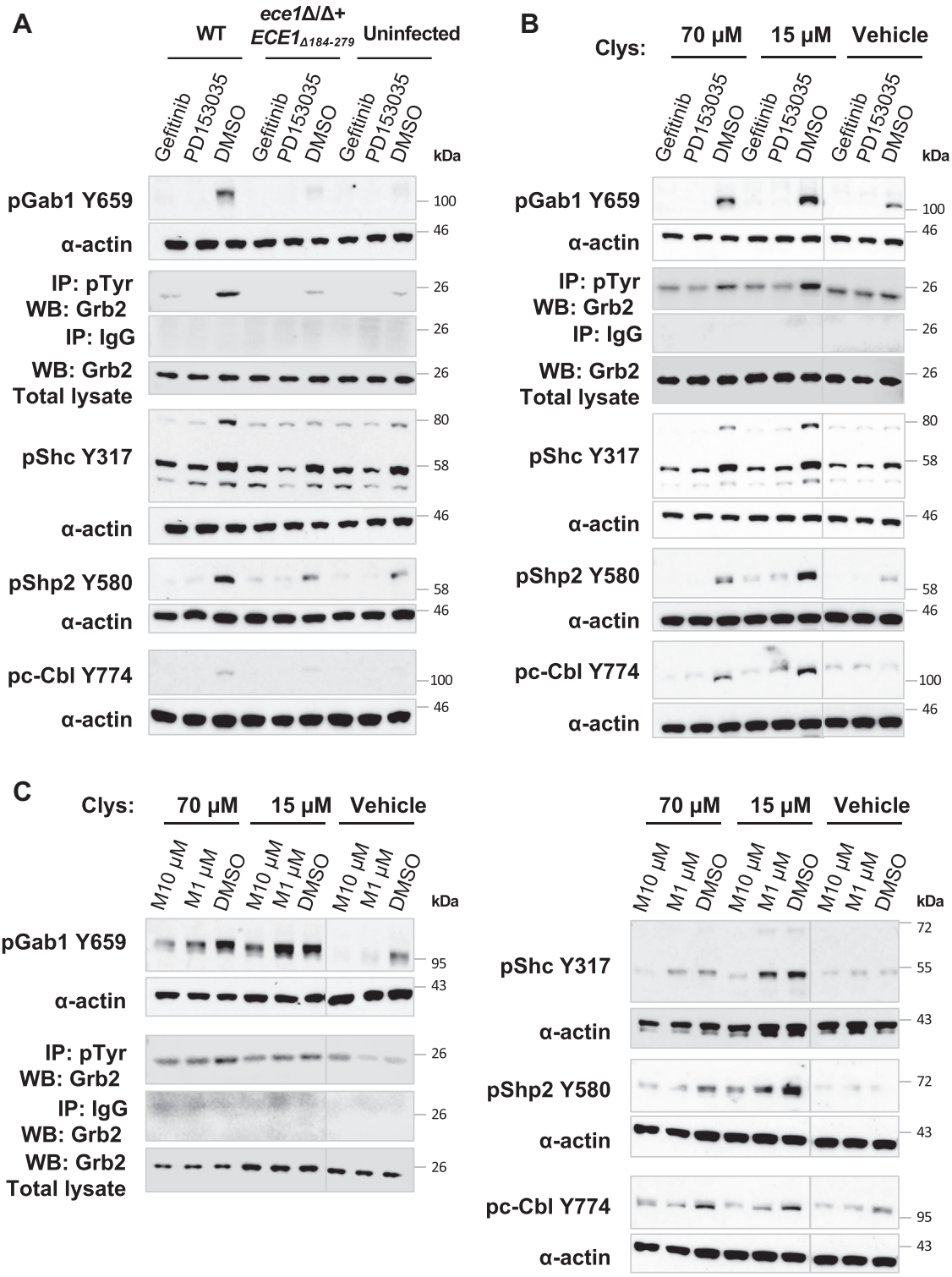

Figure 2. Candidalysin-induced adaptor activation requires EGFR and MMP activity. Inhibition of EGFR activity suppresses (*A*) *C. albicans*- and (*B*) candidalysin (Clys)-induced adaptor activation. Use of Gefitinib or PD153035 EGFR TK inhibitors suppresses WT *C. albicans*–induced and Clys-induced activation of Gab1, Grb2, Shc, Shp2, and c-Cbl. *C*, MMP inhibition suppresses candidalysin-induced adaptor activity. Pretreatment of TR146 cells with Marimastat (10 or 1 μM, designated M10 or M1) suppressed Clys-induced phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl. Protein lysates were isolated 2 h post stimulation. Blots are representative of two (Grb2) or three (Gab1, Shc, Shp2, and c-Cbl) independent experiments. (Note: because data from other experiments presented in this article were originally run on the same gel, the α-actin loading control is reused in Fig. 2, *A*–*C*). MMP, matrix metalloproteinase.

demonstrating that Clys-induced activation of adaptors is mediated by MMPs, which in turn mediate ligand-induced activation of EGFR. Previously, calcium influx induced by Clys activity was also shown to contribute to EGFR phosphorylation (13). Accordingly, pretreatment of TR146 cells with the calcium chelator, BAPTA-AM (30 or 10 μM), also

suppressed Clys-induced and *C. albicans*–induced EGFR adaptor activation (Fig. S2). These data show that Clys secreted by *C. albicans* activates EGFR-related adaptors and downstream signaling programs in OECs *via* a mechanism that requires calcium influx, MMP activity, and EGFR ligands.

EGFR adaptors mediate Clys-induced MAPK signaling responses but are dispensable for cell survival and damage protection

Clys permeabilizes target cell membranes, resulting in damage (release of lactate dehydrogenase (LDH)) and the activation of PI3K/Akt/mTOR and MAPK (MKP1/c-Fos) signaling pathways (5, 26). Notably, Akt/mTOR is important in mediating cell survival and protection against damage (26), and MAPK is required for induction of immune responses (5) during epithelial *C. albicans* infection. Furthermore, Gab1, Grb2, and Shp2 adaptors can mediate the cell survival pathway (27).

