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Review

Unbelievable but True: Epigenetics and Chromatin in Fungi

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Evolutionary innovations in chromatin biology have been recently discovered through the study of fungi. In *Saccharomyces cerevisiae*, a prion form of a deacetylase complex assembles over subtelomeric domains that produces a heritable gene expression state that enables resistance to stress. In *Candida albicans*, stress triggers adaptive chromosome destabilization via erasure a centromeric histone H3, CENP-A; a process that cooperates with a newly evolved H2A variant lacking a mitotic phosphorylation site. Finally, in *Cryptococcus neoformans*, the loss of a cytosine DNA methyltransferase at least 50 million years ago has enabled the Darwinian evolution of methylation patterns over geological timescales. These studies reveal a remarkable genetic and epigenetic evolutionary plasticity of the chromatin fiber, despite the highly conserved structure of the nucleosome.

Highlights

Fungal epigenetic phenomena include some that seem unbelievable.

A chromatin-associated prion enables heritable stress resistance in *S. cerevisiae*.

Stress-induced centromeric histone eviction enables adaptation via aneuploidy in *C. albicans*.

C. neoformans DNA methylation evolves via a Darwinian process over million-year timescales.

Why Study Epigenetics in Fungi?

Cells respond to their internal and external environment by changing their gene expression. Clones of cells can arise that persistently display such changes even after the initial trigger is removed. Most instances of heritable changes in cell state do not involve changes in DNA sequence and are thus termed epigenetic [1]. In humans, epigenetic mechanisms specify cell type, silence repeated sequences, program parent-of-origin expression of monoallelic genes, and go awry in certain diseases such as some cancer. Many molecular mechanisms can underpin such processes, ranging from positive-feedback loops mediated by transcription factors, DNA methylation, and histone modification [2–4]. Most work in this area has focused on mammalian systems. However, many remarkable mechanisms have been unearthed elsewhere on the evolutionary tree. Evolutionary innovations involving fungal epigenetics are the focus of this piece. These findings, some of which seem unbelievable, include chromatin prions, centromeric nucleosome erasure leading to kinetochore inactivation, and the Darwinian evolution of DNA methylation patterns over million-year timescales [5–8].

There are numerous reasons to study processes in fungi. One is experimental tractability, which enables facile testing of hypotheses. Studies of brewer's yeast *Saccharomyces cerevisiae* have enabled much of our understanding of the eukaryotic cell. Nonetheless, it is increasingly appreciated that not all highly conserved aspects of eukaryotic cell behavior can be modelled in this one species, in part due to ancestral evolutionary loss events. Mechanistic studies in a relatively small number of other fungi have yielded insights and true surprises. A celebrated example is the finding that mutations in the RNAi machinery of *Schizosaccharomyces pombe* impacts repressive histone H3 lysine 9 methylation [9]. However, the fungal kingdom is vast with perhaps over 1 million species [10]. Thus, research into this ancient kingdom has barely scratched the surface of its secrets. While the genome sequences of fungi can now be easily obtained through high throughput DNA sequencing, a mechanistic understanding of their biology will requires experimental investigations, which in turn require communities of scientists willing to do the painstaking work of tool development and discovery.

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Introduction to the Players

S. cerevisiae and *S. pombe* are members of the Ascomycota phylum, which harbors a major portion of the fungal kingdom [11]. Many species in this phylum are not yeast, defined as single-celled fungi. Rather, the majority are either dimorphic fungi (that alternate between yeast and filamentous mycelial forms) or strictly filamentous [11]. The filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* have served as dominant models because of their long history, strong research communities, and the development of powerful tools and resources. *Neurospora* in particular has been used to investigate DNA methylation and DNA methylation-coupled processes [12]. More recently, it has served as a model for **Polycomb** (see Glossary) silencing [13]. Another ascomycete, *Candida albicans*, is the most common fungal pathogen of humans as well as a normal commensal [14]. As detailed in the following text, it has evolved a chromatin-programmed system for regulated chromosome destabilization.

