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A matter of scale and dimensions: Chromatin of chromosome landmarks in the fungi

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Abstract

Chromatin and chromosomes of fungi are highly diverse and dynamic, even within species. Much of what we know about histone modification enzymes, RNA interference, DNA methylation, and cell cycle control was first addressed in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Aspergillus nidulans* and *Neurospora crassa*. Here, we examine the three landmark regions that are required for maintenance of stable chromosomes and their faithful inheritance, namely origins of DNA replication, telomeres and centromeres. We summarize the state of recent chromatin research that explains what is required for normal function of these specialized chromosomal regions in different fungi, with an emphasis on silencing mechanism associated with subtelomeric regions, initiated by sirtuin histone deacetylases and histone H3 lysine 27 (H3K27) methyltransferases. We explore mechanisms for the appearance of “accessory” or “conditionally dispensable” chromosomes, and contrast what has been learned from studies on genome-wide chromosome conformation capture in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Neurospora crassa* and *Trichoderma reesei*. While most of the current knowledge is based on work in a handful of genetically and biochemically tractable model organisms, we suggest where major knowledge gaps remain to be closed. Fungi will continue to serve as facile organisms to uncover the basic processes of life because they make excellent model organisms for genetics, biochemistry, cell biology and evolutionary biology.

CHROMATIN: AN ASSEMBLY OF DNA, PROTEINS, AND RNA

Chromosomes of fungi are linear segments of DNA, covered by a diverse assembly of RNA and proteins. They contain three landmarks required for function, namely origins for DNA replication, centromeric DNA as attachment points for kinetochores and telomere repeats to circumvent the end replication problem for linear DNA. Because fungi have been excellent model organisms for trail-blazing basic research since the adoption of *Neurospora crassa* as one of the workhorses for genetics in the 1940s [1], much of the foundation for general knowledge of eukaryotes was first uncovered with fungi, specifically the four species uniquely suited for genetics, biochemistry and genomics, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *N. crassa*. This has certainly also been true for studies on chromatin and chromosomes.

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Chromatin, the building material for chromosomes, is an assembly of DNA, proteins and RNA, organized into nucleosome units, most of which contain octamers of canonical core histone proteins [2]. The three landmarks on chromosomes mentioned above, origins of replication, telomeres and centromeres, are characterized by different specialized chromatin modifications, for example DNA base modifications, like cytosine or adenine methylation, and posttranslational modifications of histones, and it is the set of gene silencing modifications at those chromosome landmarks that will be examined here in more detail. Occupancy of proteins binding specialized nucleosomes, and presence of regulatory RNA species also affects regional chromatin composition. Because this chromatin composition is generally heritable through mitosis or meiosis yet the underlying DNA base sequence is not changed, these variable chromatin compositions are considered by some as “epigenetic”, acting above the DNA level to effect gene regulation, including large-scale changes in chromosome structure [2].

Since the isolation of the first histone acetyltransferase enzyme from the ciliate *Tetrahymena thermophila* [3], chromatin biology has undergone a revolution, reflecting the importance of studying transactions carried out on DNA within the normal cellular context rather than with purified DNA. In the model organisms, studies on transcriptional and post-transcriptional gene silencing, DNA methylation, cell cycle control, and the production of secondary metabolites have driven the work on chromatin structure and modification. Much of what we know about histone modification enzymes, RNA interference and cell cycle control was first addressed in *S. cerevisiae* and *S. pombe* [4, 5]. Thus fungi have been at the cutting edge of this emerging discipline and there are many recent reviews available to dive into the details of yeast chromatin and epigenetics [4–13], or chromatin of other, mostly filamentous, fungi [14–26]. One important purpose of this contribution is to suggest where additional data are necessary to better define the structure and function of chromatin and chromosomes in the fungi.

The scale of observation is important to develop a complete picture of chromatin and chromosome dynamics: are we observing a single gene, a region of co-regulated genes, specific landmarks like telomeres or centromeres, whole chromosomes or the entire set of chromosomes? Scale will affect the representation of and our thinking about the segments observed, as genes or even whole chromosomes are often drawn as simple scalar plots or histograms, even though in the nucleus chromosomes fold and interact in a non-linear way and in three-dimensional space. High resolution cytology [27] and sequencing based techniques, such as chromosome conformation capture (3C) followed by high-throughput sequencing (Hi-C) [28] allow unprecedented studies on fungal chromosome dynamics. Lastly, one important goal for chromatin and chromosome research in the near future will be to capture dynamic processes across time, and also at different scales, for example milliseconds for changes in transcription upon induction by small molecules, to minutes or hours during mitosis and cytokinesis, or across a whole day when studying circadian rhythms. Under these various conditions metastable or stable re-arrangements of chromatin complexes occur, some of which alter the structure of whole chromosomes in the nucleus. This is where traditional model species excel and are exceedingly useful, because of their large arsenal of methods and strains.

Of course, the kingdom Mycota is much more diverse than the current representation by a handful of models suggests. Luckily, technological advances, especially in high-throughput DNA sequencing, proteomics and nanoscopy, make almost any organism a facile “model”. Chromatin scaffold proteins, like core histones, are ancient and conserved in the fungi [29]. Genes for the enzymes that modify DNA or histones are also largely conserved [14, 18, 26]. One recent major discovery is the rather widespread distribution of 6-methyladenine observed in the basal lineages that is apparently absent from the Dikarya [30]. Similarly, the Saccharomycetaceae can no longer methylate cytosines or adenines in DNA or methylate lysines of histone H3 at positions 9 and 27 (Figure 1). Based on the overall phylogeny, these losses occurred more than once in different lineages, so that the ancient state is predicted to be a full complement of DNA and histone modification capabilities, similar to that observed in many plants and animals. The purpose of this chapter is to make some of the deep knowledge garnered from a small group of organisms from restricted phylogenetic backgrounds accessible, in combination with findings from emerging model fungi, always remembering that it may be premature to derive hard and fast rules for larger groups. Ultimately, comparative biology -especially on the poorly understood chytrids and zygomycetes- will yield a much broader understanding of nature to allow us to separate the general from the specific in answering the question on how fungi assemble and maintain dynamic chromosomes.

It is far beyond the scope of this review to go into great detail on all pathways of DNA or protein modifications that can affect chromatin states. In the section on subtelomeric gene silencing we will, however, discuss two examples in more depth, namely the histone deacetylase Sir2, and fungal homologues of the *Drosophila* histone H3K27 methyltransferase Enhancer of Zeste (E[z]), because they inform work in all eukaryotes.

CHROMOSOME LANDMARKS: ORIGINS, TELOMERES, AND CENTROMERES

Origins of replication

DNA replication in eukaryotes is initiated at several loci along the chromosome, short segments called “replication origins”, which are selected by binding of the Origin Recognition Complex (ORC) [31, 32]. During the late M and early G1 phase, ORC, Cdc6 and Cdt1 direct the loading of “Mini-Chromosome Maintenance” complexes (MCM), assembling pre-replication complexes that, at entry into S phase, trigger DNA unwinding and allow DNA replication. While replication origins differ in timing and frequency of activation, the maximum is only once per each nuclear cycle. While much progress has been made in a few organisms, a complete understanding of initiation efficiency and timing is still elusive, even in *S. cerevisiae*, which has been thoroughly studied [33–35]. Nucleosome positioning, post-translational histone modifications, and absence of active transcription all have some influence on the selection of ORC binding sites and replication initiation [10, 32, 36].

In some eukaryotes, including *S. pombe*, sequence requirements for replication origins are not strict, and almost any highly AT-rich sequence of ~1 kilobase (kb) is sufficient [37, 38].

Specific sites are selected by interaction with the AT-hooks of the Orc4 subunit in *S. pombe* [39]. This is different in *S. cerevisiae* where specific loci function as replication origins, initially called Autonomously Replicating Sequences (ARS), which contain consensus sequences called ARS Consensus Sequence (ACS) that are required to allow ORC binding [40–42] (Figure 2). Functional replication origins in *S. cerevisiae* have now been mapped by several techniques, including ChIP followed by genome-wide mapping via microarrays, revealing between 430 and 530 sites [33–35].

