Genetic control of *Candida albicans* biofilm development

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Abstract | *Candida* species cause frequent infections owing to their ability to form biofilms — surface-associated microbial communities — primarily on implanted medical devices. Increasingly, mechanistic studies have identified the gene products that participate directly in the development of *Candida albicans* biofilms, as well as the regulatory circuitry and networks that control their expression and activity. These studies have uncovered new mechanisms and signals that govern *C. albicans* biofilm development and associated drug resistance, thus providing biological insight and therapeutic foresight.

Pseudohypha
A chain of attached, elongated cells with constrictions at the septa.

The medically relevant *Candida* species[1] are mainly commensal fungi that reside on mucosal surfaces and in the gastrointestinal and genitourinary tracts. Although these organisms are usually benign, they can cause infection if immune function in the host is impaired or if an environmental niche becomes available[2]. Many *Candida* spp. infections arise as a result of the organisms' ability to grow as a biofilm on implanted medical devices[3–6]. The use of these devices — such as venous catheters, urinary catheters and artificial joints — is now routine, with more than 10 million recipients per year[7]. Device-associated *Candida* spp. infections have mortality rates as high as 30%[7,8], and the annual cost of antifungal therapies in the United States alone is estimated at US$2.6 billion[9]. Like the biofilms formed by bacterial pathogens, *Candida* spp. biofilms are resistant to many antimicrobial agents, so treatment can require surgical removal and later replacement of the infected device[5,7]. Here, we review *Candida* spp. biofilm development with a focus on *Candida albicans*, the most frequently isolated *Candida* pathogen[10].

Candida albicans biofilm development

C. albicans biofilms consist of two main kinds of cells: small oval yeast-form cells (also called blastospores) and long tubular hyphal cells. *C. albicans* biofilms grown *in vitro* often have a foundation of yeast cells, from which a hyphal layer emanates[5] (FIG. 1a). Extracellular matrix material is also clearly evident and is bound to both yeast and hyphal cells. It is typically interspersed throughout the biofilm, although in FIG. 1a it is mainly apparent at the top of the sample. Biofilms from *in vivo* catheter infection models seem to be more complex, with yeast cells and hyphae being interspersed[11] (FIG. 1b). Genetic analyses indicate that both yeast cells and hyphae are crucial for biofilm formation, which suggests that each cell type has a unique role in the process[5].

In vitro experiments allow *C. albicans* biofilm development to be viewed as a series of sequential steps[5,12] (FIG. 2). Biofilm formation begins with adherence of yeast cells to a substrate (the adherence step; FIG. 2). Soon afterwards, the yeast cells proliferate across the surface and produce elongated projections that grow into filamentous forms, including hyphae and pseudohyphae (the initiation step). Extracellular matrix accumulates as the biofilm matures, and high-level drug resistance is also acquired (the maturation step). Finally, non-adherent yeast cells are released from the biofilm into the surrounding medium (the dispersal step). Although these steps might occur concurrently rather than sequentially during natural biofilm development *in vivo*, they provide a useful framework with which to guide a mechanistic analysis of *C. albicans* biofilm development.

Simple inferences from biofilm genetics

Recent progress in expression profiling and genetic manipulation has increased our understanding of the regulatory pathways and mechanisms that govern *C. albicans* biofilm development and biofilm-based drug resistance. In addition, such analyses have pointed to an intriguing relationship between biofilm formation and mating. On the basis of known mutant phenotypes, it is clear that *C. albicans* genes can have net positive or negative roles in biofilm development. This distinction is useful when thinking about the relationships between genes, because a gene product with a negative effect can function by inhibiting a gene product with a positive effect, for example. In addition, it is worth bearing in mind that biofilm dispersal involves an unravelling of the steps involved in biofilm formation. Therefore,

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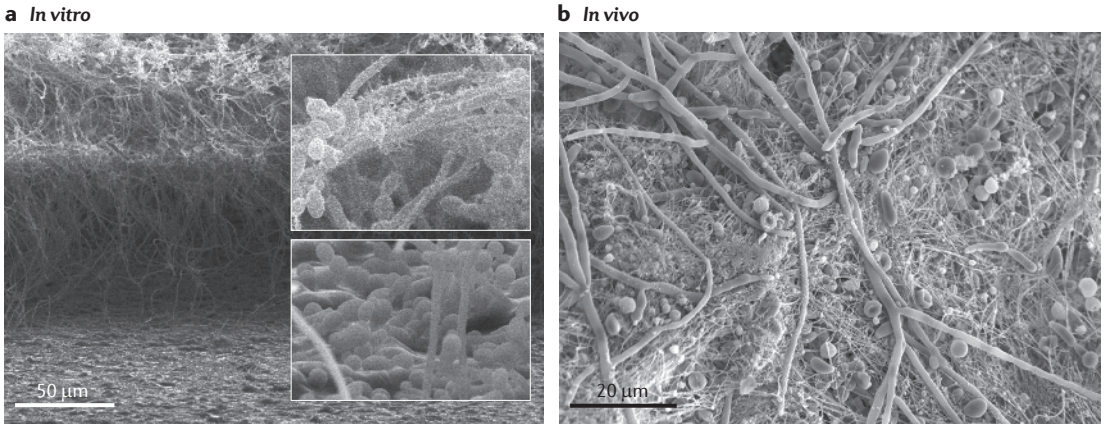

