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The mechanistic basis of pH-dependent 5-flucytosine resistance in *Aspergillus fumigatus*

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Running title: Mechanistic insight into 5-flucytosine resistance in Aspergillus fumigatus

**Keywords:** *Aspergillus fumigatus*, Antifungal Drug Resistance, pH regulation, 5-flucytosine, transcription factors.
ABSTRACT

The antifungal drug 5-flucytosine (5FC), a derivative of the nucleobase cytosine, is licenced for treatment of fungal diseases however it is rarely used as a monotherapeutic to treat Aspergillus infection. Despite being potent against other fungal pathogens, 5FC has limited activity against A. fumigatus when standard in vitro assays are used to determine susceptibility. However, in modified in vitro assays where the pH is set to pH 5 the activity of 5FC increases significantly.

Here we provide evidence that fcyB, a gene that encodes a purine-cytosine permease orthologous to known 5FC importers is downregulated at pH 7 and is the primary factor responsible for the low efficacy of 5FC at pH 7. We also uncover two transcriptional regulators that are responsible for repression of fcyB and consequently mediators of 5FC resistance, the CCAAT binding complex (CBC) and the pH regulatory protein PacC. We propose that the activity of 5FC might be enhanced by perturbation of factors that repress fcyB expression such as PacC or other components of the pH sensing machinery.

INTRODUCTION

5FC is a synthetic compound that was originally assessed along with 5-fluorouracil (5FU) and other fluorinated pyrimidine analogue to treat tumours (1). The antifungal effect of 5FC was subsequently reported in 1963 (2) and it was first used to treat candidiasis and cryptococcosis in humans in 1968 (3). The apparently rapid development of resistance of both Cryptococcus and Candida to 5FC has limited its use as a mono-therapeutic agent, however, it remains a mainstay in the treatment of cryptococcal diseases being recommended, in combination with Amphotericin B (AmB)
deoxycholate, as a primary therapy (4) and is recommended for treatment of certain presentations of candidiasis in combination with other agents (5, 6). Its use has been restricted in the treatment of aspergillosis due to an apparent lack of activity of 5FC in vitro (7), and is not recommended for treatment of any form of aspergillosis by the Infectious Diseases Society of America (8, 9).

5FC is a prodrug, lacking any intrinsic anti-mycotic activity without first being processed through the pyrimidine salvage pathway. The mechanism of action of 5FC has been extensively studied in Cryptococcus and Candida. After active transport into fungal cells mediated primarily by the action of the proton/cytosine symporter, FCY2, 5FC is rapidly metabolised to 5FU by the action of cytosine deaminase (FCY1, also known as FCA1 in C. albicans), which is absent in mammalian cells (10). 5FU is subsequently converted to 5-fluorouridine monophosphate (5FUMP) via the action of uracil phosphoribosyltransferase (FUR1), and then to 5-fluorouridine triphosphate which is either incorporated into RNA, resulting in the disruption of protein synthesis, or converted to 5-fluoro deoxyuridine-monophosphate which inhibits thymidylate synthetase and consequently DNA synthesis (11, 12) As FCY1, FCY2 and FUR1 are essential for the activity of 5FC yet are dispensable for fungal viability (as UMP can be synthesised de novo from L-glutamine), it is unsurprising that the primary mechanisms of clinical resistance observed in both Candida and Cryptococcus are directly linked to mutations in this pathway (11, 13, 14).

5FC is often cited as having no activity against Aspergillus species (15, 16), however it would be more accurate to state that 5FC has limited activity against most Aspergillus isolates, with one published study showing MICs ranging from 0.25 to >256 mg/L (n=21 isolates) (7). The limited activity of 5FC against A. fumigatus in vitro contradicts evidence showing that it is able to significantly improve outcomes in a murine model of
infection (17, 18). One explanation for this lack of concordance is the variable activity that 5FC exhibits at pH 5 and pH 7. Reducing the pH has been shown to increase the activity of 5FC in a strain dependent manner up to 4000-fold (128 mg/L at pH 7 and 0.03 mg/L at pH 5) against *A. fumigatus* (19). The MIC at low pH has been shown to better reflect the result of *in vitro* models of infection for *A. fumigatus* (17).