To determine whether this panel of EGFR adaptors can modulate these cellular responses, TR146 cells were transfected with siRNAs for Gab1, Grb2, Shc, Shp2, and c-Cbl for 48 h and stimulated with *C. albicans* or Clys for 2 h. LDH release from TR146 cells was unaffected by knockdown of all adaptors following treatment with *C. albicans* (Fig. S3*A*) or Clys (70 or 15 μM) (Fig. S3*B*). Accordingly, a Clys null strain (*ece1*Δ/Δ+$ECE1_{\Delta 184\text{-}279}$) caused no apparent change in epithelial integrity. Assessment of the Akt/mTOR pathway similarly showed no change in phosphorylation upon adaptor knockdown (Fig. S3*C*). Thus, these EGFR adaptors do not appear to be required to protect epithelial cells from damage or for cell survival in response to *C. albicans* infection and Clys. In contrast, siRNA knockdown of Gab1 and Grb2 had a marked impact on c-Fos protein levels and suppressed

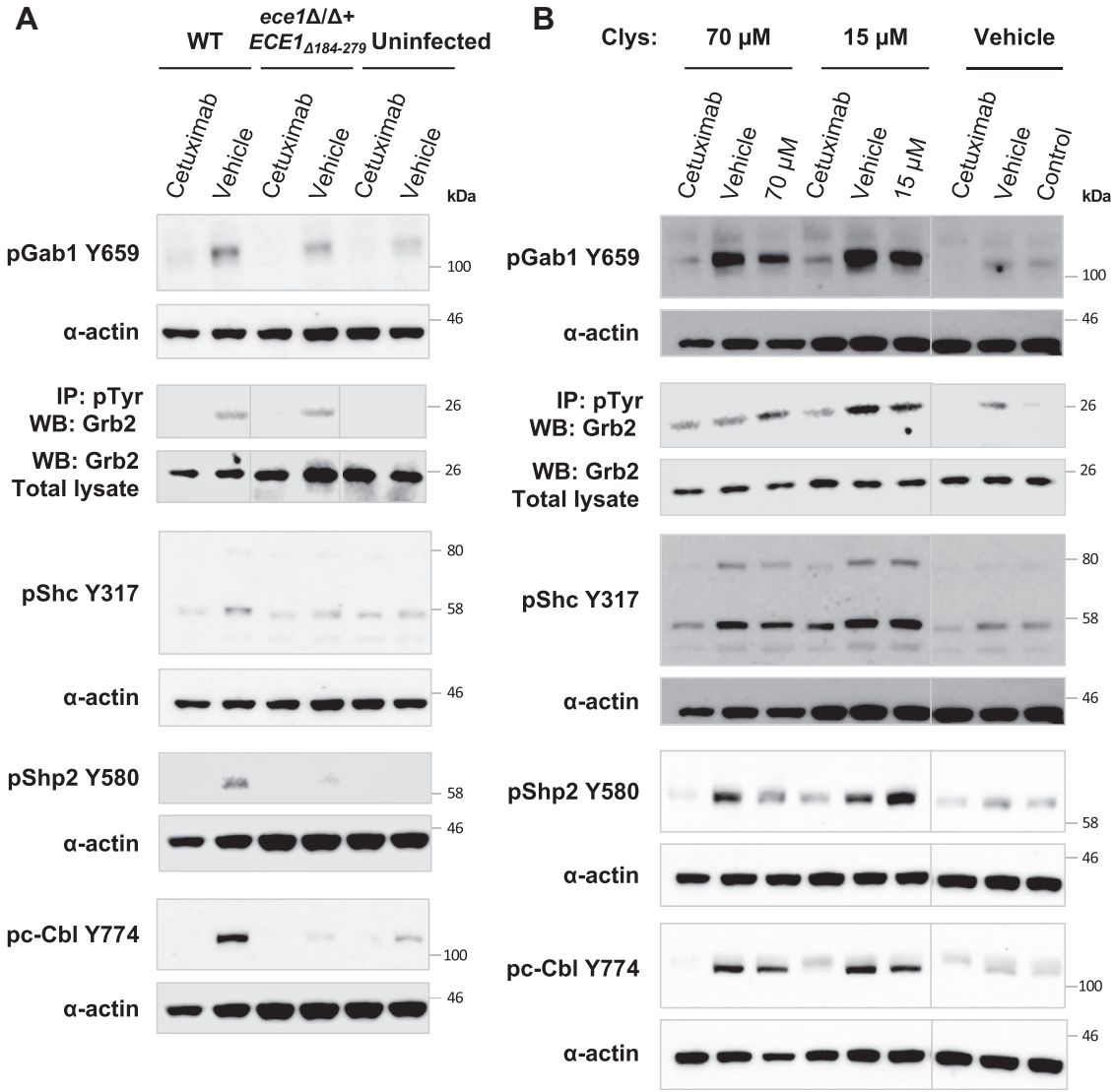

Figure 3. EGFR ligand binding is required for *C. albicans* and candidalysin-induced adaptor activation. Pretreatment of TR146 cells with the anti-EGFR mAb, Cetuximab suppressed phosphorylation of Gab1, Grb2, Shc, Shp2, and c-Cbl following (*A*) *C. albicans* infection or (*B*) candidalysin (Clys) stimulation. Blots are representative of two (Grb2) or three (Gab1, Shc, Shp2, and c-Cbl) independent experiments. (Note: because data from other experiments presented in this article were originally run on the same gel, the α-actin loading control is reused from Fig. 3, *A* and *B*).

phosphorylation of MKP1/DUSP1 in response to *C. albicans* and Clys (Fig. 4, *A* and *B*). Knockdown of Shc had only a minor impact on c-Fos and MKP1/DUSP1 activation, and the effects of Shp2 or c-Cbl knockdown *via* siRNA were negligible.