Figure 1 | ***Candida albicans* biofilm structure *in vitro* and *in vivo*. a** | Scanning electron micrograph (SEM) of an *in vitro* *Candida albicans* biofilm. The biofilm sample was sliced to show three layers in a cross-sectional view. The basal layer primarily includes yeast cells, as is evident in the lower enlarged inset. The central layer is mainly hyphae. The upper layer has yeast cells budding from the hyphae. The upper enlarged inset shows the extracellular matrix material, which seems fibrous in this preparation. **b** | SEM of an *in vivo* *C. albicans* biofilm from the rat catheter model[11]. Yeast cells, hyphae and some pseudohyphal cells are evident, along with extracellular matrix material. Images courtesy of J. Suhan (Carnegie Mellon University, Pittsburgh, Pennsylvania, USA), and J. Nett and D. Andes (University of Wisconsin–Madison, USA).

the negative function of yeast-form cell wall protein 1 (Ywp1) in the adherence step that leads to biofilm formation might go hand in hand with a positive function in biofilm dispersal, for example.

The genes that govern *C. albicans* biofilm development (TABLE 1) fit into several broad functional categories. Many of these genes are required for the production of hyphae (filamentation). Some of the first *C. albicans* biofilm genetic studies indicated that hyphae are required for stable biofilm formation[13,14]. In addition, several biofilm genes are involved in the response to the quorum sensing molecule farnesol[15,16]. Farnesol is an inhibitor of filamentation[15,16] and, as might be expected, it inhibits biofilm formation *in vitro*[17]. In fact, several quorum sensing molecules accumulate in mature biofilms (BOX 1) and the addition of such molecules to biofilm cultures *in vitro* indicates that they can promote biofilm dispersal[18–20].

Several noteworthy classes of gene products govern the properties of *C. albicans* biofilms, including known or predicted cell wall proteins. These proteins are of special interest because they might have a direct role in cell–substrate or cell–cell adherence. Indeed, heterologous expression studies indicate that a putative cell wall adhesin, enhanced adherence to polystyrene 1 (Eap1), as well as hyphal wall protein 1 (Hwp1), agglutinin-like sequence 1 (Als1) and Als3 have such roles[21–23]. Surface proteins are of further interest as accessible therapeutic targets. Finally, it has become increasingly evident that cell heterogeneity is a crucial feature of biofilms[24]. This attribute is obvious from the different cell types that are seen in *C. albicans* biofilms (FIG. 1), and the genes that encode fungal cell wall proteins are subject to both genetic and epigenetic mechanisms that further contribute to cell heterogeneity[25,26].

Many of the *C. albicans* genes involved in biofilm development encode predicted transcription factors or protein kinases. These regulatory proteins must function indirectly to control biofilm properties, but can be informative indicators of the internal and external signals that influence biofilm development. For example, a transcription factor, biofilm and cell wall regulator 1 (Bcr1), is required for biofilm formation, and its expression is upregulated in hyphae, thus indicating that Bcr1-dependent gene products might be the hyphal components that are required for biofilm formation[27,28]. Similarly, the zinc-responsive transcription factor Zap1 (also known as Csr1 and Sur1) is a regulator of extracellular matrix accumulation, indicating that alterations in zinc levels might alter matrix formation[29]. The signals that influence the activity of many of the other biofilm regulators listed in TABLE 1 are not well understood, which presents an opportunity for future study.

Several alcohol dehydrogenases and aryl-alcohol dehydrogenases have an impact on biofilm development. The fact that both positive and negative roles have been found for these proteins indicates that substrate specificity is crucial for their biological function[29,30]. Although it is possible that their substrates and products function primarily through effects on intermediary metabolism, we note that the aryl-alcohol dehydrogenases in particular have been implicated in the synthesis of amino acid-derived alcohols, which might function in quorum sensing[19,31,32].

We have assembled a model that connects the steps involved in *C. albicans* biofilm development with biofilm genes and regulatory pathways (FIG. 2). The regulatory relationships shown are based on diverse lines of evidence, which include mutant phenotypes, genetic epistasis tests, microarray analyses, gene overexpression phenotypes and chromatin immunoprecipitation assays. Thus, some pathway relationships are indirect or tentative. The model is intended as a framework for the identification of new areas of inquiry and for interpretation of future studies.

Quorum sensing
Communication between neighbouring cells, carried out through secreted signalling molecules, allowing populations to sense organism density and alter gene expression accordingly.