Understanding the mechanistic basis for the reduction in the MIC of *A. fumigatus* at low pH will further enhance our knowledge of the basis of 5FC resistance and may enable more appropriate use of such compounds. In this study we show that at pH 7 two transcriptional regulators, PacC and the CCAAT-binding complex (CBC) orchestrate 5FC resistance via negative regulation of the gene encoding the purine-cytosine transporter and FCY2 orthologue, FcyB. We also demonstrate that FcyB is critical for 5FC activity and reduced expression of *fcyB* at pH7 is the major mechanism conferring intrinsic 5FC resistance in *A. fumigatus*.

**MATERIALS & METHODS**

**Oligonucleotides, strains and growth conditions**

Strains used in this study are listed in supplementary Table 1. Conidia were harvested from four day old cultures grown on SAB agar. To assess radial growth, $10^4$ spores in a total volume of 5µl were spotted onto *Aspergillus* minimal medium (AMM) (20) agar with varying pH in the absence and presence of different concentrations of 5FC (details are given in the respective figures). MIC testing was carried out following the EUCAST broth microdilution reference method (21) with modifications to account for adequate ionic buffering. Low pH medium (pH 5) was buffered using 100 mM citrate-phosphate buffer whereas 100 mM MOPS was used to buffer high pH medium (pH 7).
Table 1. Strains used in this study.

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Generation of gene deletion constructs

Oligonucleotides used for the genetic manipulation of strains are listed in Table S2. For the generation of knock-out mutants in the A1160P+ background, genes were disrupted by homologous recombination using deletion fragments generated by Fusion PCR following the procedure described recently (24). Briefly, for the generation of gene deletion fragments, around 1 kb of 5’ and 3’ flanking region were PCR amplified using primers with designations -1 and -2 (5’) and -3 and -4 (3’) and fused to an antibiotic resistance cassette (the hygromycin and zeocin resistance cassettes were amplified with hph-FW and hph-RV using pAN 7-1 and pAN 8-1 as template, respectively, as previously described (24) and the pyrithiamine resistance cassette with primers ptrA 5 and ptrA 3 using ptrII as template) using the nested primers with designations –N1 and –N2.

The hapC deletion mutant in the ATCC46645 background (ΔhapCATCC) was kindly provided by Prof Dr Hubertus Haas. For the generation of this mutant strain, the bipartite marker technique was used (25). Briefly, the pyrithiamine resistance cassette was released from pSK275 using PstI and HindIII. In the next step hapC 5’ and 3’ flanking regions were amplified using primer pairs hapC-1/2 and hapC-3/4, respectively. Subsequently, amplicons were digested with PstI (5’) and HindIII (3’) and individually ligated to the resistance conferring cassette. In a final step hapC deletion constructs were amplified with primer pairs hapC-N1/ptrA-RV and hapC-N2/ptrA-FW. The two fragments were simultaneously transformed into ATCC46645 in order to disrupt the coding sequence of the gene via homologous recombination.

Generation of a conditional fcyB expression strain

The conditional fcyB expression fragment was generated similar to the Fusion PCR approach used for the construction of gene deletion constructs. In this case, the xylose
inducible promoter \textit{P}_{\text{xyIP}} (26) was linked downstream of the hygromycin resistance
cassette (Fig S1, \textit{hph-P}_{\text{xyIP}}). Subsequently, a construct including 5' \textit{fcyB} flanking region,
the linker cassette and \textit{fcyB} coding sequence plus 3' flanking region were amplified \textit{via}
Fusion PCR and transformed into A1160P+.

