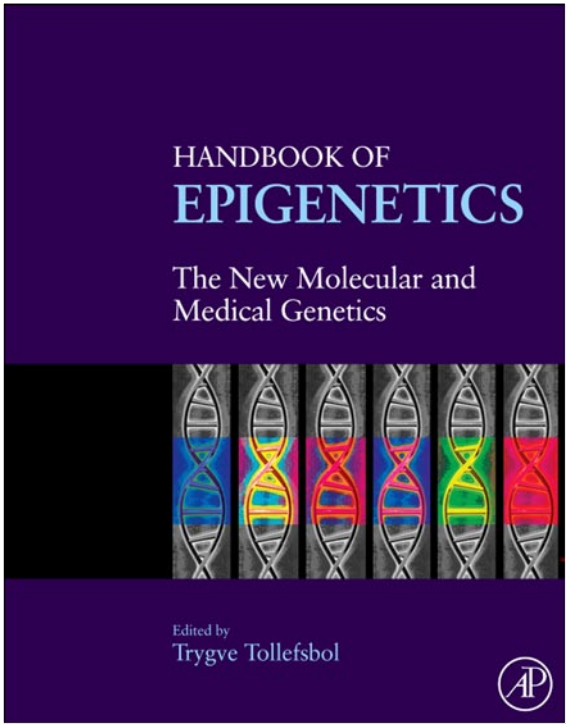

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CHAPTER 13

Epigenetics of Eukaryotic Microbes

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INTRODUCTION

Eukaryotic microbes encompass the vast majority of the eukaryotic diversity [1]. Although some have been used as laboratory models for decades and other are important plagues to humankind, their biology is often less well-known than that of animals and plants. This applies to the knowledge of epigenetic processes, which is scarce in most microbial eukaryotes. Nevertheless, key discoveries regarding the molecular processes involved in epigenetic inheritance have been made with these organisms, especially model fungi, such as *Schizosaccharomyces pombe* and *Neurospora crassa*. Here, we will not attempt to provide an exhaustive overview of epigenetic phenomena in eukaryotic microbes, due to space constraint, but rather give a close look at the major contributions brought by these model organisms, especially filamentous fungi. Readers interested in other epigenetic phenomena such as prions and related phenomena in eukaryotic microbes are invited to read Chapter 5 by Lalucque et al. in this book.

To date, silencing phenomenon can be divided into two categories, transcriptional gene silencing (TGS), when no transcript of the targeted gene is produced, and post transcriptional gene silencing (PTGS), when transcripts are produced but specifically degraded before translation could occur. The latter, PTGS, is known as RNA interference (in animals) or co-suppression (in plants). But, PTGS and TGS also exist in fungi and protists, as will be illustrated below.

POST-TRANSCRIPTIONAL GENE SILENCING

Phylogenetic surveys of proteins involved in PTGS have shown that they are present in all lineages of eukaryotes [2,3], and thus that the ancestors of the eukaryotes were likely endowed with some primitive PTGS mechanisms. However, some organisms lack the PTGS machinery (see below), indicating that PTGS is not mandatory for efficient survival. In these early eukaryotes, PTGS could either degrade mRNA with the help of small guide RNA (e.g. siRNA and related molecules) or modify histones leading to transcriptional gene silencing, two functions that are nowadays widely conserved among eukaryotic microbes [2]. In the RNAi world, a tremendous body of work has been accomplished by taking advantage of the nematode *Caenorhabolitis elegans* [4–8]. This outstanding scientific adventure made Greg Mello and Andrew Fire Nobel Price laureates in 2006. But fungi, especially the bread mould *N. crassa*, although less famous have been instrumental in deciphering PTGS at the molecular

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level. For years, this species has been a great contributor to research in many scientific fields, but as regards homologous-based control of gene expression, it shows outstanding features. In *N. crassa*, two PTGS mechanisms have been extensively studied so far: quelling and meiotic silencing of unpaired DNA. But *N. crassa* also presents a TGS mechanism, the repeat induced point mutation (RIP) phenomenon, which will be discussed at the end of this chapter.

QUELLING IN *N. crassa*

Quelling, as designated by Romano and Macino in 1992 [9], was the first truly reversible homology dependent gene silencing process, discovered in fungi. Indeed, these authors showed that endogenous expression of the *al-1* gene, involved in *N. crassa* carotenoid biosynthesis, could be silenced after transformation with homologous *al-1* sequences. This silencing was easily detected as transgenic lines ranged from wild-type orange color to light yellow and even pure white, the latter being the phenotype of *al-1* null mutant strains. But, upon vegetative growth, silenced genes were reactivated at high frequency, which often correlated with genomic rearrangements leading to partial losses of the transgenic repeats. Since then, it has been demonstrated that quelling, which is triggered during the vegetative phase, affects expression of both transgenic and endogenous homologous copies. Heterokaryons made from *al-1* silenced transgenic nuclei mixed together with wild-type nuclei revealed that quelling is dominant [10]. At the time, this latter feature strongly suggested that quelling relies on diffusible molecule(s), acting *in trans*, rather than on a DNA-DNA pairing mechanism. When the transcriptional status of the silenced loci was investigated, initiation appeared normal but no accumulation of transcripts could be detected [10]. Although DNA methylation is often detected on repeats, this epigenetic modification is not required for quelling, since silencing is fully efficient in *dim-2* mutant strains that show no DNA methylation [10]. However, methylation of lysine 9 of histones H3 (H3K9me), which is also a common epigenetic modification of chromatin, has an indirect effect on quelling [11]. Mutants defective for *dim-5*, a gene encoding a H3K9 methyltransferase [12], were unable to properly maintain quelling, because of the frequent loss of transgenes in tandem.

