Neurospora crassa, a Model System for Epigenetics Research

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SUMMARY

The filamentous fungus *Neurospora crassa* has provided a rich source of knowledge on epigenetic phenomena that would have been difficult or impossible to gain from other systems. *Neurospora* sports features found in higher eukaryotes but absent in both budding and fission yeast, including DNA methylation and H3K27 methylation, and also has distinct RNA interference (RNAi)-based silencing mechanisms operating in mitotic and meiotic cells. This has provided an unexpected wealth of information on gene silencing systems. One silencing mechanism, named repeat-induced point mutation (RIP), has both epigenetic and genetic aspects and provided the first example of a homology-based genome defense system. A second silencing mechanism, named quelling, is an RNAi-based mechanism that results in silencing of transgenes and their native homologs. A third, named meiotic silencing, is also RNAi-based but is distinct from quelling in its time of action, targets, and apparent purpose.

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OVERVIEW

Fungi provide excellent models for understanding the structure and function of chromatin both in actively transcribed regions (euchromatin) and in transcriptionally silent regions (heterochromatin). The budding yeast, *Saccharomyces cerevisiae*, has been an invaluable eukaryotic model for studying chromatin structure associated with transcription at euchromatic regions and providing a paradigm for silent chromatin (Grunstein and Gasser 2013). The fission yeast, *Schizosaccharomyces pombe*, has some epigenetic machinery that is absent from *S. cerevisiae* but common in higher organisms—most notably for RNA interference (RNAi) and for methylation of lysine 9 of histone H3 (H3K9me). As described in Allshire and Ekwall 2014, research using *S. pombe* has provided invaluable information on the structure and function of heterochromatin, principally found in regions of the centromeres, telomeres, and silent mating-type genes. This article focuses on a third model system, namely the filamentous fungus *Neurospora crassa*. Although not as commonly studied as the yeasts, *Neurospora* has proved to be a remarkably rich source of knowledge that would have been difficult or impossible to

gain from other systems. *Neurospora* sports features found in higher eukaryotes, including DNA methylation and the H3K27 methylation (“Polycomb”) system that both budding and fission yeasts lack, as well as RNAi and other epigenetic processes found in the yeasts. This has provided an unexpected wealth of information on gene silencing systems, some of which operate at distinct stages of its life cycle. The first such mechanism, named repeat-induced point mutation (RIP), has both epigenetic and genetic aspects and provided the first example of a homology-based genome defense system. The second, named quelling, is an RNAi-based mechanism that results in silencing of transgenes and their native homologs. The third, named meiotic silencing (or meiotic silencing by unpaired DNA), is also RNAi-based but is distinct from quelling in its time of action, targets, and apparent purpose. Although we are still in the early days of epigenetic studies in all organisms, it is already clear that yeasts and filamentous fungi such as *N. crassa* will continue to serve as rich sources of information on epigenetic mechanisms operative in a broad range of eukaryotes.

1 NEUROSPORA CRASSA: HISTORY AND FEATURES OF THE ORGANISM

The filamentous fungus *N. crassa* (see Figs. 1 and 2) was first developed as an experimental organism by Dodge in the late 1920s and about 10 years later was adopted by Beadle and Tatum for their famous “one gene–one protein” studies linking biochemistry and genetics (Davis and de Serres 1970). Beadle and Tatum selected *Neurospora*, in part, because this organism grows fast and is easy to propagate on defined growth media, and because genetic manipulations, such as mutagenesis, complementation tests, and mapping are simple. Although not as widely studied as some other model eukaryotes, *Neurospora* continues to attract researchers because of its moderate complexity and because it is well suited for a variety of genetic, biochemical, developmental, and subcellular studies (Borkovich et al. 2004). *Neurospora* has been especially useful for studies of photobiology, circadian rhythms, population biology, morphogenesis, mitochondrial import, DNA repair and recombination, DNA methylation, and other epigenetic processes (Borkovich et al. 2004).

