RNAi epimutations conferring antifungal drug resistance are inheritable

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Summary

Epimutations modify gene expression and lead to phenotypic variation while the encoding DNA sequence remains unchanged. Epimutations mediated by RNA interference (RNAi) and/or chromatin modifications can confer antifungal drug resistance and may impact virulence traits in fungi (1–5). However, whether these epigenetic modifications can be transmitted across generations following sexual reproduction was unclear. This study demonstrates that RNAi epimutations conferring antifungal drug resistance are transgenerationally inherited in the human fungal pathogen *Mucor circinelloides*. Our research revealed that RNAi-based antifungal resistance follows a DNA sequence-independent, non-Mendelian inheritance pattern. Small RNAs (sRNAs) are the exclusive determinants of inheritance, transmitting drug resistance independently of other known repressive epigenetic modifications. Unique sRNA signature patterns can be traced through inheritance from parent to progeny, further supporting RNA as an alternative molecule for transmitting information across generations. This study marks a significant advance in understanding epigenetic inheritance, highlighting RNAi-exclusive epimutations as a widespread phenomenon in the pathogenic *M. circinelloides* species complex. Understanding how epimutations occur, propagate, and confer resistance may enable their detection in other eukaryotic pathogens, provide solutions for challenges posed by rising antimicrobial drug resistance (AMR), and also advance research on phenotypic adaptability and its evolutionary implications.

RNA | epimutation | gene silencing | inheritance | drug resistance | epigenetics | Mucorales | fungal pathogens

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Main

Whether epigenetic traits can be transmitted across generations –a process known as transgenerational inheritance– remains a subject of ongoing debate (6–8). RNAi epimutations conferring AMR were first reported in *Mucor circinelloides* formae *lusitanicus* and *circinelloides* (1), recently identified as separate phylogenetic species (PS10 and PS14) (9). However, neither species can produce viable progeny through sexual reproduction, presenting a challenge to study genetic and epigenetic inheritance. Therefore, we studied epimutations in two fertile, opposite mating types (– and +) of *M. circinelloides* phylogenetic species 15 (referred to as PS15). PS15 was selected as it is the only species among 16 in the *Mucor circinelloides* complex able to complete the sexual cycle under laboratory conditions (9). Despite this advantage, epimutation, RNAi proficiency, and meiotic recombination had not been assessed in this species, and it was unclear if the observed progeny resulted from bona fide sexual reproduction. To address these limitations, whole-genome assemblies and gene annotations for both opposite mating types (PS15– and PS15+) were generated with Oxford Nanopore Technologies (ONT) long reads (Extended Data Table 1). Each genome assembly consists of ~ 37 Mb distributed among fourteen and fifteen contigs, showing an N50 of 3.29 and 2.90 Mb for PS15– and PS15+, respectively. Both assemblies exhibit a high level of completeness, with 97.3 % of conserved eukaryotic genes present. Protein-protein similarity searches identified homologs of the principal RNAi components involved in establishing and maintaining epimutations (1, 3, 10, 11) (Extended Data Fig. 1a). Small RNA (sRNA) sequencing of both PS15– and + confirmed the presence of canonical small interfering RNAs (siRNAs), characterized by discrete lengths of 21 to 24 nt and 5' uracil (3) (Extended Data Fig. 1b).

After confirming RNAi proficiency, RNAi-targeted *fkbA* epimutants were isolated by challenging PS15– and + fungal spores with FK506, both alone and in combination with rapamycin (Fig. 1a). Both drugs target FKBP12 encoded by the *fkbA* gene, causing similar fungistatic effects: FK506 enforces yeast-like, decreased growth by inhibiting the calcineurin pathway, whereas rapamycin compromises growth by inhibiting TOR signaling (Extended Data Fig. 1c). This dual drug approach enabled efficient screening for loss of FKBP12 function. By this approach, 14 FK506-resistant isolates were obtained after five days of continuous exposure to the drug (Extended Data Fig. 1d). Seven PS15– (E1– to E6– and M1–) and six PS15+ isolates (E10+, E12+, E15+, M1+ to M3+) were selected for their resistance to FK506 and rapamycin (Fig. 1b and Extended Data Table 2), indicating loss of FKBP12 function. Sanger-sequencing revealed *fkbA* missense mutations in four isolates, M1– and M1+ to M3+ (and so named Mutants). No DNA sequence changes were found in the remaining nine isolates, E1– to E6–, or E10+, E12+, and E15+. Following mitotic passage without drug, these resistant isolates reverted to wildtype susceptibility, indicating resistance is unstable and transient (Fig. 1c). Because a similar unstable resistance due to RNAi was previously reported in other *Mucor* species (1, 12), northern blot analyses were conducted, revealing antisense sRNAs targeting *fkbA* in these unstable resistant colonies. These sRNAs were undetectable in wildtype and *fkbA* mutant negative control strains, suggesting epimutation-driven resistance (Epimutants E1– to