To further characterize the molecular bases of quelling, a mutant screen was set up by Cogoni and Macino, generating a series of quelling-deficient mutants (qde) [13]. The *qde-1* mutant was defective in an RNA-dependent RNA polymerase (RdRP) [14]. This gave the first clue that RNA components were involved in quelling. Afterwards, it was demonstrated that *Arabidopsis thaliana* and *C. elegans* homologous genes [15,16], both encoding RdRP, are required for PTGS and RNAi, respectively, indicating that the silencing machinery is evolutionarily conserved. The RNA mediated silencing model was further supported by the identification of the second gene, *qde-2*, as encoding a protein with a piwi-Paz domain that is also found in the Argonaute protein family previously characterized in plants [17]. Again, the Argonaute proteins, through the RNA-induced silencing complex (RISC) are now known to be essential for the RNA silencing pathway in numerous eukaryotes. The last of the *qde* mutants, *qde-3*, was impaired in a gene coding for a RecQ DNA helicase, suggesting the involvement of a nucleic acid pairing step [18]. Later on, DCL-1 and DCL-2, two *N. crassa* Rnase III dicer-like proteins partially redundant, were reported to be both involved in quelling by producing siRNAs of 21–25 nucleotides [19]. Biochemical purification of QDE-2 led to the identification of the exonuclease QIP [20]. QIP is thought to degrade the passenger strand of siRNA duplexes, and strains deleted for the corresponding gene are deficient for quelling. Looking for proteins that physically interact with the QDE-1 RdRP led to the discovery of the replication protein A (RPA) [21]. This finding is the first link that has been established between RNA silencing and DNA replication and opens a new field of investigations.

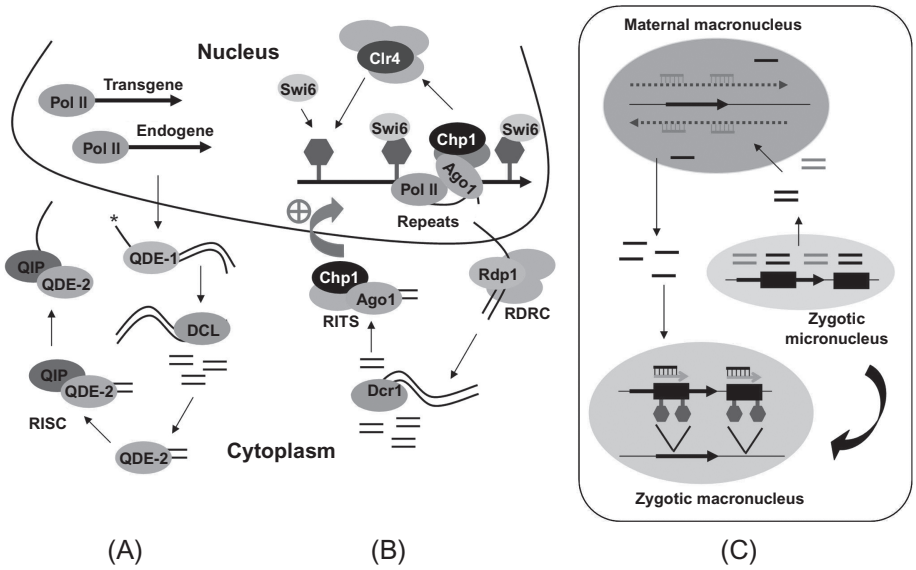

FIGURE 13.1

Models of RNAi in various eukaryotic microbes. (A) Quelling in *N. crassa*. Aberrant RNAs (*) are produced at loci that present repeats in large tandem arrays. Features of these aberrant RNAs are unknown, but they must be recognized by the RdRP QDE-1 and then convert into double stranded RNA molecules (dsRNA) [147]. dsRNA molecules are the typical substrate of the Dicer-like proteins DCL-1 and DCL-2 that chop them into siRNAs of 21–25 nucleotides. These siRNAs are integrated into the RISC complex, along with the Argonaute QDE-2 protein. They are then processed by the QIP nuclease and used as specific guides to target homologous mRNAs, which, once trapped, are most likely degraded by QDE-2. (B) RNAi silencing in *S. pombe*. The nascent transcript model proposes that RNA pol II continuously generates non-coding transcripts (*) from reverse promoter of heterochromatic repeats [36]. These aberrant RNAs are first cleaved by Ago1 and then recruited by the RNA-directed RNA polymerase complex (RDRC) to be converted into dsRNA by Rdp1 [148]. Using these dsRNAs as substrate, Dcr1 produces siRNA, which then bind to RNA-induced transcriptional silencing (RITS) complex, by means of Ago1 [149,150]. While RISC complexes target and degrade cytoplasmic mRNA, the RITS complex is tethered to chromatin through protein-protein interactions established between the chromodomain protein Chip1 and the H3K9me nucleosomes [35] (hexagons). The close association of the RITS complex and chromatin allows base-paring interactions between siRNA loaded on Ago1 and the nascent non-coding transcript soon to be cleaved by this protein. This amplification step of siRNA is likely to form a positive-feedback loop (plus arrow), which is believed to ensure the heterochromatin inheritance through cell divisions. As long as siRNA from a specific genomic region are produced, they continuously target the Clr4 histone methyltransferase complex (CLRC) to nucleosomes [151,152]. Thus, using H3K9me as signposts, heterochromatin spreads to large genomic territories in a sequence-independent but Swi6-dependent manner. As a result, transcription of the forward strand is silenced as in classical TGS systems. Gray ovals: known additional effectors. (C) Genome-scanning model in *Paramecium*. Because the micronucleus genome is unrearranged (rectangles represent IESs), it produces both IES-homologous (black) and non-IES-homologous (gray) scnRNAs. These diffusible molecules would enter and scan the IES-free maternal macronucleus. As a result of pairing with the maternal ncRNAs (dotted arrows), the non-IES-homologous scnRNAs would be sequestered. The remaining pool of scnRNA, highly enriched with IES-homologous scnRNAs, would be free to reach the developing zygotic macronucleus and pair with the nascent transcripts. At the IES targeted loci, chromatin shows H3K9 methylation [153] (hexagons), suggesting that this excision mechanism might have a TGS component. As for *S. pombe*, chromatin modifications could be used as signposts to direct an endonuclease towards the IESs to be excised. The curved arrow indicates that zygotic micronuclei develop into zygotic macronuclei throughout the course of the sexual phase. (Please refer to color plate section)

With the quelling mutants, *N. crassa* led the way to establish the molecular bases of RNAi-mediated gene silencing (Fig. 13.1A), that we now know is widely conserved among eukaryotes. However, to date, how genomic repetitive elements are identified as quelling targets is still unclear.