N. crassa is commonly observed growing on burned wood after a forest fire (Fig. 1A). It comes in two mating types (*A* and *a*), which are morphologically indistinguishable from each other (Fig. 1B). The vegetative phase is initiated when either a sexual spore (ascospore) or an asexual spore (conidium) germinates, giving rise to multinucleate cells that form branched filaments (hyphae; Fig. 1C). In the wild, the heat of the fire provides the activation required for ascospore germination (Figs. 1D and 2). In contrast,

Figure 1. Images of *Neurospora crassa*. (A) Vegetative growth in the wild on sugarcane (photo by D. Jacobson, Stanford University). (B) Slants of vegetative cultures of *N. crassa* in the laboratory (photo by N.B. Raju, Stanford University). (C) Hyphae of *N. crassa* stained with 4′6-diamidino-2-phenylindole (DAPI) to show abundant nuclei (photo by M. Springer, Stanford University). (D) A rosette of maturing asci showing ascospore patterns (photo by N.B. Raju; reprinted, with permission, from Raju 1980, © Elsevier).

conidial cells germinate spontaneously. The hyphal system spreads out rapidly (linear growth >5 mm/h at 37°C) to form a “mycelium.” After the mycelium is well established, aerial hyphae (“conidiophores”) develop, leading to the production of the abundant orange conidia that are characteristic of the organism (Figs. 1A,B and 2). The conidia, which contain one to several nuclei each, can either establish new vegetative cultures or fertilize strains of the opposite mating type. If nutrients are limiting, *N. crassa* activates its sexual phase by producing nascent fruiting bodies (“protoperithecia”). When a specialized hypha (“trichogyne”) projecting from the protoperithecium contacts tissue of the opposite mating type, a heterokaryon can form and an acquired “male” nucleus is transported back to the protoperithecium. Strains of either mating type can act as “females” or “males.” The process of fertilization transforms a protoperithecium into a young perithecium. The sexual phase of *N. crassa* and other filamentous ascomycetes differs from that of yeasts in that the filamentous fungi have a prolonged heterokaryotic phase between fertilization and karyogamy (nuclear fusion). The heterokaryotic cells resulting from fertilization proliferate in the developing perithecium, which contains a mixture of both ascogenous (heterokaryotic) and maternal (homokaryotic) tissues. Development of ascogenous tissue involves a transition to strictly dikaryotic (binucleate) cells containing one nucleus of each mating type, which undergo synchronous nuclear divisions culminating in formation of the hook-shaped cells called “croziers” (Fig. 2). Croziers develop into three cells. Karyogamy, meiosis, and postmeiotic mitosis take place in the middle cell, also known as the ascus mother cell. It is noteworthy that the diploid nucleus formed by karyogamy immediately enters into meiosis. Thus, the diploid phase of the life cycle is brief (~ 24 h) and limited to a single developing cell. The eight nuclei that result from the first postmeiotic mitosis are compartmentalized, resulting in an ascus cell that contains eight haploid spores (“ascospores”) arrayed in an order that reflects their lineage (Raju 1980, 1992). Ascospores are ejected from the beak of the perithecium and can germinate after exposure to high temperature to produce vegetative mycelia, completing the sexual cycle. One perithecium may contain up to 200 developing asci. Meiotic segregation and recombination can be studied in *Neurospora* by analyzing individual asci (“tetrads”) or random spores ejected from numerous asci (Perkins 1966, 1988; Davis and de Serres 1970). Genetic analyses have indicated that, in general, all the asci of a perithecium derive from a single maternal nucleus and a single paternal nucleus.

The ~ 40 -megabase *N. crassa* genome consists of seven chromosomes with approximately 10,000 predicted protein coding genes (Galagan et al. 2003), and a total genetic map

Figure 2. Life cycle of *N. crassa*. Half of the sexual spores (ascospores) are mating type A (red) and half are mating type a (blue). Sexual spores (ascospores) and vegetative spores (conidia) germinate and form mycelia, from which asexual fruiting bodies (conidiophores) emerge. Conidiophores form conidia, which are typically multinucleate. In response to nitrogen starvation, mycelia of either mating type form specialized female structures called protoperithecia. Vegetative tissue (e.g., a conidium) of the opposite mating type serves as the “male” to fertilize and initiate development of fruiting bodies (perithecia). After fertilization, male- and female-derived nuclei coexist in the same cytoplasm, where they undergo mitoses and eventually become organized into a dikaryotic tissue in which each cell has one nucleus of each mating type. The nuclei then pair and undergo a series of synchronous mitoses until the tip of the hyphal cell in which they reside bends to form a hook-shaped cell called a crozier. Fusion of haploid nuclei is immediately followed by meiosis and a mitotic division such that one crozier gives rise to one ascus containing eight ascospores. The approximate stages in which the epigenetic processes described in the text occur are indicated.