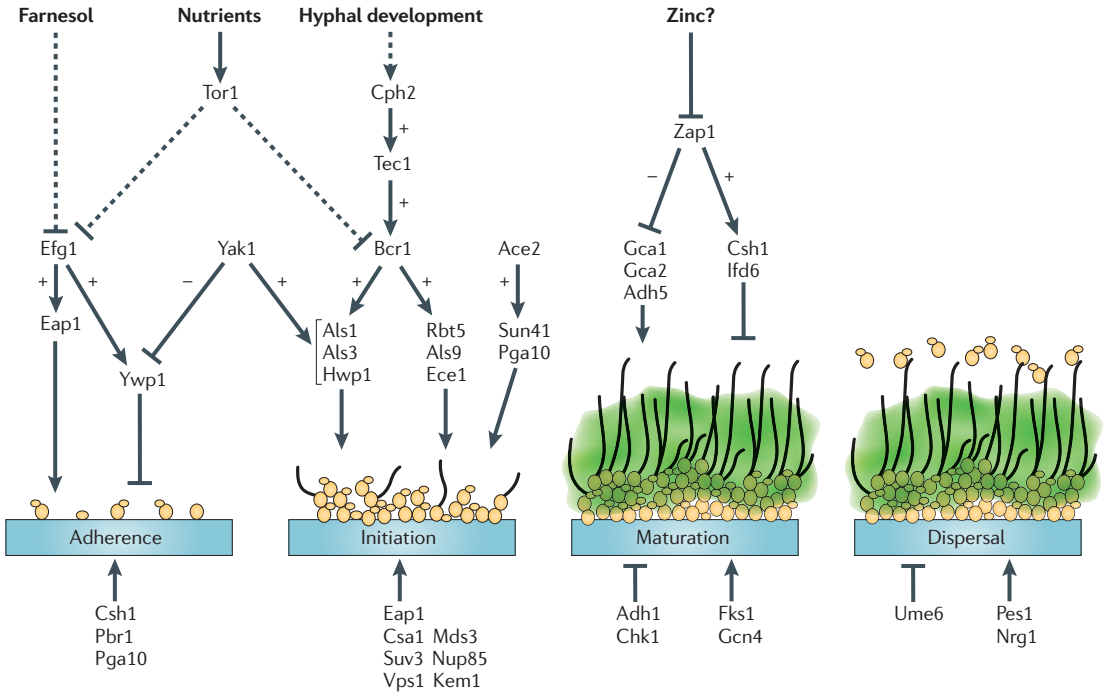

Figure 2 | **Proteins that function in biofilm development.** In the adherence step, yeast-form cells adhere to the substrate. In the initiation step, the cells propagate to form microcolonies, and germ tubes form to yield hyphae. In the maturation step, the biofilm biomass expands, the extracellular matrix (green) accumulates and drug resistance increases. In the dispersal step, yeast-form cells are released to colonize the surrounding environment. The upper half of the diagram depicts several known pathway relationships. The lower half includes proteins that function in a specific step, but might not be connected to a known pathway. For simplicity, some known pathway relationships have been omitted. Proteins are presented more than once if they have roles in more than one step of biofilm development. Dashed T-shaped bars indicate repression by an indirect mechanism. Plus and minus symbols indicate that the upstream gene or signal stimulates (+) or inhibits (–) the expression of the downstream target. See main text for details about some of the specific proteins in the model.

Perhaps the best use of a model for gene function in biofilm development is its application to *in vivo* models of biofilm formation on implanted devices[11,33–35]. *In vivo* models are crucially important because the nature of the device surface, the presence of host-derived conditioning film, the amounts of oxygen and carbon dioxide, and liquid flow all affect biofilm development[12,36–38]. Thus, it is impossible to duplicate *in vitro* all the conditions that are relevant *in vivo*.

Diversity of biofilm environments and cohabitants

Many studies have focused on the formation of *C. albicans* biofilms on implanted vascular catheters, because this is a major source of infection[7]. However, biofilms can also be formed on many other devices. A rat denture biofilm model that recapitulates features of denture stomatitis has been described recently[33]. Microscopic and microbiological analyses showed the signature features of biofilm development: adherent cells, the presence of extracellular matrix material, and high-level drug resistance. The biofilms in one other recently described rat model, involving subcutaneously implanted catheters, also had characteristic matrix material and an abundant hyphal population[34]. This model provides both ethical and technical advantages because a single animal can be used to culture numerous biofilms.

Biofilms also form on tissue surfaces, including the oral and vaginal mucosa. In such infection models, *C. albicans* produces dense three-dimensional biofilms that are embedded in extracellular matrix material[39,40]. The level of biofilm drug resistance in these models has not been tested directly, although drug resistance is seldom a clinical problem with vaginal candidiasis[40].

Is biofilm formation in these new environments mechanistically distinct from the more commonly studied *in vitro* and *in vivo* models? There are certainly some conserved features. For example, biofilm formation in most of the models described here depends on the transcription factor Bcr1[33,34,40]. Where tested, strains that were defective for filamentation were also defective for biofilm formation[34,40]. However, on the basis of results from the systematic manipulation of *in vitro* biofilm environments[38], and given the pronounced gene expression responses of *C. albicans* to distinct host niches[41], it is likely that distinct genetic requirements and mechanisms will emerge for each system.

For any one type of biofilm, the environment can be altered by the presence of co-infecting microorganisms. The overall frequency of mixed-species biofilm infections has not been reported, but >20% of bloodstream infections involving *Candida* spp. are polymicrobial[42]. In a recent analysis of 24 cases of endocarditis

Table 1 | **Selected genes involved in *Candida albicans* biofilm development**

Molecular function of gene products*	Role of gene product	Genes	Refs
Transcription factors	Positive	*ACE2*‡, *BCR1*, *CPH1*, *CZF1*‡, *EFG1*‡, *FLO8*‡, *GCN4*, *TEC1*‡, *UME6*‡ and *NRG1*‡	14,27,28,90,98, 99,118–122
	Negative	*ZAP1*	29
Cell wall-related proteins	Positive	*ALS1*, *ALS2*‡, *ALS3*, *ALS4*, *ALS5*, *ALS7*, *ALS9*, *CSA1*, *EAP1*, *FKS1*, *HWP1*, *HWP2*, *OCH1*, *PGA1*, *PGA10*‡, *PMT1*‡, *PMT2*‡, *PMT4*, *PMT6*, *RBT1*, *RBT5* and *SUN41*‡	22,23,28,38,56, 67,71,73–76,97, 123–125
	Negative	*YWP1*	126
Alcohol dehydrogenases	Positive	*ADH5*	127
	Negative	*ADH1*, *CSH1* and *IFD6*	29,30
Protein kinases	Positive	*CBK1*‡, *GIN4*‡, *IRE1*‡, *MKC1* and *YAK1*‡	64,78,128
	Negative	*CHK1* and *TOR1*,	129,130
Drug efflux pumps	Positive	*CDR1*, *CDR2* and *MDR1*	89
Glucoamylases	Positive	*GCA1* and *GCA2*	29
Other functions§	Positive	*CAT2*, *ECE1*, *KEM1*‡, *MDS3*‡, *NDH51*, *NUP85*‡, *PBR1*, *PES1*, *PDX1*, *RIX7*, *SUV3*‡, *VAM3*‡ and *VPS1*‡	28,68,98,131–136

