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Implications of the Evolutionary Trajectory of Centromeres in the Fungal Kingdom

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Keywords

CENP-A, centromere clustering, chromosome conformation capture, karyotype evolution, heterochromatin, RNAi

Abstract

Chromosome segregation during the cell cycle is an evolutionarily conserved, fundamental biological process. Dynamic interaction between spindle microtubules and the kinetochore complex that assembles on centromere DNA is required for faithful chromosome segregation. The first artificial minichromosome was constructed by cloning the centromere DNA of the budding yeast *Saccharomyces cerevisiae*. Since then, centromeres have been identified in >60 fungal species. The DNA sequence and organization of the sequence elements are highly diverse across these fungal centromeres. In this article, we provide a comprehensive view of the evolution of fungal centromeres. Studies of this process facilitated the identification of factors influencing centromere specification, maintenance, and propagation through many generations. Additionally, we discuss the unique features and plasticity of centromeric chromatin and the involvement of centromeres in karyotype evolution. Finally, we discuss the implications of recurrent loss of RNA interference (RNAi) and/or heterochromatin components on the trajectory of the evolution of fungal centromeres and propose the centromere structure of the last common ancestor of three major fungal phyla—Ascomycota, Basidiomycota, and Mucoromycota.

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INTRODUCTION

The fungal kingdom constitutes approximately 2.2 to 3.8 million species (40) ranging from free-living microbes to deadly pathogens that thrive in diverse host and environmental niches including soil, water, plants, and animals. The development of genetic tools along with the ease of laboratory culturing and faster growth rate facilitated the use of fungi to study evolutionarily conserved biological processes. One such well-explored process is chromosome segregation, mediated by centromere DNA and the associated kinetochore protein complex. The primary constrictions, first described by Walther Flemming (28) and later identified as centromeres, serve as the chromosomal binding sites for spindle microtubules. In most organisms, centromeres are localized chromosomal domains, present only once on every chromosome. The centromere-kinetochore complex ensures timely and accurate attachment of the spindle microtubules to facilitate the faithful segregation of sister chromatids.

Fast and efficient short- and long-read sequencing techniques and analytic tools enabled complete genome assembly of a large number of organisms and the study of the evolution of key molecular components involved in the maintenance of genome stability. The budding yeast *Saccharomyces cerevisiae* was the first eukaryote to have its entire genome sequenced (32). A recent boom in DNA sequencing efforts has resulted in the availability of over 2,000 representative fungal genomes, providing us with the broadest spectrum of assembled genomes among various eukaryotic kingdoms (**https://www.ncbi.nlm.nih.gov/genome/browse#!/overview/fungi**).

To date, the identity of centromeres from over 60 fungal species has been predicted by DNA sequence analyses; a majority of them have been validated by genetic and/or biochemical experiments (**Figure 1**). The critical analyses of the structural and functional properties of the fungal centromeres have extended our understanding of the evolutionary forces acting on these chromosomal elements. What has evoked interest is the nonuniversality of the factors that define and regulate centromere structure and function. In this review, we provide a comprehensive picture of the diverse classes of fungal centromeres to project a holistic view of their evolutionary trajectories. We summarize the factors influencing centromere establishment and maintenance. The influence of DNA sequence, DNA replication timing, and spatial positioning of centromeres that facilitates cross talk between chromosome segregation machinery and components of other molecular machinery within the nucleus is also explored. We discuss emerging evidence that suggests centromeres are mediators of chromosome rearrangements, a paradoxical contribution that imparts karyotypic diversity in fungi. Based on the structural conservation of centromeres identified from three major fungal phyla (Ascomycota, Basidiomycota, and Mucoromycota) (**Figure 1*a***), features of an ancestral centromere are proposed.

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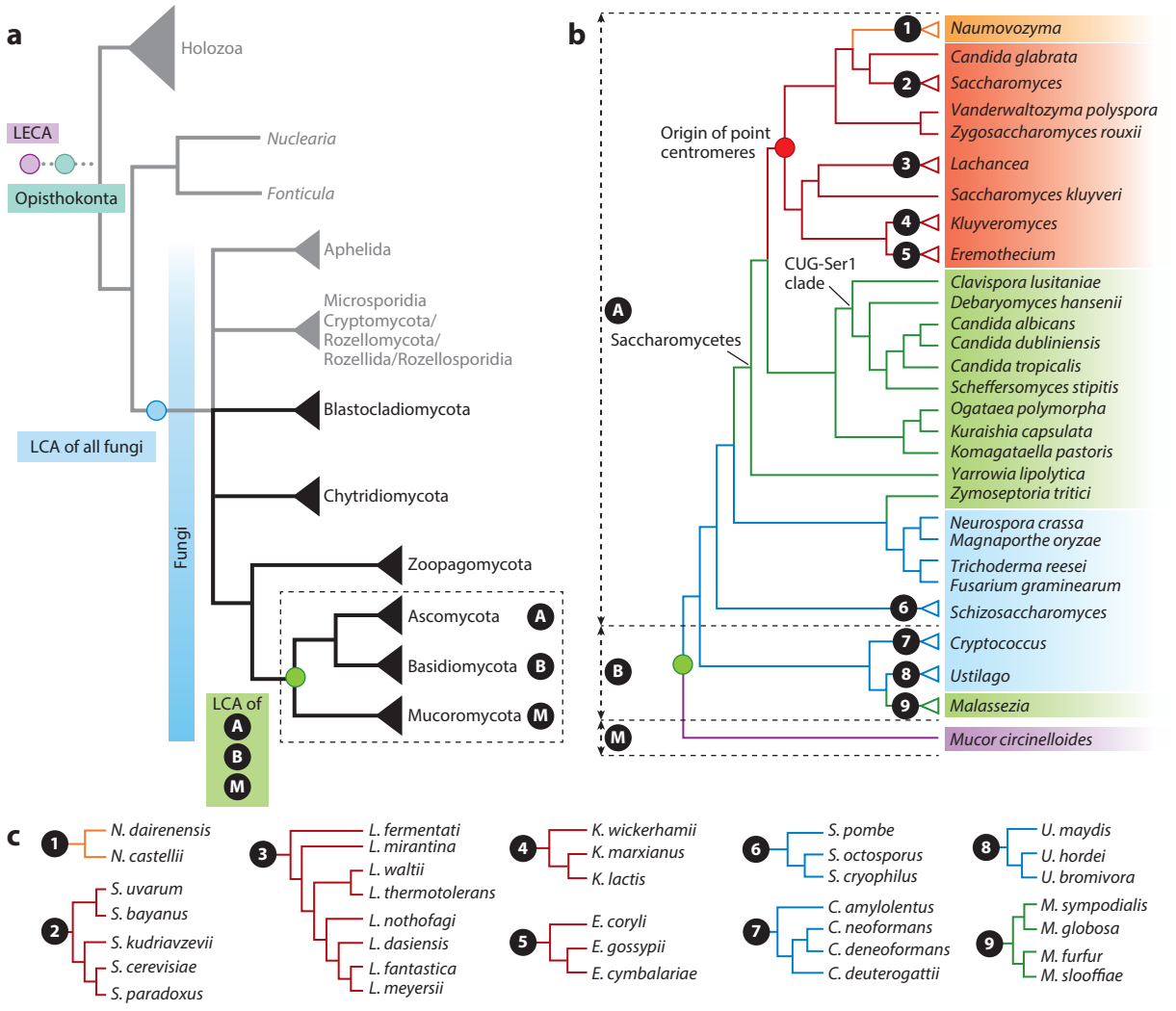

