Supporting Information

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SI Methods

Strains and Culture Conditions. *Candida albicans* strains SC5314, WO1, 1006, and NUM114 were used in this study. Methylated DNA from *C. albicans* was isolated from strain SC5314 grown in Lee's medium at 37 °C containing either low or high nitrogen concentrations favoring yeast or hyphal growth, respectively (1). To study the effect of iron dosage on DNA methylation and transcription, strain SC5314 was grown in a defined medium containing either low (1 μM) or high (100 μM) ferrous ammonium sulfate. The composition of the iron-defined medium and growth conditions were used as described (2). Phenotypic switching strain WO1 was used to examine the DNA methylation in white and opaque cells using medium and growth conditions as described (3). To examine the effect of loss of methylation, liquid medium was supplemented with 100 μM AzaC (Sigma).

Preparation of a Methyl DNA Binding Column. A methyl DNA binding column was prepared essentially as described (4) with some modifications. Briefly, the methyl-CpG binding domain (MBD) of the mouse MeCP2 gene was amplified by PCR with primers that contained an NdeI site at the 5′ end and a BamHI site at the 3′ end of the DNA fragment. PCR conditions and primers were used as described (4) except one primer was modified to introduce an NdeI site (5′-ccgggatccgctccctctcccagttaccgt-3′). The PCR product was digested with BamHI and NdeI restriction enzymes, gel purified, and cloned into the NdeI and BamHI sites of expression vector pET19b (Novagen) to give pET19bhMBD. The recombinant hMBD protein was expressed in *Escherichia coli* strain Rosetta 2 (DE3) pLysS (Novagen) and was concentrated by using a Qiagen Ni-NTA Kit following the manufacturer's protocol (Qiagen). The hMBD protein was coupled to Ni^{2+}-NTA agarose beads in a chromatography column (Qiagen). As a control, methylated Sau3AI-digested pBluescript KS(−) vector DNA (Agilent) was prepared in vitro by using CpG methylase (New England Biolabs). Methylated and unmethylated vector DNA fragments were loaded onto the column in buffer B (4) and eluted with a step gradient of 0.1–1 M sodium chloride in buffer B. The eluted DNA fractions were extracted with phenol-chloroform and precipitated with ethanol. Resuspended DNA from each fraction was electrophoresed and transferred to a Zeta-Probe GT nylon membrane (Bio-Rad) by using standard Southern blotting procedure and probed with [α-^{32}P]dCTP-labeled methylated and unmethylated vector DNA.

Identification of the Methylated Fraction of *C. albicans* Genomic DNA. Sau3AI-digested total genomic DNA from *C. albicans* strain SC5314 was passed through the hMBD column and eluted with a step gradient of 0.1–1 M NaCl in buffer B as described above. Putative methylated DNAs that eluted with high salt (≥0.6 M NaCl) were cloned into BamHI-digested pBluescript and propagated in *E. coli*. Plasmid DNA from each clone was used in slot blot analysis to identify clones enriched by the hMBD column. The blots were probed separately with [α-^{32}P]dCTP-labeled hMBD-fractionated DNA eluted with high salt (≥0.6 M NaCl) and [α-^{32}P]dCTP-labeled total genomic DNA. The probe hybridization intensities of each clone were measured by Alpha Ease imaging software. Enriched DNA clones were sequenced by using M13 forward and reverse primers at the Iowa State University DNA sequencing facility.

DNA Extraction and Methylation Assay Using Isoschizomers. Total DNA was prepared from *C. albicans* by using a DNeasy Kit following the manufacturer's instructions (Qiagen). Methylation-sensitive and -insensitive restriction endonucleases were used to determine the methylation status of *C. albicans* genomic regions. The isoschizomer pairs HpaII/MspI and Sau3AI/DpnII were used. Genomic DNAs (2 μg) were separately digested with isoschizomers Sau3AI (blocked by GAT^{me}C hemimethylation) and DpnII (cuts GAT^{me}C), HpaII (blocked by ^{me}CCGG and C^{me}CGG), and MspI (blocked by ^{me}CCGG; cuts C^{me}CGG) at 37 °C for 3 h, size fractionated by electrophoresis on a 1% agarose gel, and Southern transferred as described above. The blot was hybridized with a [α-^{32}P]dCTP-labeled DNA probe. DNA probes were PCR-amplified from SC5314 genomic DNA using primers as described (*SI Appendix*).