*Molecular functions have been inferred from protein sequence homology, in most cases. For specific gene product functions and details, see main text and Supplementary information S1 (table). ‡Indicates a regulator of filamentation. §Gene product functions that do not fit into any of the categories listed here.

associated with an implanted device, ~25% of the infections were found to be polymicrobial[43]. In biofilms grown *in vitro*, the interactions between bacteria and *C. albicans* are diverse[44]. Symbiotic interactions can result in augmented adherence and antibiotic resistance[45,46]. However, most of the known interactions are inhibitory. Among the most intriguing examples are those that arise from trans-kingdom responses to quorum sensing molecules. For example, farnesol produced by fungi inhibits the formation of *Staphylococcus aureus* biofilms and increases the antibiotic susceptibility of the bacterium[47,48]. Bacteria can fight back, however; for example, the *Pseudomonas aeruginosa* quorum sensing molecule homoserine lactone mimics farnesol, inhibiting *C. albicans* filamentation and thus preventing the formation of *C. albicans* biofilms[49]. Other inhibitory interactions arise from broader environmental manipulations; for example, vaginal bacteria inhibit *C. albicans* growth and virulence by producing H_2O_2 or lactic acid[44,50]. The importance of further study in this area is demonstrated by the fact that the presence of combined infection by both bacteria and *C. albicans* can result in increased mortality[51,52].

Adherence and attachment responses

The gene products that have been assigned to the adherence step (FIG. 2) have been shown by either null-mutant analysis or heterologous-expression studies to affect the binding of *C. albicans* to a plastic or protein-coated substrate. One of the most clearly defined biofilm adhesins that mediates surface binding is Eap1[21,53]. Eap1 has sequence features that are commonly found in fungal cell surface proteins[54,55], including a signal sequence and an amino acid composition that is rich in the prospective glycosylation acceptors serine and threonine. The protein also contains internal repeats of a peptide motif, Trp-Pro-Cys-Leu, that is found in numerous fungal cell surface proteins. Finally, it has a short carboxy-terminal sequence that directs the attachment of a glycosylphosphatidylinositol (GPI) anchor. GPI-linked proteins are found in many eukaryotes, in which the GPI moiety tethers proteins to the plasma membrane. However, *C. albicans* and many other fungi can cleave this anchor and then transfer the cleavage product and attached protein to form a covalent linkage with β-glucan in the cell wall[54,55]. Several approaches indicate that Eap1 is indeed a GPI-linked cell wall protein[53]. Three observations indicate that Eap1 functions directly in biofilm adherence: expression of the protein in a non-adherent *Saccharomyces cerevisiae* strain confers adherence to polystyrene; a *C. albicans eap1*$^{-/-}$ deletion mutant has reduced adherence to polystyrene; and a *C. albicans eap1*$^{-/-}$ deletion mutant is defective in biofilm formation, as assayed both *in vitro* and in an *in vivo* catheter model[53,56].

The closely related cell wall proteins Als1 and Als3 might also function in biofilm surface attachment[57]. Expression of Als1 or Als3 in *S. cerevisiae* promotes binding to several different protein-coated substrates[58], which may resemble the conditioned surface of an implanted device. In addition, a *C. albicans* mutant lacking both *ALS1 and ALS3* is defective in biofilm formation *in vitro* and *in vivo*[22]. In particular, catheter surfaces inoculated with the double mutant are virtually devoid of cells after incubation *in vivo*[22], as would be expected if the mutant has a severe substrate adherence defect.

The idea that Eap1 and Als1 might function in the initial adherence step is consistent with the fact that expression of both genes is detectable in cells grown as either yeast or hyphal cell types[56,59]. This is not the case for Als3, which is expressed primarily or exclusively in hyphae[59]. It is possible that the initial adherence step that leads to biofilm formation *in vivo* can be carried out by

Box 1 | **Quorum sensing and *Candida albicans* biofilms**

Quorum sensing phenomena are those in which microbial behaviours or responses are governed by cell density. Such community behaviours are usually determined by secreted signalling molecules, the accumulation of which is a measure of cell density[100]. Quorum sensing has a pivotal role in biofilms of all kinds[101,102]. The best studied quorum-sensing molecule in *Candida albicans* is *E*,*E*-farnesol, an inhibitor of hyphal formation. Exogenous farnesol inhibits biofilm formation if provided early during adherence[17,18]. The limited biofilms that form in the presence of farnesol comprise mainly yeast and pseudohyphal cells, rather than hyphae. Farnesol also accumulates in supernatants of mature biofilms[20], where stimulation of yeast cell production might promote biofilm dispersal. Tyrosol, an alcohol derived from tyrosine, has the opposite activity to farnesol: it stimulates hyphal formation. The addition of exogenous tyrosol does not have a measurable effect on overall biofilm development but can partially overcome the inhibition of biofilm formation by exogenous farnesol[18]. Tyrosol also accumulates in mature biofilm supernatants[18,20], and the overall inhibition of hyphal formation by such supernatants[17,18,20] seems to reflect the dominant activity of farnesol[18]. Several other small molecules are detectable in biofilm supernatants, including phenylethyl alcohol, dodecanol and nerolidol[20]. Each of these compounds can inhibit hyphal formation, and thus all might aid in biofilm dispersal by promoting yeast cell formation. It will be of interest to block the synthesis of, or response to, individual molecules in order to assess their biological functions, and to test their roles in biofilm development *in vivo*.

either yeast-form cells, which express Als1, or hyphae, which express Als3.