Figure 1

Phylogenetic distribution of fungal species with known centromeres. (*a*) Phylogenetic tree showing the divergence of various fungal phyla from the last eukaryotic common ancestor (LECA, *purple circle*). The fungal phyla Ascomycota (A), Basidiomycota (B), and Mucoromycota (M), which originated from a hypothetical last common ancestor (LCA, *green circle*), are placed within the black dashed box. Phylogenetic tree adapted from Reference 75. (*b*) A maximum-likelihood-based tree of 55 fungal species with known centromeres was generated from the conserved orthologs identified in these species using OrthoFinder (23), MAFFT (50), and FastTree (71). *Blastobotrys adeninivorans*, *Epichloë festucae*, and some species belonging to the genera *Lachancea* and *Malassezia* with known centromere loci are not included in this phylogenetic tree, as the complete annotation of open reading frames is not available. The branches and the groups of species are color-coded based on the centromere type: orange and red, unconventional and conventional point centromeres, respectively; green, short regional; blue, long regional; and purple, mosaic-type centromeres. Nine nodes, marked with black circles numbered from one to nine, containing species with similar centromere type, are collapsed and represented as triangles. (*c*) The internal species-level tree topology of the collapsed nodes is expanded, and branches are color-coded as in panel *b*.

A POINT THAT IS OFTEN STRETCHED: DIVERSITY IN CENTROMERE STRUCTURE

Molecular understanding of centromere DNA was initiated by cloning of centromeres in *S. cerevisiae*, which led to the construction of the first artificial minichromosome (18). The 125-bp point centromere of *S. cerevisiae*, roughly the same length as that of DNA wrapped around a single nucleosome, consists of conserved DNA elements (CDEs): CDEI, CDEII, and CDEIII (44). CDEI and CDEIII share conserved but degenerate motifs of 8 and 26 nucleotides, respectively (27). Although the highly AT-rich CDEII (78–86 bp) (31) is not conserved, its length is important for centromere function (24). A single–base pair mutation in the CCG motif in CDEIII is sufficient to abolish the centromere function. Centromeric nucleosomes contain centromere-specific histone H3 variant CENP-A^{Cse4} (97). Binding of kinetochore proteins facilitates bending of the DNA flanking CDEII, which has an intrinsic ability to form curves (8, 69). These physical properties and DNA sequence recognition by the point centromere–specific protein complexes contribute to the genetic identity of centromere DNA, enabling these sequences to mediate de novo assembly of kinetochore components.

Approximately 25 closely related Saccharomycetes species in the fungal phylum of Ascomycota have been found to contain conventional CDE-like elements at their centromeres (33) (**Figure 1*b***). In these organisms, the length of CDEII varies from 93 bp in *Lachancea waltii* to 161 bp in *Kluyveromyces lactis* (33, 42, 61). These conserved structural features of centromere DNA shared by organisms in the subphylum Saccharomycotina indicate a single origin of the point centromere (**Figure 1*b***). More recently, unconventional point centromeres that harbor CDEs different from those of *S. cerevisiae* have been reported in *Naumovozyma castellii* and *Naumovozyma dairenensis* (53). In contrast to the case of other species with point centromeres, gene synteny analysis suggests a unique and separate origin of point centromeres in *N. castellii* and *N. dairenensis*. The genetic identity of these unconventional point centromeres also revealed a rapid coevolution of the CBF3-complex components Ndc10 and Cep3, which recognize diverged point centromere DNA sequences (53).

Most other fungal species have regional centromeres spanning beyond a single nucleosome and are not strictly defined by the underlying DNA sequence (**Figures 1*b*,*c*** and **2**). The short regional centromere (<20 kb) was first identified in a CUG-Ser1 clade species, *Candida albicans*, and these centromeres feature central CENP-A^{Cse4}-bound centromeric chromatin spanning 3 to 5 kb embedded within unique sequences (77, 78). Lack of sequence conservation and the inability of centromere DNA to stabilize a centromeric plasmid carrying an autonomously replicating sequence (ARS) suggested DNA sequence–independent inheritance of centromere function (7). Centromeres of *Candida dubliniensis* also share similar features, containing unique DNA sequences that are remarkably diverged from their *C. albicans* counterparts (68). AT-rich short regional centromeres with unique DNA sequences were identified in another CUG-Ser1 clade species, *Candida lusitaniae* (49) (**Figure 1*b***). Using various genetic, genomic, and biochemical approaches, short regional centromeres were identified in other species including *Kuraishia capsulata* (59), *Ogataea polymorpha* (72), *Blastobotrys adeninivorans* (57), and *Yarrowia lipolytica* (30). Unusual short regional centromeres of *Y. lipolytica* carry conserved blocks of 9–14-bp regions with dyad symmetry (96).