To better understand how Gab1 and Grb2 activate c-Fos and MKP1/DUSP1, we next assessed the three major branches of the MAPK signaling cascade, ERK1/2, p38 and SAPK/JNK. In response to *C. albicans*, knockdown of Gab1 and Grb2 had a marked effect on ERK1/2 phosphorylation but not p38 or SAPK/JNK (Fig. 4, *A* and *B*). While Grb2 knockdown also showed a decrease in pERK1/2 in response to Clys, Gab1 knockdown showed a modest increase in ERK1/2, p38, and SAPK/JNK phosphorylation. As expected, a Clys-null strain (*ece1Δ/Δ+ECE1*$_{Δ184-279}$) did not trigger MAPK phosphorylation. Thus, Gab1 and Grb2 are required for MAPK activation during *C. albicans* infection, but the Gab1 activation pattern differs when responding to exogenously-applied synthetic Clys.

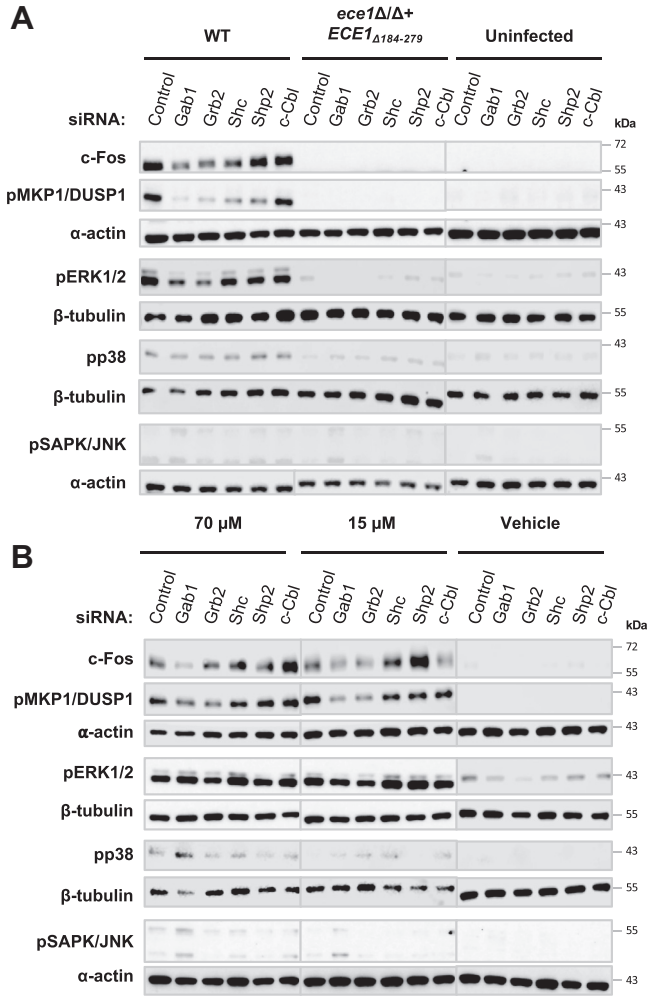

Figure 4. Gab1, Grb2, and Shc adaptors mediate candidalysin-induced MAPK signalling responses. Knockdown of Gab1, Grb2, or Shc suppresses c-Fos expression, MKP1/DUSP1 and ERK1/2 phosphorylation. Following siRNA transfection, cells were infected with the indicated strains (*A*) or stimulated with candidalysin (*B*) and lysates were collected after 2 h for Western blotting for total c-Fos, phospho-MKP1/DUSP1, phospho-ERK1/2, phospho-p38, phospho-SAPK/JNK, or α-actin. Data are representative of three independent experiments.

Impairing Shp2 *via* siRNA had no detectable effect on *C. albicans* and Clys-induced signals; however, a caveat is that Shp2 knockdown was inefficient (60%, Fig. S4). Therefore, we also addressed this question by treating TR146 cells with SHP099 HCL, an allosteric inhibitor that selectively and potently targets Shp2. Cells were pretreated with SHP099 HCL (10 or 5 μM) for 1 h and then assessed for cell damage, MAPK and Akt/mTOR pathway activation after infection. Similar to observations with Shp2 siRNA, LDH release and mTOR phosphorylation from TR146 OECs was unaffected by Shp2 inhibition (Fig. S5). However, treatment with SHP099 HCL suppressed *C. albicans*–induced and Clys-induced levels of c-Fos, pMKP1/DUSP1, and pERK1/2, though not pSAPK/JNK or pp38 (Fig. 5, *A* and *B*). Thus, in addition to a key role for Gab1 and Grb2 adaptors, Shp2 also contributes to ERK1/2 activation in response to *C. albicans* and Clys.

Gab1, Grb2, and Shp2 are required for production of inflammatory effectors in response to Clys

A characteristic endpoint of Clys activity and EGFR activation is the production of inflammatory cytokines and chemokines. Using siRNA knockdown, we evaluated the role of this panel of EGFR adaptors in driving the production of G-CSF, GM-CSF, IL-6, IL-1α, and IL-1β from TR146 cells 24 h after *C. albicans* or Clys treatment. Knockdown of Gab1, Grb2, or Shc reduced secretion of GM-CSF, G-CSF, and IL-1α in response to *C. albicans* (Figs. 6, *A*, *B* and S6*A*) and Clys (Fig. S6, *B*, *C*, and *E*). Notably, only Gab1 or Grb2 knockdown reduced release of IL-1β and IL-6 in response to *C. albicans* (Fig. 6, *C* and *D*), suggesting that distinct pathways dictate adaptor-mediated cytokine responses during fungal signaling on OECs. Interestingly, knockdown of c-Cbl appeared to increase the release of G-CSF in response to *C. albicans* and GM-CSF and IL-6 in response to Clys (Figs. 6*A*, S6, *C* and *D*), possibly suggesting a negative regulatory role for c-Cbl in immune signaling. The Shp2 inhibitor, SHP099 HCL, impaired G-CSF, GM-CSF, IL-1α, and IL-β secretion in response to *C. albicans* treatment (Figs. 6*E*, S7, *A*, *C*, and *D*). Similarly, Shp2 inhibition significantly reduced G-CSF, GM-CSF, and IL-β release in response to Clys (Fig. S7, *E*, *F*, and *H*). Interestingly, Shp2 inhibition resulted in a significant increase in IL-6 release in response to Clys (Fig. 6*F*).

