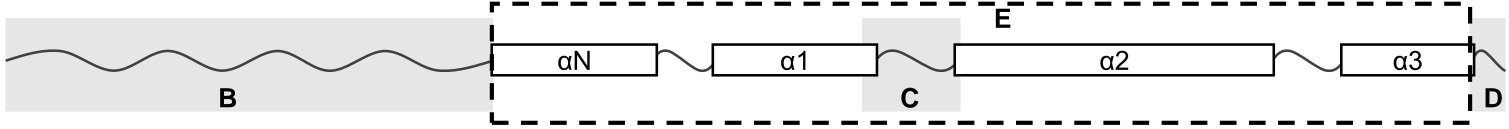
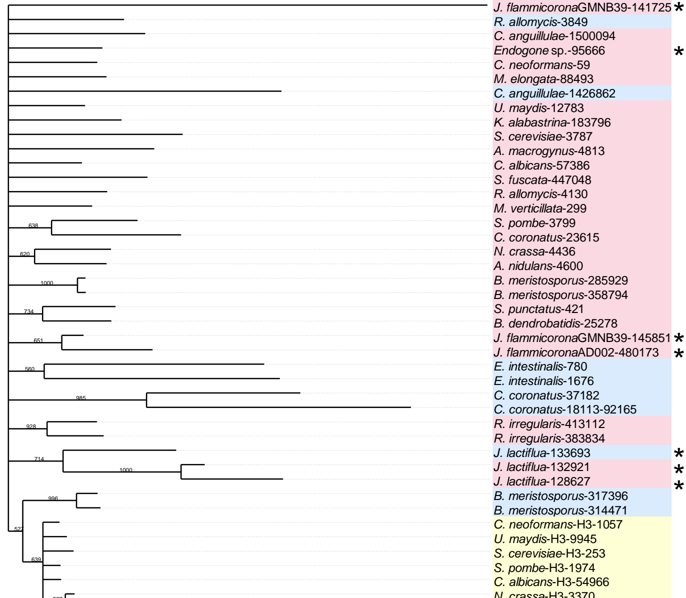


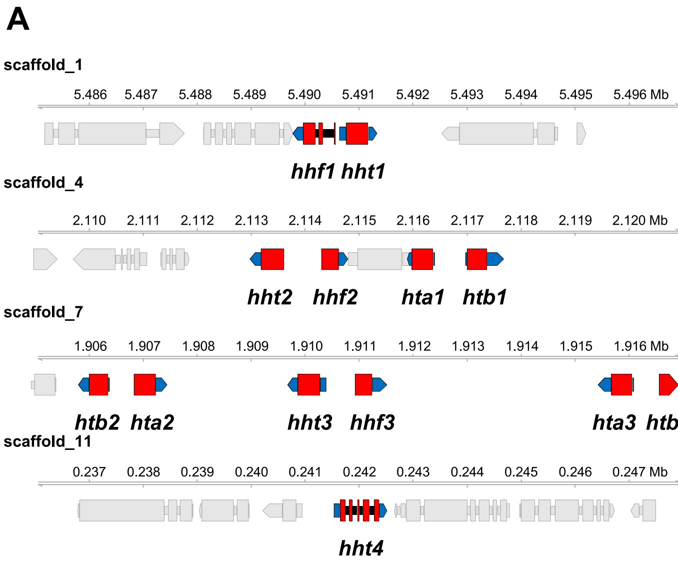


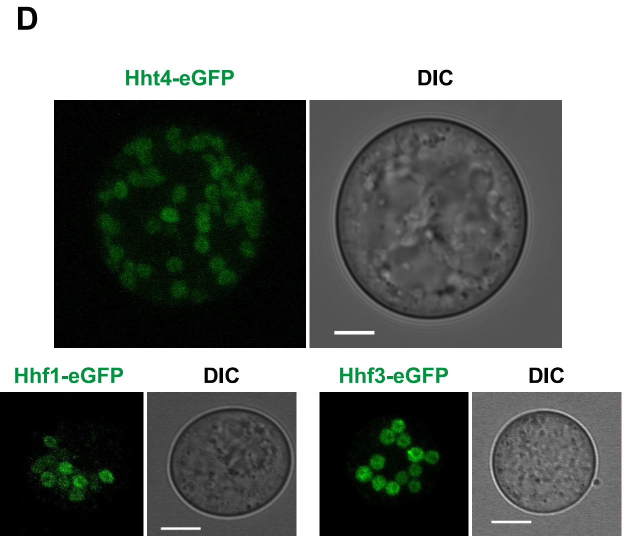
B

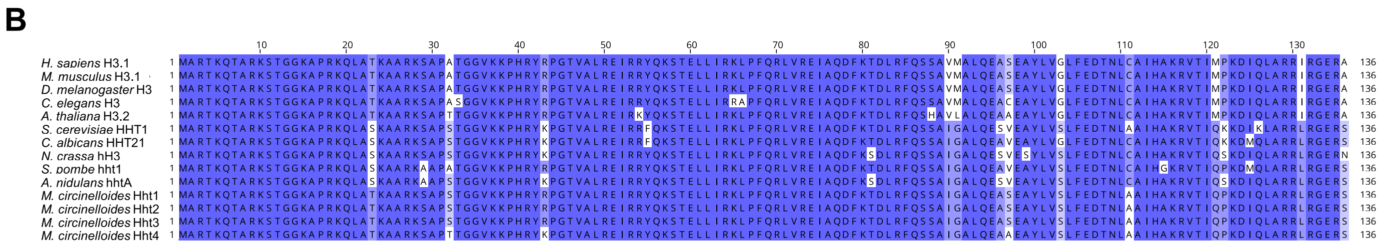
C

D

Figure S1. Mucorales and Umbelopsidales lack CENP-A. **Related to Figure 1. (A)** Schematic of canonical histone H3 and CENP-A shared protein features. **(B-D)** Multiple protein sequence alignments of the N-tail **(B)**, Loop 1 **(C)**, and C-terminal **(D)** regions. The scale above the comparisons indicates the amino acid positions with *S. pombe* sequence as the reference. Red arrows mark relevant amino acid positions. Amino acids are colored in increasing shades of blue to show conservation within each group. **(E)** Neighbor-joining phylogenetic tree (JTT model) of the Histone Folding Domain (HFD), showing phylogenetic distance (branch length) and branch support (1000 bootstraps). Branches with < 50% bootstrap support are collapsed. The protein sequences analyzed in **B** to **E** are distributed among three groups: well-studied histone H3 (yellow), rare histone H3 (blue), and the predicted CENP-A proteins in this study (red). Asterisks (*) indicate proteins from the Mucoromycotina.







