The Janus transcription factor HapX controls fungal adaptation to both iron starvation and iron excess

Fabio Gsaller¹,†, Peter Hortschansky²,†, Sarah R Beattie³, Veronika Klammer², Katja Tuppatsch²,⁴, Beatrix E Lechner¹, Nicole Rietzschel⁵, Ernst R Werner⁶, Aaron A Vogan⁷, Dawoon Chung³, Ulrich Mühlenhoff⁶, Masashi Kato⁸, Robert A Cramer³, Axel A Brakhage²,⁴,* & Hubertus Haas¹,**

Abstract

Balance of physiological levels of iron is essential for every organism. In Aspergillus fumigatus and other fungal pathogens, the transcription factor HapX mediates adaptation to iron limitation and consequently virulence by repressing iron consumption and activating iron uptake. Here, we demonstrate that HapX is also essential for iron resistance via activating vacuolar iron storage. We identified HapX protein domains that are essential for HapX functions during either iron starvation or high-iron conditions. The evolutionary conservation of these domains indicates their wide-spread role in iron sensing. We further demonstrate that a HapX homodimer and the CCAAT-binding complex (CBC) cooperatively bind an evolutionary conserved DNA motif in a target promoter. The latter reveals the mode of discrimination between general CBC and specific HapX/CBC target genes. Collectively, our study uncovers a novel regulatory mechanism mediating both iron resistance and adaptation to iron starvation by the same transcription factor complex with activating and repressing functions depending on ambient iron availability.

Keywords fungi; iron regulation; sensing; siderophores; transcription factor complex

Subject Categories Metabolism; Microbiology, Virology & Host Pathogen Interaction

DOI 10.15252/embj.201489468 | Received 8 July 2014 | Revised 15 July 2014 | Accepted 15 July 2014 | Published online 4 August 2014


Introduction

The redox-active metal iron is an indispensable cofactor in a variety of essential cellular processes such as oxidative phosphorylation, biosynthesis of numerous metabolites, and detoxification of oxidative stress. Paradoxically, the same redox property makes this metal potentially toxic by causing oxidative stress (Halliwell & Gutteridge, 1984; Lin et al., 2011). Thus, iron homeostasis requires precise regulation of iron uptake and storage to satisfy the cellular needs but to avoid toxic iron excess.

In Aspergillus fumigatus, iron homeostasis is maintained by two central transcription factors, which are interconnected in a negative transcriptional feed-back loop: the GATA-factor SreA and the bZIP-factor HapX (Haas, 2012). During iron sufficiency, SreA represses iron uptake, including reductive iron assimilation and siderophore-mediated iron uptake, to avoid toxic effects (Schrettl et al., 2008). During iron starvation, HapX activates siderophore-mediated iron acquisition and represses iron-consuming pathways, including heme biosynthesis and respiration, to spare iron (Schrettl et al., 2010). As shown in Aspergillus nidulans, HapX functions via physical interaction with the CCAAT-binding complex (CBC) (Hortschansky et al., 2007). The CBC is a heterotrimeric DNA-binding complex, which is conserved in all eukaryotes. In A. nidulans, inactivation of either one of its subunits, phenocopied HapX inactivation with respect to defects in adaptation to iron starvation (Hortschansky et al., 2007). However, the CBC has HapX-independent functions in Aspergillus spp. (Kato, 2005; Thon et al., 2010). Humans lack HapX and genome-wide identification resulted in 5,000–15,000 CBC-binding sites depending on the type of human cell analyzed (Fleming et al., 2013).

Deficiency in HapX, but not SreA, attenuates virulence of A. fumigatus in murine models of aspergillosis (Schrettl et al., 2008, 2010), which emphasizes the crucial role of adaptation to iron limitation in pathogenicity. With the exception of Saccharomyces
ceresiae and closely related Saccharomyces species, most fungal species possess orthologs to SreA and HapX (Haas et al., 2008; Kaplan & Kaplan, 2009). The important role of HapX orthologs in virulence is conserved in Candida albicans, Cryptococcus neoformans, and Fusarium oxysporum (Jung et al., 2010; Chen et al., 2011; Hsu et al., 2011; Lopez-Berges et al., 2012). Both SreA and HapX appear to be regulated post-translationally by iron, blocking HapX function and activating SreA function (Haas et al., 1999; Hortschansky et al., 2007). In Schizosaccharomyces pombe, post-translational iron sensing by the HapX and SreA orthologs involves the monothiol glutaredoxin Grx4 (Labbe et al., 2013).

Recently, iron resistance of A. fumigatus was shown to involve SreA-mediated repression of iron uptake and vacuolar iron storage mediated by the vacuolar iron importer CccA (Gsaller et al., 2012). In A. nidulans and A. fumigatus, inactivation of both HapX and SreA is synthetically lethal underlining the critical role of iron homeostasis in cellular survival (Hortschansky et al., 2007; Schrettl et al., 2010). In agreement with their expression pattern and characterized mode of action, the detrimental effects of SreA or HapX inactivation identified so far were confined to growth during iron deficiency or starvation, respectively, which does not explain the synthetic lethality of their inactivation. Here, we provide an explanation for this synthetic lethality by demonstrating that HapX mediates both repression of vacuolar iron storage during iron starvation and activation of vacuolar iron storage during iron excess, i.e. HapX displays inverse activities depending on the ambient iron availability. In line, we identified protein domains that are essential for mediating adaptation to iron starvation or iron excess, exclusively. Moreover, we demonstrate for the first time that HapX not only acts via protein–protein interaction with the CBC but also directly recognizes an evolutionary conserved motif in the cccA promoter. As the CBC has HapX/iron-independent targets, the latter data reveal the mechanism for discrimination of general CBC and specific HapX/CBC target genes.

Results and Discussion

HapX mediates iron resistance by activating CccA-mediated vacuolar iron storage

HapX functions were analyzed in A. fumigatus ATCC 46645 (Schrettl et al., 2010) and, to facilitate the studies, in its ΔakuA-derivative AIF77, which lacks non-homologous recombination (Hartmann et al., 2010). We did not observe any phenotypic differences between respective ATCC46645- and AIF77-derivative strains (data not shown). For clarity, however, the genetic background used is given for all experiments.

Previously, genome-wide transcriptional profiling revealed that during iron starvation HapX activates genes involved in iron acquisition (including siderophore transporter-encoding mirB) and represses the vacuolar iron transporter-encoding cccA as well as numerous genes involved in iron-consuming processes (see below) (Schrettl et al., 2010). CccA-mediated vacuolar iron storage was recently shown to mediate iron resistance (Gsaller et al., 2012). Consistently, the cccA transcript level is upregulated by iron and particularly by SreA-deficiency (Gsaller et al., 2012). The latter is consistent with SreA-deficiency increasing the cellular iron content (Schrettl et al., 2008) but also shows that transcriptional activation of cccA is mediated by an SreA-independent regulatory mechanism.