\textbf{Transcriptional profiling and expression analysis}

Strains were grown in a shaking incubator for 18h in liquid AMM at 37 °C 200 rpm.
Subsequently, mycelia were filtered, washed with distilled water and transferred into
RPMI pH 5 and pH 7 for 4h using the same incubation conditions. RNA was isolated
using TRI Reagent\textsuperscript{5} (Sigma). 10 µg extracted total RNA were digested using RQ1 RNase-
Free DNase (Promega) and further purified using the RNeasy Mini Kit (Qiagen). For
paired-end RNA sequencing, libraries were generated using the TruSeq\textsuperscript{®} Stranded
mRNA assay (Illumina, Inc.) according to the manufacturer’s protocol. Fastq files were
analysed with FastQC and any low-quality reads were trimmed with Trimmomatic. All
libraries were aligned to the \textit{A. fumigatus} A1163 genome assembly (GCA_000150145.1)
with the gene annotation from CADRE/Ensembl Fungi v28 using Tophat-2.1.0 and only
matches with the best score were reported for each read. Differential expression
analysis was performed using Deseq2. Genes with very low levels of expression (with
average reads across all conditions <10) were eliminated from our comparative analysis.

qPCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using
the iScript One-Step RT-PCR kit with SYBR Green (Cat# 170-8893, Bio-Rad). Primers
used for qRT-PCR analysis are listed in Table S2. Amplification reactions were
performed in a final volume of 25 µL using (1.0µL) 0.4µM forward primer, (1.0µL) 0.4
µM reverse primer, and 5 ng (5 µL) of total RNA. The PCR cycling conditions were 95°C
for 30 seconds followed by 55°C for 30 seconds and finally 72°C for 30 seconds. Samples have been assessed in triplicate. Error bars indicate the standard deviation of respective samples and p-values were calculated by Student’s T-test (reference: gpdA).

Northern analysis was carried out as described previously using digoxigenin-labelled probes (27).

**Reconstitution of ΔfcyB**

To complement the ΔfcyB mutant phenotype, the plasmid pfcyB<sub>REC</sub> was generated. The fcyB coding sequence including 1.2 kb 5’ and 3’ flanking region were amplified using primers fcyB-FW/fcyB-RV. The backbone of the pyrithiamine resistance conferring plasmid pSK275 was amplified as described previously (22). Subsequently both PCR products were fused using Gibson Assembly (NEB) and DNA was propagated in *E. coli*.

**Fungal transformation**

For all transformations 2 µg of the respective DNA constructs were used. The plasmid for reconstitution was linearised as illustrated in Fig S2. Transformation using pyrithiamine for selection (0.1µg/ml) was carried out on AMM plus 1M Sucrose, for hygromycin (200µg/ml) and zeocin (150µg/ml) based selection Sabouraud dextrose agar (SAB) supplemented with 1M Sucrose was used.

**Chromatin immunoprecipitation and ChIP-qPCR analysis**

ChIP-qPCR was performed following the same procedure as described in previously (22). Briefly, the Hap<sup>GFP</sup> expressing strain (hap<sup>GFP</sup>) was grown in AMM for 18h, 37°C and 200 rpm. The mycelia were harvested by filtration, transferred into RPMI 1640 medium (pH7.0) containing 2% glucose, and incubated for 4h at 37°C with shaking.
Cross-linking was carried out using 1% formaldehyde for 20 min. For ChIP-qPCR primers listed in Table S2 were employed. The PCR cycling conditions are as defined for the qPCR. Enrichment (% Input) of the respective peaks was determined from liquid cultures induced for 4h at pH7 in RPMI 1640. Samples have been assessed in triplicate and the results are presented together with the background signal and standard error. Error bars indicate the standard deviation of respective samples and p-values were calculated by Student’s T-test (reference: actA): *, <0.05.

RESULTS

Loss of function of two key transcriptional regulators leads to 5FC hypersusceptibility in A. fumigatus

In a recent study we identified that loss of function of the CCAAT binding complex (CBC) leads to resistance to a number of drugs that target sterol biosynthesis includingazole antifungals (22). An extended evaluation of the role of this transcriptional regulator in drug resistance led to the discovery that CBC mutants are hypersusceptible to 5FC (Fig. 1). Whereas the isogenic host strain has an MIC in excess of 400 mg/L, a strain lacking a functional CBC (ΔhapC) has an MIC of 25 mg/L in RPMI following the EUCAST broth microdilution reference method (21).

