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Tet-on, or Tet-off, that is the question: Advanced Conditional Gene Expression in *Aspergillus*

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Abstract

In *Aspergillus,* controlled gene expression is often achieved using the reverse tetracycline-controlled transactivator (rtTA) dependent Tet-on system, whereby transcription is titratably activated by addition of the tetracycline derivative doxycycline. The complementary Tet-off system utilises the tetracycline-controlled transactivator (tTA) component to quantitatively reduce gene expression. In this study, we utilized a synthetic biological approach to engineer highly optimized Tet-off conditional expression systems in *Aspergillus niger* and *Aspergillus fumigatus*. Steps for delivery of these tools include utilizing codon optimized cassette components, testing several promoters for improved genetic stability and validating two modified luciferase reporters for highly accurate measurements of gene expression. The Tet-off cassettes developed in this study enable facile and quantitative functional analysis, as validated by Tet-off analysis of genes involved in chitin synthesis and cell wall polarity in *A. niger*, and *para*-aminobenzoic acid synthesis in *A. fumigatus*. We also used a $racA^{G18V}$ dominant allele to demonstrate that Tet-off in *A. niger* enables gene over-expression and downregulation in a single isolate. Additionally, we used the improved luciferase reporters to show that the Tet-off cassette in *A. niger* enables quantification of gene oscillations. In order to demonstrate that synthetic biological approaches developed here are broadly applicable to engineering transcriptional circuits in filamentous fungi, we used our strategy for improving cassette stability by promoter replacement in the *A. niger* Tet-on system, which resulted in a modified Tet-on cassette with higher stability in recipient genomes.

Introduction

The design and reengineering of fungal systems at the molecular level has generated numerous improved tools for basic and applied research. Such synthetic biological approaches include reengineering a prokaryotic serine recombinase for site-specific marker recycling (Hartmann et al., 2010), the CRISPR-Cas9 system for genome editing (Vyas et al., 2015) (Nødvig et al., 2015), generation of RNA interference vectors for gene knock-down (Skowyra and Doering, 2012) and an impressive range of inducible promoter systems from bacteria (Meyer et al., 2011) and other kingdoms (Hörner and Weber, 2012). Such conditional expression systems, where transcript abundance is controlled by experimental parameters, are a vital tool for characterisation of essential genes, which are unable to be analysed by deletion strategies. Moreover, titratable systems allow quantitative control of gene expression, which might be an important technique for deciphering complex phenotypes by enabling tightly controlled native, reduced or over-expression levels. One such tool is the tetracycline inducible system, commonly referred to as Tet-on, whereby transcription is titratably activated by addition of the highly stable tetracycline derivative doxycycline (Dox) to growth media (Fig. 1A). In this system, a reverse tetracycline-controlled transactivator (rtTA) requires a Dox ligand for DNA binding to rtTA responsive operator sequences (*tetO*). rtTA is constitutively expressed and a gene of interest placed under control of a minimal promoter next to several *tetO* elements, which enable activation of transcription in the presence of Dox. In a seminal study, Vogt et al. established conditional gene expression in *Aspergillus fumigatus* (Vogt et al., 2005). We have optimised this system and demonstrated that Tet-on is titratable in the model organism *Aspergillus niger*, with the level of gene induction proportional to the concentration of Dox added to the media (Meyer et al., 2011). The minimal promoter of the Tet-on system, which controls the gene of interest has low/undetectable basal rates of expression in the absence of inducers. Accordingly this is a popular and versatile tool for gene functional characterisation in a variety of model and pathogenic fungi. For example, a simple application of the Tet-on system for analysis of uncharacterised gene (*cfrX*) in *A. niger* was to quantitatively elevate transcript levels using Dox and assess strain phenotypes with/without induction (Meyer et al., 2011). For natural product genome mining, Macheleidt and colleagues used Tet-on over-expression of a putative transcription factor to activate an *A. fumigatus* secondary metabolite cluster, which resulted in biosynthesis of a novel compound in induced strains (Macheleidt et al., 2015). Alternatively, replacing the native promoter of a gene with the Tet-on cassette can be used for gene functional analysis. For example, the Rho GTPase *rho1* in the pathogenic mould *A. fumigatus*

was demonstrated to be an essential gene by replacement of the native *rho1* promoter with the Tet-on cassette, which resulted in a mutant isolate which could not grow in the absence of Dox (Dichtl et al., 2012). An alternative strategy is to place genes of interest under control of the Tet-on system and subsequently delete the wild-type allele, enabling characterisation of gene function by quantitative transcript downregulation by reducing Dox in growth media, an approach validated using the *A. niger* γ-actin encoding gene (Meyer et al., 2011). An innovative application in *A. nidulans* by Wartenberg and colleagues used Tet-on to express antisense RNA for a gene encoding a putative dehydrin (Wartenberg et al., 2012). This enabled mRNA knock-down and functional characterisation of this mutant, which demonstrated a role of the product of this gene in stress resistance of dormant conidia (Wartenberg et al., 2012). Thus, the Tet-on system is a versatile tool that can be used for multiple applications in a variety of fungi.

An alternative conditional expression system, named Tet-off, utilises the tetracycline-controlled transactivator (tTA), in which tTA binding to *tetO* is prevented by tetracycline, enabling quantitative reduction of gene expression (Fig. 1B). In the diploid yeast *Candida albicans,* a high throughput approach in which one allele was deleted and the other placed under control of the Tet-off system enabled analysis of 1152 genes, of which 567 were demonstrated as essential for growth following Tet-off mediated down regulation (Roemer et al., 2003). In the corn smut *Ustilago maydis*, Tet-off replacement of the native promoter for a mating regulatory transcription factor enabled Dox controlled abolishment of sex *in vitro* (Zarnack et al., 2006). In addition to demonstrating the utility of the Tet-off system, this latter study highlights the numerous engineering steps that are required to develop highly optimised conditional expression systems in fungi, which included obviating early polyadenylation of the *tetR* gene by codon optimisation and decreasing basal activity of the Tet promoter by removal of enhancer elements in *U. maydis* (Zarnack et al., 2006). For the Tet-on system in *A. fumigatus*, further development entailed removal of repetitive sequences that were resulting in intramolecular recombination and loss of the cassette from recipient genomes (Helmschrott et al., 2013). These studies demonstrate the importance of synthetic biological approaches which are necessary to deliver optimal functionality of conditional expression systems.

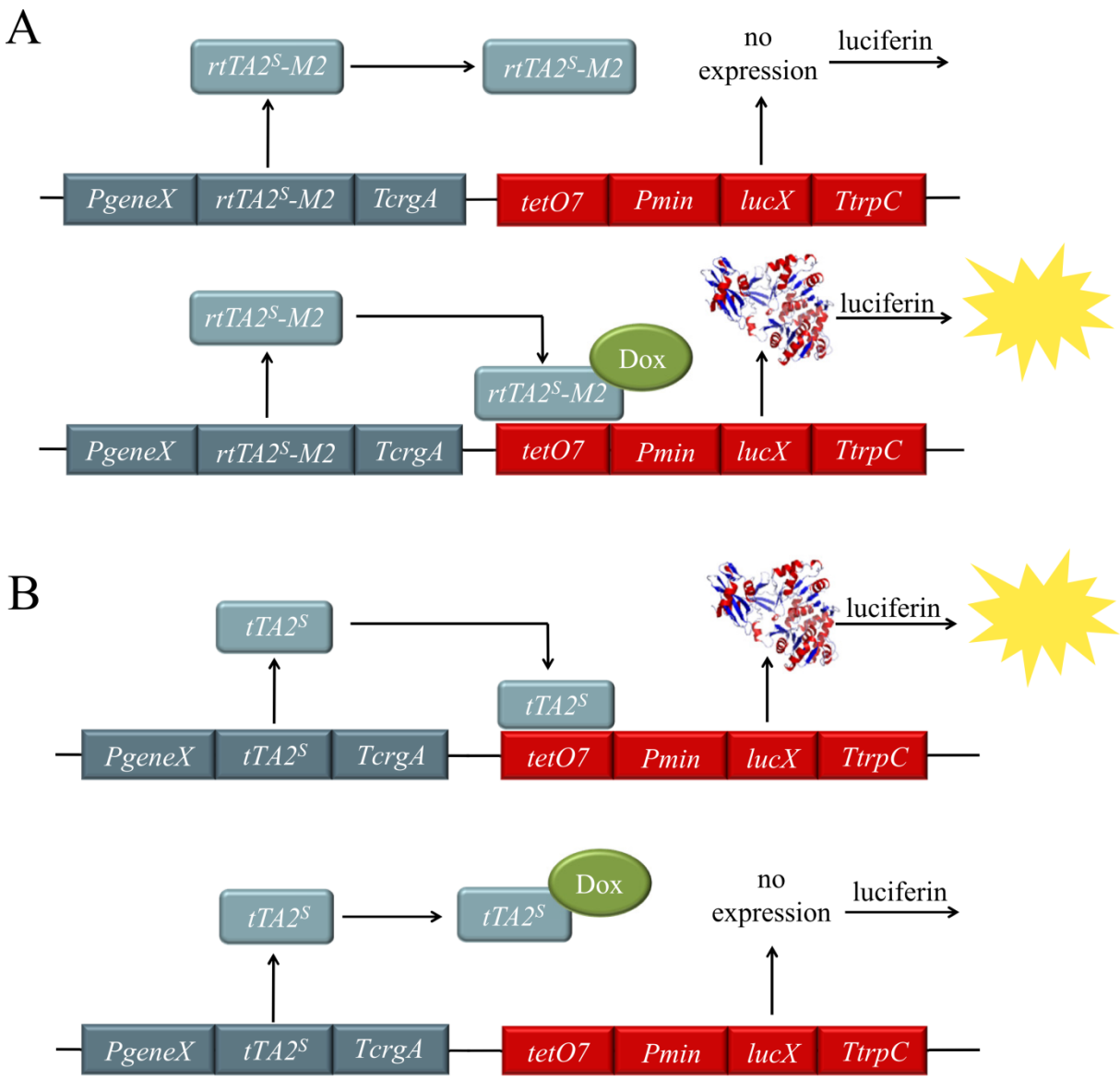

Figure 1: Design of the Tet-expression systems. (A) In the Tet-on system gene transcription is reversibly turned on by the addition of Dox. It forms a complex with the constitutive expressed transcription factor *rtTA2^S-M2*, thereby inducing association of rtTA2^S-M2 protein to its operator binding site *tetO*7. As reporter gene, behind the minimal promoter of *gpdA* (Pmin), different luciferase versions were used. (B) The Tet-off system works in opposite direction, through addition of Dox the gene transcription is reversibly turned off, because the antibiotic induced dissociation of tTA2^S from *tetO*7.