OECs respond to Clys-induced tissue injury in part by inducing damage-associated immune responses, including release of alarmins and antimicrobial peptides (AMPs) such as β-defensins (17). To investigate the role of these adaptors in AMP induction, we incubated TR146 cells with Clys following siRNA knockdown of adaptors or inhibition of Shp2 and evaluated *DEFB4A* mRNA (encoding BD2). Adaptor knockdown or inhibition had no significant effect on gene expression in response to Clys (Fig. S8, *A* and *B*). As Clys does not stimulate S100 protein release (17), their expression was not assessed. Overall, these data indicate that Gab1, Grb2, Shc, and Shp2 adaptors but not c-Cbl play pivotal roles in mediating cytokine but not alarmin innate responses to fungal infection.

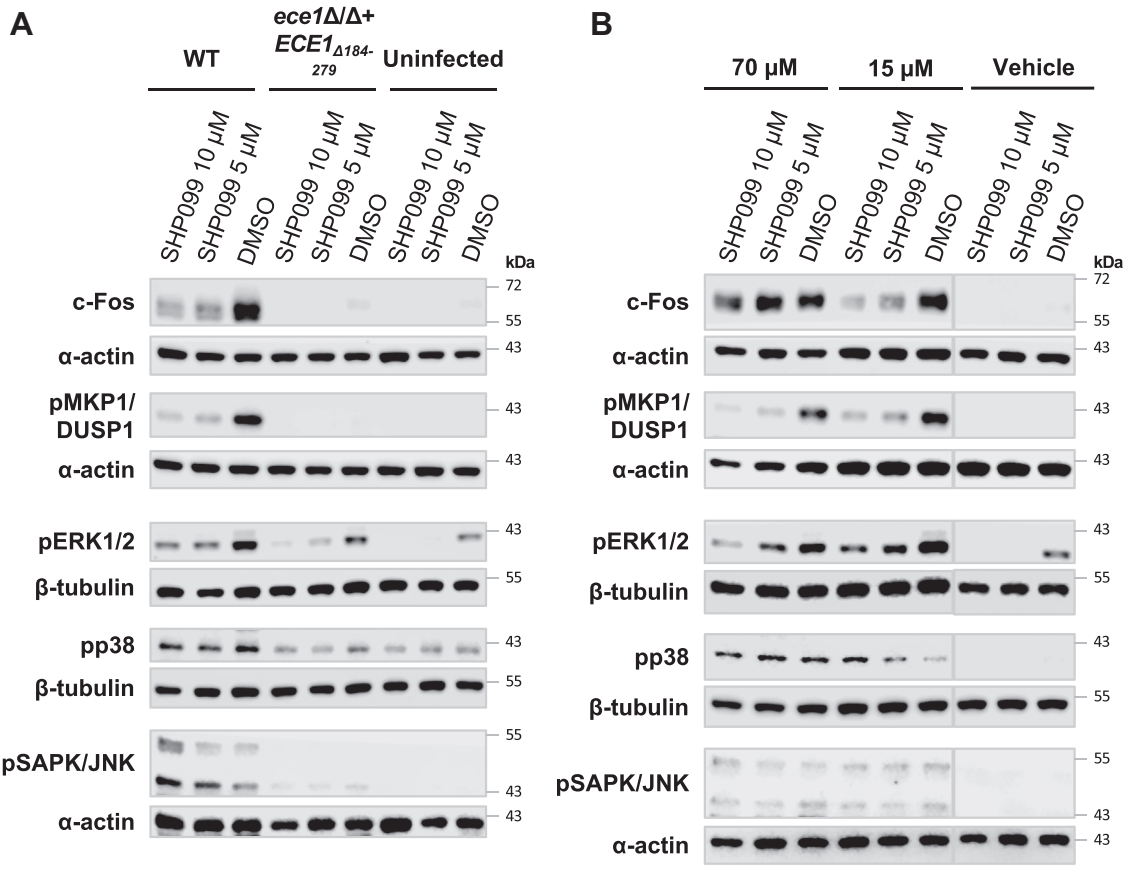

Figure 5. Shp2 mediates candidalysin-induced MAPK signaling responses. Inhibition of Shp2 activity suppresses *C. albicans*–induced and candidalysin (Clys)-induced c-Fos, pMKP1/DUSP1, and ERK1/2 activation. TR146 cells were pretreated with the Shp2 inhibitor, SHP099 HCL, for 1 h. Cells were infected with the indicated strains (*A*) or stimulated with Clys (*B*) and protein lysates isolated at 2 h for Western blotting for total c-Fos, phospho-MKP1/DUSP1, phospho-ERK1/2, phospho-p38, phospho-SAPK/JNK, or α-actin. Data are representative of three independent experiments.

Discussion

The oral epithelium coordinates the early antifungal response to infection through the release of cytokines, chemokines, and other inflammatory mediators that recruit leukocytes, infiltrating neutrophils, and inflammatory monocytes to combat infection. Interactions between epithelial cells and *C. albicans* are critical for host defense mechanisms to mucosal infection. The discovery of the *C. albicans*–derived PFT, Clys, was a paradigm shift in understanding how hyphae promote pathogenesis, simultaneously causing target cell damage but also activating innate immune responses that are essential to limit control of this commensal microbe. This discovery led to a myriad of studies revealing how Clys activity interfaces with the host immune system and balances commensalism with pathogenicity (6–10, 13, 28–33)

Clys activates the EGFR and MAPK signaling pathways (5, 13), but how these pathways are controlled remained incompletely defined. The EGFR adaptor proteins play a critical role in facilitating signaling between upstream cell surface RTKs, such as EGFR, as well as downstream cellular signaling cascades that are fundamental for a diverse array of cellular processes (34). We show that not only does OEC infection with *C. albicans* activate the Gab1, Grb2, Shc, Shp2, and c-Cbl adaptors but that this activity is mainly driven by Clys. While Clys-deficient strains reduced phosphorylation of Grb2 in OECs compared to Clys-producing strains, there were still residual levels of phosphorylation remaining, suggesting that Clys is not the only driver of Grb2 activity during infection. This may be due to interactions between fungal proteins on the surface of invading hyphae and host epithelial cell receptors, EphA2, EGFR, and globular C1q receptor (gC1qR), which are known to mediate numerous proinflammatory responses to *C. albicans* (14, 35, 36).