Bisulfite Modification, PCR, Cloning, and Sequencing. The methylation status of specific genomic regions was examined by bisulfite DNA sequencing (5). Genomic DNA (200 ng) was sonicated to an average size of 500 bp and then subjected to bisulfite treatment using the EZ DNA Methylation Gold Kit following the supplier's instructions (Zymo Research). Each bisulfite reaction included unique unmethylated pBluescript KS(−) vector DNA to determine the efficiency of bisulfite conversion. Treated DNAs were then used for PCR amplification of different loci using primers (*SI Appendix*) and amplification conditions as described (6). Primers used in PCR amplification were designed by using MethPrimer software (7). PCR products were size-fractionated on 1% agarose gels in 1× TAE buffer (0.04 M Tris acetate, pH 8.3, 1 mM EDTA), and DNA bands of the expected size were excised from the gel. PCR products were recovered by using the QIAquick gel extraction kit (Qiagen), cloned into vector pCR2.1-TOPO (Invitrogen), and transformed into *E. coli* TOP10 competent cells, and individual clones were sequenced in both directions with M13F and M13R primers at the Iowa State DNA Sequencing Facility. Bisulfite-treated control pBluescript KS(−) vector DNA sequencing indicated that bisulfite conversion reactions were efficient and converted all cytosine residues to uracil (*SI Appendix*). Sequencing data were analyzed by using BioEdit software (8). Logos were prepared by using WebLogo software (http://weblogo.threeplusone.com/). DNA sequences were deposited in the GenBank database (accession nos. ET671383–ET671921, HQ690089, HR235325–HR235465, and JJ725187–JJ725299).

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from *C. albicans* by using an RNeasy Mini kit following the manufacturer's instructions (Qiagen). RNA (15 μg) was first size fractionated by electrophoresis on a 1% agarose gel under denaturing conditions. The RNA samples were then transferred to a Zeta-Probe GT nylon membrane (Bio-Rad) by using standard Northern blotting procedures. The membrane was hybridized with [α-^{32}P]dCTP labeled DNA probes. The probe DNA sequences were generated by PCR amplification and purified with Sephadex G50 columns (Amersham Biosciences).

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4. Cross SH, Charlton JA, Nan X, Bird AP (1994) Purification of CpG islands using a methylated DNA binding column. *Nat Genet* 6:236–244.

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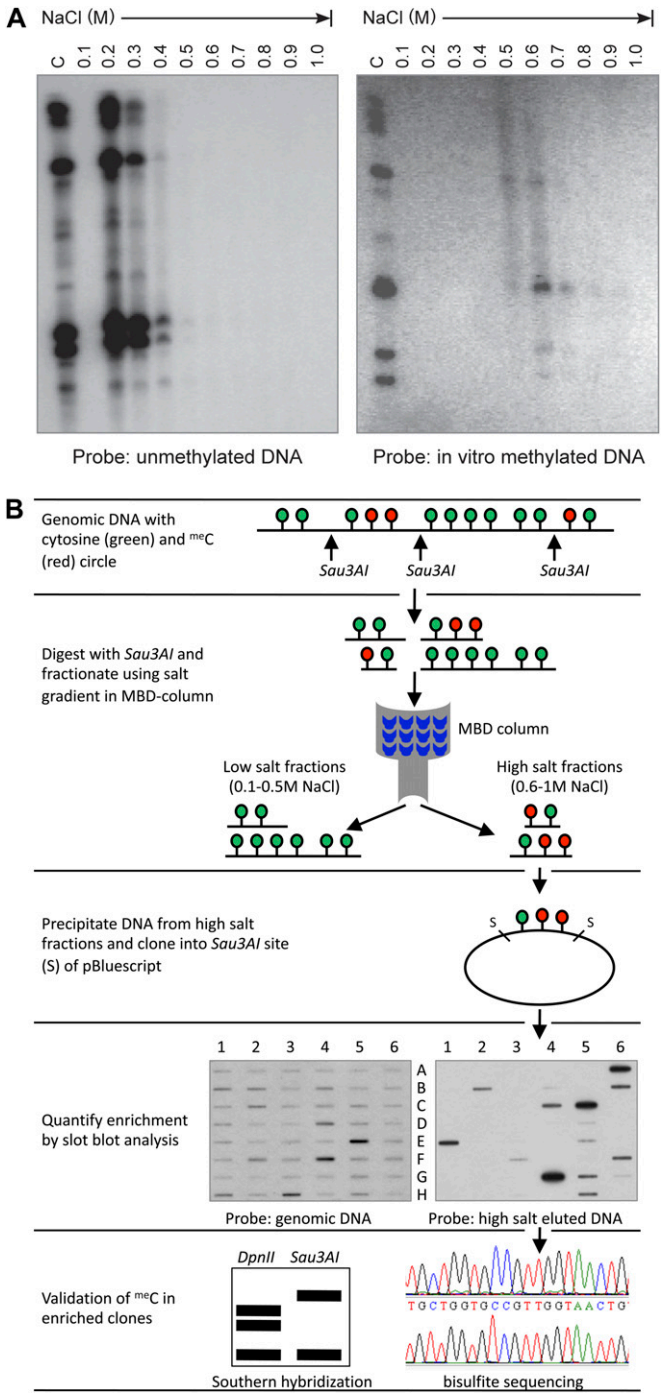

