An expanded regulatory network temporally controls *Candida albicans* biofilm formation

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Summary

***Candida albicans* biofilms are composed of highly adherent and densely arranged cells with properties distinct from those of free-floating (planktonic) cells. These biofilms are a significant medical problem because they commonly form on implanted medical devices, are drug resistant and are difficult to remove. *C. albicans* biofilms are not static structures; rather they are dynamic and develop over time. Here we characterize gene expression in biofilms during their development, and by comparing them to multiple planktonic reference states, we identify patterns of gene expression relevant to biofilm formation. In particular, we document time-dependent changes in genes involved in adhesion and metabolism, both of which are at the core of biofilm development. Additionally, we identify three new regulators of biofilm formation, Flo8, Gal4, and Rfx2, which play distinct roles during biofilm development over time. Flo8 is required for biofilm formation at all time points, and Gal4 and Rfx2 are needed for proper biofilm formation at intermediate time points.**

Introduction

A biofilm is a community of cells arranged in a complex structure where individual cells have properties different from cells grown in suspension cultures (Hawser and Douglas, 1994; Baillie and Douglas, 1999; Chandra *et al.*,

2001; Nobile and Mitchell, 2007; Bonhomme and d'Enfert, 2013). For example, cells in biofilms are more adherent and more drug resistant than free-floating cells (Hawser and Douglas, 1994; Jabra-Rizk *et al.*, 2004; Nobile *et al.*, 2006; 2008; López *et al.*, 2010; Tobudic *et al.*, 2012). Analogous to developing animal and plant tissues, biofilms undergo changes over time that require cell–cell communication, regulation of structure and cell differentiation (Baillie and Douglas, 1999; Chandra *et al.*, 2001). In *Candida albicans*, a major fungal species of the human microbiome, the initial stage is adherence, in which yeast-form cells attach to a surface and form a monolayer. Then, a subpopulation of yeast-form cells undergoes a morphological transition into elongated hyphal cells. As the biofilm develops, a protein- and carbohydrate-rich extracellular matrix forms, which encases the biofilm and plays a key role in drug resistance (Hawser *et al.*, 1998; Nett *et al.*, 2007; 2010a,b; Zarnowski *et al.*, 2014). The final stage is dispersal, where yeast-form cells are released from mature biofilms and can seed new biofilms in distant locations (Uppuluri *et al.*, 2010).

Candida albicans biofilms form on medical devices, including heart valves, catheters and prostheses (Kojic *et al.*, 2004; Cauda, 2009). As biofilms are highly adherent and drug resistant, these infections typically require costly device removal and replacement. In some cases, biofilm infections act as reservoirs for systemic infections with high morbidity and mortality rates (Kojic *et al.*, 2004; Raad *et al.*, 2007; Cauda, 2009; Seddiki *et al.*, 2013).

We examined regulation of biofilm formation over time, assessing biofilm samples immediately after adherence, at 8, 24 and 48 h. We measured gene expression levels at each time point and analyzed patterns that change throughout biofilm development. Because temperature, cell morphology and cell–cycle growth phase all affect gene expression patterns, we used four different 'reference' conditions to highlight changes that were specific to biofilms. Thus, we conducted four sets of gene expression microarray experiments, each with a different 'reference' strain grown under a particular set of conditions. These are (i) planktonic culture grown to log phase at 30°C (yeast-form cells); (ii) planktonic culture grown to log phase at 37°C (predominantly filamentous cells); (iii) planktonic culture grown to stationary phase at 30°C (yeast-form

cells); and (iv) the unadhered, planktonic cells collected after 90 min at 37°C in our biofilm assay (predominantly filamentous cells). Previous studies have monitored gene expression in *C. albicans* over time in different ways (García-Sánchez *et al.*, 2004; Murillo *et al.*, 2005; Yeater *et al.*, 2007; Nett *et al.*, 2012). García-Sánchez *et al.* (2004) compared gene expression in biofilms grown in different conditions to that in planktonic cultures grown to stationary phase; Murillo *et al.* (2005) measured gene expression in young biofilms, up to 6 h, compared with planktonic cultures of the same age; Yeater *et al.* (2007) measured gene expression in 6, 12 and 48 h biofilms, compared with planktonic cultures of the same age; and Nett *et al.* (2012) measured gene expression in biofilms grown in a rat catheter central venous model grown for either 12 or 24 h, compared with log phase planktonic cultures. Our work measures gene expression during biofilm development over time in reference to log phase, stationary phase, filamentous and yeast form planktonic cultures, encompassing a comprehensive cohort of reference conditions, including several growth conditions that have not been previously investigated. We analyzed the gene expression data to identify biofilm-specific sets of genes that are temporally regulated, with particular emphasis on expression patterns of transcription regulators, with the goal of identifying new transcription regulators important for biofilm development over time. In particular, we sought transcription regulators that are required for normal temporal biofilm development at one or multiple time points throughout the course of biofilm development, assessing biofilm samples immediately after adherence, at 8, 24 and 48 h.

Previous work showed that a network of six 'master' transcription regulators (Bcr1, Efg1, Ndt80, Rob1, Tec1 and Brg1) is required for normal biofilm formation both *in vitro* and *in vivo* in two different animal models designed to mimic human biofilm-related candidiasis (Ramage *et al.*, 2002a; Nobile and Mitchell, 2005; Fox and Nobile, 2012; Nobile *et al.*, 2012). These regulators control about one thousand genes, many of which are differentially regulated in mature (48 h) biofilms compared with planktonic cultures. The master regulators were identified by screening a transcription factor deletion library of 165 mutants (Homann *et al.*, 2009; Nobile *et al.*, 2012) for biofilm formation at 48 h, and we used our gene expression data to prioritize construction of 27 additional regulator deletion mutant strains, creating a library of 192 mutants in total. In order to identify transcription regulators required for normal biofilm development, we screened this expanded library for biofilm formation at the same four time points used for transcriptional profiling: immediately after adherence, at 8, 24 and 48 h. We then validated our results using confocal scanning laser microscopy (CSLM) *in vitro* and SEM *in vivo*. In addition to the six previously identified master

regulators (Nobile *et al.*, 2012), the screen revealed three new transcription regulators that are required for normal biofilm development over time both *in vitro* and *in vivo*, Flo8, Rfx2 and Gal4. Chromatin immunoprecipitation experiments carried out with the three newly identified regulators show that each binds upstream of at least one previously identified master regulator gene. Moreover, the upstream regions of the three new genes are each bound by at least one master regulator. Thus, the newly identified regulators are well integrated into the previously described *C. albicans* transcriptional network.