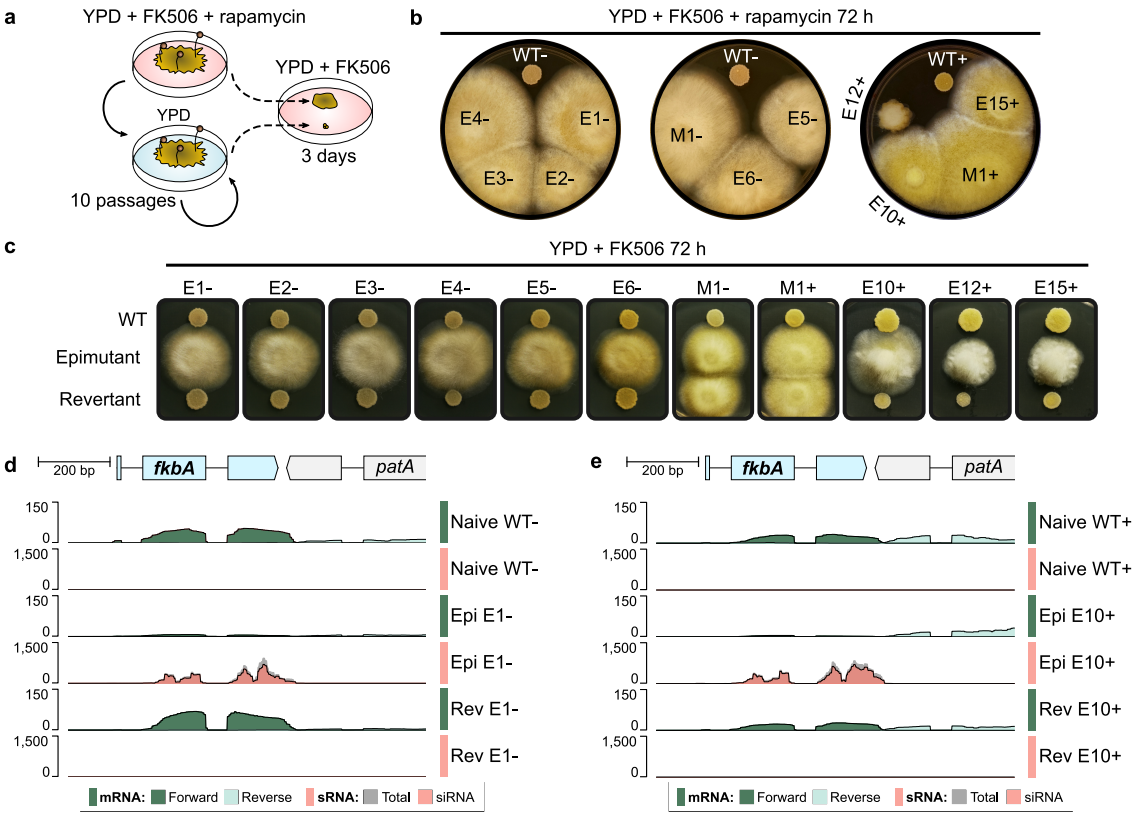

Fig. 1. Two opposite mating types from *Mucor circinelloides* Phylogenetic Species 15 (PS15) exhibit RNAi-based epigenetic resistance to FK506 and rapamycin. **(a)** Experimental design to validate FK506 reversible resistance due to loss of FKBP12 function. **(b)** Isolates from PS15 – and + mating types exhibiting FK506 resistance were challenged with both FK506 and rapamycin for 72 hours, comparing growth to wildtype strains (WT). Isolates resistant to both drugs are labelled in black, and susceptible isolates in white. Resistant isolates with wildtype *fkbA* DNA sequence are labeled as Epimutants (E); those harboring *fkbA* mutations are labeled as Mutants (M). **(c)** Epimutants and mutants were passaged on non-selective medium for ten 84-hour passages (revertants) and transferred onto FK506-selective medium with wildtype sensitive and resistant control isolates to assess reversion. **(c, d)** Genomic plots of the *fkbA* locus (1 kb) depicting the *fkbA* gene (light blue) and 3' region of the neighboring *patA* gene (light gray). Stranded messenger RNA (mRNA) and sRNA coverage tracks are color-coded for a representative Epimutant, and its corresponding WT control and Revertant isolate from the **(c)** – and **(d)** + mating type. Stranded mRNA coverage was aligned to the forward and reverse strands and overlaid in different shades of green. sRNA coverage shows all aligning reads (total, dark gray) overlaid with reads exhibiting typical small interfering RNA features (siRNA, red), i.e., antisense 21-24 nt reads with a 5'-uracil.

E6–, E10+, E12+, and E15+, Extended Data Fig. 1e). RNAi epimutations were further analyzed by sRNA and ribosomal RNA (rRNA)-depleted RNA sequencing. This revealed that resistant isolates harbored *fkbA* antisense siRNAs, resulting in a decrease of *fkbA* messenger RNA (mRNA). Upon removal of drug selective pressure, revertant isolates lost siRNAs and *fkbA* mRNA was restored to wildtype levels (Fig. 1d, e). We have now found RNAi-epimutations conferring FK506 and rapamycin resistance in three different *Mucor* phylogenetic species.

Previous studies (1, 12) and this work shows that RNAi epimutations in *Mucor* species are stable enough to be transmitted throughout multiple rounds of mitotic division before reversion. Therefore, we hypothesized that epimutations might be similarly transmitted through meiosis and sexual reproduction without drug selective pressure. To test their heritability and determine the genetic basis of the resistance trait, we obtained F1 progeny from a series of genetic crosses. First, an epimutant and a wild type were crossed, utilizing epimutants from both mating types (Fig. 2a, b). Second, two epimutants from opposite mating types were crossed (Fig. 2c). After successful mating of haploid, opposite-mating type mycelia, the reproductive structures known as zygospores were formed (13). Zygospores from each cross were individually dissected and germinated after a dormancy period of 2 to 8 weeks, exhibiting germination rates from 1 to 2%. Within each zygospore, haploid nuclei fuse into a diploid that undergoes meiosis (14, 15). Only one of the four meiotic products is mitotically amplified to form a germsporangium containing genetically identical germspores (16, 17), which were considered F1 progeny. Drug susceptibility to FK506 alone (Extended Data Fig. 2a-c) and in combination with rapamycin (Fig. 2a-c) was assessed in the progeny without any intermediate passaging. Resistance was considered inherited if observed in both conditions. This implies the resistance was present in the progeny prior to the drug challenges, rather than arising from two independent de novo epimutations due to drug exposure. The cross with the – mating type epimutant yielded 6 out of 22 resistant progeny (Fig. 2a), which is inconsistent with a 1:1 inheritance ratio expected from Mendelian inheritance of a monogenic trait in haploid organisms (Chi-square test, p-val 0.03301). Similarly, the + mating type epimutant yielded a 2 out of 11 resistance inheritance ratio (Fig. 2b, Chi-square test, p-val 0.03481). Together, these results indicate that resistance can be inherited from either mating type at similar rates, ruling out mat-