Histone H3 αN α1 α2 α3

LOGO

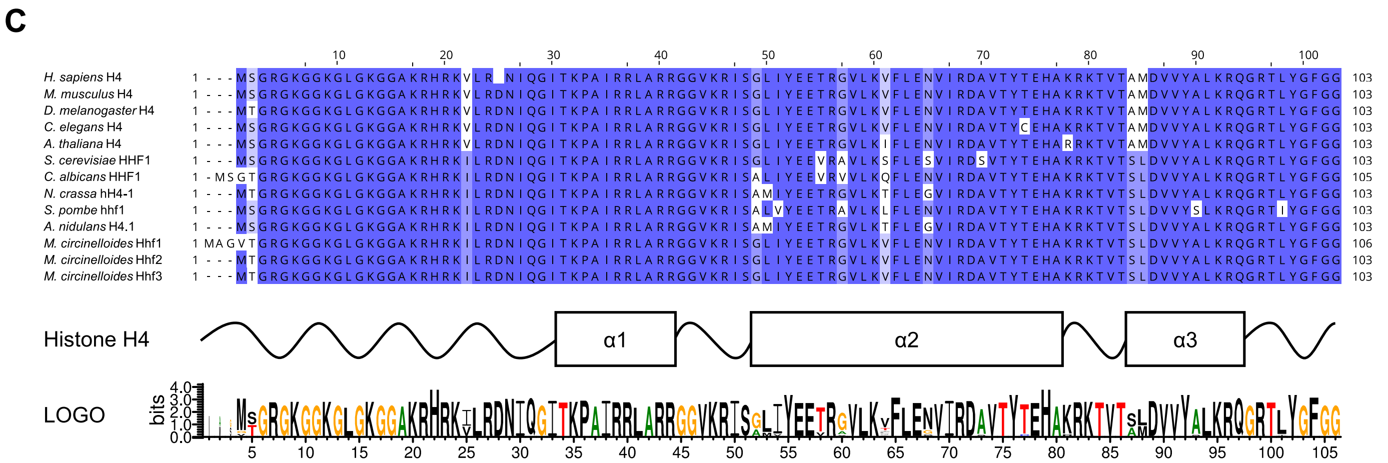


Figure S2. ***M. circinelloides*** **histones H3 and H4 are not centromere-specific binding proteins**. **Related to Figure 2.** **(A)** Location of all the histone H2A, H2B, H3, and H4-coding genes in the *M. circinelloides* genome. Genes are represented by arrows showing transcription direction; the coding sequence is depicted as larger red blocks, flanked by smaller blue blocks representing the untranslated regions, and connected by black lines as intronic sequences. Light-gray arrows indicate neighboring non-histone genes and their transcription direction. **(B, C)** Protein alignment of several well-characterized H3 **(B)** and H4 **(C)** histones with *M. circinelloides* orthologs. A scale indicates the amino acid positions taking *S. pombe* sequence as the reference. Histone fold domains (HFD) are outlined in a diagram below each alignment, as well as the N-terminal tail. Amino acids are colored in increasing shades of blue and a consensus protein logo is provided to reflect conservation. **(D)** Confocal microscopy images of *M. circinelloides* strains expressing eGFP-fluorescent fusion histone proteins Hht4, Hhf1, and Hhf3 in 4-hour pregerminated spores. The fluorescent signal is colored as green. A calibrated scale (white bar) is provided for size comparison (5 µm).

