Antifungal drug resistance evoked via RNAi-dependent epimutations

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Microorganisms evolve via a range of mechanisms that may include or involve sexual/parasexual reproduction, mutators, aneuploidy, Hsp90 and even prions. Mechanisms that may seem detrimental can be repurposed to generate diversity. Here we show that the human fungal pathogen *Mucor circinelloides* develops spontaneous resistance to the antifungal drug FK506 (tacrolimus) via two distinct mechanisms. One involves Mendelian mutations that confer stable drug resistance; the other occurs via an epigenetic RNA interference (RNAi)-mediated pathway resulting in unstable drug resistance. The peptidylprolyl isomerase FKBP12 interacts with FK506 forming a complex that inhibits the protein phosphatase calcineurin1. Calcineurin inhibition by FK506 blocks *M. circinelloides* transition to hyphae and enforces yeast growth2. Mutations in the fkbA gene encoding FKBP12 or the calcineurin A or B subunit cnbR or cnaA genes confer FK506 resistance and restore hyphal growth. In parallel, RNAi is spontaneously triggered to silence the fkbA gene, giving rise to drug-resistant epimutants. FK506-resistant epimutants readily reverted to the drug-sensitive wild-type phenotype when grown without exposure to the drug. The establishment of these epimutants is accompanied by generation of abundant fkbA small RNAs and requires the RNAi pathway as well as other factors that constrain or reverse the epimutant state. Silencing involves the generation of a double-stranded RNA trigger intermediate using the fkbA mature mRNA as a template to produce antisense fkbA RNA. This study uncovers a novel epigenetic RNAi-based epimutation mechanism controlling phenotypic plasticity, with possible implications for antimicrobial drug resistance and RNAi-regulatory mechanisms in fungi and other eukaryotes.

The pathogenic fungus *M. circinelloides* grows as hyphae aerobically, and as yeast in low oxygen and high-CO2 conditions3. FKBP12 is a prolyl isomerase conserved throughout eukaryotes that interacts with FK506 and rapamycin and mediates their antifungal activity in *M. circinelloides* and other fungi. The FKBP12–FK506 and FKBP12–rapamycin complexes inhibit the protein phosphatase calcineurin and the Tor kinase, respectively1,5. FK506 inhibition of calcineurin blocks hyphal growth of *M. circinelloides* and enforces yeast-phase growth2 (Fig. 1a and Extended Data Fig. 1a). Exposure to FK506 yields drug-resistant isolates exhibiting hyphal growth emerging from the yeast colony periphery (Extended Data Fig. 1a) at a ratio of one resistant isolate out of ~1 × 10⁶ yeast cells. A subset of FK506-resistant (FK506Δ) isolates harbour mutations in the fkbA gene encoding FKBP12 or the calcineurin A or B subunit genes cnaA and cnbR (45 of 64 isolates (~70%), Supplementary Table 1). However, several FK506Δ isolates (17 of 64 (~27%)) harboured no mutations in the fkbA or calcineurin target genes. These isolates exhibited resistance to FK506 and rapamycin, but not to cyclosporin A (CsA), which, similar to FK506, enforces largely yeast growth, Fig. 1a) or other drugs (nystatin, amphotericin B, not shown), suggesting that they had not developed multidrug resistance mechanisms. These unusual drug-resistant isolates also reverted frequently within several generations of vegetative growth on drug-free media and were restored to a wild-type phenotype (yeast growth on exposure to FK506) (Extended Data Fig. 1b, c). Expression analyses revealed a complete loss of fkbA mRNA and FKBP12 protein in these drug-resistant isolates when grown in media containing FK506. In contrast, mRNA and protein levels were reduced but detectable in some resistant isolates when grown in drug-free media (Fig. 1b, c) and were restored to wild-type levels in revertant isolates that became FK506-sensitive (FK506Δ) following growth in drug-free media (Fig. 1b, c).

**Figure 1 | RNAi-dependent epimutations confer FK506 resistance in *M. circinelloides*.** a, Wild-type (WT, NRRL3631), fkbA mutant (fkbAΔ) and epimutant strains were grown on yeast extract peptone dextrose (YPD) media alone or supplemented with FK506, rapamycin or cyclosporin A (CsA). Images are representative of two independent experiments. b, c. The epimutant strains EM1, EM2 and EM3 were grown on YPD media with FK506 (FK506 lanes) or YPD drug-free media (R lanes). The reverted strains EM1-S, EM2-S and EM3-S were grown in YPD media (S lanes) and whole cell protein and RNA extracts were prepared. b, Equivalent protein amounts (120 µg) were resolved by SDS–PAGE and analysed by western blot with an anti-Sc fkbA antibody. An S. cerevisiae extract (Sc) was included as control for antibody specificity; tubulin served as a loading control. Images are representative of seven independent experiments. c, 50 µg total RNA was analysed by northern blot employing probes specific for fkbA, act1 (loading control) and antisense fkbA mRNAs. Images are representative of six independent experiments for the fkbA and act1 probes, and two for the antisense fkbA probe.
As drug resistance was reversible and an active RNAi pathway is present in the organism, we hypothesized that drug resistance is RNAi-mediated. Notably, small RNAs (sRNAs) complementary to fkbA were detected in these unusual drug-resistant isolates (Fig. 2a and Extended Data Fig. 2a), suggesting a new role for RNAi in the development of transient resistance to antifungal drug exposure. Consistent with the expression analyses, the sRNA signal was highly abundant during growth with FK506 (100%), less abundant when the isolates were grown in drug-free media (~62% and ~25% for epimutant strains EM2 and EM3, respectively, Fig. 2a) and lost when drug resistance reverted (0–0.02%). The fkbA silencing was not associated with DNA methylation in M. circinelloides (Extended Data Fig. 2b). We term these unusual drug-resistant isolates epimutants, by analogy with studies in fungi, plants and animals, in which epimutations have been described as silencing of genes that are usually active, or vice versa.