Results

Multiple reference conditions reveal biofilm-specific expression patterns

We measured gene expression during biofilm formation using microarrays that covered the complete genome of 6,111 genes (Dataset 1). We created four sets of arrays where biofilm gene expression at four time points was compared with one of four reference conditions: (i) planktonic culture grown to log phase at 30°C; (ii) planktonic culture grown to log phase at 37°C; (iii) planktonic culture grown to saturation at 30°C; and (iv) the unadhered, planktonic cells collected after 90 min at 37°C in our biofilm assay (Fig. S1, Dataset 1). Collectively, the reference conditions enabled us to filter out changes due to a wide variety of variables in order to highlight those changes related to biofilm formation per se. The use of multiple reference conditions allowed many conclusions to be drawn from the data. We first examined genes that were upregulated at specific time points irrespective of which planktonic reference condition was used. This allowed us to identify 81 genes that are biofilm-specific and temporally regulated (Fig. 1). Eleven of these (*ALS1*, *TRY6*, *RHR2*, *RHD1*, *ORF19.2669*, *HGT10*, *CWH8*, *ORF19.3483*, *SAP5*, *NAG3* and *NAG 4*) are upregulated early and include several genes previously identified as important for biofilm formation. *TRY6* encodes a transcriptional regulator of adhesion (Finkel *et al.*, 2012) and *ALS1* encodes an adhesion protein involved in biofilm formation (Nobile *et al.*, 2006). *RHR2* encodes a glycerol biosynthetic enzyme and is required for normal biofilm formation (Desai *et al.*, 2013). *HGT10* and *ORF19.3483* are involved in glycerol acquisition (Kunze *et al.*, 2005; Kayingo *et al.*, 2009), but play no known role in biofilms. *NAG3* and *NAG4* encode drug efflux pumps, which have not been studied in biofilms (Sengupta and Datta, 2003). Of the 70 genes upregulated at later time points, 24 are uncharacterized. Several other genes identified in our study are involved in amino acid metabolism and acquisition (*ARG1*, *ARG3*, *ARG8*, *CPA1*, *CPA2*, *GLN1*, *AAP1*, *HNM3* and *CAN1*), which is consistent with previous measures of biofilm gene expression
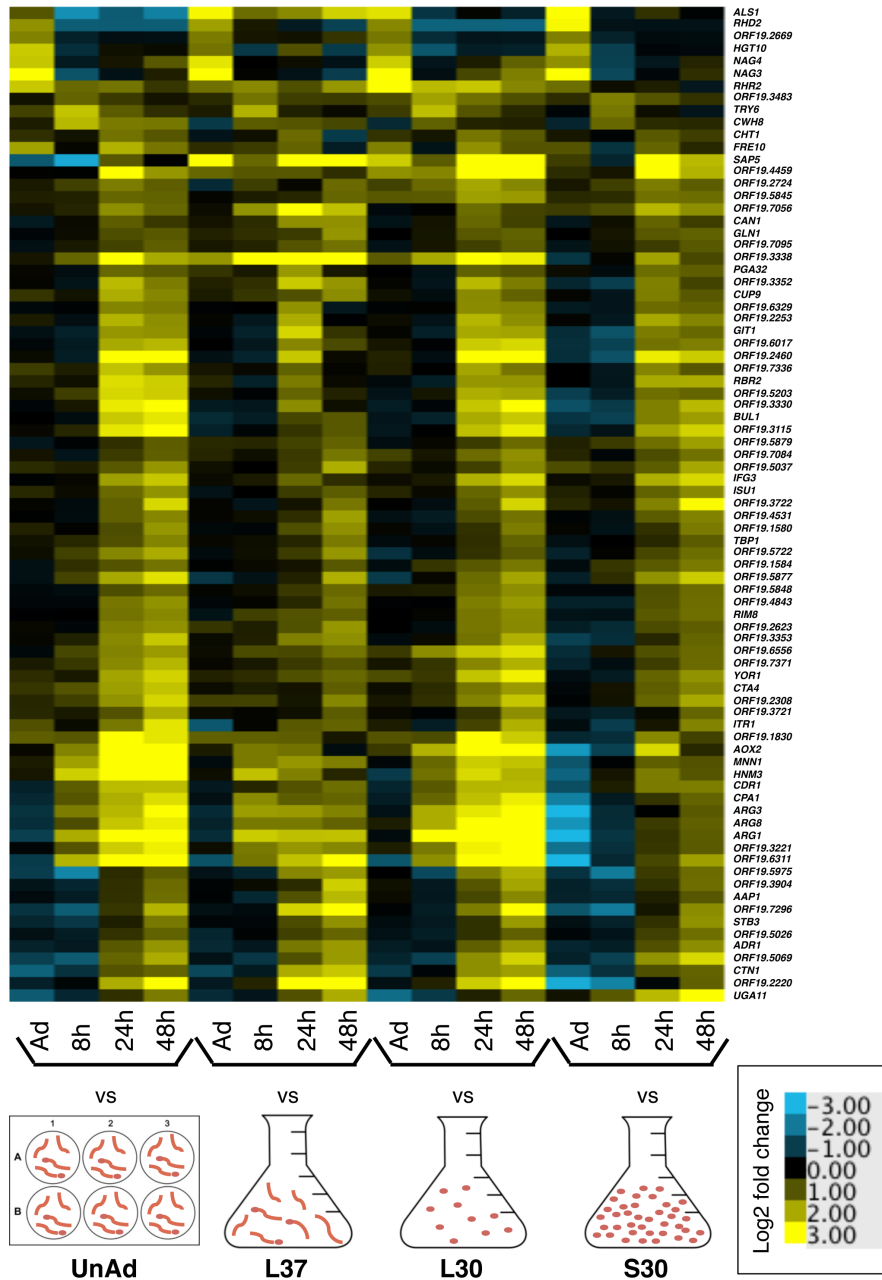
Fig. 1. Eighty-one genes are upregulated temporally in biofilms irrespective of which planktonic reference is used. Heat map of gene expression in *C. albicans* in biofilms at the indicated time points, compared with each of four reference conditions: unadhered cells (UnAd), log phase cells grown at 37°C (L37), log phase cells grown at 30°C (L30) and stationary phase cells grown at 30°C (S30). Shown are the median values of at least two biological replicates. On the *y*-axis are 81 genes that are expressed early or late in biofilms compared with each reference condition. Upregulated genes are yellow; downregulated genes are blue. See also Fig. S1.

(García-Sánchez *et al.*, 2004; Yeater *et al.*, 2007). Genes involved in fatty acid beta-oxidation and lipid catabolism were also upregulated (*ADR1* and *CTA4*).

As the majority of the characterized genes temporally regulated in biofilms were involved in adhesion and metabolism, we chose to focus on these processes for further analysis. We also used the gene expression data to identify transcription regulators that are temporally regu-

lated during biofilm formation to prioritize the construction of additional gene deletion mutant strains to test for biofilm formation. These results are described in detail below.