This discovery prompted us to further evaluate the mechanistic basis of 5FC resistance in Aspergillus species. Previous studies have highlighted that the potency of 5FC against A. fumigatus can be increased significantly by reducing the pH of culture media from pH 7 (as defined by EUCAST) to pH 5 (7, 17, 18). Assessment of our wild-type isolate confirmed this relationship with the MIC decreasing by more than 64-fold at pH 5 (Fig. 1). The pH interdependency of 5FC activity led us to hypothesise that the pH-responsive
transcription factor PacC was involved in the regulation of genetic factors responsible for 5FC resistance. To investigate the role of PacC in 5FC resistance, we generated a pacC deletion mutant (∆pacC) and determined the MIC to 5FC of this strain. Similar to the result obtained for ΔhapC, the ∆pacC strain showed increased susceptibility to 5FC, with an MIC of 12.5 mg/L at pH 7 (Fig. 1). These data suggest a novel regulatory role of the CBC and PacC in resistance to 5FC.

Commonalities in the pH, PacC and CBC transcriptional regulons identifies fcyB as the factor promoting pH-dependent 5FC resistance.

As susceptibility to 5FC was dramatically increased at low environmental pH or by loss of either PacC or the CBC we hypothesised that the genetic target or targets driving 5FC susceptibility were linked. We therefore assessed the transcriptional profiles of ΔhapC, ΔpacC and their isogenic progenitor at pH 5 and pH 7 by RNAseq to uncover common transcriptional regulons. As the quantitative difference in MIC between conditions was large, we concentrated on genes that were differentially regulated by >4 fold. There were 379 and 407 genes upregulated in ΔhapC and ΔpacC, respectively, at pH 7.0, whilst 475 and 262 genes were downregulated. 483 genes were upregulated and 412 downregulated by the transition from pH 7 to pH 5. A total of 27 genes were coordinately upregulated (Table S1) and 49 genes downregulated in all three aforementioned data sets. One of the 27 genes upregulated in all data sets was fcyB (AFUB_025700), a gene that encodes a purine-cytosine permease orthologous to yeast Fcy2p (28) which has been previously linked to 5FC uptake and resistance in Candida species but also in Aspergillus nidulans (12, 28-33).

To confirm the results of our genome-wide transcriptomic study we analysed the expression of fcyB in ΔhapC and ΔpacC mutants at pH 7 and pH 5 by qRT-PCR. As
predicted by our previous results, fcyB was significantly upregulated at pH 5 (24.5-fold) in the wild-type isolate (Fig. 2). In ΔpacC transcript levels were significantly increased during growth at both pH 5 (4.0-fold) and pH 7 (34.6-fold) when compared to the isogenic control. Similarly, fcyB expression was upregulated by 11.3-fold at pH 7 in ΔhapC, however, fcyB expression was not significantly increased in ΔhapC in low pH conditions. It is also noteworthy that pH specific regulation of fcyB expression is retained in both the hapC and pacC null mutants. To ensure the results were not unique to one strain background, we evaluated the regulatory role of PacC and HapC in 5FC resistance in a second isolate, ATCC46645. Consistent with data from the A1160P+ strain, fcyB was upregulated at low pH and repression of the gene in the respective mutant strains at neutral pH was lifted (Fig S3).

Combined our transcriptional data indicates that i) the CBC is a negative regulator of fcyB at pH 7 but not at pH 5, ii) PacC is a negative regulator of fcyB at pH 7 and pH 5 and iii) at least one other pH dependent factor is involved in modulating the expression of fcyB, either a negative regulator at pH7 or a positive regulator at pH5.

The transcriptional results for fcyB correlate with the 5FC MIC levels for the mutants at pH 5 and 7 (Fig. 1 & Fig. 2). The only exception is for ΔhapC at pH 5 where we identified a c. 12.5-25 fold decrease in susceptibility to 5FC (Fig. 1A) however the fcyB transcript was unchanged, which indicates that further factors contribute to 5FC susceptibility in the mutant (Fig. 2).