Our first hypothesis was that RNAi could be triggered via double-strand RNA (dsRNA) production from the overlap in the 3′ regions between fkbA and its convergently transcribed neighbouring gene patA (Extended Data Fig. 3 and Supplementary Table 2). But patA mutants did not show any effect on the frequency of fkbA epimutational silencing (Table 1, Supplementary Table 1 and Extended Data Fig. 4). A rapid amplification of CDNA end assays confirmed that the mRNA from fkbA gene and the pyrG marker replacing patA were not overlapping (Supplementary Table 2). Thus, expression of patA to generate overlapping RNA molecules is not necessary for fkbA silencing.

As fkbA is a highly expressed gene (Fig. 1c), and a high RNA turnover has been implicated in the production of aberrant RNA and triggering of silencing, we speculated that an fkbA antisense RNA may be generated by an RNA-dependent RNA polymerase (RdRP). Northern blot analysis with an fkbA antisense-specific probe revealed an antisense fkbA mRNA in all strains with robust fkbA expression (Fig. 1c). This fkbA antisense RNA is perfectly complementary to the intron-spliced mature fkbA sense mRNA, and is 5′ capped and polyadenylated (Extended Data Fig. 5). Thus, the antisense RNA is generated from the mature fkbA mRNA. The fkbA antisense RNA is expressed in the wild-type strain (Fig. 1c); however, RNAi is activated in only a subset of cells selected with FK506. The in vivo efficiency of dsRNA formation or its transport to the cytoplasm may limit sRNA levels restricting silencing to fewer cells. Only traces of antisense sRNAs complementary to fkbA (16 reads) were detected in the wild-type strain by high-throughput sequencing (Extended Data Fig. 6), supporting this hypothesis.

To test if mutations may have occurred to promote the formation of epimutations, the reverted and now sensitive epimutant strains were exposed to a second round of FK506 to isolate mutants/epimutants. The frequency of epimutation versus mutation to FK506 in the reverted epimutant strains was similar or even lower than in the parental wild-type strain (Extended Data Fig. 7). Thus, the underlying mechanism appears solely epigenetic and does not require any genetic change in the genome to promote epimutation.

High-throughput sequencing demonstrated abundant sRNAs complementary to the fkbA mRNA (antisense) as well as sense sRNA in three epimutant resistant isolates, but these were barely detectable in the wild-type strain or in the two corresponding FK506 revertants analysed (Fig. 2b). Some sRNA sequences spanned exon–exon junctions (Fig. 2b and Extended Data Fig. 8a), indicating that the source and target of the sRNA is mature mRNA. Most of these sRNAs average 21–24 nucleotides in length, with a bias towards 5′ terminal uridine (Fig. 2c), features typical of sRNAs that interact with Argonaute proteins. No further loci were detected exhibiting the same pattern of sRNA production: high level in epimutants and very low levels in wild-type and reverted strains (Extended Data Fig. 8b). Silencing pathways can operate constitutively on M. circinelloides endogenous genes during normal growth. In contrast,

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**Table 1** Frequency of epimutants/mutants in the wild-type, patA and RNAI mutant strains

| Strain   | Background | Total analysed | Mutations in fkbA | Mutations in cmaA/cmbR | No mutation found | Epimutants (no.) | Epimutants (%) | P value |
|----------|------------|---------------|-------------------|------------------------|------------------|-----------------|----------------|---------|
| NRRL3631 | Wild type  | 33            | 22                | 0                      | 11               | 10              | 30.3           |         |
| R7B      | leuA       | 33            | 22                | 1                      | 5                | 5               | 15.2           | 0.531   |
| MU434    | patA::pyrG | 33            | 28                | 0                      | 7                | 2              | 22.6           |         |
| MU435    | patA::pyrG | 33            | 26                | 1                      | 7                | 1              | 21.2           |         |
| MU406    | dcl1::pyrG | 25            | 23                | 1                      | 1                | 1              | 4              | 0.06    |
| MU407    | dcl1::pyrG | 21            | 18                | 3                      | 0                | 0              | <4.8           | 0.033   |
| MU410    | dcl2::pyrG | 34            | 31                | 2                      | 1                | 0              | <2.9           | 0.004   |
| MU413    | ago1::pyrG | 26            | 25                | 1                      | 0                | 0              | <3.8           | 0.012   |
| MU426    | ago1::pyrG | 21            | 27                | 0                      | 0                | 0              | <3.7           | 0.012   |
| MU416    | ago2::pyrG | 23            | 16                | 3                      | 4                | 4              | 17.4           | 0.741   |
| MU414    | ago3::pyrG | 27            | 22                | 2                      | 1                | 1              | 14.8           | 0.518   |
| MU419    | rdrp1::pyrG| 32            | 6                 | 0                      | 26               | 26             | 81.3           | 0.000004 |
| MU420    | rdrp2::pyrG| 25            | 23                | 2                      | 0                | 0              | <4.0           | 0.013   |
| MU428    | rdrp2::pyrG| 27            | 26                | 1                      | 0                | 0              | <3.7           | 0.012   |