Adhesion proteins are differentially expressed over time

We compared gene expression in cells that were adhered after 1.5 h to those cells that were inoculated in the same
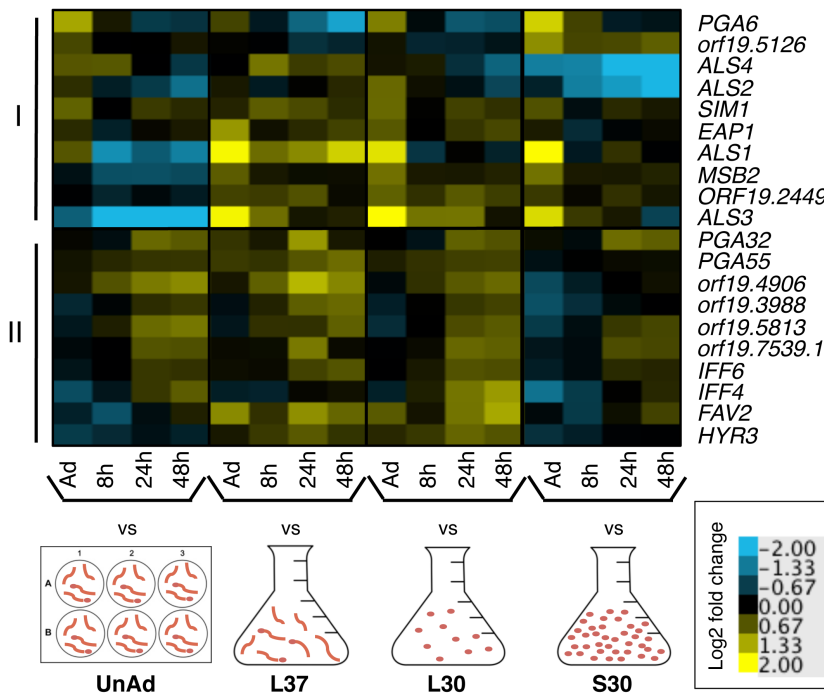
Fig. 2. Two patterns of biofilm-specific expression of adhesion genes are observed during biofilm development. Heat map of gene expression in *C. albicans* in biofilms at the indicated time points, compared with each of four reference conditions: unadhered cells (UnAd), log phase cells grown at 37°C (L37), log phase cells grown at 30°C (L30) and stationary phase cells grown at 30°C (S30). Shown are the median values of at least two biological replicates. On the *y*-axis are known or predicted adhesion-encoding genes differentially regulated at least twofold in at least one condition: I) genes upregulated early. II) genes upregulated late. Upregulated genes are yellow; downregulated genes are blue.

medium but did not adhere. The adhered vs. unadhered gene expression dataset contained 251 genes upregulated more than twofold and 157 genes downregulated more than twofold (Dataset 1). Gene Ontology analysis (Inglis *et al.*, 2012) revealed that more than half of the upregulated genes were significantly enriched for genes involved in DNA synthesis (131 genes, $P = 1.25 \times 10^{-39}$), transcription (128 genes, $P = 9.22 \times 10^{-36}$), RNA processing (110 genes, $P = 3.82 \times 10^{-61}$) or translation (109 genes, $P = 9.06 \times 10^{-82}$), indicating that physical adherence strongly upregulates DNA and protein synthesis, likely in preparation for the large shift in morphology and physiology that accompanies biofilm formation. Downregulated genes were largely of unknown function but were significantly enriched for genes involved in oxidation-reduction (33 genes, $P = 2.15 \times 10^{-7}$), carbohydrate transport (10 genes, $P = 5.11 \times 10^{-6}$), and sulfur amino acid metabolism (nine genes, $P = 2.63 \times 10^{-5}$). We conclude that as *C. albicans* yeast form cells adhere to a solid surface, they undergo extensive changes in gene expression, many of which are in preparation for the later stages in biofilm formation.

Because adhesion is a key part of biofilm formation, we examined expression of known or predicted adhesion proteins over time (Fig. 2). Based on the transcript levels of adhesion genes in biofilms compared with the multiple reference states, we can distinguish two classes of adhesion genes that are upregulated in biofilms, largely irrespective of which planktonic reference they are compared to. (i) A group of ten adhesion genes (*ALS1*, *ALS2*, *ALS3*,

ALS4, *EAP1*, *MSB2*, *PGA6*, *SIM1*, *ORF19.2449* and *ORF19.5126*) was induced early in biofilm formation. Seven of these genes (*PGA6, ORF19.5126*, *ALS4*, *ALS2*, *SIM1*, *EAP1* and *ALS1*) were upregulated compared with the unadhered planktonic reference, indicating that adhesion of cells to a solid surface was required for their expression. Three of these genes (*MSB2*, *ORF19.2449* and *ALS3*) are also induced in the unadhered planktonic reference, indicating that their expression is mediated by a cue other than contact with a solid surface, perhaps temperature or serum. This conclusion is consistent with previous work showing that *ALS3* expression is upregulated within an hour of temperature shift from 30°C to 37°C but drops again after 2 h (Kadosh and Johnson, 2005; Argimón *et al.*, 2007). (ii) A group of 10 additional adhesion genes (*FAV2*, *HYR1*, *IFF4*, *IFF6*, *PGA32*, *PGA55*, *ORF19.3988*, *ORF19.4906*, *ORF19.5813* and *ORF19.7539.1*) was induced at later times in biofilm formation. We suggest that the early adhesion proteins are involved primarily with attaching cells to the substrate and the late adhesion proteins mediate cell-to-cell contact.

Biofilm metabolism is downregulated over time compared with stationary-phase cells

It is known that metabolism is downregulated in mature biofilms (Yeater *et al.*, 2007; Nobile *et al.*, 2012); indeed, cells in biofilms are often likened to cells in stationary phase. To expose biofilm-specific changes in gene expression, we chose stationary planktonic cells as one of

the reference conditions for the microarray experiments. We examined the expression patterns of metabolic genes, specifically, those needed for glycolysis, the tricarboxylic acid cycle, the electron transport chain, fermentation, fatty acid metabolism, glycerol metabolism, galactose metabolism and ergosterol biosynthesis (Fig. 3). As might have been predicted, we found that many of these genes are downregulated over time during biofilm development; however, most are downregulated even more in mature biofilms than in stationary cultures. Compiled data from several studies indicates that glycolytic genes are expressed more highly in early biofilms and decrease over time (García-Sánchez *et al.*, 2004; Yeater *et al.*, 2007; Nobile *et al.*, 2012); this is consistent with our data. We also found that a few metabolism genes involved in glycerol degradation (*GUT1* and *GUT2*) and genes involved in fermentation (*ALD5*, *ALD6*, *ADH3* and *ADH4*) are upregulated over time in biofilms compared with stationary cultures, suggesting these processes are more important in mature biofilm cells than in stationary phase cells. Indeed, *RHR2*, which encodes a glycerol biosynthetic enzyme, is required for normal biofilm formation (Desai *et al.*, 2013), suggesting that glycerol metabolism is important for biofilm cells. The importance of upregulating fermentation in biofilms is unknown, but we recently showed that the inside of a *C. albicans* biofilm is a hypoxic microenvironment (Fox *et al.*, 2014), favorable for fermentation. We suggest that alcohols produced during fermentation may be used for energy or could be involved in cell signaling with neighboring yeast or other microbes; some alcohols have been shown to alter biofilm formation (Mukherjee *et al.*, 2006; Martins *et al.*, 2010; Rane *et al.*, 2012; Chauhan *et al.*, 2013). Overall, our analysis reveals that the regulation of metabolism genes in biofilms does not simply parallel that in stationary cultures.

Newly identified transcription regulators play roles in biofilm development

We used the gene expression data to identify 11 previously untested transcription regulators whose transcript levels change temporally over biofilm development, and we added these to an existing transcription regulator deletion library (Homann *et al.*, 2009; Fox *et al.*, 2014) (see *Materials and methods* for details). We then blindly screened the expanded library of 192 mutant strains for biofilm formation *in vitro* at four time points: immediately after 1.5 h of adherence, and at 8, 24 and 48 h of biofilm growth (Figs 4, S2A). In addition to the six regulators identified previously (Nobile *et al.*, 2012), we found seven other regulators, whose deletion resulted in a biofilm phenotype at at least one time point (Fig. 4), as measured by optical density of the cells remaining adhered to the bottom of the well after removal of the media (see *Materials and methods*). These

seven were confirmed by testing independently constructed deletion strains (Fig. S2B). We also confirmed that none of the mutants had altered growth kinetics that could contribute to the observed phenotype (Fig. S3). One regulator deletion mutant (*gal4Δ/Δ*) formed biofilms with an increased optical density at 24 h and six regulator deletion mutants (*rfx2Δ/Δ*, *rim101Δ/Δ*, *orf19.6874Δ/Δ*, *gzf3Δ/Δ*, *flo8Δ/Δ* and *rfg1Δ/Δ*) formed biofilms with a decreased optical density at one or more time points (Fig. 4). *ORF19.6874* was previously uncharacterized, and we named it B̲iofilm P̲artial R̲egulator 1 (*BPR1*).