Evidence for true replication origins from most other fungi is still lacking, except for *Candida albicans* and *Cryptococcus*. The short centromeric and pericentric regions in *C. albicans* replicate early in S phase [43], and were found to contain *bona fide* replication origins characterized by short sequence motifs [44]. In *C. deneoformans*, replication origins were mapped by enriching DNA containing replication bubbles [45]. One origin was localized in the non-transcribed spacers of rDNA repeats. Seven additional regions on five chromosomes were identified, which –based on 2D gel analyses– resemble those of *S. pombe* and humans in their relatively inefficient usage, suggesting the existence of replication zones, rather than well-defined sequence elements for ORC binding [45]. Note that taxonomy and nomenclature of *Cryptococcus* has been updated [46], and that *Cryptococcus neoformans* var. *grubii* is now referred to as *Cryptococcus neoformans*, while *Cryptococcus neoformans* var. *neoformans* is now *Cryptococcus deneoformans*.

In the other popular model species, like *N. crassa*, *A. nidulans*, and *Fusarium* spp., true functional chromosomal replication origins remain to be discovered and mapped. Early attempts to find ARS in fungi outside budding and fission yeasts utilized functional approaches to isolate DNA segments –often derived from mitochondrial plasmids– to stabilize introduced DNA and to increase transformation efficiency [47–51], or DNA segments that were preselected because of their DNA structure [52]. Domestication of a transposable element, the *Aspergillus* MATE, allowed the construction of a “fungal artificial chromosome” for the expression of secondary metabolite gene clusters [53], although strictly speaking these large elements are still metastable plasmids rather than true chromosomes as no telomere sequences were captured, unlike on the *Fusarium*, *Histoplasma* or *Cryptococcus* plasmids that have been in use but which also do not act like true artificial chromosomes [48, 54–56]. ChIP-seq with a combination of ORC and MCM subunits seems to be the most obvious approach to map true chromosomal replication origins and their dynamic chromatin context, and this would close a wide knowledge gap in fungal biology.

Telomeres

Telomere repeat sequences and the shelterin complex—Telomeres were first discovered in the ciliate *Tetrahymena thermophila* [57], but budding yeast soon became a facile model to uncover the structure and function of telomeres. Most organisms that have been examined use simple sequence repeats of various composition and length as telomeric DNA sequence. In fungi, the vast majority have 5′-TTAGGG-3′ repeats, similar to the ends of mammalian chromosomes [58], but important exceptions are found within the ascomycetous yeasts both in the Saccharomycetaceae and the Taphrinomycotina (Figure 1). One major difference between mammals and fungi is the overall length of the telomere

repeat tracts, only 100–200 bp compared to several kb in most mammalian species that have been examined [59–62].

The 16 chromosomes of *S. cerevisiae* end in 75–300 TG₂₋₃(TG)₁₋₆ repeats [63], with a 12–15 nt G-tail [64]. *Candida albicans* has longer, 23-nt tandem repeats that are overall GC-rich and contain a 5′-TGGTGT-3′; this arrangement is also found in other *Candida* species [65, 66] (Figure 2). The canonical tandem repeat in *S. pombe* is G₀₋₄GGTTACAC₀₋₁ [67, 68], while the animal and human pathogens in the Taphrinomycotina, *Pneumocystis carinii* and *P. jirovecii*, have telomere repeats composed of the more common 5′-TTAGGG-3′ sequence [69, 70]. Telomeres from Dikarya in classes outside of the Taphrinomycotina and Saccharomycotina were found to be 5′-TTAGGG-3′ repeats, like in *N. crassa* [71, 72] and *A. nidulans* [73, 74]; many additional species have been examined as part of genome sequencing projects (Figure 1), though many projects did not capture any telomere repeats, e.g. in *Rhizoctonia* or *Puccinia*. There may be exceptions even in these lineages, as *A. oryzae* uses a 12-mer repeat, 5′-TTAGGGTCAACA-3′ [59]. Direct sequence or biochemical evidence for telomere repeats is even more sparse in the early diverging lineages, e.g. among the chytrids or zygomycetes, where the standard 5′-TTAGGG-3′ repeat has been documented in *Mucor circinelloides* [75]. Based on the genome sequences available in various databases, the microsporidia, among them *Enzephalthozoon cuniculi* and numerous *Nosema* species, appear not to have consensus telomere repeat sequences.

The telomeric DNA repeats serve as platforms for single-stranded or double-stranded DNA-binding proteins, forming protective caps or more often loops at chromosome ends (Figure 3). In animals this complex is called “shelterin”, and is important for the maintenance of genome integrity, acting as a tumor suppressor involved in DNA repair [76]. In fungi analogous complexes are involved in regulated maintenance of telomere length by promoting replication at telomeres. In both *S. cerevisiae* and *S. pombe*, the telomeric repeats are free of nucleosomes and instead organized into specialized chromatin by action of the telomere-binding protein Rap1, and two Rap-interacting proteins, Rif1 and Rif2; in *S. pombe*, the DNA-binding function of Rap1 is taken over by Taz1, and Rif2 is replaced by Rap1 (Figure 3). As for telomere repeat sequence, there is great diversity among telomere binding proteins that are ill-conserved at the primary sequence level. Outside of *S. cerevisiae*, *C. albicans* and *S. pombe* there is still not much known about shelterin in the fungi, and mechanistic studies are sorely needed. Budding yeast and its close relatives seem to have derived protein complexes rather than representing the ancient state, whereas *S. pombe* shelterin shares functional similarities with shelterin identified in mammals [76–78]. While homologs for shelterin components have been found by sequence similarity searches in many fungi (Figure 3) it is unclear that they are also functionally conserved, illustrated by their different roles in *S. cerevisiae*, *S. pombe*, and *N. crassa* [76, 79–83].

Subtelomeric heterochromatin controls expression of disease-related genes—

Segments of DNA immediately neighboring the telomeres are called “subtelomeric”, and contain nucleosomes that are organized into transcriptionally silent heterochromatin in many species. Pioneering work on heterochromatin-mediated gene silencing was carried out in *S. cerevisiae*, and has been reviewed recently [4, 10]. Silent genes in the subtelomeric regions are intermingled with active genes – not all of the telomere-near segments are silent at all

times, and silent chromatin blocks are relatively short [84]. “Telomeric silencing” was first discovered by means of the *URA3* reporter gene and selection on 5-FOA medium [85]. The strength of silencing is generally correlated with the length of the 5′-TGGG-3′ repeats, and internal telomere repeats, dispersed along chromosome arms, can aid in silencing neighboring genes in budding yeast [86]. Silencing complexes restrict access to the DNA for the transcriptional machinery, i.e. switching from active to silent chromatin or *vice versa* results from a local balance between silencing and activating factors, generating so-called “facultative” heterochromatin.

In *S. cerevisiae*, three heterochromatic regions have been studied in great detail, namely the silent mating type loci, subtelomeric regions, and rDNA. All three regions require the presence of the silent information regulator protein, Sir2, a conserved NAD⁺-dependent histone deacetylase [4, 10, 11, 87]. Sir2 is recruited to silencers or proto silencers by the action of Sir1, ORC, Rap1 and Abf1. At the silent mating type loci and subtelomeric regions the specialized yeast Sir3 and Sir4 proteins are recruited, and spreading of this Sir complex is dependent on Sir2 deacetylase activity on H4K16 [88, 89]. How exactly spreading occurs is still not resolved. The conventional idea has been that spreading of heterochromatin occurs in a linear fashion along chromosomes, though interactions *in trans* are possible, similar to some enhancer-promoter interactions. Hi-C experiments may resolve this question in the near future. Lastly, generating patches of Sir2/3/4-enriched chromatin may not be sufficient on its own to silence transcription. Several studies suggest that a “maturation” step is necessary, which may be demethylation of H3K79me3, because deletion of the sole H3K79 methyltransferase Dot1 resulted in accelerated establishment of silencing [90, 91].