*P values were obtained based on a Fisher's Exact Probability Test for a 2x2 Contingency Table, comparing each of the mutant strains individually versus the R7B strain.*
the FK506-selected silencing of the fkbA gene may not affect other loci. However, no other phenotypes were selected and sRNAs are often lost without selection (Fig. 2a and Extended Data Figs 1b and 8b).

To test if silencing was driven and enhanced by a genome-wide increase in stress-activated RNAi, the wild-type strain was exposed to stress conditions before FK506 exposure (Supplementary Table 3). However, analysis of recovered FK506 isolates did not show an increase (or decrease) of epimutation frequency in fkbA (Supplementary Table 4). Thus, either stress fails to activate RNAi or we have not identified the precise activating stress. The calcineurin inhibitor CsA also did not increase epimutations conferring FK506 resistance; thus, inhibition of calcineurin (by CsA or FK506) does not activate RNAi.

To investigate if sRNAs generated against fkbA are produced by canonical RNA silencing, we screened for epimutants in mutants lacking RNAi pathway components. No epimutants were found in dcl2, ago1 or rdrp2 mutants (Table 1 and Extended Data Fig. 9a), showing that Dcl2 (Dicer), Ago1 (Argonaute) and RdRP2 are necessary for endogenous silencing of fkbA. Dcl2 and Ago1 are essential for both transgene-induced and endogenous silencing in M. circinelloides22,23. RdRP2 is essential to amplify RNAi signals for transgene-induced silencing18, but was thought to play a role secondary to RdRP1 for endogenous RNAi silencing. Instead, we find RdRP2 is crucial, suggesting that different pathways for endogenous silencing operate in M. circinelloides, which may be correlated with the four different known classes of exonic siRNA (ex-siRNA)2. Notably, we found only one epimutant in two independent dcl1 mutants (1 of 46 FK506 isolates, 2.2%, Table 1, Supplementary Table 1 and Extended Data Fig. 9a). Dcl1 is not known to have any essential role in transgene-induced or endogenous silencing7,21, but does have an auxiliary function in mutants lacking Dcl2 (ref. 19). The paucity of fkbA epimutants in the dcl2 and dcl1 mutant backgrounds suggests that both Dcl1 and Dcl2 are involved in epimutational fkbA silencing. ago2 and ago3 mutants exhibited a wild-type frequency of silencing (15–17%, Table 1 and Extended Data Figs 9a and 2a), thus Ago2 and Ago3 are dispensable for epimutation20. These results reveal that establishment of epimutants in M. circinelloides depends on Dcl2, Dcl1, Ago1 and RdRP2. Except for Dcl1, these genes are involved in biogenesis of the known class I type of ex-siRNA2. Thus, although epimutant genetic requirements could suggest that a distinct sRNA class is involved (given the role of Dcl1), we cannot exclude the possibility that fkbA silencing is due to an atypical class I ex-siRNA.

Unexpectedly, our studies revealed a novel role for RdRP1 in constraining epimutational silencing. RdRP1 is required for RNA silencing of exogenous sense transgenes in M. circinelloides. When DNA alleles producing dsRNA are introduced, this bypasses RdRP1 to evoke gene silencing18. As noted above, RdRP1 has a major role in endogenous silencing2. RdRP1 is hypothesized to be central for activating RNAi by generating dsRNA from the single-stranded RNA (ssRNA) precursor, both from sense transgenes or mRNA. In this model, RdRP1 should be essential for triggering fkbA silencing because it involves an fkbA dsRNA (Fig. 1c and Extended Data Fig. 5). Surprisingly, the rdrp1 mutant showed an elevated silencing rate of ~80% (versus ~22% in wild-type, Table 1 and Extended Data Fig. 9a and 2a) and the epimutants isolated in this mutant did not revert on drug-free media (epimutant strain EM4, Extended Data Fig. 1b). In Caenorhabditis elegans the rrf-3 mutant lacking one of several RdRPs has a similar enhanced RNAi phenotype22,23. RRF-3 has also been proposed to generate dsRNA from mRNA templates24, hence RdRP1 and RRF-3 could serve analogous roles. Furthermore, the exosome and RNAi pathways in Schizosaccharomyces pombe compete in their degradation activities. Both mechanisms share common targets25, and in the rdrp1 mutant sRNA formation is abolished and mRNAs are primarily directed to the exosome26. In Mucor, RdRP1 could have an opposing role to RNAi, promoting assembly of exosome machinery on specific mRNA targets and thereby avoiding activation of RNAi under normal conditions, as was suggested previously2.

