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The cytochrome b_5 CybE is regulated by iron availability and is crucial for azole resistance in *A. fumigatus*

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Cytochrome P450 enzymes (P450) play essential roles in redox metabolism in all domains of life including detoxification reactions and sterol biosynthesis. The activity of P450s is fuelled by two electron-transferring mechanisms, heme-independent P450 reductase (CPR) and the heme-dependent cytochrome b_5 (CYB5)/cytochrome b_5 reductase (CB5R) system. In this study, we characterised the role and regulation of the cytochrome b_5 CybE in the fungal pathogen *Aspergillus fumigatus*. Deletion of the CybE encoding gene (*cybE*) caused a severe growth defect in two different *A. fumigatus* isolates, emphasising the importance of the CB5R system in this pathogen, while the non-essentiality of *cybE* indicates the partial redundancy of the CPR and CB5R systems. Interestingly, the growth defect caused by the *cybE* loss of function was even more drastic in *A. fumigatus* strain AfS77 compared to strain A1160P+ indicating a strain-dependent degree of compensation, which is supported by azole resistance studies. In agreement with CybE being important for the assistance of the ergosterol biosynthetic P450 Cyp51, deletion of *cybE* decreased resistance to the Cyp51-targeting antifungal voriconazole and caused accumulation of the ergosterol pathway intermediate eburicol. Northern analysis indicated that CybE deficiency leads to the compensatory transcriptional upregulation of Cyp51-encoding *cyp51A* and CPR-encoding *cprA*. Overexpression of *cybE* did not affect azole resistance suggesting that CybE activity is not rate limiting. Expression of *cybE* was found to be repressed during iron starvation by the iron-regulatory transcription factor HapX demonstrating iron dependence of CybE not only at the level of enzyme activity but also at the level of gene expression.

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Significance to metallomics

Cytochrome P450 enzymes (P450) are involved in oxidative metabolism and biosynthesis in all kingdoms of life. The required electrons for this redox chemistry are provided by an iron-dependent and an iron-independent system. With a focus on the iron-dependent cytochrome b_5, this study gives new insights about the fine-tuning of electron transfer systems dependent on iron availability.

Introduction

Cytochrome P450 enzymes (P450) are found in all domains of life. They play important roles in xenobiotic metabolism/biotransformation,[1] secondary metabolism[2] and primary metabolism such as biosynthesis of fatty acids, vitamins[3,4] and sterols.[5] Although P450 amino acid sequences are extremely diverse between species, their structures are highly conserved. This structural conservation allows all P450s to bind the cofactor heme, which is essential for their function.[6] In addition to the structural conservation, members of this family share the catalytic property to activate molecular oxygen to incorporate one molecule of oxygen into a substrate, while the other is reduced to water by NAD(P)H.[4] This reductive activation is highly dependent on free electrons and on the co-factor heme of the respective cytochrome P450.[6] The heme-bound iron is a crucial factor for this chemistry, as it can adopt different oxidation states and hence serves as an electron acceptor and donor.[7] In many species including the yeast *Saccharomyces cerevisiae*, the two

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electrons, which are required for a P450 reaction, are provided by two partially redundant systems.[8,9] In one system, electrons are passed from NADPH by a cytochrome P450 reductase (CPR) to the respective P450. In the second system, electrons are supplied to P450s by the cytochrome b_5 (CYB5)/NADH cytochrome b_5 reductase (CB5R) system. It is speculated that CYB5 optimises the kinetics of P450 reactions by allosteric enhancement and by acting as an electron store. In the latter mechanism, the second electron needed for P450 reactions is supplied by CYB5 immediately after the activation of oxygen.[10] This facilitation of electron supply increases the formation of reaction products and decreases the release superoxide.

CYB5 is also involved in P450-independent reactions as an electron shuttle in systems such as the electron transfer to microsomal desaturases, which are important for unsaturated fatty acid synthesis.[11,12] CYB5 also contains heme as a prosthetic group, which is involved in shuttling electrons to the respective target.[13,14]

Ergosterol is the predominant sterol in fungal membranes.[5] One step in ergosterol biosynthesis includes sterol-C14α demethylation, which is performed by the P450 sterol-C14α demethylase CYP51.[13] This enzyme is the target of the azole class of antifungals, which bind to CYP51, and inhibit its function and thereby the biosynthesis of ergosterol, resulting in lethality.[5] In two yeast species, *S. cerevisiae* and *Candida albicans*, individual inactivation of either CPR or CYB5 was not lethal and decreased but did not block ergosterol biosynthesis.[14–16] In contrast, simultaneous inactivation of both CYB5 and CPR was lethal,[14,17] which corroborates the partial redundancy of these systems in these yeast species. In addition, the CYB5/CB5R system was found to fully support yeast CYP51 *in vitro*.[13] Due to decreased CYP51 activity, loss of CPR leads to increased azole susceptibility, and overexpression of the CYB5 encoding gene in such a setting increased azole resistance in contrast to the CPR knockout, which emphasises partial redundancy of the CYB5/CB5R and CPR systems in electron transport to CYP51 in yeast.[14]

The filamentous fungus *Aspergillus fumigatus* is an opportunistic human pathogen and is the major cause for life-threatening invasive aspergillosis.[18] Immunocompromised patients are especially at high risk for being infected. One of the most common treatments of fungal infections including aspergillosis is the inhibition of the sterol-C14α-demethylase CYP51 (termed Erg11 in yeast species) with triazole-type antifungals.[19–21] The *A. fumigatus* genome encodes two CYP51 enzymes termed Cyp51A and Cyp51B.[22] Both are able to perform sterol-C14α demethylation with similar activity.[23,24] However, most mechanisms described for azole resistance are linked to Cyp51A.[21,25]

The *A. fumigatus* genome, similar to other filamentous fungal genomes, comprises 77 genes encoding for P450s.[2,26] This quantity is striking compared to the widely used model organism *S. cerevisiae*, which only possesses three P450s: the sterol-C14α-demethylase CYP51/Erg11 and the sterol-C22-desaturase CYP61/Erg5, which are both involved in ergosterol biosynthesis, and the dityrosine hydroxylase CYP56, which is important for yeast sporulation.[13] In contrast to *S. cerevisiae*, mould species like *A. fumigatus* produce a wide range of secondary metabolites, the biosynthesis of which requires numerous P450-dependent oxygenation reactions.[27] Consequently, the expansion of P450s in moulds compared to *S. cerevisiae* is most likely linked to their complex secondary metabolism. The *A. fumigatus* genome encodes one putative CYB5, termed CybE (AFUA_2G04710), and two putative CPRs, termed CprA (AFUA_6G10990) and CprB (AFUA_2G07940), which are potentially involved in the biosynthesis of ergosterol and secondary metabolites.