In fungi closely related to *S. cerevisiae*, like *Candida glabrata*, the subtelomeric regions are enriched for genes that encode glycoprotein pathogenicity and virulence factors, similar to what has been found for parasite genomes, e.g. *Plasmodium* [92]. The study of Epa adhesins, which allow binding to human cells, revealed a staggering variety in expression of these loci near chromosome ends, and differential expression was controlled by Sir-protein-mediated transcriptional silencing [93–95]. While the Sir proteins were essential, Rif1 and the Ku70/Ku80 proteins involved in non-homologous end joining (NHEJ) showed differential effects at only some subtelomeres [96].

During the annotation of the *C. albicans* genome, a novel family of “telomere-associated” (TLO) genes was discovered [97], which encode subunits of the Mediator co-activator complex [98–100]. TLOs are involved in pathogenicity functions like filamentous growth and biofilm formation, and were considered to be adaptive by binding to specific target genes. More recent data suggest that increased protein levels of TLOs in *C. albicans* may affect regulation of virulence factors at the transcriptional level, by competition with basal transcription factors that bind to targets affecting key pathways, such as filamentous growth [101]. Lab evolution studies on TLO genes showed how quickly the repertoire of these genes can change; subtelomeric recombination generated diversity in copy number and sequence for TLO genes [102]. Earlier work had revealed that deletion of Sir2 resulted in the rapid appearance of novel phenotypes [103], resembling (but mechanistically not related to) “phenotype switching”. Indeed, Sir2 is required for recombination at specific sites, called TLO recombination elements (TREs). TREs enhance recombination but Sir2 generally

inhibits recombination, and Sir2 action can be bypassed by environmental stressors, such as azole drugs and hydrogen peroxide [104].

The *S. pombe* Sir2 homolog is important for heterochromatin formation at silent mating type loci, centromeres and subtelomeres, and it recruits Swi6 to telomeres [105]. Swi6 is a homolog of the animal Heterochromatin Protein 1 (HP1) and binds to H3K9 that is di- or trimethylated [106]. Another Sir2 homolog, Hst4, also was found to be important for subtelomeric silencing [107]. In contrast to the *Sacharomycetaceae*, *S. pombe* uses H3K9 methylation and HP1 to induce formation of heterochromatic regions [108], and the interplay between H3K9me3 and H4K16, H3K9 and H3K14 acetylation and deacetylation has received much attention in the past decade [5]. While an in-depth discussion of this topic is beyond the scope of this review, a few hallmark findings will illustrate the differences to telomeric silencing when compared to *S. cerevisiae*. Deposition of H3K9me3 and HP1 binding is dependent on the machinery that generates small interfering RNA [109], and this pathway acts at subtelomeric regions as well [110]. However, Taz1 can also recruit HP1^{Swi6} independently from the RNA-induced transcriptional silencing (RITS) pathway [111]. A Clr3 HDAC complex containing the Mit1 chromatin remodeling factor ATPase, called SHREC, regulates nucleosome positioning at heterochromatic regions [112]. Deletion of another telomere-associated protein, Sde2, results in decreased recruitment of SHREC and increased minichromosome formation, suggesting a role in telomere maintenance [113]. SHREC directly interacts with the shelterin component Ccq1, linking Taz1, RITS, and HP1-containing silencing complexes. These analyses have now come full circle, as there is accumulating evidence for the role of shelterin components Taz1 and Ccq1 in heterochromatin formation at late replicating origins that are sites for double-strand break formation during meiosis and genome organization in general [114, 115]. Data obtained from high resolution three-dimensional structured illumination fluorescence microscopy (3DSIM) suggested that the “condensed” heterochromatin at subtelomeres in *S. pombe* is less condensed than nearby “knobs” that were eliminated by ablation of H3K36me3 [116], a histone mark correlated with gene expression and enriched in the 3′ regions of genes but actually repressing transcription from cryptic promoters and controlling transcript elongation [117, 118]. Genes inserted into the condensed knobs were not silenced, whereas silent genes in the subtelomeric regions outside of the knob were less condensed, challenging our current notion of “heterochromatin” as condensed or less accessible silent region.

In *Neurospora* there are no obvious signatures of specific gene families near the telomeres. This is different in several other filamentous fungi. Before the *Candida* virulence gene families (*epa* and *tlo*) had been studied in detail, work with *Magnaporthe oryzae* had identified a family of telomere-linked RecQ-like helicases (TLH) and ectopic recombination, aided by the presence of transposons or transposons relicts had been invoked to explain the proposed terminal truncations and reshuffling of these loci observed in this species [119, 120]. In many other taxa secondary metabolite gene clusters and genes involved in pathogenicity as “effectors”, i.e. short, cysteine-rich secreted peptides or proteins, are enriched towards the telomeres, but this can encompass many hundreds of kb, and thus is not strictly “subtelomeric silencing” [121–128].

Subtelomeric silencing has been studied in *N. crassa*, especially as it relates to H3K9me3-dependent DNA methylation and H3K27me3-mediated silencing [83, 129–137]. While *S. cerevisiae* lacks H3K9, H3K27, and cytosine DNA methylation, and *S. pombe* lacks H3K27 methylation, *Neurospora* serves as an excellent model organism to decipher the roles of H3K27 and cytosine DNA methylation [23, 24], two chromatin modifications that are essential for proper development and differentiation in animals [138, 139]. The *N. crassa* Sir2 homolog, NST-1, is involved in subtelomeric silencing, that was at least partially relieved when *nst-1* or three different sirtuins, *nst-2*, *nst-3*, and *nst-5*, were mutated. Even double or triple *nst* mutants, however, were not as effective in de-repression of reporter genes as single deletions of HP1 or the gene for the H3K9 methyltransferase, *dim-5* [137]. Because DNA methylation was only partially affected, these results suggested a DNA-methylation-independent role for H3K9 methylation in *Neurospora*. Recent studies showed that wildtype importin alpha (called DIM-3 in *N. crassa*) is required for normal DIM-5 function [132], and for normal genome organization [131] but the mechanism for this is still unresolved.

H3K27 methylation is a hallmark of “facultative heterochromatin” in many fungi—Over the past five years H3K27 methylation has attracted the attention of several research groups, working with *Neurospora*, *Fusarium*, *Zymoseptoria tritici*, *Epichloë festucae*, and *Cryptococcus neoformans*. H3K27 methylation is catalyzed by the Polycomb Repressive Complex 2 (PRC2), which has three core subunits, KMT6, EED, and SUZ12 (Figure 4). The genes, E(z), Esc, and Su(z)12, respectively, were first discovered in *Drosophila* in enhancer and suppressor screens for loci affecting position effect variegation [138, 140]. Specifics of the situation in *Neurospora* and *Cryptococcus* have been recently reviewed [23, 24], so the discussion here will be limited to contrasting these results with what has been found in the other three taxa.

Like the original PRC2 from *Drosophila*, NcPRC2 contains three core subunits, SET-7 (KMT6), EED, and SUZ12 (Figure 4A); in subtelomeric regions an additional component, NcNPF (identical to *S. cerevisiae* Msi1, *Drosophila* p55, and mammalian RbAp46/48), is required for H3K27me3 [134]. The same core subunits are essential for H3K27me3 in *F. graminearum*, but the Msi1 homolog, FgMSL1, is not required for H3K27me3 (L.R. Connolly & M. Freitag, unpublished results). The *Cryptococcus* Ezh2^{EZH2} complex contains three conserved proteins (Ezh2, Eed, and Msi1) and two proteins unique to *Cryptococcus* (Bnd1, Ccc1), but it lacks a recognizable SUZ12 homologue that is usually associated with E(z) [141]. While there are no clear homologues of Bnd1 in ascomycetes, the best Ccc1 homologues in *Neurospora* and *Fusarium* are not involved in H3K27 methylation [130] (L. Connolly and M. Freitag, unpublished results). A partial crystal structure of the core PRC2 from *Chaetomium thermophilum*, a species of Sordariales relatively closely related to *Neurospora* and *Fusarium*, for the first time defined important functional motifs in any E(z) and EED homologue [142, 143]. Allosteric interactions between KMT6 and EED had been reported previously [144], but the crystal structure and *in vitro* enzyme assays further defined interaction motifs in the H3K27me3 and the H3K27 forms [143]. In KMT6, ten motifs were described [143], which are well conserved between fungal versions of the protein but are not easily discerned by sequence comparisons with proteins from animals

(Figure 4B). One clear difference in the organization of KMT6 homologues from ascomycetes is the extended sequence at the C-terminus, lacking from the *Cryptococcus* Ezh; the structure and function of this domain remain unknown as this segment was not included in the crystal structure. While the EED homologs all have seven WD40 motifs that form a seven-bladed β -propeller, fungal EED proteins are characterized by a long “insertion domain” [143]; this is a highly variable region that is not directly involved in KMT6-EED interactions and the function of this domain is unknown (Figure 4C). SUZ12 homologs are overall fairly conserved when they are present in fungal genomes (Figure 4C), though functional and structural motifs remain to be uncovered as the partial crystal structure contained only a short fragment, the VEFS motif [143].