The other major fungal phylum is Basidiomycota. Ascomycota and Basidiomycota together form a subphylum called the Dikarya [11]. Basidiomycota include mushrooms and many other species, including several plant pathogens [11] and the human pathogens, *Cryptococcus neoformans*, *Cryptococcus deneoformans*, and *Cryptococcus gattii* [15,16]. Like *S. cerevisiae*, these human pathogens grow as single-cell budding yeasts, maintain stable haploid genomes, and display a complete sexual cycle. They can also be transformed with DNA and display efficient homologous recombination [17]. In particular, *Cryptococcus* harbors all of the chromatin gene silencing systems identified in mammalian cells, including a Polycomb system [18], an H3K9 methylation system [18], and DNA methylation [19]. Not further discussed here are a number of basal fungal lineages that include several species in which tools are being developed that will enable detailed studies.

A Chromatin Prion Enables Bet Hedging

First identified as the infectious agent of scrapie, a neurodegenerative disease of sheep, prions (proteinaceous infectious agents) are protein-only infective agents [20]. Prions are now known to be widespread in biology and have been studied extensively in *S. cerevisiae,* where such nonchromosomally heritable states of protein activity are widespread [21]. While prions display epigenetic inheritance, they do so by multiple mechanisms, including the formation of protein amyloid aggregates [22]. Using the well-developed toolbox of *S. cerevisiae*, Jarosz *et al.* took advantage of a property of many prions to identify new candidates in a systemic fashion: many prions are induced by overexpression of the monomeric form of the protein that forms the prion [23]. In some cases, this triggers the assembly of protein aggregates (e.g., amyloids) that are the prion form of the protein. This form is self-propagating, can persist in the absence of the initial overexpression triggers, is heritable both mitotically and meiotically, and is often inactive. Thus, transient overexpression of protein can trigger an epigenetic change mediated by prion formation.

These investigators systematically transiently overexpressed nearly every yeast protein and then used a series of stress conditions to identify cases in which the transient overexpression altered cellular fitness. This bold approach yielded dozens of new candidate prions. Subsequently, the authors followed up on a single hit from the screen (Figure 1, Key Figure), a prion dubbed [*ESI+*]. [*ESI+*] formation is triggered by transient overexpression of *SNT1*, which encodes a subunit of the Set3 histone deacetylase complex, a yeast ortholog of the human NCOR–SMRT deacetylase complex [8]. Transient overexpression of *SNT1* triggers resistance to zinc ion stress that lasts for hundreds of generations. As with many yeast prions, overexpression of a protein chaperone, Hsp90, can cure cells of this phenotype [8].

Following mating, the [*ESI+*] prion state of a haploid parent can be transmitted to all four meiotic progeny, even when the second parent lacks the prion [8]. In addition, using an experimental trick

Glossary

Bub1: a protein kinase involved in multiple aspects of mitosis including the mitotic checkpoint. Among is substrates is a conserved serine or threonine residue on the C-terminal tail of histone H2A.
CENP-A: a centromeric histone H3 variant found in almost all eukaryotes. It forms the foundation of the kinetochore.
Chromodomain: a protein domain found in eukaryotes whose known function is to bind lysine residues on histones in a manner dependent on prior methylation of the epsilon amine group of those residues.
Lanosterol-14-demethylase: An enzyme in the cholesterol (plants and mammals)/ergosterol (fungi) biosynthesis pathway. In fungi, it is the target of the azole group of antifungal drugs.
Opportunistic pathogen: a pathogen that is virulent in hosts with compromised tissues or immunity such as cancer chemotherapy patients, organ transplant recipients, and individuals with AIDS.
Polycomb: a conserved eukaryotic gene silencing system involving modification of histone H3 on lysine 27 and chromatin compaction. It produces heritable gene silencing important for epigenetic memory during development (animals) and in response to environmental changes (plants).
Shugoshin: a conserved regulator of sister chromatid cohesion and other functions during mitosis and meiosis.