MEIOTIC SILENCING BY UNPAIRED DNA IN *N. crassa*

Besides quelling, *N. crassa* presents a second PTGS mechanism, specifically active during meiosis. First described as "meiotic transvection" (regulation dependent on pairing of alleles) [22], it causes unpaired DNA to silence all the genes homologous to it, whether or

not they are themselves paired [23]. To better characterize this fascinating process, a gene fusion between histone H1 and the green flourescent protein (GFP) was transformed into *N.* crassa [24]. The transgenic strains were then crossed. When both parental strains harbored the hH1-GFP construct at the same locus, hH1-GFP was expressed all along meiosis. However, when a wild-type strain, carrying no hHP1-GFP transgene, was crossed to a hH1-GFP strain, the transgene was silenced during meiosis since no green fluorescence could be detected. But, once sexual reproduction was over, 12 to 24 hours after spore formation, the expression of the silenced hH1-GFP transgene gradually resumed. Thus, meiotic silencing operates in a limited period of the *N. crassa* life cycle, and with respect to timing, it seems to be the opposite of quelling. Nonetheless, as with quelling, meiotic silencing affects not only the unpaired copies but any additional copy sharing homology with them. This suggested that a mobile *trans*-acting signal is involved in meiotic silencing.

Once more, genetic screens set up to select suppressors of meiotic silencing allowed Metzenberg and collaborators to clarify the links between DNA pairing and this new RNA silencing-related mechanism [23]. One of the mutant strains, *sad-1*, uncovered the first gene involved in meiotic silencing. It encodes an RdRP similar to QDE-1. The *sms-2* (suppressor of meiotic silencing-2) and *sms-3* (suppressor of meiotic silencing-3) mutants are affected in genes encoding paralogs of QDE-2 and DCL-2, respectively [25]. Characterization of the *sad-2* mutant strains unraveled a protein of an unknown function, not yet identified as a component of the RNA-based silencing pathways [24]. SAD-2 and SAD-1 likely interact together, since the perinuclear localization of SAD-1 depends on the presence of SAD-2. Altogether, these findings tell us that, although different sets of proteins are required to operate quelling or meiotic silencing, the general machinery, by itself, is very similar [26]. Interestingly enough, *sad-1* mutant strains can perform interspecific crosses, which are otherwise barren when done with wild-type strains, suggesting that meiotic silencing could be one of the mechanisms by which genetic barrier is built between species, given that interspecific crosses might display unpaired DNA due to chromosomal variation. Genes encoding SAD-1-like protein can be found in a large number of fungal genomes, but to date, meiotic silencing has been described only in *N. crassa*, and is either absent or substantially reduced in the closely related species *Neurospora tetrasperma* [27].

PTGS IN OTHER FILAMENTOUS FUNGI

More generally, in filamentous fungi other than *N. crassa*, involvement of typical RNA silencing proteins such as Dicer in homology-based silencing phenomena is known at least in *Aspergillus nidulans* [28] and *Magnaporthe grisea* [29,30]. Production of siRNAs was detected in *A. nidulans* [28], *M. grisea* [29], and *Mucor circinelloides* [31]. Recent availability of numerous fungal genomes in public databases enables searches for the typical RNA silencing components by *in silico* approaches. The discovery of homologs of genes required for PTGS shows that an ever growing number of fungi are endowed with the RNAi machinery. As a matter of fact, genes seemingly involved in PTGS can be found in the four major groups of Eumycota: Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota, although this last group appears to lack QDE-1 RdRP.

Strikingly, only a very narrow subset of species, including the basidiomycete *Ustilago maydis* and the ascomycetous yeasts, both pre-whole genome duplication (WGD) species (*Ashbya gossypii*, *Kluyveromyces lactis*, *Kluyveromyces waltii*) and post-WGD species (*Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Candida glabrata*, *Candida guilliermondii*, and *Candida lusitaniae*) lack the complete set of typical RNAi proteins [32]. This finding suggests that PTGS has been recently and repetitively lost during budding-yeast evolution and therefore might not be essential for fungal survival over long periods of time. But some other budding-yeasts including the pre-WGD species *Candida albicans* and the post-WGD *Saccharomyces castellii* and

Kluyveromyces polysporus display Argonaute proteins but no canonical dicer [32]. Recently, it has been discovered that these species are in fact endowed with Dicer proteins which present a RNAseIII domain but no helicase or PAZ domains [33]. Nevertheless, these atypical Dicers produce siRNA, which are mostly targeted to transposable elements and subtelomeric repeats [33]. By introducing *S. castellii* Dicer and Argonaute genes into *S. cerevisiae*, Drinnenberg and his colleagues were even able to obtained RNAi silenced genes [33]!

The actual role of PTGS in fungi is somewhat unclear. In *N. crassa*, it has been hypothesized that quelling and meiotic silencing would protect the genome from incoming selfish genetic elements. It is also possible that some regions of the genome need PTGS for proper structuration. Indeed, data obtained with *S. pombe* have uncovered a connection between TGS and PTGS.