The most obvious function of H3K27 methylation in *Neurospora* and *Cryptococcus* was proposed to be gene silencing, though loss of silencing does not result in drastic overt defects in either species [134, 141]. In both species, H3K27me3 is found mostly in subtelomeric regions and a few dispersed regions on the chromosome arms, covering less than 10% of the genome (Figure 5A). This situation is different in *F. graminearum*, where ~20% of all genes were newly turned on or upregulated when KMT6 was deleted, and where H3K27me3 covers almost a third of the whole genome [145]. In *F. fujikuroi* deletion of *kmt6* is lethal, and effects of derepression of secondary metabolite gene clusters were studied by RNAi-mediated downregulation of *kmt6* [146]. Again, H3K27me3 covers almost a third of the whole genome, including almost all of the smaller chromosomes (Figure 5C), the so-called “accessory” chromosomes [22]. In *E. festucae*, H3K9me3 and H3K27me3 in combination were reduced in secondary metabolite gene clusters that produce lolitrems and ergot alkaloids when the fungus was growing *in planta* [147]. Whether the situation found in *Fusarium* and *E. festucae* is common to other pathogens needs to be resolved in the near future. There are indications that in some species, like *Leptosphaeria maculans* [148], H3K9me3 may control the activity of virulence-related genes, although the idea that long clusters of secondary metabolite genes are controlled by H3K9me3 in *Aspergillus* species may need to be readressed [149].

Studies on the interplay between H3K9 and H3K27 methylation showed that in *Neurospora* HP1 is key for the proper distribution of H3K27me2/3 [130, 133, 150]. In mutants lacking H3K9me3 or HP1, almost all H3K27me3 is mislocalized to regions previously enriched with H3K9me3, most strikingly the centromeres, and deleting components of the PRC2 at least partially suppresses defects observed in *dim-5* mutants. In HP1 mutants, H3K9me3 and H3K27me2/3 are found in largely overlapping regions, a situation that is common in wildtype strains only in subtelomeric regions [130, 133, 150]. In *Cryptococcus*, Ccc1 is involved in H3K27me3 recognition and binding, and disruption of *ccc1* results in redistribution of H3K27me3 into H3K9me2 domains, again especially the centromeric regions. If H3K9me2 deposition is abolished by deletion of the Ctr4^{SUV39} homologue, H3K27me3 is also lost from these regions in the *ccc1* background [141]. These results suggest that normal binding of the Ezh2 complex to its product, H3K27me3, via Ccc1 suppresses an inherent activity towards H3K9me2-modified centromeric chromatin regions [141]. Re-distribution of either H3K9me3 into H3K27me3 regions or *vice versa* has not been observed in *Fusarium* mutants (L. Connolly and M. Freitag, unpublished results). In *Z.*

tritici H3K9me3 and H3K27me3 distribution were found to overlap not only in subtelomeric regions but in most segments that are not transposons or transposon relics [62].

In several *Neurospora*, *Fusarium*, and *Zymoseptoria* species a correlation between subtelomeric blocks of non-syntenic DNA and H3K27me3 has been found [62, 123, 134, 145, 146]. H3K27me3 seems to act like an “immune system” for the fungus, perhaps silencing novel incoming DNA sequences [134], though this idea has been difficult to test, and a sequence-based “signal” for H3K27me3 targeting has yet to emerge from studies with *Neurospora* or *Fusarium*. The four chromosomes of *F. graminearum* likely resulted from chromosome fusions of the ancestral 11 or 12 chromosomes that are present in extant sister species [151]. Recombination profiles [151–153] and chromatin structure analyses [145, 146] suggest that the epigenetically defined and usually silent or “cryptic” regions within chromosome arms maintain sequence diversity but also have been maintained as “subtelomeric-like regions” over millennia. What emerges from the combination of the studies discussed in the previous sections is a large variety of regulatory circuits in different taxa, suggesting that we are just at the beginning of uncovering how and why H3K27 methylation is necessary in fungi.

Centromeres

Centromeric DNA attracts the Kinetochore Interaction Network (KIN)—

Centromeres are essential chromosomal loci, dictating nucleation points for kinetochore and spindle assembly during chromosome segregation. While a handful of organisms, all related to *S. cerevisiae*, have sequence-dependent “point” centromeres that often involve as few as one or two nucleosomes, most other fungi, animals and plants have epigenetically defined “regional” centromeres that stretch across several kilobases or even hundreds of kilobases (Figure 1). These regional centromeres are functionally defined by the presence of a specialized histone H3 variant, CENP-A, or the presence of kinetochore complex components [154, 155]. CENP-A and another essential centromere foundation protein, CENP-C, recruit the rest of the constitutive centromere-associated network (CCAN), which forms the inner kinetochore, and the KNL1-MIS12-NDC80 complexes (KMN), which form the outer kinetochore and serve as the attachment point of chromatin to microtubule spindles. In combination, CCAN and KMN form the “kinetochore interaction network” (KIN) [156], where “centromere” denotes DNA and DNA-binding chromatin proteins, and “kinetochore” refers to proteins that do not directly contact DNA [157].

Compared to replication origins and telomere biology, much more is known about fungal centromeres. Among the earlier diverging ascomycetes in the Taphrinomycota, *S. pombe* is one of the best studied models. Well-defined pericentric flanking regions include the *outer repeat* (*otr*) and *inner most repeat* (*imr*) surround the *central core* (*cc* or *cnt*; 4–7 kb) [158], which contains the majority of nucleosomes with CENP-A^{Cnp1} [159]. The discovery of *S. pombe* centromeres showed early on that fungi can have regional centromeres that do not depend on conserved recognition sequences for kinetochore complexes, and that they can be excellent genetic models for animal centromeres [160], and by now several sister species have been studied as well [161]. The poorly conserved *cc* sequences are not sufficient to allow CENP-A^{Cnp1} recruitment [162]. To assemble a functional kinetochore, the *otr* and *imr*

repeats or similar regions producing siRNA are required to assemble *de novo* heterochromatin marked by histone H3 lysine 9 dimethylation (H3K9me2), which is also involved in recruitment of cohesins for the binding of sister chromatids [5, 109, 163, 164]. A role for centromeric repeat sequences to generate *cis*-acting short or long non-coding RNA seems to be shared by animals and plants taxa as well [165, 166].

Searches for centromere consensus sequences showed that *S. cerevisiae* has a genetically defined centromere with three conserved Centromere Determining Elements (CDEI, CDEII, and CDEIII) [167, 168]. CDEI, an 8-bp palindromic sequence, is bound by Cbf1 and CDEIII, a conserved 26-bp motif, is bound by the CBF3 complex, interrupted by 75–86 bp of AT-rich CDEII sequence; Cbf1 and CBF3 are conserved only in the Saccharomycotina. This topic has recently been reviewed in great detail, and discoveries with budding yeast facilitated trail-blazing research into the requirements for kinetochore formation and “portable” centromere signals [169]. Emergence of point centromeres seems to have occurred before the whole genome duplication event that occurred in the ancestors of *Saccharomyces*, *Candida glabrata*, *Naumovozyma castellii*, and *Vanderwaltozyma* (*Kluyveromyces polyspora*) but after divergence of the “true” *Candida* species and *Komagataella phaffii* (*Pichia pastoris*), which have short regional centromeres [170]. Thus, although sequence-specific point centromeres were discovered first, largely because of the genetic tractability of budding yeast, the combination of molecular and phylogenetic evidence suggests that point centromeres of many Saccharomycotina represent a more recently evolved state [171]. One possibility is that specific sequence elements invaded chromosomes resulting in preferred binding of centromere-associated proteins and replaced ancestral regional centromeres present in the last common ancestor.