To further investigate if increased expression of fcyB is the major cause for increased 5FC susceptibility in ΔpacC and ΔhapC mutants, the fcyB coding sequence was disrupted in both mutant backgrounds (ΔpacCΔfcyB and ΔhapCΔfcyB). Deletion of fcyB dramatically increased resistance in both double mutants to near wild-type and ΔfcyB levels at pH 7 (Fig. 1). At pH 5 although deletion of fcyB in both the ΔpacC and ΔhapC
background reduced susceptibility to 5FC by c. 400-fold, ΔpacCΔfcyB was still significantly more susceptible to 5FC than either ΔfcyB or the wt at pH 7.0.

Our results suggest that CBC and PacC-mediated regulation of fcyB is a major, but not the sole contributory factor, to 5FC resistance in *A. fumigatus.*

**CBC mediated regulation of fcyB occurs in a direct manner**

To identify a direct or indirect regulatory role of HapC in *fcyB* expression we looked for putative CBC binding sites within 1 kb of the *fcyB* promoter in ChIP-Seq data generated in our previous work (22). A single binding peak summit was located -570bp relative to the translation start (TLS) (Fig. 3A & 3B). In close proximity of the peak area we identified two putative CBC binding sites (5′-CCAAT-3′) (-663 and -568 relative to the TLS). *In vivo* binding of the CBC with the promoter was measured employing ChIP-qPCR and revealed significant enrichment of the transcription factor complex at this locus (Fig. 3C). We have also assessed interaction of PacC with three putative binding sites (-977, -1075 and -1294 relative to the TLS) located within 1.5 kb of the *fcyB* promoter by ChIP qPCR. No significant enrichment for binding at these sites was detected (data not shown).

Taken together, these data strongly suggest that the CBC acts as a direct negative regulator of *fcyB,* however no direct regulatory function could be identified for PacC.

**CBC and PacC independent overexpression of fcyB results in 5FC hypersusceptibility**

Our results strongly indicate that mutation of PacC and the CBC results in increased susceptibility to 5FC *via* upregulation of *fcyB*. To assess if upregulation of *fcyB,* independent of PacC and CBC activity, could cause reduction in resistance to 5FC, we generated an *fcyB* tunable mutant (*fcyB*<sup>XYL</sup>). This was achieved by replacing the
endogenous promoter with the *Penicillium chrysogenum* xylose-inducible, glucose-
repressible promoter *P*<sub>xylP</sub> (Fig. 4A) (26). This system was successfully tested for
tunable expression in *A. fumigatus* in previous studies (34-36).

Glucose mediated downregulation of *fcyB* expression resulted in pH independent 5FC
hyperresistance (Fig. 4B; *fcyB<sup>XYL</sup> n.i.) whereas xylose mediated induction rendered
strain *fcyB<sup>XYL</sup>* hypersusceptible (*fcyB<sup>XYL</sup> i.*) to 5FC during both low and high pH (Fig. 4B)
with MIC levels lower than 0.03 mg/L at pH 7 (Fig. 4C).

Taken together, this data further suggests that transcriptional upregulation of *fcyB*
represents a major contributory factor for 5FC hypersusceptibility.

**DISCUSSION**

In this work, we unveil novel mechanisms mediating 5FC resistance in the opportunistic
fungal pathogen *A. fumigatus*. We show that enhanced expression of the gene encoding
the orthologue of the *A. nidulans* purine-cytosine permease, FcyB, is a major factor
contributing to 5FC sensitivity. As in *A. nidulans* lack of FcyB activity confers
hyperresistance (33). We discovered that *fcyB*, is transcriptionally repressed in high pH
environments, illustrating a major reason for the intrinsic resistance to 5FC observed
when *A. fumigatus* isolates are assessed using the standard EUCAST and CLSI
methodologies (21, 37). We have, in addition, identified two transcription factors, the
CBC and the pH regulatory protein PacC, that orchestrate 5FC resistance via
transcriptional repression of *fcyB* (model see Fig. 5). Our data reveals a direct role for
the CBC in repression of *fcyB* at high pH and shows that PacC is a negative regulator of
*fcyB* expression at both low and high pH. Our data also suggest that additional, as yet
unidentified factors, transcriptionally governed by both the CBC and PacC but independent of FcyB contribute to 5FC resistance.