Fig. S1. (*A*) Methylated DNA preferentially binds to an MBD column. Sau3AI-digested unique in vitro methylated and unique unmethylated DNA fragments derived from pBluescript KS(–) vector DNA were fractionated through the MBD column with a step salt gradient. DNA from each fraction and an unfractionated sample (c) were precipitated, separated on a 1% agarose gel by electrophoresis, and blotted to Zeta-Probe GT nylon membrane. Blots were hybridized with probes made from the unique *in vitro* methylated and unique unmethylated DNA as described above. (*B*) Identification of enriched, methylated *C. albicans* DNAs. Independently cloned DNAs derived from high salt (≥0.6 M) elution fractions of the MBD column were applied to duplicate nylon membranes and were hybridized with ^{32}P-labeled probes corresponding to unfractionated total genomic DNA or high salt (≥0.6 M) eluted DNA. Enrichment is the ratio of high salt eluted DNA to total genomic DNA signals and is normalized to a value of 1 for *CEN1*, an unmethylated region. Clones showing enrichment values twofold higher than *CEN1* were further analyzed.

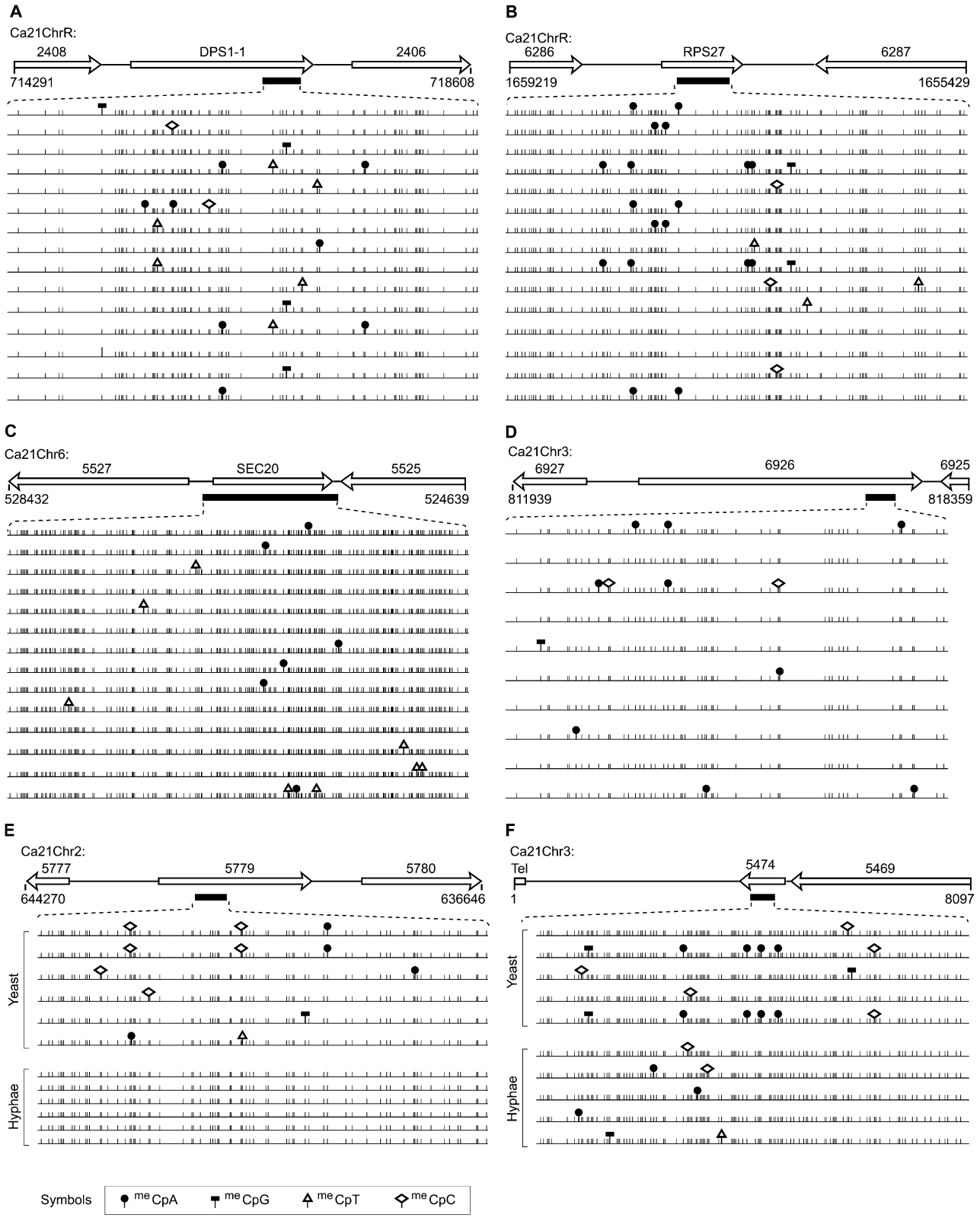

Fig. S2. Confirmation of DNA methylation in *C. albicans* genes using bisulfite sequencing. (*A–F*) Schematics are drawn to scale and are derived from *C. albicans* genome sequence assembly 21 (www.candidagenome.org). Bisulfite-converted genomic DNA corresponding to the MBD-affinity enriched DNA fragment, depicted as a filled black bar below the indicated gene, was analyzed by DNA sequencing. Sequences from independent clones were aligned in a lollipop diagram. Cytosine, vertical lines; ^{me}CpN, symbols as indicated.