Iron is essential for almost all organisms, including the heme function in P450s and CYB5. Iron acquisition and the expression of iron-consuming genes has to be tightly regulated to keep the balance between iron toxicity and iron shortage.[28] In *A. fumigatus*, this includes gene regulation by the iron-responsive transcription factor HapX, which represses iron-consuming pathways during iron starvation in order to spare iron and activates the respective genes during iron excess.[29] Adaptation to iron starvation including high-affinity acquisition *via* siderophores and HapX-mediated transcriptional reprogramming has been demonstrated to be crucial for the virulence of *A. fumigatus*.[30] This iron-responsive reprogramming also involves the SREBP (sterol regulatory element-binding protein)-type regulator SrbA, which links the control of iron metabolism, ergosterol biosynthesis and adaptation to low oxygen conditions.[31]

In this study, we characterised the function of CybE in *A. fumigatus via* gene inactivation and overexpression, including its role in ergosterol biosynthesis, demonstrating its regulation by iron availability *via* the transcription factor HapX.

Experimental

Growth conditions

In liquid cultures, strains were grown at 37 °C and 200 rpm in *Aspergillus* minimal medium (MM),[32] containing 1% glucose (carbon source), 20 mM glutamine (nitrogen source) and 30 μM $FeSO_4$ (if not otherwise indicated). For plate assays, MM agar or agarose was used (agarose for −Fe conditions to avoid iron contaminations). Plates were grown for 48 h at 37 °C. For low oxygen experiments, strains were grown for 48 h at 37 °C with 1% O_2 and 5% CO_2.

A. fumigatus strains, generation of *cybE* deletion mutants, *cybE* inducible strains and reconstitution of Δ*cybE*

The coding sequence of *cybE* (AFUA_2G04710) was replaced by a hygromycin (*hph*) resistance conferring cassette in the AfS77 and A1160P+ strains. The deletion construct was generated by fusion PCR following an approach previously described.[33] In the first step, around 1 kb of the *cybE* 5′-flanking region and 1 kb of the 3′-flanking region were amplified by PCR using primers the AACCCATCCTGTCTGACCGA & TAGTTCTGTTACC GAGCCGGCGGTTTATCTGGATTGGTGACG and GCTCTGAACG ATATGCTCCCGCAAGGGGAAAGGAGGTCTG & GGCGTCATCCC TTCCATTGA. Subsequently, fragments were linked to a hygromycin resistance cassette (amplified with the primers CCGGCTCGGTAA CAGAACTAACGGCGTAACCAAAAGTCAC and GGGAGCATATCGTT CAGAGCTCTTGACGACCGTTGATCTG) by PCR fusion using the

primers CCCTGAAGATGTGCTGCTGA and GTTGTCGATCAC AGCGATGC.

To reconstitute Δ*cybE*, plasmid pcybE^{REC}, comprising a pyrithiamine resistance cassette, was generated. The backbone of the pyrithiamine resistance cassette carrying plasmid pSK275 (syn. pME3024[34]) was amplified using the primers ACCGGTCAAA GCTAAAGAGG and ACTAGTTCTAGAGCGGCCG.

The *cybE* coding sequences including 1.5 kb 5′- and 1.9 kb 3′-flanking regions were amplified with the primers CGGCCGC TCTAGAACTAGTCTGCTCTGTGGCCTGTGATA and CCTCTTTA GCTTTGACCGGTCGCGTGCAGATAAAGATCAA. Both fragments were connected *via* Gibson Assembly®.

To generate a strain in which *cybE* is inducible by the addition of xylose to the growth medium (*cybE*^{xylP}), the promoter of *cybE* was separated from the coding sequence by a hygromycin cassette and a xylose inducible *xylP* promoter.[35] The construct was generated using the NEBuilder® kit (New England Biolabs), with the primers MM118 (AATTCGAGCTCGGTACAGCGGAAGAC CATTC), MM119 (TACCTAGGTGTGTAGAAATTATTCGGTTT), MM120 (TCTACACACCTAGGTACAGAAGTCC), MM121 (GCGGA CATGGTTGGTTCTTCGAGT), MM122 (AACCAACCATGTCCGCC TCCAAG), MM123 (GCCAAGCTTGCATGCCCACTCCACTGTTGA GAG), and linear pUC19 (amplified with the primers GGCATG CAAGCTTGGCGT and GTACCGAGCTCGAATTCACTG) as a plasmid backbone. MM118/119, MM120/121 and MM122/123 were used to amplify the 5′-homologous region, a hygromycin-*xylP* cassette and the 3′-homologous region, respectively.

Linear knockout- and promoter exchange constructs, as well as pcybE^{REC}, were transformed into *A. fumigatus* protoplasts and integrated into the genome by homologous recombination. Integration was proven by Southern blot analysis (Fig. 1). pcybE^{REC} restored the wildtype phenotype in the Δ*cybE* strain, just as *cybE* induction did in the *cybE*^{xylP} strain (Fig. 1). Two different *A. fumigatus* isolates were used for this study, AfS77[36] and A1160P+.[33] The strains used in this study are listed in Table 1.

RNA isolation and northern blot analysis

RNA was isolated using TRI Reagent® (Sigma) according to the manufacturer's manual. 10 μg of RNA was used for electrophoresis on 2.2 M formaldehyde agarose gels and subsequently blotted onto Hybond N membranes (Amersham). DIG labelled probes were amplified by PCR.