Adherence itself can activate a gene expression response. For example, a microarray comparison of planktonic cells and substrate-adherent cells revealed that, interestingly, a change in gene expression was established as little as 30 minutes after adherence and was maintained for hours for several genes[60]. In addition, the expression of fusion proteins consisting of GFP fused to the drug efflux proteins Cdr1 or Mdr1 was upregulated following a few minutes of adherence to a glass slide[61]. Thus, *C. albicans* can sense and respond to surface contact. The regulators that promote attachment responses are unknown, but the transmembrane protein Dfi1 and the mitogen-activated protein kinases Mkc1 and Cek1 (also known as Erk1) are mediators of other surface-dependent responses[62–64] and are, thus, excellent candidates. Mkc1 is required for normal biofilm formation[64], and it would be interesting if this requirement were to reflect an effect on adherence-induced gene expression.

Adherence is also highly regulated through a new mating factor response pathway (BOX 2). Interestingly, this pathway operates in cells that do not mate but rather assist in mating through biofilm formation[65–68]. The responsive cells are of mating type a/a and thus have the potential to mate with α/α cells. However, they are unable to do so, because they have not made the epigenetic transition from the white, mating-incompetent state to the opaque, mating-competent state[69]. They nonetheless respond to α-factor through a newly evolved hybrid signal transduction pathway[66] to create a biofilm. Four white-cell genes that are induced by α-factor are required for full adherence of this biofilm: the genes that encode the cell surface proteins Eap1 and the predicted GPI-anchored protein 10 (Pga10), the predicted secreted protein Pbr1 and the putative aryl-alcohol dehydrogenase cell surface hydrophobicity 1 (Csh1)[68]. Csh1 has been detected on the *C. albicans* cell surface[70], so the nature of its role in adherence is uncertain. Most *C. albicans* isolates are a/α cells and do not secrete or respond to mating factor; a/α strains have been used for most of the biofilm studies described in this Review. However, the fact that Eap1, Pga10 and Csh1 all have roles in biofilm formation in a/α cells indicates that both kinds of *C. albicans* biofilm might use similar gene products[68].

Biofilm initiation and filamentation

The gene products that have been assigned to the initiation step (FIG. 2) have a range of functions. This diversity results, in part, from our broad definition: these are genes in which mutations cause the production of only a small, rudimentary biofilm *in vitro*. Overexpression of some of the gene products in this group (Rbt5, Als9 and extent of cell elongation 1 (Ece1)) improves biofilm formation in a *bcr1*$^{-/-}$ mutant, which is defective in biofilm initiation (see below). We note that a mutant with a partial adherence defect might be categorized as an initiation mutant; thus, our assignment of gene products to this step is tentative.

The production of hyphae is a hallmark of initiation, and many initiation-defective mutants grow solely as yeast cells under biofilm conditions (TABLE 1). Bcr1 expression is upregulated in hyphae, although this protein is required for biofilm initiation but not for the production of morphologically normal hyphae[27,28]. Rather, it is required for normal production of several cell surface proteins, some of which (such as Als3 and Hwp1) are induced in hyphae. The failure to express these surface proteins is the cause of the biofilm defect in the *bcr1*$^{-/-}$ mutant, because increased expression of *ALS3* or *HWP1* in this mutant restores the ability to form biofilms, both *in vitro* and *in vivo*[23,28] (FIG. 3). Moreover, expression of *BCR1* or its target genes can even permit biofilm formation by mutants that are defective in hyphal morphogenesis: specifically, increased expression of *BCR1* in a hyphal-defective *tec1*$^{-/-}$ mutant permits *in vitro* formation of a biofilm, albeit a fragile one[28]. In addition, the expression of a surface-directed Als3 fusion protein permits biofilm formation *in vitro* in a hyphal-defective enhanced filamentous growth 1 (*EFG1*) deletion mutant[71]. Therefore, the main way that hyphae promote biofilm formation is through expression of their surface protein complement.

Interestingly, the Bcr1 orthologue in the biofilm-forming species *Candida parapsilosis* is also required for biofilm formation in this species[72]. Because *C. parapsilosis* does not form hyphae, the regulatory pathway upstream of Bcr1 may be divergent. Nonetheless, this finding points to the possibility that Bcr1 orthologues in other species might also govern biofilm formation.

What do these hyphal surface proteins do? Hyphae are extremely 'sticky', and both Als3 and Hwp1 are adhesins in some contexts[54], so it seems reasonable to assume that they might promote cell–cell or cell–substrate binding. In fact, Als3 (along with the closely related Als1) and Hwp1 seem to function as complementary cell–cell adhesins, analogous to the mating agglutinins of *S. cerevisiae* that promote binding between a-cells and

Box 2 | ***Candida albicans* mating**

One of the most exciting discoveries in *Candida albicans* research in recent years is the finding that this organism, which was long considered to be asexual, can mate. A mating-type locus called *MTL* determines sexual identity through regulatory relationships with some similarity to those of *Saccharomyces cerevisiae*: *MTLa/MTLa* cells can mate with *MTLα/MTLα* cells, and *MTLa/MTLα* cells cannot mate[103]. The mating response is induced by secreted mating pheromones: *MTLa/MTLa* cells secrete a-factor, and *MTLα/MTLα* cells secrete α-factor[104]. However, mating involves more than just *MTL*-specified sexual identity; cells must switch from the mating-incompetent white cell type to the mating-competent opaque cell type[105–109]. The epigenetic white–opaque switch responds to numerous genetic and environmental signals, but it does not seem to be regulated by mating factors[110,111].