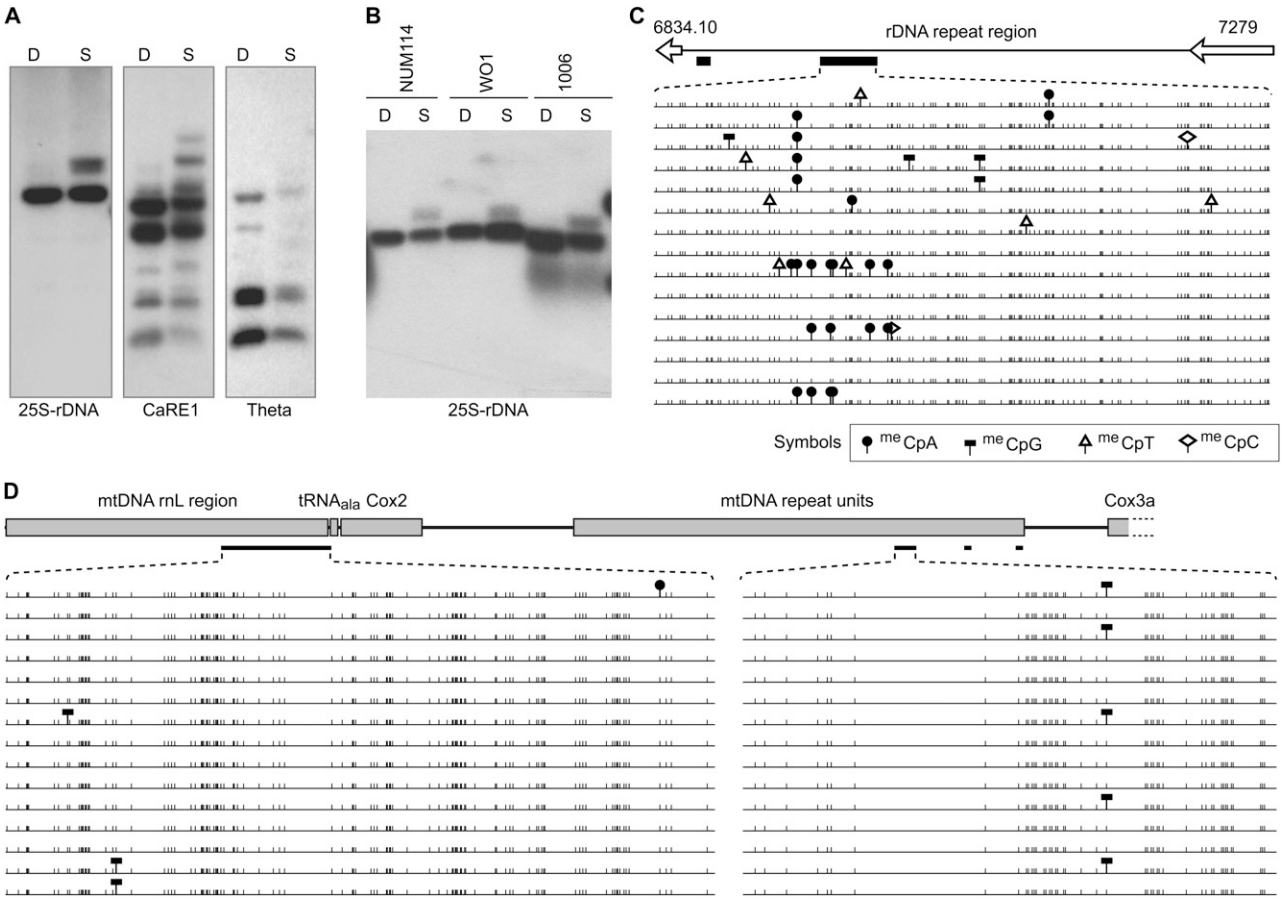

Fig. S3. DNA methylation in repeated DNA of *C. albicans*. (*A*) Genomic DNA from *C. albicans* strain SC5314 was digested with isoschizomers DpnII (D) and Sau3AI (S), fractionated by gel electrophoresis, blotted to a nylon membrane, and hybridized with radiolabeled probes derived from 25S-rDNA, CaRE1, and LTR Theta. (*B*) Genomic DNAs from *C. albicans* strains NUM114, WO1, and 1006 were analyzed by Southern blotting as described in *A* and hybridized with 25S-rDNA probe. (*C* and *D*) Validation of DNA methylation using bisulfite sequencing in 25S-rDNA (*C*), and mtDNA rnL and mtDNA repeat sequences (*D*). Schematics are drawn to the scale and are derived from *C. albicans* genome sequence assembly 21 (www.candidagenome.org). Bisulfite-converted genomic DNA corresponding to the MBD-affinity enriched DNA fragment, depicted as