The objective of this study was to develop, optimise and validate a functional Tet-off system in the model genus *Aspergillus*, represented by the industrially important model organism *A. niger* and the pulmonary pathogen *A. fumigatus*. We describe a synthetic biological approach in which the Tet-on cassette was reengineered to a titratable, stable, tightly regulated Tet-off conditional expression system in both organisms. These inducible downregulation systems significantly expand the toolkit of *Aspergillus* spp. and provide an engineering framework for adapting any given promoter system in filamentous fungi.

Results

Engineering of the Tet-off system in *Aspergillus niger*

The fully functional *A. niger* Tet-on cassette was optimized previously and is encoded on plasmid pVG4.1 (Meyer et al., 2011). This vector also enables *A. niger* transformation using a *pyrG* auxotrophic marker (Meyer et al., 2011). Given that promoter and terminator sequences are common to both Tet-on and Tet-off conditional expression systems (Fig. 1), it was reasoned that simple exchange of the pVG4.1 reverse transactivator (*rtTA2^S-M2*) with a transactivator (*tTA*) would yield a derivative vector encoding a functional Tet-off cassette. Accordingly, a *tTA* sequence was PCR amplified from p473 (Vogt et al., 2005), which was used to replace the entire *rtTA2^S-M2* coding sequence in pVG4.1 to give plasmid pMA247. This vector also contains a luciferase reporter encoded by the *mluc* gene under control of the minimal promoter so that functionality of the cassette can be validated. This vector was transformed into *A. niger* strain AB4.1, to give strain MA241.1, expressing a single copy of the putative Tet-off cassette at the *pyrG* locus. However, in microtiter assays this isolate demonstrated very low *mluc* expression as measured by LCPS values (data not shown). Strain SB1.16, which contains multiple integrations of the putative Tet-off expression system at random loci, did not sufficiently improve *mluc* expression (data not shown).

Hence, a transactivator optimized for mammals (*tTAS^2*, see Material and methods) was used to replace the *rtTA2^S-M2* sequence in pVG4.1, which gave derivative plasmid pFW9.1. Transformation of *A. niger* with this plasmid generated strain FW13.11, which expressed a single copy of the Tet-off modified cassette at the *pyrG* locus. In microtiter assays, this isolate demonstrated strong luciferase activity following 8 hours growth, which was not observed in growth media supplemented with various concentrations of Dox, indicating successful Tet-off mediated downregulation of this gene (Fig. 2A). Interestingly, non-induced FW13.11 shows higher luciferase values than the Tet-on expressing strain VG8.27 following induction with 20 µg/ml Dox (Fig. 2A). Importantly, induction of the Tet-off cassette with higher concentrations of Dox resulted in faster downregulation of luciferase, indicating this system is titratable (Fig. 2A). Bioreactor cultivation of Tet-off expressing strain FW13.11, pictured in Fig 2B, shows that downregulation using the expression system works on a large scale and with low concentrations of Dox induction (5 µg/ml). These data also demonstrate high expression of the *mluc* gene results in slower growth rates of *A. niger*, an effect which is abolished by Tet-off mediated downregulation of this gene (Fig. 2B). During development and testing the Tet-off system, we observed that not all PCR and Southern blot confirmed *A. niger* transformants

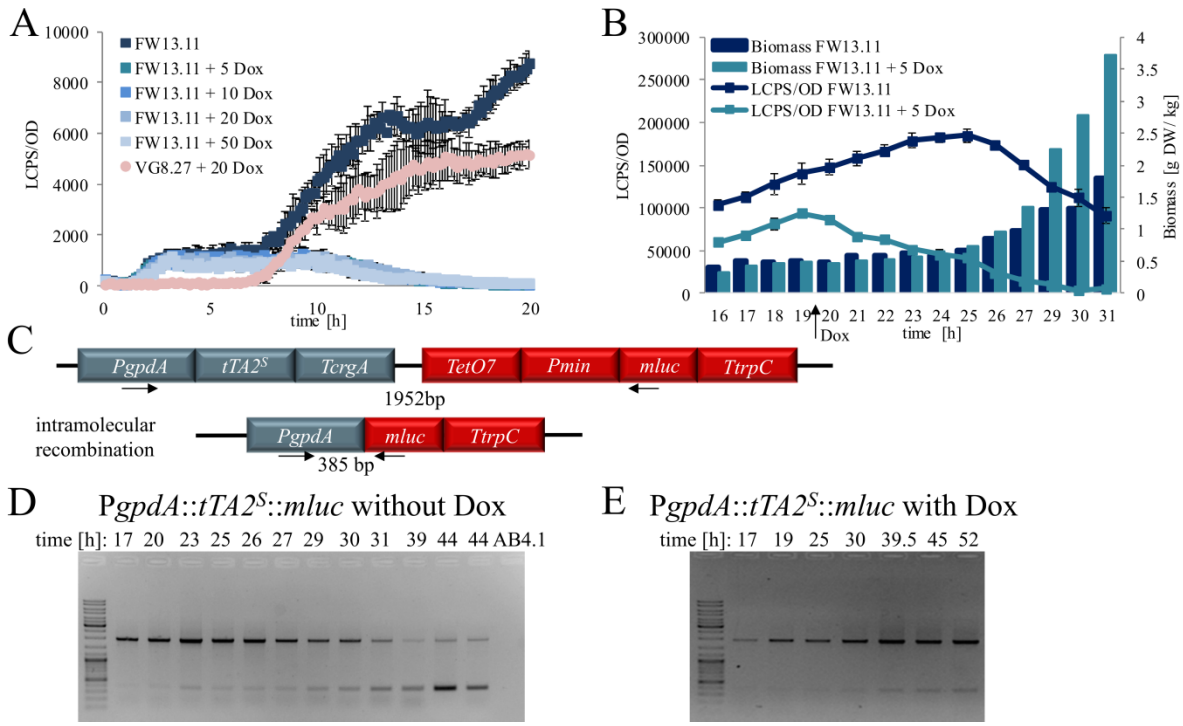

Figure 2: Microtiter plate assay and bioreactor cultivation and proof of genetic stability of Tet-off *PgpdA::tTA2^S::mluc* strain. (A) 5x10^4 spores/ml of the Tet-off strain FW 13.11 and the control Tet-on strain VG8.27 were inoculated in 300 µl complete medium with 0, 5, 10, 20 and 50 µg/ml Dox for 20 h (30 °C). Luciferase activity was measured in LCPS and normalised through division of measured optical density at 595 nm (OD) during cultivation in microtiter plate. (B) Two batch cultivations of FW13.11 were run, inoculated with 10^9/l spores, one was induced after ~19.5 h with 5 µg/ml Dox. The diagram shown on the left side the luciferase activity per OD printed with lines and on the right side the biomass accumulation was figured by blocks. The cultivation with gene down regulation, show a decrease luminescence after induction and higher biomass values in comparison to the non-induced luciferase producing strain. (C) Schematic overview of the analytic PCR, the forward primer annealed on the P*gpdA* and the reverse primer on the modified luciferase (Supplementary Table S1). The integration of the whole Tet-off expression system in the *A. niger* genome resulted in ~2 kbp PCR fragment. If an intramolecular recombination event occurred the assumed PCR fragment of ~ 400 bp will be visible. (D) The Tet-off strain (P*gpdA*) of the bioreactor cultivation without Dox induction shown an increase in intramolecular recombination event PCR fragment over cultivation time. The 44 h time point was analysed in technical duplicate, and additionally the recipient strain AB4.1 was used a control. (E) The cultivation of the Tet-off strain with 5 µg/ml Dox induction lead to constant but small amount of an intramolecular recombination event over cultivation time.

expressing the Tet-off *mluc* reporters demonstrated down regulation following induction. Moreover, isolates in which the conditional expression system was functional lost Tet-off mediate gene regulation following storage on MM plates. Given that other groups utilizing the Tet-on system in *A. fumigatus* demonstrated genome instability of this cassette due to intramolecular recombination events (Helmschrott et al., 2013), we investigated this possibility for the Tet-off cassette in *A. niger* using diagnostic PCR (primers listed in Supplementary Table S1). We extracted genomic DNA from bioreactor culture samples throughout a time series of growth, and PCR using primers spanning a 1.9 kb fragment of the Tet-off cassette demonstrated loss of a 1.5 kilobase pair sequence over time (Fig. 2C),

indicating the Tet-off cassette is not genetically stable due to a intramolecular recombination event which was observed to increase throughout cultivation (Fig. 2D). The cultivation with Dox induction in Fig. 2E shows that this event occurs in a small but consistent subset of the population. Given that there is 176 bp sequence homology between the minimal *gpdA* promoter and constitutive *gpdA* promoter, we reasoned that this was resulting in intramolecular recombination and loss of functionality in *A. niger*.

Accordingly, we decided to replace the constitutive *gpdA* promoter, which in vector pFW9.1 originates from *A. nidulans*. Firstly, we utilised the *A. niger gpdA* promoter (*gpdAn*), with the rationale that this gene would offer similar expression levels to the *A. nidulans* orthologue encoded in the Tet-off cassette. This was verified to lack homologous sequences to the minimal *gpdA* promoter as determined by DNA alignment analysis (data not shown). Initial experiments using a *gpdAn* promoter resulted in a functional Tet-off system, but ~15% of transformants demonstrated loss functionality as measured by luciferase activity in the presence of Dox (data not shown). By PCR amplification of the Tet-off cassette from these isolates and subsequent sequencing we demonstrate this is due to another recombination event between this promoter and the *crgA* terminator (primers describe in Supplementary Table S1).

In order to identify a further constitutive promoter which lacks any sequence homology to other regions of the cassette, we interrogated an in-house compendium of microarray data for genes which have comparable transcriptional profiles to *gpdAn*. This analysis yielded one gene, which we term *fraA*, encoding a putative ribosomal subunit (An16g04690) which demonstrated comparable transcriptional profiles with *gpdAn* in a variety of experiments (data not shown). Exchange of the constitutive *gpdA* promoter with the *fraA* promoter resulted in a stable strain as repeated purifications of transformants on MM plates did not result in loss of function of the expression system, and evaluation of southern blots demonstrated no detectable intramolecular recombination event (data not shown). In microtiter plate assays, the resulted new Tet-off strain FW25.35 demonstrated titratable downregulation of luciferase after induction with Dox, but the strain is about 50 % less active in the absence of induction than the established Tet-on system (VG8.27) induced with 20 µg/ml Dox (Fig. 3A). Batch cultivations in a bioreactor demonstrated that biomass accumulation was comparable under both inducing and non-inducing conditions (Fig. 3B), which was a notable improvement when compared to isolate FW13.11, which demonstrated reduced growth under inducing conditions (Fig. 2B). Using the P*fraA* Tet-off system, gene downregulation occurred in the presence of 5 µg/ml Dox, and we observed rapid decrease in reporter luminescence after induction (< 11 minutes, Fig. 3B). Figure 3D and 3E demonstrate that in both bioreactor cultivations with and without inducer there was no intramolecular recombination event as determined by diagnostic

PCR (Fig. 3C). Taken together, these data indicate the *fraA* Tet-off system will be a useful tool for functional gene characterization in *A. niger*.