Southern blot analysis

DNA was isolated by PCI extraction and isopropanol precipitation. To confirm the gene-specific restriction pattern of the genetic manipulations, the DNA was digested with PstI (AfS77 strains) or BamHI (A1160P+ strains). The resulting restriction fragments were separated on an agarose gel and transferred to a Hybond N membrane (Amersham) by capillary blotting with NaOH. The deletion of *cybE* caused the deletion of a PstI site in the CDS and the insertion of a BamHI site. The *xylP*-promoter insertion elongated the space between the PstI sites. Integration of pcybE^{REC} generated a second 5′-region of *cybE*. These differences in fragment size were detected with a DIG-labelled probe binding to the 5′-region of *cybE*. A successful genetic manipulation leads to the following fragments: wt PstI 1752 bp, Δ*cybE* PstI 3368 bp, *cybE*^{xylP} PstI 2641 bp, wt BamHI 8107 bp, Δ*cybE* BamHI 5804 bp, *cybE*^{REC} BamHI 5804 and 10 521 bp.

Susceptibility tests

For diffusion tests, conidia were mixed with 25 mL of MM to a final concentration of 10^6 conidia per mL. These plates with 25 mL of MM were poured, and a paper disc was placed in the middle or a hole was pricked out, respectively. For oxidative stress tests, the hole was filled with 100 μL H_2O_2 (34 mg mL^{-1}).

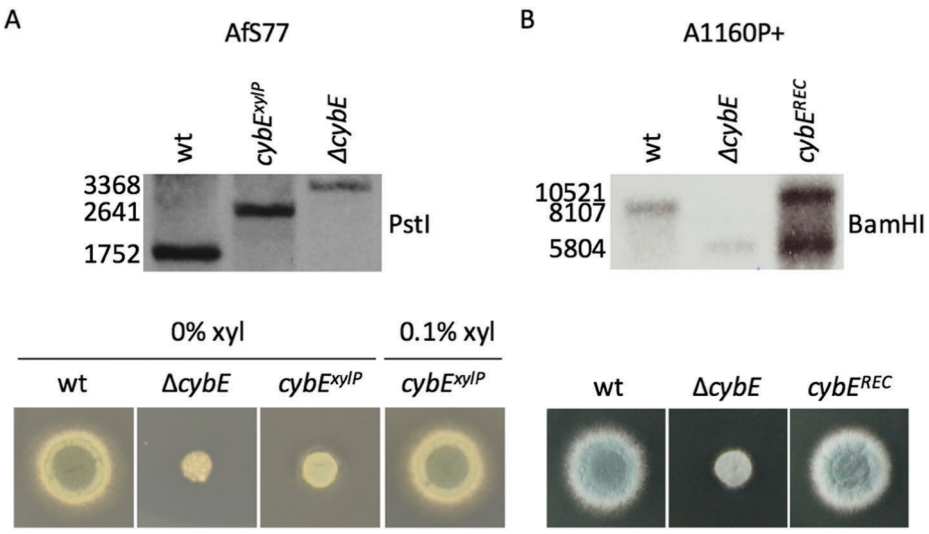


Fig. 1 Southern blot analysis and growth of *cybE* mutant strains on solid iron-replete minimal medium (MM). Strains were generated in the two *A. fumigatus* isolates: (A) AfS77 and (B) A1160P+. Δ*cybE* strains are *cybE* gene deletion strains; in strain *cybE*^{REC}, the *cybE* gene was re-integrated at the *cybE* deletion locus in Δ*cybE*; in strain *cybE*^{xyl}, *cybE* expression is controlled by the xylose-inducible *xylP* promoter. Strains have been validated using Southern blot analysis revealing the expected pattern (see the Experimental section). Δ*cybE* and *cybE*^{xyl} under non-inducing conditions display reduced growth, while *cybE*^{REC} and *cybE*^{xyl} under inducing conditions (0.1% xylose) show growth similar to wt.

Table 1 Strains used for this study

Strain	Genotype	Ref.
wt, A1160P+	A1160, Δ*akuB::pyrG+*	33
Δ*cybE*, A1160P+	A1160P+, Δ*cybE::hph*	This study
$cybE^{REC}$	A1160P+, Δ*cybE::hph*, p$cybE^{REC}$, *ptrA*	This study
wt, AfS77	ATCC46645, Δ*akuA::loxP*	36
Δ*cybE*, AfS77	AfS77, Δ*cybE::hph*	This study
$cybE^{xylP}$	AfS77, $_{p}$*cybE::hph-*$_{p}$*xylP*	This study
Δ*srbA*	AfS77, Δ*srbA::hph*	This study

For antifungal susceptibility tests, the paper disc was drenched with voriconazole (10 μL of a 320 μg mL^{-1} solution in DMSO) or with terbinafine (10 μL of a 100 μg mL^{-1} solution in DMSO).

Sterol measurements with gas chromatography ion trap mass spectrometry (GC-IT-MS)

Sterols were analysed as their corresponding trimethylsilyl (TMS) ethers. The sterols were identified by their relative retention time and mass spectra.[37–39] Strains were grown in MM for 24 h at 37 °C and 200 rpm. Mycelia were harvested, freeze-dried, ground and dissolved in 2 M NaOH. Subsequent procedures were performed according to Müller *et al.*[38] The content for each sterol was calculated according to Müller and Bracher.[40] The samples were measured in biological triplicates.

Results and discussion

Deletion of *cybE* causes severe growth defects in *A. fumigatus*

To analyse the function of cytochrome b_5, mutant strains were generated lacking CybE (AFUA_2G04710), here referred to as Δ*cybE*, by replacing the encoding gene *cybE* with a hygromycin resistance cassette, as described in the Experimental section. This gene deletion was performed in the *A. fumigatus* isolate A1160P+,[33] which is an *akuB::pyrG* derivative of CEA10 (termed wt A1160P+). To confirm the *cybE* specificity of this genetic manipulation, the *cybE* gene was reinserted in the Δ*cybE* mutant strain in single copy at the *cybE* locus yielding $cybE^{REC}$ (see the Experimental section). Correct integration of the deletion construct and the reconstitution construct, respectively, was proven by PCR analysis (not shown) and Southern blot analysis (Fig. 1). In addition, a Δ*cybE* strain was generated in AfS77,[36] which is an *akuA::loxP* derivative of ATCC46645 (termed wt AfS77). Gene deletion in both genetic backgrounds caused a clear reduced growth phenotype. Reinsertion of *cybE* in Δ*cybE* in the A1160P+