a filled black bar below the indicated repeat, was analyzed by DNA sequencing. Sequences from independent clones were aligned in a lollipop diagram. Cytosine, vertical lines; ^{me}CpN, symbols as indicated.

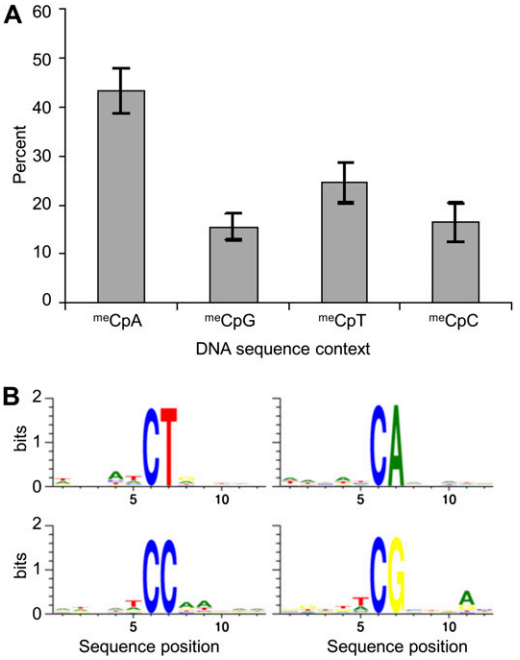

Fig. S4. DNA methylation in *C. albicans* occurs in both symmetric (CpG) and asymmetric (CpN) sequence contexts. (*A*) Relative amounts of the four ^{me}CpN dinucleotides were determined from bisulfite DNA sequences of 40 independent methylated DNA loci. Means (±SE) are shown. (*B*) Logos of 12 nucleotide sequence contexts that are preferentially methylated; ^{me}C is at the sixth position. The logos show the sequence enrichment, measured in bits, at a particular position in the alignment of 12 nucleotides for each sequence context. The maximum sequence conservation per site is 2 bits; no preference for a nucleotide is 0.