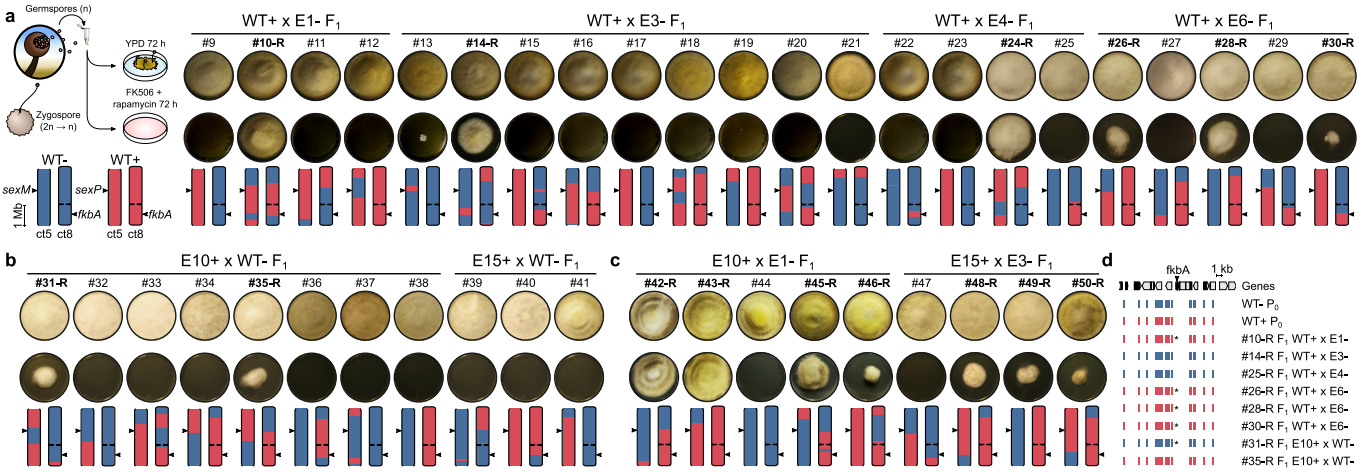

Fig. 2. FK506 resistance displays non-mendelian inheritance ratios in progeny from sexual reproduction. (a-c) *M. circinelloides* PS15 progeny from sexual crosses are shown: **(a)** – mating type epimutant crossed with + mating type wildtype strain, **(b)** + mating type epimutant crossed with – mating type wildtype strain, and **(c)** + mating type epimutant crossed with – mating type epimutant. Growth on YPD as a viability control and on FK506+Rapamycin medium is shown for each progeny, along with two contigs containing the *sex* (ct5) and *fkbA* (ct8) loci, indicated by arrows. The contig drawings depict the inheritance pattern of SNVs (– mating type in blue and + mating type in red), demonstrating meiotic recombination and/or independent chromosome assortment. Ct5 is depicted as an incomplete chromosome arm lacking telomeric repeats (open ends), whereas ct8 is a full chromosome depicting a centromere (constriction) and telomeric repeats on both ends (closed ends). **(d)** Genomic plot showing a 20 kb region centered on the *fkbA* gene. Genetic variation (shown as SNVs) inherited from either mating-type wildtype parent (WT P_0) is shown for each resistant (-R) progeny isolated from the crosses of epimutants with wildtype.

ing type bias or uniparental inheritance. Importantly, resistance was inherited at significantly lower ratios than those expected from Mendelian inheritance, supporting RNAi epimutations as epigenetic traits. The remaining progeny showed wildtype susceptibility to FK506, suggesting the epimutation was either lost or not inherited. On the other hand, crossing two epimutants yielded a higher frequency of drug-resistant progeny (7 out of 9, Fig. 2c) but still inconsistent with a 100% inheritance expected from a Mendelian monogenic trait present in both haploid parents (Chi-square test, *p*-val = 0.01776). Control crosses between two naïve wildtype isolates yielded only susceptible progeny (8 out of 8, Extended Data Fig. 2d). Similarly, F1 progeny from an *fkbA* mutant showed resistance ratios consistent with Mendelian inheritance (3 out of 5, Extended Data Fig. 2e), highlighting that mutations and epimutations affecting *fkbA* show distinct inheritance rates.

Molecular analysis demonstrated that the progeny isolated from these crosses resulted from bona fide sexual reproduction including meiosis. We analyzed genetic variation across the PS15– and PS15+ genomes, identifying a total of 18,731 single nucleotide variants (SNVs) across 13 genomic blocks that exhibited full synteny and contiguity in both opposite mating types (Extended Data Fig. 3). These synteny blocks include six telomere-to-telomere, complete chromosomes and seven contigs, with nine annotated centromeres. By identifying inherited genetic variation across these synteny blocks, we were able to show meiotic recombination events and independent chromosome assortment in all of the F1 progeny isolates (Fig. 2a-c and Extended Data Fig. 4). The distribution of the inherited SNVs revealed the source of the *fkbA* allele in the progeny. Resistant progeny inherited the *fkbA* locus at ratios consistent with Mendel's laws (5:3, Chi-square test, *p*-val 0.4795). The results demonstrate that the epimutation resistance is not genetically linked to the *fkbA* locus, as half of the progeny that inherited resistance from the epimutant parent inherited the *fkbA* allele from the naïve wildtype parent (Fig. 2d, asterisks).Similarly, susceptible progeny inherited the *fkbA* allele from either parent, regardless of their susceptibility.

Substantial evidence supports epigenetic inheritance across a range of eukaryotes, including animals, plants, fungi (8), and other eukaryotic microorganisms (18). This inheritance mainly relies on chromatin modifications, such as histone H3 lysine 9 methylation (H3K9me) and/or DNA 5-methylcytosine (5mC). Although *Mucor* spp. lack the necessary components for 5mC (19–21), heterochromatin can form and be maintained through H3K9me (20). Therefore, we hypothesized that RNAi and H3K9me could be coupled to stabilize epimutations, allowing their inheritance in the progeny. This hypothesis was tested by chromatin immunoprecipitation (ChIP) targeting H3K9me2 in PS15 epimutant parents and progeny. H3K9me2-based heterochromatin is abundant in repeats, particularly at centromeres and telomeres (Fig. 3a), consistent with previous findings in *Mucor lusitanicus* PS10 (20). No H3K9me2 was detected at either the *fkbA* locus in epimutant parents or their drug-resistant progeny (Fig. 3a, b). Instead, abundant siRNAs target *fkbA* in these epimutant isolates, and this results in a decrease in *fkbA* mRNA levels (Fig. 3b). Although decreased, *fkbA* transcription is still active during epimutation, and RNA polymerase II (RNAP) binding was detected across this locus (Fig. 3b). Whole-genome analysis of the progeny revealed neither changes in the *fkbA* DNA sequence nor chromosomal rearrangements contributing to the observed resistance (Extended Data Fig. 5), supporting RNAi as the exclusive cause for epimutational resistance. Taken together, these findings indicate that *Mucor* epimutations act posttranscriptionally and depend exclusively on RNAi.