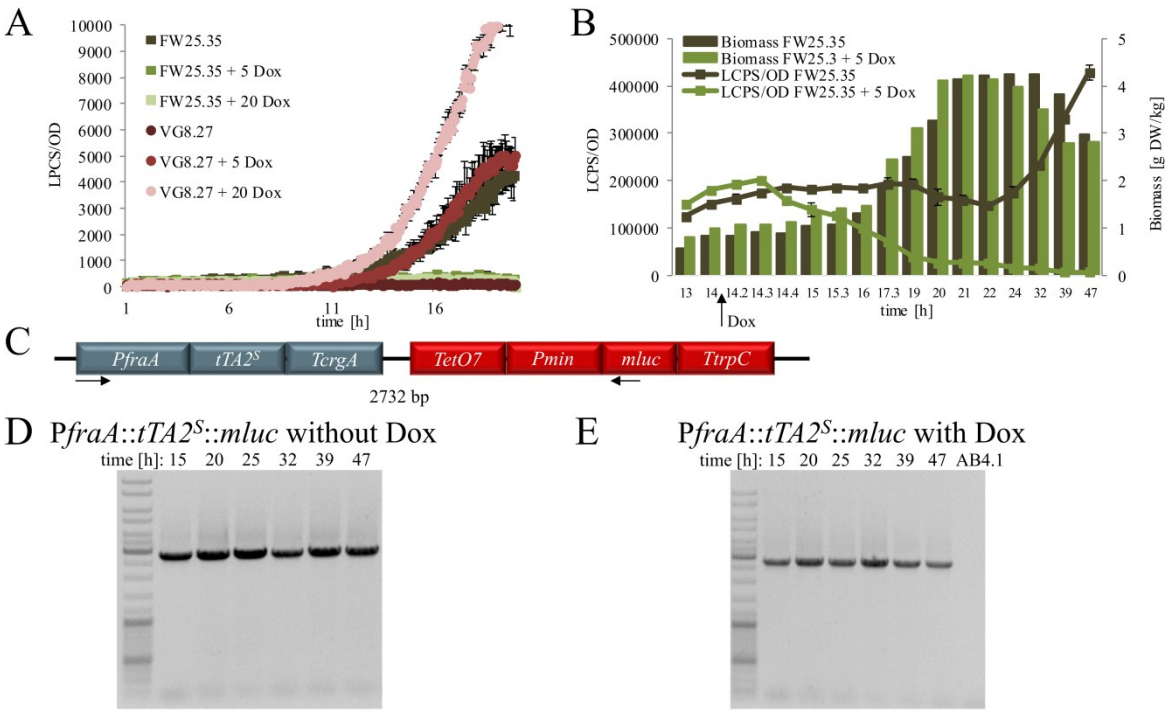

Figure 3: Microtiter plate assay, bioreactor cultivations and proof of genetic stability of Tet-off *PfraA::tTA2^S::mluc*. (A) The Tet-off strain FW 25.35 and as control the Tet-on strain VG8.27 were inoculated with 5x10^4 spores/ml in 300 µl complete medium with 0, 5 and 20 µg/ml Dox and measured for 20 h (30 °C). Luciferase value per optical density at 595 nm (OD) indicated a less expression strength of the Tet-off system compared with the induced Tet-on system. (B) The luciferase activity per OD of the strain FW25.35 in a bioreactor run induced after ~19 h with 5 µg/ml Dox and in another run without Dox is printed with lines and additionally the biomass accumulation is marked by blocks over cultivation time of 2 days. (C) The analytic PCR is pictured, where by the forward primer anneals on the P*fraA* and the reverse primer on the modified luciferase (Supplementary Table S1). The integration of the whole Tet-off expression system in the *A. niger* genome resulted in ~2,7 kbp PCR fragment, if an intramolecular recombination event of the transactivator take place the assumed PCR fragment would be a minimum of 800 bp shorter. (D) The Tet-off with P*fraA* indicates without and (E) with Dox addition no an intramolecular recombination event problem during 48 h batch cultivation.

Engineering improved genetic stability of the *A. niger* Tet-on cassette

After demonstration of an intramolecular recombination event during Tet-off construction and re-engineering a stable cassette (Fig. 2D and E), we reasoned that similar design flaws might be present in the original Tet-on system. Using a similar diagnostic PCR approach (Supplemental Table ST 1, Fig. 2C), we confirmed that during bioreactor cultivation of Tet-on strain VG8.27 a similar recombination event was occurring, in media with and without Dox (Supplemental Figure S1A&B). Accordingly, to obviate recombination between *gpdA* promotor and Pmin, we exchanged P*gpdA* with P*fraA* in pFW20.1 (Supplementary Table S1), and generated the strain FW36.1 (Table 1). This strain demonstrated no further recombination

of the Tet-on cassette (data not shown), yet luminescence MTP analysis demonstrated less strength and a different reporter expression profile when compared to isolate FW32.14 containing the P*gpdA* promoter (Supplemental Figure S1C). These variations in vector functionality (i.e. improved genome stability or maximal expression level) must be taken into consideration for optimal use in downstream applications.

Table 1: *Aspergillus* strains used in this study.

Strain	Relevant genotype	Source
N402	*Aspergillus niger* wild type	(Bos et al., 1988)
AfS35	*Aspergillus fumigatus* recipient [*akuA::loxP*]	(Krappmann et al., 2006)
AB4.1	*A. niger pyrG*$^{-}$ isolate	(Hartingsveldt et al., 1987)
VG8.27	AB4.1, *pyrG*$^{+}$ (transformed with pVG4.1 (Tet-on *PgpdA*::*rtTA2*^{S}*-M2*::*mluc*), single copy)	(Meyer et al., 2011)
MA241.1	AB4.1 transformed with pMA247 (Tet-off *Pgpd*::*tTA*::*mluc pyrG**), *pyrG*$^{+}$, single copy	this work
SB1.16	AB4.1 transformed with pSB1.1 (Tet-off *PgpdA*::*tTA*::*mluc*) and co-transformation with pAB4.1 (*pyrG*), *pyrG*$^{+}$,~ 7 copies	this work
FW13.11	AB4.1 transformed with pFW9.3 (Tet-off *PgpdA*::*tTA2*^{S}::*mluc pyrG**), *pyrG*$^{+}$, single copy	this work
FW25.35	AB4.1 transformed with pFW15.1 (Tet-off *PfraA*::*tTA2*^{S}::*mluc pyrG**), *pyrG*$^{+}$, single copy	this work
FW28.1	AB4.1 transformed with pFW19.7 (Tet-off *PfraA*::*tTA2*^{S}::*gfaA pyrG**), *pyrG*$^{+}$,single copy	this work
FW29.37	FW28.1 transformed with Δ*gfaA*::*hyg* single integration	this work
MA37.29	AB4.1, Δ*gfaA ::AopyrG*	(Ram et al., 2004)
FW26.5	transformed with pFW17.1 (Tet-off *PfraA*::*tTA2*^{S}::*racA pyrG**), *pyrG*$^{+}$, single copy	this work
FW30.37	FW26.5 transformed with Δ*racA*::*hyg*, single integration	this work
MA80.1	MA70.15 transformed with Δ*racA*::*AopyrG*	(Kwon et al., 2011)

FW27.7	AB4.1 transformed with pFW18.1 (Tet-off *PfraA*::*tTA2*^{S}::*racA*^{G18V} *pyrG**), *pyrG*$^{+}$, single copy	this work
FW31.14	FW27.7 transformed with Δ*racA*::*hyg*, single integration	this work
MA61.24	AB4.1 transformed with *PinuE*::*racAG18V*	(Kwon et al., 2011)
FW35.1	AB4.1 transformed with pAB4.1 (*pyrG*), *pyrG*$^{+}$, single copy	this work
AfS191	AfS35 transformed with pSK606 (*A. fum.* Tet-on::*pabaA*) HpaI fragment replacing the endogenous *pabaA* promoter region	this work
XM1.7	AB4.1 transformed with pXM1.1 (Tet-on *PgpdA*::*rtTA2*^{S}-*M2*::*luc pyrG**), *pyrG*$^{+}$, single copy	this work
FW32.14	AB4.1 transformed with pFW20.1 (Tet-on *PgpdA*::*rtTA2*^{S}-*M2*::*luc-PEST pyrG**), *pyrG*$^{+}$, single copy	this work
FW33.23	AB4.1 transformed with pFW21.8 (Tet-off *PfraA*::*tTA2*^{S}::*luc-PEST pyrG**), *pyrG*$^{+}$, single copy	this work
FW36.1	AB4.1 transformed with pFW22.1 (Tet-On *PfraA*::r*tTA2*^{S}-*M2*::*luc-PEST pyrG**), *pyrG*$^{+}$, single copy	this work

Validation of the Tet-off system in *A. niger*

In order to validate that the Tet-off inducible expression system developed in this study will enable gene functional characterization, we utilised a strategy where genes which mediate easily detectable phenotypes in *A. niger* were placed under control of the Tet-off system, followed by deletion of the wild-type allele. In these isolates, Tet-off mediated transcript downregulation should result in comparable phenotypes to previously published null isolates. Firstly, we substituted the coding sequence for the *mluc* reporter in the *fraA* Tet-off system with gene *gfaA*, which encodes a glutamine: fructose- 6- phosphate amidotransferase (Ram et al., 2004). This gene is responsible for the first step in chitin synthesis and null isolates are unable to grow on media without exogenous supplementation of glucosamine, which is the metabolite produced by the enzyme gfaA (Ram et al., 2004). Additionally, the RhoGTPase *racA* coding sequence was cloned into *fraA* Tet-off. Deletion of this gene results in reduced colony sporulation and a hyphal hyperbranching phenotype in simple growth assays (Kwon et al., 2011).

In order to demonstrate that the Tet-off system can also be used for gain-of-function studies under non-induced conditions, we cloned the dominant activation allele of the *racA* RhoGTPase *racA*^{G18V} into *fraA* Tet-off. *racA*^{G18V} confers a clavate germling phenotype when

over-expressed in *A. niger* (Kwon et al., 2011). We therefore hypothesised that by deletion of the wild-type *racA* in a *PfraA*::*tTA2*^{S}::*racA*^{G18V} background, this single isolate can be used for both gain-of-function and loss-of-function analysis during non-inducing and inducing growth conditions respectively, thus proving a further technique for use with the Tet-off system.

Accordingly, *A. niger* strains expressing *gfaA*, *racA*, *racA*^{G18V} under Tet-off control at the *pyrG* locus were generated (Table 1). These Tet-off expression strains were used as recipient isolates in which the respective native gene was deleted using a split marker approach. We used Δ*gfaA* and Δ*racA* isolates as positive controls for the predicted growth deficiencies of Tet-off downregulation strains (Table 1). All isolates were phenotypically screened on solid media +/- Dox induction (Fig. 4). For all strains macroscopic colony morphology was assessed, and for analysis of *racA* mutant isolates cell morphology was analysed microscopically to determine apolar growth phenotypes.