Northern analysis demonstrated that HapX-deficiency (strain ΔhapX) impairs not only repression of cccA during iron starvation but also induction of cccA during a 1-h shift from iron starvation to iron sufficiency as well as during growth in high-iron medium (Fig 1A). As shown previously (Schrettl et al., 2010), HapX-deficiency caused downregulation of mirB during iron starvation, but did not affect repression of mirB by iron (Fig 1A).

The role of HapX in transcriptional control of cccA during iron excess implicated a role of HapX in iron detoxification. In agreement, HapX-deficiency not only decreased sporulation on agar plates in the presence of the iron starvation-inducing, iron-specific chelator bathophenanthroline disulfonate (BPS) and decreased biomass production in liquid cultures during iron starvation, as shown previously (Schrettl et al., 2010), but also dramatically decreased growth on solid and in liquid high-iron media (Fig 1B and C). As reported previously (Schrettl et al., 2010), HapX-deficiency did affect neither growth rate nor sporulation under iron-replete conditions.

A mutant strain lacking both HapX and CccA, ΔhapXΔccCA, displayed the same growth pattern as ΔhapX on solid and in liquid media (Fig 1B and C). The epistasis of HapX- to CccA-deficiency strongly suggests that lack of cccA expression is responsible for the ΔhapX growth defect during iron excess.

Taken together, HapX acts as a Janus-type transcription factor mediating both repression and activation of cccA and consequently vacuolar iron storage depending on the ambient iron availability.

HapX additionally controls CccA-independent mechanisms involved in iron detoxification

Notably, HapX-deficiency rendered A. fumigatus more susceptible to iron toxicity than CccA-deficiency on solid (Fig 2A) and in liquid (Supplementary Table S1) high-iron media. These data indicate that HapX is also required for the activity of iron detoxification mechanisms other than CccA-mediated iron storage. In support, conditional expression of cccA using the xylose-inducible xyIP promoter (Zadra et al., 2000; Gsaller et al., 2012) increased iron resistance of ΔhapX under inducing but not repressing conditions (Fig 2A; compare strains ΔhapX and ΔhapXΔccCA). However, the radial growth of this strain did not reach that of the wild-type or the ΔcaccΔccCA strain (a cccA deletion mutant expressing cccA under control of the inducible xyIP promoter). Compared to ΔhapX, ΔhapXΔccCA also displayed a significant decrease in growth and sporulation on BPS- and low-iron agar plates, demonstrating that activation of vacuolar iron storage is particularly detrimental in a HapX-deficient background.

As previously indicated by genome-wide transcriptional profiling (Schrettl et al., 2010), apart from cccA numerous other genes involved in iron-consuming processes are repressed by HapX during iron starvation. Northern analysis revealed that in a 1-h shift from iron-limited to iron-replete conditions, which reflects short-term iron excess, HapX-deficiency impairs the transcriptional activation not only of cccA but also of genes encoding iron-consuming functions (Fig 2B). These proteins include the iron–sulfur cluster-containing LeuA (α-isopropylmalate isomerase) and AcoA (aconitase), involved in leucine biosynthesis and the TCA cycle, respectively, the
Northern analysis did not detect increase the sensitivity of transcript detection, despite the HapX requirement under this condition (Fig 1A). To

In contrast to iron sufficiency, HapX levels are significantly higher during iron starvation (A. nidulans, F. oxysporum, C. albicans, S. pombe, A. fumigatus hapX derivative of yellow fluorescent protein) (Nagai et al, 2012), the hapX transcript level was about threefold increased compared to iron sufficiency, and 17-fold downregulation after a 1-h shift from iron starvation to iron sufficiency. As reported previously (Schrettl et al, 2008), sreA expression was increased (about threefold) during iron sufficiency compared to iron starvation and highly upregulated (about 29-fold) during a 1-h shift from iron starvation to iron sufficiency. During iron excess, a condition in which SreA was previously found to be important for iron resistance (Schrettl et al, 2008; Gsaller et al, 2012), the sreA transcript level was about threefold increased compared to that of hapX (data not shown), i.e. hapX was clearly expressed, although below the Northern sensitivity level.

To analyze the expression and localization of A. fumigatus HapX at the protein level, we generated an A. fumigatus strain expressing HapX N-terminally tagged with the Venus fluorescent protein (a derivative of yellow fluorescent protein) (Nagai et al, 2002), under the control of the endogenous hapX promoter in single copy at the hapX locus in ΔhapX (strain hapXVENUS). This cured all mutant phenotypes on solid and in liquid media, indicating that the

Figure 1. HapX is important for adaptation to both iron limitation and iron excess.

A HapX represses cccA during iron starvation and activates cccA during iron excess. Northern analysis was performed with liquid cultures under conditions of iron starvation (~Fe), iron sufficiency (+Fe, 0.03 mM FeSO₄), and high-iron availability (hFe, 3 mM FeSO₄) at 37°C for 24 h or from mycelia shifted for 1 h from ~Fe to +Fe (~Fe).

B On agar plates, HapX-deficiency impairs sporulation on BPS-plates, and growth during iron excess. Growth pattern of wild-type (wt), ΔhapX and ΔhapXΔcccA on solid minimal media containing the indicated iron concentration is shown after 48 h at 37°C. The greenish color of the fungal colonies originates from the spore pigment, and its decrease indicates reduced sporulation. The original size of fungal colony photographs is 2.3 × 2.3 cm in all figures.

C HapX-deficiency impairs submerged growth during both iron starvation and iron excess. Liquid biomass production was monitored after a 1-h shift from iron starvation to iron sufficiency. During iron starvation, i.e. 33-fold higher compared to iron sufficiency, and 17-fold downregulation after a 1-h shift from iron starvation to iron sufficiency. As reported previously (Schrettl et al, 2008), sreA expression was increased (about threefold) during iron sufficiency compared to iron starvation and highly upregulated (about 29-fold) during a 1-h shift from iron starvation to iron sufficiency. During iron excess, a condition in which SreA was previously found to be important for iron resistance (Schrettl et al, 2008; Gsaller et al, 2012), the sreA transcript level was about threefold increased compared to that of hapX (data not shown), i.e. hapX was clearly expressed, although below the Northern sensitivity level.

The greenish color of the fungal colonies originates from the spore pigment, and its decrease indicates reduced sporulation. The original size of fungal colony photographs is 2.3 × 2.3 cm in all figures.