These findings were generalized to other *Mucor* phyloge-

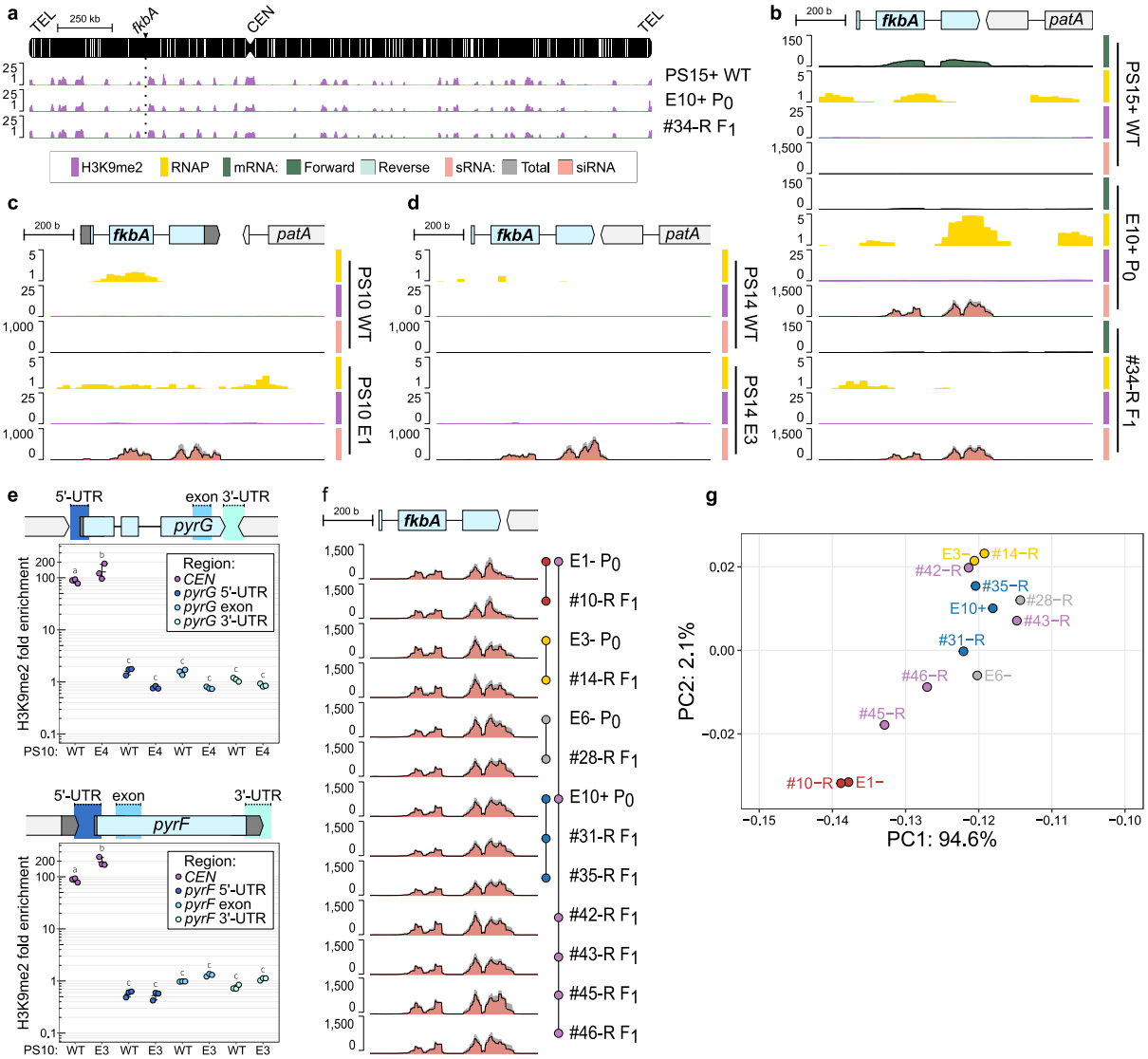

Fig. 3. Inherited epigenetic resistance is exclusively driven by RNAi and independent of heterochromatin marks. (a-d) Genomic plots showing H3K9me2 enrichment (purple), RNA Polymerase II enrichment (yellow), stranded mRNA (overlaid shades of green for forward and reverse strand), and/or sRNA (dark gray for total sRNA overlaid with red for siRNA) in **(a-b)** *M. circinelloides* PS15 wild type, an epimutant parent (P_0) and its F_1 progeny across **(a)** the full chromosome containing the *fkbA* gene (arrowhead and dotted line). Repeats are depicted as black vertical lines, the centromere as a constriction (*CEN*), and telomeric sequences as rounded ends (*TEL*); **(b)** a magnified view of the *fkbA* locus (1 kb); **(c, d)** a wild type (WT) and *fkbA* epimutant (E) from **(c)** *Mucor lusitanicus* PS10 and **(d)** *M. circinelloides* PS14. **(e)** Lack of H3K9me2 ChIP enrichment across *pyrG* and *pyrF* regions is shown for *fkbA* susceptible (WT) and epimutant (EM) *M. lusitanicus* PS10 isolates. For each gene, amplicons corresponding to the 5'- and 3'-UTRs and exonic regions were tested by qPCR, as indicated in the gene model. A highly enriched centromeric amplicon (*CEN*) was also included for comparison. Average values and standard deviations were derived from technical triplicates. Significant differences were determined using a one-way ANOVA and Tukey HSD tests. Average values not showing significant differences are indicated by the same letter. **(f)** The *fkbA* genomic plot shows total and siRNA coverage for a representative set of epimutant parental (P_0) and F_1 resistant progeny (#-R) isolates. Parents and progeny are represented by colored circles connected by lines. **(g)** Principal component analysis of the relative abundance of sRNA sequences complementary to the *fkbA* locus in epimutant parents and progeny shown in **(f)**. Progeny from a single epimutant donor cross share the same color as their parent, while progeny from a double epimutant donor cross display mixed colors from both parents (e.g., purple for red and blue parents).

netic species that exhibit RNAi epimutations targeting *fkbA*, *M. lusitanicus* PS10 and *M. circinelloides* PS14 (1). Similarly, we found siRNAs indicative of active *fkbA* posttranscriptional silencing, but no H3K9me2 modification at this locus and no changes in RNAP occupancy between wildtype and epimutant strains (Fig. 3c and e, PS10 and PS14, respectively). In addition to *fkbA*, previously identified epimutations conferring 5-Fluoroorotic acid (5-FOA) resistance by silencing the *pyrG* and *pyrF* pyrimidine biosynthetic genes in *M. lusitanicus* PS10 (12) were also tested by ChIP followed by quantitative PCR. Neither *pyrG* nor *pyrF* exhibited any enrichment in H3K9me2 during epimutation (Fig. 3e).

Because chromatin marks are not repressing *fkbA* transcription during epimutation, siRNAs are apparently functioning as exclusive epigenetic agents of inheritance. We therefore questioned whether siRNAs might be traceable from parents to their progeny. To test this, we analyzed the siRNA profiles and features in a representative set of epimutant parents and their progeny (Fig. 3f). All drug-resistant epimutant progeny harbored abundant sRNAs targeting *fkbA*, which displayed features consistent with canonical siRNAs. These features include being mainly antisense to the transcript exons, having a defined length of 21 to 24 nt, and possessing uracil at the 5' position (Fig. 3f and Extended

Data Fig. 6a-c). There is also a notable bias in accumulating siRNAs towards the last exon of the *fkbA* gene. But more importantly, a striking similarity was observed in the relative abundance of siRNAs between epimutant parents and their epimutant progeny (Fig. 3f and Extended Data Fig. 6c). The progeny from two epimutant parents displayed a blend of both parental siRNA profiles, usually favoring one parent. To validate these similarities, Principal Component Analysis (PCA) was conducted on the relative abundances of siRNA molecules complementary to the *fkbA* transcript from each epimutant. Progeny from a single epimutant donor closely clustered with their epimutant parent (Fig. 3g, red, blue, yellow, and black), whereas progeny from two epimutants were positioned between the parents, or aligned more closely to one parent (Fig. 3g, progeny in purple, red and blue parents). Taken together, these findings suggest that individual epimutants exhibit a detectable pattern of siRNA, similar to a fingerprint.