Inverted repeat (IR)-associated short regional centromeres were identified in the CUG-Ser1 clade species *Candida tropicalis* (15), which diverged ~39 million years ago from *C. albicans*. Unlike the unique centromeres in *C. albicans*, all seven centromeres of *C. tropicalis* are highly homogeneous (56) (**Figures 1*b*** and **2**), containing a 2–3-kb-long CENP-A^{Cse4}-bound *mid* core flanked by 3–5-kb-long IRs. Intriguingly, the entire *mid* core flanked by IRs present on a plasmid can facilitate the de novo recruitment of CENP-A^{Cse4} and improve its mitotic stability, albeit at a lower frequency

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than that of *S. cerevisiae* (15). Similar IR-associated centromeres were identified in *Komagataella phaffii* that consist of ~2-kb IRs flanking ~1-kb central core (*mid*) regions (20). *Zymoseptoria tritici*, a filamentous ascomycete, contains 5.5–13.5-kb CENP-A^{CenH3}-enriched centromeric chromatin (80). Apart from the ascomycetes described above, organisms of the *Malassezia* species complex

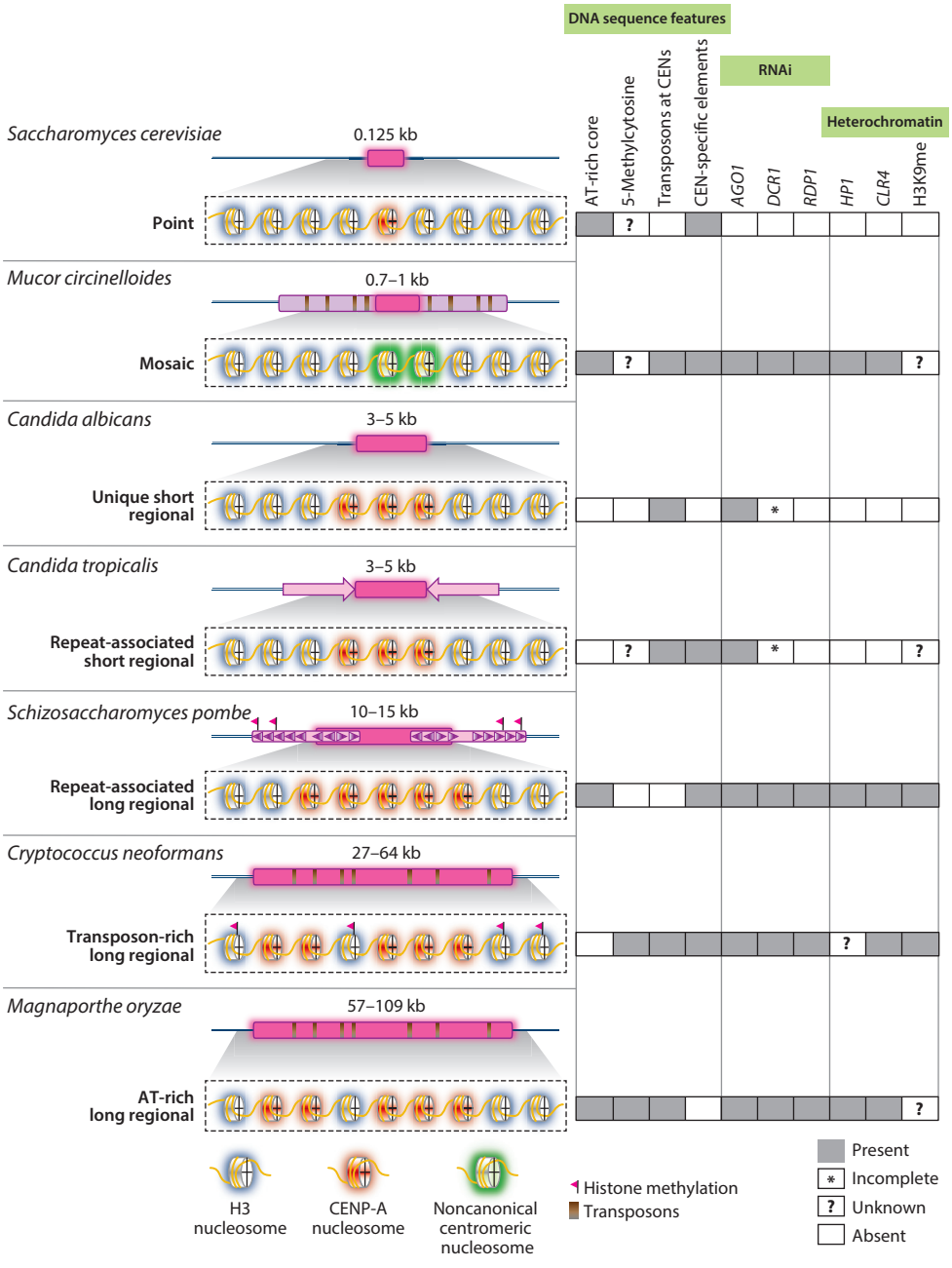

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Figure 2 (*Figure appears on preceding page*)

DNA sequence and structural and chromatin properties of seven major fungal centromere types. (*Left*) Schematic of the centromere organization highlighting the centromeric chromatin domain (*purple*) and flanking pericentric region (*pink*) in each representative type of fungal centromere. Line diagrams are not drawn to scale. A representative nucleosomal arrangement of each type of centromere is shown in a dashed box. (*Right*) The presence or absence of various determinants of centromere structure and function is shown. Transposon refers to the presence of either a full-length transposon or a truncated version of it in at least one centromere of a given species. Centromere-specific DNA sequence elements include conserved DNA sequences present exclusively at the centromeres, but not necessarily common to all centromeres. Centromere-specific elements include conserved DNA elements (*Saccharomyces cerevisiae*), an AT-rich motif (*Mucor circinelloides*), pericentric repeats (*Candida tropicalis* and *Schizosaccharomyces pombe*), and full-length Tcn retrotransposons (*Cryptococcus neoformans*) (14). Abbreviations: CEN, centromere; RNAi, RNA interference.

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of the fungal phylum Basidiomycota (**Figure 1**) also possess short regional centromeres that are highly AT-rich, with 2–5-kb-long centromeric chromatin (76) (**Figure 2**).

A class of DNA sequence–dependent long regional centromeres (>20 kb) was identified in the fission yeast *Schizosaccharomyces pombe* (16, 26, 64). The length of fission yeast centromeres ranges from 40 to 110 kb, encompassing the kinetochore-bound central core (*CC*) region flanked by various types of repeats (17) (**Figure 2**). The central regions of *CEN1* and *CEN3* of *S. pombe* share homology, whereas the central region of *CEN2* is unique (17). The pericentric region consists of *dg* and *dh* classes of repeats (16). However, a part of *CC* and one arm of pericentric chromatin proved to be sufficient for the establishment of centromere identity and proper segregation of minichromosomes (6). Similar repeat-associated long regional centromeres were identified in closely related *Schizosaccharomyces* species: *Schizosaccharomyces cryophilus*, *Schizosaccharomyces octosporus*, and *Schizosaccharomyces japonicus* (74, 93).