Data information: The iron-sensitive phenotype of ΔcccA was previously analyzed in Gsaller et al (2012) and was further characterized in Fig 2. Moreover, the response of hapX transcript levels to a 1-h shift from iron starvation to sufficiency (~Fe) was analyzed in Fig 4. Strains are derivatives of A. fumigatus Af293.
HapXVENUS protein is fully functional (Supplementary Table S1). In agreement with the transcriptional data, in epifluorescence microscopy HapXVENUS was detectable during iron starvation but not during iron sufficiency, iron excess or a 1-h shift from iron starvation to iron sufficiency (Fig 3B). As previously observed in A. nidulans and S. pombe (Hortschansky et al., 2007; Mercier & Labbe, 2009), A. fumigatus HapXVENUS localized to the nucleus during iron starvation. These data indicate that lower protein levels of HapX are required for its functions during iron excess compared to iron starvation. Consistently, S-tagged HapX (strain hapXs) was detectable only during iron starvation (Fig 4E) but not during iron excess (data not shown) in Western blot analyses. These data also demonstrate that expression pattern-based prediction of gene functions can be misleading. In order to increase the sensitivity of HapX protein detection, we enriched HapXVENUS by GFP-trap, a commercially available GFP pull-down (Rothbauer et al., 2008), before Western blot analysis with a GFP-directed antiserum was applied. This way, the HapXVENUS protein was detected in mycelia grown under iron starvation, iron-replete as well as high-iron conditions with the lowest amount present under high-iron conditions (Fig 3C). Under iron starvation, significant HapX proteolyses was found. Most likely, the Venus-HapX degradation was caused during the non-denaturating GFP enrichment procedure. The highly increased degradation during iron starvation conditions can be explained by the strong induction of protease activity during iron starvation conditions (data not shown and Supplementary Table S2). However, it cannot be ruled out that this proteolysis reflects a higher HapX turnover during iron starvation, which might be related to the increased transcript level under this condition. The reduced HapX protein content during iron excess compared to iron starvation might be explained by the reduced number of target genes expressed under this condition.

Two cysteine-rich regions (CRR), CRR-A and CRR-B, are crucial for HapX-mediated iron resistance

Aspergillus fumigatus HapX, 491 amino acid residues in length, contains the following domains: a “b(ZIP)” basic and a “coiled-coil” domain, which together mediate DNA-binding in bZIP-type transcription factors, and an N-terminal CBC-binding domain that is essential for HapX function due to its requirement for interaction with the CBC subunit HapE (Hortschansky et al., 2007). Moreover, HapX harbors the enormous number of 19 cysteine residues (Cys), whereby 16 are organized in 4 clusters, termed CRR-A, CRR-B, CRR-C, and CRR-D containing four Cys each (Fig 4). Two single Cys (Cys115 and Cys126) are localized in the coiled-coil region, and another one (Cys422) is localized in the C-terminus. The importance of these Cys is supported by their evolutionary conservation, for example all Cys are conserved in seven Aspergillus species (Supplementary Fig S1); CRR-A, CRR-B, CRR-C as well as two C-terminal Cys are conserved even in distantly related fungal species such as C. albicans (Fig 4A and Supplementary Fig S2).

Due to the potential role of Cys in iron sensing (Lill et al., 2012), we studied the impact of 11 of these Cys on HapX functions by site-directed mutagenesis replacing Cys by alanine residues (Fig 4 and Supplementary Fig S3). This analysis included all three single as well as two Cys from each CRR. For simplicity, only one mutant per CRR is shown in Fig 4, the respective, phenotypically identical second mutant is shown in Supplementary Fig S3. All analyzed hapX versions, including the non-mutated (strain hapX0), were expressed under the control of the endogenous promoter, contained a C-terminal S-tag (Terpe, 2003) and were integrated at the hapX locus in the ΔhapX strain.

Mutations in CRR-B (strains hapXB1C277A and hapXB3C286A) dramatically decreased adaptation to iron excess, similar to...
HapX-deficiency (ΔhapX), reflected by decreased radial growth under high-iron conditions, decreased biomass production in high-iron media as well as impaired transcriptional induction of cccA and leuA during a shift from iron starvation to iron sufficiency (Fig 4 and Supplementary Fig S3). Compared to CRR-B mutations, CRR-A mutations (strains hapXA2C203A and hapXA3C208A) similarly impaired the transcriptional response of cccA and leuA to iron, but the growth of the mutant strain was significantly increased on solid as well as in liquid high-iron media (Fig 4 and Supplementary Fig S3). Mutations in CRR-C (strains hapXC2C350A and hapXC3C353A) led to a slightly decreased growth on solid and in liquid high-iron media, but did not affect the transcription pattern of cccA and leuA to iron, but the growth of the mutant strain was significantly increased on solid as well as in liquid high-iron media (Fig 4 and Supplementary Fig S3). Mutations in CRR-C (strains hapXC2C350A and hapXC3C353A) led to a slightly decreased growth on solid and in liquid high-iron media, but did not affect the transcription pattern of cccA and leuA to iron, but the growth of the mutant strain was significantly increased on solid as well as in liquid high-iron media (Fig 4 and Supplementary Fig S3). Notably, mutation of two different Cys in the same CRR impaired iron resistance to the same degree in CRR-A, CRR-B, and CRR-C (Fig 4 and Supplementary Fig S3) suggesting that the Cys in the same CRR act as a domain rather than individually. Remarkably, neither of the mutations in CRR-A, CRR-B and CRR-C did affect the growth during iron sufficiency or limitation (Fig 4). Consistently, siderophore production and transcript levels of HapX-repressed genes (cccA, leuA, cycA) as well as HapX-activated mirB were wild-type-like (strain hapX0) in all these mutants under iron limitation (Fig 4). Taken together, these data demonstrate that mainly CRR-B, but to a lower degree also CRR-A and even less CRR-C are required for the HapX functions in iron detoxification, while these CRR are dispensable for the HapX functions in adaptation to iron starvation.

Mutation of Cys126 (strain hapXC126A) slightly decreased liquid biomass production and TAFC production during iron starvation but did not impact HapX functions in any other assays performed (Fig 4). Mutation of Cys422 (strain hapXC422A) slightly decreased liquid biomass production under both iron starvation and excess but did not cause any other alterations (Fig 4). Mutations in CRR-D (strains hapXD2C380A and hapXD3C389A) were phenotypically inconspicuous in all analyses performed (Fig 4).

In contrast, mutation of the Cys115 (strain hapXC115A) phenocopied HapX-deficiency during both iron limitation and iron excess (Fig 4). The most likely explanation is that this mutation results in the loss of the HapX protein as seen in Fig 4E, possibly due to improper folding of this HapX derivative. Noteworthy, this mutation results in a decrease of hapX transcript level during iron starvation suggesting positive transcriptional autoregulation of HapX.
The HapX C-terminus is essential for the adaption to iron starvation

To further characterize HapX domains, we generated A. fumigatus strains expressing different C-terminal hapX truncations (strains hapX\(^{246\rightarrow158}\) – hapX\(^{158}\)), here untagged, under the control of the endogenous promoter and integrated at the hapX locus in ΔhapX (Fig 5D).