DNA is classically regarded as the molecular basis of inheritance, but substantial evidence supports the existence of an RNA world that preceded the current central dogma of molecular biology (22). In addition to catalyzing chemical reactions, RNA might have stored genetic information and transmitted it across generations. Presently, RNA viruses transmit genetic information by hijacking the synthesis machinery of their hosts, including capsid-free, vertical RNA transmission (23). But there are more examples beyond viral mechanisms, as there is also transmission of maternal RNA and proteins to oocytes, influencing early zygotic development (24), and traumatic stress can alter microRNA expression in sperm (25). Our research demonstrates that spontaneous epigenetic drug resistance can be transgenerationally inherited in living organisms, relying exclusively on RNA as the information molecule.

Our current understanding of epigenetic inheritance is mostly based on chromatin modifications, which affect DNA directly, e.g. 5mC, or DNA-binding histone proteins. However, RNA-based epigenetic inheritance remains poorly understood. Nuclear-independent, engineered RNAi epimutation inheritance has been observed across generations in the nematode *Caenorhabditis elegans* (26), but it is challenging to untangle the effects of chromatin modifications because sRNAs guide heterochromatin formation in this organism (27), and many others (28). H3K9me2 coupled with RNAi drives epimutations conferring caffeine resistance in *Schizosaccharomyces pombe* (4), but whether these epimutations are inheritable following sexual reproduction has not been reported. It is also unclear to what extent either heterochromatin or RNAi contributes, i.e., which is the initial driver of the epimutation. Recently, we showed that RNAi is not required to maintain H3K9me-based heterochromatin at transposable elements, suggesting that these two mechanisms function independently in Mucorales (20). Our findings build on this and indicate that de novo RNAi is not triggering heterochromatin formation in either epimutants or their drug-resistant progeny. More importantly, siRNAs can function as the sole molecular determinants that cause, maintain, and transmit RNAi epimutations, uncoupled from chromatin modifications commonly associated with epigenetic inheritance in other organisms.

RNAi inheritability is independent of mating type. Notably, it is also independent of the genetic locus targeted by the epimutation, as shown by multiple epimutant progeny that inherited the *fkbA* allele from a naïve, drug susceptible wildtype parent. Mucoralean sexual reproduction begins with the contact of sexually determined, engorged hyphae called zygophores, which develop into progametangia. As progametangia differentiate into gametangia, they become separated from the mycelium by septae, and fuse to form a zygosporangium and zygospore (13, 29). Plasmogamy and shared cytoplasmic contribution from both gametangia, combined with our findings, suggest cytoplasmic inheritance is the most parsimonious model for siRNA transmission. Cytoplasmic inheritance would explain the higher inheritance rates observed with two epimutant parents, implying that siRNA availability at the tip of the progematangia prior to septa formation is a crucial determinant for inheritance. Previous studies have shown that the RNAi components Dcl2, Ago1, Qip1, and Rdrp2 are essential for initiating and maintaining both epimutations and canonical gene silencing (1, 10, 11). Rdrp2 functions as the primary RdRP, amplifying secondary siRNAs and thereby propagating RNAi throughout multiple mitotic divisions (30). However, the role of Rdrp2 in siRNA inheritance is challenging to ascertain as *rdrp2*Δ mutants are unable to mate and produce zygospores (31). Given that heritable sRNAs in the epimutant progeny harbor typical features of canonical siRNAs, we propose that the canonical RNAi mechanism may extend beyond vegetative growth to preserve epimutations during sexual reproduction, similarly to *C. elegans* (32, 33).

Our findings contribute to the growing evidence supporting epigenetic transgenerational inheritance, which allows organisms to adapt rapidly to environmental pressures by transmitting epigenetic modifications across generations before genetic changes can occur. Once the environmental pressure is relaxed, epimutations can readily revert, providing remarkable adaptive phenotypic plasticity. This phenomenon is particularly concerning in pathogenic microbes that maintain epigenetic modification (5, 34, 35) and/or RNAi machinery (36, 37). Fungal infections already impose a significant burden on global health and agriculture –a concern highlighted by the World Health Organization to encourage more research into antifungal resistance (38). In recent years, epimutations have been identified as a novel mechanism that confers AMR (1, 4), posing a significant threat as they may evade detection due to their transient and adaptive nature. Understanding how epimutations are inherited and the epigenetic mechanisms driving resistance may enable their detection in clinical settings and offer solutions to the growing challenges posed by AMR.

Methods

Fungal strains, culture, and drug susceptibility testing. Extended Data Table 2 lists the fungal strains utilized in this work. All generated strains were derived from *M. circinelloides* CBS394.68 (PS15–) and/or CBS172.27 (PS15+) (9). To conduct experiments, asexual spores were freshly harvested after 4-6 days incubation in yeast-peptone-dextrose (YPD) at room temperature. Spores were challenged with FK506 (1 μg/ml) and/or rapamycin (100 ng/ml) to obtain resistant isolates, test drug susceptibility, and maintain epimutations. Unstable resistant isolates were reverted to drug susceptibility by 84-hour, non-selective passages in YPD.

Nucleic acids isolation, blotting, immunoprecipitation, and sequencing. Mycelium cultures were collected from YPD –with or without FK506 as needed–, flash froze in liquid N_2 and ground into a powder for nucleic acid isolation. Genomic DNA samples for Illumina sequencing were prepared using Norgen Biotek Yeast/Fungi Genomic DNA Isolation Kit from overnight, broth cultures. Genomic DNA libraries were constructed with Roche KAPA HyperPrep Kit. 150-bp paired-end reads were obtained utilizing the NovaSeq X Plus sequencing system. Concurrently, *fkbA* sequences were PCR-amplified using primer pairs listed in Extended Data Table 3 and analyzed by Sanger-sequencing. Ultra-high molecular weight DNA was isolated following a phenol/chloroform-based DNA extraction method (21) from PS15– and + 6-hour pregerminated spores. DNA was ligated with barcodes (EXP-NBD104, NB10 for PS15– and NB12 for PS15+) and sequencing adapters (SQK-LSK110) to prepare ONT MinION libraries, and loaded into a single MinION flowcell (FLO-MIN106) for a 72-hour sequencing run.

Total RNA samples from 72-hour cultures in YPD agar were prepared with QIAGEN miRNeasy Mini Kit and divided into small and long RNA preparations. sRNAs were subjected to electrophoresis in urea acrylamide gels (Invitrogen), transferred, and chemically crosslinked (EDC) to neutral nylon membranes (Hybond NX, Amersham) as previously described (39). *fkbA* sense, $\alpha[^{32}$P]UTP-labelled radioactive probes were digested into small fragments using an alkaline solution and hybridized to detect *fkbA* antisense sRNAs, using similarly prepared *5.8S* rRNA antisense digested probes as a loading control. sRNA libraries were amplified using QIAseq miRNA library kit and 75-bp single-end reads were sequenced with Illumina NextSeq 1000 High-Output sequencing system. rRNA-depleted RNA libraries (long RNA) were prepared using Illumina Stranded Total RNA Prep and Ribo-Zero Plus rRNA Depletion Kit using *M. circinelloides* rDNA-specific probes, and 150-bp paired-end reads were obtained with NovaSeq X Plus sequencing system.