For strain FW29.37 (*PfraA*:*tTA2*^{S}::*gfaA,* Δ*gfaA*) growth on MM plates was identical to the control isolate FW35.1 (Fig. 4A). Induction of *gfaA* Tet-off by supplementation of the growth media with more than 5 µg/ml Dox resulted in complete absence of growth in FW29.37 (10-10^3 spores), which is consistent with loss of chitin synthesis due to *gfaA* down-regulation. The *gfaA* knock out strain was unable to grow on MM or MM+Dox plates, but supplementation with 10 mg/ml glucosamine enabled growth of this isolate. The plate assay also shows rescue of FW29.37 growth on MM+Dox plates through addition of glucosamine. Down-regulation of gene *gfaA* was titratable with various Dox concentrations as determined by colony growth on plate assays (Fig. 4A).

Growth of strain FW30.37 (*PfraA*::*tTA2*^{S}::*racA,* Δ*racA*) was identical to control isolate FW35.1 on MM (Fig. 4 B). Tet-off downregulation resulted in radial colony growth which was indistinguishable from that of a Δ*racA* strain but with notably less spores than the control isolate (Fig. 4 B). Microscopic inspection revealed a clear hyberbranching phenotype in both Δ*racA* and following Tet-off downregulation in strain FW30.37 (Fig. 4 C).

In FW31.14 (*PfraA*::*tTA*::*racA*^{G18V}, Δ*racA*) dominant activation of RacA^{G18V} under non-inducing conditions resulted in growth inhibition due to the previously documented actin mis-localisation defect in this over-expression strain (Fig. 4D and 4E). Following Tet-off downregulation, FW31.14 demonstrated reduced growth rates and hyphal hyperbranching consistent with loss of *racA* function. These data collectively demonstrate that Tet-off is a versatile tool for both gain-of-function and quantitative downregulation studies in *A. niger*.

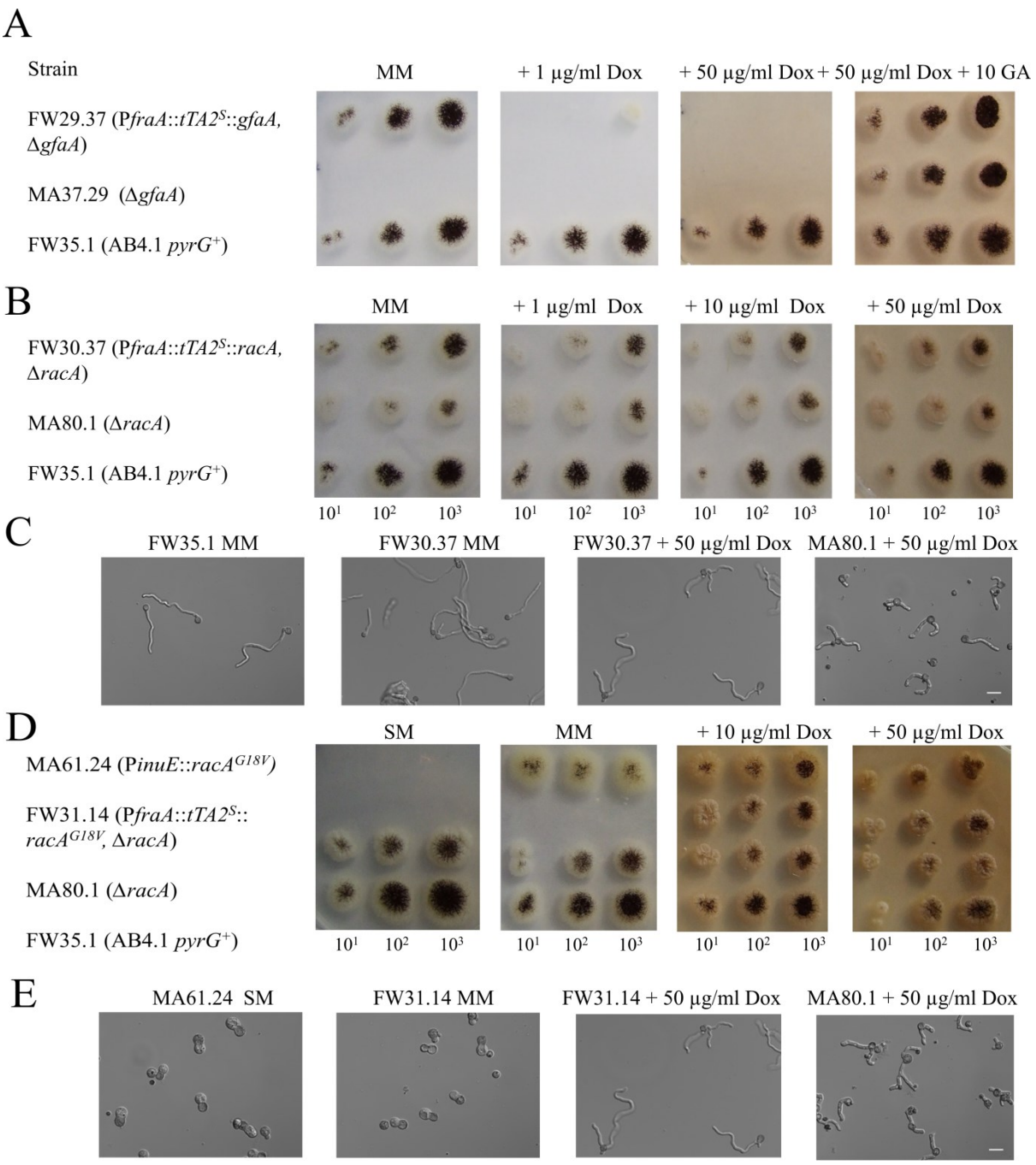

Figure 4: Phenotypical growth assay and microscopic analysis of *gfaA*, *racA* and *racA*^{G18V} under control of the new Tet-off (P*fraA*) in *A. niger*. Minimal medium plates (MM) and a serial spore dilution (10, 10^2, 10^3) were used. (A) Tet-off mediate down regulation of the *gfaA* gene (FW29.37) encoding glutamine fructose-6-phosphate amidotransferase. Deletion of *gfaA* is lethal (MA29.37) but can be rescued by addition of glucosamine (GA). (B) The Rho GTPase *racA* integrated in the Tet-Off system (FW30.37) shows a down regulation with low Dox concentrations (1 µg/ml), which was a comparable phenotype to the knock out strain (MA80.1), with reduced diameter and spore formation. (C) The strains grown on coverslips in MM for microscopy. Microscopic studies confirmed that FW30.37 showed hyperbranching similar to the knock out mutant (MA80.1) under induced conditions in the Tet-off system, and under uninduced conditions a phenotype comparable with the wild type (FW35.1). Scale bar, 10 µm. (D) The strain FW31.14 included the dominant activation allele *racA*^{G18V}, without Dox the overexpression of *racA*^{G18V} confers a lethal clavate germling phenotype similar to the control strain MA61.24, which shown the overexpression phenotype only on saccharose medium plates (SM) because of the used *inuE* promoter. Under induced conditions, the downregulation of

racA^{G18V} in the Tet-off system introduced reduced macroscopic growth rates and hyphal hyperbranching consistent with loss of *racA* function as in B. (E) The clavate germling overexpression phenotype of the Tet-off *racA*^{G18V} system looked similar to the induced control strain MA61.24 in microscopic analysis with a magnification of 40x and also the hyperbranching took place under induced condition similar to C. Scale bar, 10 µm.

Conditional gene silencing in *A. fumigatus* by an alternative Tet-off module

In a parallel effort, the established Tet-on system as validated in the human-pathogen *A. fumigatus* was remodeled to a Tet-off version. Initial attempts after replacing the *rtTA2*^{S}-*M2* sequence in the established Tet-on module of pVG4.1 (Meyer et al., 2011) by a formerly validated *tTA* sequence (Vogt et al., 2005) (Gossen and Bujardt, 1992) were unsuccessful, presumably due to toxicity this transactivator when expressed at high levels in the host cell. Accordingly, we made use of the recently modified version of the Tet-on system (Helmschrott et al., 2013), in which *rtTA2*^{S}-*M2* expression is driven by the *tpiA* promoter. Moreover, we used a synthetic *tTA2* transactivator that might be tolerated in *A. fumigatus* at higher intracellular concentrations (Baron et al., 1997) (Urlinger et al., 2000). The resulting Tet-off module of plasmid pSK606 was then used to assemble a conditional promoter replacement (Hu et al., 2007) cassette in order to target the *pabaA* gene in *A. fumigatus* (Fig. 5A). Respective recombinant strains were screened for their growth behavior in the presence and absence of Dox with respect to *para*-aminobenzoic acid (PABA) necessity, a vitamin K precursor that is formed by the action of the *pabaA*-encoded PABA synthetase (Fig. 5B). Inoculation of the *Tet-off::pabaA* strain AfS191 on solid culture medium lacking PABA revealed that the presumed auxotrophy depends on the presence of the inducer, as the isolate was unable to grow significantly when Dox was supplemented at a concentration of 50 µg/ml and this conditional auxotrophy could rescued by the presence of PABA.