length of roughly 1000 map units (Perkins et al. 2001). Only ~9% of the genome consists of repetitive DNA and, aside from a tandem array of approximately 170 copies of the ~10-kb recombinant DNA (rDNA) unit encoding the three large ribosomal RNAs, most of the repetitive DNA consists of inactivated transposable elements. That most strains of *N. crassa* lack active transposons and have very few close paralogs almost certainly reflect the operation of RIP, the first homology-dependent genome defense system discovered in eukaryotes (Selker 1990a,b). We now know that *Neurospora* has at least three gene silencing processes that act to conserve the structure of the genome: RIP, quelling, and meiotic silencing (Borkovich et al. 2004). All of these processes have epigenetic aspects and have direct or indirect connections with DNA methylation, a basic epigenetic mechanism found in *Neurospora* and many other eukaryotes. We will discuss DNA methylation and then RIP, quelling, and meiotic silencing.

2 DNA METHYLATION IN NEUROSPORA

Since its discovery decades ago, DNA methylation in eukaryotes has remained remarkably enigmatic. Basic questions are still debated, such as “What determines which chromosomal regions are methylated?” and “What is the function of DNA methylation?” *Neurospora* revealed itself to be an excellent system to study the control and func-

tion of DNA methylation. Some model eukaryotes, including the nematode *Caenorhabditis elegans* and the yeasts *S. cerevisiae* and *S. pombe*, lack detectable DNA methylation and isolated reports of DNA methylation in another model organism, *Drosophila melanogaster*, remain controversial. In some organisms such as mammals, DNA methylation is essential for viability, complicating certain analyses.

In *N. crassa* DNA, ~1.5% of the cytosines are methylated, but this methylation is dispensable, facilitating genetic studies. Although one must be cautious when extrapolating from one system to another, at least some aspects of DNA methylation appear conserved. For example, all known DNA methyltransferases (DMTs), the enzymes that methylate cytosine residues, including those from both prokaryotes and eukaryotes, show striking homology in their catalytic domains (Goll and Bestor 2005). Findings from *Neurospora*, *Arabidopsis*, mice, and other systems in the last decade have revealed important similarities and interesting differences in the control and function of DNA methylation, demonstrating the value of performing investigations in multiple model systems.

Discovery of DNA methylation in *Neurospora* initially attracted interest because it was not limited to symmetrical sites, such as CpG dinucleotides or CpNpG trinucleotides (see articles by Li and Zhang 2014; Pikaard and Mittelsten Scheid 2014). Riggs, and Holliday and Pugh had proposed

an attractive model for the “inheritance” or “maintenance” of methylation patterns that relied on the symmetrical nature of methylated sites observed in animals (see Fig. 2 in Li and Zhang 2014). Although results of a variety of in vitro and in vivo studies have supported the “maintenance methylase” model, mechanisms for maintenance methylation that do not rely on faithful copying at symmetrical sites can be imagined and may be operative in a variety of organisms (e.g., Selker 1990b; Selker et al. 2002). The possibility that the observed methylation at asymmetric sites represented “de novo methylation” was exciting because mechanisms that blindly propagate methylation patterns can complicate the determination of which sequences are methylated in the first place. Indeed, results of DNA-mediated transformation and methylation inhibitor studies with *Neurospora* showed reproducible de novo methylation (e.g., Singer et al. 1995). More recently, genomic experiments with methylation mutants revealed widespread and rapid de novo methylation after genetic reintroduction of the wild-type allele corresponding to the defective gene (Lewis et al. 2009). Additional studies defined, in part, the underlying signals for de novo methylation (e.g., see Tamaru and Selker 2003).