Of the seven additional regulator deletion mutants with optical density biofilm phenotypes, five of these formed biofilms morphologically distinct from those of wild type when examined by CSLM *in vitro* (Figs 5, S4); the phenotypes were rescued by adding back a wild-type copy of each gene in the gene deletion background (Fig. S5). The *flo8Δ/Δ* mutant biofilm was severely deficient at all four time points, similar in severity to the biofilm phenotypes observed for the previously identified six master biofilm regulator mutants. At the 8 h time point, the *rim101Δ/Δ* mutant biofilm was thinner than the wild type and had fewer hyphae. The biofilm formed by *rfg1Δ/Δ* detached entirely from the surface and broke apart by 48 h. The *gal4Δ/Δ* mutant biofilm was thicker than wild type at the 24 h time point, in agreement with the optical density assay. The *rfx2Δ/Δ* mutant biofilm was thicker than wild type at 24 h, which is opposite of the optical density assay results. This discrepancy may be explained by the differences between the CSLM assay, which is performed on silicone squares in 12 well plates, and the OD assay, which is performed in 96 well plates. In the 96 well assay, it was observed that the *rfx2Δ/Δ* mutant strain formed a very thick pellicle at the liquid–air interface. This pellicle was not observed in the 12 well CSLM assay. Either the different substrate (silicone rather than polystyrene) or the larger wells may have allowed for additional cells that are more mobile and better able to incorporate into the biofilm to become a part of the biofilm in the larger-well assay, resulting in an enhanced phenotype in the CSLM assay.

Because two of the mutant strains identified in the initial screen did not show clear differences from the reference strain by microscopy, we did not consider them further in this study. We next tested the five remaining regulator deletion strains with clear morphological phenotypes (*flo8Δ/Δ*, *rim101Δ/Δ*, *gal4Δ/Δ*, *rfx2Δ/Δ* and *rfg1Δ/Δ*) for biofilm formation *in vivo*, in a rat central venous catheter model (Andes *et al.*, 2004). After the catheter was removed, SEM was used to examine biofilm morphology (Fig. 6). In order to minimize the number of animals used, we measured biofilm formation only at the time points at which a clear *in vitro* phenotype was observed, 8 h for *rim101Δ/Δ*, 24 h for *gal4Δ/Δ*, *rfx2Δ/Δ* and *flo8Δ/Δ* and 48 h for *rfg1Δ/Δ*. We found that three of these regulator mutant
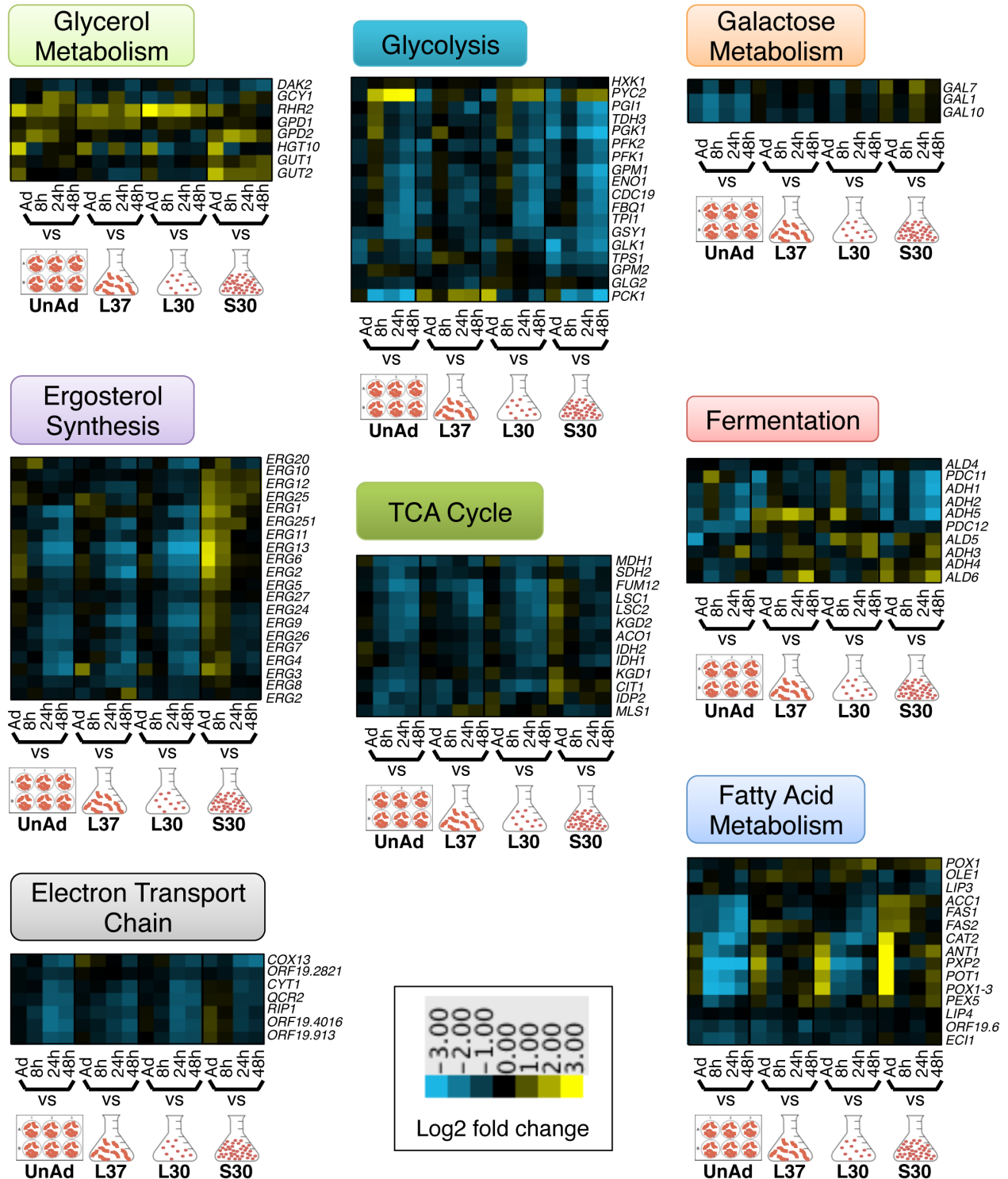
Fig. 3. Metabolism is downregulated over time during biofilm development. Heat maps of gene expression in *C. albicans* in biofilms at the indicated time points, compared with each of four reference conditions: unadhered cells (UnAd), log phase cells grown at 37°C (L37), log phase cells grown at 30°C (L30) and stationary phase cells grown at 30°C (S30). Shown are the median values of at least two biological replicates. Each heat map shows expression of genes involved in the metabolic processes of glycolysis, the tricarboxylic acid (TCA) cycle, the electron transport chain, fermentation, glycerol metabolism, galactose metabolism, fatty acid metabolism or ergosterol biosynthesis. Upregulated genes are yellow; downregulated genes are blue.
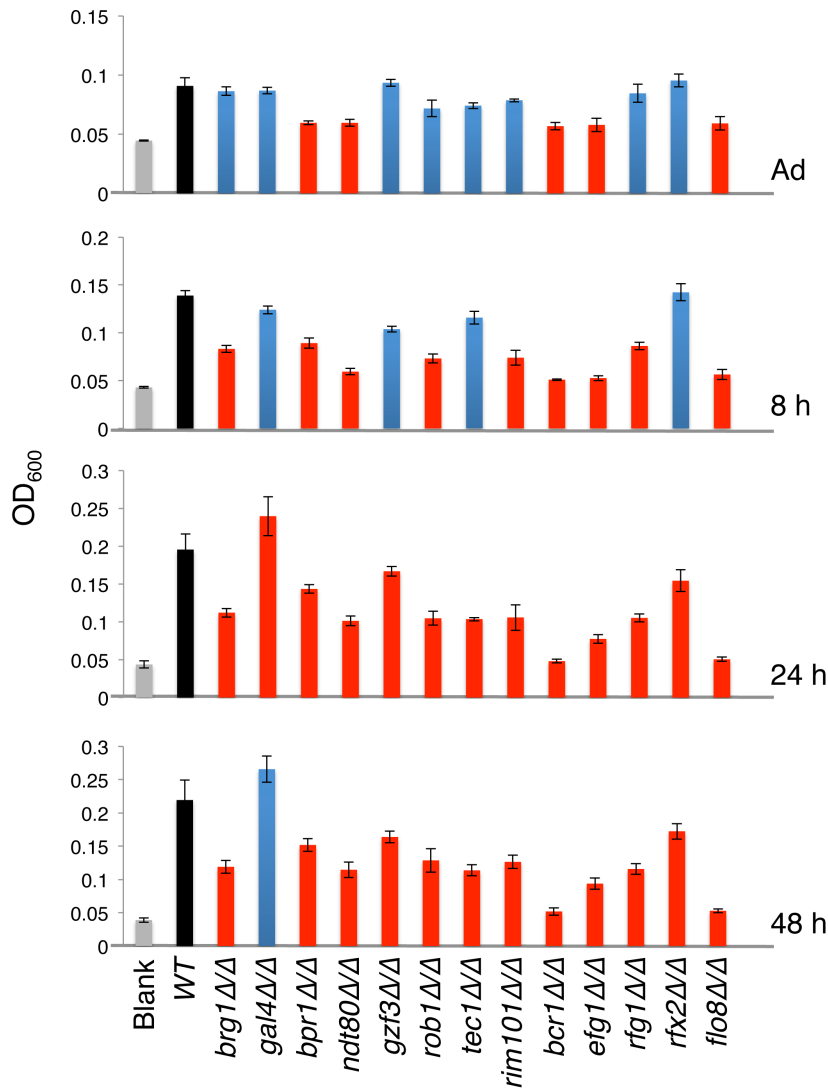
Fig. 4. Thirteen transcription regulators are required for normal biofilm development *in vitro* in an optical density assay. The indicated wild type or mutant strains were adhered in a 96 well polystyrene plate for 90 min (labeled 'Adhered'), unadhered cells were removed and adhered cells developed into biofilms for 8, 24 or 48 h. Biofilm formation was quantified by measuring OD_{600} of the cells adhered to the bottom of the well. Shown is the mean of at least six replicates, error bars are standard deviation. Gray bars are blank wells, black bars are wild type biofilms, blue bars are deletion strain biofilms not significantly different from wild type, red bars are deletion strain biofilms significantly different from wild type, $P < 0.001$ by Student's paired *t*-test. See also Figs S2 and 3.