The distinct temporal activity of these EGFR adaptors suggests they may mediate the early, coordinated response of OECs to ensure appropriate signaling during fungal infection. Notably, no studies have implicated EGFR adaptor activation in response to fungal infection and very few in bacterial infections (21, 22, 37). In addition to EGFR, Gab1, Grb2, Shc, Shp2, and c-Cbl are essential for signaling responses of RTKs, including EphA2, c-Met, and other Her-family members (38–40). The near abolishment of adaptor activation in response to EGFR inhibition confirmed EGFR is a critical receptor for adaptor protein activation and downstream responses to *C. albicans via* Clys. Previously, we reported that inhibition of EGFR reduces MAPK signaling and subsequent immune responses during infection (13). Interestingly, our results show that some, but not all, adaptors orchestrate the

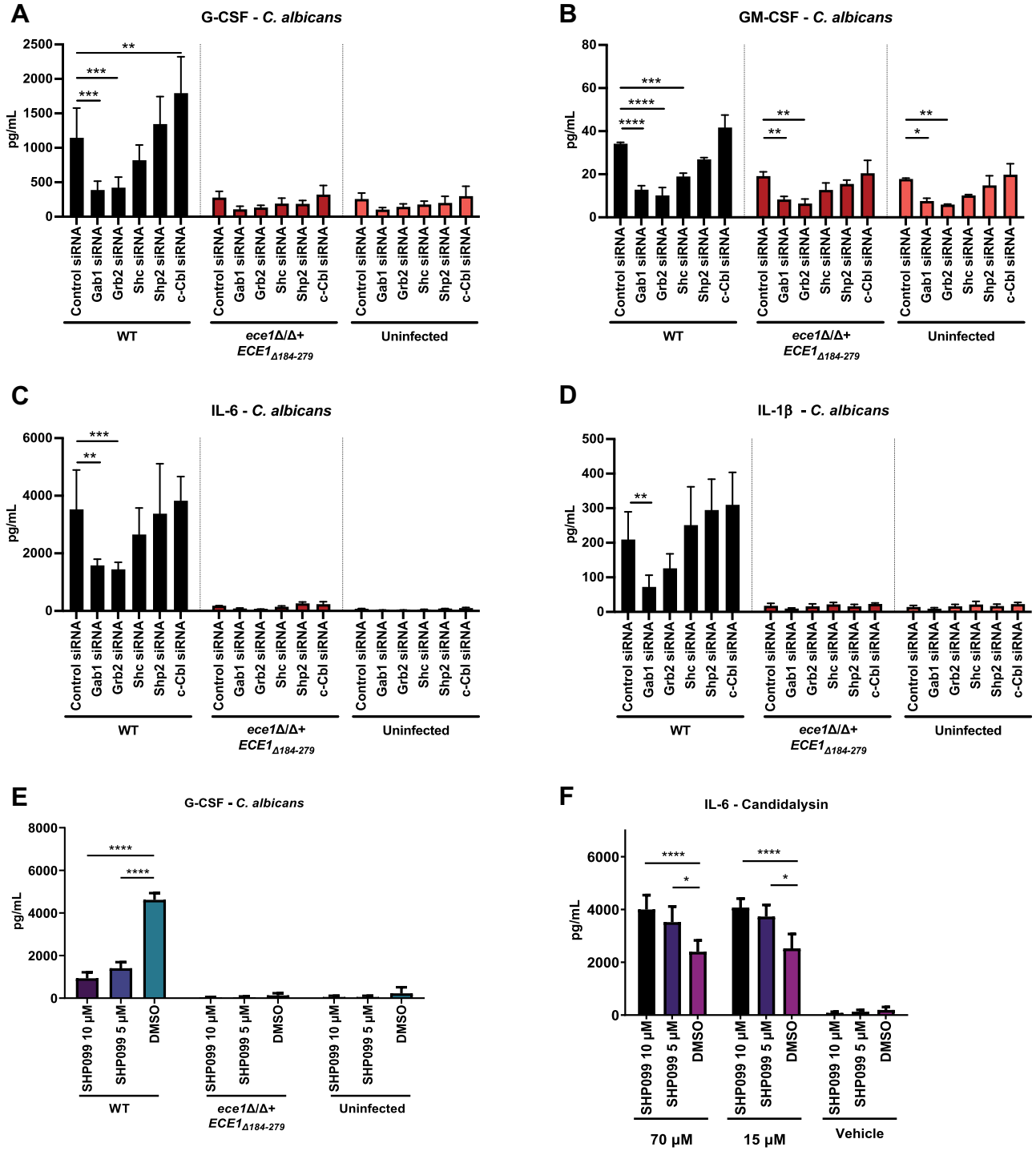

Figure 6. Gab1, Grb2, Shc, and Shp2 adaptors mediate *C. albicans*–induced *and* candidalysin-induced cytokine secretion in oral epithelial cells. Knockdown of Gab1, Grb2, or Shc or inhibition of Shp2 suppresses G-CSF, GM-CSF, IL-6, and IL-1β secretion. *A–D*, following siRNA knockdown or (*E* and *F*) 1 h pretreatment with the Shp2 inhibitor SHP099 HCL, cells were infected with the indicated strains or stimulated with candidalysin and supernatants collected after 24 h. Cytokines were evaluated by Luminex. Data show the mean (±SD) of three replicates. Significance was assessed by one way ANOVA with Bonferroni's multiple comparisons test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