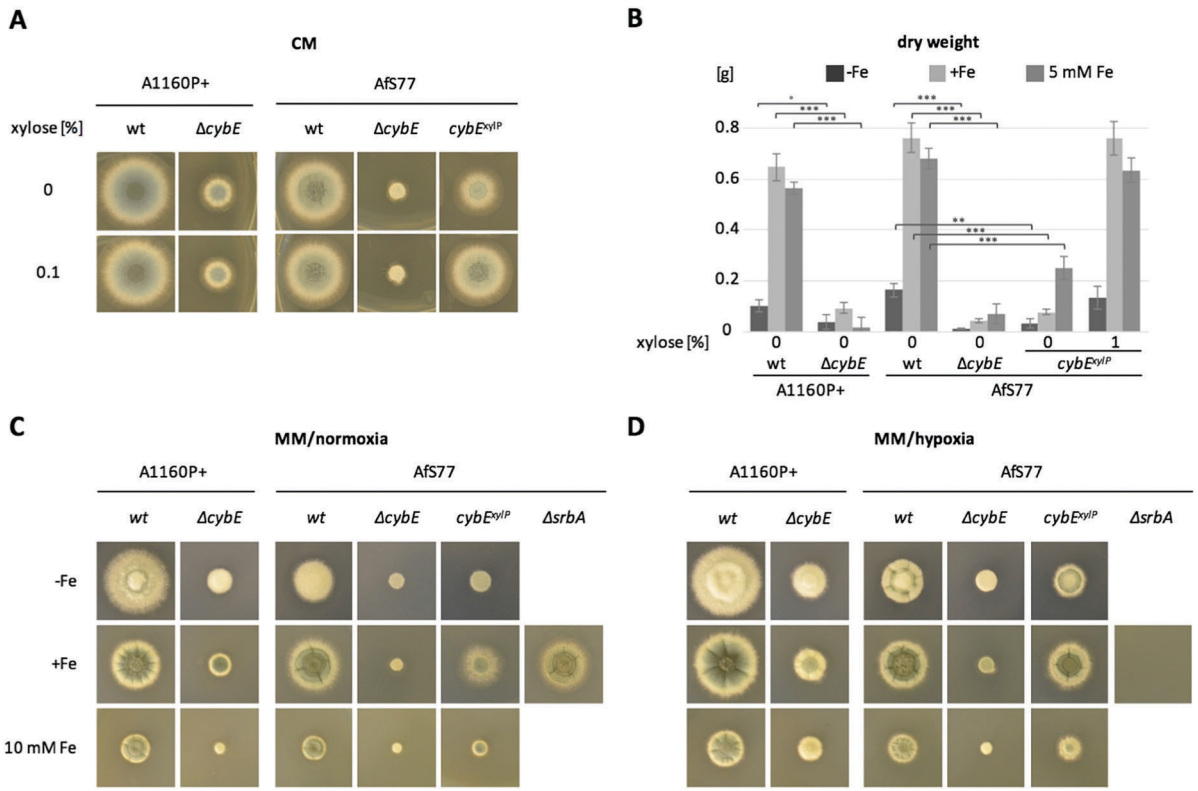


Fig. 2 Deletion of *cybE* caused severe growth defects in solid and liquid media. (A) 10^4 spores were spotted in the solid complex medium (CM) containing 0% ($cybE^{xylP}$ repressing conditions) or 0.1% xylose ($cybE^{xylP}$ inducing conditions). (B) 10^8 spores were inoculated in 100 mL minimal medium (MM) containing no (−Fe), 30 μM (+Fe) and 5 mM iron. $cybE^{xylP}$ was grown under *cybE* repressing (0% xylose) and overexpressing (1% xylose) conditions. Error bars indicate the standard deviation. Asterisks indicate the level of significance according to the *p*-values of an unpaired two-tailed Student's *t*-test: ° for $p = 0.054$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. Cultures were analysed in triplicate. (C and D) 10^4 spores were spotted on solid MM containing −Fe, +Fe and 10 mM iron. Plates were incubated for 48 h at 37 °C under normal oxygen conditions (C) and in parallel under low oxygen conditions (D, 1% O_2, 5% CO_2). In plate cultures, the mycelial density of the colonies is significantly lower during −Fe compared to +Fe, and therefore the radial growth is not informative for the growth rate in this case. Iron starvation is reflected by the decreased sporulation.

genetic background (strain *cybE*^{REC}) restored the wt phenotype, which proves that the growth defect is *cybE* deletion specific and not an off-target effect (Fig. 1). To study the effects of *cybE* overexpression, the gene was expressed in AfS77 under the control of the xylose-inducible *xylP*-promoter.[35] Growth of this strain, termed strain *cybE*^{xylP}, under non-inducing conditions (0% xylose) resulted in decreased growth, albeit less extreme compared to *cybE* deletion, indicating decreased *cybE* expression compared to the wt strain (Fig. 1). Induction of *cybE* gene expression (0.1% xylose) again fully restored the wt phenotype.

In both genetic backgrounds (A1160P+ and AfS77), the deletion of *cybE* led to severe growth defects on plates, as well as in liquid cultures in both minimal (MM) and complex (CM) growth media (Fig. 2A–C). Interestingly, the growth defect caused by *cybE* deletion was more severe in the AfS77 genetic background compared to A1160P+.

CybE has a heme-binding domain and is supposed to be a hemoprotein like other cytochrome b_5 proteins.[9] Consequently, it requires iron for proper function. Therefore, we compared the growth of wt, Δ*cybE* and *cybE*^{xylP} strains under conditions of different iron availabilities. Despite the growth defect, both the Δ*cybE* and uninduced *cybE*^{xylP} strains responded to starvation and excess of iron similar to the wt strain (Fig. 2B–D). This underpins an important role of CybE independent of iron availability.

Taken together, the severe growth defect caused by CybE deficiency in two different *A. fumigatus* isolates emphasises the importance of the CB5R system in this species while the non-essentiality of *cybE* indicates the partial redundancy of the CPR and CB5R systems. Moreover, the difference in the degree of growth impairment in the two *A. fumigatus* strains demonstrates the strain-dependent degree of compensation.