The genus *Candida* includes some of the best-studied human pathogens within the fungi and recent work has thoroughly examined the centromeres of *C. albicans*, *C. dubliniensis*, *C. lusitaniae* and *C. tropicalis*. *Candida albicans* centromeres were identified by immunoprecipitation of DNA with antibodies against CENP-A^{Cse4}, followed by cloning and sequencing [172]. This direct identification of centromeric DNA showed that *C. albicans* has small (~3–5 kb) centromeres composed of non-repetitive sequence elements that are not conserved, even on chromosomes of the same strain or species. Syntenic centromeric regions have different sequences in *C. dubliniensis* and *C. tropicalis* [173, 174] and centromere cores of *C. tropicalis* are flanked by inverted repeats, similar to the organization found in *S. pombe* and *K. phaffii* [173]. The seven centromeres of *C. tropicalis* are more similar than those of the other species, suggesting ongoing homogenization by gene conversion. The core regions have the highest CENP-A^{Cse4} occupancy but when (inverted) repeats are present they are often enriched with CENP-A^{Cse4}. At the same time, *Candida* centromeres have –like *S. cerevisiae*– a single kinetochore-microtubule spindle attachment per chromosome [175, 176], thus blurring the line between point and regional centromeres.

The filamentous fungi comprise most taxa in the early diverging lineages and the Dikarya (Figure 1). In *N. crassa*, a 16 kb region that mapped to the centromeric region of LG VII was identified in a YAC library and sequenced [177, 178]. The approximate sizes of all centromeres were determined after the genome had been nearly completely assembled by traditional shotgun Sanger sequencing [14, 179]. Assembly of the AT-rich regions in

Neurospora is aided by the presence of Repeat-Induced Point mutation (RIP), a pre-meiotic mutator system that affects duplicated sequences as short as 150 bp [180, 181], and whose effect is heterogenization and inactivation of populations of identical retrotransposons. *Neurospora* has large (170–300 kb) regional centromeres, enriched with inactivated retroelements. This is similar to plants and animals but satellite repeats are absent [182]. Based on ChIP-seq with CENP-A^{CenH3} the centromeric core regions are surrounded by short (2–45 kb) regions of pericentric heterochromatin. Limited examination of the *N. crassa* Mauriceville wildtype strain showed large differences in the architecture of centromeric DNA segments, initially by analysis of AT-rich segments that also had cytosine DNA methylation [183], and later by comparison of near-complete centromeric DNA sequence [184]. The patterns of changes suggested that mitotic or meiotic recombination events, perhaps anchored in the near-identical repeats of nonfunctional transposons, result in large scale insertions or deletions.

Taxa most closely related to *Neurospora* all have shorter regional centromeres, with active or incapacitated retrotransposons, similar to those found in the putative centromeric regions of *Magnaporthe oryzae* [185] and *Verticillium* [127, 186]. For many of these species CENP-A^{CenH3} has not yet been mapped by ChIP-seq, though several species in the genus *Fusarium* (*F. graminearum*, *F. asiaticum*, *F. oxysporum*, *F. solani*, *F. fujikuroi*) have been examined more closely and revealed centromeric DNA of 30–50 kb. All have centromeres that are enriched with retrotransposons, some of which appear to be active. Centromeres of the Eurotiomycetes, a medically and industrially important class of ascomycetes, have still not been studied in detail, so centromeric sequences from *Penicillium*, *Aspergillus*, *Histoplasma* and *Coccidioides* remain unknown or unassembled. In *A. nidulans*, repeat elements, e.g. the *Dane1* and *Dane2* LTR elements have been found [187] and based on comparative genomics, centromeric regions are thought to be between 8–80 kb long [188]. Like in *Candida*, centromeres of the sister species are embedded in regions with high synteny, even though centromeric sequence has diverged. In the Dothideomycetes the location of centromeres had been predicted based on the longest AT-rich regions on each chromosome. Surprisingly, such predictions turned out to be wrong for the genus *Zymoseptoria*, where most centromeres are not associated with AT-rich DNA [62]. Instead, ChIP-seq with *Z. tritici* CENP-A^{CenH3} revealed short (5–10 kb) CENP-A enriched regions without distinct sequence patterns; some centromeres contain expressed genes, while others harbor active or silent retroelements.

Among the Basidiomycota only *Cryptococcus neoformans* has been examined in any detail for centromere sequences, both by sequence comparisons and ChIP-seq with CENP-C [45, 189]. Together the pericentric and centromeric regions are between 20–65 kb long and enriched for active or disabled Tcn1-Tcn6 retrotransposons. Mapping of CENP-C showed that this centromere foundation protein binds to a core region within the centromeric and pericentric region, covering ~5 kb on *CEN14*. The flanking regions found in *C. neoformans* and *C. deneoformans* are mostly syntenic, but the chromosome numbering is different for the two subspecies; a similar arrangement was observed with the more distantly related *C. gattii* [45]. The well-studied *Ustilago maydis* seems to have short centromeres that are associated with ARS sequences [190, 191] but, again, no detailed studies have been undertaken.

The last common ancestor of fungi split from the animal lineage ~1.5 billion years ago, and the most basal fungal lineages, the Microsporidia and Cryptomycota, separated from the precursors of the zygomycetes, Ascomycota and Basidiomycota, ~1.3 billion years ago (Figure 1) [192, 193]. No molecular studies have investigated the position of centromeres in the basal taxa and standard genome sequencing alone will likely prove insufficient as it is unclear that all centromeres are in long AT-rich regions. The genome of the industrially important *Trichoderma reesei* has been assembled by chromosomes conformation capture (3C) followed by high-throughput sequencing (Hi-C) [194]. This method makes no *a priori* assumptions about centromere clustering [131], thus application of Hi-C-aided assembly will allow the precise mapping and assembly of centromeres of many fungi. While this method will provide important information, the extent of centromeres should always be confirmed by localization of the defining epigenetic marker, CENP-A.

What emerges from investigations on short point or extended regional centromeres in the fungi is the realization that genes surrounding the centromeric core or repeat regions of related species are syntenic while the core regions are highly divergent. This suggests that centromeric sequences undergo mutation without repair at a higher frequency than surrounding sequences. It remains to be determined whether this implies positive selection towards highly adapted sequence signals aiding deposition of CENP-A [195, 196], or rather genetic drift because CENP-A deposition is determined entirely epigenetically by pre-existing CENP-A nucleosomes, thus making DNA sequences immaterial.

Centrochromatin—That centromeric chromatin is different from bulk chromatin became clear when CENP-A was identified as histone H3 variant [197–199]. The sparsity of genes, enrichment with repeat elements and active or disabled retrotransposons, transcriptional repression, and compaction during interphase, pegged centromeric chromatin as constitutive heterochromatin, but this idea was challenged by results that showed the presence of histone modifications commonly associated with active transcription in fission yeast, *Drosophila*, and human cells [110, 200, 201]. Thus, along with transcriptionally active euchromatin and silent heterochromatin, “centrochromatin” may constitute a distinct, third form of chromatin.

CENP-A is rapidly evolving, especially the N-terminal tail, Loop 1 of the histone fold domain (HFD), and the CENP-A targeting domain (CATD), which are all involved in CENP-A localization and kinetochore interactions. In fungi this has been studied in the Taphrinomycotina [202], Saccharomycetaceae [203] and Sordariomycetes (P. Phatale, S. Friedman and M. Freitag, unpublished results). The C-terminal tail is important for recruitment of two essential CCAN components, CENP-C and CENP-N [204–207]. The controversy about the shape and size of centromeric nucleosomes across the cell cycle has been reviewed [169], and the balance of CENP-A nucleosomes and post-translationally modified H3 nucleosomes is a topic of ongoing investigations; earlier studies uncovered roles for histone modifications in *de novo* establishment of stable fission yeast centromeres [162]. How histone modifications may aid in centromere maintenance, however, remains to be uncovered in most organisms. Because the complement of histone genes is very simple in fungi -there are single genes for H2A, H2B and H3, and only two genes encoding identical H4 proteins [208]- and because most fungi are genetically and molecularly tractable

organisms this may be the area where they can contribute the most to advances on centromerichromatin in the near future.