Key Figure

A Stress-Induced Chromatin Prion in *Saccharomyces Cerevisiae*

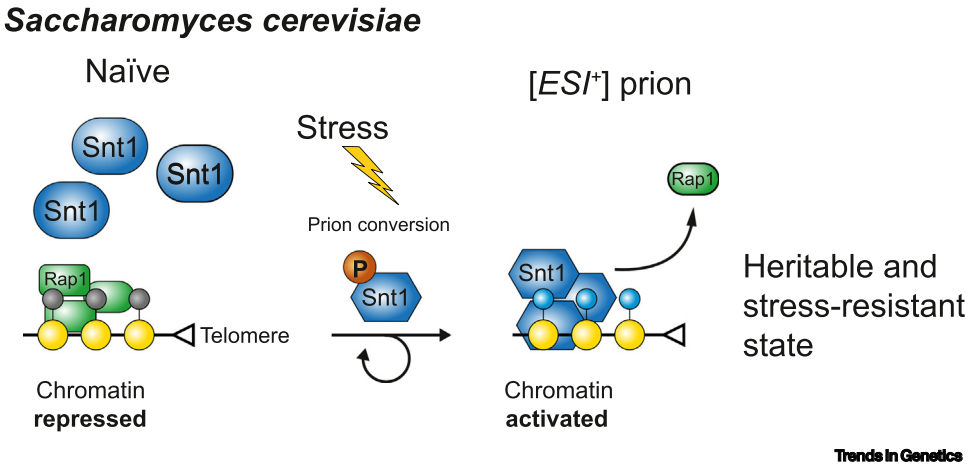

Figure 1. Depicted is a model for how repressed subtelomeric heterochromatin is activated via the formation of a prion involving a subunit of the Set3 histone deacetylase complex called Snt1.

called cytoduction in which cytoplasm and organelles but not nuclei are introduced from one cell to another, it was shown that the [*ESI+*] state can be transferred from cell to cell in this manner [8]. To definitively demonstrate protein-only inheritance, Jarosz and colleagues introduced an aggregated form of the protein (produced by recombinant expression in *Escherichia coli* followed by purification) into yeast lacking the prion phenotype, and found that it was sufficient to trigger the [*ESI+*] phenotype in a significant fraction of transformants [8]. Thus, transient *SNT1* overexpression can trigger prion activity that can mediate zinc ion tolerance.

Further work demonstrated that *SNT1* harbors several predicted disordered regions, which may help to promote the formation of the prion state. In addition, the protein is phosphorylated on multiples sites and phosphomimic mutations promote prion formation [8]. Although not proposed by the authors, these properties raise the possibility that the prion form of *SNT1* is a coacervate, that is, a droplet formed by liquid–liquid phase separation [8], as these can be driven by interactions between charged regions in disordered domains [24]. Genome-wide transcript profiling revealed that the prion form of *SNT1* results in increased expression of subtelomeric genes. Strikingly, binding of the Set3 complex increases at subtelomeric domains when *SNT1* is in the prion form [8]. Moreover, subtelomeric binding of the Set3 complex anticorrelates with binding of a nucleator of subtelomeric silencing, the sequence-specific DNA-binding protein Rap1. Thus, a prion form of a histone deacetylase complex associates with chromatin and activates subtelomeric gene expression by antagonizing gene silencing [8].

Much remains to be learned here. In particular, we need to learn how precisely the prion form leads to increased gene. It is also unclear whether the deacetylase activity of the complex important and whether histones the relevant target for this activity. More generally, it will be important to test whether other chromatin-modifying complexes act by forming chromatin-associated prions and whether phase-separation involved in this mechanism. The chromatin-associated prion identified by Jarosz *et al.* might portend a new principle and mechanism in chromatin biology; namely, the heritable polymerization of a chromatin component with associated functional consequences.

Such a principle, if demonstrated to be general, would represent a fundamentally novel way of thinking about how a chromatin fiber can operate in cells. In the next section, I describe additional aspects of chromatin fiber composition and regulation that may demand some rethinking.

Centromeric Nucleosome Erasure and a Newly Evolved Defective Histone H2A Enable Adaptive Aneuploidy

C. albicans is part of a group of human pathogenic fungi that have several shared characteristics including a nonuniversal genetic code and a sexual cycle that does not involve meiosis [25]. *C. albicans* cells are diploid. Like diploid *S. cerevisiae*, they do not mate because they are heterozygous at the mating type locus, displaying a *MAT*a/*MAT*α genotype [26]. However, loss of one copy of the chromosome harboring the mating type locus, chromosome V, results in *C. albicans* cells that are sexually analogous to haploid a and α cells of *S. cerevisiae* [26]. These are capable of differentiating into mating-competent cells called opaque cells [26]. Mating between *MAT*a and *MAT*α *C. albicans* cells results in tetraploid cells [25]. However, this is where the unusual aspect of the sexual life cycle occurs: rather than undergoing meiosis, under certain conditions, *C. albicans* undergoes massive, apparently random chromosome loss, which results in massive cell death and ultimately the selection of a new diploid cell population [25]. The ability of *C. albicans* cells to lose chromosomes easily under particular conditions suggested that it harbored a mechanism to destabilize its chromosome segregation machinery.