CybE deficiency increases susceptibility to voriconazole, terbinafine, oxidative stress, and cellular accumulation of the ergosterol biosynthetic pathway intermediate eburicol

The ergosterol biosynthetic P450 enzyme sterol-C14α-demethylase, termed Cyp51, is the target for triazole-type antifungals such as

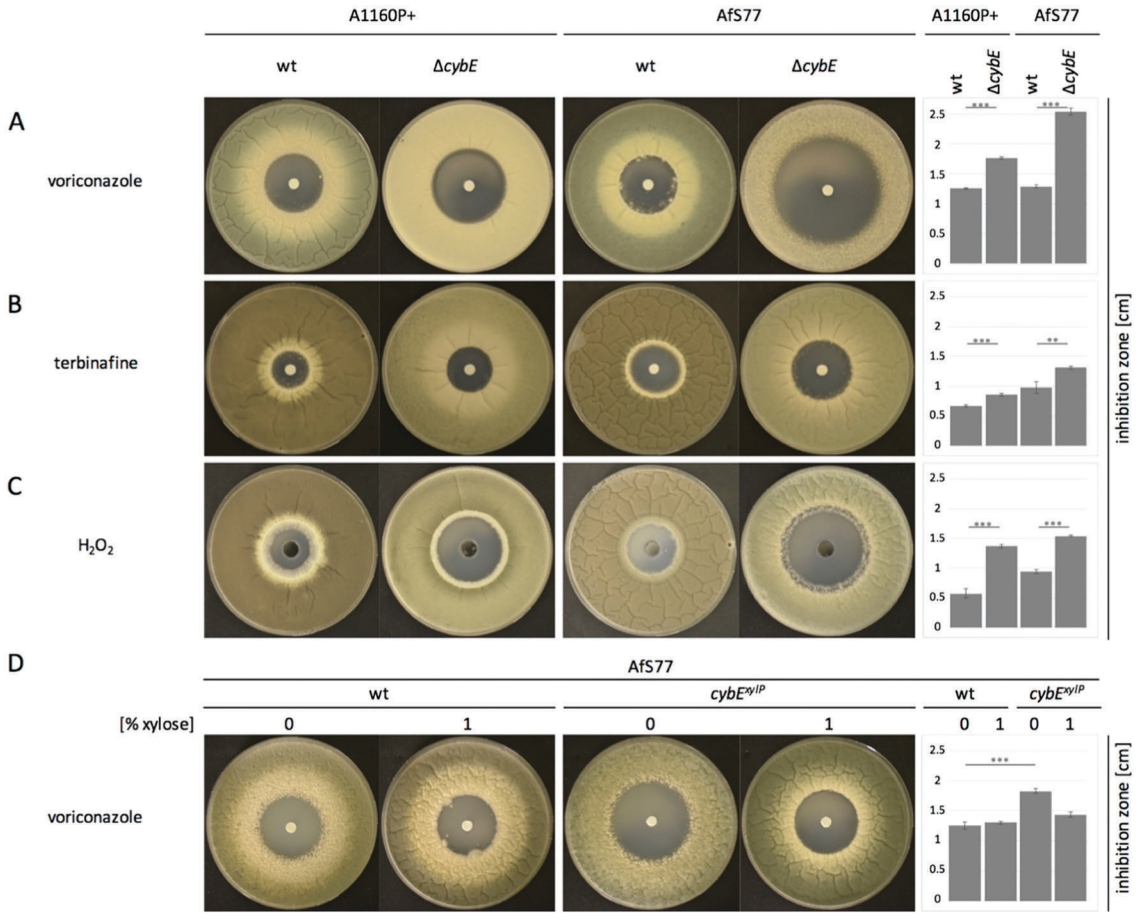
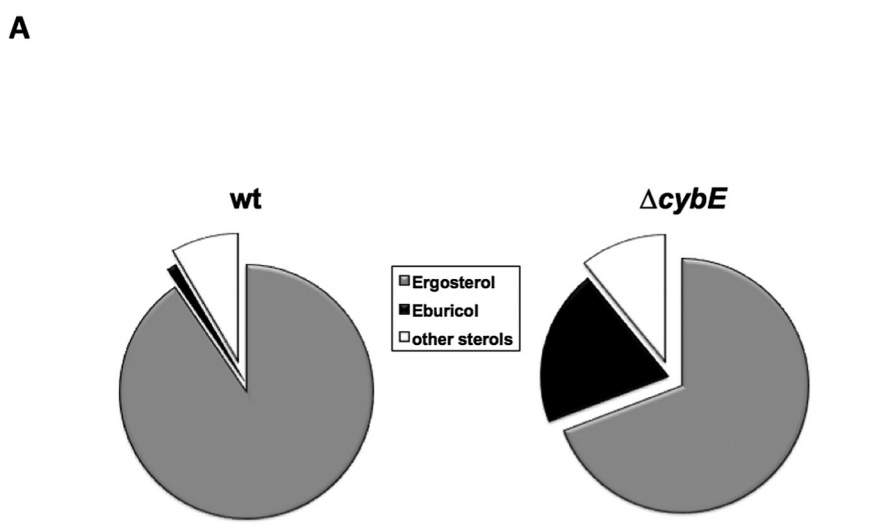


Fig. 3 CybE deficiency increases the susceptibility to voriconazole, terbinafine and H_2O_2. For inhibitor diffusion assays, plates containing 25 mL of MM were inoculated homogeneously with 2×10^7 spores. (A) 10 μL of voriconazole (320 μg mL^{-1}) or (B) terbinafine (100 μg mL^{-1}) was administered onto a filter paper disk in the centre. (C) For oxidative stress tests, 100 μL of H_2O_2 (34 mg mL^{-1}) was added to a well in the centre. (D) Voriconazole resistance was also examined under *cybE* repressing and overexpressing conditions. Pictures were taken after 48 h of incubation at 37 °C. From three biological replicates, the mean size of the inhibition zone, which reflects the compound susceptibility, is shown as bar graphs on the right of each row. Error bars indicate the standard deviation. Significance levels are indicated as asterisks according to the *p*-values of an unpaired two-tailed Student's *t*-test: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