Long regional centromeres, which are rich in transposons, have been reported in both the Ascomycota and Basidiomycota (**Figure 2**). Centromeres of *Neurospora crassa*, *Magnaporthe oryzae*, and *Cryptococcus neoformans* are highly repetitive and harbor active and/or truncated transposable elements (13, 100, 101). The length of centromeres ranges from 150 to 300 kb of heterochromatic DNA in *N. crassa* (13). The repeats at the centromeres of *N. crassa* contain numerous C:T and G:A transitions introduced by recurring cycles of repeat-induced point mutations (RIPs) leading to centromere DNA sequence divergence (12, 82). AT-rich centromeres of *M. oryzae* contain 57–109-kb centromeric chromatin (101). Chromosome conformation capture (3C) analysis revealed putative centromeric regions containing clusters of retrotransposon element Tdh5 spanning 18–27-kb regions on every chromosome in the ascomycete *Debaryomyces hansenii* (59). The RNA interference (RNAi)-proficient species of the *Cryptococcus* species complex harbor 20–110-kb-long centromeric chromatin. RNAi seems to help maintain full-length retrotransposons at centromeres by suppressing their expression in these organisms (100). It has been proposed that in the absence of RNAi, increased transposition and recombination between retrotransposable elements led to reduction in centromere length. A correlation between accumulation or loss of retrotransposons with alteration in centromere length has been reported in the *Cryptococcus* as well as the *Ustilago* species complexes (100).

Most fungal centromeres studied to date are enriched with CENP-A (99). The loss of CENP-A has been described in kinetoplastid kinetochores present in trypanosomes (1). In addition, certain insect lineages that lack CENP-A (21) harbor holocentric chromosomes, implying an independent transition to holocentricity (diffuse centromeres along the entire length of a chromosome) upon CENP-A loss in these lineages (1, 21). Among fungi, CENP-A loss has been recently reported in an early diverging subphylum, Mucoromycotina (66, 94). Strikingly,

Mucor circinelloides has monocentric chromosomes despite lacking CENP-A. The average kinetochore binding length is 941 bp, with a conserved AT-rich motif, in this organism. These centromeres are of a mosaic type given their point centromere–like kinetochore binding domain and unusually long pericentric regions. These pericentric regions range between 15 and 75 kb and are interspersed with Grem-LINE1 elements, which are repeats of LINE1-like non-LTR retrotransposable elements (**Figure 2**). The diversity in both the length and the structure of fungal centromeres hints at the presence of additional factors beyond centromeric DNA for the establishment and propagation of centromeric chromatin.

CENTROMERE ESTABLISHMENT VERSUS PROPAGATION

Establishment of centromeric chromatin involves interactions between kinetochore proteins and centromere DNA that can be at the level of primary DNA sequence, chromatin architecture, and/or three-dimensional conformation of the genome (**Figures 2** and **3**). Factors required for maintenance of centromeric chromatin include heterochromatin components, transcriptional status, replication timing, and spatial chromosomal interactions (**Figure 3**).

Establishment of centromeric chromatin on naked DNA sequences was first demonstrated by high mitotic stability of minichromosomes in *S. cerevisiae* (18). However, in many fungal species, the mode of centromere establishment is independent of the underlying DNA sequence. In *S. pombe*, a heterochromatic environment facilitated by the HP1 homolog Swi6 and RNAi-mediated machinery helps in the efficient recruitment of CENP-A^{Cnp1} to the central regions (29, 37) (**Figure 3*a***). On the other hand, the epigenetic nature of centromeres in *C. albicans* that lacks RNAi and conventional heterochromatin does not permit the stabilization of a kinetochore on an externally introduced centromeric plasmid (7). This raises the possibility that species-specific factors are involved in centromere establishment. This plasticity of centromeric chromatin has been exemplified in experiments carried out in fungal species studying neocentromere formation, transgene silencing at the centromere, artificial centromere construction, and dicentric inactivation.

Neocentromeres, which are sites acquiring centromeric properties in the event of native centromere inactivation, act as an excellent tool to study factors contributing to centromere establishment. Systematic deletion of CENP-A^{Cse4}-binding and -flanking DNA sequences in *C. albicans* resulted in the formation of neocentromeres in close proximity to the deleted region (92) (**Figure 3*c***). An independent study reported the activation of both proximal and distal neocentromeres in *C. albicans* (51). Strikingly, Hi-C studies indicated that even the distal neocentromere clusters with other native centromeres of various chromosomes. This indicates that proximity to the CENP-A–rich zone or CENP-A cloud where endogenous centromeres cluster together at the nuclear periphery is a stronger determinant than the DNA sequence itself for neocentromere establishment in this organism (92). This was seen to be consistent in *C. dubliniensis* as well (92). On the other hand, the conditional deletion of a centromere in *S. pombe* produced survivors in which chromosomes were largely rescued by telomeric fusions with another chromosome or in rare cases activated a neocentromere at a subtelomeric region (46) (**Figure 3*c***). The similarities in the heterochromatin environment at both of these loci and the presence of sequences homologous to the *dg* and *dh* elements identified in the subtelomeric regions explain the preferential activation of neocentromeres at these loci (38).

Reversible transgene silencing is a unique feature of centromeric chromatin. When transgene *URA4* or *ADE2* is integrated at the central region of centromeres in *S. pombe*, the transgene undergoes reversible transcriptional silencing, rendering variable expression patterns (2, 35,

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102). However, the expression of the same transgene integrated at the outer repeats was efficiently turned off due to the highly heterochromatic nature of these repeats (3). The boundary of centromeric heterochromatin that retains the property of reversible silencing was determined in *C. albicans* by integrating *URA3* as a transgene at the core and centromere-flanking regions. This study suggested flexible positioning of CENP-A^{Cse4} within a domain that permits neocentromere activation when the native centromeric DNA sequence is deleted (85). In *S. pombe*, however, the tRNA genes were identified as the boundary elements between CENP-A^{Cnp1} chromatin and flanking heterochromatin regions (81). These studies indicate that low levels of CENP-A^{Cse4} can be present beyond the 3–5-kb region of centromeric chromatin in the absence of any boundary elements in *C. albicans*, while CENP-A^{Cnp1} is restricted by defined boundary elements in *S. pombe*. Structural boundary elements are not identified in other classes of regional centromeres, and thus