strains had phenotypes *in vivo* that matched those observed *in vitro*: *gal4Δ/Δ* and *rfx2Δ/Δ* were enhanced and *flo8Δ/Δ* was deficient at 24 h.

Three new transcriptional regulators fit in the biofilm regulatory network

As described above, three newly identified regulators, Gal4, Rfx2 and Flo8, are required for biofilm formation *in vivo*. To determine how they fit within the existing biofilm regulatory network, we tagged Gal4, Rfx2 and Flo8 and performed chromatin immunoprecipitation experiments followed by qPCR (ChIP-qPCR), using probes to the intergenic regions upstream of each of the nine biofilm regulators (*GAL4*, *RFX2*, *FLO8*, *BCR1*, *EFG1*, *NDT80*, *BRG1*, *TEC1* and *ROB1*) (Fig. 7). As shown in the figure, all three bind to the upstream regions of at least one of the previously identified master biofilm regulators. Moreover, the

intergenic regions upstream of *GAL4*, *RFX2* and *FLO8* are each bound by at least one of the six previously identified master biofilm regulators (Nobile *et al.*, 2012) – *GAL4* and *RFX2* are bound by Ndt80, and *FLO8* is bound by Ndt80, Brg1 and Efg1, providing further evidence that these newly identified regulators are an integral part of the biofilm regulatory network.

Discussion

Previous work identified six 'master' transcription regulators of biofilm formation in *C. albicans* (Ramage *et al.*, 2002b; Nobile and Mitchell, 2005; Fox and Nobile, 2012; Nobile *et al.*, 2012). In this paper, we describe three additional transcription regulators (Gal4, Rfx2 and Flo8) that are needed for proper biofilm formation both *in vitro* and in a rat *in vivo* model. These genes were identified by screening an expanded library of deletion mutants, based
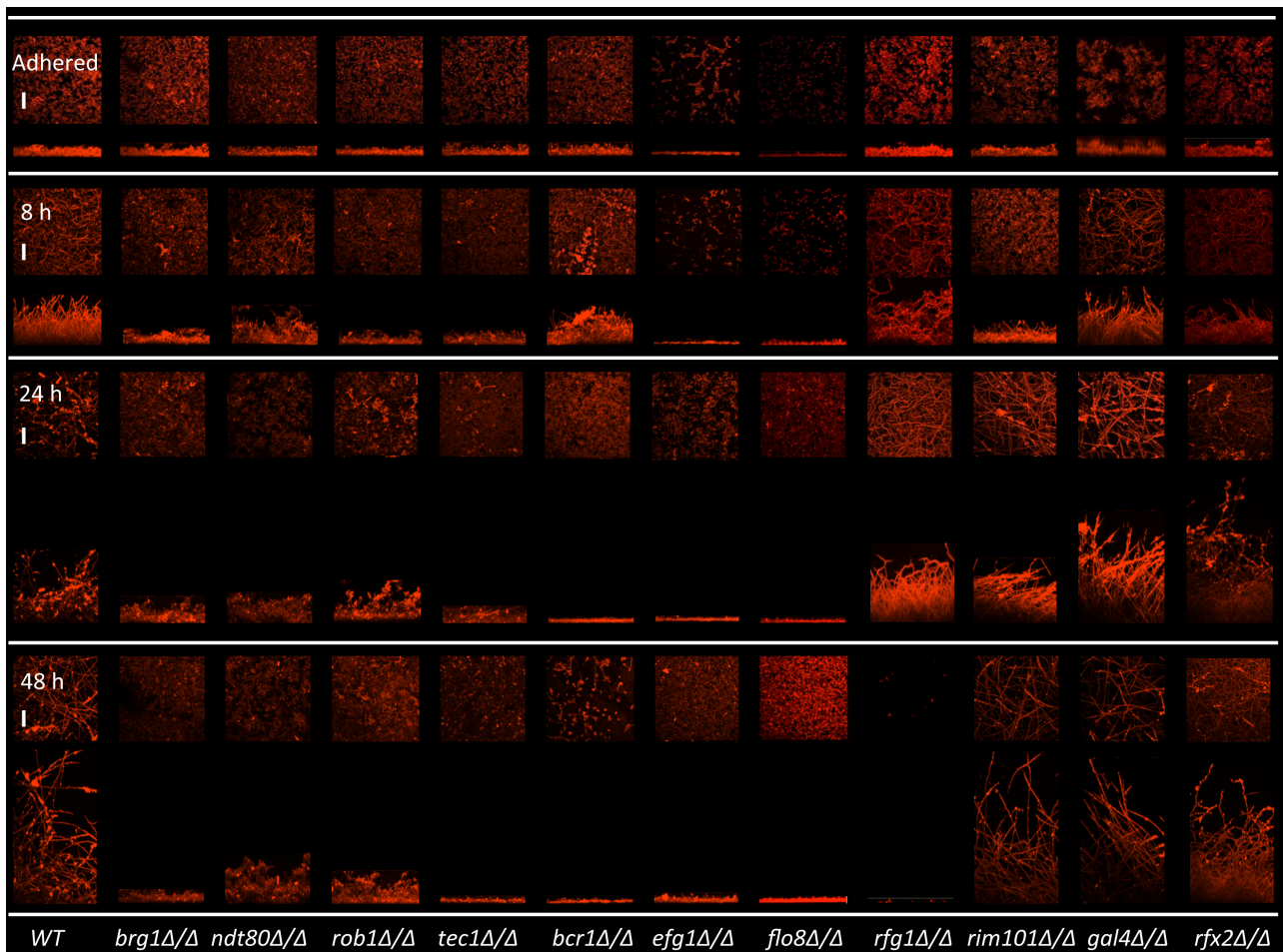
Fig. 5. Eleven transcription regulators are required for normal biofilm development *in vitro* by CSLM. The indicated wild type or mutant strains were adhered to silicone substrates for 90 min (labeled 'Adhered'), unadhered cells were removed and adhered cells developed into biofilms for 8, 24 or 48 h. Biofilms were stained with conconavalin A – Alexa 594 and Syto 13 dyes, then imaged by CSLM. Images are maximum intensity projections of the top and side view. Representative images of at least three replicates are shown. Scale bars are 50 μm. See also Figs S4 and 5.