Truncation of the C-terminal 27 amino acid residues (strain hapX\(^{246\rightarrow158}\)) decreased sporulation on BPS agar plates (Fig 5A). In iron-limited liquid media, this truncation decreased biomass production, TAFC production, mirB transcript levels but increased protoporphyrin IX (PpIX) accumulation and transcript levels of cccA and leuA (Fig 5). Taken together, this truncation was similar to a ΔhapX phenotype during iron starvation, however, less severe (with regard to biomass and TAFC production as well as the cycA transcript level), but did not affect growth and transcription of iron-related genes under iron excess, i.e. cccA and leuA (Fig 5).

Truncation of the C-terminal 93, 117, and 195 amino acid residues (strains hapX\(^{290\rightarrow53}\), hapX\(^{254\rightarrow53}\) and hapX\(^{290}\), respectively) perfectly phenocopied ΔhapX under iron limitation but did not affect iron detoxification (Fig 5). The latter is consistent with the presence of CRR-A and CRR-B that are crucial for iron resistance in the respective HapX versions (see above). The functionality of the C-terminus is supported by the high evolutionary conservation not only in Aspergillus species (Supplementary Fig S1) but also for example in C. albicans with 41% identical amino acids in the C-terminal 66 amino acid residues (data not shown).

Truncation of the C-terminal 333 amino acid residues (strain hapX\(^{385}\)) impaired iron detoxification to the same extent as HapX-deficiency, which is in agreement with the lack of CRR-A and CRR-B (Fig 5). This truncation also reduced adaptation to iron starvation, but not to the same extent as seen in hapX\(^{246\rightarrow158}\) – hapX\(^{290}\) or ΔhapX, i.e. during iron limitation liquid biomass and TAFC production as well as the mirB transcript level were higher, while the PpIX accumulation was lower. These data indicate that this HapX version, comprised of only the CBC-binding domain and the bZIP region, still executes residual functions in activation of siderophore biosynthesis and repression of iron consumption. These functions appear to be masked in the HapX versions encoded by hapX\(^{246\rightarrow158}\) – hapX\(^{290}\).

Notably, hapX\(^{398\rightarrow158}\) displayed not only decreased transcriptional activation of mirB but also decreased hapX transcript levels as seen in the HapX loss of function hapX\(^{C158\Delta}\) mutant (see above). These data indicate positive transcriptional autoregulation of HapX.

Taken together, both the cysteine-to-alanine mutations and the C-terminal truncations indicate that CRR-A and CRR-B are required for HapX-mediated iron detoxification, while the C-terminal 93 amino acid residues are crucial for both activation as well as repression functions that are required for adaptation to iron starvation.

An evolutionarily conserved cccA promoter element is recognized by a protein complex consisting of the CBC and a HapX homodimer involving direct DNA binding by both the CBC and HapX

To identify putative, evolutionarily conserved, regulatory motifs in the cccA promoter, the 1-kb 5'-upstream regions of cccA homologs from 28 fungal species including A. fumigatus, A. nidulans and F. oxysporum were subject to MEME analysis (Bailey & Elkan, 1994). The identified sites and their positions in the promoters of the different species are shown in Supplementary Fig S4. The highest scoring sequence (e-value of 3.4 × 10\(^{-115}\)), present in all 28 species, was a bipartite motif separated by a spacer region with low conservation (Fig 6A). Consistent with the HapX-independent regulation, the highest scoring motif was not found in the promoter of the S. cerevisiae cccA homolog (data not shown). The 5'-conserved submotif matches the CBC consensus DNA-binding motif (CCAAT box), CCAAT(C/T)(A/G) (Huber et al., 2012). This is in perfect agreement with the previous finding that HapX acts via physical interaction with the CBC (Hortschansky et al., 2007). Interestingly, binding of the CCAAT box by CBC would cover the entire spacer region as identified in the CBC/DNA binary complex crystal structure (Huber et al., 2012), which indicates that the 3'-submotif is the first accessible region for binding of another DNA-binding protein. The 3'-conserved non-palindromic submotif does not match any known transcription factor consensus binding sequence. This is intriguing, because bZIP proteins usually bind short palindromic or pseudo-palindromic target sequences. Furthermore, based on the amino acid signature sequence of its basic region NXXAQXXFR (Supplementary Fig S1), HapX belongs to the Pap1/Yap1subfamily of bZIP transcription factors that are known to recognize TTAAGCTAA and TTACCTAA consensus motifs (Fujii et al., 2000).

Nevertheless, we postulated that the 3'-submotif might be recognized by HapX. To address this hypothesis, we overexpressed and purified the A. fumigatus CBC (comprising the conserved domains of subunits HapB, HapC, and HapE) as well as a peptide corresponding to residues 24–158 of HapX, which includes the CBC-binding domain, basic region, and coiled-coil domain (Supplementary Fig S2).
Fabio Gsaller et al HapX controls adaptation to low iron and iron excess

The EMBO Journal

Published online: August 4, 2014

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- leuA
- cycA
- cccA
- mirB
- hapB
- rRNA

sFe

- leuA
- cycA
- cccA
- mirB
- hapB
- rRNA

E

S-tag

Porin

66 kDa
Figure 5. The HapX C-terminus is crucial for adaption to iron starvation but not iron detoxification.

A Strains were grown for 48 h at 37°C on agar plates with the given iron concentration.
B Production of biomass during iron starvation (−Fe), iron sufficiency (0.03 mM, +Fe), and iron excess (3 mM, hFe), as well as production of siderophores and PpIX under iron starvation was monitored after liquid growth for 24 h at 37°C. The data represent the mean ± SD of biological triplicates; the values were normalized to the wild-type. Statistically significant differences compared to the wild-type are shown in red (two-tailed, unpaired t-test; P < 0.05). Original data ± SD are found in Supplementary Table S1.
C Northern blot analyses were performed after liquid growth for 24 h at 37°C under iron limitation (−Fe) or after a subsequent 1-h shift to iron sufficiency (+Fe). rRNA is shown as a control for RNA quantity and quality.
D Schematic view of the HapX truncations analyzed.

Data information: Strains are derivatives of A. fumigatus AfS77.

Figure 6. HapX binds in vitro and in vivo to an evolutionary conserved motif identified in promoters of cccA homologs.