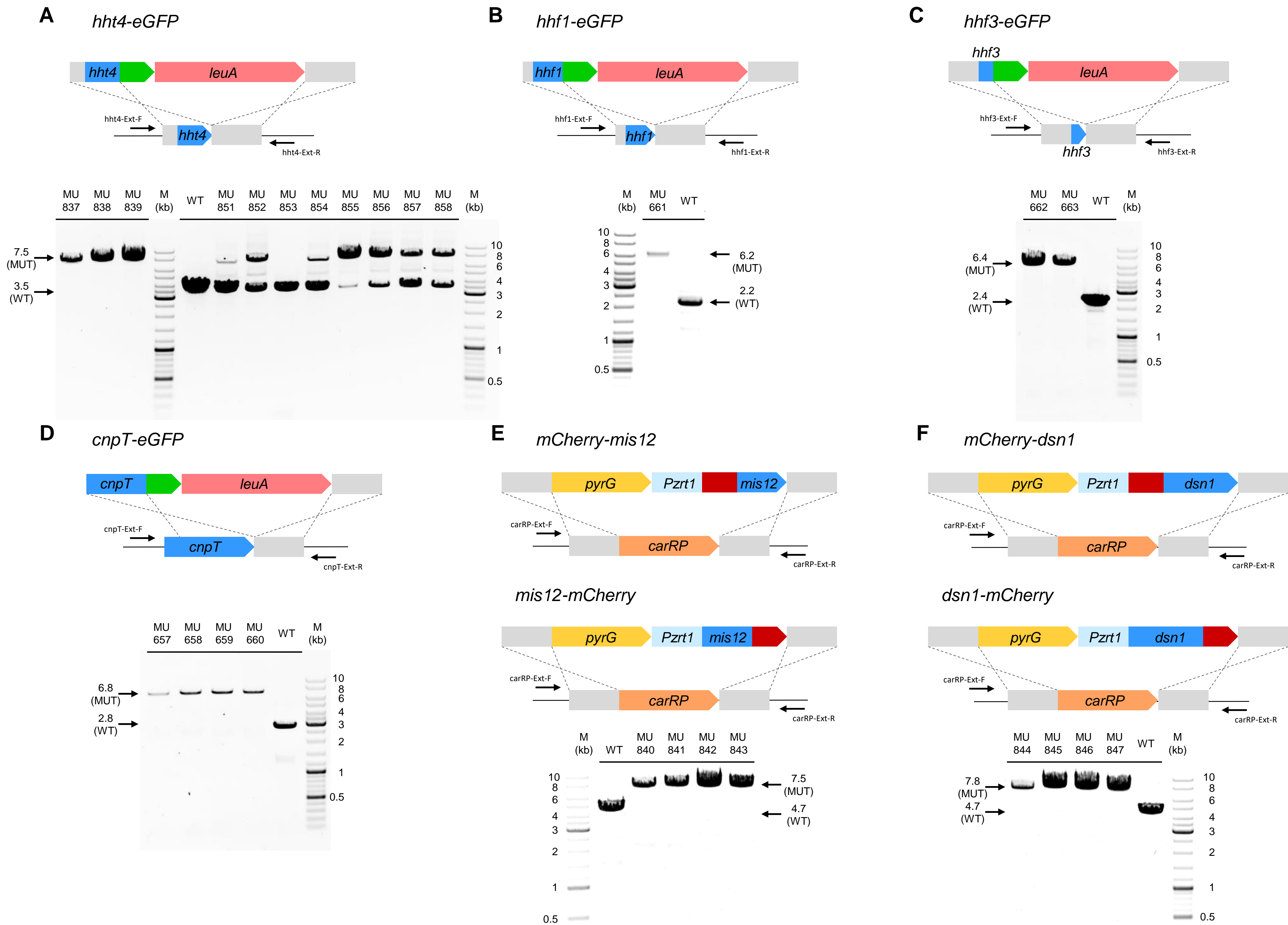
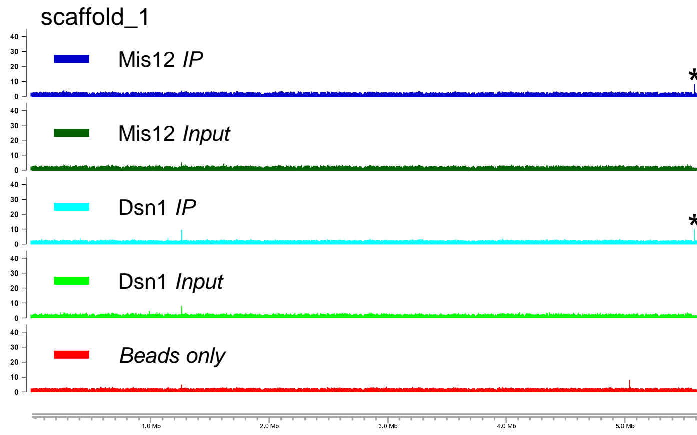
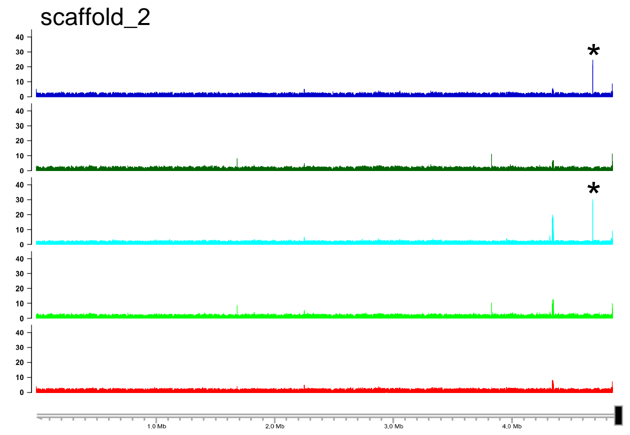
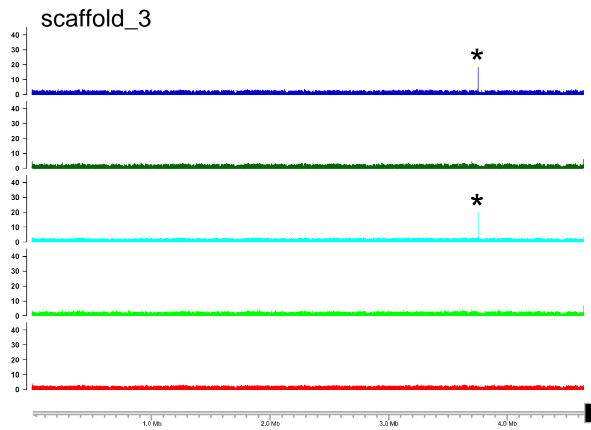
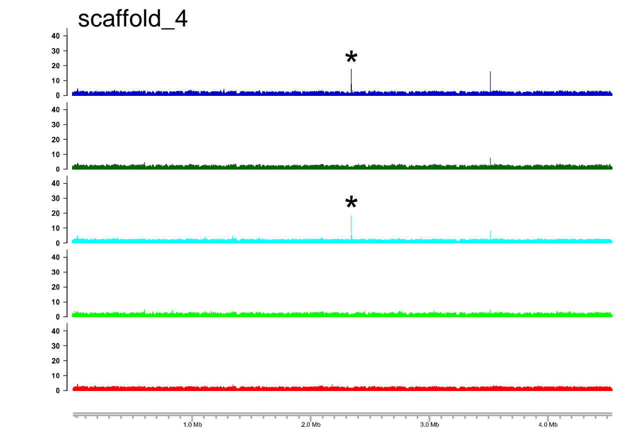