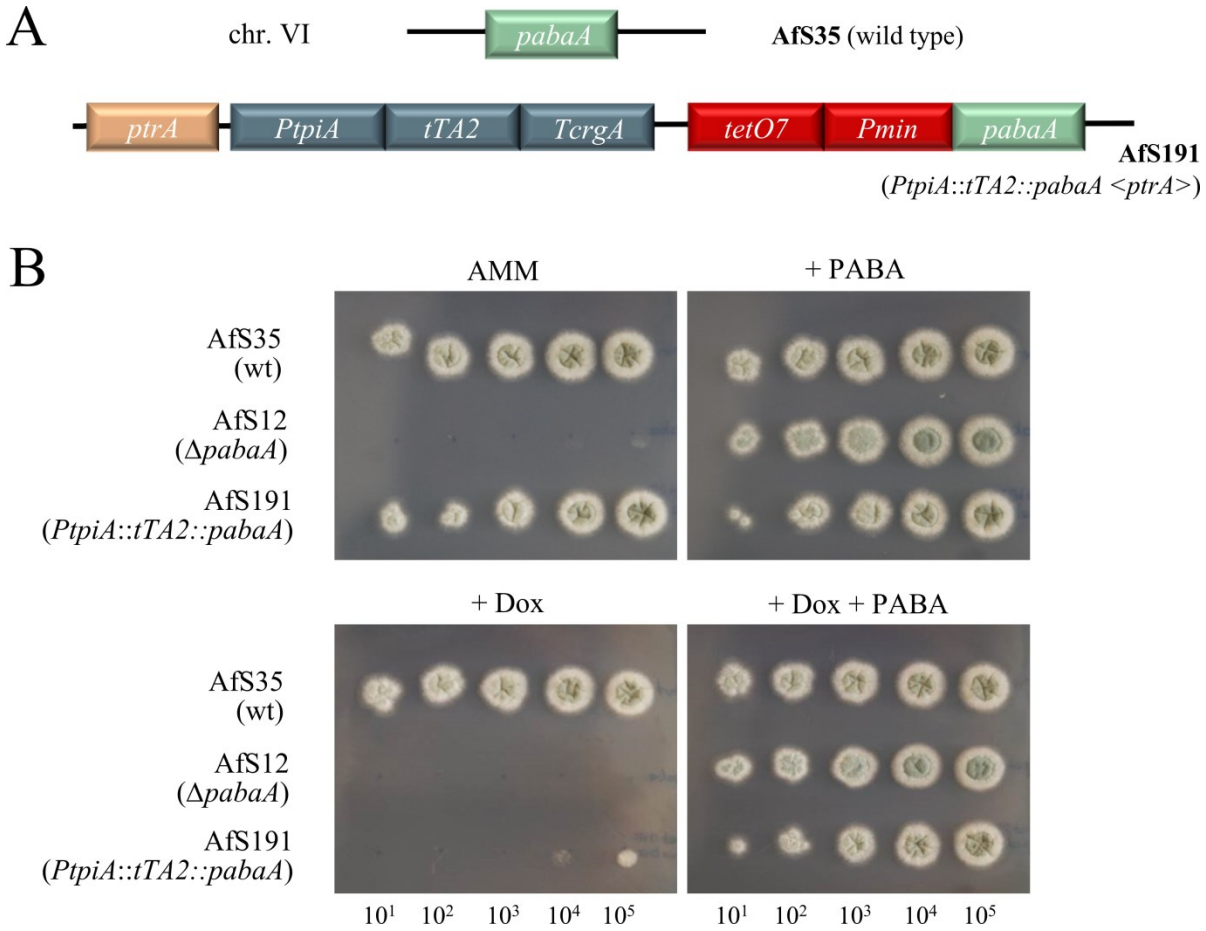

Figure 5: Conditional Tet-off promoter replacement in *A. fumigatus*. (A) Schematic outline of the *A. fumigatus pabaA* locus and the conditional promoter replacement allele carrying the functional Tet-off module, in which expression of the doxycycline-responsive trans-activator (tTA2) is driven by the *tpiA* promoter. Transcription of the *pabaA* gene is initiated from a minimal promoter comprising *tetO* sequences that the tTA2 factor binds to in the absence of the tetracycline derivative Dox; the pyrithiamine resistance-conferring *ptrA* gene is used as selection marker after genetic recombination. (B) Growth phenotype of the *Tet-off::pabaA* strain AfS191 in dependency of Dox and supplementation of *para*-aminobenzoic acid (PABA) compared to its wild-type progenitor and an auxotrophic *pabaAΔ* deletion strain. Indicated amounts of conidia were spotted on *Aspergillus* minimal culture medium (AMM) in the presence or absence of supplements and growth was monitored after three days of incubation at 37 °C. A conditional requirement for PABA is evident for the conditional promoter replacement strain AfS191, demonstrating functionality of the Tet-off system.

Application of the Tet-on and Tet-off system for induced gene oscillations

In order to conduct gene oscillatory studies in *Aspergillus* spp., we decided to test two modified luciferase reporters, including a fungal codon optimized version *luc* (Gooch et al., 2008) and a reporter encoding a proline, glutamic acid, serine, and threonine (PEST) protein degradation sequence *luc*-PEST (Cesbron et al., 2013). We reasoned that these modifications would increase fluorescent intensity and decrease luciferase half-life, which is essential for accurate measurement of gene expression during oscillations. We firstly used the Tet-on system [4] to compare both *luc* and *luc-PEST* with the conventionally used *mluc* (for

construction of plasmids see Supplementary Table S1, and strain generation see Table 1). We used a standard luciferase microtiter plate assay to measure luciferase activity in all Tet-on isolates expressing the various luciferase genes (Fig. 6A). Following induction, strains expressing the codon optimized *luc* and *luc-PEST* demonstrated an average of 5 and 1.5 times higher LCPS/OD values when compared to those expressing *mluc* respectively. This indicates both performed favourably with regards to fluorescent intensity.

Next, we compared *mluc* with *luc-PEST* using the Tet-off system to determine luciferase half-life following conditional expression (Fig. 6B). Downregulation by addition of Dox to growth media was comparable for *mluc* with *luc-PEST* in MTP format. Under non-inducing conditions, luciferase in *luc-PEST* expressing strains rapidly decreases following ~14 h growth. However, in *mluc* expressing strains we observed increased LCPS/OD values beyond this timepoint, which we hypothesised in due to accumulation of luciferase with an unacceptably high half-life rather than active transcription of this gene in stationary phase cells.

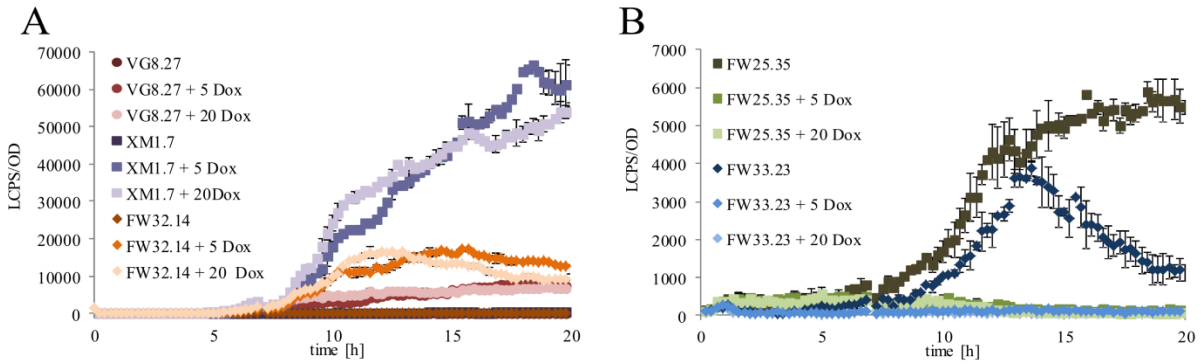

Figure 6: Comparison of different luciferase variants in Tet-on and off system. (A) The Tet-on expression system (P*gpdA*) was used to compare the different luciferase version *mluc* (VG8.27), *luc* (XM1.7) and *luc-PEST* (FW32.14) in *A. niger* in a microtiter plate assay. Induction with 5 or 20 µg/ml Dox occurred at time point 0. (B) In the Tet-off system (P*fraA*) *mluc* (FW25.35) was compared with *luc-PEST* (FW33.23) and repression of both systems induced with 5 or 20 µg/ml Dox at time point 0.

In order to corroborate this, we inhibited protein translation by addition of cycloheximide to culture media following 10 hours of growth of Tet-off strains in microtiter plate assay (Supplementary Figure S2). *Mluc* expressing isolates demonstrated that decrease in luciferase activity following cycloheximide treatment is not so fast with a half-life of mluc with 90 min (Supplementary Figure S2A). In contrast, we observed rapid decrease of luciferase per optical density activity in *luc-PEST* strains and estimated the half-life of luc-PEST with 30 min (Supplementary Figure S2B). The identified half-lifes were independent of cycloheximide concentration (Supplementary Figure S2). Reporter half-lifes estimated using cycloheximide

inhibition of *mluc* and *luc-PEST* reporters under control of the Tet-on cassette induced with 5 µg/ml Dox were comparable to values observed with the Tet-off cassette (data not shown).

A *luc-PEST* reporter has previously been used to quantify circadian rhythms in *Neurospora crassa*, where improved sensitivity enabled accurate reporting of oscillatory transcriptional patterns (Cesbron et al., 2013). In order to test if luc-PEST in *A. niger* was similarly sensitive, we simulated circadian oscillations by addition of Dox to growth medium, which was followed by a growth period of 5h and subsequent removal of Dox by washing cultures with fresh media. We used strain FW33.23 expressing *luc-PEST* under control of P*fraA* Tet-off in this assay (Fig. 7A) which demonstrated clear Dox dependent oscillations in luciferase activity. At later time-points (19 – 30 h) LCPS/OD was reduced when compared to earlier time-point (10.7 h) which is consistent with increased optical density of the culture. Direct comparisons of strains expressing Tet-off controlled *luc-PEST* (FW33.23) with *mluc* (FW25.35) demonstrated that Dox induced repression was faster using PEST modified luciferase and with lower LCPS/OD values (Fig. 7B). This indicates that *luc-PEST* will enable improved detection of both small and rapid transcriptional changes.

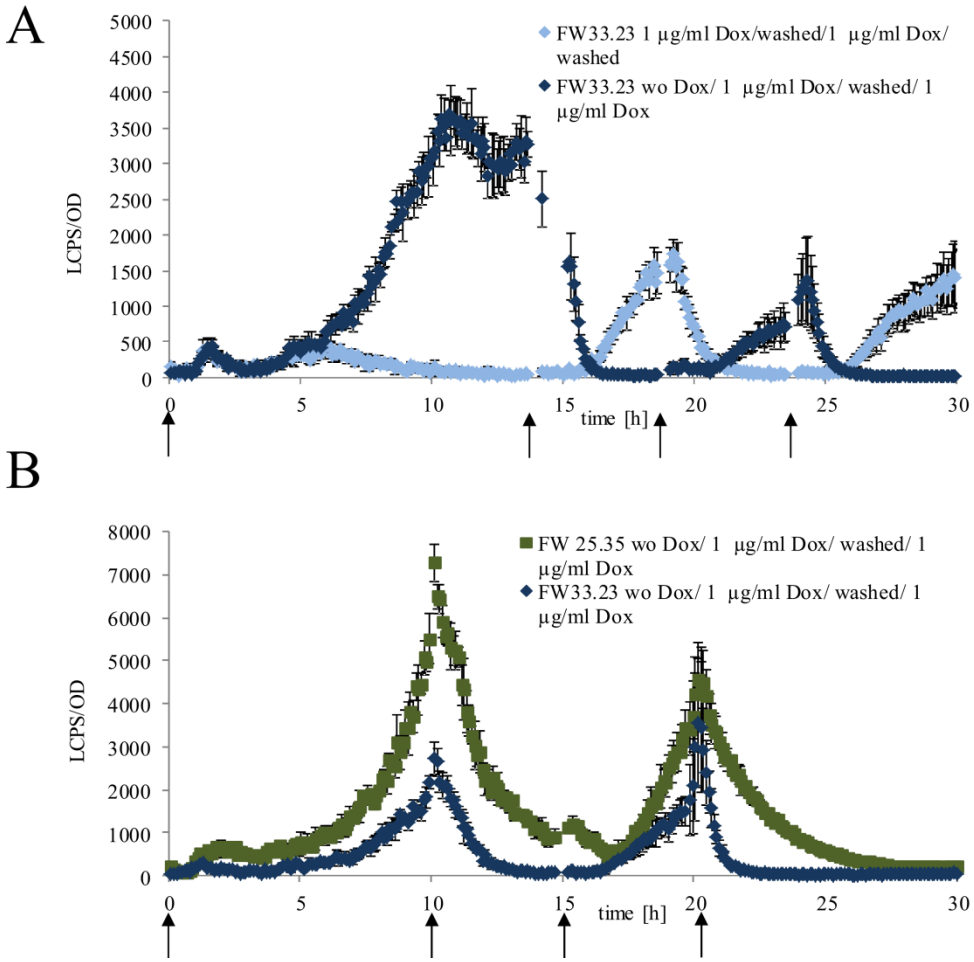

Figure 7: Gene oscillation studies used the Tet-off system. (A) The microtiter plate assay was inoculated with $5x10^4$ spores/ml of the Tet-off strain with P*fraA luc-PEST* (FW33.23) for oscillation studies. Following 14 h growth the culture was induced with 1 µg/ml Dox, and after further 5h the

wells were washed with fresh medium to enable gene expression. Following 5 h further growth, we induced the culture again. For comparison the same experiment was started directly with induction through 1 µg/ml Dox. (B) To show the advantage of *luc-PEST,* FW25.35 (*mluc*) was used in microtiter plate assay for comparison. After 10 h growth both cultures were induced with 1µg/ml Dox, after 5h the cultures were washed and let grown again for 5 h and after further 5 h induced the last time. The LCPS per OD values indicated that FW33.23 show a faster decrease after gene repression and enable vales closed to zero.