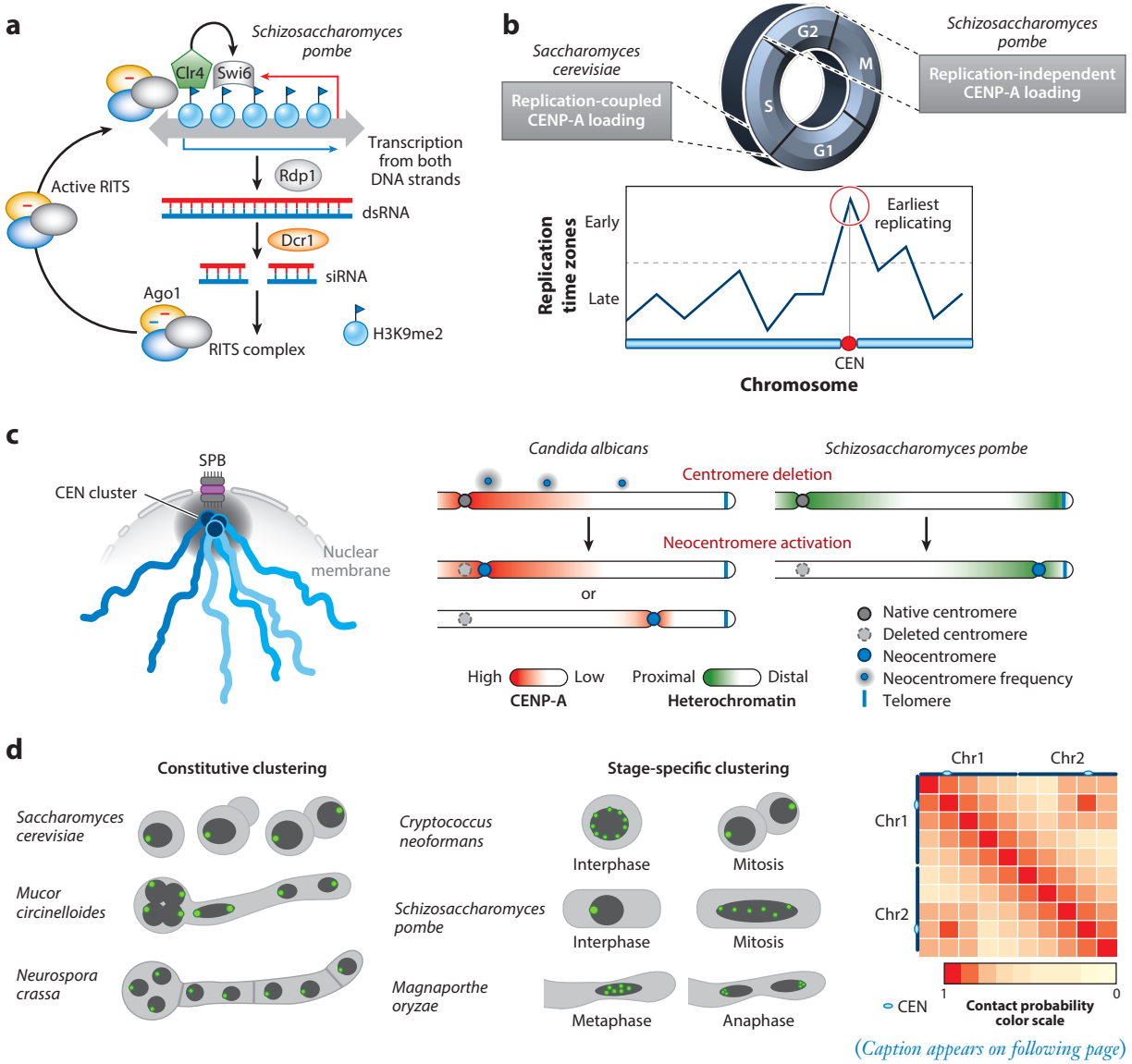

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Figure 3 (*Figure appears on preceding page*)

Molecular determinants of centromere formation in fungi. (*a*) Maintenance of centromeric heterochromatin at the outer repeats in *Schizosaccharomyces pombe* is mediated by RNA interference–dependent machinery (60) where both strands of the outer repeats are transcribed by RNA polymerase II. Double-stranded RNA (dsRNA) molecules are generated with the help of RNA-dependent RNA polymerase I (Rdp1) and processed by Dicer (Dcr1) to yield small interfering RNAs (siRNAs). The resulting duplex siRNAs are loaded onto the Argonaute (Ago1) complex and converted into single-stranded siRNAs after cleavage and released as the passenger strand in the RNA-induced transcriptional silencing (RITS) complex. The RITS complex also recruits the H3K9 methyltransferase Clr4. H3K9 methylation stabilizes the association of RITS with centromeric chromatin and also provides binding sites for Swi6. (*b*) Fungal centromeres replicate early in S phase. The time of incorporation of new CENP-A molecules on the replicated centromere DNA strands differs between point and regional centromeres. While a replication-coupled loading of CENP-A occurs in point centromeres of *Saccharomyces cerevisiae*, CENP-A loads at G2 phase in a replication-independent manner in regional centromeres of *S. pombe*. (*c*) The location of the centromere (14) cluster at the nuclear periphery is evolutionarily conserved across fungal species. A drawing representing the Rabl-like organization of chromosomes is shown. Centromeres are clustered close to the spindle pole bodies (SPBs). The centromere cluster is enriched with CENP-A molecules that form a CENP-A-rich zone or CENP-A cloud. The concentration of CENP-A is gradually reduced from the core centromere to the peripheral regions. The size of the gray area around the blue circles is proportionate to the frequency of neocentromere activation. Upon deletion of a native centromere, the frequency of neocentromere activation is higher at a centromere-proximal location than at the centromere-distal sites in *Candida albicans*. In *S. pombe*, a heterochromatin-mediated mechanism guides the activation of neocentromeres at subtelomeric regions. (*d*) The spatial clustering of centromeres, either constitutive or cell cycle stage–specific, is a unique feature across fungal species. (*Left* and *middle*) Clustering patterns for representative fungal species have been depicted by kinetochores (*green*) arranged at the periphery of the nuclear mass (*dark gray*). (*Right*) The microscopic observations of spatial clustering have been supported by 3C-seq and derived techniques. A drawing of a genomic contact probability matrix representing enhanced intercentromeric interactions, as reported in several fungal species, is shown. Other abbreviation: CEN, centromere.

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it is not well understood what restricts the length of the functional centromeric chromatin that seeds kinetochore assembly.