voriconazole, and previous studies have indicated a link between the roles of CybE and Cyp51.[5,13] To investigate a potential involvement of CybE in ergosterol biosynthesis in *A. fumigatus*, we compared the voriconazole susceptibility of wt, Δ*cybE* and *cybE*^{xylP} strains in paper disc diffusion assays (Fig. 3A and D). Both wt strains displayed about the same susceptibility to voriconazole. CybE deficiency significantly increased the susceptibility to this antifungal in both genetic backgrounds. However, the increase in susceptibility was more dramatic in AfS77 compared to A1160P+ (Fig. 3A) underlining the strain-specific effects already seen at the level of growth (see above). In line with the fact that CybE deficiency was more detrimental at the level of growth in AfS77 compared to A1160P+, the voriconazole susceptibility data demonstrate that mechanisms that compensate for CybE deficiency are more efficient in the A1160P+ strain compared to AfS77. The *cybE*^{xylP} strain displayed an increased voriconazole susceptibility under non-inducing conditions (without xylose, Fig. 3D), although the increase was lower compared to *cybE* gene deletion indicating leaky expression below the wt level (Fig. 3A and D), which is in line with the growth pattern of this strain (see above). Inducing conditions (1% xylose), which causes huge overexpression of *cybE* as shown in Fig. 5C, did not increase voriconazole resistance of the *cybE*^{xylP} strain compared to the wt strain (Fig. 3D), which indicates that CybE activity is not a limiting factor for the Cyp51 activity in *A. fumigatus*, even under triazole stress. These data also indicate that mutations leading to gain of function of CybE are not expected to play a role in the development of resistance to triazoles in clinical settings.

The increase in the voriconazole susceptibility caused by loss of CybE indicates the role of this enzyme in sterol biosynthesis, perhaps *via* Cyp51. This is corroborated by the analysis of the cellular sterol content of wt and Δ*cybE* strains (Fig. 4). In line with CybE assisting Cyp51 enzyme activity, CybE deficiency caused a 0.76-fold decrease in the cellular ergosterol content and an 18-fold increased accumulation of eburicol, the substrate of Cyp51. Such a change of the cellular sterol profile is also caused by deletion of one of the two Cyp51-encoding genes or by triazole treatment.[5] Reintegration of *cybE* into Δ*cybE* (strain *cybE*^{REC}) restored the wt sterol profile (Fig. 4), proving that the changes observed are indeed caused by gene deletion.

The viability and ergosterol production of CybE-deficient strains indicate that alternative systems are able to compensate for this defect. In agreement, CprA has been shown to be able to



	relative sterol composition [%]							
sterols	1	2	3	4	5	6	7	8
wt	2.3%	90.5%	0.6%	0.8%	1.0%	2.5%	1.1%	1.1%
Δ*cybE*	4.4%	69.2%*	0.0%	0.8%	1.6%	2.7%	19.9%#	1.4%
cybE^{REC}	4.4%	89.6%	0.2%	0.2%	0.3%	2.9%	1.6%	0.8%

p-value: *0.000001 #0.00000004

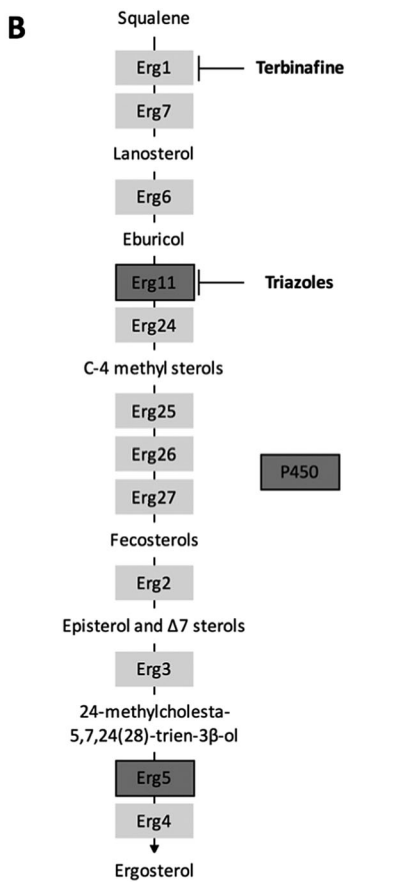


Fig. 4 CybE deficiency decreases the cellular ergosterol content and increases accumulation of eburicol. (A) Sterol levels of strains incubated in MM for 24 h at 37 °C and at 200 rpm were quantified by GC-MS. The sterol content has been normalised to total sterol levels. Samples were assessed in biological triplicates. Sterols: 1, ergosta-5,7,9(11),22-tetraen-3β-ol; 2, ergosterol; 3, 5,6-dihydroergosterol; 4, ergosta-5,7,24(28)-trien-3β-ol; 5, episterol; 6, lanosterol; 7, eburicol; and 8, 4,4-dimethylergosta-8,24(28)-dien-3β-ol. Significance levels were calculated by employing an unpaired two-tailed Student's *t*-test. (B) The ergosterol biosynthesis pathway. The two P450 enzymes and the targets of terbinafine and triazoles are indicated.

assist the activity of both *A. fumigatus* Cyp51A and Cyp51B *in vitro*.[41] Our data provide the first proof of involvement of CybE in *A. fumigatus* Cyp51 enzyme activity.

The role of CybE in sterol biosynthesis is further exemplified in susceptibility tests to the allylamine-type antifungal terbinafine, which inhibits squalene epoxidase (Erg1), involved in an early step in ergosterol biosynthesis (Fig. 4B), and is used therapeutically against dermatophytes.[42] The two wt isolates demonstrated a distinct susceptibility to terbinafine, with AfS77 being more susceptible compared to A1160P+ (Fig. 3B), again illustrating differences between these two strains. CybE deficiency slightly increased the susceptibility to terbinafine in both genetic backgrounds. Similarly, deletion of the *cybE* homolog in *C. albicans* increased the susceptibility to both triazoles and terbinafine.[16] It has been shown that squalene epoxidase, although not a member of the P450 family, accomplishes its function in a CPR-dependent manner, *i.e.* CPR provides electrons to squalene epoxidase using NADPH.[43] As the CybE homolog appears to be able to compensate for CPR deficiency in yeast species with azole susceptibility as the read out,[15,17] the modest increase in terbinafine susceptibility caused by CybE deficiency in *C. albicans*[16] and here in *A. fumigatus* might indicate a role of CybE in assisting squalene epoxidase activity.

AfS77 was also found to be less resistant to hydrogen peroxide, and in both genetic backgrounds CybE deficiency increased the susceptibility to this oxidative stressor (Fig. 3C). *A. fumigatus* must cope with oxidative stress in various environments. Detoxification of oxidative stress is particularly important during host infection, as reactive oxygen species are produced by phagocytes during the immune defense.[44] A huge part of intrinsic oxidative stress is caused by respiration and by P450 reactions, which form reactive oxygen species as by-products that harm cell components.[45] During P450 reactions, two electrons are required for substrate oxidation. In P450 reactions, unwanted superoxide is formed due to the premature release of the superoxide anion before the second electron can be acquired for proper catalysis.[10] CybE is believed to support the donation of the second electron and thereby reduces uncoupling of superoxide anions. As *A. fumigatus* possesses 77 P450s[2,26] including Cyp51, the oxidative-stress susceptibility caused by CybE deficiency might indicate that CybE is important to avoid the generation of intrinsic oxidative stress.