Discussion

The rational redesign of fungal genetic circuits has huge promise for industrial applications and fundamental research. Such optimised systems might enable production of a greater variety of bioactive products at higher yields by experimentally and genetically tractable fungi. For characterisation of gene function in basic research, applications of reengineered conditional expression systems have enabled interrogation of multiple attributes of fungal biology, including gene essentiality (Roemer et al., 2003), secondary metabolism (Skowyra and Doering, 2012) and mating (Zarnack et al., 2006). In this study we deliver a functional Tet-off conditional expression system in *A. niger* and *A. fumigatus* which are model /industrially important and pathogenic moulds respectively.

In *A. niger* we re-engineered the established Tet-on cassette to a functional Tet-off conditional expression system by sequential molecular modifications which included: (i) replacement of the *rtTA2*^{S}*-M2* with a codon optimised *tTA2S*; (ii) testing several promoters for improved genetic stability of the cassette and (iii) validating two modified luciferase reporters for highly accurate measurements of transcription.

During validation experiments, poor genetic stability of both Tet-off and Tet-on cassettes was identified. Previous studies have demonstrated that the strength of promoter has an influence on the stability of the expression system, where an overexpressed transactivator interacts with a variety of essential components of the transcriptional machinery, which can be deleterious to cell metabolism, a phenomena described as squelching (Gill and Ptashne, 1988). To improve stability of the Tet-off cassette, we exchanged the *gpdA* promoter with the *fraA* promoter, so that an intramolecular recombination event was undetectable in various laboratory cultures. Importantly, this Tet-off system maintained titratable downregulation of a luciferase reporter.

In order to demonstrated the utility of the new Tet-off expression system for gene functional analysis, we could confirmed roles of *gfaA* and *racA* in chitin biosynthesis and regulation of polar growth respectively. Thus, distinct processes which include fungal metabolism and signalling cascade components can be assessed using the Tet-off system described in this study. We also used a dominant *racA*^{G18V} allele to prove that the Tet-off cassette will enable

both gain-of-function and conditional gene downregulation in a single strain background. Concomitant gene over-expression and downregulation enables comprehensive characterisation of gene function, yet has previously required time-consuming generation of multiple mutant isolates. That a single background can be used for both experimental approaches greatly enhances the available toolkit for gene functional analysis in *Aspergillus* spp.

Probing the cellular function of gene products in the context of fungal virulence is a valuable and promising application of conditional gene expression in *Aspergillus*. Successful establishment of the Tet-on system in *A. fumigatus* made such studies possible, in which the expression of distinct genes during infection could be manipulated by Dox feeding of the inoculated animals (our unpublished results). Accordingly by addressing the role of presumably essential genes during pathogenesis of aspergillosis, the conditional promoter replacement approach employing the Tet-on or Tet-off system is valid and supportive in defining novel targets of antifungal therapy. When titrating the minimal amount of Dox that would result in auxotrophy in the *Tet-off::pabaA* strain AfS191, concentrations of 3 µg/ml turned out to be sufficient when inoculating 10^3 conidia (data not shown), prompting successful application of the Tet-off system in infection series where concentrations of 0.2% in the drinking water of susceptible mice are routinely applied.

With regards to modification of luciferase reporters for optimal measurement of gene expression, we tested the fully codon optimised luciferase (*luc*) (Gooch et al., 2008) and short half-life luc-PEST (Cesbron et al., 2013). Using a gene oscillation approach in *A. niger* with the Tet-off system, we demonstrate approximately 5 times higher luciferase values using *luc*, which is therefore a useful tool for experiments which assess activity of lower activity promoters. With regards to improved sensitivity, our results determined the average half-life of luc-PEST as 30 mins, which is comparable to the half-life published in *Neurospora crassa* (Cesbron et al., 2013) . In contrast, the conventionally used *mluc* (Morgan et al., 2003) demonstrated an extended half-life of 90 min with cycloheximide microtiter plate determination. Faster degradation of luciferase reporters presented here will enable more accurate measurements of conditional expression systems in future synthetic biological applications.

Conclusions

In this study we engineered a titratable Tet-off system in *A. niger* and *A. fumigatus*. This conditional expression system enabled gene functional analysis as determined by quantitative

downregulation of *racA* and *gfaA* in *A. niger* and *pabaA* in *A. fumigatus*. These data provide proof of principle that this tool will be useful for assessing essential genes in both these organisms. Using a $racA^{G18V}$ dominant activation allele in *A. niger*, we were able to confirm that the Tet-off conditional expression system enabled downregulation and overexpression in a single isolate, an approach which obviates experimentally costly generation of multiple mutant strains. We conducted several quality control experiments in which genetic stability of the Tet-off encoding cassette was maximised by replacement of a *gpdA* promoter with a *fraA* promoter. Accordingly, the Tet-off system is a versatile and robust tool for gene functional analysis in industrially important and pathogenic *Aspergilli*. Expansion of this synthetic biological approach enabled improvement of genetic stability in the previously published *A. niger* Tet-on cassette, demonstrating that tools and techniques described in this study can broadly be applied to engineering transcriptional circuits in filamentous fungi. Finally, in order to conduct gene oscillatory studies, we describe two improved luciferase reporters which can be used for accurate measurement of gene transcription in *Aspergillus* spp.

Material and methods

Cloning

The Gibson assembly method was utilized for plasmid construction (Gibson et al., 2009). Briefly, PCR products or restriction endonuclease digested DNA fragments to be recombined were designed with 20 over-lapping base pair regions to facilitate homologous recombination (all plasmid constructions are listed in Supplementary Table S1). 5 µl DNA fragments were mixed with 15 µl Gibson master mix consisting of 4 µl 5x isothermal reaction buffer (25 % PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 1 mM of each four dNTPs and 5 mM NAD), 0.08 units T5 exonuclease (Epicentre), 0.5 units Q5 DNA polymerase (NEB) and 80 units Taq DNA ligase (NEB), made up to 15 µl with sterile water. Samples were incubated at 50 °C for 60 minutes, before 3 minutes cooling at room temperature, subsequent 3 minute incubation on ice, after which 3 µl aliquots were transformed into chemically competent *Escherichia coli* strain TOP 10. Bacteria were grown in LB medium supplemented with ampicillin at 50 µg/ml where appropriate.

Construction of Tet-off cassettes

Several versions of the *tTA* were used throughout this study. Initial experiments utilized the *tTA* gene provide from p473 (Vogt et al., 2005), which was then cloned into the Tet-On

system of pVG4.1 plasmid (Meyer et al., 2011) (all plasmid constructions are listed in Supplementary Table S1). In subsequent work we used the commercially available *tTA2* ^{S} codon optimized for eukaryotic translation (pTet-Off® Advanced Vector, Clontech).

To validate the functionality of other promoters instead of *gpdA*, 5' upstream regions from two genes were rationally selected based on comparable gene expression profiles to *gpdAn* transcripts from an in-house compendium of *A. niger* microarray experiments (*gpdAN* An16g01830, *fraA* An16g04690). In both cases we choose 1000 bp in front of the annotated gene as promoter region.

To determine whether the new Tet-off system allowed gene function assays, the gene *racA* (An11g10030), *racA*^{G18V} (dominant activation of *racA*) and *gfaA* (An18g06820) were used to verify the concept. The reporter gene *mluc* was PmeI restriction digested from pFW15.1 and replaced by DNA sequences encoding one of the above genes using Gibson cloning (racA: pFW17.1, racA^{G18V}: pFW18.1, gfaA: pFW19.7), in detail see Supplementary Table S1.

A Tet-off module based on the pCH008 construct of Wagener and co-workers (Helmschrott et al., 2013) was generated by replacing the *rtTA2*^{S}*-M2*-encoding sequence by the synthetic *tTA2* sequence isolated from the plasmid pUHT61-1 (Urlinger et al., 2000) by sequence and ligation independent cloning (SLIC) using the Seamless Cloning system of Life Technologies. A fragment of the resulting plasmid pSK606 was then used to assemble a conditional promoter replacement cassette targeting the *pabaA* locus of *A. fumigatus*, using PCR amplicons covering 1.5 kb of the 5' region and the coding sequence together with the 3' region. A 7 kb HpaI fragment from this conditional promoter replacement vector pSK607 was used for transformation of the *A. fumigatus* recipient strain AfS35.

Furthermore both *luc-PEST* (1879 bp) and *luc* (1741 bp) DNA encoding sequences were amplified by Q5-polymerase from pFH62 (Cesbron et al., 2013) and Gibson cloned into the recently used Tet-on system (pVG4.1) at PmeI restriction locus. *luc-PEST* was also cloned into the Tet-off plasmid using this approach (pFW15.1). Additionally, the published Tet-on cassette (pVG4.1) was also optimised with the new *fraA* promotor (Tab.1) and *luc-PEST* resulting in pFW22.1.

A. niger transformation

A. niger transformation protocols, selection procedures, fungal chromosomal DNA isolation, diagnostic PCR and Southern analyses were performed as described in Meyer et al., 2010.

We observed that expression of the Tet-off reporter cassettes resulted in slow growth of positive clones during transformation. Accordingly, transformation media was supplemented with Dox (1-5 µg/ml) in order to prevent heterokaryotic colonies overgrowing positive

transformants. In the case of the transformation with pFW18.1 (Tet-off with *racA*^{G18V}) it was necessary to add Dox in the transformation and purification plates, because the overexpressed racA^{G18V} transformant is non-viable.