on transcriptional profiling described in this paper. Two of the newly identified genes (*RFX2* and *FLO8*) were not represented in the libraries that had previously been screened; the third (*GAL4*) was revealed because we monitored multiple time points during biofilm formation. ChIP-qPCR experiments showed that the new regulators are integrated into the complex biofilm transcription circuitry (see Fig. 7).

All three regulators were previously studied in contexts other than biofilm formation. Flo8 plays a role in hyphal growth and regulates a subset of genes also regulated by Efg1 (Cao *et al.*, 2006), one of the biofilm master regulators. The *flo8Δ/Δ* mutant is defective in hyphal formation and is avirulent in a systemic tail vein mouse model (Cao *et al.*, 2006). Our work also shows that the *flo8Δ/Δ* mutant is highly defective in biofilm formation *in vivo* in a rat central venous catheter model. The *flo8Δ/Δ* deletion phenotype was the most severe of the new regulators; the strain was

defective in biofilm formation at all time points, and it closely resembled the biofilms formed by strains in which one of the six previously identified master regulators is deleted. Flo8 also plays a role in CO_2 sensing during white-to-opaque morphological switching (Du *et al.*, 2012), a finding that may provide a link between biofilm formation and white-to-opaque switching. *FLO8* is expressed increasingly over time regardless of which planktonic reference condition is used (Dataset 1). Therefore, *FLO8* expression appears to be biofilm specific, and upregulation is not dependent on whether the reference cells are filamentous or yeast form.

In the *gal4Δ/Δ* and *rfx2Δ/Δ* mutant strains, biofilm formation was enhanced rather than deficient, implying that these are negative regulators of biofilm formation. Consistent with this idea, both regulators are downregulated in biofilms relative to at least two of the reference conditions used in our expression array experiments (Dataset 1).
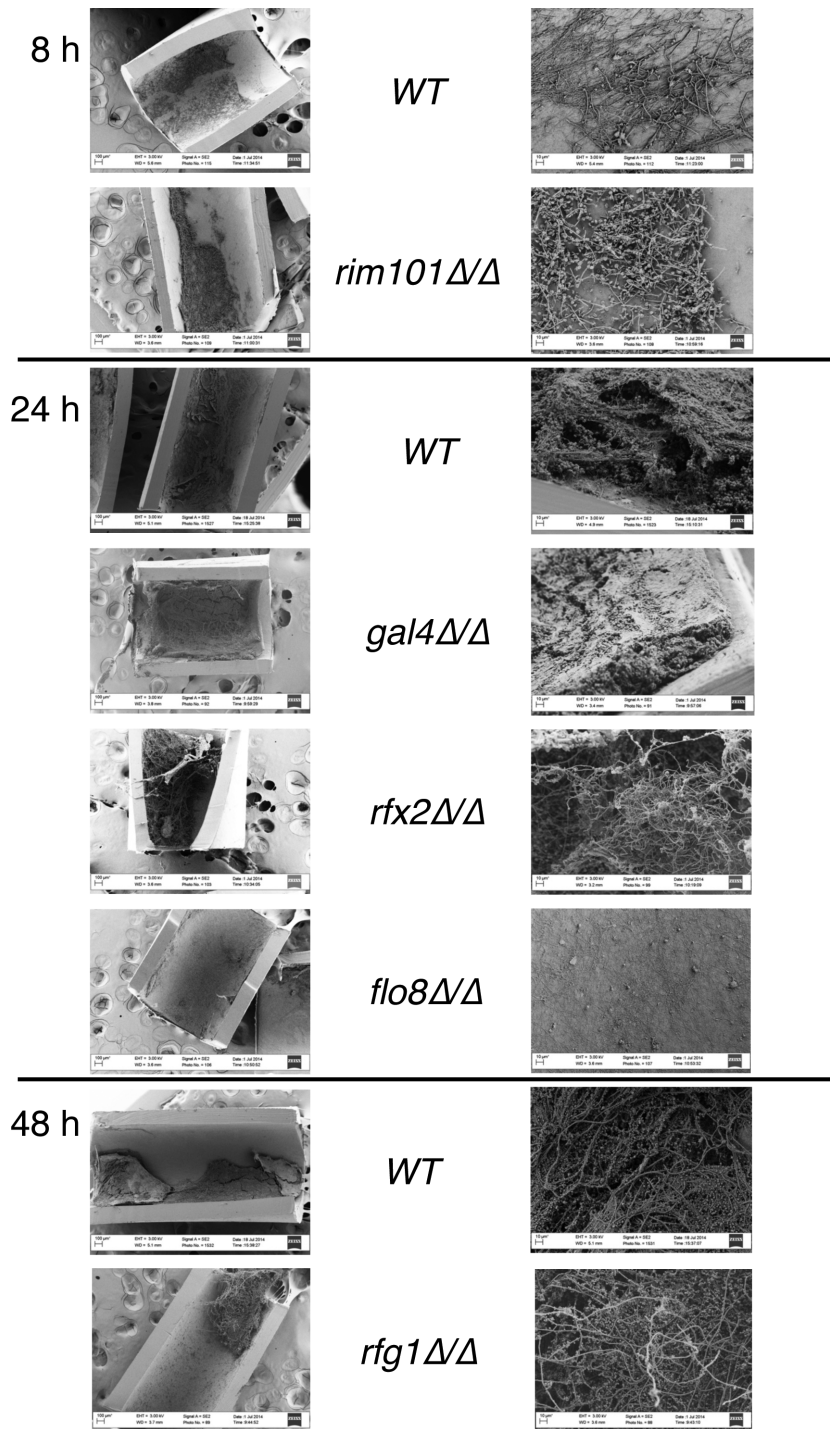
Fig. 6. Three new transcription regulators are required for normal biofilm development *in vivo* in a rat central venous catheter model. The indicated wild type or mutant strains were inoculated into rat intravenous catheters and developed into biofilms for 8, 24 or 48 h prior to visualization by SEM. The left column shows biofilms at 100×; the right column is at 1000×.