A An evolutionary conserved, bipartite motif in promoters of cccA homologs identified by MEME analysis. The underlined nucleotides would be covered upon CBC-binding as can be predicted based on the identified CBC/DNA binary complex crystal structure (Huber et al., 2012).
B Real-time SPR characterization of in vitro formation of the CBC/DNA-HapX ternary complex on the conserved cccA promoter motif from A. fumigatus. SPR analyses included binding of the CBC to DNA (panel 1), HapX to DNA (panel 2) and HapX to preformed CBC/DNA complexes (panel 3). The SPR sensograms are shown from sensor-immobilized 37 base pair duplexes covering the full as well as 3′-truncated duplexes. Nucleotides marked in blue represent the HapX consensus binding site in fungal cccA promoters identified by MEME analysis. Binding responses of the indicated CBC or HapX concentrations injected in duplicate (black lines) are shown overlaid with the best fit derived from a 1:1 interaction model including a mass transport term (red lines). Binding responses of CBC/DNA-HapX ternary complex formation (panel 3, blue lines) were obtained by concentration-dependent co-injection of HapX on preformed binary CBC/DNA complexes after 200 s within the steady-state phase. Sensograms in panel 4 depict the association/dissociation responses of HapX on preformed CBC/DNA and were generated by CBC response (co-injection of buffer instead of HapX) subtraction from HapX co-injection responses. Dissociation constants (K_D) are plotted inside the graphs.
C ChIP analysis demonstrating in vivo binding of HapX to the conserved cccA promoter motif from A. fumigatus. ChIP qPCR was performed on wild-type or the strain containing Venus-tagged HapX (hapXVENUS) grown for 18 h, then shifted to fresh media with no iron (−Fe), 0.03 mM iron (+Fe), or 3 mM iron (hFe) for 8 h. DNA was immunoprecipitated with either a control IgG Antibody, or anti-GFP polyclonal antibody that recognizes the Venus protein. Binding of HapXVENUS to the DNA region was assessed by qPCR. HapX binding is represented as percent enrichment of input control samples ± SD from triplicates. The actA (actin) promoter served as a negative control.
Light scattering analysis of purified HapX (24–158) revealed a molar mass of 31.38 kDa, demonstrating that this domain is dimeric in solution (theoretical mass of 31.36 kDa), as expected for a bZIP protein (Supplementary Fig S5B). To examine the putative in vitro interaction of the CBC, HapX and the identified common promoter element of cccA homologs, we applied surface plasmon resonance (SPR) analyses (Fig 6B). These measurements indicated high-affinity ($K_D = 0.7$ nM) recognition of the CCAAT box by the $A.\ fumigatus$ CBC independent of the presence of the 3'-submotif, i.e. its binding affinity was not affected by truncation of the 3'-submotif (compare first column in Fig 6B). This affinity is similar to that found for the interaction of the $A.\ nidulans$ CBC with a CCAAT motif from the $sreA$ promoter (Thon et al., 2010). HapX binds the 3'-submotif with low affinity ($K_D = 170.6$ nM) as its
binding was abolished by truncation of the 3′-submotif (compare second column in Fig 6B). However, on CBC-coated DNA, the binding affinity of HapX was 87-fold increased ($K_D \approx 1.97 \text{nM}$), whereby the binding again strictly depended on the presence of the 3′-submotif (compare fourth column in Fig 6B). Furthermore, by taking advantage of the fact that SPR responses correspond to bound masses, we were able to unravel the stoichiometry of the CBC/DNA-HapX ternary complex. Saturating CBC responses on the 37-bp DNA duplex reached a value of $\approx 100 \text{ RU}$ (upper left sensorgram in Fig 6B) and nearly the same responses were observed by co-injection of HapX on preformed CBC/DNA complexes (upper right sensorgram in Fig 6B) due to the similar molecular masses of the CBC (33.44 kDa) and the HapX dimer (31.36 kDa). Therefore, we conclude that one binary CBC/DNA complex is bound by one HapX dimer. Taken together, our data demonstrate that HapX interacts in vitro not only with the CBC but also with DNA with 2:1 stoichiometry by recognizing the 3′-submotif located adjacent to the CCAAT box in the evolutionary conserved cccA promoter element.

Chromatin immunoprecipitation (ChIP) analysis confirmed that HapX also binds to this promoter element in vivo, notably independent of the ambient iron concentration (Fig 6C). The constitutive presence of HapX at the cccA promoter suggests that transcription of cccA is primarily mediated by post-translational modification of HapX, i.e. iron sensing, rather than by transcriptional regulation of hapX. In agreement, the potential HapX iron-sensing CRR-B motif, CGFCX$_2$XCX, is essential for the transcriptional activation of cccA in response to high-iron stress (see above).

In summary, we show that HapX not only physically interacts with the CBC but also directly recognizes a distinct DNA motif. As the CBC has numerous HapX/iron-independent functions (Kato, 2005; Fleming et al, 2013), these data reveal for the first time the mechanism for discrimination of general CBC and specific HapX/CBC target genes.

Both functions, adaptation to iron limitation and excess, are evolutionary conserved in HapX orthologs

Similar to A. fumigatus, HapX orthologs repress iron-dependent pathways and the cccA orthologs during iron starvation in A. nidulans, F. oxysporum, S. pombe, C. neoformans, and C. albicans (Mercier et al, 2006; Hortschansky et al, 2007; Jung et al, 2010; Schrettel et al, 2010; Hsu et al, 2011; Lopez-Berges et al, 2012). Here, we found that HapX-deficiency also impairs growth of A. nidulans and F. oxysporum on high-iron media (Fig 7) demonstrating that the function of HapX in iron detoxification is evolutionarily conserved. Inspection of genome-wide transcriptional profiling data indicated that the cccA ortholog FOXG$_04047$ in F. oxysporum is repressed similar to A. fumigatus during iron starvation and upregulated during iron sufficiency in a HapX-dependent manner (Lopez-Berges et al, 2012). These data suggest that in F. oxysporum the decreased iron resistance caused by HapX-deficiency also results from impaired vacuolar iron storage.

In agreement with the evolutionary conserved function in iron resistance, CRR-A and CRR-B, which were identified in this study to be crucial for transcriptional activation of cccA and consequently iron detoxification in A. fumigatus, are conserved in most HapX orthologs (Fig 4A and Supplementary Fig S2).

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Php4, which transcriptionally represses iron-dependent pathways in S. pombe (Vachon et al., 2012), is an atypical HapX ortholog comprising a CBC-binding domain and a coiled-coil domain, but lacking the basic DNA-binding region of the bZIP domain. Only three Cys are present with two being clustered in the sequence CNSVEGClLys (Fig 4A). In a two-hybrid approach, the first of these Cys, Cys172, was found to be essential for iron-dependent association of Php4 with the monothiol glutaredoxin Grx4, indicating a role in iron sensing via iron–sulfur cluster availability. However, the function of this Cys has not been analyzed directly in Php4.