To test which RdRP might generate dsRNA from fkbA mRNA, we tested for fkbA antisense RNA in silencing mutants (Extended Data Fig. 9b). All strains analysed expressed the antisense RNA at similar levels, even in the rdrp1 and rdrp2 mutants. The two RdRP polymerases could have a redundant role in generating fkbA antisense RNA (rdrp1 rdrp2 double mutants appear inviable, precluding analysis, not shown), or other rdrp genes may participate.

Together these results provide evidence for a different route for the processing of endogenous sRNA, wherein RdRP2, Ago1 and both Dcl proteins are necessary to silence mRNA expression via epimutation, and RdRP1 has an unexpected role constraining epimutational silencing. We consider two possible models. In the first, sRNAs are produced constitutively and stochastically at low levels against the entire genome or some designated loci, allowing adaptation to environments through an RNAi-based pathway. In the second model, some mechanism activates RNAi under adverse or novel physiological conditions, facilitating genomic and phenotypic plasticity. Either could explain the broad range of environments in which M. circinelloides grows, and the limited antifungal drug susceptibility.

Previous studies showed that the M. circinelloides species complex includes three distinct subspecies: M. circinelloides f. lusitanicus, M. circinelloides f. cinereofuscus, and M. circinelloides f. griseoexcavus27. Matings barriers and phylogenetic separation provide evidence that these three lineages are different enough to represent distinct species. To generalize our findings, two M. circinelloides f. cinereofuscus strains (Muc, 1006PhL28) and an M. circinelloides f. griseoexcavus strain (ATCC1207a) were tested in addition to the M. circinelloides f. lusitanicus strains. Only the M. circinelloides f. cinereofuscus strains grew as yeast in the presence of FK506 (Extended Data Fig. 10a), enabling the recovery of FK506 isolates. The two M. circinelloides f. cinereofuscus strains exhibited different patterns of genomic plasticity; FK506 isolates appeared earlier in 1006PhL than Muc (5–7 days versus 5–15 days) and 2.5-fold more isolates were recovered from 1006PhL (Supplementary Table 5). Epimutants silencing fkbA occurred in the pathogenic isolate 1006PhL at a surprisingly higher rate (90%) than in Muc (~7.7%) or the M. circinelloides f. lusitanicus strains (~20–30%) (Supplementary Table 5 and Extended Data Fig. 10b). M. circinelloides f. cinereofuscus is the most common Mucor species associated with human infection. The enhanced ability to activate RNAi exhibited by the 1006PhL virulent isolate suggests that RNAi may enable this fungal pathogen to readily adapt both in nature and the host. Further studies are required to elucidate whether our observations are generally applicable, or if not, how specificity is brought about.

This study underscores the ability of M. circinelloides to adapt to the environment through two different routes of phenotypic variation, one stable (mutation) and one transient (epimutation). This plasticity evokes a broader phenotypic repertoire including the ability to reverse epimutations when selective pressures are relaxed. This is, to our knowledge, the first known example of epimutations involving an endogenous gene in fungi identified in a standard genetic screen; however, given the ubiquity of RNAi it is unlikely to be unique. While this example involves resistance to an antifungal drug in a human fungal pathogen, these findings could have implications beyond novel modes of transient antimicrobial resistance for the broader evolutionary trajectory of this and other eukaryotes with active RNAi pathways.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** S.C., C.S.-W., S.T.M., R.M.R.-V., M.E.C. and J.H. performed experiments, interpreted data and wrote the paper. S.C., C.S.-W., R.J.B., S.C.L. and F.E.N. performed experiments. P.M. sequenced the sRNA library. J.A.G. analysed deep-sequencing data. S.T.M., R.M.R.-V., M.E.C. and J.H. provided materials.

**Author Information** Sequences for the fkbA gene from WT strain NRRL3631 and epimutant strains (EM1, EM2 and EM3) were deposited in GenBank with accession numbers KF203228, KF203229, KF203230 and KF203231. Raw data from high-throughput sRNA sequencing of WT, epimutant and revertant strains have been deposited in NCBI’s Gene Expression Omnibus and are accessible through accession numbers GSE56353. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.H. (heitm001@dukes.edu).
METHODS

Strains and growth conditions. The leu4 leucine auxotrophic strain R7B, derived from M. circinelloides L. usitatus CBS277.49 (syn. Mucor racemosus, ATCC21126), was used as the wild-type strain to compare with the patA and silencing mutant strains, as they were grown in the R7B background [14–16]. Strain NRRL3631 is used as wild type in the rest of the experiments. The strains were grown at room temperature (−26°C) on yeast extract peptone dextrose agar (YPD, 10 g·L−1 yeast extract, 20 g·L−1 dextrose, 2% agar), MMC medium pH = 4.5 (1% casamino acids, 0.05% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose), or YNB minimal medium pH 4.5, supplemented with 1 μg·mL−1 of FK506 (Prograf), 100 μg·mL−1 of rapamycin, or 100 μg·mL−1 of CsA when needed. The cultures were routinely incubated for 48 h except when noted otherwise or for the isolation of FK506-resistance patches, when the cultures were incubated for as long as 4 weeks in some cases. The media to test the different stress conditions was prepared by adding the specific compounds to the concentrations indicated (see Supplementary Table 3) except for trisporic acid, which was sprayed over the spores (approximately 100 μg of trisporid as suggested by previous studies [36]). All of the compounds were added to YPD medium unless otherwise specified.