The first methylated patch characterized in detail was the 1.6-kb ζ – η (zeta–eta) region, which consists of a diverged tandem duplication of a 0.8-kb segment of DNA, including a 5S rRNA gene (Selker and Stevens 1985). Comparison of this region with the corresponding chromosomal region from strains lacking the duplication initially led to the idea that repeated sequences can somehow induce DNA methylation, and ultimately led to the discovery of the genome defense system named RIP (Fig. 3) (Selker 1990b). Elucidation of RIP revealed that repeated sequences do not directly trigger DNA methylation, at least in *Neurospora*; instead, repeats trigger RIP, which is closely tied to DNA methylation, as described below. Both the ζ – η region and the $\psi 63$ (psi-63) region, the second methylated region discovered in *Neurospora*, are products of RIP. Moreover, subsequent genome-wide analyses of DNA methylation revealed that nearly all methylated regions in *Neurospora* are relics of transposons inactivated by RIP (Selker et al. 2003; Galagan and Selker 2004). Indeed, the only DNA methylation in *Neurospora* that may not have resulted from RIP is at the tandemly arranged rDNA genes (Perkins et al. 1986).

3 RIP, A GENOME DEFENSE SYSTEM WITH BOTH GENETIC AND EPIGENETIC ASPECTS

RIP was discovered as a result of a detailed analysis of progeny from crosses of *Neurospora* transformants (Selker 1990b). It was noticed that duplicated sequences, whether

Figure 3. Repeat-induced point mutation (RIP). For clarity, only two chromosomes are illustrated. The open box represents a gene or chromosomal segment that was duplicated in one strain (*top, right*). Duplications are subject to RIP (symbolized by lightning bolt) between fertilization and karyogamy. Results of genetic experiments revealed that duplications can be repeatedly subjected to volleys of C to T transitions (symbolized by filled boxes) during this period of approximately 10 mitoses, right up to the final premeiotic DNA synthesis (Selker et al. 1987; Watters et al. 1999). The four possible combinations of chromosomes in progeny are indicated. Pink “Me” represents DNA methylation, which is frequently (although not always) associated with products of RIP.

native or foreign, and whether genetically linked or unlinked, were subjected to numerous polarized transition mutations (G:C to A:T) in the haploid genomes of the special heterokaryotic cells resulting from fertilization. When the stability of a gene was tested when it was unique in the genome or else combined with an unlinked homolog, it was found that RIP is not simply repeat-associated; it is truly repeat-induced. In a single passage through the sexual cycle, up to ~30% of the G:C pairs in duplicated sequences can be mutated. Frequently (but not invariably), the sequences that are altered by RIP become methylated de novo. It is likely that the mutations arising from RIP occur by enzymatic deamination of 5-methylcytosines (5mC) or by deamination of Cs followed by DNA replication (Selker 1990b). This was postulated partly because cytosine methylation involves a

reaction intermediate that is prone to spontaneous deamination, suggesting that the putative deamination step of RIP might be catalyzed by a DMT or DMT-like enzyme. Consistent with this possibility, one of the two DMT homologs predicted from the *Neurospora* genome sequence—RID (RIP defective)—is involved in RIP (Freitag et al. 2002). Progeny from homozygous crosses of *rid* mutants show no new instances of RIP. *Rid* mutants have no noticeable defects in DNA methylation, fertility, growth, or development. In contrast, the second *Neurospora* DMT homolog (DIM-2), is necessary for all known DNA methylation, but is not required for RIP (Kouzminova and Selker 2001).

All indications are that every sizable duplication (>~400 bp for tandem duplication or ~1000 bp for unlinked duplication) is subject to RIP in some fraction of the special heterokaryotic ascogenous cells. Nevertheless, typically <1% of tandem duplications and ~50% of unlinked duplications escape RIP. Even duplications of chromosomal segments containing numerous genes are sensitive to RIP (Perkins et al. 1997; Bhat and Kasbekar 2001). Although RIP is limited to the sexual phase of the life cycle, the existence of this process raised the question of whether *Neurospora* can use gene duplications to evolve. The genome sequence revealed gene families, but tellingly, virtually all paralogs were found to be sufficiently divergent that they should not trigger RIP (Galagan and Selker 2004). Thus, RIP may indeed limit evolution through gene duplication in *Neurospora*. Interestingly, some fungi, such as *Ascobolus immersus*, show what appear to be milder genome defense systems that are similar to RIP. The most notable example is MIP (methylation induced premeiotically), a process that detects linked and unlinked sequence duplications during the period between fertilization and karyogamy, like RIP, but which relies exclusively on DNA methylation for inactivation; no evidence of mutations has been found in sequences inactivated by MIP (Rossignol and Faugeron 1994).