Recently, sulfur starvation caused by deficiency in the sulfur regulator MetR was found to increase the cellular iron content, iron sensitivity and transcript levels of genes involved in iron uptake but to decreased the cccA transcript level (Amich et al., 2013). These data demonstrate that sulfur homeostasis is required for proper iron regulation, most likely via iron-sulfur cluster and/or glutathione biosynthesis as shown for S. cerevisiae and S. pombe (Li & Outten, 2012). Together with the results presented here, these data furthermore indicate that sulfur homeostasis is required not only for SreA-mediated regulation of iron uptake but also HapX-mediated regulation of vacuolar iron detoxification.

Taken together, CRR-A and -B, but not the C-terminus, are required for sensing of high-iron conditions by Php4 to mediate activation of vacuolar iron detoxification via a conformational change of and/or interaction with different protein partners compared to iron starvation conditions. In contrast, the C-terminal 93 amino acid residues, but not CRR-A and CRR-B, are crucial for both the activating (e.g. mirB) as well as repressing (e.g. cccA) functions of HapX during iron starvation. The intriguing question why some promoter interactions result in repression and some in activation has still to be resolved; again this might be dependent on interaction with different partners present in different promoter sets.

Strikingly, certain domains of HapX and Yap5 are also found in the Ustilago maydis peroxide sensor, named Yap1 (Molina & Kahmann, 2007). This protein was identified as an ortholog of Yap1 from S. cerevisiae and regulates the oxidative stress response of the plant pathogen. A closer inspection of the Yap1 amino acid sequence revealed the presence of common HapX domains, i.e. the N-terminal CBC-binding and bZIP domains, a central HapX/Yap5 CRR-B type motif and the C-terminal CRR-C domain. Two additional Cys (CPC-motif) are located close to the C-terminus, whereas HapX-like domains CRR-A and CRR-D are lacking. Within the CRR-B domain, Cys399, and Cys407 were found to be crucial for nuclear localization and function of U. maydis Yap1, probably due to oxidative masking of a putative nuclear export sequence located between CRR-B and CRR-C (Fig 4A and Supplementary Fig S2) (Delaunay et al., 2000). Taken together, it seems that different fungi use a toolbox-like set of domains for sensing distinct environmental stimuli. Furthermore, these data indicate a possible role of Yap1 as a Yap1/HapX chimera in regulation of U. maydis‘ iron homeostasis.

Conclusion

Collectively, our study uncovered a novel regulatory mechanism in A. fumigatus and other filamentous fungi mediating both iron resistance and adaptation to iron starvation by the same transcription factor complex, comprising the CBC and a HapX homodimer, with activating and repressing functions depending on ambient iron availability. Comparison of HapX target promoter regions coupled with protein-DNA interaction analysis identified an evolutionary conserved CBC/HapX binding motif and revealed the discriminatory mechanism for CBC and CBC/HapX targets. Moreover, mutational analysis identified HapX protein domains that are essential for adaptation to either limitation or excess of iron, which will be instrumental for the further characterization of iron sensing.

Most fungal species encode HapX orthologs indicating wide evolutionary conservation of this iron regulatory mechanism. In contrast, the role model S. cerevisiae lacks a HapX ortholog and employs iron regulatory transcription factors that are not found in most other fungal species (Haas et al., 2008; Kaplan & Kaplan, 2009). In addition to the high-iron-sensing Yap5, iron regulation in S. cerevisiae involves the low-iron-sensing paralogous transcription factors Aft1 and Aft2. Aft1 and Aft2 transcriptionally activate iron acquisition as well as Chl1 and Chl2. These two paralogous mRNA-binding proteins mediate post-transcriptional repression of iron consumption by promoting respective mRNA degradation, including the homolog of A. fumigatus cccA (Martinez-Pastor et al., 2013). These data demonstrate complete regulatory rewiring of vacuolar iron storage in this yeast compared to A. fumigatus, with different regulators being required for activation and repression.Nevertheless, the mechanistically different iron regulatory systems of A. fumigatus and S. cerevisiae seem to employ common protein motifs for mediation of iron regulation. As an example of the modular toolbox using a common protein motif for transmitting different signals, the N-terminal CBC-binding domain of HapX is also present in S. cerevisiae Hap4. Hap4 was recently suggested to participate in iron regulation (Ihrig et al., 2010). In contrast to HapX, however, Hap4 lacks DNA binding and CRRs and therefore its mode of action is significantly different from the HapX mechanism (McNabb & Pinto, 2005; Hortschansky et al., 2007). In mammals, iron acquisition (transferrin receptor) and iron detoxification (ferroportin-mediated iron export and ferritin-mediated iron storage) are coupled at the post-transcriptional level by responding inversely to binding of iron regulatory proteins (IRPs) to iron-responsive elements in the untranslated regions of respective mRNA’s (Wang & Pantopoulos, 2011).

Taken together, the comparison of different organisms underlines the essentiality of iron handling with similar readouts mediated by different regulatory mechanisms.

Materials and Methods

Strains, oligonucleotides, and growth conditions

All strains and oligonucleotides used in this study are listed in Supplementary Tables S3 and S4, respectively. Generally, A. fumigatus strains were cultivated at 37°C in Aspergillus minimal medium (AMM) according to Pontecorvo et al. (1953) containing 1% (w/v) glucose and 20 mM glutamine as carbon and nitrogen sources, respectively. For iron-depleted conditions, iron was omitted and iron amounts used in this study are given in the figures. To increase iron starvation in solid media, the ferrous iron chelator BPS was
used at a final concentration of 0.2 mM in iron-depleted media. Production of conidia was performed on AMM agar plates containing 0.03 mM FeSO₄. Depending on the transformed resistance gene, *A. fumigatus* strains were selected on media containing 0.1 µg/ml pyrithiamine or 0.2 mg/ml hygromycin B.

**Generation of a hapX mutant strain with conditional cccA expression**

For heterologous expression of cccA, the plasmid pxylPcdcCC (Gsaller et al., 2012) was transformed in *A. fumigatus* ΔhapX (ATCC 46645) (Supplementary Fig S6). The plasmid harbors cccA under control of xylP, a xylan/xyllose-inducible promoter derived from the xylanase *xylP* of *Penicillium chrysogenum* (Zadra et al., 2000). pAN7.1 containing a hygromycin resistance cassette (*hph*) was co-transformed for selection of transformants.