A split marker approach enabled directed deletion of the target genes *gfaA* and *racA* at endogenous loci, with a hygromycin resistance gene (from pAN7.1 (Punt et al., 1987)) used as a selectable marker. Following transformation, agar plates were supplemented with 200 µg/ml and 100 µg/ml hygromycin in subsequent purification plates (Arentshorst et al., 2015).

In order to confirm single cassette integration in the recipient genome at the *pyrG* locus, transformant genomic DNA was restriction endonuclease digested and probed using a DIG labelled DNA amplicon, which was a homologous sequence to 538 bp in *AnpyrG** at 3` of the Tet-off construct (described in detail in Supplementary Table S1). Two independent restriction endonucleases were used for confirmation of each strain. Similarly, for confirmation of *gfaA* and *racA* gene deletion events, two DIG labelled DNA probes were used which targeted either the promoter or terminator region of each gene respectively (Supplementary Table S1).

Strains and culture conditions

Aspergillus strains used in this study are given in Table 1. In all instances where *A. niger* was modified with derivative plasmids of pVG4.1, we used AB4.1 (Table 1) as recipient strain using its uracil-auxotrophy for selection. This enabled comparison to established Tet-On strains (Meyer et al. 2011) and AB4.1 may show a better genomic stability in comparison to NHEJ-inactivated strains (Zhang et al., 2011). *A. niger* strains were routinely grown on minimal medium (MM) containing 1 % glucose, 1 x ASP+N (50 x ASP+N: 3.5 M $NaNO_3$, 550 mM KH_2PO_4, 350 mM KCl, pH5.5), 2 mM $MgSO_4$ and 1x trace elements solution **(**modified from composition given by Vishniac and Santer, 1957, 1000 x trace elements solution: 10 g of EDTA, 4.4 g of $ZnSO_4 \cdot 7H_2O$, 1.01 g of $MnCl_2 \cdot 4H_2O$, 0.32 g of $CoCl_2 \cdot 6H_2O$, 0.315 g of $CuSO_4 \cdot 5H_2O$, 0.22 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, and 1 g of $FeSO_4 \cdot 7H_2O$) or on complete medium (CM) consisting of MM supplemented with 0.5 % yeast extract and 0.1 % casamino acids. For the growth assay we used saccharose medium plates (SM) composed of the same ingredients as MM, except 1 % saccharose replacing 1 % glucose. For growth on solid plates, media was supplemented with 1.5 % agar.

All bacterial and fungal strains were routinely stored at −80 °C in 50 % (v/v) glycerol. For short term storage of fungal strains, spores were suspended in physiological sodium chloride solution and kept at 4 °C.

Bioreactor cultivation

Bioreactor cultivation was conducted as described previously (Jørgensen et al., 2010). Briefly, glucose-limited batch cultivations were performed with 5 l reactor minimal medium containing the following 22.5 g of NH_4Cl, 7.5 g of KH_2PO_4, 2.5 g of KCl, 2.5 g of $MgSO_4 \cdot 7H_2O$, 5 ml of trace metal solution (described above) and 0.8 % glucose, with pH adjusted to 3. The 5 l bioreactor cultivation was inoculated with a spore density of 10^9 /liter in BioFlo3000 bioreactors (6.6 liter, New Brunswick Scientific, NJ, USA). Temperature of 30° C and pH 3 through computer controlled addition of 2 M NaOH or 1 M HCl were kept constant. The addition of base was used as an indirect growth measurement, after consumption of ~12,5 ml 2M NaOH (correlated with 1 $g_{biomass}$ dryweight/kg, early exponential phase), we induced or repressed our expression systems Tet-On and Tet-off with 5 µg/ml Dox.

Luciferase Measurement in Microtiter plate

Luciferase reporter activity was measured in microtiter ViewPlates (96 white with transparent bottom, from Perkin Elmer) using a Victor3X plate reader. Two different types of luminescence assays to evaluate the conditional expression systems were performed. In the first type of assay, the strains were grown directly in the microtiter plates for ~20 h. In the second assay, strains were grown in bioreactor cultivation, after which aliquots were taken and measured in microtiter plates. For microtiter growth, wells were inoculated with $4x10^5$sp/ml, 70 µl luciferin solution (diluted with CM to 1.4 mM, Promega), Dox were stated (ranging from 0 – 20 µg/ml) and a final volume of 300 µl made up by addition of CM medium. For every condition, triplicate biological replicates were performed. The measurement protocol determines luminescence (LCPS) and optical densitiy (OD) values at 595 nm. For determination of the half-life of the different luciferase proteins (from *mluc*, *luc*, *luc-PEST*) cycloheximide in a final concentrations of 10, 20, 30 and 100 µg/ml was added 10h after inoculation of the respective strains. For oscillation studies, we used the P*fraA* Tet-off system *mluc* and *luc-PEST*. Microtiter samples were inoculated with spores 10h in media without Dox. Then cultures were induced with 1 µg/ml Dox, and after 5h the inoculated Dox was removed by washing MTP wells (through discarding the used CM, addition of fresh CM, pipette mixing, centrifugation of the plates 5min 1000 x g followed by exchange with fresh CM plus luciferin solution). After a further 5 h the cultures were induced again. For bioreactor growth assays, triplicate biological replicates were conducted and LCPS and OD measured as described above. In this experiment 230 µl samples were mixed with 70 µl

luciferin (diluted with reactor minimal medium to 1.4 mM) and directly measured in Victor3X.

Phenotypical growth assays on plates

A dilution series of *A. niger* spores (10, 10^2, 10^3) and *A. fumigatus* spores (10, 10^2, 10^3, 10^4, 10^5) of indicated strains (listed in Table 1) were spotted on either MM, SM or AMM plates, supplemented where indicated with 10 mg/ml glucosamine, 1/ 10/ 50 µg/ml Dox concentrations or 50 µg/ml *para*-aminobenzoic acid. Plates with *A. niger* strains were incubated at 30 °C and *A. fumigatus* plates at 37 °C for 3 days. All plates were photographed to visualize macroscopic colony morphology.

Microscopy

Two coverslips were disinfected and placed onto the bottom of a small Petri Dish, after which 5 ml of liquid MM or SM supplemented with 0.003 % yeast extract and 0 or 50 µg/ml Dox were added. Petri dishes were inoculated with 10^6 spores of *A. niger* strains and incubated for 7 h at 30 °C. Coverslips with adherent germlings were placed upside down on an object slide and analysed by microscopy. Light microscopic pictures (using DIC settings) were captured with a 40x objective using a Leica DMI5000 CS equipped with a Leica DFC365 FX camera and processed with GIMP 2.8 afterwards.

Authors' contributions

FW, SK and VM designed the study. FW, SB, AH, CB and XM performed the experiments. FW, TC, SK, and VM interpreted the results and were involved in writing the manuscript. All authors read and approved the final manuscript.

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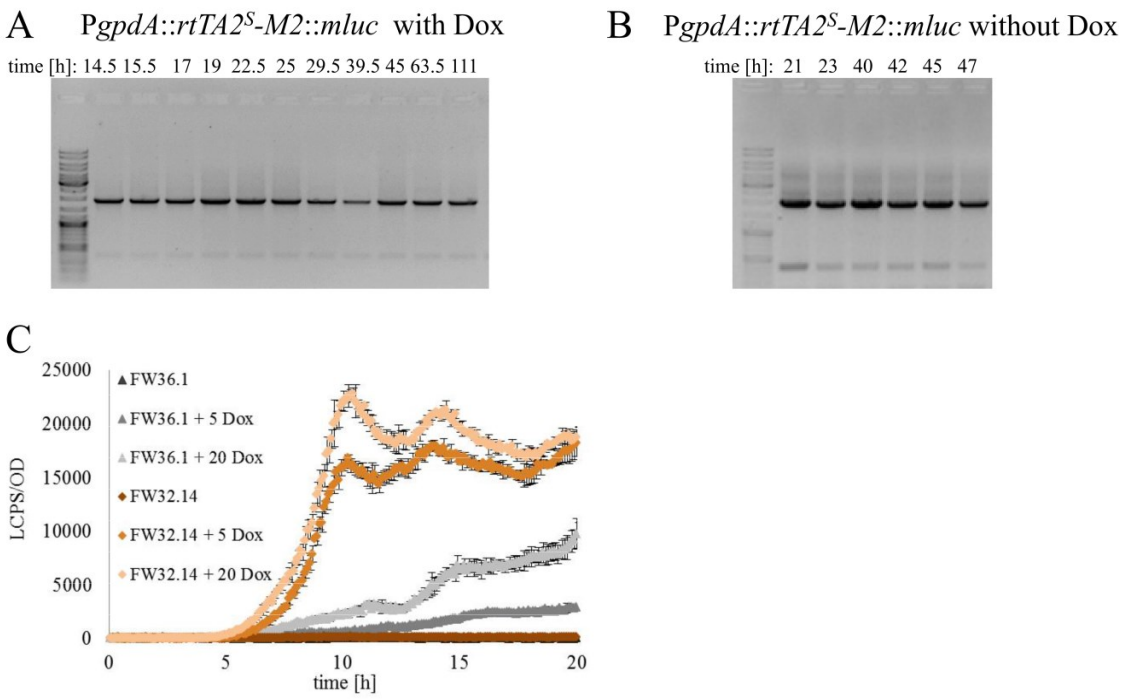
Supplementary Table S1: Expression vectors and split markers designed and used in this study.