A
hht4-eGFP
hht4
leuA
hht4-Ext-F
hht4
hht4-Ext-R
MU 837
MU 838
MU 839
M (kb)
WT
MU 851
MU 852
MU 853
MU 854
MU 855
MU 856
MU 857
MU 858
M (kb)
7.5 (MUT)
3.5 (WT)
10
8
6
4
3
2
1
0.5
B
hhf1-eGFP
hhf1
leuA
hhf1-Ext-F
hhf1
hhf1-Ext-R
M (kb)
MU 661
WT
6.2 (MUT)
2.2 (WT)
C
hhf3-eGFP
hhf3
leuA
hhf3-Ext-F
hhf3
hhf3-Ext-R
MU 662
MU 663
WT
M (kb)
6.4 (MUT)
2.4 (WT)
D
cnpT-eGFP
cnpT
leuA
cnpT-Ext-F
cnpT
cnpT-Ext-R
MU 657
MU 658
MU 659
MU 660
WT
M (kb)
6.8 (MUT)
2.8 (WT)
E
mCherry-mis12
pyrG
Pzrt1
mis12
carRP-Ext-F
carRP
carRP-Ext-R
mis12-mCherry
M (kb)
WT
MU 840
MU 841
MU 842
MU 843
7.5 (MUT)
4.7 (WT)
F
mCherry-dsn1
pyrG
Pzrt1
dsn1
carRP
dsn1-mCherry
MU 844
MU 845
MU 846
MU 847
7.8 (MUT)
4.7 (WT)

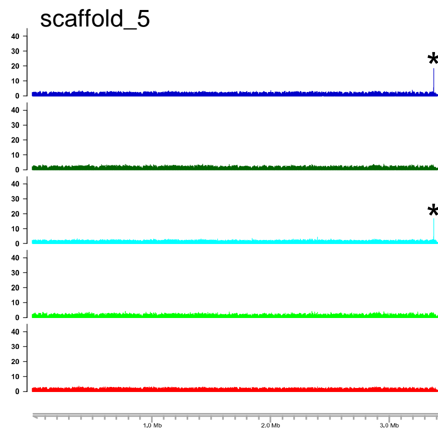
Figure S3. Generation of mutant strains expressing fluorescent fusion histones and kinetochore proteins. Related to Figure 2 and Table S2. Diagram (above) and agarose gel images (below) showing the integration of the *hht4* **(A)**, *hhf1* **(B)**, and *hhf3* **(C)**, and *cnpT* **(D)** fusion alleles into their wild-type loci; and the *mis12* **(E)** and *dsn1* **(F)** fusion alleles (both N-terminal and C-terminal fusions) into the *carRP* locus. Discontinuous crosses indicate homologous recombination and arrows mark the annealing regions for specific primers used to confirm the integration by PCR, amplifying both the wild-type (WT) and mutant (MUT) alleles shown in the gel images. M lanes were loaded with the GeneRuler DNA Ladder Mix (Thermo Fisher) to estimate the fragment sizes. Red and green regions indicate mCherry and eGFP genes, respectively.