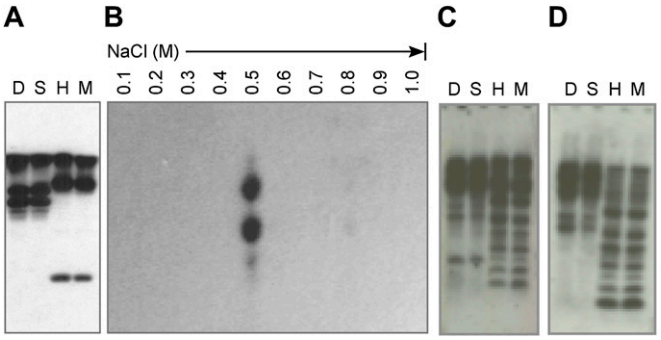

Fig. S5. DNA methylation is not detected at major repeat sequences (MRS) and *ALS* genes. Genomic DNA from *C. albicans* strain SC5314 was digested with isoschizomer pairs, DpnII (D)/Sau3AI (S) and HpaII (H)/MspI (M), fractionated by gel electrophoresis, blotted to a nylon membrane, and hybridized with probes derived from repeated DNA sequences. (*A*) Blot probed with the RPS1 region of MRS. (*B*) Validation that MRS eluted at 0.5 M salt using RPS1 probe. (*C*) Blot probed with *ALS3*. (*D*) Blot probed with *ALS4*.