downstream mechanisms of epithelial immunity to infection. Recent work has demonstrated that Grb2 is associated with EGFR in response to *C. albicans* infection (36), and sustained activation of both EGFR and EphA2 by Clys and the hyphal adhesin, Als3, is required for proinflammatory responses during *C. albicans* infection (14). However, unlike Clys, recombinant Als3 does not induce cell damage, activate c-Fos/MKP1/DUSP1, or promote cytokine responses (41), which are downstream of EGFR signaling, suggesting Als3 does not directly activate these EGFR adaptors. Additionally, it should be noted that an *ALS3*-deficient strain is poor at forming the invasion pocket, which is required to enable efficient delivery

and activity of Clys (8). Together, these data indicate that Als3 does not directly activate EGFR adaptors and that its role in driving epithelial signaling and/or immune responses is probably due to its role in facilitating the formation of the invasion pocket to permit efficient secretion of Clys, which activates these processes.

Clys induces EGFR activation by activating MMPs, which cleave EGFR proligands that subsequently bind EGFR, and inducing calcium influx (5, 13). Various MMPs activate EGFR in response to a diverse array of stimuli (42, 43). Additionally, MMP activation and subsequent cleavage and release of EGFR ligands are involved in the activation of EGFR in response to *Helicobacter pylori* and *Clostridium difficile* (44, 45). The role of MMPs in infectious diseases, such as mycobacterial infection and *Paracoccidioides brasiliensis* infection, has also been reported (46, 47). We now demonstrate that MMPs mediate antifungal immune responses *via* EGFR through the adaptors described herein.

Clys is the first PFT identified in a human fungal pathogen. Like analogous toxins in other organisms, Clys causes a rapid influx of intracellular calcium, thought to be a pivotal step in the activation of downstream signaling events (48). Calcium influx triggered by bacterial PFTs is also a key factor in the induction of cellular responses that limit infection and promote survival (49, 50). We found that chelation of calcium reduced the activation of adaptors in response to *C. albicans* and Clys. As such, our results demonstrate a role for calcium flux induced by Clys in inducing EGFR adaptor activation and epithelial immune responses during *C. albicans* infection.

Clys induces cell stress in OECs, with the release of alarmins, ATP, and AMPs (17). Our findings reveal that these adaptors play a negligible role in expression of a key host-protective AMP, *DEFB4A* (human BD-2). Interestingly, Shc activation has been associated with cellular stress responses (51), and Shp2 is critical in regulating the stress response to aryl hydrocarbon receptor activation and calcium dynamics in mast cells (52). Though this aspect of adaptor activation *via* cellular stress was not assessed here, stress-induced calcium flux is a key biological response to bacterial PFTs, and it is likely that Clys induces stress-associated signals in OECs during *C. albicans* infection, which may activate EGFR adaptors to mediate this specific response to Clys.

This work establishes roles for multiple EGFR-related adaptors in the response to Clys secreted from *C. albicans*. Notably, four of these proteins, Gab1, Grb2, Shc, and Shp2, were required for G-CSF and GM-CSF secretion (Figs. 6, *A*, *B*, S7, *A*, *E*, and *F*). The secretion of IL-1α was reduced when Gab1 or Grb2 were knocked down *via* siRNA (Fig. S6*A*) or when the activity of Shp2 was inhibited chemically (Fig. S7*C*). In a similar manner, IL-1β and IL-6 secretion were reduced upon siRNA knockdown of Gab1 but not Grb2 in response to infection or Clys treatment (Fig. 6*D*). Inhibition of Shp2 effectively reduced IL-1β but not IL-6. These distinct roles for each adaptor in mediating the secretion of IL-1α, IL-1β, and IL6 indicate a potentially extensive and complex network of regulation of these cytokines in response to infection (Fig. 7) (Table 1).

Few studies show that Gab1, Grb2, or Shp2 mediate such specific cytokine responses. However, activation of Gab1 and Shp2 can transmit signals to the ERK1/2 signaling cascade to activate the cytokine receptor, gp130 (53), and activation of Shc, Grb2, and Shp2 mediate MAPK activation *via* the G-CSF receptor (54). Both G-CSF and GM-CSF are required for the resolution of *C. albicans* infections (55, 56). The decrease in ERK1/2 phosphorylation in combination with G-CSF and GM-CSF reduction upon Gab1 and Grb2 knockdown suggests that both adaptor proteins control the OEC immune response to Clys, which may subsequently mediate neutrophil mobilization during *C. albicans* infection *in vivo*.

EGFR adaptor activation and the OEC response to fungal infection are not well understood. Our data show that ERK1/2 is the major pathway activated by the EGFR adaptors, rather than p38 or SAPK/JNK, in agreement with work showing that Clys activates p38 independently of EGFR and ERK1/2 and that the EGFR-ERK1/2-c-Fos pathway is downstream of p38 (11). During infection with *C. albicans*, MAPK signaling is regulated by MKP1/DUSP1, with ERK1/2 mediating a negative feedback loop to coordinate the innate immune response (25). These data suggest a dual role for ERK1/2 signaling in response to *C. albicans* and Clys (i) as a mediator of epithelial immune responses and (ii) a regulator of MAPK signaling responses. Interestingly, we only observed a partial reduction in c-Fos total protein when Gab1, Grb2, Shc, or Shp2 were knocked down or inhibited during fungal exposure, suggesting that additional unidentified factors can promote OEC responses against *C. albicans* and Clys.