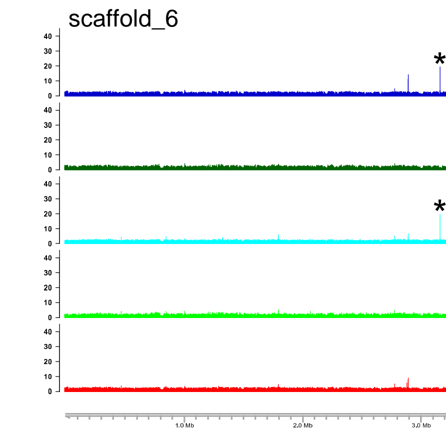

scaffold_1
Mis12 IP
Mis12 Input
Dsn1 IP
Dsn1 Input
Beads only

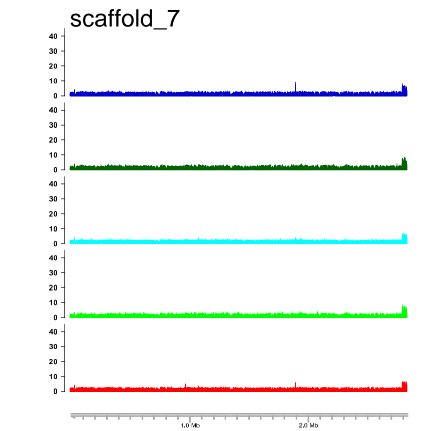

scaffold_2

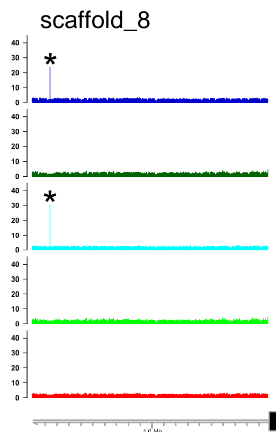

scaffold_3

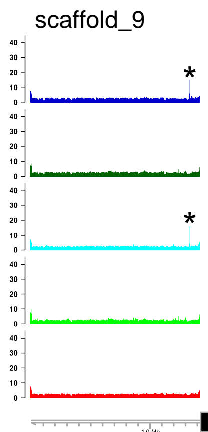

scaffold_4

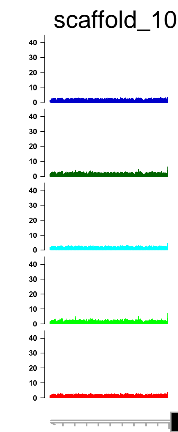

scaffold_5


scaffold_6


scaffold_7


scaffold_8


scaffold_9


scaffold_10

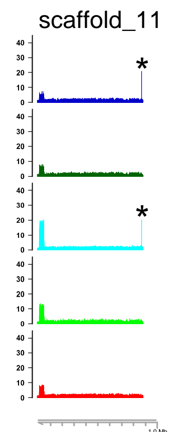
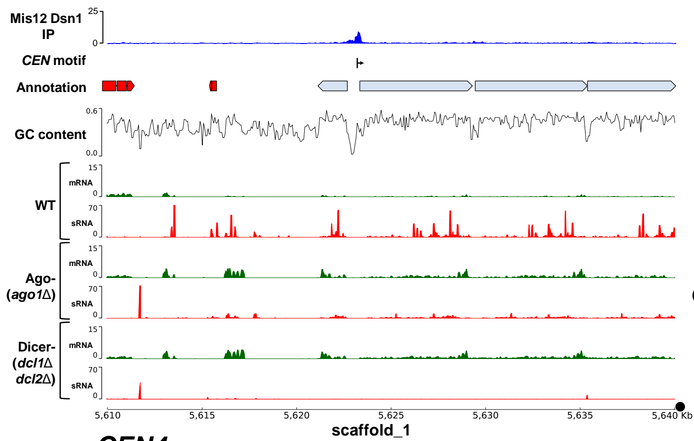
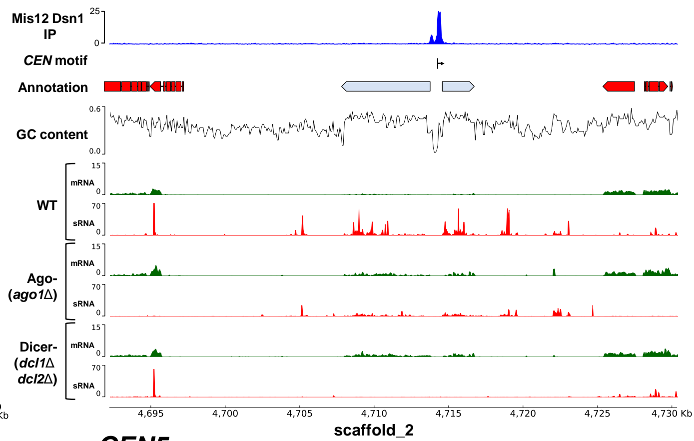
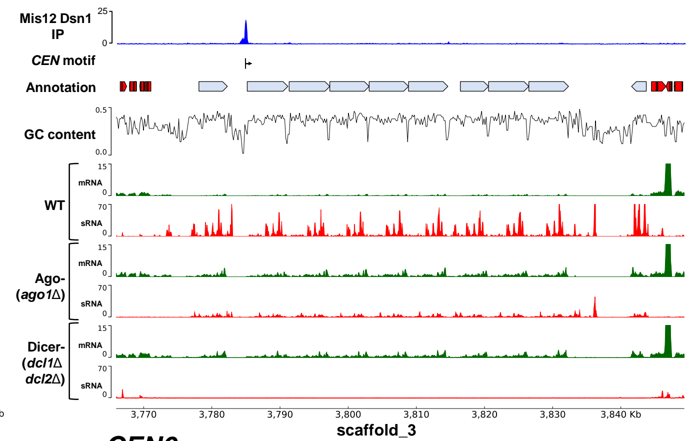
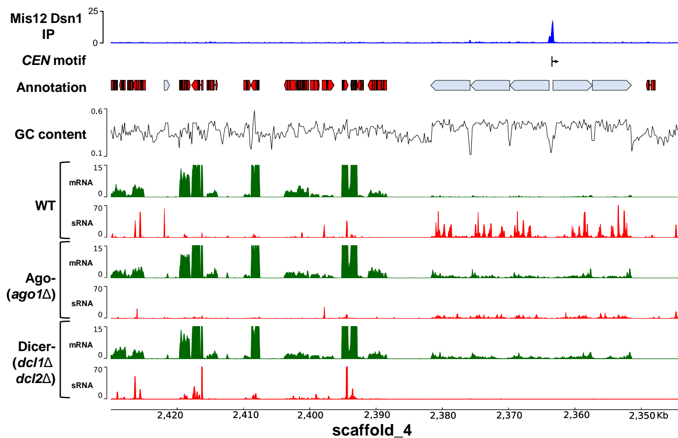

scaffold_11

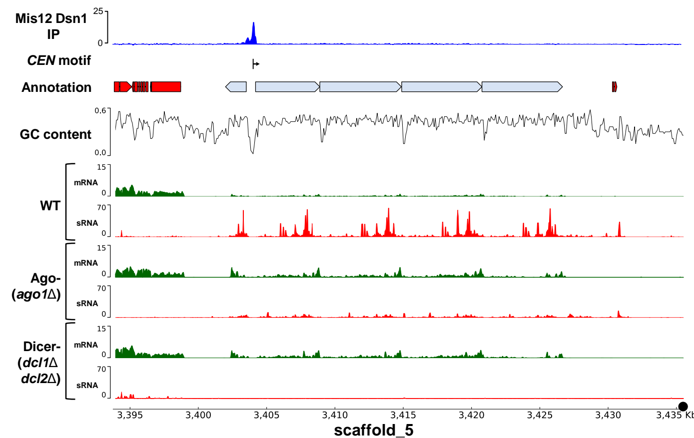
Figure S4. ***M. circinelloides*** **kinetochore protein-bound regions. Related to Figures 3 and 4, and Data S1.** *IP* enrichment (coverage x1) of immunoprecipitated DNA (*IP* DNA) from Mis12 and Dsn1 mCherry-tagged strains compared to their corresponding input (*Input* DNA) and binding controls (*beads only* DNA), shown as color-coded tracks across the whole sequence of scaffolds 1-11. Asterisks (*) mark significant peaks (FDR $\leq 5 \times 10^{-5}$, fold enrichment ≥ 1.6) in both *IP* DNA samples that are not present in the controls, indicating the putative centromeric regions. Each kinetochore-protein data was obtained from a pool of duplicated IP DNA samples. Black rectangles at either end of the genomic axis marks a repeated telomeric sequence.