Mating of two *C. albicans* diploid cells yields a tetraploid that breaks down through chromosome loss to yield recombinant diploid progeny[112–115]. Normal functioning of the chromosome loss pathway depends on Spo11[116], orthologues of which function in meiosis in other organisms, but there is no evidence that *C. albicans* has a complete meiotic pathway.

Although white cells do not mate, they do respond to mating pheromones. The response can be assayed through changes in gene expression and increases in cell–cell and cell–substrate adherence, which stimulate biofilm formation[65–68,104,117]. Interestingly, only a proportion of the opaque pheromone response pathway is used in white cells; they use a hybrid pathway with new downstream components[66–68]. Opaque cells may be rare in many niches, and the biological role of this white cell biofilm seems to be to facilitate mating among disperse opaque cells[65,110,115].

α-cells. Two main observations support this idea. First, both a *hwp1*$^{-/-}$ mutant and an *als1*$^{-/-}$*als3*$^{-/-}$ double mutant are defective in biofilm formation, but a mixture of the two mutant strains produces a robust biofilm *in vitro* and *in vivo*[22]. This finding indicates that Hwp1, Als1 and Als3 have distinct and complementary roles in biofilm formation. Second, expression of *HWP1* in *S. cerevisiae* promotes the adherence of this yeast to hyphae of wild-type *C. albicans*[22], but adherence is diminished when tested with hyphae of a *C. albicans als1*$^{-/-}$ *als3*$^{-/-}$ double mutant. These findings point towards a function of Hwp1, Als1 and Als3 as mediators of cell–cell adherence.

At this juncture, it is possible to propose a minimal pathway of biofilm formation. First, yeast cells express Eap1 and Als1, which mediate cell–substrate binding. Second, surface-bound cells propagate and express Als3 and Hwp1, which mediate cell–cell binding. Hyphae formation might provide a simple pathway that leads to Als3 and Hwp1 accumulation. Als3 would also augment cell–substrate binding, as discussed above. In addition, it has been shown that Eap1 mediates cell–cell binding and cell–substrate binding, so this protein would participate in both processes. Many of the proteins required for biofilm initiation are also required for hyphae formation; in the minimal model, their functions are explained as ultimately being required for *ALS3* and *HWP1* expression.

However, the roles of some biofilm initiation proteins are less readily explained by the minimal model: the additional cell surface proteins, including Sun41, Csa1, Pga10, Rbt5, Hwp2 and Rbt1 (TABLE 1). Analysis has been challenging for several of these proteins, because they belong to families with overlapping or compensatory functions (Csa1, Pga10 and Rbt5; Hwp2, Rbt1 and Hwp1; and Sun41 and Sun42)[67,73–76]. To study the genes encoding these proteins, strains with mutations in multiple genes are required, but the construction of such mutants is not trivial. In any case, current observations indicate that some of these proteins might function as adhesins. In particular, the additive effects of *hwp2* or *rbt1* mutation with a *hwp1* mutation, along with the known role of Hwp1 as an adhesin, indicate that Hwp2 and Rbt1 are adhesins[67]. For example, they might contribute to a threshold level of cell–cell binding that is required for biofilm stability. In *C. parapsilosis*, the Bcr1 homologue promotes the expression of *RBT1*, so perhaps *C. parapsilosis* Rbt1, which has an important role in biofilm formation[72,77], has assumed a predominant adhesin function in this species. A second suggestion is that some of these cell surface proteins have general roles in cell wall structure, and that perturbation of the cell wall architecture impairs adherence through effects on either post-translational modification or the expression of adhesins. It should be noted that loss of Sun41 or Pga10 confers hypersensitivity to cell wall inhibitors, an expected consequence of a general cell wall defect[73–76]. It is also noteworthy that the amounts of *ALS1* RNA are reduced in the biofilm- and cell wall-defective protein kinase mutants *gin4*$^{-/-}$, *ire1*$^{-/-}$ and *cbk1*$^{-/-}$, thus indicating that adhesin gene expression might be regulated through cell wall regulatory pathways[78]. The mechanistic contribution of so many cell surface proteins to biofilm formation is a key aspect to be addressed, particularly because such proteins are inviting therapeutic targets.

Biofilm maturation and the extracellular matrix

Biofilm maturation includes continued growth of the biofilm and the accumulation of extracellular matrix material. Genes assigned to this category (FIG. 2) include those that affect matrix production or overall biofilm biomass.

The composition of the matrix that is produced *in vitro* includes carbohydrate, protein, hexosamine, phosphorus and uronic acid[79]. One main extracellular carbohydrate constituent is β-1,3 glucan, increased production of which is associated with biofilm cells rather than planktonic cells[80]. A proteomic analysis showed the presence of specific proteins associated with the biofilm cell surface; these proteins may include matrix components[30,81]. Finally, a recent study reported the detection of extracellular DNA[82], as has been found in bacterial biofilms[83]. Indeed, the addition of DNase to a mature biofilm partially disrupts the biofilm[79,82], and the addition of extracellular DNA at the beginning of biofilm development results in mature biofilms with increased biofilm biomass. Thus, extracellular DNA in the matrix contributes to the structure and stability of a mature biofilm.