Centromere inactivation or deletion experiments in *C. albicans* demonstrated the utility of this system for the study of “neocentromere” formation and inheritance [209]. Neocentromeres are previously naïve chromatin regions that give rise to functional kinetochores competent for chromosome segregation. Upon inactivation or deletion of the original centromeres, *Candida* neocentromeres form almost anywhere on the chromosome, though there is some preference for transcriptionally silent pericentric and subtelomeric regions [43, 210–212]. Like in *S. cerevisiae*, centromeres and neocentromeres in *Candida* interfere with transcription of nearby genes [210], also suggesting heterochromatin characteristics. The role of histone modifications for centromerichromatin in *Candida* is unresolved; like *S. cerevisiae*, *Candida* lacks the conserved heterochromatin pathways that rely on methylation of H3K9 and H3K27.

Constitutive heterochromatin, defined in many eukaryotes by the presence of H3K9 di- or trimethylation (H3K9me_{2/3}) and cytosine DNA methylation, is also a characteristic of most regional pericentric or centromeric regions. In *S. pombe*, the RNAi machinery is essential to recruit H3K9me₂ to the pericentric repeat and help to incorporate CENP-A^{Cnp1} on naïve plasmid sequences [162]. Once assembled, however, the heterochromatin machinery, including the histone methyltransferase Clr4^{SUV39} and the H3K9me₂ adapter protein Swi6^{HP1}, is no longer required for CENP-A^{Cnp1} inheritance. Nevertheless, heterochromatin is essential for proper chromosome segregation and chromosome structure, likely by the recruitment of cohesins [163, 213–215]. Early genome-wide ChIP studies showed that the *imr* and *cc* (*cnt*) regions were enriched for a euchromatic mark, H3K4me₂ [110], but recent experiments suggest that H3 nucleosomes are depleted from the centromeric core and that CENP-A^{Cnp1} nucleosomes and the CENP-T complex dominate [159].

There have been far fewer studies on centromerichromatin of other clades in the fungi. Centromeric regions of *N. crassa* contain blocks of canonical nucleosomes that are enriched with H3K9me₃ interspersed with blocks of CENP-A^{CenH3} nucleosomes [182, 216]. In mutants lacking the H3K9me₃ methyltransferase DIM-5^{SUV39} and the H3K9me₃-binding protein HP1 the regions enriched for CENP-A^{CenH3} were smaller. H3K4me_{2/3} did not associate with the formerly H3K9me₃-enriched nucleosomes though overall nucleosome occupancy seemed unaltered [182]. Further studies revealed re-localization of H3K27me_{2/3} to regions usually occupied by H3K9me₃ in DIM-5^{SUV39} and HP1 mutants, suggesting that HP1 prohibits H3K27 di- and trimethylation in H3K9me₃-regions [130, 133]. Single mutants lacking DIM-5^{SUV39} and HP1 show growth and chromosome segregation phenotypes and are homozygously sterile or result in aberrant progeny [217, 218], while SET-7^{EZH2} mutants show no overt defects (Jamieson et al. 2013). In DIM-5^{SUV39} SET-7^{EZH2} or HP1 SET-7^{EZH2} double mutants these phenotypes are largely suppressed [130, 133], suggesting that it is the presence of H3K27me_{2/3} at centromeres or its absence from normal facultative heterochromatin that results in the chromosome segregation defects. A link between the RNAi and meiotic silencing pathways and heterochromatin establishment in *N. crassa* has not been found [219], unlike in fission yeast. In *F. graminearum* centromerichromatin is similar to *Neurospora*, although the re-localization of

H3K27me_{2/3}, which has been observed in *Neurospora* DIM-5^{SUV39} and HP1 mutants [130, 133] as well as *C. neoformans* *ccc1* mutants [141], does not occur in *F. graminearum* (L. Connolly and M. Freitag, unpublished results). Overt phenotypes are also drastically different from those observed in *Neurospora* [134], as H3K9me₃-defective strains have no discernable phenotypes under standard growth conditions while single mutants lacking H3K27me₃ or double mutants lacking H3K9me₃ and H3K27me₃ show numerous developmental and other defects [145]. In *Z. tritici*, centromeres cannot easily be defined because CENP-A-enriched regions do not show any obvious DNA sequence or chromatin pattern [62]. So far only H3K4, H3K9, and H3K27 methylation have been tested but none of these marks overlap reliably with CENP-A localization. The only basidiomycete whose centromeres has been studied is *C. neoformans* [45, 189]. H3K9me₂ is found almost exclusively in the centromeric, pericentric, and subtelomeric regions. Overall, fungi represent diverse opportunities to test centromere plasticity that is still difficult to carry out in many other organisms. All known variants of centromeres in fungi are more similar to heterochromatin than euchromatin, whether due to the presence of silencing marks (e.g., H3K9me_{2/3}) or the involvement of the RNAi machinery in *S. pombe*.

NOT ALL CHROMOSOMES ARE EQUAL

How to tell core from accessory chromosomes

Many eukaryotes carry extra “B” chromosomes in addition to the set of core (“A”) chromosomes [220]. By definition, B chromosomes are not essential; in fact, most are harmful to the host, and at best they are neutral elements. In fungi, however, an overwhelming majority of B chromosomes seems to confer benefits by carrying genes involved in plant or animal colonization, or involved in detoxifying host defense compounds, explaining the term “pathogenicity” chromosomes [221, 222]. Selective advantages through pathogenicity determinants do not hold for all of these extra chromosomes, however [122, 223]. In some fungi, B chromosomes are found only in specific accessions of one species, hence the name “lineage-specific” chromosomes [221]; other monikers are “conditionally dispensable” (CD), “supernumerary” or “accessory” chromosomes [224–226].

Animal and plant B chromosomes use various forms of meiotic drive and self-accumulation mechanisms to propagate, and many of the inheritance patterns are non-Mendelian. While there is much variation, some rules seem to apply [220, 227, 228]. The more B chromosomes in a single nucleus, and the harsher the environment, the more pronounced the negative effects of B chromosomes. B chromosomes can affect recombination frequency on A chromosomes, and in plants inbreeding promotes the accumulation and spread of rare beneficial B chromosomes. Overall, the presence of B chromosomes is positively correlated with low ploidy, low chromosome numbers and large genomes (and therefore much larger domains of repetitive DNA). B chromosomes are also more prevalent in genomes with acrocentric chromosomes, and smaller Bs are mitotically less stable than large Bs [220, 227, 228]. In fungi the rule seems to be that accessory chromosomes are rarely larger than the smallest A chromosome, and this is true in most eukaryotes. B chromosomes are small, containing sometimes only the elements required for propagation, i.e. telomeres,

centromeric DNA for kinetochore and spindle attachment, and origins of replication, but there is a large diversity in B chromosomes in plants and animals.

So what separates “accessory” and “B” chromosomes? The chromosome structure of *Fusarium* and *Zymoseptoria* species has been studied extensively, because they have a large number of accessory chromosomes [62, 224–226]. Overall accessory chromosomes are depleted for genes and enriched for repeated elements, mostly active and mutated transposons that are shared or rarely of different types than on the core chromosomes [221, 225, 229]. Very few –if any– genes have been found on B chromosomes in plants or animals; it appears that increase of gene density on accessory chromosomes is thus positively correlated with benefit to the host organism. Matching the finding of few active genes, cytological data from plants and animals suggest that B chromosomes are heterochromatic, and most studies suggest that they are enriched for H3K9me2/3 [230, 231]. In fungi the hallmark chromatin feature of accessory chromosomes is H3K27me3 [22, 62, 145, 146], though transposable elements are still covered by H3K9me3 in *Fusarium* and *Z. tritici* (Figure 5). Thus, the separating characteristic –at least for now– seems to be the presence of H3K27me3 and facultative heterochromatin on accessory chromosomes.

How do B chromosomes or accessory chromosomes arise?