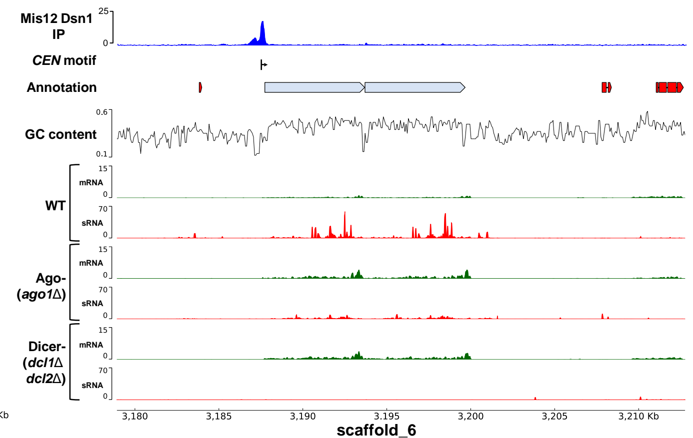

CEN1
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_1

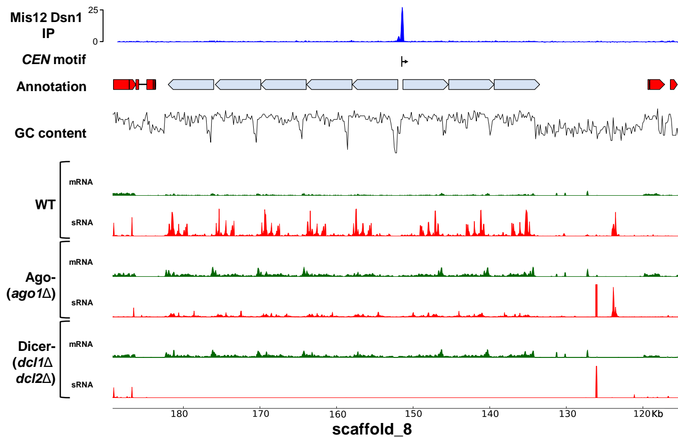

CEN2
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_2

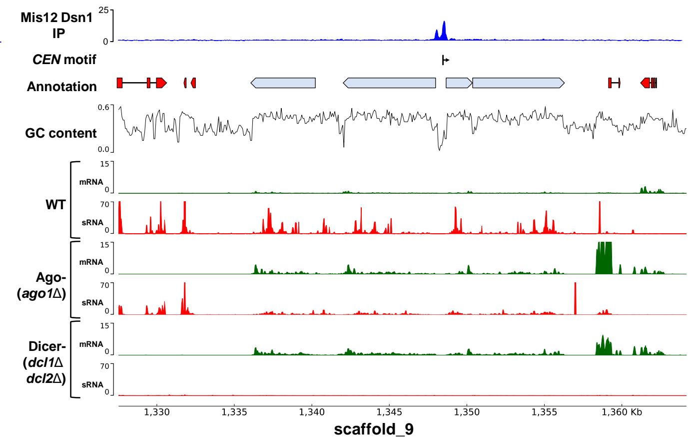

CEN3
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_3


CEN4
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_4


CEN5
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_5


CEN6
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_6


CEN8
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_8


CEN9
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_9

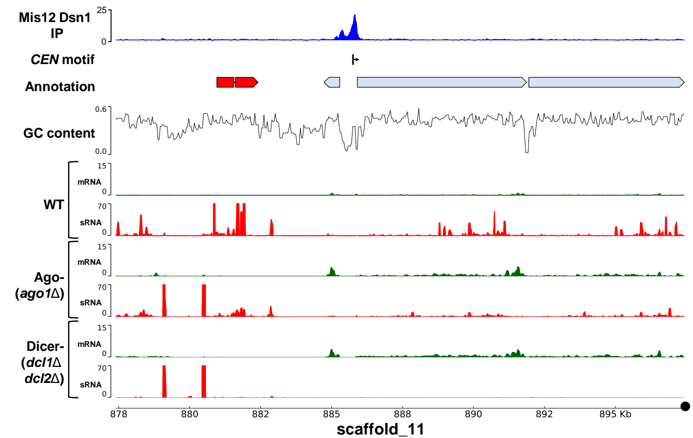
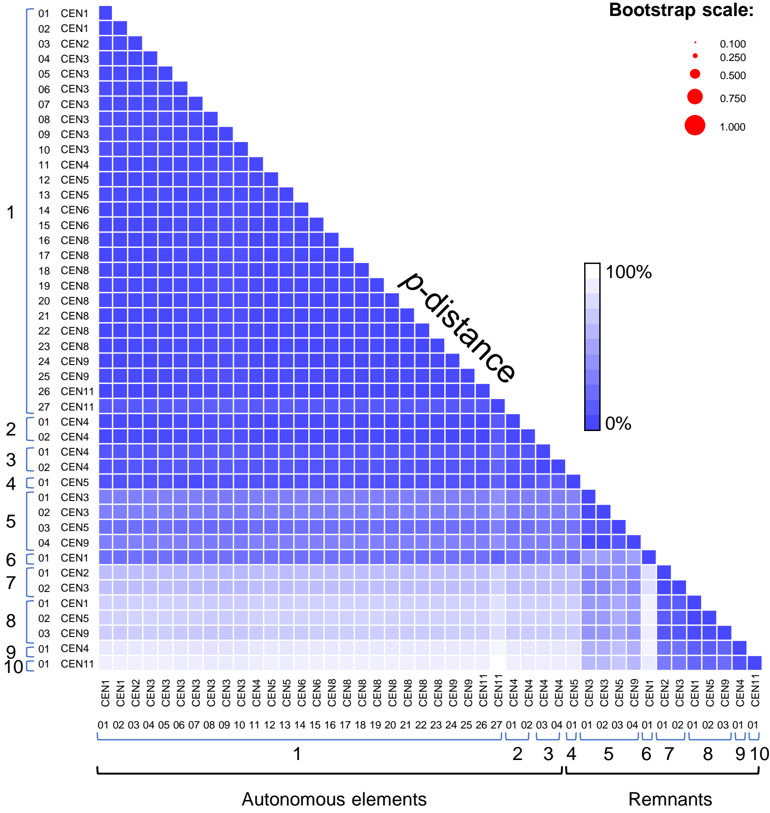
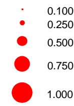

CEN11
Mis12 Dsn1 IP
CEN motif
Annotation
GC content
WT
Ago- (ago1Δ)
Dicer- (dcl1Δ dcl2Δ)
mRNA
sRNA
scaffold_11

Figure S5. ***M. circinelloides*** **pericentromeric regions. Related to Figure 5 and Table S3.** A genomic view of the pericentromeric regions of all nine centromeres. Each region shows the kinetochore-binding region enrichment (IP, an average of both *IP* signals minus *Input* and *Beads only* controls), annotated genes (red blocks) and transposable elements (light blue blocks), *CEN*-specific DNA motif position (black vertical line) and its direction (arrow), GC content, and transcriptomic data of mRNA (green) and sRNAs (red) in *M. circinelloides* wild-type strain, and *ago1* and double *dcl1 dcl2* deletion mutants after 48 h of growth in rich media. A black circle at either end of the genomic axis indicates an abrupt, non-telomeric end of that scaffold.