SILENCING IN *S. pombe*, WHEN PTGS MEETS TGS

S. pombe is the yeast of choice to study heterochromatin assembly, partly because its genome contains a large array of heterochromatic regions (pericentric and subtelomeric regions, rDNA, and silent mating-type loci). By contrast to euchromatin, the chromatin of these regions shows enrichment for Swi6 (the *S. pombe* HP1 homolog), Clr4 (the *S. pombe* homolog of Su(var)39 histone methyltransferase), and hypoacetylated H3K9me. Reporter genes inserted into these heterochromatic regions are silenced. Evidence for a functional link between RNAi machinery and heterochromatic gene-silencing assembly first came from deletion mutants of RNAi components. Indeed, deletions of Argonaute (*Ago1*), Dicer (*Dcr1*), or RNA-dependent RNA polymerase (*Rdp1*) genes impair epigenetic silencing at centromeres and the initiation of heterochromatin assembly at the mat locus, resulting in a loss of H3K9 methylation and Swi6 localization from these loci [34–36]. These findings were somehow puzzling since RNAi requires transcription while heterochromatin assembly results in TGS, as shown by silenced reporter genes. Nonetheless, small RNAs [20–22 nt) sharing homology with repeats present in the pericentric region could be detected [37]. Soon after, it was demonstrated that the RNA Pol II subunit Rpb7, contrary to other Pol II subunits, promotes pre-siRNA transcription of the so-called aberrant RNA required for RNAi-directed chromatin silencing [38,39]. Schematic representation of RNAi silencing and heterochromatin assembly in *S. pombe* is given in Figure 13.1B. The proposed model postulates that RNAi-mediated heterochromatin assembly in fission yeast appears to require initial nucleation sites that are then used as platforms to spread, but this spreading is *cis*-restricted. Boundary elements, such as that of inverted repeat (IR) of the mating-type region, prevent heterochromatin from invading the neighboring euchromatic regions [40]. This *cis* restriction is under the control of the ribonuclease Eri1, presumably by local degradation of excess siRNA [41].

To date, despite a good understanding of the involvement of the RNAi pathway in heterochromatin assembly, how histone-modifying activities, such as methylation and deacetylation, are localized in the first place remains to be determined. Addressing this question will help to understand the partition at a whole genome scale of heterochromatic regions versus euchromatic regions.

PTGS IN PROTISTS

Among protozoa and algae, PTGS has been demonstrated to be functional in alveolata (ciliates), discicristata (trypanosomes [42] and possibly *Leishmania* [43]), and unicellular green algae [44]. In many instances, the discovery of PTGS processes has led to their utilization in gene knockdown [45–47], with little study on the molecular modalities of gene silencing, some exceptions being *Trypanosoma brucei* [48,49] and *Chlamydomonas reinhardtii* [44,50–52]. PTGS pathways have been most extensively studied in ciliates in which, as in the worm *C. elegans*, silencing of gene expression can be obtained after either (i) transformation

of the somatic nucleus with transgenes critically lacking a 3′ untranslated region leading to the production of dsRNA (23–24-nt siRNA) and subsequent degradation of homologous mRNA [53]; (ii) direct injection of dsRNA; or (iii) even feeding with bacteria expressing ciliate homologous dsRNA [54]. In *Tetrahymena thermophila*, production of the 23–24-nt siRNA has been shown to be dependent upon Dicer Dcr2 and the RNA-dependent RNA polymerase Rdr1 [55], as canonical RNAi pathways are. However, it is from studies focused on sexual development of this organism and *Paramecium tetraurelia* that a fascinating genome editing system, mediated by a second and distinct small RNA pathway, has been discovered.

RNA MEDIATED DEVELOPMENT IN CILIATES

P. tetraurelia is a unicellular eukaryote that contains two functionally distinct nuclei, namely germline micronuclei and somatic macronuclei. The diploid germline micronuclei, which undergo meiosis, are transcriptionally inactive during vegetative growth, whereas the highly polyploid somatic macronuclei (∼800 n) are responsible for gene expression all along the life cycle, but are lost after fertilization. The mating process of *P. tetraurelia* is also very peculiar. Indeed, right after meiosis, three of the four haploid nuclei degenerate. In each conjugating partner, the remaining nucleus is then duplicated through a mitotic division. This duplication allows a reciprocal exchange of haploid nuclei between the mating paramecia. Once karyogamy has occurred, the resulting zygotes present a diploid micronucleus and deliquescent macronuclei. Therefore, brand new zygotic macronuclei have to be built up. This is achieved, after two micronucleus divisions, by massive endoreplication and extensive rearrangements of two of the four nuclei, the ones that lie at the posterior side of the cell. Chromosomes are heavily fragmented into shorter molecules capped by *de novo* telomere addition [56], but the most striking feature of those rearrangements is the precise excision of tens of thousands of single-copy short non-coding internal sequences (IESs) [57,58], which makes the macronuclei an expurgated version of the micronuclei. How can such an astonishing editing effort be performed?

First hints of an epigenetic compound implicated in that genome-wide rearrangement process came from transformation experiments on *P. tetraurelia* [59–61]. When an IES sequence is integrated into vegetative macronuclei, excision of the corresponding IES in the new macronuclei of sexual progeny is specifically inhibited [62]. The IES retention, which makes it present in all macromolecular copies is then maternally (cytoplasmically) inherited in the following sexual generations. This was clearly reminiscent of an epigenetic homology-based mechanism. Later on, in *T. thermophila*, developmental rearrangements were shown to depend on the *TWI1* gene, which encodes a protein homologous to Piwi-like proteins [63], on the *DCL1* gene, encoding a Dicer-like protein [64] and on Ema1p a putative RNA helicase [65]. In *P. tetraurelia*, identification of the Nowa1 and Nowa2, two RNA binding proteins required to remove the IESs from the developing macronuclei [66], further indicated that the cross-talk between nuclei at work during genome rearrangements is related to an RNAi pathway. Thus, unlike the canonical RNAi pathway, this second homology-dependent silencing system is restricted to sexual development, precisely when germline DNA rearrangements take place. It produces a specific class of 25-nt siRNA, called "scan RNAs" (scnRNAs) [67,68]. Microinjection of a 25-nt synthetic RNA duplex mimicking the structure of scnRNAs was shown to actually promote excision of the homologous IESs in the developing zygotic macronuclei [69]. Furthermore, in *Paramecium*, non-protein-coding transcripts (ncRNAs) produced from the somatic maternal macronucleus (devoid of IESs) are essential for IES excision in the developing zygotic macronucleus [69]. From this set of data, a whole "genome-scanning" model [70,71] has been proposed (Fig. 13.1C). According to this model, the epigenetic developmental program resulting in massive but precise DNA elimination would be based on a genomic subtraction between deletion-inducing scnRNAs and protective non-coding transcripts.