Plant and animal B chromosomes are likely predominantly generated within the host by aberrant chromosome segregation during division; “Horizontal Chromosome Transfer” (HCT), similar to horizontal gene transfer in bacteria but involving entire chromosomes, is considered a less likely pathway [220]. The finding that species with acrocentric chromosomes are more likely to generate B chromosomes supports this idea, providing a mechanism that suggests breakage within centromeric DNA regions and addition of telomere repeats to generate a minichromosome that can be segregated in mitosis. This may be inefficient, however, especially when centromeric regions are insufficient for optimal kinetochore and spindle attachment, resulting in stochastic inheritance of B chromosomes, which may result in extinction at accelerated rates [220].

For fungi, HCT has been long considered as one model for the acquisition of accessory chromosomes, but HCT may mean different things to scientists from various disciplines. Most authors have made no distinction about species boundaries when discussing HCT [221, 222, 232–235], using “horizontal” or “lateral” transfer merely to describe a process distinct from the “vertical” transfer through the germline. Others may argue that transfer of new chromosomal material has to occur between different species before it is truly “horizontal”. This very specific condition is only rarely fulfilled; even horizontal gene transfer between species is difficult to detect, although there are some cases where genes encoding host-specific toxins have been transferred [236]. The hurdles for HCT even within species are high; no satisfying mechanistic explanation for how this may occur in *Fusarium* or *Alternaria* has been conclusively validated.

Chromosomes with drastically altered gene sequences, transposon or repeat content, and codon bias that may no longer resemble those commonly found in the same species have been observed in some species [221, 225, 237]. Interspecific hybridization may occur between fungal genera, yet endogenous sources for the generation of accessory

chromosomes seem more likely, especially in light of recent results obtained with *Fusarium* and *Zymoseptoria*. While HCT has been shown to occur within the “species” *F. oxysporum* between different *formae specialis* under strong selection [221, 238, 239], this is more difficult to show for different species or across different genera. The emerging consensus is that accessory chromosomes in *Fusarium oxysporum* are not only “lineage-specific” but appear to be *bona fide* pathogenicity factors because of the reliable conversion of non-pathogenic strains into pathogenic strains [240–244]. How many traits or specificity factors can be assembled into one such chromosome? One wonders whether one pathogenicity gene or set of pathogenicity genes on each accessory chromosome is specific to only one host. In other words, are pathogenicity chromosomes inherited as a single trait?

Studies with *Fusarium* and *Zymoseptoria* also showed that core chromosomes with large segments of facultative chromatin typical of accessory chromosomes exist [62, 221]. These cases suggest that either subtelomeric H3K27me3-enriched regions increase and spread to encompass longer segments, or –perhaps the more likely scenario– translocations between accessory and core chromosomes occur on a regular basis (Figure 5B). Some evidence for the second idea is provided by mapping of *Z. tritici* Chromosome 7, where the boundary between core and accessory chromosome characteristics falls almost precisely in the regions where the rDNA cluster was mapped [62, 245]. This suggests that there may be preferred regions for reciprocal or non-reciprocal translocations, which predominantly seem to be linked to H3K9me3-enriched transposable elements [62, 126].

While intra-species HCT and translocations from pre-existing accessory chromosomes can explain some of the variation within a species, these mechanisms still do not explain how relatively gene-rich fungal accessory chromosomes arise in the first place. Endogenous generation of a novel chromosome from two existing accessory chromosomes by chromosome fusion followed by degenerative breakage, as proposed by the “breakage-fusion-bridge” (BFB) model [246, 247], may serve as a general explanation for how accessory chromosomes in *Z. tritici* arise [226]. Repetitive DNA may result in non-allelic homologous recombination between repeats to generate a dicentric and acentric chromosome. The dicentric chromosome (in the case of *Z. tritici* Chromosome 17) may have undergone BFB cycles [226], while the acentric chromosome was simply lost in subsequent divisions. There are indications that chromosome loss in this species is quite common [223], especially in meiosis where short chromosomes may not support the necessary cross-over events for successful segregation [248].

Successive cycles of translocations or BFB may result in chromosomes that are successively more enriched in H3K27me3 facultative heterochromatin and depleted in H3K4me2/3 euchromatin (switch from panel B to C in Figure 5), and further degeneration of chromosomes may result in extremely short accessory chromosomes that have a mixture of H3K27me3 and H3K9me2/3 marks but no longer carry any essential genes that would be enriched with H3K4me2/3. If driven to the extreme this may result in true B chromosomes that carry only H3K9me2/3 (Figure 5E), like in some plant or animal species, though this type of chromosome has not yet been observed in fungi.

CHROMATIN INTERACTIONS WITHIN THE WHOLE NUCLEUS

On the previous pages we examined landmark regions required for chromosome function in a reductionist fashion but the various pieces must work together. There are various ways to examine chromosome dynamics, one being cytology [4, 12], which has now entered into the age of high resolution “nanoscopy” [249]. Another way to resolve contacts between chromosomal regions at high resolution is chromosome conformation capture (3C) followed by high-throughput sequencing (Hi-C) [28, 250] and its many variants [251]. Several studies in budding and fission yeast, as well as *N. crassa*, resolve the importance of chromosome landmarks for normal growth in fungi. Hi-C and cytological studies with wild type and mutants defective in constitutive (H3K9me2/3-enriched) or facultative (H3K27me2/3-enriched) heterochromatin showed that, like in budding [252] and fission yeast [214, 253], all centromeres of *N. crassa* are co-localized within the nucleus, revealing Rabl orientation [129, 131]. Surprisingly, all mutants that disturb features of transcriptional silencing, or “heterochromatin”, namely those that are lacking HP1, the H3K9 methyltransferase DIM-5 (i.e., KMT1, SUVAR39), or the H3K27 methyltransferase SET-7 (i.e., KMT6, E[z]) revealed only minor changes in overall chromosome organization (Figure 6). Cytology showed altered position and increased numbers of centromere foci in the SET-7 but not the DIM-5 and HP1 single or *set-7; dim-5* double mutants [129]. These data suggest a role for H3K27me2/3 in the control of centromere maintenance. In fission yeast, cohesins and condensins seem to play a major role in maintaining overall chromosome structure [13, 214, 254]. Recent studies revealed surprising roles of condensins in organizing centromeric regions in fission yeast [255, 256]. Thus, Hi-C studies suggest that there is higher-order organization to chromatin, though the precise role of heterochromatin, cohesins, condensins or other scaffolding proteins is still uncertain.

In summary, while it is clear that chromatin modification enzymes are important for establishment of large chromatin segments, more work must be done to determine what, if any, negative effects on chromosome segregation occur in a large number of chromatin mutants. Cytology has been carried out for a long time but 3C-based studies carried out across the cell cycle in synchronized cells will be necessary to provide a much deeper understanding of the relationships between chromatin state and chromosome landmark functions.

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Figure 1. Presence and absence of selected histone H3 and cytosine DNA methylation marks, structure of centromeres, and sequence of telomere repeats in selected fungi

Representative fungi from various clades were selected to show the phylogenetic distribution of chromatin characteristics. Species in which presence (check) or absence (cross) were experimentally validated are largely found within the Ascomycota, while species for which only genome sequencing-based evidence for the presence (plus) or absence (minus) of genes is available are in the Basidiomycota and the large group of early diverging lineages. No experimental data on chromatin modifications in chytrids and microsporidia are available; some chytrids have predicted DNA methyltransferases (DNMTs) that are similar to those in animals, while some zygomycetes (e.g., *Phycomyces*) have DNMTs similar to those in ascomycetes [257]. *Zymoseptoria tritici* has no cytosine methylation but sister species have intact genes for DNMTs. No obvious DNMTs are found in the *Candida* genome, yet there have been reports on cytosine DNA methylation. *Pneumocystis*, unlike *S. pombe*, appears to carry genes to carry out all modifications listed here, suggesting large diversity in the Taphrinomycotina. More recently an entirely new class of cytosine DNA methyltransferases has been identified in fungi, demonstrating DNA methylation in species that were long thought to be devoid of methylation, like *C. neoformans* [258]. The overall distribution pattern suggests that genes necessary to catalyze the two major gene silencing histone modifications, H3K9me and H3K27me, are ancient and have been lost in several branches over evolutionary time. Presence of conserved genes does not necessarily mean presence of the expected chromatin modification. Centromeric DNA segments (Cen) are defined as

regions with CENP-A or CENP-C enrichment, are highly variable in size, and even for some of the best-studied fungi, like *Aspergillus*, we still do not have experimental data. Pericentric regions, flanking the Cen regions, are larger and also of variable size. Most fungi use the mammalian and human (*Hs*) consensus telomeric repeat sequence, 5'-TTAGGG-3', sometimes with a variable number of Gs, like in *Cryptococcus*. Data on telomere repeats were compiled from the literature [54, 61, 65, 70, 72, 73, 75, 119, 124, 151, 194, 259–263].