Overnight, YPD broth cultures were processed for ChIP as described in previous research (20, 40). Briefly, cultures were crosslinked, lysed, and chromatin sonicated throughout 35 cycles 30 s ON/OFF in a Bioruptor (Diogenode) to obtain 100-300 bp chromatin fragments. Sheared chromatin aliquots were stored as input DNA controls. ChIP-grade antibodies α-H3K9me2 (ab1220, Abcam) and α-RNA pol II (39497, Active Motif), as well as protein A or G magnetic beads accordingly, were utilized to immunoprecipitate chromatin-bound DNA. After washing and decrosslinking, DNA was purified by a phenol/chloroform-based extraction method. Input and ChIP DNA duplicates from each strain and condition were pooled together. Libraries were prepared using Roche KAPA HyperPrep Kits and sequenced with Illumina NovaSeq X Plus sequencing system for 150-bp paired-end reads. For quantitative PCR, ChIP and Input DNA were amplified using SYBR Green PCR Master Mix (Applied Biosystems) with specific primer pairs (Extended Data Table 3). qPCRs were performed in triplicate using a QuantStudio™ 3 real-time PCR system. Fold enrichment was determined as a relative quantification to the Input DNA ($2^{-\Delta CT}$) and to the negative control *vma1* values ($2^{-\Delta\Delta CT}$), a constitutively active gene lacking heterochromatin marks.

Genome assembly, annotation, and homolog identification. Long, ONT-based, FASTQ raw reads were obtained from MinION raw output and demultiplexed into PS15– and + reads with ONT basecaller Dorado v0.7.0 (https://github.com/nanoporetech/dorado/). Draft assemblies were generated using Flye v2.9.3-b1797 (41) and polished with Racon v1.5.0 (42). Assemblies were further corrected with NextPolish1 v1.4.1 (43) and 2 v0.2.0 (44) using long, ONT-based and Illumina-based, adapter-trimmed short reads. Long and short reads were aligned with Minimap2 v2.26-r1175 (45) to examine coverage as a measure of assembly correctness. Similarly, contig sequences were aligned and visualized using Circos v0.69-8 (46) to examine synteny between both assemblies (Extended Data Fig. 3). 13 alignment blocks that showed perfect synteny and contiguity between both assemblies –referred to as synteny blocks– were selected for further analyses. Repeated sequences were identified utilizing RepeatModeler2 v2.0.5 (47) and classified by iterative similarity searches against the RepBase database for RepeatMasker (Edition-20181026) (48) and already classified repeats. After masking the genomes with these repeat libraries and RepeatMasker v4.1.6 (http://www.repeatmasker.org/), gene annotation was conducted using the Funannotate pipeline v1.8.15 (49) and training models generated with our RNA data. Genome assemblies were evaluated using QUAST v5.2.0 (50) for contiguity metrics and the eukaryotic ortholog database (eukaryota_odb10) from BUSCO v5.7.1 (51) for completeness (Extended Data Table 1). RNAi components were identified by NCBI BLASTp v1.12.0 (52) searches using *M. lusitanicus* protein sequences as queries, returned positive reciprocal BLASTp hits, and their protein domain configuration was predicted with InterProScan v5.70-102.0 (53) and examined to evaluate their putative function (Extended Data Fig. 1a).

Mating assays, zygospore isolation, and progeny germination. Opposite mating-type spores were inoculated onto four spots in whey agar plates, allowing them to grow facing each other in dark conditions and room temperature. After 7 days, zygospores that formed at the intersection of opposite mating types were scrapped from the agar using tweez-

ers and suspended in distilled water. Zygospores were germinated according to previous research (9, 14, 16). Briefly, the suspensions were successively pass through 40 and 100 µm cell strainers, discarding asexual spores and hyphal fragments and therefore, enriching in zygospores. Single zygospores were manually dissected onto agarose 1% plates at pH 4.0 and incubated at room temperature for 2-8 weeks until germination. Germspores from germinating zygospores were collected in water using a wet micropipette tip.

Genetic variation analyses. Low quality and adapter sequences were removed from genomic DNA raw reads using fastp v0.23.4 (54). For this and subsequent analyses, unless otherwise stated, the newly generated PS15- genome was selected as the reference genome to directly compare all of the samples. Processed reads were aligned with BWA-MEM v0.7.17-r1188 (55) ensuring Picard compatibility, and resulting aligments were sorted and further processed to mark duplicates and assigned read groups using the GATK4 suite v4.4.0.0 (56). Variant calling was performed on PS15– and + by GATK4 HaplotypeCaller. Low quality variants (QD < 20.0, QUAL < 30.0, SOR > 3.0, FS > 60.0, MQ < 40.0) and variants that were concordant between PS15– and PS15+ were filtered by GATK4 VariantFiltration, as well as variants that overlapped with repeated sequences using Bedtools v2.30.0 (57), generating high-quality, PS15+ specific SNVs derived from non-repetitive genomic sequences. A total of 18,731 SNVs overlapped with the synteny blocks previously identified and were used to characterize genetic variation in the progeny datasets. Parent and progeny processed alignments were piled up against this set of variants, establishing a threshold of $\geq$ 95 % read support towards either reference (PS15–) or alternate (PS15+) position to be considered inherited SNVs. Same parent variants closer than 25 kb were merged to avoid overplotting during rendering (Extended Data Fig. 4). DNA coverage was assessed with Deeptools2 bamCoverage v3.5.4 (58) and coverage plots were centered to approximately whole-genome average coverage, which was assumed to represent a haploid genome (Extended Data Fig. 5).

RNA-sequencing analyses. sRNA raw reads were trimmed by Trim Galore! v0.6.10 (https://github.com/FelixKrueger/TrimGalore) and stringent parameters (--stringency 4) to avoid random 3'-end trimming. Processed reads were aligned using Bowtie v1.3.1 (59). Coverage files were generated using bamCoverage and normalized to counts per million (CPM). Alternatively, processed reads were aligned with STAR v2.7.11b (60) to assess splice junction read support. Samtools v1.10 (61) and Bioawk v20110810 (https://github.com/lh3/bioawk) were used to count and/or filter reads harboring specific siRNA features. Relative abundances of each 21-24 nt, *fkbA* antisense unique read harboring a 5'-uracil (siRNAs) were computed ($\text{read number} \setminus \text{total fkbA reads}$), and this standardized relative abundances were utilized to perform a PCA to assess similarities among samples.

Long RNA raw reads were trimmed with fastp and aligned with STAR. Alignments were split into forward and reverse strand to generate stranded coverage files with bamCoverage and normalized to counts per million reads (CPM).