4 STUDIES OF RELICS OF RIP PROVIDED INSIGHTS INTO THE CONTROL OF DNA METHYLATION

4.1 Noncanonical Maintenance Methylation

The finding that a single DMT, DIM-2, is responsible for all detected DNA methylation was surprising. No previously identified DMT was known to methylate cytosines in a variety of sequence contexts. An obvious but important question was: Does methylation at nonsymmetrical sites necessarily reflect the potential of the corresponding sequences to induce methylation de novo? Early transformation experiments were consistent with this possibility;

methyated sequences that were stripped of their methylation (e.g., by cloning) regained their normal methylation when reintroduced into vegetative cells. A surprise came when eight alleles of the *am* gene that were generated by RIP were tested for their capacity to induce methylation de novo (Singer et al. 1995). Consistent results were obtained from experiments performed in two ways: (1) Sequences were scored for remethylation after being stripped of methylation by treatment with a demethylating agent (5-azacytidine), and (2) unmethylated sequences introduced by transformation were scored for methylation de novo. Some products of RIP with relatively few mutations (Fig. 4; *am*^{RIP3} and *am*^{RIP4}) did not become remethylated, even at their normal locus, suggesting that the observed methylation represented propagation of methylation established earlier. Importantly, their methylation, like other observed methylation in *Neurospora*, was not limited to symmetrical sites, did not significantly spread with time, and was “heterogeneous” in the sense that the pattern of methylated residues was not invariant within a clonal population of cells. Thus this methylation, although dependent on preexisting methylation established in the sexual phase (perhaps by RIP), could not reflect the action of a “maintenance methylase” of the type envisioned in the original model for inheritance of methylation patterns.

The capacity of *Neurospora* to perform “maintenance methylation” was confirmed experimentally (Selker et al. 2002). Interestingly, propagation of methylation was found to be sequence-specific (i.e., it did not work on all sequences), adding a new dimension to the maintenance methylation concept. It is noteworthy that MIP in *Ascobolus* also provided evidence for propagation of DNA methylation in

Figure 4. Mutations from RIP and methylation status of eight different *am* alleles (adapted from Singer et al. 1995). Vertical bars indicate mutations. Alleles shown in black were not methylated. Alleles in blue were initially methylated, but after loss of methylation induced by 5-azacytidine, or by cloning and gene replacement, did not become remethylated. Alleles shown in red were not only initially methylated, but also triggered methylation de novo.

fungi (Rossignol and Faugeron 1994). Although a number of potential schemes that would result in propagation of DNA methylation can be imagined, the actual mechanism operative in *Neurospora* remains unknown. In principle, maintenance of methylation at nonsymmetrical sites could depend on methylation of nearby symmetrical sites, but the observed heterogeneous methylation, including at CpG sites, renders this possibility unlikely. Feedback mechanisms involving proteins associated with the methylated DNA could result in methylation that depends on pre-existing methylation (i.e., maintenance methylation). As discussed below, findings from *Neurospora* (and other organisms) implicate histone modifications in the control of DNA methylation, raising the possibility that histones play a role in the maintenance of DNA methylation.

4.2 Involvement of Histones in DNA Methylation

The first indication of a role of histones in DNA methylation came from the observation that blocking histone deacetylation in *Neurospora* reduced DNA methylation in some chromosomal regions (Selker 1998). This was performed by treatment with the histone deacetylase inhibitor trichostatin A (TSA). The selectivity of demethylation by TSA could reflect differential access to histone acetyltransferases (Smith et al. 2010), but has not been thoroughly investigated. Studies of the *dim-5* gene in *Neurospora* unambiguously tied chromatin to the control of DNA methylation. A *dim-5* mutant, like *dim-2* strains, shows a complete loss of DNA methylation, yet it is a SET domain protein that acts as a histone H3 lysine methyltransferase (HKMT), specifically trimethylating lysine 9 (Tamaru and Selker 2001; Tamaru et al. 2003). Confirmation that histone H3 is the physiologically relevant substrate of DIM-5 came from two demonstrations: (1) Replacement of lysine 9 in H3 with other amino acids caused loss of DNA methylation, and (2) trimethyl-lysine 9 (H3K9me3) was found specifically at DNA methylated chromosomal regions.