FK506-resistant strain isolation. FK506-resistant isolates were obtained after growing the different strains on YPD containing 1 μg·mL−1 of FK506 for 3 days to 3–4 weeks at room temperature, until patches with hyphal growth were observed. Each isolate was derived from an independent subculture grown on a different Petri dish. The isolates were analysed after at least three generations of vegetative growth in the presence of FK506 to ensure a high proportion of the nuclei in the mycelium syncytium were mutant or silenced.

DNA/RNA extraction and analysis. The isolates were grown on MMC media pH = 4.5, supplemented with 1 μg·mL−1 of FK506 when necessary, before DNA or RNA purification. The FKBP12 gene fkbA and calcineurin genes calA, calB, calC and cnbR were sequenced from DNA purified with CTAB and chloroform extraction from lyophilized mycelia. The FK506-resistant isolates obtained in a mutant background were verified by junction PCR for the deletion of the proper gene. Small and total RNAs were extracted using Trizol as described previously [37] from frozen mycelia in liquid nitrogen. 25–35 μg of sRNAs were separated by electrophoresis on 15% TBE-Urea gels (Invitrogen), electrod transferred to Hybond N + filters for 400 mA in 0.1X TBE, and cross-linked by irradiation with ultraviolet irradiation (2 pulses at 1.2 × 108 J·cm−2). Prehybridization and hybridization were carried out with ultrasonicated hybridization buffer UltraHyb (Ambion). The fkbA antisense-specific and 5S rRNA riboprobes were prepared by in vitro transcription using the Maxiscript transcription kit (Ambion) following supplier-recommended protocols. Riboprobes were treated as described previously [38] to result in an average size of 50 nucleotides (nt).

Protein cell extracts and analysis. Whole-protein cell extracts were prepared from mycelia grown on MMC media pH = 4.5 and frozen in liquid nitrogen as described previously [37] with some modifications. The sample was ground to a powder with a mortar and pestle and transferred to TSA extraction buffer (10 mM Tris-HCl pH = 8.0 and 0.15 M NaCl) with protease inhibitors (complete Mini from Roche), and 1 mM of benzamidine. After 30 min incubation on ice and 30 min centrifugation at 10,000 × g at 4°C, the supernatant was removed by aspiration. The ^14C-labeled fkbA was determined by computing the Kendall correlation coefficient (that is, most closely correlated with fkbA) were plotted using R [44] for comparison to fkbA.

GenBank accession numbers. Sequences for the fkbA and calcineurin genes from the wild-type strain NRRL3631 and the epimutant strains (EM1, EM2 and EM3) were deposited in GenBank with accession numbers wild-type fkbA, KF203228; EM1 fkbA, KF203229; EM2 fkbA, KF203230; EM3 fkbA, KF203231; wild-type calA, KJ668831; wild-type calB, KJ668832; wild-type calC, KJ668833; wild-type cnbR, KJ668834; EM1 calA, KJ668835; EM1 calB, KJ668836; EM1 calC, KJ668837; EM1 cnbR, KJ668838; EM2 calA, KJ668839; EM2 calB, KJ668840; EM2 calC, KJ668841; EM2 cnbR, KJ668842; EM3 calA, KJ668843; EM3 calB, KJ668844; EM3 calC, KJ668845; and EM3 cnbR, KJ668846.

P-value calculation. P-values listed in Table 1 are based on a Fisher’s exact probability test for a 2 by 2 contingency table, comparing each of the patA and silencing mutant strains individually versus the R7B WT strain, from which all of the mutants were generated.

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a

Yeast growth on YPD

Hyphal growth on YPD

+FK506

4 days

4 days

10 days

16 hours

16 hours

8 days

b

| Strain          | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 8 | Passage 11 | Passage 15 |
|-----------------|-----------|-----------|-----------|-----------|-----------|------------|------------|
| R7B             | Y         | Y         | Y         | Y         | Y         | Y          | Y          |
| NRRL3631 (WT)   | Y         | Y         | Y         | Y         | Y         | Y          | Y          |
| fkbΔ           | H         | H         | H         | H         | H         | H          | H          |
| SM2 (fkbΔ')     | H         | H         | H         | H         | H         | H          | H          |
| EM1             | H         | H         | H         | H         | Y         | Y          | Y          |
| EM2             | H         | Y         | Y         | Y         | Y         | Y          | Y          |
| EM3             | H         | H         | H         | H         | Y         | Y          | Y          |

| Strain          | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 8 | Passage 9 | Passage 22 | Passage 23 | Passage 26 |
|-----------------|-----------|-----------|-----------|-----------|-----------|-----------|------------|------------|------------|
| R7B             | Y         | Y         | Y         | Y         | Y         | Y         | Y          | Y          | Y          |
| NRRL3631 (WT)   | Y         | Y         | Y         | Y         | Y         | Y         | Y          | Y          | Y          |
| fkbΔ           | H         | H         | H         | H         | H         | H         | H          | H          | H          |
| SM2 (fkbΔ')     | H         | H         | H         | H         | H         | H         | H          | H          | H          |
| EM1             | H         | H         | H         | H         | H         | H         | H          | Y          | Y          |
| EM2             | H         | H         | H         | Y         | Y         | Y         | Y          | Y          | Y          |
| EM3             | H         | H         | H         | H         | H         | H         | Y          | Y          | Y          |
| EM4 (relp1Δ)    | H         | H         | H         | H         | H         | H         | H          | H          | H          |

c

YPD

FK506

Rapamycin

CsA

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Extended Data Figure 1 | *M. circinelloides* can develop resistance to FK506 by two mechanisms, one stable (mutation) and one transient (epimutation).