Likewise, multiple studies have shown that *C. albicans* can develop resistance to an antifungal drug by evolving an aneuploidy of a chromosome encoding the target of the drug (the drug being fluconazole and the target being the *ERG11* gene, encoding **lanosterol-14-demethylase**). Studies of this process led to the model that fluconazole stress itself induces chromosome instability, suggesting mode of evolution in which the selection induces a higher mutation rate in this case via increased frequency of aneuploidy [27,28]. However, the chromosome-destabilizing mechanisms underlying such an unconventional mechanism remained unclear.

Noble *et al.* identified two underlying mechanisms that appear to act in parallel to enable induced chromosome destabilization (Figure 2). In the process of characterizing the histone genes of *C. albicans*, these authors noticed that homozygous deletions of either of two distinct histone H2A genes yielded distinct colony phenotypes [5]. They further noticed that one of the H2A genes displayed an amino acid substitution on a universally conserved phosphorylation site for

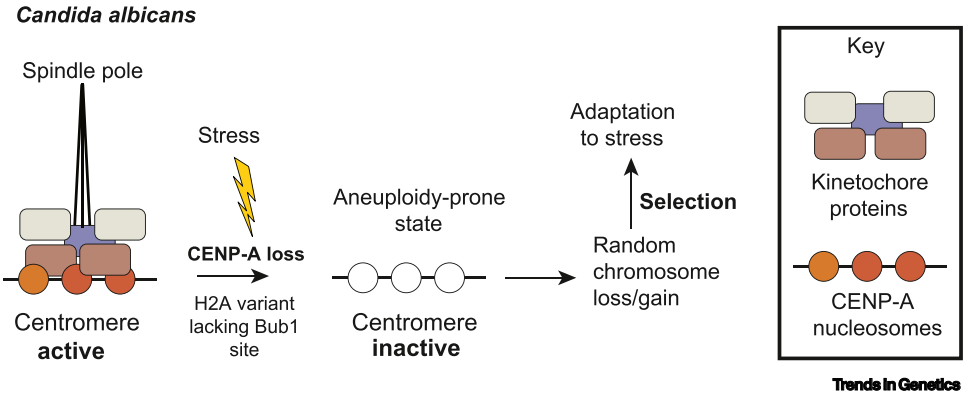

Figure 2. Adaptive Induction of Mitotic Chromatin Destabilization in *Candida Albicans*. Depicted is the stress-induced kinetochore inactivation enabled by loss of CENP-A (centromeric histone H3 variant) in the context of a newly evolved histone H2A variant that lacks the otherwise conserved phosphorylation site for the Bub1 mitotic kinase.

the **Bub1** mitotic kinase [5]. Phylogenetic analysis revealed that this change occurred in a shared ancestor of *C. albicans* and two of the related *Candida* species that share a noncanonical genetic code and a nonmeiotic sexual cycle [5]. Both the noncanonical and canonical H2A genes are expressed and, as expected, replacement of the canonical with the noncanonical coding sequences result in decreased chromosome stability while replacement of the noncanonical H2A coding sequence with the canonical resulted in increased chromosome stability [5]. Thus, it is clear that *C. albicans* has evolved to have nonoptimal chromosome segregation, which is a striking finding. The impact of H2A was demonstrated to be relevant to drug resistance as cells harboring only the noncanonical H2A displayed increased rates of fluconazole tolerance as do mutations in the phosphorylation site in the canonical H2A as well as knockouts of Bub1 or its downstream effector **Shugoshin** [5]. Likewise, efficient chromosome loss after mating in tetraploids also required the noncanonical H2A [5]. These studies make clear that what would appear to be a defective histone missing an otherwise universally conserved phosphorylation site for a mitotic kinase has been actively maintained in a clade of organisms for the purpose of promoting adaptive chromosome loss. As described in the next paragraph, this is not the only such mechanism that has evolved in *C. albicans*.