The discovery that histone methylation controls DNA methylation, at least in *Neurospora*, led to two important questions: (1) What instructs DIM-5 which nucleosomes to methylate? (2) What reads the trimethyl mark and transmits this information to the DMT, DIM-2? It has been easier to answer the second question than the first, in part because of information from other systems. In particular, knowledge that HP1, a protein first identified in *Drosophila*, binds H3K9me3 in vitro (discussed in Elgin and Reuter 2013), motivated a search for an HP1 homolog in *Neurospora*. A likely homolog was found and its involvement in DNA methylation was tested by gene disruption (Freitag et al. 2004a). The gene, named *hpo* (HP one), was indeed essential for DNA methylation. As another test of whether

Neurospora HP1 reads the mark generated by DIM-5, its subcellular localization was examined in wild type and *dim-5* strains. In wild type, HP1-GFP localized to heterochromatic foci, but this localization was lost in *dim-5*, confirming that *Neurospora* HP1 is recruited by the H3K9me3 mark generated by DIM-5. A yeast two-hybrid screen, and subsequent coimmunoprecipitation experiments, revealed that the chromoshadow domain of HP1 interacts directly with DIM-2 through PXVXL-related motifs in its amino-terminal region (Honda and Selker 2008).

The question of how DIM-5 is controlled has proved more difficult and is not yet fully answered. A combination of genetic, biochemical, and proteomic approaches has yielded insights, however. Results of such studies have culminated in the discovery that the localization and action of DIM-5 depends on a multiprotein complex, DCDC (DIM-5/-7/-9, CUL4/DDB1 complex; diagrammed in Fig. 5), which resembles an E3 ubiquitin ligase (Lewis et al. 2010a; Lewis et al. 2010b). Although all five core members of DCDC are essential for methylation of H3K9 and DNA, only DIM-7 is required to bring DIM-5 to heterochromatic regions. Interestingly, the *S. pombe* H3K9 MTase (Clr4) responsible for heterochromatin formation is in a similar complex, CLRC, comprised of Clr4, Cul4, Rik1, Raf1, Raf2, and Rad24 (Jia et al. 2005; Li et al. 2005; Thon et al. 2005), although CLRC and DCDC have significant structural and functional differences. No ubiquitination substrate of CLRC or DCDC has yet been identified, despite intensive efforts in multiple laboratories, raising the possibility that these H3K9 MTase complexes do not function as ubiquitin ligases in vivo.

DCDC may be controlled by one or more proteins that recognize products of RIP. It is noteworthy that most methylated sequences in the *Neurospora* genome are relics of RIP; most relics of RIP are methylated (Selker et al. 2003) and sequences resembling products of RIP are potent triggers of DNA methylation (Tamaru and Selker 2003). Indeed, analyses of the genomic distribution of 5mC, histone H3K9me3, HP1, and sequences showing evidence of RIP (high A + T content with unexpectedly high densities of TpA dinucleotides) in *Neurospora* revealed that these are all tightly correlated (Fig. 6). Extensive tests on synthetic and natural sequences that trigger DNA methylation led to the suggestion that an unidentified “A:T-hook”-type protein may mediate DNA methylation in *Neurospora*. Consistent with this idea, Distamycin A, an analog of the A:T-hook motif, interferes with de novo methylation in *Neurospora* (Tamaru and Selker 2003).

It is interesting to consider the possible implications of controlling DNA methylation through histones. First, the fact that DNA methylation patterns are relatively stable (i.e., they do not normally spread or shift significantly

implies that the underlying histones, and the H3K9me3 mark, are similarly stable. Second, it raises the possibility that other histone modifications may play regulatory roles. Indeed, *in vitro* studies showed that DIM-5 is inhibited by phosphorylation of H3S10 and methylation of H3K4 (Adhvaryu et al. 2011). Thus, DIM-5 can integrate information relevant to whether DNA in a particular region should be methylated. This provides a possible explanation for the observation that TSA can inhibit DNA methylation in certain regions (Selker 1998).