Chromatin immunoprecipitation-sequencing analyses. Raw read samples were processed with fastp to remove adapter and low-quality sequences. Processed reads were aligned with BWA-MEM. Coverage of ChIP-enrichment was determined as the IP/Input DNA ratio using bamCompare v3.5.4 and normalized to CPM. Broad ChIP-enriched regions were identified by MACS2 v2.2.9.1 (62).

Statistical information. Inheritance ratios in the progeny were compared to the expected values of monogenic trait inheritance in haploid organisms with only one parent exhibiting the trait (1:1 ratio, $p = 0.5, 0.5$), or both parents exhibiting the trait (1:0 ratio, $p = 0.95, 0.05$ to avoid expected frequency = 0 and to account for other biological variation, e.g., spontaneous mutations). Statistical significance was assessed by Chi-square tests with a 95% confidence interval (CI), using 2 by 2 contingency tables and 1 degree of freedom.

One-way ANOVA testing was conducted to determine significant differences among ChIP qPCR fold enrichment values (n = 3), using Tukey's Honestly Significant Distance (HSD) to avoid false positives due to multiple pairwise comparisons. Tests were performed with a 95% CI and pairwise comparison results were summarized by a compact letter display using the R package multcomp (63).

Data availability. Genome assemblies, gene annotation, and raw ONT and Illumina-based raw reads will be accessible under NCBI's Sequence Read Archive (SRA) project accessions PRJNA1168935 (PS15–) and PRJNA1168941 (PS15+). The remaining raw sequencing data will be accessible under PRJNA1170303, including small RNA, rRNA-depleted RNA, ChIP, and Illumina Whole-genome sequencing. Sequencing raw data will be available upon publication. In addition, *M. lusitanicus* PS10 wildtype ChIP and small RNA data was retrieved from the publicly available project accession PRJNA903107 (20) and used to compare our findings.

Author contributions

C.P.-A, M.I.N.-M, and J.H. conceptualized the project; C.P.-A and M.I.N.-M designed the experiments; C.P.-A, M.I.N.-M, and Z.X. performed the experiments; G.W. provided biological resources and critical advice on mating; C.P.-A and M.I.N.-M assembled and annotated the genomes; C.P.-A and M.I.N.-M analyzed and interpreted the data; C.P.-A and M.I.N.-M generated figures; C.P.-A and M.I.N.-M wrote the original draft; C.P.-A, M.I.N.-M, and J.H. edited the original draft; C.P.-A, M.I.N.-M, Z.X., J.H., and G.W. reviewed the final manuscript.

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the decision to submit the work for publication. J.H. is Codirector and Fellow of the CIFAR program Fungal Kingdom: Threats & Opportunities.