a, The wild-type strain (NRRL3631) grows as hyphae (white, upper left panel) on YPD and as a yeast (yellow, upper centre panel) on YPD containing 1 μg ml⁻¹ of FK506. An FK506-resistant patch that emerged from the south-eastern edge of the yeast patch is shown after 10 days of incubation (arrow, upper right panel). Microscopic images corresponding to the culture plates at the top were taken at different incubation periods as indicated and are shown in the lower panels. For yeast growth, cells from the colony were dispersed in water on a microscope slide. The black patch (arrow) in the microscopic image in the lower right panel corresponds to the edge of the compact yeast colony. Images are representative of all of the FK506-resistant isolates obtained (see Supplementary Tables 1, 3 and 5).

b, Epimutant strains revert during passage on drug-free media. Y, yeast; H, hyphal. Shaded areas indicate reversion of epimutants to the wild-type phenotype (yeast growth on YPD supplemented with 1 μg ml⁻¹ FK506). SM2 strain harbours an A-to-G substitution (A316G) in the acceptor splice site of intron 2. Darker vertical bars indicate intervals in which some passages are not depicted.

c, Reverted epimutant strains from b lost their resistance to FK506 and rapamycin (central panels) and remained sensitive to CsA whose mechanism of action does not involve FKBP12 (right panel). The images were taken after 48 h of incubation at room temperature (~26 °C) on YPD or YPD media supplemented with the different drugs. Images are representative of two independent experiments. EM1-S, EM2-S and EM3-S, epimutants 1, 2 and 3 reverted to restore FK506-sensitivity; fkbAΔ, fkbA null mutant.
Extended Data Figure 2 | Epimutations are generated by the RNAi pathway, and are not associated with DNA methylation in *M. circinelloides.*

**a,** Confirmation of the presence of sRNAs in all of the remaining epimutant isolates from the different strains lacking mutations in the *fkbA* and calcineurin genes (*cnaA, cnaB, cnaC* and *cnbR*) not shown in Fig. 2a or Extended Data Fig. 9. The numbers of the isolates correspond to those in Supplementary Table 1. sRNA blots were hybridized with an antisense-specific probe to detect *fkbA* sRNA. 5S rRNA served as a loading control. Abundant sRNAs were detected in all of the strains with the exception of three of the isolates (NRRL3631 isolate 9, R7B isolate 8 and MU410 isolate 26). These isolates also do not show any mutations in the genes analysed (*fkbA,* *cnaA,* *cnaB,* *cnaC* and *cnbR*) and the mechanism by which they have developed FK506-resistance remains to be established.

**b,** Genomic DNA (~40 μg) from the wild-type strain (NRRL3631), the three epimutants (1, EM1; 2, EM2; 3, EM3), and the three reverted strains (1-S, EM1-S; 2-S, EM2-S; 3-S, EM3-S) was treated with the methylated-DNA-specific restriction enzyme McrBC (NEB) with or without previous treatment with the CpG methyltransferase SssI (NEB), following the manufacturer’s protocols. PCR amplification of the *fkbA* locus (~1.6 kilobases = 732 base pairs (bp) 5’ upstream *fkbA*, 457 bp *fkbA* ORF and 435 bp 3’ downstream *fkbA*) was carried out using 100 ng of purified DNA. PCR amplification after McrBC treatment yielded similar levels of product as the untreated samples. Virtually no product was obtained by PCR amplification in any of the samples after treatment with the CpG methyltransferase SsI followed by McrBC treatment, indicating that McrBC digested the newly methylated DNA, preventing its amplification. These results indicate that RNAi silencing does not involve DNA methylation of the *fkbA* locus in *M. circinelloides.* Image is representative of two independent experiments.
Extended Data Figure 3 | mRNAs from fkbA and its neighbouring gene patA overlap in their 3' regions by 92 bp.  

a, The fkbA and patA genes are convergently oriented. The intergenic region is only 40 bp, and the mRNA overlap of their 3’ UTR regions spans 92 nucleotides.  