Figure 2. Chromosome landmarks in four model organisms
Characteristics of DNA sequences for replication origins, centromere and telomere repeats are compared between budding yeast (*S. cerevisiae*), fission yeast (*S. pombe*), *N. crassa* and the basidiomycete yeast, *C. neoformans*. Few origins have been mapped in *C. neoformans* so it seems premature to say whether they share specific characteristics [45]. ARS, autonomously replicating sequence.

Figure 3. Telomere-repeat binding complexes homologous to mammalian shelterin

(A) The budding yeast *S. cerevisiae* has a CST (Cdc13, Stn1, Ten1) complex that binds to single-stranded 3'-G-rich-tail overhangs. The nucleosome-free double-stranded DNA is bound by Rap1, which in turn forms complexes with Rif1 and Rif2. Subtelomeric regions are transcriptionally silent because of hypoacetylation initiated by Sir2, and propagated by the Sir complex. (B) The fission yeast *S. pombe* has poorly conserved proteins serving similar functions as the CST complex, namely Pot1, Tpz1 and Ccq1. Poz1 creates a bridge to the Rap1/Taz1 complex, but Rap1 has different functions than in *S. cerevisiae*, even though there is slight sequence conservation. There is no Sir2-3-4 complex, instead fission yeast uses H3K9me2-mediated silencing catalyzed by the Clr4 complex and recognized by HP1 (called Swi6 in *S. pombe*). (C) The shelterin complex first identified in mammals by purification of the first telomere-repeat factors (TRFs) is very similar to the *S. pombe* complex, though Ccq1 is apparently missing. In both *S. pombe* and mammals HP1 acts on the CST complex homologs, and in *S. pombe* a histone deacetylase complex (SHREC) is involved.

A. Fungal PRC2 complexes

B. KMT6 (E[z], EZH2, SET-7, Ezh)

C. EED (Esc)

D. SUZ12 (Su[z]12)

Figure 4. Polycomb Repressive Complex 2 (PRC2) from three fungi has different components (A) Facultative heterochromatin, enriched with H3K27me2/3, is generated by PRC2 complexes. Approximate arrangement of complex subunits is based on published structures of human [264] and *Chaetomium* PRC2 [143]. *Fusarium* has a core PRC2 complex that lacks a homolog of the *S. cerevisiae* Msi1 homolog (crossed out MSL1) that is found in PRC2 of *Neurospora* (NPF) and *Cryptococcus* (Msl1). While genes for KMT6, EED and SUZ12 homologs are found in many taxa, the CnCccl and CnBnd1 proteins are restricted in distribution, suggesting diversification of PRC2 across the fungi. (B) *Chaetomium thermophilum* Ezh (*Fusarium* KMT6, *Drosophila* E[z], human EZH2, *Neurospora* SET-7, *Cryptococcus* Ezh) contains ten structurally distinct motifs (adapted from Jiao and Li, 2015), named SBD (SANT1L-binding domain, 1), EBD (Eed-binding domain, 2), BAM (b-addition motif, 3), SAL (SET activation loop, 4), SRM (stimulation-responsive motif, 5), SANT1L (SANT1-like, 6), MCSS (motif connecting SANT1L and SANT2L, 7), SANT2L (SANT2-like, 8), CXC (cysteine-rich pre-SET domain) and the catalytic SET domain. The SANT motifs are the least conserved surfaces in the *Chaetomium* crystal structure. (C) Fungal EED proteins (*Drosophila* Esc) contain WD40 (WD) domains that generate a seven-bladed propeller structure, for which the C-terminus folds back towards the N-terminus to generate propeller 1. The function of the extended C-terminal insertion domain is unknown. The accessory Msl1/NPF subunit of *Cryptococcus* and *Neurospora* is conserved in humans (RBAp46/48); all Msi1-like proteins share the WD40 propeller structure with EED. (D) SUZ12 (*Drosophila* Su[z]12) contains an Eed-binding domain (2), a Zn-finger region (Z) and a conserved VEFS domain that in the crystal structure is wedged between KMT6 and EED.

Figure 5. Histone modifications associated with transcriptionally active euchromatin and transcriptionally silent heterochromatin on four different types of chromosomes found in many fungi

(A) Core or "A" chromosomes have a mixture of transcriptionally active euchromatin (green), constitutively silent heterochromatin (grey) that remains densely packaged even in interphase, and facultative heterochromatin (orange) that becomes transcriptionally active upon external or internal cues. Modifications on core chromosomes most often correlated with euchromatin are H3K4 di- and trimethylation (H3K4me2/3), which are usually found in sharp peaks around the nucleosome-free transcriptional start sites or in the 5' regions of genes. In constitutive heterochromatin, which is often found in repetitive DNA sequences like centromeric regions that also contain CenH3 nucleosomes (purple), pericentric (dark grey) regions or in transposable elements (light grey), H3K9 is di- or trimethylated (H3K9me2/3) and DNA is often methylated at cytosines. In facultative heterochromatin H3K27 is di- or trimethylated (H3K27me2/3) and controls the expression of genes in a time- and space-dependent manner. Telomeric repeats (blue) have specialized chromatin structures in many fungi, some are free of nucleosomes and bound by shelterin-like complexes. In addition to the histone modifications shown here, lysines in the H3 and H4 tails of euchromatic regions are hyperacetylated (H3ac, H4ac), H3K79 and H3K36 are trimethylated (H3K79me3, H3K36me3), and H2BK120 is mono-ubiquitylated (H2BK120ub1); canonical H2A is replaced by the variant H2AZ. In heterochromatin, H3 and H4 lysines are hypoacetylated and H2AK119 is mono-ubiquitylated (H2AK119ub1). (B) In several *Fusarium* species and in *Z. tritici*, complete or segments of chromosomes arms from accessory chromosomes that are enriched for H3K27 methylation have translocated onto core chromosomes, generating bi-partite chromosomes with different histone modification environments. (C) Most accessory chromosomes from *Fusarium* and *Zymoseptoria* species that have been studied show almost complete coverage with H3K27me3. A very minor fraction of genes is active and enriched with H3K4me2/3, while pericentric regions and centromeric regions in *Fusarium* species are enriched with H3K9me3. In *Z. tritici*,

H3K9me3 and H3K27me3 are partially overlapping in repeat-rich regions but H3K27me3 is mostly found at silent genes. In this species no clear correlation with centromeric chromatin and any tested histone modification has been found. (D) The shortest accessory chromosomes have no active genes and show equal fractions of H3K27me3 and H3K9me3. (E) Predicted structure of true “B” chromosomes similar to those that have been found in plants and animals. These simplest chromosomes are completely gene-free, have only constitutive H3K9me3-enriched heterochromatin, centromeres and telomeres. No such true B chromosomes have been documented in fungi.

Figure 6. Three-dimensional models of *Neurospora crassa* linkage group (LG) VII based on Hi-C data

Chromosomes are represented as wire diagrams, where the wire path runs through the center of a series of 50 kb “spheres” determined by the contact frequencies calculated from Hi-C datasets for wildtype (WT) and three chromatin mutants (*dim-5*, *hpo*, *dim-3*). The chromosome path is calculated by attractive or repulsive forces between each sphere so that the system relaxes to a low energy state. Regions that are enriched with one heterochromatic mark, H3K9me3, in wildtype are shaded in red. Centromeres and subtelomeres are separated but telomeres are closer to each other than to the centromere (adapted from [131]).