Gal4 regulates glycolysis and carbohydrate metabolism, along with Tye7 (Askew *et al.*, 2009), and our array data show that glycolysis and most metabolic pathways are downregulated over time during biofilm development. Tye7 also plays a role in biofilm formation; biofilms formed by the *tye7Δ/Δ* mutant contain more hyphae but are more fragile than wild-type biofilms (Bonhomme *et al.*, 2011). One possible explanation for the biofilm phenotypes exhibited by the *gal4Δ/Δ* and *tye7Δ/Δ* mutant strains is that the altered morphology is related to metabolism. As is proposed in Bonhomme *et al.* (2011) and Rossignol *et al.* (2009), glycolysis is an important part of survival during hypoxia and its misregulation could affect biofilm structure, which normally contains a hypoxic internal microenvironment (Fox *et al.*, 2014). Rfx2 plays a role in hyphal formation; the *rfx2Δ/Δ* deletion mutant is hyperfilamentous, invasive and
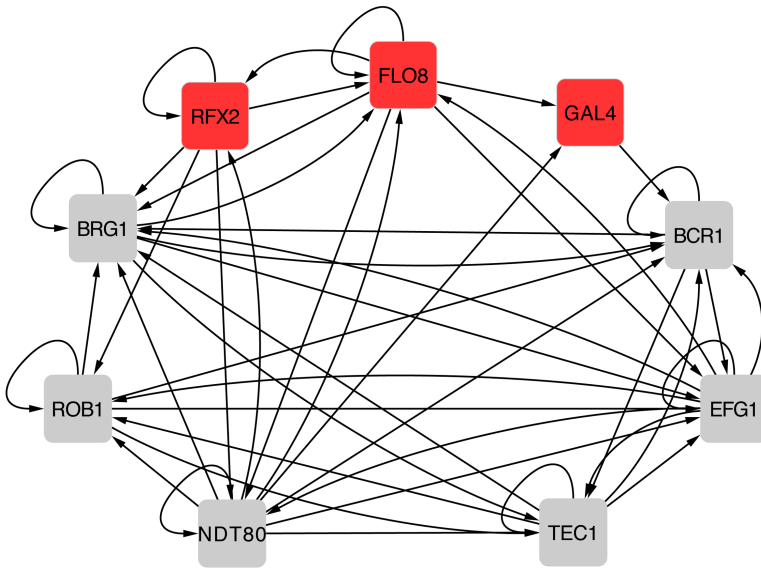
Fig. 7. The expanded biofilm regulatory network model contains three newly identified regulators. Intergenic region binding data generated for Flo8, Rim101 and Gal4 was combined with binding data for Brg1, Bcr1, Rob1, Efg1, Ndt80 and Tec1 from Nobile *et al.* (2012) to determine which regulators bind upstream of the genes encoding each of the other regulators. An arrow indicates a binding event of the gene it points to. For example, Gal4 binds upstream of *BCR1* and the region upstream of *GAL4* is bound by Flo8 and Ndt80.

constitutively expresses several hyphal-specific genes, including *HWP1*, *ALS3*, *HYR1*, *ECE1* and *CEK1* (Hao *et al.*, 2009). Given these observations, it is perhaps not surprising that deletion of *RFX2* results in enhanced biofilm formation.

Concurrent with the genetic approach, we examined gene expression in biofilms, measuring expression at four time points and comparing expression to four different planktonic reference conditions. García-Sánchez *et al.* (2004) compared gene expression in mature biofilms with that of planktonic cultures and varied flow, aerobiosis and glucose concentration, whereas Murillo *et al.* (2005) compared gene expression in 6 h biofilms to that of planktonic culture. Yeater *et al.* (2007) measured gene expression in 6, 12 and 48 h biofilms, compared with planktonic cultures of the same incubation time. Nobile *et al.* (2012) measured gene expression in mature biofilms compared with equivalently aged planktonic cultures. In the only *in vivo* transcriptional profiling biofilm study to date, Nett *et al.* (2009) measured gene expression in biofilms grown in a rat central venous catheter model compared with *in vitro* planktonic cells. Our gene expression data adds a new set of comparisons to this body of *C. albicans* biofilm gene expression data. Our work is distinguished from previous studies in that we measured gene expression in newly adhered cells, 8, 24 and 48 h biofilms and compared these patterns with those of unadhered planktonic cells collected from the biofilm assay or to planktonic cells grown to log phase at 30°C, log phase at 37°C or stationary phase. Although there are many specific types of analyses that can be performed using our data (which is freely available), we discuss only two here. First, we found that there are two distinct expression classes of adhesion proteins involved in biofilm development: those that are enriched earlier and

those that are enriched later during biofilm development. There is a third group of adhesion proteins whose expression does not differ between biofilm and planktonic cultures. These results indicate that the nature of adhesion in biofilms changes over time, consistent with the idea that biofilm cells must first adhere to a surface, then to one another, both in the yeast and filamentous forms. Moreover, at late times, at least some biofilm cells must detach from one another or from the surface to disperse to new locations. Second, we examined metabolism in biofilms over time and found that, regardless of which reference was used, metabolism is largely downregulated as a biofilm ages, consistent with the idea that a significant proportion of mature biofilm cells are in a metabolically quiescent state. This relative downregulation is observed even when mature biofilms are compared with stationary phase planktonic cultures; thus, cells in a mature biofilm are not metabolically equivalent to those in stationary phase.

Biofilms are a dynamic community of multiple cell types whose formation over time is orchestrated by a network of transcription regulators. In this paper, we have expanded the network of six master regulators to now include three additional regulators, Flo8, Gal4 and Rfx2, and have documented key gene expression changes that occur specifically in *C. albicans* cells that are forming biofilms.

Experimental procedures

Culture growth and maintenance

Candida albicans was streaked from a glycerol stock onto Yeast Peptone Dextrose (YPD) agar plates and grown at room temperature or 30°C. Suspension cultures were grown in YPD media at 30°C, with aeration.

Strain construction

Strains used are listed in Table S1 and include 165 strains published in Homann *et al.* (2009) as well as 19 strains published in Fox *et al.* (2014), and here we add eight additional homozygous gene deletion strains (some of which were previously published in Noble *et al.* (2010)), for a total of 192 strains in the updated transcription regulator knockout library. Gene deletion strains and complementation strains were constructed as described in Noble and Johnson (2005) and Fox *et al.* (2014), and Myc-tagged strains were constructed as described in Nobile *et al.* (2012). Primers are listed in Table S2.