Studying sexual development in *P. tetraurelia* and *T. thermophila* has brought a lot to epigenetic fields, especially by giving intriguing new insights of how diverse homology-dependent mechanisms can be. The recruitment of the PTGS machinery in ciliates to help shape a new somatic genome free of selfish DNA elements is reminiscent of the roles attributed to PTGS in protecting the filamentous fungus genomes and in defining genomic heterochromatin territories of *S. pombe*. In other protists, such as in *T. brucei*, it was shown that transposons are reactivated in PTGS deficient mutants, confirming a role of PTGS in defending the genome against expression, and possibly expansion, of junk DNA [72].

TRANSCRIPTIONAL GENE SILENCING

Chromatin and chromatin-based gene regulation is present in many eukaryotes [73]. Again, a phylogenetic survey of chromatin proteins show that they are widely conserved [74], an argument in favor of an ancient origin of chromatin-based gene silencing. Yet, some eukaryotes have lost all chromatin, arguing that, like PTGS mechanisms, TGS pathways are not mandatory for survival. The best known of these organisms lacking typical chromatin are dinoflagellates. Indeed, these highly successful protists are considered to be one of the three major constituents of the phytoplankton. They have no nucleosomes [75] and have huge genomes condensed in the liquid crystal state [76,77]. For eukaryotic organisms that have lost canonical histones, this liquid crystal state of DNA may be the only option for retaining the necessary chromosomal compactness with segregation capability.

TGS modulates gene expression for various purposes, including antigen variability, mating type switching, protection against transposons and, possibly, development. As with PTGS mechanisms, fungi have greatly contributed to elucidating TGS mechanisms. Due to lack of space we are not able to discuss gene extinction in *S. cerevisiae*, where TGS is known to regulate silencing at mating-type cassettes, variegation in expression of telomere-located genes and recombination at the rDNA repeats. Importantly, *S. cerevisiae* lacks the HP1 protein, involved in the other eukaryotes in packaging heterochromatin. The production of heterochromatin in this yeast relies on a different set of proteins. Readers interested in *S. cerevisiae* TGS can refer to recent reviews [78–81].

We will discuss two TGS mechanisms of filamentous ascomycetes (Pezizomycotina), *A. immersus* and *N. crassa*. Although the *N. crassa* RIP process [82] was discovered before the Methylation Induced Premeiotically (MIP) process [83] of *A. immersus*, the latter will be dealt with first as it is truly a TGS system, but it is important to note that much of what was discovered about MIP was aided by the prior discovery of RIP. *N. crassa* and *A. immersus* are haploid during their vegetative growth phase. But when two haploid strains of compatible mating type encounter each other, sexual reproduction takes place. This first results in the formation of a transient dikaryotic cell. This feature, where two haploid nuclei are brought together, after mating, during an extended period within the same cell, is unique to higher fungi, the Dikaryomycota. Once karyogamy occurs a diploid cell is formed that undergoes meiosis immediately, which is then followed by post-meiotic mitosis generating asci with eight haploid ascospores. TGS in both *A. immersus* and *N. crassa* has been detected in the progeny after meiosis and affects genes present in two copies or more, in the same nucleus, during the dikaryotic phase.

METHYLATION INDUCED PREMEIOTICALY IN *A. immersus*

DNA methylation is a common epigenetic modification that can be detected in eubacteria, protists, fungi, plants, and animals. In eukaryotes, DNA methylation is restricted to the cytosine residues, either to any cytosine residues in plant and fungal genomes, or cytosine located within CpG dinucleotides in genomes of animals. As do cumented in other chapters of this book, DNA methylation has a strong impact on gene expression. Namely, in association with chromatin remodeling factors, it acts as a switch that can reversibly turn ON and OFF gene transcription. Methylation as a regulator of gene expression has been especially

well studied in *A. immersus*. In this fungus, genes present in more than one copy, the so-called repeats obtained after integrative transformation, frequently lost their expression after the first round of sexual reproduction [83–85]. In the 1990s, Rossignol and his co-workers were able to demonstrate that this spontaneous inactivation, clearly triggered by repeats, was not due to mutations but rather to epimutations, since systematic DNA sequencing showed no mutation in the inactivated strains [86]. The observed silencing of gene expression was faithfully maintained throughout numerous mitotic and meiotic divisions, even if the repeats had segregated away from each other, but was proved to be reversible under selective pressure. With no exception, the silenced repeated genes were found heavily methylated. Most of their cytosine residues were modified. Furthermore, in all cases, the methylation pattern was strictly co-extensive with the length of the duplication. Since the repeats have to be present in the same haploid nucleus for the silencing to occur (a single copy present in the other nucleus was not inactivated), it was inferred that this inactivation process takes place in the dikaryotic nuclei, in a period between fertilization and karyogamy during which the two haploid nuclei involved in the cross are both present in the same cell but have not yet fused.