New insights into factors required for centromere function could be gained by studying the fate of dicentric chromosomes. In *S. cerevisiae*, dicentric chromosomes are unstable but are stabilized exclusively by DNA rearrangements when one of the two centromeres becomes inactivated (47). The artificial dicentric chromosome generated in *S. pombe* using site-directed recombination led to cell cycle arrest at interphase. Less than 1% of the survivors were shown to inactivate one of the centromeres either by DNA sequence rearrangement or by heterochromatinization of the centromere DNA sequence leading to epigenetic inactivation (79). The fact that the native centromere always serves as the sole functional centromere despite the presence of several potential neocentromere sites indicates the existence of an active suppression mechanism to keep neocentromeres dormant.

Maintenance of centromeric chromatin involves efficient propagation of already established centromeric chromatin marks. Even the genetically determined point centromere in *S. cerevisiae* displays an epigenetic mode of maintenance. Chl4 is a nonessential kinetochore protein in *S. cerevisiae*. A centromeric plasmid introduced into *chl4* mutants displays reduced mitotic stability. Whereas if the same mutation is introduced after the centromere is allowed to establish on the plasmid centromere, 50% of the cells show high mitotic stability, indicative of the semi-essential role of Chl4 in centromere maintenance (63). In *S. pombe*, when various centromeric plasmids with incomplete centromere DNA sequences were transformed, the mitotically unstable plasmid switched to a stable state by epigenetic means. Strikingly, this stable state was efficiently propagated in subsequent cell divisions (87).

THE ENIGMATIC CHROMOSOMAL HUB: CENTROMERE SPECIFICATION IN TIME AND SPACE

Centromeres are spatially and temporally distinguishable from the rest of the genome owing to their distinct clustering patterns and replication timing, respectively. Centromeres are replicated

in the earliest part of S phase in certain *Saccharomyces* species (70), *C. albicans* (54), and *S. pombe* (73) (**Figure 3*b***). What is the significance of fungal centromeres being early replicating? Early replication timing ensures proper kinetochore assembly at the centromeres (52) and helps to maintain the viability of cells in the face of any replication stress in *S. cerevisiae* (25). Early replication of centromeres due to the early firing of the centromere-proximal origins can be attributed to their characteristic clustering and nuclear subpositioning (4). The relocation of a centromere to a late firing region enhances the replication timing of the latter, emphasizing that the mere presence of a centromeric sequence can modulate replication timing (70). Pausing of the DNA replication fork at centromeres helps in maintaining centromere DNA loop formation, which is essential for sister centromere separation and kinetochore assembly in *S. cerevisiae* (19, 34). In *S. pombe*, centromeres and the subtelomeric regions have a similar heterochromatin environment but differ in their replication timing. The heterochromatin protein Swi6 helps in early replication of centromeres (5, 39), exhibiting the prominent role played by heterochromatin in influencing replication timing and the consequent effect on centromere function.

The temporal effect on DNA replication origin firing has also been studied in *C. albicans*, in which deletion of a native centromere gives rise to a neocentromere with the activation of an early firing neo-origin (54). This clearly shows that centromeric location positively influences replication timing of the adjacent regions. In *Y. lipolytica*, a centromere-linked replication origin helps to maintain plasmid stability (30). Hence, the role of centromere-proximal origins seems to be more than just acting as initiation sites for DNA replication.

Apart from the temporal regulation of centromere replication, the positioning of centromeres at the nuclear periphery near spindle pole bodies (SPBs) in a transcription-poor zone facilitates spindle attachment and shields the centromere from pervasive transcription (62). Centromeres are clustered throughout the cell cycle in *S. cerevisiae* (48) and *C. albicans* (78) (**Figure 3*c***), and the existence of a CENP-A-rich zone or CENP-A cloud at centromere-proximal regions has been proposed (92). In *S. cerevisiae*, a locally enriched population of CENP-A^{Cse4} molecules at pericentromeres helps in the rapid incorporation of CENP-A^{Cse4} in the event of untimely eviction of CENP-A^{Cse4} from the centromeres (36). Localization of CENP-A^{Cse4} molecules as a single punctum per nucleus suggests that a CENP-A-rich zone exists. The CENP-A cloud hypothesis explains the activation of native centromere-proximal neocentromeres in *C. albicans* (11, 92). Unlike the case of budding yeast, centromeres in fission yeast cluster during interphase and uncluster for a brief period during mitosis (90). These clustered centromeres are attached to the nuclear envelope near the site of SPBs during interphase (45). In *C. neoformans*, unclustered centromeres in interphase eventually cluster at the mitotic onset in a microtubule-dependent manner (55).

Apart from unicellular yeasts, centromere clustering has also been observed in filamentous fungi like *Fusarium graminearum*, *N. crassa*, and *M. oryzae*, wherein with the exception of *M. oryzae*, all centromeres were found to constitutively cluster by fluorescence microscopic analyses (84, 101) (**Figure 3*d***). Despite the differences in the centromere clustering patterns across fungal species examined to date, it has been consistently shown that centromere clustering is important for proper kinetochore-microtubule attachment during mitosis (45, 91, 98).

Recent progress in microscopic imaging and sequencing techniques has enabled the successful mapping of distinct compartments within the nucleus to address fundamental questions regarding the structure and functional states of chromosomes. 3C-sequencing in *S. cerevisiae* revealed that the clustered centromeres are present in close spatial proximity, leading to physical interactions between different chromosomes (22) (**Figure 3*d***). In *S. pombe*, where heterochromatin is a major determinant of centromere organization, centromere-proximal regions interact with each other at higher contact frequency, as revealed by Hi-C analysis. A similar correlation supported by Hi-C

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analysis in *N. crassa* revealed predominant interactions across constitutively heterochromatic regions enriched with H3K9me3 and HP1. Due to the conserved clustering features of fungal centromeres, Hi-C and related techniques have been used to accurately predict centromere loci in fungal genomes (95).

What determines the clustering of centromeres in the absence of heterochromatic marks and well-defined DNA sequences remains an enigma. Clustering of *C. albicans* centromeres, which are devoid of conventional heterochromatin, indicates additional factors are involved that facilitate this process (85). As discussed previously, centromere clustering favors the site of centromere formation in subsequent cell cycles, possibly by CENP-A nucleation. Surprisingly, even in the CENP-A-deficient species *M. circinelloides*, centromeres are constitutively clustered in both the spore and the germination tube (66) (**Figure 3*d***).