The transcription factor Zap1, a regulator of zinc acquisition[84,85], is a net negative regulator of biofilm matrix production (FIG. 2). A *zap1*$^{-/-}$ mutant forms a biofilm with elevated levels of matrix β-1,3 glucan *in vitro* and *in vivo*[29]. Zap1 activates the expression of *CSH1* and *IFD6*, which have inferred negative roles in matrix production, and represses the expression of the glucoamylase 1 gene (*GCA1*), *GCA2* and the alcohol dehydrogenase 5 gene (*ADH5*), which have inferred positive roles[29]. Gca1 and Gca2 might function through the hydrolytic release of soluble β-1,3 glucan fragments

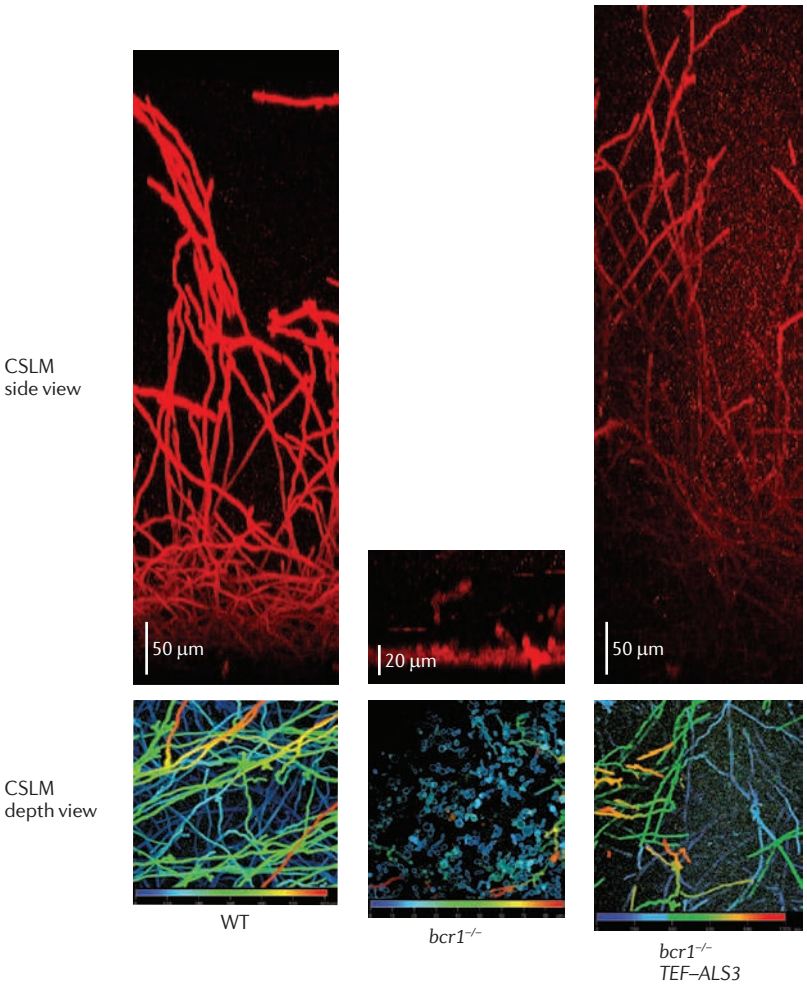

Figure 3 | **Restoration of biofilm formation in a biofilm and cell wall regulator 1 (BCR1)-null background by overexpression of surface protein gene agglutinin-like sequence 3 (*ALS3*).** Confocal scanning laser micrographs (CSLMs) of biofilms stained with concanavalin A–Alexa Fluor, grown under standard *in vitro* conditions[28]. The upper panels are side views; the lower panels are pseudocolour depth views, in which blue represents cells closest to the substrate and red represents cells farthest from the substrate. The wild-type (WT) biofilm has a dense mixture of yeast cells and hyphae that gradually becomes predominantly hyphae at the top of the biofilm. The *bcr1*$^{-/-}$ biofilm forms a basal layer of yeast cells attached to the substrate, with few or no hyphae. Increased expression of *ALS3* (under the control of a strong promoter, *TEF*) in the *bcr1*$^{-/-}$ strain permits substantial biofilm formation.

Ergosterol
The main sterol in the fungal cell membrane. Ergosterol is responsible, and essential, for structural and regulatory membrane features such as fluidity and permeability (equivalent to cholesterol in mammalian cells).

Azole
A class of antifungal drug that inhibits a late step in the biosynthesis of ergosterol; this includes the triazoles (for example, fluconazole, voriconazole and posaconazole) and the imidazoles.

from longer glucan chains. The precise functions of the alcohol dehydrogenase-related proteins Csh1, Ifd6 and Adh5 are unknown, but several similar *S. cerevisiae* alcohol dehydrogenases function in the synthesis of acyl and aryl alcohols[31,32,86,87]. These alcohols have roles in quorum sensing and cell signalling (BOX 1), as indicated by effects on hyphal growth, for example[15–18,20,88]. Thus, a possible mechanistic role for these dehydrogenases is to promote the biogenesis of biofilm-associated acyl and aryl alcohols, which, in turn, would control matrix synthesis. Csh1 and Ifd6 might function preferentially to yield a matrix-inhibitory signal, whereas Adh5 might function preferentially to yield a matrix-stimulatory signal[29]. The known role of Zap1 in zinc-responsive gene expression indicates that ambient zinc levels could be a crucial determinant of biofilm matrix formation.