Tandem repeats as short as 400 pb, and ectopic duplications of 600 pb in length, can be efficiently targeted by MIP [87]. In addition to *de novo* methylation of the cytosine residues within the MIPed alleles, silencing was accompanied by either the absence of transcripts or the presence of truncated transcripts [88]. This was indicative of a TGS type of silencing mechanism. Sequencing and mapping of truncated transcripts made it clear that, once initiated, transcription can progress up to the boundary of the adjacent duplicated and methylated region, but reaching this point, the transcription elongation stops abruptly, leading to the production of unusual shorten transcripts. Thus, even though TGS is a conserved process among eukaryotes, effects on transcription are quite different between fungi on one hand, and plants and animals on the other hand. In plants and mammals, methylation of promoter regions correlates with lack of transcription initiation. In *A. immersus*, methylation of promoters does not prevent initiation of transcription, but methylation in the body of a duplicated gene inhibits transcription elongation from both copies. To date, no explanation has been found to account for this discrepancy. The chromatin states of the MIPed alleles was investigated [89]. Partial micrococcal nuclease digestion evidenced that the sensitive sites present along the unmethylated regions are no longer observed along the MIPed ones. Hence MIP is able to change the chromatin compaction of its genomic targets. Again, the extent of methylation and chromatin remodeling are alike. What role DNA methylation plays in these changes remains to be determined. In addition, these chromatin changes are associated with an increase in dimethylation on H3K9, and a decrease in dimethylation on H3K4 [89]. Contrary to the case with other organisms that display TGS, such as plants, no decrease in acetylation of histones H4 was observed. Is this why transcription initiation in *A. immersus* seems independent of the chromatin states and methylation status of the promoters? Or is it because promoters are not as well defined in filamentous fungi as in plants and animals?

Because it was so easy to get portions of DNA methylated through MIP, transfer of methylation between alleles was investigated in the *A. immersus* genome. This transfer was shown to be as frequent and polarized as gene conversion is [90]. This was a first indication that methylation transfer and recombination might be mechanistically related. A second clue came when crossing-over frequency was measured between two markers flanking an *A. immersus* spore color gene [91]. When the two homologs were methylated, the crossing-over frequency was reduced several hundredfold. This demonstrates that DNA methylation strongly inhibits homologous recombination. This also supports, on experimental bases, the hypothesis that methylation prevents homologous recombination between dispersed DNA repeats and therefore contributes to genome integrity.

The only MIP mutant that has been characterized is impaired in a gene, *masc1*, encoding a protein that bears all motifs of the catalytic domain of eukaryotic C5-DNA-methyltransferases

(DMT) [92]. However, despite its canonical DMT structure, no enzymatic activity was ever detected in standard *in vitro* assays. Although methylation was fully maintained on previously MIPed alleles, the *masc1* mutation prevents the *de novo* methylation of newly formed DNA repeats through MIP. Interestingly, crosses involving *masc1* mutant strains of the compatible mating types were arrested at an early stage of sexual reproduction and therefore barren. This indicates that the Masc1 protein, in addition to being required for the MIP process, plays a crucial role in sexual development. Curiously, DmtA, the Masc1 ortholog of *A. nidulans,* a fungus thought to have no DNA methylation and no TGS system, is also essential for early sexual development [93]. Is there a class of DMT-like proteins involved in early steps of fungal sexual reproduction? Is MIP a mechanism that evolved to protect the spreading of repeats across the *A. immersus* genome, in order not to have to deal with unpaired DNA during meiosis, as meiotic silencing does in *N. crassa*? To date, these questions remain to be addressed.

REPEAT INDUCED POINT MUTATION IN *N. crassa*

N. crassa, in addition to quelling and meiotic silencing, also displays a TGS-related mechanism, RIP. It was first discovered by Selker and collaborators in 1987 [82]. Like MIP, this premeiotic silencing process takes place at the dikaryotic stage of the sexual cycle. DNA repeats longer than 400 pb [94] that share a nucleotide identity greater than 80% are irreversibly mutagenized via C:G to T:A transitions. As an outcome of RIP, the *Neurospora* genome reveals a complete absence of intact mobile elements [26,95] and natural repeats display an AT-rich content. Interestingly, while the bulk of the *N. crassa* genome is unmethylated, RIPed repeats are heavily methylated. Furthermore it was shown that these AT-rich regions are by themselves a positive signal that promotes DNA methylation [96–98]. Whether DNA methylation is installed before the mutagenesis as the first step of RIP or only after the cross, in vegetative cells, is still not elucidated. Nonetheless, DNA methylation is associated with most of the sequences affected by RIP, and methylated cytosines are not limited to CpG dinucleotides [99]. If the RIPed sequences encompass genes, their expression is silenced, due to a strong reduction in transcription [100]. Run-on experiments have demonstrated that transcripts are initiated, even from methylated promoters but that elongation is blocked when the RNA polymerase II stalls in methylated regions lying in the body of the RIPed genes. However, DNA methylation alone is not sufficient to block transcription, which strongly suggests that others factors, likely linked to chromatin remodeling, might turn the RIPed region into silent heterochromatin. Altogether, these features define a two component system. Before meiosis, RIP introduces true mutations in the *N. crassa* genome and is therefore non-reversible. Reversibility is a property exhibited by most of the proper epigenetic phenomena, MIP included. But during vegetative life, DNA and H3K9 methylation [12], two genuine epigenetic modifications, maintain the transcriptional silencing of the RIPed alleles.

Mechanistically, majors questions remain to be answered [101]. One of them is how repeats identify each other. Since none or all the copies of repeated DNA are RIPed, the idea that this silencing mechanism can involve a DNA–DNA pairing step has been proposed. Moreover, the fact that RIP cannot be transmitted from one nucleus to the other in the dikaryotic cells suggests that it may not work through a diffusible signal [102]. Indeed, the *N. crassa qde* mutants impaired in the RNAi machinery – see the earlier text on "quelling" – can establish and maintain DNA or H3K9 methylation very well [103]. Thus, it is very unlikely that RNA intermediates can participate in the RIP homologously-based gene expression silencing. Another crucial question is how RIP mutations occur. It has been proposed that methylated cytosines are prone to be spontaneously deaminated at high frequency which would result in a cytosine to thymidine conversion. Alternatively, a DNA-cytidine deaminase might directly perform the conversion [101]. But so far, no experimental clue has arisen to confirm any of these hypotheses.