FcyB is a member of the high-affinity nucleobase cation symporter family 1 (NCS1) and requires H+ ions for the import of its substrate molecules, including the antifungal prodrug 5FC (33, 38, 39). It has therefore been suggested, that proton availability in acidic environments is a key factor driving increased activity of FcyB and ultimately uptake of 5FC. Our transcriptional data however, cast doubt on this hypothesis. Using a xylose-inducible expression system to uncouple fcyB expression from CBC and PacC regulation, we have demonstrated that overexpression of fcyB alone is sufficient to increase 5FC activity independent of pH (Fig. 5).

More than a decade ago the interdependency of 5FC activity with environmental pH was revealed in clinical yeast and mould species including *A. fumigatus* (7, 19). Despite studies demonstrating the efficacious, monotherapeutic use of 5FC in murine models of aspergillosis, this drug is typically not applied in clinical settings (9, 40). To our knowledge, no data is available to indicate the pH in the lung during aspergillus infection however from studies of other pathological states it has been shown that cellular damage, inflammation and infection can lead to a localised reduction of pH below pH 7 (41-43). This coupled with the ability of *Aspergillus* sp. to acidify their local environments (44) may indicate that 5FC may be variably active in the host depending on the acidity of the local environment. This raises the interesting possibility that treatment using 5FC will only be effective for those sites with relatively acidic environments. Pockets of persister cells could remain in neutral or basic environments providing a reservoir for the development of resistance. Our findings suggest it would be theoretically possible to enhance the clinical activity of 5FC by chemical disruption of
the pH sensing mechanisms used by *A. fumigatus* such as PacC or proteins of the pH signalling cascade upstream of PacC.

**FUNDING**

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**DECLARATIONS**

MB is a consultant to Synlab GMBH and is the director and shareholder of Syngenics Limited.

**REFERENCES**


24. Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, Ramage G, Denning DW, Bowyer P. 2013. The cdr1B efflux transporter is associated with non-


FIGURE LEGENDS

Fig. 1 PacC and the CBC regulate 5FC resistance via fcyB. Susceptibility of mutant strains were determined by (A) following the EUCAST broth microdilution reference method in RPMI (21) and (B) on AMM agar. For both experiments isolates were incubated for 48 h at 37 degrees.

Fig. 2 fcyB expression is repressed at neutral pH. Deletion of either hapC or pacC leads to transcriptional upregulation of fcyB. Low pH results in a significant increase in fcyB transcript levels in wild-type. In contrast to mutation of the CBC (∆hapC), ∆pacC also shows significant upregulation of the gene at low pH.

Fig. 3 The CBC directly interacts with the fcyB promoter in vivo. (A) ChIP-seq peak (22) with putative CBC binding regions (red, CCAAT boxes) are located -663 and -568 relative to the translation start (TLS). (B) Based on the peak summit (-620 relative to the TLS) direct in vivo binding of the CBC to the promoter of fcyB was validated employing ChIP-qPCR (see Material and Methods).

Fig. 4 Overexpression of fcyB results in pH independent 5FC hypersusceptibility. (A) For the conditional overexpression of fcyB, its native promoter region (wt) was replaced by the PxylP-based, xylose inducible promoter system (fcyBXYL). (B) AMM was supplemented with 1% xylose (i., induced) to overexpress fcyB. To downregulate expression of the gene in fcyBXYL xylose was omitted (n.i., non-induced). (C) MIC levels
during inducing and non-inducing conditions have been determined according to EUCAST (21). For both experiments strains were incubated for 48h at 37°C.

Fig. 5 **Proposed regulatory mechanism determining pH dependent 5FC activity in *A. fumigatus.*** The major route of 5FC entry into *A. fumigatus* cells occurs via uptake through the purine-cytosine permease FcyB. *fcyB* gene expression is repressed at high pH but transcriptionally upregulated at low pH. At pH 7 *fcyB* is repressed by the CBC and PacC, which confers intrinsic resistance to 5FC. The CBC acts as direct repressor of the gene. In contrast to the CBC, PacC also exerts a negative regulatory function on *fcyB* gene expression at low pH.