A



B



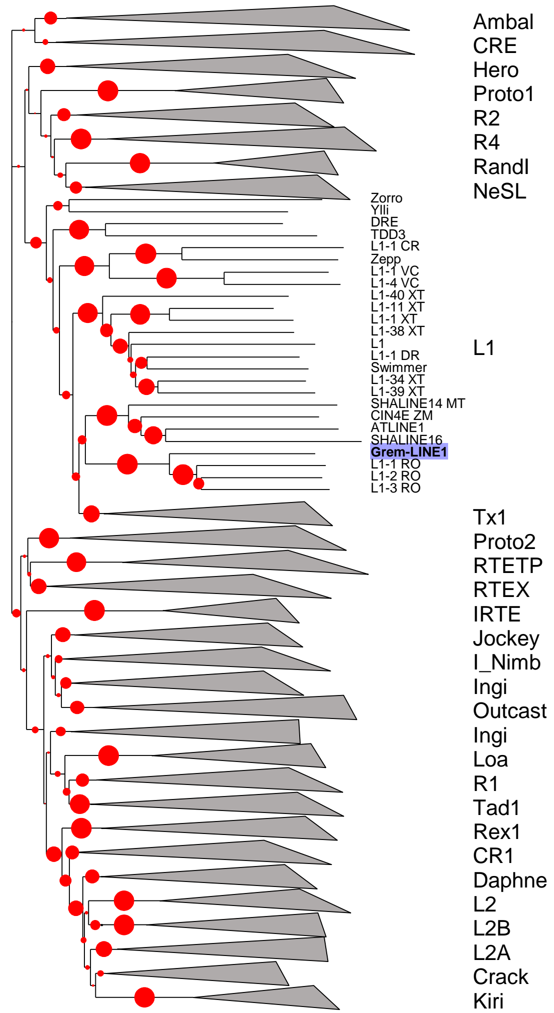


Figure S6. Centromere-specific non-LTR L1-like retrotransposable elements. Related to Figure 5, and Tables S3 and S4. (A) Matrix showing pairwise *p*-distance (% changes per site) across all 44 repetitive elements flanking the centromeres of *M. circinelloides*. Elements sharing ≥ 95.0% identity are clustered together, generating 10 groups that comprise full-length elements and incomplete sequences (remnants). Elements belonging to the same centromere have been clustered together within each separated group. **(B)** Neighbor-joining phylogeny (JTT model) of the RVT domain of well-known non-LTR elements. The RVT domain of the centromere-specific element (Grem-LINE1) identified in this study is marked inside a blue box. Phylogenetic distance (branch length) and branch support (1000 bootstraps, size-coded circles) are shown. Leaves within the same clade of non-LTR retrotransposons are collapsed, forming a scalene triangle (top and bottom corners show the shortest and longest phylogenetic distance, respectively); except for the L1 clade which contains the Grem-LINE-1.

Name	Sequence	Use
mCherry-F	ATGGTGAGCAAGGGCGAGGA	*mCherry* and *eGFP* amplification
mCherry-R	CTTGTACAGCTCGTCCATGC	*mCherry* and *eGFP* amplification
mCherry-R-*leuA*	gaatagagttggtagggagcaTTACTTGTACAGCTCGTCCATGC	*eGFP* tagging with marker *leuA*
*leuA*3kbFow	TGCTCCCTACCAACTCTATTC	*leuA* amplification
*leuA*3kbRev	GTCGAGTTGACCAGAATGTAC	*leuA* amplification
*pyrG*Fow2kb	TGCCTCAGCATTGGTACTTG	*pyrG* amplification
*pyrG*Rev2Kb	GTACACTGGCCATGCTATCG	*pyrG* amplification
P*zrt1*-F	cgatagcatggccagtgtacATCATCATCGATGTTTGTGCTGTC	Construction of pMAT1915
P*zrt1*-R	CTCGAGATTTAGTTATTTTG	Construction of pMAT1915
carRP-Inv-F	caagtaccaatgctgaggcaCCATATTGAGTCATCCTGCAACG	Construction of pMAT1915
carRP-Inv-R	TACCACACATTGCAGACAGG	Construction of pMAT1915
hht4-1	tacatgggcccTCGCAATCATCCATGAAGTG	Hht4 tagging with eGFP at C-terminus
hht4-2	tcctcgcccttgctcaccatAGAGCGTTCACCACGAAGA	Hht4 tagging with eGFP at C-terminus
hht4-3	gtacattctggtcaactcgacATCATCATCTGATGTCTTTCTT	Hht4 tagging with eGFP at C-terminus
hht4-4	tacatccgcggATGGCTCTGAAGTGATCCAC	Hht4 tagging with eGFP at C-terminus
hhf1-1	tacatgtcgacACCAACAAACGTCACCTAGAGTAG	Hhf1 tagging with eGFP at C-terminus
hhf1-2	tcctcgcccttgctcaccatTCCACCGAAACCGTAGAGGG	Hhf1 tagging with eGFP at C-terminus
hhf1-3	gtacattctggtcaactcgacATCAAATCCCTCTGCTCATTCAC	Hhf1 tagging with eGFP at C-terminus
hhf1-4	tacatctgcagCAACACGGGTGGTTTGGAG	Hhf1 tagging with eGFP at C-terminus
hhf3-1	tacatgtcgacCCTAAAAGGGACAAAGATTATGGC	Hhf3 tagging with eGFP at C-terminus
hhf3-2	tcctcgcccttgctcaccatTCCACCGAAACCGTAGAGGG	Hhf3 tagging with eGFP at C-terminus
hhf3-3	gtacattctggtcaactcgacATGCAATTCATCATGCTTCTCAC	Hhf3 tagging with eGFP at C-terminus
hhf3-4	tacatctgcagTGACAGGGCTTTCGCTTAGC	Hhf3 tagging with eGFP at C-terminus
cnpT-1	cgaggtcgacggtatcgataCACGGCAGCAGCAGCAACATAC	CENP-T tagging with eGFP at C-terminus
cnpT-2	tcctcgcccttgctcaccatTTCGTCTTCATTATCGTATCCACCG	CENP-T tagging with eGFP at C-terminus
cnpT-3	gtacattctggtcaactcgacCTGTGATTGGTTGCCATGGTGG	CENP-T tagging with eGFP at C-terminus
cnpT-4	caggaattcgatatcaagcTTGCTCGTGTATAGAACGAATCCAG	CENP-T tagging with eGFP at C-terminus
mis12-1	tcctcgcccttgctcaccatTGGATCTGATGGCTGCTGG	Mis12 tagging with mCherry at C-terminus
mis12-2	caaaataactaaatctcgagGATGCAAACCGACGAAAGCTA	Mis12 tagging with mCherry at C-terminus
mis12-3	cctgtctgcaatgtgtggtaTAGTTTGAGAAGATTGTGGAGC	Mis12 tagging with mCherry at N-terminus
mis12-4	ggcatggacgagctgtacaagATGCAAACCGACGAAAGCTA	Mis12 tagging with mCherry at N-terminus
dsn1-1	tcctcgcccttgctcaccatTGGATCCTCCATCACAGAAGAT	Dsn1 tagging with mCherry at C-terminus
dsn1-2	caaaataactaaatctcgagATGTCGGATAGACGCTTAAG	Dsn1 tagging with mCherry at C-terminus