A motivation for searching for such an additional mechanism is the observation that the H2A genes appear to be constitutively expressed and not induced by mating or stress. Thus, it seemed likely that there exist chromosome-destabilizing mechanisms induced by mating and/or stress conditions and that cooperate with the presence of a noncanonical H2A that would respond to conditions thought to trigger chromosome loss [5]. **CENP-A** is a centromeric histone H3 variant that forms the foundation of the kinetochore in nearly all eukaryotes. Thus, Noble *et al.* examined CENP-A deposition in *C. albicans* using ChIP-seq [5]. These studies revealed that CENP-A is significantly depleted at the kinetochores of tetraploid cells compared to diploids, which correlates with the reduced chromosome stability of tetraploids [5]. Strikingly, the condition that triggers chromosome loss in tetraploids (pre-Spo medium) triggers the near elimination of CENP-A at centromeres [5]; similar to the result in diploid cells exposed to fluconazole [5]. Likewise, treatment of diploid cells with fluconazole also resulted in depletion of CENP-A from centromeres. As CENP-A is the platform of the eukaryotic kinetochore, its loss provides a plausible explanation for the inducibility of chromosome loss in *C. albicans*. It is also striking that lineages can evolve mechanisms to eliminate a highly conserved cellular feature to enhance evolvability.

These findings suggest the existence of a signal-regulated mechanism to control centromere activity via CENP-A. It is unclear what programs the loss of CENP-A or whether noncanonical H2A required for CENP-A removal. Addressing some of these questions will benefit from identifying the machinery responsible for CENP-A depletion at centromeres. Other key issues in need of study include defining the signal transduction pathway upstream of CENP-A removal, determining how CENP-A is redeposited in the correct place after nonstress conditions have returned, whether there is natural variation in this process, and whether this process is relevant to virulence and commensalism. In the next section, I discuss a feature of centromeric chromatin in a fungus that is only distantly related *S. cerevisiae* and *C. albicans*. This yeast, *C. neoformans*, is a member of the Basidiomycota.

Darwinian Evolution of DNA Methylation Patterns Over Million-Year Timescales

Before delving into the specifics of centromeric chromatin in *Cryptococcus*, a brief introduction into a common feature of centromeric and subtelomeric chromatin in mammals and plants, namely DNA methylation, is warranted since the species discussed up to this point lack this otherwise common DNA modification.

The methylation of cytosine at position 5 in mammals and plants is a critical genome defense and regulatory mechanism involving two types of enzymes: *de novo* enzymes that can methylate unmethylated DNA and maintenance enzymes that prefer hemimethylated DNA [29]. The latter recognize sequences in palindromic contexts (e.g., CG or CHG) which, after DNA replication, harbor methylcytosine on the parental strand and unmethylated cytosine on the newly synthesized daughter strains [29]. Maintenance methylases restore the original state enabling epigenetic propagation of DNA methylation patterns that were established by the prior action of *de novo* methylases [30]. This paradigm in widespread in animals and plants. In fungi, the best-characterized DNA methylation system is that of the ascomycete filamentous fungus *N. crassa*, which is known colloquially as bread mold. Foundational work by Selker and colleagues demonstrated that all cytosine methylation in *Neurospora* is mediated by a single enzyme, DIM-2 [31]. DIM-2 acts downstream of a repressive histone modification H3-K9 methylation [32]. Although DIM-2 has not been purified in active form, *in vivo* experiments indicate that it appears to have both *de novo* and maintenance capabilities [31,33]. Studies of another ascomycete, *Ascobolus immersus*, demonstrated that memory systems exist in fungi: in this species, duplicated DNA sequences become methylated prior to meiosis, in a process called methylation-induced premeiotically (MIP) [34].