TABLE 13.1 Repeat Induced Point Mutation (RIP) in Fungi

Organism	Evidence	Reference
Neurospora crassa	Experimental	[82,101]
Podospora anserina	Experimental	[131,132]
Leptospheria maculans	Experimental	[110,133]
Magnaporthe grisea	Experimental	[134,135]
Magnaporthe oryzae	*In silico*	[136]
Aspergillus fumigatus	*In silico*	[137]
Aspergillus nidulans	*In silico*	[138]
Aspergillus niger	*In silico*	[139]
Fusarium oxysporum	*In silico*	[140–142]
Fusarium graminearum	Experimental	[143]
Nectria haematococca	Experimental	[144]
Microbotryum violaceum	*In silico*	[145]
Penicillium chrysogenum	*In silico*	[139]
Stagonospora nodorum	*In silico*	[146]

Experimental: functional RIP has been evidenced by experimental methods. *In silico* signatures: genomic sequences show typical C:G to T:A transitions, mostly by sequencing DNA repeats such as transposons; there is no experimental proof of functional RIP.

To date, only one RIP defective mutant has been characterized, whereas several mutations that impair DNA methylation with no effect on RIP are known [12,103–106]. Mutation in the *rid-1* gene encoding a putative DNA methyltransferase protein results in fertile but RIP defective strains [107]. As for *Ascobolus* Masc1 protein, *in vitro* assays did not reveal any DNA methyltransferase activity. Again, function of this DMT-like protein remains mysterious.

RIP/TGS IN OTHER FILAMENTOUS FUNGI

RIP has also been observed in numerous filamentous ascomycetes (Table 13.1). Although a common feature, this silencing system appears less efficient in other fungi than in *N. crassa* and has still no clear physiological role besides its impact upon genomic plasticity. Indeed, on one hand, RIP counters selfish DNA and therefore protect genomes from expansion of junk DNA, but on the other hand, it has significant cost on genome evolution by preventing the appearance of paralogs, as illustrated by the *N. crassa* genome, where creation of new genes through duplication is almost impossible [26]. By contrast, in *P. anserina*, where RIP is weak, numerous segmental duplications are detected [108]. The fact that large genes may duplicate is not contradictory to the presence of RIP, since, when moderately efficient, it can accelerate gene divergence as described for the het-D/E family [109]. Interestingly, in a field population of *L. maculans*, multiple independent RIP events were shown to be responsible for evolution of the AvrLm6 locus toward virulence, within a single season [110].

CHROMATIN-BASED REGULATION OF SECONDARY METABOLITE GENE CLUSTER EXPRESSION

Aspergilli are fungi of particular importance both as pathogens (human and plants) and as industrial organisms used in a wide range of productions. Synthesis of an amazing number of secondary metabolites, some of economic value, others poisonous, is one of the most remarkable properties of these fungi. The genes encoding secondary metabolites are generally grouped into clusters. It is difficult to monitor the production of these compounds since some clusters may be silenced [111]. Deletion of the *A. nidulans laeA* gene encoding an O-methyltransferase blocks the expression of the sterigmatocystin, penicillin, and lovastatin gene clusters [112,113]. Conversely, overexpression of *laeA* leads to increased penicillin and lovastatin gene transcription [112]. Recently, mutants of *A. nidulans* impaired for hdaA [114], a histone deacetylase, and CclA [115] involved in H3K9 methylation showed activation of

several otherwise cryptic secondary metabolite clusters. These results led to the hypothesis that epigenetic mechanisms based on histone modifications might be crucial regulators for secondary metabolite clusters and provide a framework to attempt to control their expression.

TGS IN PROTISTS

Data on TGS in protozoa and algae are scarce. Some are available for the green algae *C. reinhardtii* [52,116,117]. However, most of them come from studies on *Plasmodium falciparum*, the malaria parasite (an apicomplexan), and on *T. brucei*, the agent of sleeping sickness (a discicristatan). A common fascinating property of these evolutionary very divergent intracellular parasites is their ability to perform antigenic variation. The multigenic VAR family of *P. falciparum* and VSG family of *T. brucei* both encode glycoproteins that coat the surface of the cells. The VSG genes and the VAR genes are localized at subtelomeric loci [118]. Only one gene of the family is expressed at a time. Moreover, using a periodic switch of the expressed gene, parasites can alter their antigenic signature and thus escape the immune system of the host [119]. Antigenic variation is the main reason that makes malaria or sleeping sickness chronic diseases. But to establish such an unusual mono-allelic expression, the parasites must dispose of a mechanism that tightly regulates *in situ* the switching and the mutually exclusive transcription of the VAR and VSG genes. Among others, epigenetic regulation has been postulated [120]. Notably, while the available apicomplexa genomes [121] show very few DNA-binding factors, it seems that numerous non-coding RNA are expressed in these parasites [122]. Another uncommon feature is that the VSG and VAR families are transcribed by Pol I, a polymerase exclusively involved in ribosomal DNA transcription in other eukaryotes.

In *T. brucei*, RNAi mediated knock-down of *ISWI*, a gene encoding a chromatin remodeling factor, results in derepression of the silenced VSG genes [123]. In the same organism, deletion of *DOT1B*, a gene encoding an enzyme responsible for trimethylation of H3K76, also leads to tenfold derepression of silent VSG genes [124]. But the link between TGS-based telomeric silencing and VSG regulation of expression is not so straightforward. Indeed, mutants impaired in the gene encoding SIR2rp1, a sirtuin, show activation of Pol I reporter constructs, but not of the endogenous VSG genes [125].

In *P. falciparum*, activation and silencing of VAR genes correlate with specific histone tail marks: H3K9 acetylation and H3K4 methylation have been shown to be associated with VAR gene activation [126], whereas tri-methylation of H3K9 is associated with VAR gene silencing [127]. Conversely to *T. brucei*, *P. falciparum* homologs of the histone deacetylase Sir2 are involved in the regulation of antigenic variation, in both mutual exclusion and silencing [128–130]. Further characterization of TGS pathways in such parasites might provide therapeutic prospects.