**SUPPORTING INFORMATION LEGENDS**

Fig. S1 **Schematic representation for the replacement of the native *fcyB* with an inducible *fcyB* construct.**

Fig. S2 **Schematic representation for the reconstitution of *fcyB.*** The plasmid containing a functional copy of *fcyB* and pyrithiamine resistance conferring cassette were reintroduced into the *fcyB* deletion locus.

Fig S3. **pH dependent expression of *fcyB* in wild-type as well as ΔpacC and ΔhapC mutants of the ATCC46645 background.** (A) Expression of *fcyB* was monitored in both A1160P+ and ATCC46645 wild-type backgrounds to demonstrate evolutionary conservation of pH dependent expression of the gene and to validate the role of PacC and CBC as repressors of the gene at neutral pH in a further background isolate. (B) Expression of the gene was quantified using ImageJ.

wt, wild-type; ΔC, ΔhapC; ΔP, ΔpacC; ATCC, ATCC46645; A1160, A1160P+. 
Fig. 1 PacC and the CBC determine 5FC resistance via fcyB, particularly at neutral pH. Susceptibility of mutant strains was analysed in 1 x RPMI (A) following the EUCAST broth microdilution reference method (24). (B) 5FC susceptibility was further monitored on solid AMM containing the given concentration 5FC. For both experiments isolates were incubated for 48 h at 37 degrees.

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Fig. 2 *fcyB* expression is repressed at neutral pH. Deletion of either *hapC* or *pacC* leads to transcriptional upregulation of *fcyB*. Low environmental pH results in a significant increase in *fcyB* transcript levels in wild-type. In contrast to mutation of the CBC (Δ*hapC*), Δ*pacC* also shows significant upregulation of the gene at low pH. To determine pH dependent expression strains liquid cultures were incubated for 18h at 37°C, 200 rpm. Subsequently, mycelia were filtered, washed with water and transferred into fresh 1x RPMI pH 5 (100mM citrate buffer) or 1x RPMI pH 7 (100mM MOPS).
Fig. 3 The CBC directly interacts with the fcyB promoter in vivo. (A) ChIP-seq peak (23) with putative CBC binding regions (red, CCAAT boxes) are located -663 and -568 relative to the translation start (TLS). (B) Based on the peak summit (-620 relative to the TLS) direct in vivo binding of the CBC to the promoter of fcyB was validated employing ChIP-qPCR. Enrichment (% Input) of the respective peaks was determined from liquid cultures induced for 4h at pH7 in RPMI 1640. Samples have been assessed in triplicate. Error bars indicate the standard deviation of respective samples and p-values were calculated by Student’s T-test (reference: actA): *, p < 0.05.
Fig. 4 Overexpression of fcyB results in pH independent 5FC hyper-susceptibility. (A) For the conditional overexpression of fcyB, its native promoter region (wt) was replaced by the PxylP-based, xylose inducible promoter system (fcyB\textsuperscript{XYL}). (B) AMM was supplemented with 1% xylose (i., induced) to overexpress fcyB. To downregulate expression of the gene in fcyB\textsuperscript{XYL} xylose was omitted (n.i., non-induced). (C) MIC levels during inducing and non-inducing conditions have been determined according to EUCAST (24). For both experiments strains were incubated for 48h at 37°C.
Fig. 5 Proposed regulatory mechanism determining pH dependent 5FC activity in A. fumigatus. The major route of 5FC entry into A. fumigatus cells occurs via uptake through the purine-cytosine permease FcyB. fcyB gene expression is repressed at high pH but transcriptionally upregulated at low pH. At pH 7 fcyB is repressed by the CBC and PacC, which confers intrinsic resistance to 5FC. The CBC acts as direct repressor of the gene. In contrast to the CBC, PacC also exerts a negative regulatory function on fcyB gene expression at low pH.