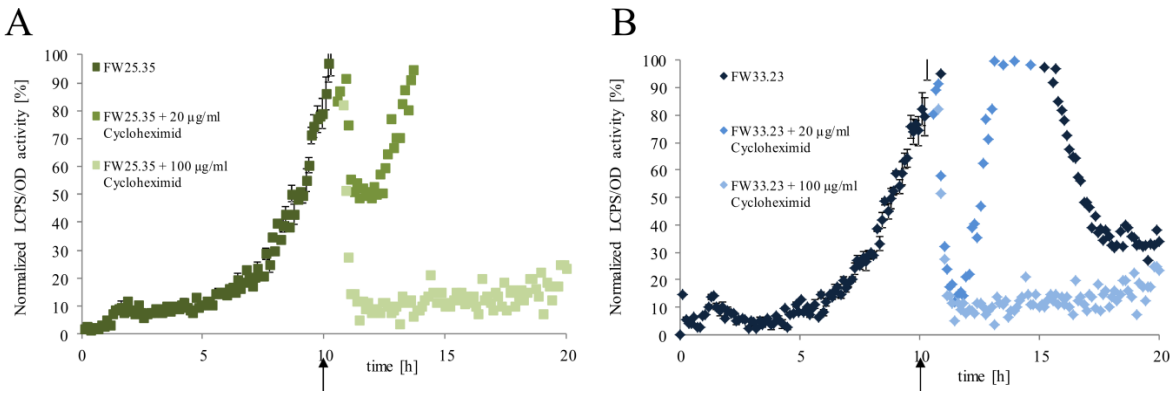
Plasmid/ split marker (SM)	Description	Source
pVG4.1	Tet-on *PgpdA*::*rtTA2^{S}-M2*::*TcrgA*::*TetO7*::*Pmin*::*mluc*::*Ttrpc*	(Meyer *et al.*, 2011)
p473	synthetic *tTA* transactivator	(Vogt *et al.*, 2005)
pMA247	Exchange *rtTA* with *tTA* from p473 in pVG4.1, by digestion with EcoRI-BamHI in 2 steps, because of the extra BamHI site in pVG4.1. Southern probe for *pyrG* was amplified with 5`TCTCGCGCAGAAGCACAACT and 3`GCAGCCTGCACCGGATCG.	This work
pSB1.1	pMA247 was digested with AscI (flanked pyrG*), the linear DNA without the *pyrG** sequence ligated again.	This work
pTet-off® Advanced Vector	synthetic *tTA2^{S}* transactivator	Clontech
pFW9.3	pVG4.1 was digested with BamHI (2 restrictions sites) and EcoRI, two linear fragments without *rtTA-M2* was cloned together with cutted *tTA2^{S}* from pTet-off® Advanced Vector (BamHI/ EcoRI) in a three-way ligation. To proof in the genome the recombination event between P*gpdA* and Pmin 5` TTCCTGCTCTCCCCACCAG (in P*gpdA*) and 3` TGTCCACCTCGATATGTGCA (in *mluc*) were used.	This work
pFW15.1	pFW9.3 was digested with EcoRI and BspLUII, the backbone was used for Gibson cloning. And also the promoter of *fraA* (1000 bp in front of An16g04690) was amplified from AB4.1 genome with 5` CCCTCGGCTGGTCTGTCTTA and 3` agtctagacatggtgaattcTTTGGCGGTTTGTTGCTGGC and additionally a PCR part which was amplified with 5` TTTTGCTGGCCTTTTGCTCA and 3` taagacagaccagccgagggAAGCTTATCGATACCGTCGA from pFW9.3 as template. To proof in the genome the recombination event between P*fraA* and Pmin 5` CCCTCGGCTGGTCTGTCTTA (in P*fraA*) and 3` TGTCCACCTCGATATGTGCA (in *mluc)* were used.	This work
pAT1.11	pFW9.3 was digested with EcoRI and BspLUII, the backbone was used for Gibson cloning. And also the Syer of *gpdAn* (1000 bp in front of An16g01830) was amplified from AB4.1 genome with 5`TAAGAATGGGGAAGGCGAAG and 3` cagtctagacatggtgaattcTGTTTAGATGTGTCTATGTG and additionally a PCR part which was amplified with 5` TTTTGCTGGCCTTTTGCTCA and 3` cttcgccttccccattcttaAGCTTATCGATACCGTCGAC from pFW9.3 as template. To proof in the genome the recombination event between P*gpdAn* and Pmin 5` TAAGAATGGGGAAGGCGAAG and 3` TGTCCACCTCGATATGTGCA (in *mluc*) were used.	This work
pFW17.1	With PmeI cut out the backbone (8138 bp) of FW15.1 and amplified *racA* with 5` gacatcaccgtttaaacaccATGGCCACTGGTCCAGCT and 3` tcggcatctactgtttaaacCTACAGAATCACGCATTTCTTGTTCT from AB4.1 genome and Gibson-cloned together.	This work

pFW18.1	With PmeI cut out the backbone (8138 bp) of FW15.1 and amplified *racA*^{G18V} with 5` gacatcaccgtttaaacaccATGGCCACTGGTCCAGCT and 3` tcggcatctactgtttaaacCTACAGAATCACGCATTTCTTGTTCT from MA61.24 genome and Gibson-cloned together.	This work
pAN7.1	*PgpdA*::*hph* (hygromycin resistence)::*Ttrpc*	(Punt *et al.*, 1987)
SM 1 and SM2 for ΔracA	Construction of 2 split markers for the deletion of *racA*. For split marker 1 we amplified GOI 5` of *racA* with 5`AGCAGCAGCAGCAACACTAA and 3` ccagaaagagtcaccggtcaGTCGAATTGAGGCGAGG from AB4.1genome and the hygromycin resistance with 5`aagccgctgctggaattgGGCTCTGAGGTGCAGTGGAT and cgatggataattgtgccgtgTTGGGTGTTACGGAGCATTCA from pAN7.1. Following we fusioned GOI 5` and *hph* together with a PCR with 5`ACCTGTCCAGTGGCTATCTT and 3´ GAAATTGCCGTCAACCAA. With the same approach we constructed the splitmarker 2, we amplified GOI 3` part with 5` acgagactgaggaatccgctGCCAAACCGAAGAACAAGAA and 3` CAACTACGACCGCATGAAGA from AB4.1 genome and fusioned it together with *hph* in a PCR with 5`AGAGCCTGACCTATTGCATCT and 3` CAACTACGACCGCATGAAGA . By recombination of the two parts of the selection marker and homologous integration of the cassette in the genome, a successful gene deletion mutant can be obtained. Probe for the promoter was amplified with 5` ACCTGTCCAGTGGCTATCTTT and 3`ccagaaagagtcaccggtcaGTCGAATTGAGGCGAGGG and for the terminator with 5` acgagactgaggaatccgctGCCAAACCGAAGAACAAGAA and 3` CAACTACGACCGCATGAAGA.	This work
pFW19.1	With PmeI cut out the backbone (8138 bp) of FW15.1 and amplified *racA* with 5` gacatcaccgtttaaacaccATGTGGTATGTATGGCTCCAAAG and 3` gtcggcatctactgtttaaacGCTCTCTATTCAACAGTAACCGAC from AB4.1 genome and Gibson-cloned together.	This work
SM 1 and SM2 for ΔgfaA	Construction of 2 split markers for the deletion of *gfaA*. For splitmarker 1 we amplified GOI 5` of *gfaA* with 5`AGCAGGTCACCACTACCATC and 3` caattccagcagcggctATGTGATTACTCGGAGGCGT from AB4.1 genome and the hygromycin with 5`aagccgctgctggaattgGGCTCTGAGGTGCAGTGGAT and 3`cgatggataattgtgccgtgTTGGGTGTTACGGAGCATTCA from pAN7.1. Following we fusioned GOI 5` and *hph* together with a PCR with 5`AGCAGGTCACCACTACCATC and 3`GGCGTCGGTTTCCACTATC. With the same approach we constructed the splitmarker 2, we amplified GOI 3` part with 5`acacggcacaattatccatcgGTGGGCACGAGACTGGGA and 3`ATCTGGGAAGCCGCGTATAA from AB4.1 genome and fusioned it together with *hph* in a PCR with 5`AAAGTTCGACAGCGTCTCC and 3`ATCTGGGAAGCCGCGTATAA. By recombination of the two parts of the selection marker and homologous integration of the cassette in the genome, a successful gene deletion mutant can be obtained. Probe for the promoter was amplified with 5`AGCAGGTCACCACTACCATC and 3` caattccagcagcggctATGTGATTACTCGGAGGCGT and for the	This work

	terminator with 5` acacggcacaattatccatcgGTGGGCACGAGACTGGGA and 3` ATCTGGGAAGCCGCGTATAA.	
pCH008	Tet-on module ^{p}*tpiA::rtTA2*^{S}*-M2::cgrA*^{t}*-tetO-*^{p}*min*	(Helmschrott *et al.*, 2013)
pUHT61-1	synthetic *tTA2* transactivator	(Urlinger *et al.*, 2000)
pSK606	Tet-off module ^{p}*tpiA::tTA2::cgrA*^{t}*-tetO-*^{p}*min*: 800 bp PCR amplicon with *tTA2* sequence from pUHT61-1 assembled with 540 bp BstBI/XbaI fragment from pCH008 carrying *tpiA* promoter assembled in SpeI/PstI vector pCH008 backbone	This work
pSK607	*Tet-off::pabaA* conditional promoter replacement cassette: assembly of 1.5 kb 5' *pabaA* flanking region, 4.1 kb SfiI fragment of pSK606, and 1.5 kb *pabaA* cds and 3' flanking region in pUC19	This work
pXM1.1	With PmeI cut out the backbone (7818 bp) of pVG4.1 and *luc* sequence (1741 bp) was amplified from pFH62 (Cesbron et al. 2013). Primer 5` ttgagcagacatcaccgtttaaacaccATGGAGGACGCCAAGAACA and 3`ccggtcggcatctactgtttaaacttaGAGCTTGGACTTGCCGCCCT were used, with a stop codon encoded in the 3' region of primer. Backbone and PCR product were fusioned by Gibson cloning.	This work
pFW20.1	With PmeI cut out the backbone (7818bp) of pVG4.1 and *luc-PEST* sequence (1879 bp) was amplified from pFH62 (Cesbron et al. 2013) with primers 5` ttgagcagacatcaccgtttaaacaccATGGAGGACGCCAAGAACA and 3` atcccggtcggcatctactgtttaaacTTAGACGTTGATCCTGGCGCT and fusioned by Gibson cloning.	This work
pFW21.8	With PmeI cut out the backbone (8138bp) of pVG15.1 and *luc-PEST* sequence (1879 bp) was amplified as describe above (see pFW20.1) and fusioned by Gibson cloning.	This work
pFW22.1	pFW20.1 was digested with EcoRI and BspLUII, the backbone (8589 bp) was used for Gibson cloning together with the same two PCR products as for construction of pFW15.1. To proof in the genome the recombination event between P*fraA* and Pmin 5` CCCTCGGCTGGTCTGTCTTA (in P*fraA*) and 3` CTCGAAGTACTCGGCGTAGG (in *luc-PEST*) were used.	This work



Supplementary Figure S1: Genetic Stability of Tet-on P*gpdA::rtTA2^S-M2* strain and MTP assay of Tet-on P*fraA::rtTA2^S-M2* strain. (A) Samples from bioreactor cultivation with strain VG8.27 (P*gpdA*) +/- Dox induction were extracted throughout a time series of growth. A diagnostic PCR demonstrated that with Dox induction, an intramolecular recombination event was occurring. (B) This recombination event was also observed in culture without Dox over the time period shown. (C) In microtiter plate assays the Tet-on system with *fraA* promotor (FW36.1) was compared with the established one (P*gpdA*) (FW32.14), under conditions with 0, 5 and 20 µg/ml Dox. The expression strength of FW36.1 was less compared to FW32.14.



Supplementary Figure S2: Determination on half-life of luciferase variants in Tet-off system. (A) In a microtiter plate assay the Tet-off system with *mluc* (P*fraA*) is cultivated without Dox, after 10 h growth 20 and 100 µg/ml cycloheximide was added to the wells. The time point were the normalized LCPS/OD activity [%] decrease 50 % related to the values at addition time point was estimated as half-life of the protein. (B) The Tet-off strain with *luc-PEST* (FW33.23) shown after addition of cycloheximide a fast decrease and reduced normalized LCPS/OD activity [%] up to 10.