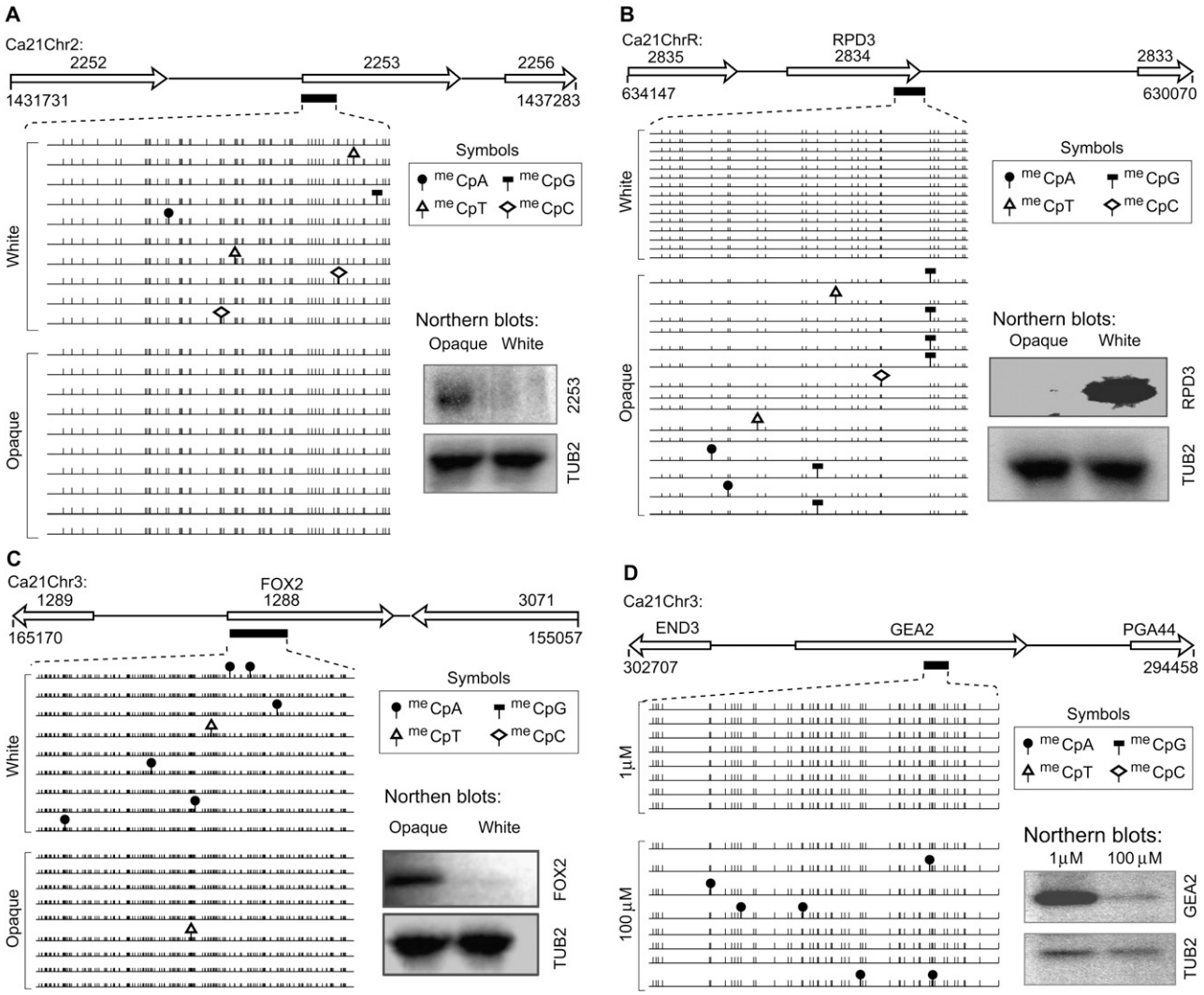

Fig. S6. DNA methylation directs transcriptional repression dependent upon morphological phenotype or iron dosage. Schematics are drawn to scale and are derived from *C. albicans* genome sequence assembly 21 (www.candidagenome.org). Bisulfite-converted genomic DNA corresponding to the MBD-affinity enriched DNA fragment, depicted as a filled black bar below the indicated gene, was analyzed by DNA sequencing. Sequences from independent clones were aligned in a lollipop diagram. Cytosine, vertical lines; ^{me}CpN, symbols as indicated. (*A*) DNA methylation in *orf19.2253* (predicted gene of unknown function) determined by bisulfite sequencing in white and opaque cells of *C. albicans* strain WO1. Northern blot of total RNA from white and opaque cells probed with *orf19.2253* or *TUB2* (loading control) are shown. (*B*) DNA methylation in *orf19.2834* (*RPD3*) determined by bisulfite sequencing in white and opaque cells of *C. albicans* strain WO1. Northern blot of total RNAs from white and opaque cells probed with *RPD3* or *TUB2* (loading control) are shown. (*C*) DNA methylation in *orf19.1288 (FOX2)* determined by bisulfite sequencing in white and opaque cells of *C. albicans* strain WO1. Northern blot of total RNAs from white and opaque cells probed with *FOX2* or *TUB2* (loading control) are shown. (*D*) DNA methylation in the *GEA2* gene determined by bisulfite sequencing of DNA derived from *C. albicans* strain SC5314 grown in low (1 μM) and high (100 μM) iron medium. Northern blot of total RNAs prepared from cultures grown in low (1 μM) and high (100 μM) iron probed with *GEA2* or *TUB2* (loading control) are shown.