Expression of *cybE* is HapX-dependent regulated by iron availability

In *A. fumigatus* as in other fungal species,[46,47] transcription of numerous genes that encode iron-dependent proteins or genes that are involved in iron-dependent pathways is downregulated by the transcription factor HapX in cooperation with the CCAAT-binding complex (CBC). Moreover, transcription of this gene set is induced by the HapX–CBC complex within 30 minutes in a shift from iron starvation to iron sufficiency.[29] Northern blot analysis demonstrated that *cybE* expression is also responsive to iron availability (Fig. 5A). Compared to iron sufficiency (+Fe), the *cybE* transcript level was decreased to undetectability during iron starvation (−Fe) in both wt genetic backgrounds, while the repression of *cybE* transcription was impaired by HapX deficiency in both genetic backgrounds. These results demonstrate that *cybE* expression is repressed by HapX during iron starvation. As a control for iron starvation conditions, the expression of the iron-repressed, siderophore transporter-encoding *mirB* gene was monitored.[48] The transcript levels of *mirB* are lower in *hapX*-deficient backgrounds because HapX is also involved in the activation of iron acquisition during iron starvation.[38,39] In both genetic wt backgrounds, *cybE* transcription was induced within 30 minutes after the addition of iron to a final concentration of 30 μM (iron shift, sFe), while this induction was abrogated by HapX deficiency in the AfS77 background suggesting that the short-term activation of *cybE* transcription is mediated by HapX. In contrast, HapX deficiency did not abrogate *cybE* induction in the A1160P+ genetic background. As a control for the HapX-mediated short-term activation of transcription in the iron shift, we monitored transcript levels of *cccA*, which encodes a vacuolar iron transporter that is essential for iron detoxification.[49]

The transcription of *cccA* was induced in the iron shift in both genetic backgrounds in a HapX-dependent manner, thus demonstrating the general functionality of this regulatory circuit in both genetic backgrounds. The difference in the short-term response of *cybE* transcript levels in the two HapX-deficient genetic backgrounds reveals that *cybE* expression is subject to different regulatory circuits in the different strains, which might mask the HapX-dependency in A1160P+. The undetectability of *cybE* transcripts in Δ*cybE* strains in both genetic backgrounds confirms the successful gene deletion.

Taken together, *cybE* expression was found to be transcriptionally repressed during iron starvation in a HapX-dependent manner in both genetic backgrounds, while the short-term iron-induction was dependent on HapX in the AfS77, but not the A1160P+ background, revealing an interesting strain-specificity of this regulation.

CybE deficiency causes transcriptional upregulation of the CPR system and *cyp51A*

Northern blot analysis revealed that CybE deficiency leads to upregulation of *cprA* transcript levels in both genetic backgrounds, indicating that compensation for loss of CybE involves the upregulation of the alternative CPR system (Fig. 5B). Remarkably, the second CPR encoding gene, *cprB*, was expressed at a significantly lower level (detectable only after long exposure of the blot) and not affected by CybE deficiency (Fig. 5B).

Moreover, CybE deficiency resulted in the transcriptional upregulation of *cyp51A* in both genetic backgrounds (Fig. 5B). This compensatory transcriptional upregulation of cyp51A emphasises the link between CybE, ergosterol biosynthesis and Cyp51. This transcriptional response is reminiscent of the increase of *cyp51A* transcription level upon treatment with triazoles,[50] which also impairs Cyp51 activity.

Taken together, these data emphasise the links of CybE with CPR and Cyp51A *via* transcriptional responses caused by the CybE defect and demonstrate the cells' effort to compensate for defects by transcriptional upregulation of a target gene and the CybE alternative system.