C. neoformans is an **opportunistic pathogen** responsible for ~200 000 deaths annually due to its ability to cause a lethal meningoencephalitis [35]. We recently investigated cytosine DNA methylation, an epigenetic mechanism involved in genome defense, in this organism [6]. CpG methylation in *C. neoformans* is mediated by Dnmt5, the only apparent DNMT (cytosine DNA methyltransferase) encoded by the *C. neoformans* genome. Dnmt5 is a member of a clade of cytosine methyltransferases that harbor a Snf2 ATPase domain in addition to a DNMT domain. These enzymes are found in specific lineages in the fungal and protist kingdoms. Our analysis of CG methylation in *C. neoformans* revealed that, as in *N. crassa*, it acts downstream of H3K9 methylation via two different readers of this chromatin mark: the **chromodomain** of Dnmt5 itself and the *C. neoformans* ortholog of HP1/Swi6 [6]. In addition, *C. neoformans* harbors an ortholog of a human methylation cofactor that recognizes hemimethylated DNA called UHRF1. Like its metazoan relative, *C. neoformans* Uhrf1 selectively bind hemimethylated CG-harboring DNA *in vitro* [6]. Biochemical analysis revealed the purified *C. neoformans* Dnmt5 to be an exquisitely specific maintenance-type enzyme that only recognizes hemimethylated DNA [6]. *In vitro,* Dnmt5 requires ATP for activity, an unprecedented requirement for a cytosine DNA methylase [6]. *In vivo*, methylation is not globally restored once the gene for Dnmt5 is removed from cells and then re-introduced. This was shown in three ways: (i) using a regulated promoter; (ii) deleting the catalytic domain of *DMT5* gene and re-introducing it by homologous recombination; and (iii) by re-introducing the wild-type gene through a genetic cross [6]. Moreover, it was shown that integration of a fragment of foreign DNA that had been methylated *in vitro* using the HpaII methylase resulted in maintenance of DNA methylation at HpaII sites without detectable spread to nearby CG sites [6]. This indicates that Dnmt5 acts as a maintenance enzyme in cells. In other words, Dnmt5 requires preexisting methylation to propagate methylation.

If Dnmt5 is the only DNMT in *C. neoformans*, how was methylation ever established in the first place? Our evolutionary studies revealed that the ancestor of *C. neoformans* harbored both a gene for Dnmt5 but also a gene for a second Dnmt, DnmtX [6]. Introduction of DnmtX from extant species triggered *de novo* methylation that could then be maintained by Dnmt5, indicating that DnmtX has *de novo* activity *in vivo* [6]. The gene for DnmtX was lost between 50 and 150 million years ago, suggesting the startling conclusion that DNA methylation has been maintained in this species for millions of years without a *de novo* enzyme (Figure 3).

As it seems unlikely that an original pattern imparted by DnmtX could be propagated without change for millions of years, we performed two types of experiments aimed at understanding

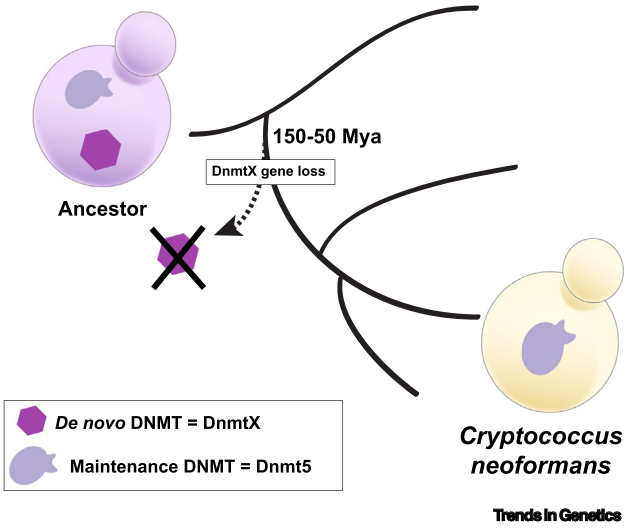

Figure 3. Epigenetic Evolution of *Cryptocccus Neoformans*. Loss of a gene encoding a *de novo* DNA cytosine methyltransferase (DNMT) resulting in a long-lived lineage harboring only a maintenance-type DNA cytosine methyltransferase. This lineage gave rise of the human pathogenic yeast, *Cryptococcus neoformans*. Cytosine methylation patterns have been undergoing Darwinian evolution that is mediated by rare random loss events, rarer random gain events and the action of natural selection on transposable elements.

how methylation might evolve over such long time periods without the agency of a dedicated *de novo* system. The first set of experiments consisted of experimental evolution experiments in which a culture was propagated for ~140 generations and then the methylation patterns of two colonies were examined and compared to the parental strains using whole-genome bisulfite sequencing (WGBS). This analysis revealed apparently random losses of methylation at a rate of about 10^{-4}/generation per site [6]. Considerably rarer gain events were observed at a 20-fold lower rate. They also appeared to be random [6]. These rare gain events may reflect a trace of *de novo* activity produced by Dnmt5. Because the rate of loss was considerably greater than the rate of gain, the system is not in equilibrium. We concluded from this analysis that selection for methylation levels is required to maintain the steady state [6].