b, Alignment of the overlapping fkbA and patA 3' regions based on 3' RACE analysis. The direction of the transcripts are the same as in the upper figure, where the patA transcript is 5' to 3' end (top sequence) and the fkbA transcript is in the opposite orientation (3' to 5', bottom sequence). The poly(A) tails of both mRNAs are shown.
Extended Data Figure 4 | patA expression to generate overlapping RNA molecules is not necessary for fkbA silencing. a, Two independent patA null mutants (M1, MU434 and M2, MU435) were generated by homologous recombination, employing pyrG as the selectable marker. The patA ORF was replaced with the pyrG gene after electroporation of protoplasts with a gene deletion cassette, generated by overlap PCR, containing the selectable marker pyrG flanked by 5’ upstream and 3’ downstream sequences flanking the patA ORF. Almost 400 bp from the 3’ end of patA were preserved to keep intact the 3’ UTR of fkbA. PCRs from 5’ and 3’ junctions (P1/P3 and P4/P2, respectively), the patA ORF (P5/P6) and spanning the patA and fkbA loci (P1/P2) were performed to confirm the deletion of the patA ORF and correct insertion of the pyrG disruption cassette (bottom). Image is representative of two independent experiments. 3’ RACE assays were performed to verify that the pyrG 3’ UTR and fkbA 3’ UTR do not overlap in the patA null mutants (see Supplementary Table 2). b, Confirmation of the presence of sRNAs in epimutants derived from two independent patA null mutants. The numbers of the isolates correspond to those in Supplementary Table 1. An antisense-specific probe was used to detect fkbA sRNAs by northern blot. 5S rRNA served as a loading control. Abundant sRNAs complementary to fkbA were detected in all of the FK506r strains that lacked Mendelian mutations isolated from the two independent patA null mutants. The numbers of the isolates correspond to those in Supplementary Table 1. An antisense-specific probe was used to detect fkbA sRNAs by northern blot. 5S rRNA served as a loading control. Abundant sRNAs complementary to fkbA were detected in all of the FK506r strains that lacked Mendelian mutations isolated from the two independent patA null mutants.
Extended Data Figure 5 | fkbA antisense RNA is complementary to mature fkbA RNA. The complete sequence of the antisense RNA was determined based on 5’ and 3’ RACE analyses (bottom sequence) and compared to the fkbA DNA (top sequence). These analyses indicate the antisense RNA is 5’ capped and 3’ polyadenylated. The fkbA introns were absent in the antisense sequence and the 3’ end matched the beginning of the 5’ UTR found on fkbA mRNA by 5’ RACE analysis, indicating that mature spliced fkbA RNA is used as a template by an RdRP to generate the complementary strand. The antisense RNA 5’ end is located 7 nucleotides upstream of the STOP codon. The 3’ end is located 40 nucleotides upstream of the ATG codon. The fkbA DNA sequence includes the sequenced 5’ and 3’ UTR regions in blue and the introns in black. The fkbA coding region is indicated in red. The ATG start and TAA stop codons are shown in green boxes.
Extended Data Figure 6 | Very few fkbA sRNAs were detected by high-throughput sequencing in the wild-type strain. In the wild-type strain, the total count of antisense sRNA complementary to fkbA is extremely low (sixteen reads), and below the detection limit of northern blot. All but one of the antisense sRNA detected have a uridine at the 5′ terminus, features typically found in sRNA that interact with Argonaute proteins, suggesting that they may represent authentic sRNAs but are present at insufficient levels to trigger RNAi silencing. Sense sRNA does not show any bias (data not shown). Sequence logos summarizing all sixteen reads are shown, with one logo for each size antisense read observed (x axis). The unit ‘bits’ on the y axis of the sequence logos indicate how much the frequency of a base in each position departs from the expected random distribution. The height of a letter represents the frequency with which the base is observed in that position, and the total height of the letters in a position indicates how strong the bias is for specific bases in that position. Positions in the logo with zero bits indicate a random distribution of bases. Bit values at or near two indicate that the same base is always present in that position. Bit values of one indicate that two bases have equal distribution in the position. Note that because the total number of fkbA antisense reads in the wild-type strain is very small, they are probably not representative of the true distribution of sRNAs, so the logos probably overestimate any sRNA sequence bias.
Extended Data Figure 7 | Reverted strains (EM1-S, EM2-S and EM3-S) exposed to a second round of FK506 selection undergo epimutations at the same frequency as the wild-type strain. Epimutant strains (EM1, EM2 and EM3) that had reverted to an FK506-sensitive wild-type phenotype (yeast growth in the presence of FK506) after several passages on drug-free media were exposed a second time to 1 μg ml⁻¹ FK506 to ascertain if genomic mutations had occurred that enhance epimutant formation. a, The numbers of the isolates correspond to those in Supplementary Table 1. The new FK506r isolates that lacked a Mendelian mutation in any of the target genes showed abundant sRNAs complementary to fkbA based on northern blot of sRNA hybridized with an fkbA antisense-specific probe. 5S rRNA served as a loading control. Images are representative of two independent experiments. EM1-S, EM2-S and EM3-S, epimutants 1, 2 and 3 reverted to restored FK506-sensitivity; C+, EM1 before reversion of FK506-resistance. b, The frequency of epimutation was similar or lower in the reverted epimutant strains compared to the wild type, which suggests that no mutations arose promoting epimutation. In addition, because rdrp1 mutations enhance epimutation frequency and stability, the rdrp1 gene was sequenced in EM3 and found to be wild-type with no mutations. P values were obtained based on a Fisher’s exact probability test for a 2 by 2 contingency table, comparing each of the mutant strains individually versus the wild-type strain NRRL3631.
Extended Data Figure 8 | sRNAs were detected by high-throughput sequencing in the epimutant strains, but not in the wild-type and reverted strains. This pattern was not conserved in any other loci in the genome.