**Generation of a hapX deletion mutant in ΔahuA background and a ΔhapXΔcccA double deletion strain**

The *hapX* coding sequence was deleted in AfS77 using the bipartite marker technique (Supplementary Fig S7A) (Nielsen et al., 2006). AfS77 was transformed with two DNA fragments each containing overlapping but incomplete fragments of the pyrithiamine resistance-conferring gene ptrA as described previously (Schrett et al., 2010) yielding strain ΔhapX (Supplementary Fig S7A). Deletion constructs were amplified with primers oAfptrA5/oAoPtrA2 (PCR1, 2.6 kb) and oAfhapX-6/oAoPtrA1 (PCR2, 2.2 kb) using genomic DNA of ΔhapX (ATCC 46645 derivative) as template.

Disruption of cccA in ΔhapX background (AfS77) was also carried out using the bipartite marker technique (Supplementary Fig S7B). For this purpose, the cccA 5′-flanking region was amplified from genomic DNA using primers oAfccAc1/oAfccAcAr4. For amplification of the 3′-flanking region, primers oAfccAc3A/oAfccAcAr2 were employed. Generated DNA fragments were digested with *Stu*I (5′-flanking region) and *Hind*III (3′-flanking region). The hygromycin resistance cassette was released from plasmid pAN7.1 by digestion with *Stu*I and *Hind*III and ligated with the 5′- and 3′-flanking region, respectively. The transformation construct A (3.3 kb, fusion of the cccA 5′-flanking region and the *hph* split marker) was amplified from the ligation product using primers oAfcccA5 and ohph14. For amplification of the transformation construct B (2.8 kb, fusion of the cccA 3′-flanking region and the supplementary *hph* split marker) primers oAfcccAr6 and ohph15 were employed. For transformation of *A. fumigatus* strains, both constructs A and B were used simultaneously. ΔhapX (AfS77) was transformed with the fragments each containing overlapping but incomplete fragments of the hygromycin resistance gene *hph*, yielding strain ΔhapXΔcccA.

**ΔhapX complementation, site-directed mutagenesis, C-terminal truncation, S-tagging, and venus-tagging of HapX**

For these studies, the basic plasmid phapX-hph was generated employing the fusion PCR-technique (Nielsen et al., 2006). The resulting plasmid contains the *hapX* coding sequence C-terminally linked with an S-tag under control of its native promoter and terminator region as well as a hygromycin resistance cassette. The different plasmid versions were integrated at the *hapX* deletion locus in AfS77 ΔhapX (Supplementary Fig S8A).

As a first step, the sequence encoding the S-tag (72 bp), including a 5′-sequence coding for a GA2 linker and a 3′-stop codon (TAA), was PCR amplified from plasmid pAO81 (Yang et al., 2004) using primers oAfhapX-S1 and oAfhapX-S2 (Supplementary Table S4). These primers carry extensions, which are complementary to sequences 30 bp upstream (oAfhapX-S1) and downstream (oAfhapX-S2) of the *hapX* stop codon (TGA), thereby generating a 132-bp-long PCR product. Primers oAfhapX-1 and oAfhapX-S3 were employed to amplify 2.9 kb of genomic DNA sequence, comprising 5′-flanking region (including the *hapX* promoter) and the *hapX* gene lacking the endogenous stop codon (TGA). Primers oAfhapX-S2 and oAfhapX-S4 were used to amplify 1.0 kb of genomic sequence, comprising 3′-flanking region (*hapX* terminator region). After gel purification, equal molar amounts of the PCR products were applied as template for fusion PCR. By using the (nested) primers oAfhapX-7 and oAfhapX-8 a 3.7 kb long fragment was amplified, which contains the *hapX* promoter, the *hapX* coding sequence comprising the S-tag, and the *hapX* terminator region. Subsequently, 3′ A-overhangs were added and the construct was subcloned into pGEM-T-Easy (Promega) via T/A cloning, yielding phapX6 (6.7 kb). Finally, a hygromycin resistance cassette was subcloned in phapX6, yielding plasmid phapX6-hph. Therefore, a DNA fragment containing the resistance cassette (2.4 kb) was amplified with primers ohyg-1/ohyg-2 using pAN7.1 as template. Next, phapX6 was opened with *SpI*I and blunt-ended using the Klenow enzyme (NEB). Eventually, the amplified DNA fragment was phosphorylated with polynucleotide kinase (NEB) and ligated into the blunt-ended plasmid backbone.

In order to substitute specific amino acids by site-directed mutagenesis the QuikChange kit (Stratagene) and oligonucleotides listed in Supplementary Table S4 were used. For the introduction of each mutation (Fig 4B and Supplementary Fig S3), complementary primers, around 40 bp in length including the desired mutation in its center, were designed. Plasmid phapX6-hph was amplified with respective primers in a PCR with a total volume of 50 µl (18 cycles, \(T_{\text{Den}} = 95^\circ \text{C}, T_{\text{Mel}} = 56^\circ \text{C}, T_{\text{long}} = 72^\circ \text{C}\). Next, 1 µl *Dpn*I restriction enzyme was added to the solution followed by 3 h of incubation at 37°C. *E. coli* DH5α cells were transformed with 10 µl of the digested solution. After amplification of plasmid DNA, plasmids containing mutated codons were screened by digestion. Resulting plasmids were named phapXCI15A-hph, phapXCI20A-hph, phapXAS240A-hph, phapXAS243A-hph, phapXB1277A-hph, phapXB2386A-hph, phapXC2530A-hph, phapXC3133A-hph, phapXD2380A-hph, phapXD3249A-hph, and phapXCI422A-hph.

For C-terminal truncation of HapX, reverse primers were designed that anneal to the *hapX* coding sequence at specific sites (Supplementary Table S4). Additionally, reverse primers comprise a STOP codon and a *Bsr*BI recognition site. Each PCR was performed with the same forward primer – hapXtrunc-f. Amino acid lengths of truncated HapX proteins (full-length in Af293 = 491 aa) are listed in Supplementary Table S3 and Fig 5D. After amplification, PCR fragments were digested with XbaI/BsrBI and subcloned into XbaI/BsrBI opened phapX6-hph. Plasmids comprising the truncated *hapX* versions were designated phapX464-hph, phapX598-hph, phapX74 -hph, phapX296 -hph, and phapX158 -hph.