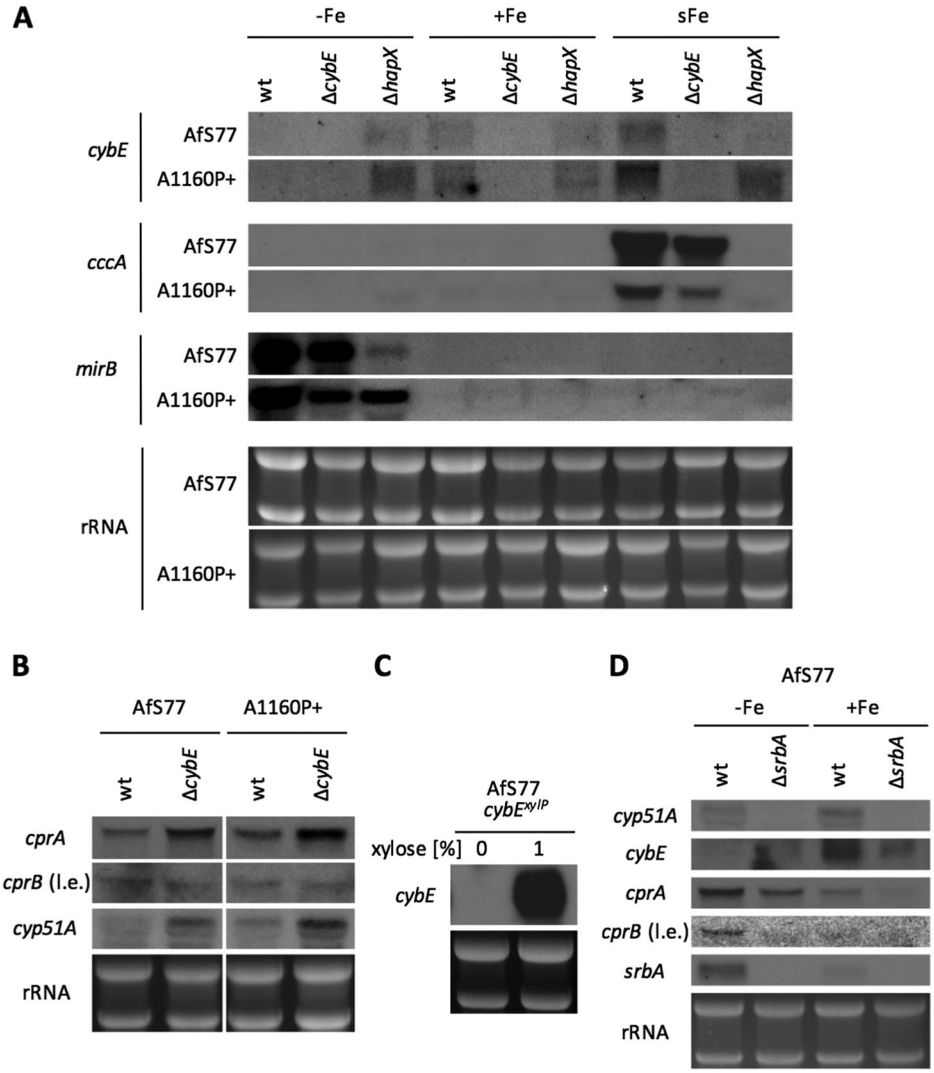


Fig. 5 *cybE* expression is iron-regulated and its deletion triggers compensatory transcriptional changes. RNA was isolated from the strains grown for 20 h in MM containing no iron (−Fe) or 30 μM iron (+Fe) at 37 °C. Δ*cybE* strains were grown for 40 h to ensure similar biomass and growth phase. For the iron shift (sFe), iron was added to a final concentration of 30 μM for 30 min after pregrowth for 20 h (wt)/40 h (Δ*cybE*) under iron starvation conditions. Ethidium bromide-stained ribosomal RNA (rRNA) of the gels is shown as a control for the loading and quality of RNA. (A) HapX-mediated iron regulation of *cybE* transcript levels. *cccA* and *mirB* were included as controls for correct iron conditions and genetic backgrounds (see text). (B) Deletion of *cybE* causes compensatory upregulation of genes, which are involved in P450 enzyme reactions. In contrast to the other genes, *cprB* transcripts were detectable only after long exposure ("l.e.") indicating the low level of expression. (C) Expression of *cybE* can be repressed and induced by xylose addition to the medium in $cybE^{xylP}$. (D) SrbA is involved in the regulation of P450 associated gene expression.

The transcription factor SrbA regulates not only *cyp51* expression but also Cyp51 associated genes

SrbA is a SREBP-type transcriptional activator that plays an important role in the regulation of sterol biosynthesis, and adaptation to hypoxia and iron starvation in *A. fumigatus.*[51,52] One of the SrbA target genes is *cyp51A* and, consequently, SrbA deficiency leads to a dramatically increased susceptibility to triazole-type antifungals.[53,54] In agreement, northern blot analysis confirmed the transcriptional downregulation of *cyp51A* in response to SrbA deficiency in the AfS77 background during both iron sufficiency and starvation (Fig. 5D). Furthermore, SrbA deficiency was found to result in the downregulation of transcript levels of *cprA* during iron starvation and sufficiency, *cybE* during iron sufficiency, and *cprB* during iron starvation (Fig. 5D). These data indicate that these Cyp51-assisting protein-encoding genes belong to the SrbA regulon in order to support the coordination of sterol biosynthesis. In accordance with previous findings,[31] *srbA* was transcriptionally upregulated during iron starvation (Fig. 5D). Interestingly, also *cprA* and *cprB* were found to be transcriptionally upregulated during iron starvation, while *cybE* was downregulated under this condition as already discussed above (Fig. 5D). The inverse regulation of *cybE* and *cprA/B* genes in response to iron availability indicates the cellular optimisation of partially alternative iron-dependent and iron-independent systems to iron availability.

CybE is not essential under low oxygen conditions

A. fumigatus can cope with extremely low oxygen conditions, which is important to adapt to certain niches, including inflamed or necrotic tissue during host infection.[52] During hypoxia, several enzymes involved in ergosterol biosynthesis are transcriptionally elevated.[55] This might be due to the fact that oxygen is required in several steps of ergosterol biosynthesis including Cyp51 enzyme activity.[56] Decreased availability of the substrate oxygen is expected to decelerate the reaction speed of the involved enzymes. Increasing the amount of the enzymes would compensate for this to maintain the rate of ergosterol production when oxygen is rare. As CybE supports the Cyp51/Erg11 reaction kinetics,[10] we addressed the question of whether CybE is also involved in this adaptation to hypoxia. We tested whether limited oxygen availability worsens the growth of Δ*cybE* strains with plate growth assays performed at low oxygen conditions (1% O_2, 5% CO_2). As a control under low oxygen conditions, we included a mutant strain (Δ*srbA*) lacking SrbA, a transcription factor that is essential for adaptation to low oxygen conditions including the transcriptional activation of the ergosterol biosynthetic pathway.[55] In comparison to normoxic conditions, low oxygen conditions did not decrease the growth of the *cybE*-deficient strains, in contrast to Δ*srbA* (Fig. 2C and D). These data demonstrate that CybE is not essential during low oxygen conditions and that alternative electron-shuttling systems are also able to partially compensate for the loss of CybE under this condition.

Conclusion

This study illustrates that CybE assists Cyp51 enzyme activity in *A. fumigatus* and is consequently important for resistance to ergosterol biosynthesis pathway targeting antifungals. However, overexpression of *cybE* does not lead to increased resistance to these antifungals, making this enzyme an unlikely cause for resistance in clinical settings. Expression of *cybE* was found to be regulated by iron availability mediated by the transcription factor HapX. Recently, the CBC–HapX complex has been identified as a repressor of *cyp51A*/*erg11* gene expression in *A. fumigatus.*[54] Consequently, our data demonstrating the HapX-dependent iron regulation of *cybE* expression expand and underline the link between ergosterol biosynthesis, HapX and iron. Northern blot analysis revealed the compensation of CybE deficiency by the transcriptional upregulation of the alternative CPR system and the target gene *cyp51A*. Moreover, this study revealed remarkable differences between the two analysed *A. fumigatus* isolates, which highlights the extent to which different isolates of *A. fumigatus* can vary with respect to sterol homeostasis. This phenomenon has recently been observed regarding virulence and photoresponse in other *A. fumigatus* strains.[57–59]

Conflict of interests

The authors declare no conflict of interest.

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