CONCLUSION

Although still patchy, the available data concerning gene silencing show that, in many eukaryotic microbes, both PTGS and TGS occur with modalities similar to those described in animals and plants. However, differences may occur as exemplified by the complete loss of the PTGS machinery in some fungi, the lack of HP1 in *S. cerevisiae*, and the lack of true chromatin in dinoflagellates. Gene silencing is involved in a variety of unrelated physiological processes in the form of clonal regulation of gene expression (antigen variation in parasites), genome defense (RIP and MIP), and genome structuration (macronuclei formation in ciliates and PTGS in *S. pombe*). Interestingly, the PTGS phenomenon of meiotic silencing could participate in the formation of species by an original mechanism. We expect that the exploration of these mechanisms both in well-tracked models and in more exotic species is likely to provide further original modalities and roles for both TGS and PTGS.

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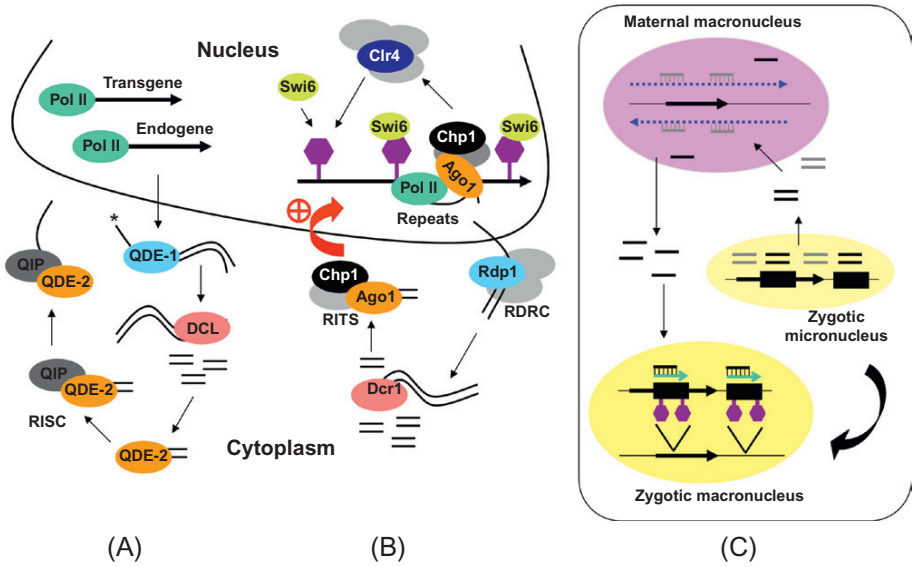

FIGURE 13.1

Models of RNAi in various eukaryotic microbes. (A) Quelling in *N. crassa*. Aberrant RNAs (*) are produced at loci that present repeats in large tandem arrays. Features of these aberrant RNAs are unknown, but they must be recognized by the RdRP QDE-1 and then convert into double stranded RNA molecules (dsRNA)[147]. dsRNA molecules are the typical substrate of the Dicer-like proteins DCL-1 and DCL-2 that chop them into siRNAs of 21–25 nucleotides. These siRNAs are integrated into the RISC complex, along with the Argonaute QDE-2 protein. They are then processed by the QIP nuclease and used as specific guides to target homologous mRNAs, which, once trapped, are most likely degraded by QDE-2. (B) RNAi silencing in *S. pombe*. The nascent transcript model proposes that RNA pol II continuously generates non-coding transcripts (*) from reverse promoter of heterochromatic repeats [36]. These aberrant RNAs are first cleaved by Ago1 and then recruited by the RNA-directed RNA polymerase complex (RDRC) to be converted into dsRNA by Rdp1 [148]. Using these dsRNAs as substrate, Dcr1 produces siRNA, which then bind to RNA-induced transcriptional silencing (RITS) complex, by means of Ago1 [149,150]. While RISC complexes target and degrade cytoplasmic mRNA, the RITS complex is tethered to chromatin through protein-protein interactions established between the chromodomain protein Chip1 and the H3K9me nucleosomes [35] (hexagons). The close association of the RITS complex and chromatin allows base-paring interactions between siRNA loaded on Ago1 and the nascent non-coding transcript soon to be cleaved by this protein. This amplification step of siRNA is likely to form a positive-feedback loop (plus arrow), which is believed to ensure the heterochromatin inheritance through cell divisions. As long as siRNA from a specific genomic region are produced, they continuously target the Clr4 histone methyltransferase complex (CLRC) to nucleosomes [151,152]. Thus, using H3K9me as signposts, heterochromatin spreads to large genomic territories in a sequence-independent but Swi6-dependent manner. As a result, transcription of the forward strand is silenced as in classical TGS systems. Gray ovals: known additional effectors. (C) Genome-scanning model in *Paramecium*. Because the micronucleus genome is unrearranged (rectangles represent IESs), it produces both IES-homologous (black) and non-IES-homologous (gray) scnRNAs. These diffusible molecules would enter and scan the IES-free maternal macronucleus. As a result of pairing with the maternal ncRNAs (dotted arrows), the non-IES-homologous scnRNAs would be sequestered. The remaining pool of scnRNA, highly enriched with IES-homologous scnRNAs, would be free to reach the developing zygotic macronucleus and pair with the nascent transcripts. At the IES targeted loci, chromatin shows H3K9 methylation [153] (hexagons), suggesting that this excision mechanism might have a TGS component. As for *S. pombe*, chromatin modifications could be used as signposts to direct an endonuclease towards the IESs to be excised. The curved arrow indicates that zygotic micronuclei develop into zygotic macronuclei throughout the course of the sexual phase. (Please refer to Chapter 13, page 187).