To investigate the evolution of methylation over longer time periods, we analyzed across a phylogeny of *C. neoformans* isolates that shared a common ancestor ~5 Mya. This required the long-read assembly of eight *C. neoformans* genomes using Nanopore sequencing. By combining WGBS and methylation information obtained from the Nanopore platform, the methylation landscapes of the eight genomes was obtained [6]. A standard phylogenetic approach to evaluate methylation evolution was not feasible as rearrangement of the methylated sequences precluded multiple sequence alignment. Nonetheless, local pairwise alignment revealed higher sharing of methylation patterns than expected by chance [6]. Moreover, annotation of putative full-length transposons revealed that they displayed a considerably higher fraction of methylated CGs than other centromeric sequences [6].

Taken together, these studies indicate that methylation patterns have been maintained for millions of years by a Darwinian process in which random rare losses and rarer gains of methylation occur and are then maintained by natural selection for methylation of transposable elements. This Darwinian mode of methylation pattern evolution indicates that DNA sequence is not the only substrate for evolution.

Numerous questions remain. A number of these relate to mechanism. In particular, we do not understand the *in vivo* determinants of accurate maintenance methylation, what determines the exquisite specificity of Dnmt5, why the enzyme requires ATP hydrolysis to function, and precisely

how do cofactors such as Uhrf1 function with Dnmt5. Also of interest is how *de novo* methylation controlled in species that harbor both DnmtX and Dnmt5 and whether maintenance methylation patterns also evolve via a Darwinian process in species harboring DnmtX. A clue that such evolution might occur in human cells is provided by recent evidence indicating that Dnmt1 can display *de novo* activity *in vivo* [36].

Concluding Remarks

The beauty of evolution lies in its ability to modify the existing to yield the rich variety of forms and mechanisms that is life. Core cellular functions not immune to evolutionary tinkering, and the case studies described in the preceding text demonstrate new ways in which fundamental organizers of the eukaryotic genome, the nucleosome and ensuing chromatin fiber, can be regulated. One intriguing question concerns how such mechanisms have evolved (see Outstanding Questions). Answering this question will require defining the underlying sequence of events and driving forces. If such mechanisms are easily evolvable, then one would have to consider the possibility that such events occur frequently during evolution. Such convergently evolved mechanisms may be widespread and could even occur in human cells. However, their identification will require carefully designed experimental strategies. The approaches pioneered in fungi provide a clear roadmap for such tests. Conversely, the development of experimental tools in a much larger variety of organisms will pave the way for those aiming to identify new unexpected mechanisms through which the genetic material is controlled.

Acknowledgments

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Outstanding Questions

How precisely does the prion form of [*ESI1*+] lead to increased gene expression?

How does the prion form of [*ESI1*+] interfere with DNA binding by Rap1?

Is the deacetylase activity of the Set3 complex important for the action of [*ESI1*+]?

Do other chromatin-modifying complexes act by forming chromatin-associated prions?

Is phase-separation involved in this mechanism of formation of [*ESI1*+]?

What programs the loss of CENP-A in *C. albicans*?

Is the noncanonical *C. albicans* H2A required for CENP-A removal?

What is the mechanism for CENP-A depletion at centromeres in *C. albicans*?

What is the signal transduction pathway upstream of CENP-A removal *C. albicans*?

How is CENP-A re-deposited in the correct place after nonstress conditions have returned *C. albicans*?

It is centromere inactivation in *C. albicans* relevant to virulence and commensalism?

What are the determinants of accurate maintenance methylation in *C. neoformans*?

What determines the exquisite specificity of Dnmt5 in *C. neoformans*?

Why does Dnmt5 require ATP hydrolysis to function in *C. neoformans*?

How do cofactors such as Uhrf1 in *C. neoformans* function with Dnmt5?

How is *de novo* methylation controlled in species that harbor both DnmtX and Dnmt5?

Does maintenance methylation also evolve via a Darwinian process in species harboring DnmtX?

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