Evidence that RNAi is important for heterochromatin formation and maintenance in *S. pombe* raised the question of whether the RNAi machinery of *Neurospora* is involved in HP1 localization and/or DNA methylation. *Neurospora* has homologs of a variety of genes implicated in RNAi (Galagan et al. 2003; Borkovich et al. 2004). Studies of mutants with null mutations in all three RNA-dependent RNA polymerase (RdRP) genes, in both Dicer genes, or in other presumptive RNAi genes revealed no evidence that RNAi is involved in methylation of H3K9, heterochromatin formation, or DNA methylation in *Neurospora* (Chicas et al. 2004; Freitag et al. 2004a; Lewis et al. 2009). However, as discussed in Sections 6 and 7, the *Neurospora* RNAi genes are involved in at least two other silencing mechanisms with epigenetic aspects, quelling, and meiotic silencing.

4.3 Modulators of DNA Methylation

An expectation is that DNA methylation would be subject to regulation. As mentioned above, there is already evidence from *Neurospora* that the HKMT underlying DNA methylation (DIM-5) is sensitive to histone modifications. It would not be surprising to find that other elements of the methylation machinery, such as HP1 and the DMT (DIM-2), would also be sensitive to histone modifications, and that DNA methylation would be subject to regulation in other ways. Indeed, we know that the extent of DNA methylation depends on environmental variables such as temperature and the composition of growth medium (Roberts and Selker 1995). Forward and reverse genetic studies have also identified proteins that modulate DNA methylation patterns. A notable example is DNA methylation modulator-1 (DMM-1) and its partner protein, DMM-2 (Honda et al. 2010). Mutants lacking either of these proteins show aberrant methylation of DNA and histone H3K9, with both epigenetic marks frequently spreading into genes adjacent to transposable elements. *Dmm-1* mutants grow poorly but growth can be restored by reduction or elimination of DNA methylation using the drug 5-azacytosine or mutation of the DNA methyltransferase gene, *dim-2*. The observation that *dmm-1*, *dim-2* double mutants

Figure 5. Basic components of the DNA methylation machine of *Neurospora*. Chromatin associated with DNA substantially mutated by RIP (orange spiral decorated with pink mC moieties) is subjected to methylation of H3K9 by the histone methyltransferase DIM-5, whose localization and action depends on a multiprotein complex, DCDC (DIM-5/-7/-9, CUL4/DDB1 complex; Lewis et al. 2010a,b). The CUL4 subunit of the DCDC complex is associated with the small protein Nedd (N), which resembles the E3 ubiquitin ligase complex. Trimethylated H3K9 (K9me3) is recognized by HP1, which is involved in at least three heterochromatin-associated complexes: (1) It recruits the DNA methyltransferase DIM-2 (Honda and Selker 2008); (2) it is required for localization and function of the HCHC silencing complex, which contains HP1, the chromodomain protein CDP-2, the histone deacetylase HDA-1, and a CDP-2/HDA-1-associated protein, CHAP (Honda et al. 2012); and (3) it is required to guide the DMM complex, which serves to block the spreading of heterochromatin into neighboring transcribed regions (Honda et al. 2010).

Figure 6. Epigenomic features of *N. crassa* genome. The genomic distributions of H3K9me3 (orange), HP1 (yellow), 5-methylcytosine (green), H3K27me3 (medium blue), and H3K4me3 (dark blue) are displayed for each of *N. crassa*'s seven linkage groups (OR74A NC10 sequence assembly, <http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html>) using the Integrative Genomics Viewer (<http://www.broadinstitute.org/igv>) (Jamieson et al. 2013; MR Rountree and EU Selker, unpubl.). Base composition is shown at the top of each linkage group as the moving average of %GC (red) calculated for 500-bp windows in 100-bp steps, whereas the positions of predicted genes (purple) and repeats (black) are indicated below. The predicted gene file was downloaded from The Broad Institute (<http://www.broadinstitute.org/annotation/genome/neurospora>) and repeats were determined using the RepeatMasker program (<http://www.repeatmasker.org>).

display normal H3K9me3 patterns implies that the spread of H3K9me3 involves DNA methylation. The DMM complex is preferentially localized to edges of methylated regions in an HP1-dependent manner, as cartooned in Fig. 5. A conserved residue within the JmjC domain of DMM-1 is essential for its function, raising the possibility that the complex functions as a histone demethylase.