In vitro *planktonic and biofilm culture conditions*

Planktonic cultures and biofilms were grown in Spider medium (Liu, 1994) at 37°C at 200 rpm in an ELMI incubator (ELMI, Ltd. Riga, Latvia) for biofilms and 225 rpm in a New Brunswick Scientific incubator for planktonic cultures, as described in Nobile *et al.* (2012). Saturated overnight cultures grown at 30°C in YPD (2% Bacto Peptone, 2% dextrose, 1% yeast extract) were inoculated into Spider medium ($OD_{600} = 0.02$ in 50 ml for planktonic cultures or $OD_{600} = 0.5$ in 4 ml in a bovine serum-treated 6 well plate for biofilm cultures). Stationary phase planktonic cultures were grown to an $OD_{600} = 20$ at 30°C and log phase planktonic cultures were grown to an $OD_{600} = 0.8$ at either 30°C or 37°C, shaking. Biofilm cultures were adhered for 90 min, washed with 4 ml PBS, then fresh 4 ml of Spider media was added, and biofilms were grown for the indicated times (8, 24 or 48 h). Adhered and unadhered cells were collected after the 90 min adherence step.

Biofilm formation assay by optical density at 600 nm

Biofilms were grown as described above but without bovine-serum treating the plates, and in a volume of 200 μl, in 96 well polystyrene plates (BD Falcon 351172). The plates were sealed with breathable seals (Excel Scientific BS25) to prevent cross-contamination between wells and plates were incubated at 350 rpm, 37°C. At least six replicates were performed for each mutant strain. At each time point, wells were washed with 200 μl PBS, all liquid was aspirated off, and optical density was read at 600 nm on a Tecan Infinite M200 plate reader (Tecan Systems).

Growth assay

The growth curves were performed using methods previously described in Wells *et al.* (2015). Briefly, cells from an overnight culture grown in YPD at 30°C were inoculated into 100 μl of YPD media at a starting $OD_{600} = 0.01$. The assay was performed in flat-bottom 96 well plates (BD Falcon) with $n = 3$ for each strain and grown in a Tecan Infinite M1000 reader (Tecan Systems) at 30°C with 408 rpm orbital shaking. An optical density (OD_{595}) measurement was taken every 15 min for 24 h.

Confocal scanning laser microscopy

Biofilms were imaged as described in Nobile *et al.* (2012). In brief, biofilms were formed on silicone squares for various time points. Biofilms were stained with 50 μg ml^{-1} of concanavalin A Alexa Fluor 594 conjugate (Molecular Probes C11253) for 1 h, 200 rpm, in the dark. Silicone squares were placed in a 6 well plate and covered with water for imaging by Confocal Scanning Laser Microscopy (CSLM) (Nikon Instruments Inc, Melville, New York, USA) under a 40X/0.08W Nikon water-dipping objective lens (Nikon Instruments Inc, Melville, New York, USA). A 561 nm laser line was used, and images were acquired with Nikon EX-C1 Version 3.80 software. Images were taken every 0.8 μm through the Z axis of the biofilm, and images were assembled into maximum intensity Z-stack projections with Nikon NIS Elements Version 3.00 software (Nikon Instruments Inc, Melville, New York, USA).

In vivo *rat catheter biofilm model*

The rat central-venous catheter infection model was used as described previously (Andes *et al.*, 2004; Nobile *et al.*, 2012). In brief, specific-pathogen-free male Sprague-Dawley rats weighing 400 g (Harlan Sprague-Dawley) were used. A heparinized (100 U ml^{-1}) polyethylene catheter with 0.76 mm inner and 1.52 mm outer diameters was inserted into the external jugular vein and advanced to a site above the right atrium. The catheter was secured to the vein with the proximal end tunneled subcutaneously to the midscapular space and externalized through the skin. The catheters were inserted 24 h prior to infection to permit a conditioning period for deposition of host protein on the catheter surface. Infection was achieved by intraluminal instillation of 500 μl *C. albicans* cells (10^6 cells ml^{-1}). After a 4 h dwelling period, the catheter volume was withdrawn and the catheter flushed with heparinized 0.15 M NaCl. Catheters were removed after 8, 24 or 48 h of *C. albicans* infection to assay biofilm development on the intraluminal surface by SEM. Catheter segments were washed with phosphate buffered saline (PBS), pH 7.2, fixed in 1% glutaraldehyde/4% formaldehyde, washed again with PBS for 5 min and placed in 1% osmium tetroxide for 30 min. Following PBS washing, the samples were dehydrated in a series of 10 min ethanol washes (30%, 50%, 70%, 85%, 95% and 100%), followed by critical point drying. Specimens were mounted on aluminum stubs, sputter coated with gold, and imaged using a Leo 1530 scanning electron microscope (Leo Electron Microscopy Ltd, Cambridge, Cambridgeshire, United Kingdom) in the high-vacuum mode at 3 kV. Images were assembled using Adobe Photoshop Version 7.0.1 software (Adobe Systems Inc, San Jose, California, USA).

Gene expression microarrays

Planktonic and biofilm cells were grown as described above in flasks (for planktonic) or in bovine-serum coated 6 well plates (for biofilms). Biofilm cells were harvested with a sterile transfer pipet, biofilm or planktonic cells were centrifuged at 3,000× *g* for 5 min, supernatant was removed and cell pellets were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the Ambion RiboPure-Yeast RNA kit (AM1926) (Life Technologies, Grand Island, New York, USA) according to the manufacturer's protocol. Synthesis of cDNA and dye coupling was performed as described in Nobile *et al.* (2009). Gene expression microarrays from Agilent Technologies

(AMID #020166) were used as described in Nobile *et al.* (2012). LOWESS normalization of microarray data was performed as previously described (Lohse and Johnson, 2010). At least two independent replicates were performed at each time point. Microarray data are reported in Dataset 1, and raw gene expression array data are stored at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, accession #GSE61143).

Chromatin immunoprecipitation quantitative real-time PCR (ChIP-qPCR)

The Flo8, Gal4 and Rfx2 Myc-tagged strains (Table S1) were grown under standard biofilm conditions for 24 h, and the biofilm cells were harvested for chromatin immunoprecipitation as described in Nobile *et al.* (2012). ChIP-qPCR was performed using primers in the promoter regions of *FLO8*, *GAL4*, *RFX2*, *BCR1*, *TEC1*, *EFG1*, *NDT80*, *BRG1* and *ROB1* (Table S2).

Identification of transcription regulators with temporal expression patterns

We used an algorithm (see Supporting information) to search for gene expression trends in which the expression values were increasing or decreasing over time in at least two out of four of the datasets, using a starting list of 286 regulators. We also scanned the data by eye to include genes with expression values that did not fit the slope used in the algorithm but still showed patterns of increasing or decreasing expression. This analysis resulted in a list of 91 regulator candidates. We then applied the criteria that at least one gene expression value must represent at least a twofold difference from the reference (79 regulators), have not been previously tested and have no known biofilm phenotype (30 regulators), have not been annotated as essential (27 regulators) and must have their promoter bound by at least one of the six 'master' transcription regulators identified in Nobile *et al.* (2012). This resulted in 11 candidate transcription regulators (*ORF19.7521*, *ORF19.4342*, *RFX2*, *WOR3*, *ZPR1*, *MDM34*, *ECM22*, *ORF19.7098*, *ADR1*, *ORF19.5953*, and *ORF19.7397*), which were included in the target gene set for constructing deletion mutant strains.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.