Although Gab1, Grb2, and Shc mediate cytokine responses in response to *C. albicans* and Clys, knockdown of c-Cbl showed either no effect or an increase in G-CSF, GM-CSF, and IL-1β release. Additionally, inhibition of Shp2 resulted in an increase in IL-6 secretion, most notable with Clys. This suggests a role for c-Cbl and Shp2 as possible negative regulators of EGFR signaling and immune induction. Shp2 has been shown to promote bacterial clearance following post-influenza *Staphylococcus aureus* pneumonia (57) and orchestrate macrophage function against pulmonary bacterial infection (58), but its role in fungal infections is largely undefined. However, Shp2 is known to negatively regulate IL-6 release in macrophages in response to TLR ligands (59); thus, we anticipate that Shp2 has a similar function during OEC infections. The c-Cbl adaptor is important in EGFR trafficking and regulation (60, 61); notably, ubiquitination of EGFR by c-Cbl is implicated in clathrin-independent endocytosis of EGFR and downstream signaling (62). Additionally, though the p38 MAPK pathway is pivotal in EGFR endocytosis (63), little is known about the role of p38 in EGFR cellular localization in response to infection. It is likely that the complex dynamics of EGFR trafficking during fungal infection impacts EGFR signaling and consequently the OEC immune response.

In summary, these data indicate that the EGFR- and MAPK-associated adaptors Gab1, Grb2, Shc, Shp2, and c-Cbl play distinct roles in regulating OEC responses to *C. albicans* infections (Fig. 7). Though these adaptors are key mediators

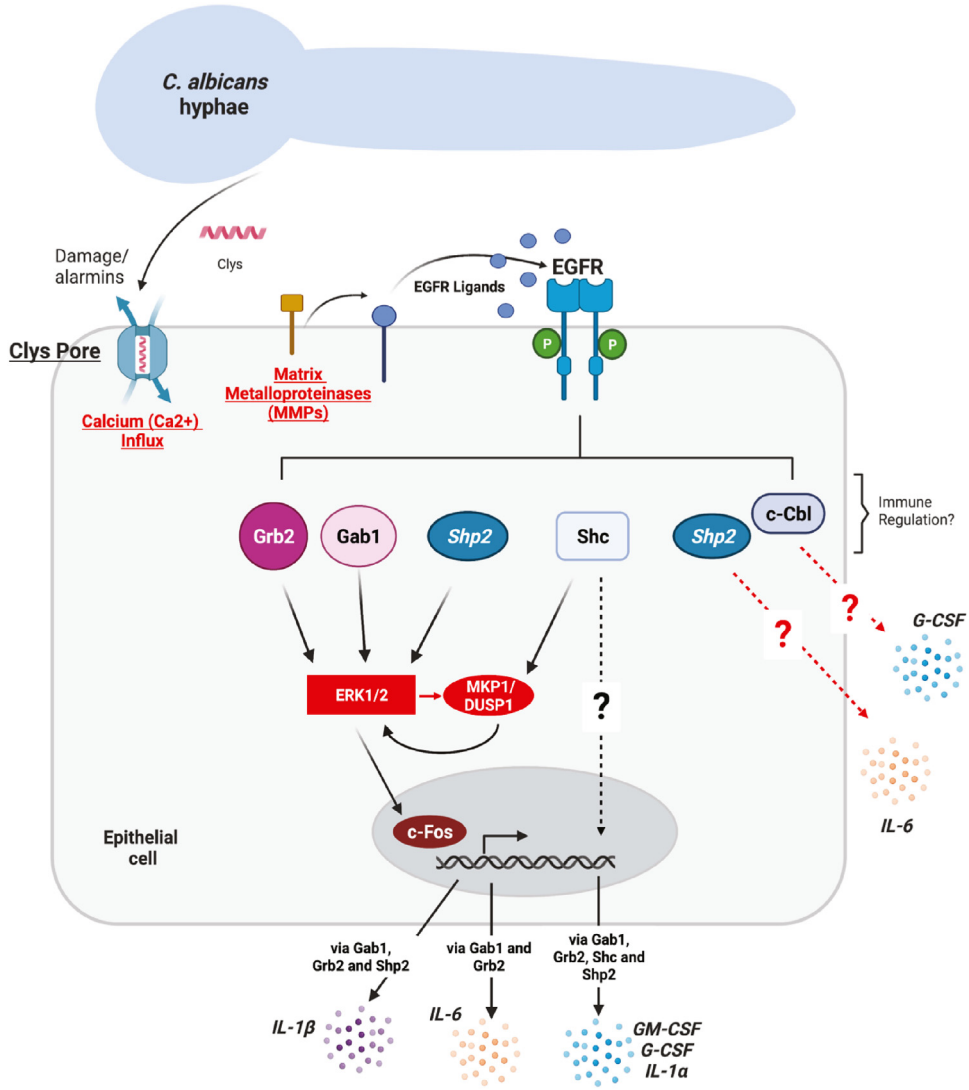

Figure 7. Gab1, Grb2, Shc, and Shp2 adaptors mediate candidalysin-induced signaling events that trigger oral epithelial cytokine responses. *C. albicans* infections are initiated by increased fungal burden, associated hypha formation, and secretion of the pore-forming toxin candidalysin (Clys). When accumulated at high concentrations, Clys interacts with an epithelial cell membrane to form pore-like structures that result in membrane damage, alarmin release, and calcium influx. These events lead to activation of matrix metalloproteinases (MMPs) and the cleavage/release of epidermal growth factor receptor (EGFR) ligands. EGFR activation leads to the recruitment and activation of the Gab1, Grb2, Shc, Shp2, and c-Cbl adaptor proteins which in turn mediate MKP1/DUSP1 and c-Fos activation *via* the MAPK-ERK1/2 signaling pathway. Cumulatively, this leads to induction of GM-CSF and G-CSF, as well as IL-6, IL-1α, and IL-1β, which mediate the subsequent stages of the innate and adaptive immune responses. Figure created with Biorender.com

supporting microbial infection, relatively few studies have evaluated them as regulators of an epithelial immune response. As such, it is likely that EGFR-associated adaptors act as sentinel regulators of epithelial immune responses during mucosal infections.