a, sRNAs were found to span exon–exon junctions. Antisense and sense sRNAs from EM1 that span intron 1 are shown at the top and bottom, respectively. The numbers on the left are the normalized read counts (reads per million) for each specific sRNA. Only sRNAs with five or more read counts are shown. The reference sequence is in red and sRNAs spanning the intron are in black.

b, Distribution of antisense sRNA for the *fkbA* gene across the different strains and the 50 genes with sRNA patterns most closely correlated with *fkbA*. sRNA read count distribution for the *fkbA* gene is represented with a heavy grey line (left panel). The 50 genes with the closest correlated pattern are represented with thin colored lines. The bottom part of the left panel has been expanded to elucidate the read count patterns of the correlated genes (right panel). While some of these genes show an apparent similarity to the pattern of sRNA in the *fkbA* silenced and revertant strains, the levels of read counts are in most cases ~100-fold lower than that for the *fkbA* gene, and were not detected by sRNA blots (data not shown). WT, strain NRRL3631; EM1-R, EM2-R and EM3-R, epimutants 1, 2 and 3 resistant to FK506; EM1-S and EM3-S, epimutants 1 and 3 reverted to restored FK506 sensitivity.
Extended Data Figure 9 | sRNA and antisense fkbA RNA detection in the different mutant strains lacking RNAi pathway components. a, The numbers of the isolates correspond to those in Supplementary Table 1. No epimutants (absence of sRNA complementary to fkbA) were found in the dcl2 (MU410), dcl1 (MU407), ago1 (MU413, MU426) or rdrp2 (MU420, MU428) null mutants based on sRNA northern blots hybridized with an fkbA antisense-specific probe. Only one epimutant was recovered in the second independent dcl1 mutant (MU406). We reconfirmed this result (sRNA blot, no fkbA mutation) and also validated that the isolate was dcl1Δ by PCR. ago2 (MU416) and ago3 (MU414) null mutants showed a frequency of epimutation similar to the wild-type strain (R7B). The rdrp1 null mutant (MU419) showed an elevated epimutation frequency. The conclusion that Dcl2, Dcl1, Ago1 and RdRP2 are required for epimutation is supported by the congruence of phenotype, and the analysis of two independent null mutants each for ago1, rdrp2 and dcl1. R7B served as wild type for this experiment as all of the RNAi-silencing mutants were generated in this background. 5S rRNA served as a loading control. C+, EM1 strain. Images from dicer mutant blots are representative of two independent experiments. The remaining blots were generated once. b, Total RNA was isolated from the wild type, fkbA mutant, patA mutant, R7B and the indicated RNAi pathway mutants, and 50 μg of total RNA were used to ensure signals could be detected from all of the mRNA analysed, as the level of patA expression is low. All of the silencing mutant strains have the same level of expression of the fkbA antisense RNA based on northern blot. Antisense- and sense-specific probes were used to detect antisense and sense fkbA RNA. The northern blot was first probed for antisense RNA to avoid residual signal from fkbA mRNA. patA expression was not affected in any the fkbAD or the RNAi mutant strains, but as expected was absent in the two independent patAD strains. Actin served as a loading control. Images are representative of three independent experiments.
Extended Data Figure 10 | *M. circinelloides f. circinelloides* (Mcc) and *M. circinelloides f. griseocyanus* (Mcg) strains were tested for generation of FK506r and fkbA silencing. a, The indicated *Mucor* strains, plus *M. circinelloides f. lusitanicus* (Mcl) wild-type strain used as a control, were incubated on YPD media for 3 days (top panel) and on YPD supplemented with 1 μg ml⁻¹ of FK506 for up to 10 days (lower panels). The *M. circinelloides f. lusitanicus* and 1006PhL strains grew as a yeast colony until FK506r sectors started to grow as hyphae. The Mucho strain grew as a yeast colony for several days, and in some of the plates a resistant patch appeared, but after several days the colonies developed aerial hyphae on top of the yeast colony, producing FK506-sensitive spores that grew as yeast after falling on the media, preventing the development of more FK506r patches. The *M. circinelloides f. griseocyanus* strain was more sensitive to FK506 without a visible colony until day 6 when the spores started to germinate as a mixture of yeast and hyphae that did not produce any FK506r growth. Images are representative of ~40 independent colonies from each strain. b, Confirmation of the presence of sRNAs in epimutants derived from one of the two pathogenic *M. circinelloides f. circinelloides* strains. The numbers of the isolates correspond to those in Supplementary Table 5. An antisense strain-specific probe was used to detect fkbA sRNAs by northern blot from both strains (using 30 μg of sRNA). 5S rRNA served as a loading control. Abundant sRNAs complementary to fkbA were detected in all of the FK506r strains that lacked Mendelian mutations isolated from the 1006PhL strain, but not from the Mucho strain. Images from the lower blots are representative of two independent experiments. Images from the upper blots were generated once since sRNA-positive signals were detected from all samples analysed.