THE ACHILLES' HEEL: GENOME REARRANGEMENTS INVOLVING CENTROMERES

The mechanisms contributing to the rapid evolution of centromere DNA, especially in asexual fungi, remain unclear. Genome synteny analyses in *C. albicans* and *C. tropicalis* helped identify genomic rearrangements near centromeres suggesting intercentromeric translocations in the last common ancestor (15, 35a). One can hypothesize that centromere-type transition between *C. tropicalis* and *C. albicans* was initiated due to such translocation events. A similar intercentromeric translocation has been observed in the common ancestor of *S. cryophilus* and *S. octosporus* (93). Centromeres are also involved in karyotypic evolution of fungal species. One such example is found in *Eremothecium gossypii*, where a break at the centromere followed by fusion of the broken arms with two other chromosomes in the ancestor led to chromosome number reduction (33). Centromere breaks resulting in chromosome number reduction have been reported recently in the *Malassezia* species complex (76) (**Figure 4**).

Chromosomal rearrangements may lead to reproductive isolation and speciation (10). However, the altered karyotype may also confer fitness advantages for it to be selected over the

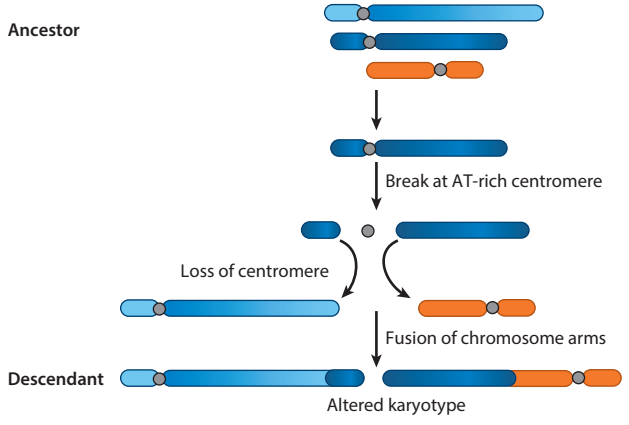

Figure 4

Centromere-mediated karyotype evolution in fungal species. A possible consequence of chromosome breakage at an AT-rich centromere. The resulting acentric fragments can be stabilized by fusion with other chromosomes, eventually leading to an altered karyotype as observed in the species complexes belonging to Ascomycota and Basidiomycota. Each of the three colors (*blue*, *light blue*, and *orange*) indicates a distinct chromosome.

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ancestral karyotype. Because it is difficult to predict the factors driving speciation, the fitness advantages conferred by species-specific rearrangements are not well understood. One of the ways to achieve karyotypic alteration is through centromere-mediated chromosomal rearrangements. An example of such a translocation includes the bipolar-to-tetrapolar mating-type transition in the *Cryptococcus* species complex, which involves a pericentric inversion, thereby rewiring the regulation of the mating-type locus (89). Another instance where karyotype alteration provides a specific fitness advantage involves the generation of an isochromosome of chromosome 5L in *C. albicans* that confers fluconazole resistance (83). Thus, centromere DNA, one of the guardians of genome stability, may contribute to chromosomal rearrangements and possibly speciation.

SUMMARY AND FUTURE DIRECTIONS

In this review we highlight the diversity of fungal centromeres and the conserved factors for centromere structure and function (**Figure 5**). Over the last few decades, the number and types of centromeres identified within the fungal kingdom hinted that centromere specification cannot be explained by a unifying factor. The contribution of the centromere DNA sequence alone encoding centromere identity holds true only for certain species. In fact, identification of centromeres in closely related *Saccharomyces*, *Schizosaccharomyces*, and *Candida* species revealed that the centromeres are one of the fastest-evolving loci even in the absence of asymmetric meiosis (9, 68, 74). Therefore, a rapid coevolution of centromere DNA and the associated kinetochore proteins seems to be the only plausible explanation for fungal centromeres, similar to what was originally proposed in flies (41). It may be possible that the structural and sequence elements present in the ancestral lineages established centromere identity but were not essential for its propagation and were eventually lost in their successors (**Figure 5**). RIP is one such mechanism reported in *N. crassa* to account for such rapid changes in centromere DNA sequences. The presence of similar mechanisms can be probed in other fungal species.

Independent of centromere DNA sequences or the presence of RNAi and/or heterochromatin components, spatial positioning of the centromere cluster and a favorable chromatin environment contribute to kinetochore formation. This necessitates in-depth studies to understand the centromere-kinetochore interactions in 3D across closely related species. Recent advances in Hi-C analyses have significantly enriched our understanding of the interactions between centromeres and flanking regions in fungal species. Roles of replication-associated proteins in centromere maintenance have been studied in *S. cerevisiae* (65) and *C. albicans* (86). It is tempting to speculate that despite their having different centromere types, a similar spatial memory guides centromere location and regulates its activity (7, 11). The CENP-A cloud hypothesis was proposed to explain the importance of physical location that regulates neocentromere establishment (92). The conserved Rabl conformation is also associated with a gradient of replication timing. The chromosomes are arranged in a predetermined way where the clustered centromeres and the flanking regions are early replicating and replication timing is increasingly delayed from centromeres toward telomeres (58). The impact of spatial genome organization in fungi can be better understood by studying physical processes like phase separation (88). Formation of heterochromatin domains is mediated by phase separation, a phenomenon that gives rise to non-membrane-bound nuclear, cytoplasmic, and extracellular components (88). It is plausible to imagine centromeric chromatin as a phase-separate biomolecular condensate owing to its distinct chromatin properties, its subnuclear positioning, and most importantly the presence of an ensemble of a hundred different kinetochore proteins occupying its binding site.