Experimental procedures

C. albicans *strains and Clys*

C. albicans strains were previously described (5) and were maintained on yeast extract peptone dextrose agar and cultured in yeast extract peptone dextrose at 30 °C. Fungal cells were harvested by centrifugation, washed twice with PBS, and diluted in appropriate media. The Clys peptide (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK; also termed Ece1-III$_{62\text{-}92K}$) was synthesized by Peptide Protein Research Ltd and stored frozen at 3.021 mM (10 mg/ml) in sterile water.

Cell culture

TR146 human buccal epithelial squamous cell carcinoma cells (64) were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 HAM (DMEM/F-12) (Gibco) supplemented with 15% fetal bovine serum (Gibco) and 1% (v/v) penicillin–streptomycin) (Sigma–Aldrich). Serum-free DMEM/F-12 was used to replace normal growth medium 24 h before and during cell stimulations.

Table 1
Summary of EGFR-MAPK adaptor knockdown and inhibition phenotypes

	Adaptor knocked down/inhibited				
Cytokine	Gab1	Grb2	Shc	Shp2	c-Cbl
G-CSF	↓↓	↓↓	↓↓	↓↓	↑↑
GM-CSF	↓↓	↓↓	↓↓	↓↓	-
IL-6	↓↓	↓↓	-	↑↑	-
IL-1α	↓↓	↓↓	↓	↓	↓
IL-1β	↓↓	↓	-	↓	-

↓↓ Significant reduction.
↓ Reduced.
- No change.
↑ Increased.
↑↑ Significant increase.

Reagents

Inhibitors were from Santa Cruz Biotechnology (Gefitinib), Selleckchem (PD153035, SHP099 HCL, BAPTA-AM), and Tocris Bioscience (Marimastat) and were reconstituted in dimethyl sulfoxide. Cetuximab was a gift from Guy's Hospital Cancer Centre. TR146 cells were incubated with inhibitors for 1 h prior to *C. albicans* infection or Clys treatment. Antibodies sources were as follows: Cell Signaling Technology: pGab1 Y659 (#12745S), Gab1 (#3232S), Grb2 (#3972S), pShc Y317 (#2431S), Shc (#2432S), pShp2 Y580 (#3703S), Shp2 (#3752S), pc-Cbl Y774 (#3555S), c-Cbl (#8447S), pMKP1/DUSP1 S359 (#2857S), c-Fos (#2250S), pp44/42 MAPK (ERK1/2) T202/Y204 (#4370S), pp38 MAPK T180/Y182 (#4511S), pSAPK/JNK T183/Y185 (#4668S), pAkt S473 (#4060S), pMTOR S2448 (#5536S) and β-Tubulin (#2146S); Millipore: mouse α-actin (#MAB1501); Jackson ImmunoResearch: goat antimouse IgG (#115-035-062), goat anti-rabbit IgG (#111-035-144) horseradish peroxidase.

RNA silencing

RNAi primers were from Thermo Fisher Scientific. TR146 cells were transfected with 5 or 10 nM of Gab1 (s5463), Grb2 (s226232), Shc (s12813), Shp2 (s11525), c-Cbl (s2476), or a negative control siRNA (Silencer Negative Control No. 2 siRNA) using HiPerfect Transfection Reagent (Qiagen) per manufacturers' instructions. The extent of knockdown was verified after 48 h by immunoblotting or quantitative PCR (qPCR).

qPCR

RNA was isolated with RNeasy Mini Kits (Qiagen). Complementary DNA was synthesized using iScript cDNA synthesis Kit (Bio-Rad). Real-time qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX Opus 96 real-time PCR instrument (Bio-Rad), normalized to *GAPDH*. Primers were from QuantiTect Primer Assays (Qiagen).

Immunoblotting

TR146 cells were incubated with the indicated fungal strains or treated with Clys (70, 15, or 3 μM) for the indicated time points. Cells were washed with PBS and lysed with radioimmunoprecipitation assay buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS, 20 mM EDTA) containing protease and phosphatase inhibitors (Sigma–Aldrich). Cells were manually detached, and lysates incubated on ice for 30 min. Five to ten micrograms of protein extracts were separated by SDS-PAGE on Bolt 4% to 12% Bis–Tris precast gels (Bio-Rad) using Immobilon chemiluminescent substrate (Millipore). Images were visualized on an Odyssey Infrared Imaging System (LI-COR).

Immunoprecipitation

TR146 cells were serum starved and infected with *C. albicans* at an multiplicity of infection of 10 or treated with Clys (70, 15, or 3 μM). For inhibitor experiments, cells were pretreated 1 h prior to infection or stimulation. Cells were washed with PBS and lysed with buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5% glycerol) supplemented with protease and phosphatase inhibitors (Sigma–Aldrich) for 30 min. Approximately 100 μl of total lysate was saved for use as loading controls and the remainder was precleared with 25 μl of Protein A/G magnetic beads (Pierce, Thermo Fisher). Lysates (250 μg) were immunoprecipitated with phosphotyrosine Ab-magnetic beads (#8095S, Cell Signaling Technology) or control IgG (#5873, Cell Signaling Technology).

Quantification of cell damage (LDH assay)

A Cytox 96 nonradioactive cytotoxicity assay kit (Promega) was used to measure the activity of lactate dehydrogenase (LDH) in culture supernatants as described (13, 17) according to the manufacturer's instructions, and recombinant L-Lactic Dehydrogenase from porcine heart (Sigma–Aldrich) was used to generate a standard curve.

Cytokine secretion

Cytokine levels in culture supernatants were assessed by Luminex assay (Bio-techne) and a Bioplex-200 machine (Bio-Rad). Bioplex manager 6.1 software (Bio-Rad) was used to determine analyte concentrations.

Statistics

Data were analyzed by ANOVA with Bonferroni's multiple comparisons test using GraphPad Prism (v. 8) software (GraphPad Software Inc). p values < 0.05 were considered significant.

Data availability

All data on adaptor phosphorylation, inhibition and knockdown assays, and cytokine expression are contained within this article.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: AMP, antimicrobial peptide; Clys, candidalysin; MMP, matrix metalloproteinase; OEC, oral epithelial cell; PFT, pore-forming toxin; qPCR, quantitative PCR.

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