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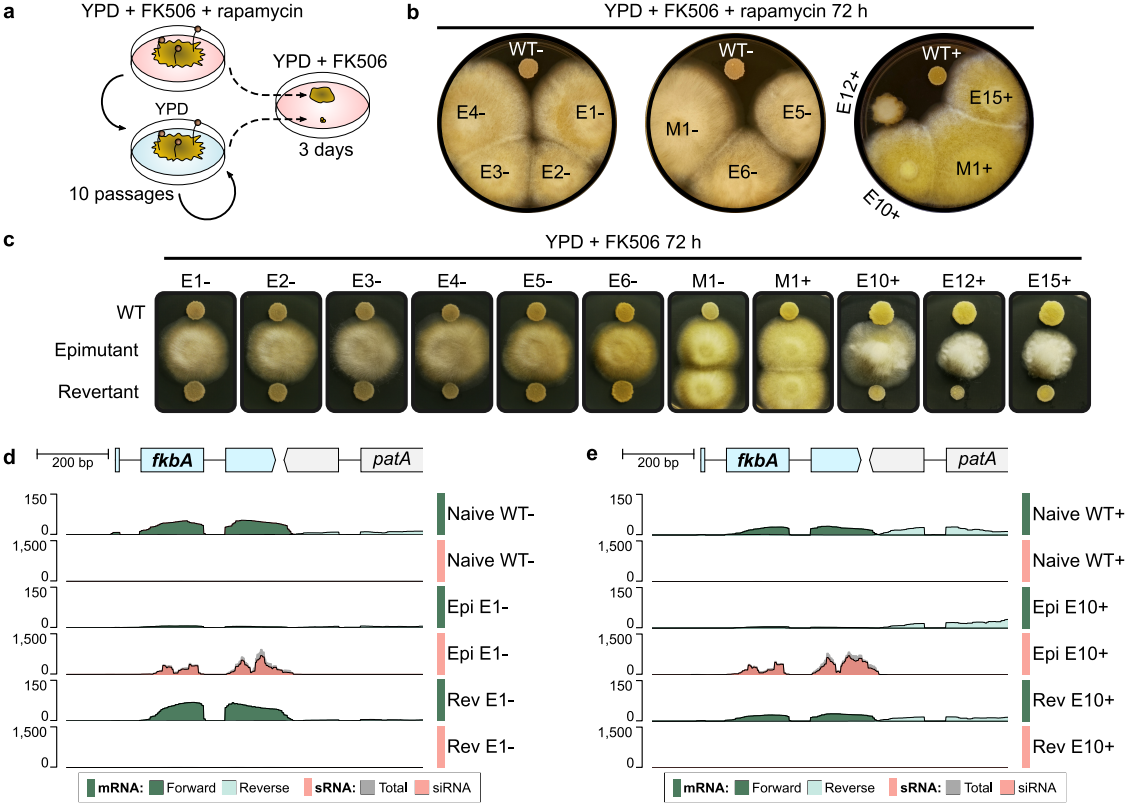
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YPD + FK506
YPD
3 days
10 passages
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E2-
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E6-
WT+
E12+
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c
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E3-
E4-
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M1+
E10+
E12+
E15+
WT
Epimutant
Revertant
d
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patA
150
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0
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Epi E1-
Rev E1-
Rev E1-
mRNA: Forward Reverse
sRNA: Total siRNA
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fkbA
patA
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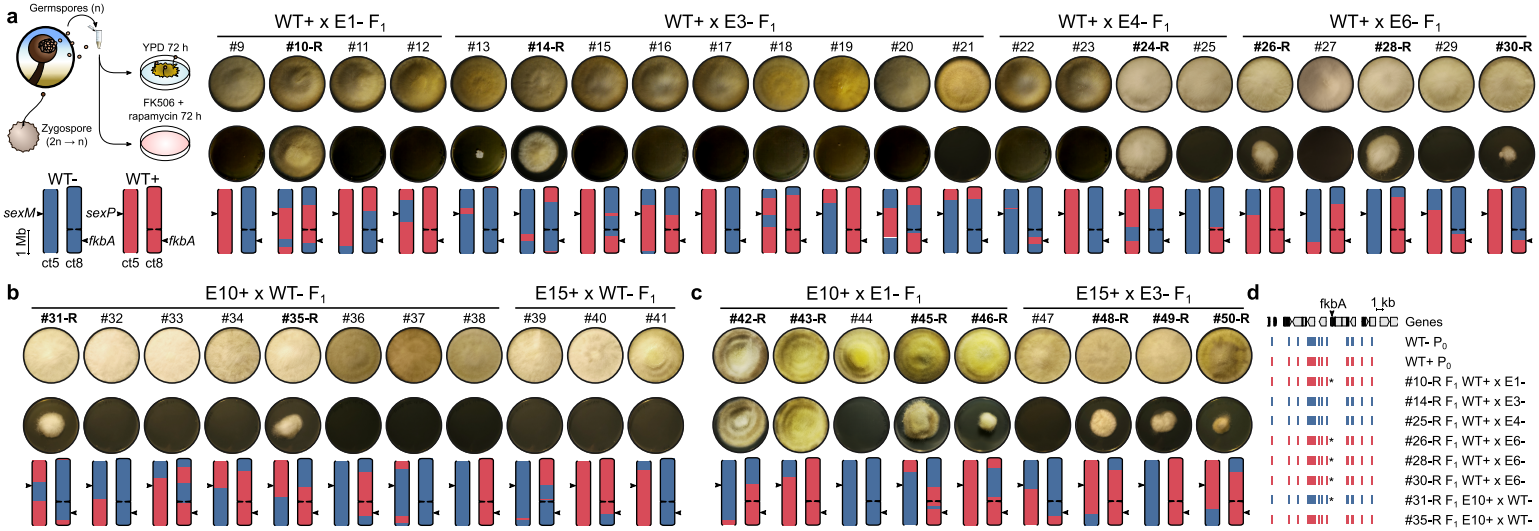
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sexP
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ct5 ct8
WT+ x E1- F1
#9 #10-R #11 #12
WT+ x E3- F1
#13 #14-R #15 #16 #17 #18 #19 #20 #21
WT+ x E4- F1
#22 #23 #24-R #25
WT+ x E6- F1
#26-R #27 #28-R #29 #30-R
b
E10+ x WT- F1
#31-R #32 #33 #34 #35-R #36 #37 #38
E15+ x WT- F1
#39 #40 #41
c
E10+ x E1- F1
#42-R #43-R #44 #45-R #46-R
E15+ x E3- F1
#47 #48-R #49-R #50-R
d
fkbA
1 kb
Genes
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WT+ P0
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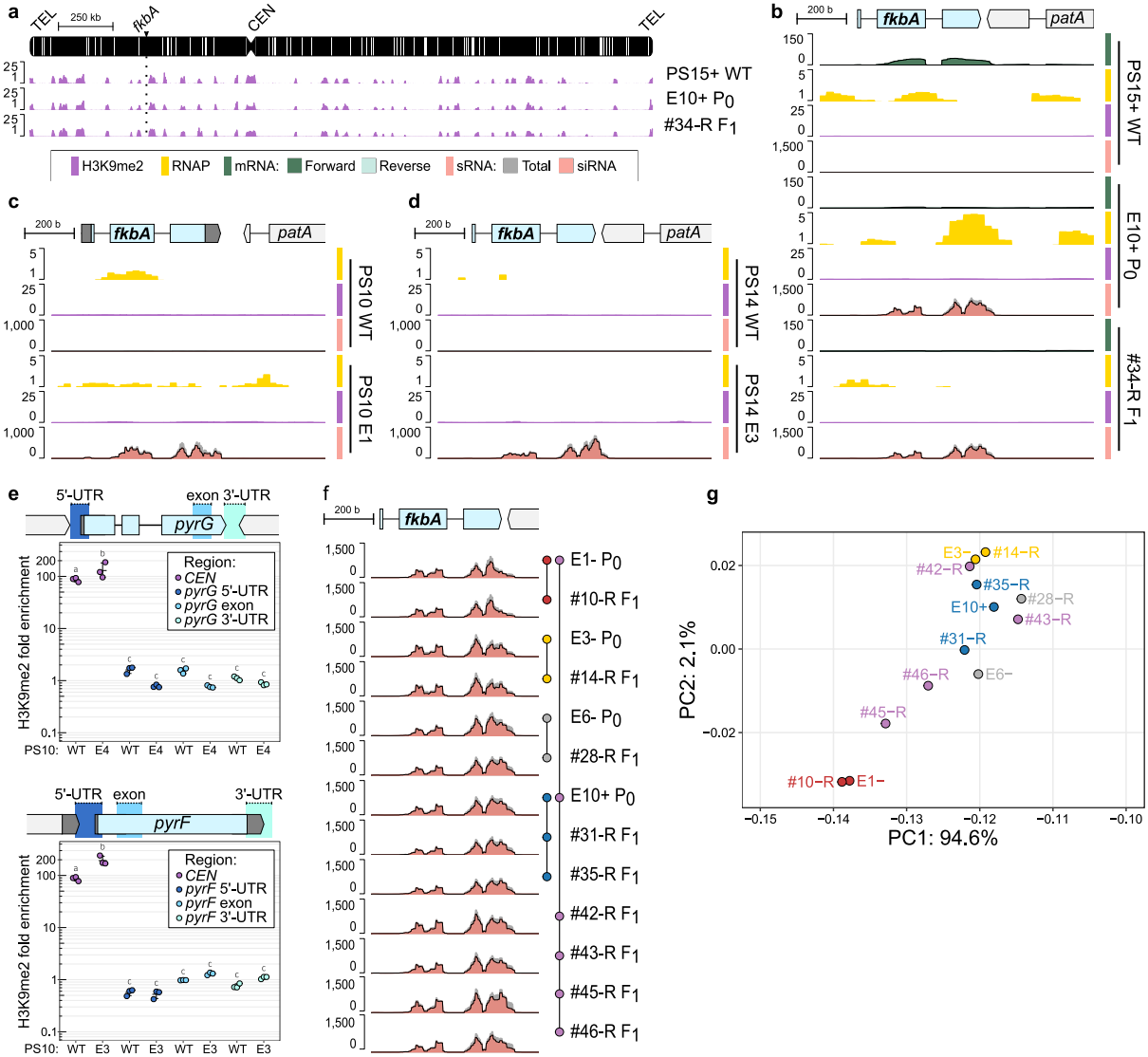
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pyrF 3'-UTR
H3K9me2 fold enrichment
PS10: WT E3 WT E3 WT E3 WT E3
f
200 b
fkbA
E1- P0
#10-R F1
E3- P0
#14-R F1
E6- P0
#28-R F1
E10+ P0
#31-R F1
#35-R F1
#42-R F1
#43-R F1
#45-R F1
#46-R F1
g
PC2: 2.1%
PC1: 94.6%
E3−
#14−R
#42−R
#35−R
E10+
#28−R
#43−R
#31−R
#46−R
E6−
#45−R
#10−R
E1−