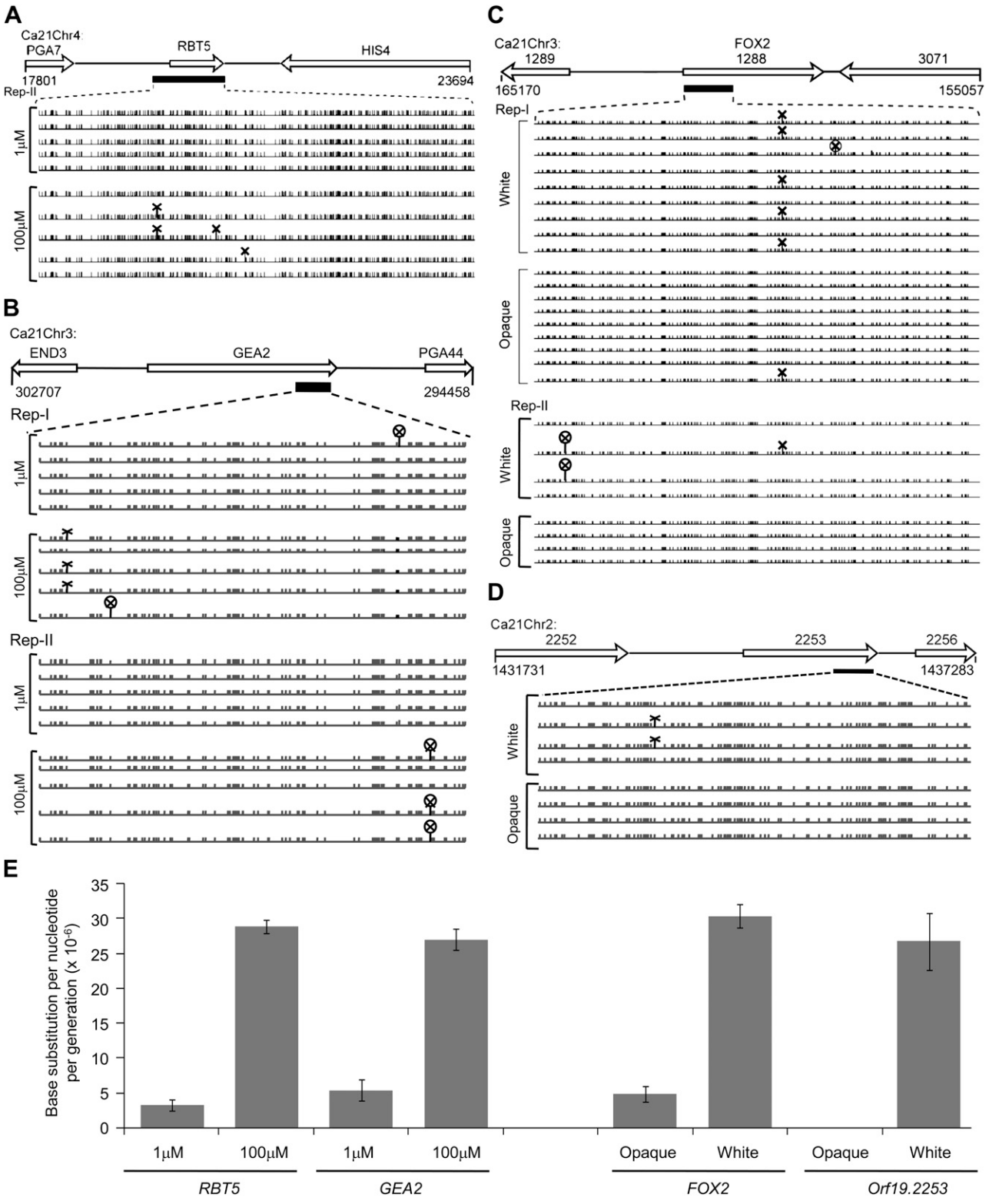

Fig. S7. Transcriptionally repressed methylated genes show increased frequency of cytosine to thymine transitions. Schematics are drawn to scale and are derived from *C. albicans* genome sequence assembly 21 (www.candidagenome.org). Sequences from independent clones are aligned. Cytosine, vertical lines; C-to-T mutation, X; G to A mutation, circled X. (*A* and *B*) Strain SC5314 was grown for ~50 generations in low (1 μM) or high (100 μM) iron conditions. Total genomic DNA was extracted and used in PCR to amplify *RBT5* (*A*) and *GEA2* (*B*) genes using proofreading Taq DNA polymerase. Independent PCR products were cloned into *E. coli* and sequenced. Biological replicates (Rep-I, Rep-II) from two independently grown cultures were performed. (*C* and *D*) Strain WO1 was grown as white or opaque cells for ~50 generations; total genomic DNA was extracted and used in PCR to amplify *FOX2* (*C*) and *orf19.2253* (*D*) genes using proof reading Taq DNA polymerase. Independent PCR products were cloned into *E. coli* and sequenced. Two independent biological replicates were performed for *FOX2*. (*E*) Mutation rates measured as base substitutions per nucleotide per generation for gene sequences derived from strains grown under the conditions described above in *A–D* are shown. Error bars indicate SEMs.

Other Supporting Information Files

SI Appendix (PDF)
Dataset S1 (XLS)