dsn1-3	cctgtctgcaatgtgtggtaCCCAACAGTAGAGCATCTTGG	Dsn1 tagging with mCherry at N-terminus
dsn1-4	ggcatggacgagctgtacaagATGTCGGATAGACGCTTAAG	Dsn1 tagging with mCherry at N-terminus
hht4-ext-F	GCTACCTTGGATACCTGGAACA	*hht4* PCR confirmation
hht4-ext-R	CGAGTAAGGACGCCGTAGAC	*hht4* PCR confirmation
hhf1-Ext-F	GCTTCTTGACACCACCAGTAGAG	*hhf1* PCR confirmation
hhf1-Ext-R	GAATAGGTGGACAAGATGGGACT	*hhf1* PCR confirmation
hhf3-Ext-F	TATCTGTGAGGCTTCTTGACACC	*hhf3* PCR confirmation
hhf3-Ext-R	CGCTAAGTCCAAAGCAACTCTC	*hhf3* PCR confirmation
cnpT-Ext-F	CTTTTACCCTCTCAACCACGAG	*cnpT* PCR confirmation
cnpT-Ext-R	GCCTGTTTCAGATTGAGGGAAT	*cnpT* PCR confirmation
carRP-Ext-F	GGGCACATTGACGTAGAAGG	*mis12* and *dsn1* integration in the *carRP* locus PCR confirmation
carRP-Ext-R	GCTGTTGCTGTGCTAACATCAT	*mis12* and *dsn1* integration in the *carRP* locus PCR confirmation
CEN2 core-F	GTTTCCTGAACGGGCTATTTG	104 bp amplicon for ChIP-qPCR
CEN2 core-R	ACTGACAAAGTGTCCAAACCGA	104 bp amplicon for ChIP-qPCR
CEN2 1L-F	GTACTGATGAAGCAAGAGGCG	118 bp amplicon for ChIP-qPCR
CEN2 1L-R	AGCTCTTTGTTCTCTGCTACCTTG	118 bp amplicon for ChIP-qPCR
CEN2 2L-F	CCTTCCTGTTTTGATTGGCGG	99 bp amplicon for ChIP-qPCR
CEN2 2L-R	TTGGCTTGCTAGAAGCACTTG	99 bp amplicon for ChIP-qPCR
CEN2 1R-F	GCAAACGTTCATGGTAGTGCAAG	123 bp amplicon for ChIP-qPCR
CEN2 1R-R	CTTCAGGTTGTGACAGATGTATGCA	123 bp amplicon for ChIP-qPCR
CEN2 2R-F	TGGGAAATTCATCAAGGCCAGTGC	98 bp amplicon for ChIP-qPCR
CEN2 2R-R	GAACCTCCTTTAGGGCCATGTTG	98 bp amplicon for ChIP-qPCR
CEN2 ORF-L-F	AAATTGCAGGACAGAAAAGACGC	126 bp amplicon for ChIP-qPCR
CEN2 ORF-L-R	CATGTCCAGCGCATCGCTTGATA	126 bp amplicon for ChIP-qPCR
CEN2 ORF-R-F	CAATTGACACGATGGGACTTTGAC	102 bp amplicon for ChIP-qPCR
CEN2 ORF-R-R	CAGTTTGACGCCGTATTGGAATG	102 bp amplicon for ChIP-qPCR
Far-*CEN* ORF-F	CCTTGCCACTACCATCTGCTTC	107 bp amplicon for ChIP-qPCR
Far-*CEN* ORF-R	ATCATCCATTCCCTCTTGTGCC	107 bp amplicon for ChIP-qPCR

Table S2. Primers used in this study. Related to Figure 2, 5, and S3.

[a]Lowercase bases do not anneal to the the gene locus indicated in the table

[b]Underlined sequences are restriction sites