Zap1 could have a broader role in biofilm maturation than simply the control of matrix accumulation. The *zap1*$^{-/-}$ mutant has reduced expression of several genes that are normally upregulated in mature biofilms, including those encoding ergosterol biosynthesis enzymes and putative hexose transporters[29]. Thus, Zap1 seems to govern several aspects of biofilm maturation. It will be interesting to see whether any of these mutant phenotypes reflect the postulated alteration of the levels of quorum sensing molecules.

A unique feature of mature biofilms, in addition to matrix accumulation, is the acquisition of high-level resistance to antifungals[5], notably the azoles and polyenes that target membrane sterols. The nature of biofilm drug resistance may reflect four distinct mechanisms. First, compared with non-biofilm cells, mature biofilm cells have reduced amounts of membrane sterols[89] and elevated expression of several ergosterol biosynthesis genes[60,90,91], which perhaps reflects hypoxia in these cells[38,77]. The ability of mature biofilm cells to survive with low sterol levels, combined with elevated levels of biosynthetic enzymes, could contribute to azole and polyene resistance. Indeed, a recent study showed that a polyene-resistant biofilm cell subpopulation displayed substantially increased expression of ergosterol biosynthesis genes[92]. Second, the azole efflux genes *CDR1, CDR2* and *MDR1* are induced early in biofilm formation and might contribute to overall azole resistance. However, their phenotypic contribution is detectable only in early biofilm cells[89]. Third, as with several bacterial biofilms[93], *C. albicans* biofilms contain a subpopulation of persisters that are tolerant to a range of otherwise cidal treatments[94]. The importance of this phenomenon is highlighted by the presence of persisters in human oral *C. albicans* populations[95]. Persisters do not have the long-term stability of mutants, but are phenotypic variants that may arise from an epigenetic change or, perhaps, transient aneuploidy[96]. Finally, the β-1,3 glucan found in the biofilm matrix binds to and sequesters azole drugs[80]. The physiological importance of this has been demonstrated by the analysis of a *C. albicans* strain with reduced β-1,3 glucan biosynthesis capacity (genotype *FKS1*/*fks1*$^{-}$ (also known as *GSC1*/*gsc1*$^{-}$))[97]. Biofilms formed by this strain have reduced matrix β-1,3 glucan and reduced azole resistance levels in both *in vitro* and *in vivo* models. The dramatic sensitivity of the mutant biofilm cells to azole treatment, particularly in the *in vivo* biofilm model, indicates that this sequestration mechanism is a major contributor to azole resistance in *C. albicans* biofilms[97].

Cell dispersal

Ultimately, a biofilm releases cells that can initiate the formation of new biofilms or disseminate into host tissues. Recent studies have examined the quantitative and qualitative properties of cells released from a *C. albicans* biofilm[98,99] and have yielded three important findings. First, most dispersed cells are yeast cells[98], as depicted in FIG. 2. This observation indicates that the transition from yeast cells to hyphae that occurs during biofilm initiation might be reversed for dispersal. Second, three

new transcriptional regulators of dispersal (Ume6, Pes1 (also known as Nop7) and Nrg1) were identified. Overexpression of *UME6* reduced the release of cells from a biofilm, and overexpression of either *PES1* or *NRG1* increased release[98,99]. Thus, changes in the expression or activity of Ume6, Pes1 or Nrg1 during biofilm maturation — perhaps in response to the accumulation of quorum sensing molecules — might control cell dispersal. Finally, dispersed cells have phenotypes that are distinct from those of planktonic cells: dispersed cells display elevated adherence and filamentation capacity, and increased pathogenicity in a disseminated infection model, when compared with planktonic cells. Thus, the dispersal step releases cells that are uniquely equipped to seed new biofilms and sites of infection[98,99].

Polyene
A class of antifungal drug that intercalates into ergosterol-containing fungal membranes, thereby forming membrane-spanning channels that lead to the leakage of cellular components and cell death.

Persister
A metabolically quiescent cell that neither grows nor dies when exposed to cidal concentrations of antimicrobial compounds.

Concluding remarks

C. albicans biofilm formation on implanted devices is a major source of disseminated *C. albicans* infection. The past decade has seen key advances in the identification of the *C. albicans* genes that govern biofilm development. In many cases, we have moved forwards from gene discovery to defining pathway relationships and, in some cases, we now have a mechanistic understanding. In addition, numerous quorum sensing molecules with potential roles in biofilm maturation have been defined. Moreover, there are now several animal models for the analysis of biofilm development *in vivo* that have validated the importance of key biofilm genes discovered *in vitro*. However, a summary of the progress that has been made to date also shows major gaps in our understanding. How can so many cell wall proteins participate in biofilm formation; are they all adhesins? Which quorum sensing molecules are actually active *in vivo*, and can we harness their activities for the development of therapeutics? Can we use our understanding of biofilm drug resistance to develop better therapeutics and more focused assays of biological activity? What are the dynamics of the formation of — and key molecular players in — mixed-species biofilms? And, perhaps most difficult to answer and most interesting to ponder, what selective pressures caused the evolution of the ability to form biofilms — was it for mating, or for mucosal surface adherence and persistence in the host? There has never been a more interesting time to study *C. albicans* biofilms.

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Competing interests statement
The authors declare no competing financial interests.

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