For N-terminal tagging of HapX with Venus fluorescent protein plasmid phapXVENUS35-hph was generated (Supplementary Fig S8B).
Therefore, hapX promoter DNA was amplified using primers 5’hapX-l/5’hapXvenus-r (PCR1, 1.3 kb; template: phapX5’-hph) (Supplementary Table S4). Primers 5’hapXvenus-l/venus-r were employed to amplify the codon optimized venus coding sequence (PCR2, 0.7 kb; template: pMA-Venus). In a third PCR, hapX coding sequence comprising the 3’ untranslated region was amplified using primers venushapX-l/hapX3’-r (PCR3, 1.6 kb; template: phapX5’-hph). Subsequently, the hapX promoter region and Venus coding sequence were combined via fusion PCR using primers 5’hapX-I2/venushapX-r (PCR4, 2.1 kb; template: PCR1 & PCR2). In the final PCR, hapX promoter DNA linked to venus coding sequence was fused to hapX coding sequence including the 3’ terminator region with primers hapXtrunc-f/hapX-r (PCR5, 3.5 kb; template PCR3 & PCR4). The resulting DNA fragment was digested with XbaI/BstBI and subcloned into XbaI/BstBI opened phapX5’-hph. The 5’hapX promoter region includes an XbaI recognition site and primer hapX-r contains the palindromic recognition sequence for BstBI.

For transformation, 5 μg of the respective plasmid was linearized through digestion with SnaBI, the recognition site of which is located 761 bp downstream of the stop codon.

Fluorescence microscopy, PpiX analysis, siderophore analysis, Northern analysis, and qRT-PCR analysis

For imaging of hyphae, cells were grown on coverslips in 0.3 ml AMM containing 1% (w/v) glucose, 20 mM glutamine and the desired iron concentration. Microscopy images were captured using an Axio Imager. M2 microscope (Carl Zeiss) equipped with a 63× oil immersion objective lens (numerical aperture, 1.40), a HPX 120 V compact light source (Carl Zeiss) and the AxioCam MRm camera (Carl Zeiss). Images were processed using ZEN 2012 imaging software (Carl Zeiss). DAPI was used to stain nuclei. PpiX content, siderophore production, RNA isolation, and Northern analysis were carried out as described previously (Hortschansky et al., 2007). The hybridization probes used in this study were generated by PCR using DIG-labeled nucleotides. For qRT-PCR analysis, RNA was digested with DNase I and column eluted using RNA Clean and loaded onto 4× SDS-PAGE sample buffer, and loaded onto 4–12% NuPAGE Bis-Tris gels. Proteins were transferred to a PVDF membrane using the iBlot system (Invitrogen). After antibody incubation, the membrane was developed using 1-Step Ultra TMB-Blotting substrate (Thermo). The following antibodies were used: anti-GFP antibody (ab290, Abcam); HRP-conjugated anti-rabbit IgG antibody (GGHL-15P, ICL, Inc.).

Western blot detection of S-tagged HapX

For Western blots, 100 μg dry weight of lyophilized mycelia were rehydrated in 1 ml of TNETG buffer (20 mM Tris, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM PMSF). Cells were lysed by 5 bursts of 1 min each in the presence of 1/3 volume of glass beads. Cell debris were removed by centrifugation. For Western blots, 50 μg protein were loaded per lane and separated on a 12.5% SDS-PAGE. Blots were immunodecorated with a polyclonal anti-S-tag antibody (ICL, Inc.) for detection of S-tagged HapX or a polyclonal antibody directed against S. cerevisiae Porin (Por1).

MEME analysis

Protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the most similar homologs of A. fumigatus CcaA (XP_751578.1). The 1-kb 5’-upstream sequences of the top 26 hits from unique species (Supplementary Fig S6) as well as A. fumigatus and F. oxysporum, which is known to require HapX for iron detoxification (see above), were subject to MEME analysis (Bailey & Elkan, 1994) to identify putative common motifs of size 6–35 bp, occurring once or zero times.

Bacterial expression and purification of the CBC and HapX for SPR analysis

The A. fumigatus core CBC was produced and purified as described for the CBC from A. nidulans (Huber et al., 2012). Briefly, synthetic genes coding for HapC(40–137) and HapE(167-174) were cloned in the pnCS vector (Diebold et al., 2011) for expression of a bicistronic transcript. A synthetic gene encoding HapB(230-299) was cloned into pET39b (Novagen) and E. coli BL21(DE3) cells were co-transformed with both plasmids. After overnight autoinduction and cell lysis, the heterotrimeric CBC was purified to homogeneity by subsequent cobalt chelate affinity and size-exclusion chromatography.

A cDNA fragment encoding A. fumigatus HapX(24–158) (covering the CBC-binding domain, basic region, and coiled-coil domain) with an extended N-terminus including a cleavage site for tobacco etch virus (TEV) protease was amplified and subcloned into the pMAL-c2X (New England Biolabs) vector. The resulting plasmid was transformed into E. coli Rosetta 2 (DE3) cells for overnight autoinduction. Crude bacterial lysates were purified by Dextrin Sepharose affinity chromatography (GE Healthcare). The maltose-binding
protein HapX(24–158) fusion was cleaved with TEV protease and further purified sequentially using CelluloseSulfate (Millipore) affinity chromatography, (NH₄)₂SO₄ precipitation (50% w/v), and Superdex 75 prep grade (GE Healthcare) size exclusion chromatography. The absolute molecular mass of HapX(24–158) was determined by static light scattering experiments on a miniDawn TREOS monitor in series with an Optilab T-REX differential refractometer (Wyatt). HapX(24–158) was chromatographed on a Superdex 200 10/300 GL column (GE Healthcare), and molar mass was calculated using ASTRA 6 software (Wyatt).

**Surface plasmon resonance measurements**

Real-time analyses were performed on a Biacore T200 system (GE Healthcare) at 25°C. DNA duplexes containing CCAAT box at position −369 in the S′-upstream region of the A. fumigatus cccA gene were produced by annealing complementary oligonucleotides using a fivefold molar excess of the non-biotinylated oligonucleotide. The dsDNA was injected on flow cells of a streptavidin (Sigma)-coated CM3 sensor chip at a flow rate of 10 μl/min until the calculated amount of DNA that gives a maximum CBC-binding capacity of 100 RU had been bound. CBC and HapX(24–158) samples containing 10 μg/ml poly(dl-dc) were injected in running buffer (10 mM phosphate pH 7.4, containing 2.7 mM KCl, 137 mM NaCl, 1 mM DTT and 0.005% (v/v) surfactant P20) at a flow rate of 30 μl/min. Co-injection of HapX(24–158) on preformed binary CBC/DNA complexes within the equilibrium phase was performed by using the dual injection command. Each injection was performed at least two times. The chip surface was regenerated with 10 mM Tris/HCl pH 7.5, containing 0.5 M NaCl, 1 mM EDTA and 0.005% (w/v) SDS for 1 min. Refractive index errors due to bulk solvent effects were corrected with responses from DNA-free flow cell 1 as well as subtracting blank injections. Kinetic raw data were processed and globally fitted with Scrubber 2.0c (BioLogic Software) using a 1:1 interaction model including a mass transport term.


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The EMBO Journal Vol 33 No 19 2014

2275


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