The loss of functional RNAi machinery within the same species complex leads to shortening of the centromere (100). RNAi has been suggested as a centromere length maintenance mechanism

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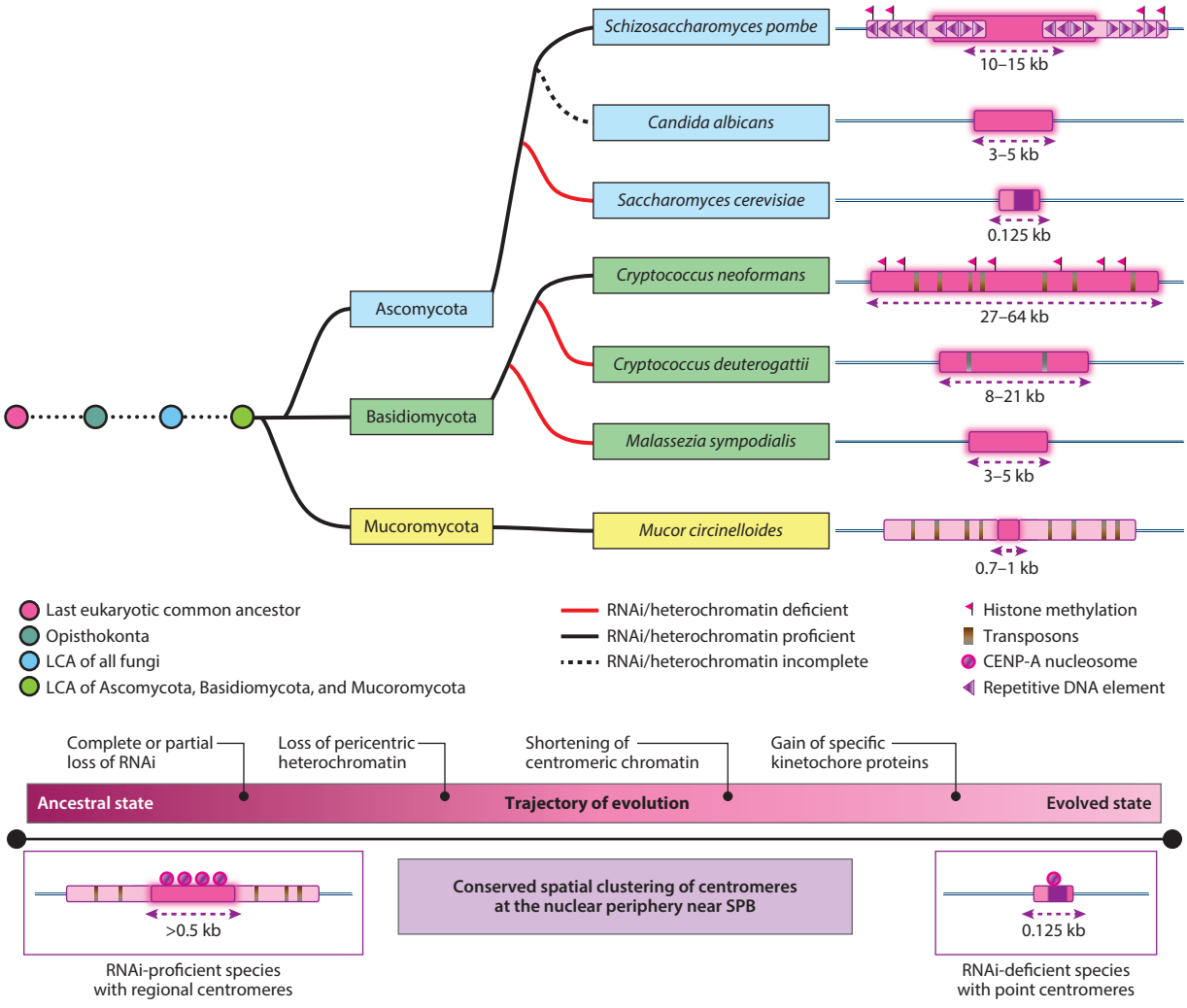

Figure 5

Trajectory of centromere evolution in fungi. A possible trajectory of events leading to transition in centromere types in fungal species during evolution. Presence of RNA interference (RNAi) and/or heterochromatin in species from each of the three major fungal phyla represented above suggests that the ancestral species harbors transposon-rich regional centromeres with >700-bp-long kinetochore-bound regions flanked by pericentric regions. Recurrent loss of RNAi and/or heterochromatin led to a reduction in centromere length and emergence of point centromeres. The only feature that remains conserved in spite of the diversity in the centromere structure is the spatial clustering of centromeres. This evolutionarily conserved nature of spatial clustering of centromeres near spindle pole bodies (SPBs) away from the active transcription zone may play a significant role in centromere specification and its subsequent propagation. Other abbreviation: LCA, last common ancestor.

for long transposon-rich centromeres. On the other hand, the absence of RNAi within the Saccharomycotina is correlated with their short centromeres devoid of transposons. The loss of HP1 and other components of heterochromatin in these budding yeast species (43) compromised the possibility of an RNAi-induced heterochromatin pathway, which is a predominant mechanism for heterochromatin formation in species like *S. pombe* (**Figure 5**). Further analysis of more fungal species will reveal whether this dependency is universal. RNAi can be thought to be a defense

mechanism that functions in fungi to combat mycoviruses (67). In addition, it is not clear whether the transposons hitchhiked on the centromeres for their efficient survival or whether centromeres formed on the transposons to silence them.

An emerging hypothesis suggests an involvement of the centromeres during karyotype evolution among closely related fungal species. One of the possibilities for this unusual process is that the difficult-to-replicate centromeres are hotspots for replication fork stalling (34). The presence of IRs at the centromeres and the possible secondary structure formation by the AT-rich centromere DNA may render centromere DNA prone to occasional breakage, as proposed for the *Malassezia* species (76). Extensive plasticity of many fungal genomes, especially the predominantly asexual and pathogenic ones, may accommodate such karyotypic changes associated with speciation.

Finally, one may wonder what the ancestral type of centromere was in the last eukaryotic common ancestor (LECA). Based on the centromeres identified, we speculate the last common ancestor of the Ascomycota, Basidiomycota, and Mucoromycota had a regional centromere with a kinetochore binding region longer than 500 bp surrounded by pericentric heterochromatin. Because such an ancestral species harbored the RNAi machinery, retrotransposons at the centromeres may also have been present. We hypothesize that RNAi and heterochromatic components were lost either gradually or concomitantly during evolution of some Basidiomycota and Ascomycota, paving the path of centromere evolution from regional-type transitioning to point centromeres (**Figure 5**). Identification of centromeres from other fungal phyla such as the Zoopagomycota, Chytridiomycota, and Blastocladiomycota will shed light on the centromere type of the last common ancestor of all fungi.

FUTURE ISSUES

1. How are the neocentromere sites repressed when the native centromere is active?
2. Does phase separation favor the spatial clustering of centromeric chromatin?
3. What could be the structure and nucleotide composition of the centromeres in the last common ancestor of fungi and the LECA?
4. How is the unconventional pericentric heterochromatin in *C. albicans* and related species different from the conventional heterochromatin seen in large regional centromeres?
5. What are the elements restricting the length of centromeric chromatin in fungi?

DISCLOSURE STATEMENT

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