Other proteins also affect the distribution of DNA methylation in *Neurospora*. For example, mutation of any of the genes encoding components of another HP1 protein complex, HCHC (HP1, CDP-2, HDA-1, and CHAP) cause hyperacetylation of centromeric histones, loss of centromeric silencing, increased accessibility of DIM-2 to centromere regions, and hypermethylation of the associated DNA. Interestingly, loss of HCHC also causes mislocalization of the DIM-5 H3K9 methyltransferase at a subset of interstitial methylated regions, leading to selective DNA hypomethylation (Honda et al. 2012). Figure 5 illustrates our current understanding of key known elements of the *Neurospora* DNA methylation/heterochromatin machinery.

5 HISTONE H3K27 METHYLATION

Trimethylated lysine 27 on histone H3 (H3K27me3) is present in metazoans such as *Arabidopsis*, *Drosophila*, and mammals, in which it is known to be involved with gene repression. H3K27me3 is absent from yeasts that have been examined, but is present in *Neurospora* (Smith et al. 2008), rendering the organism an attractive model to study basic aspects of this histone modification. H3K27me3 occupies ~7% of the *N. crassa* genome and is segregated into approximately 230 domains that are particularly enriched near telomeres, but are also found dispersed in the genome (Fig. 5). Approximately 700 predicted genes are covered by H3K27me3, all of which are normally silent. *Neurospora* possesses homologs of the four core components of the Polycomb repressive complex 2 (PRC2) but lacks clear homologs of members of the PRC1 complex found in *Drosophila*, mammals, and plants. Three of the PRC2 core components are required for H3K27 methylation, whereas the fourth, NPF (*Neurospora* homolog of *Drosophila* P55 and mammalian RbApP46/RbApP48), is not absolutely required for H3K27me3. Nevertheless, NPF is critical for H3K27me3 regionally, particularly in telomeric and subtelomeric domains (Jamieson et al. 2013). Loss of H3K27me3, caused by deletion of PRC2 genes, results in upregulation of a small subset of genes in both H3K27me3 and non-H3K27me3 regions. The emergence of *Neurospora* as a model system to explore the control and function of H3K27me3 promises to provide insights into this fascinating, but still largely mysterious, epigenetic mechanism.

Important open questions include: What governs the distribution of H3K27me3? Do sequence elements akin to PREs (Polycomb response elements) in *Drosophila* regulate this epigenetic mark in *Neurospora* and other systems? To what extent are RNAs involved in H3K27me3 regulation? How much of the observed H3K27me3 reflects epigenetic “inheritance” and what is the actual mechanism of this process? What is the detailed function of H3K27me3 in gene silencing?

6 QUELLING

Soon after transformation techniques were established for *Neurospora*, researchers in several laboratories noticed that a sizable fraction (~30%) of *Neurospora* transformants showed silencing of transforming DNA, and more surprisingly, silencing of native sequences homologous to those of the transforming DNA. The latter form of vegetative phase silencing was named “quelling” by the Macino laboratory, which performed most of the pioneering research on this phenomenon (Pickford et al. 2002). Quelling is most apparent with visible markers such as the *albino* genes, which encode enzymes required for carotenoid biosynthesis (Fig. 7), and is comparable to “cosuppression” or PTGS (posttranscriptional gene silencing) in plants (Pikaard and Mittelsten Scheid 2014). Interestingly, genes seem to vary in their sensitivity to quelling. For genes that are sensitive, quelling seems most common in transformants bearing multiple copies of transforming DNA in a tight array. Nuclei flow freely in hyphae of *Neurospora*, allowing for “heterokaryosis” in which genetically distinct nuclei share a common cytoplasm. Thus, it was easy to show that quelling is “dominant”—that is, a transformed nucleus can silence homologous sequences in nearby nuclei (Cogoni et al. 1996).

The ability to silence nearby nuclei implicated a cytoplasmic silencing factor, which we now know is RNA, or small RNAs, to be more precise. The roles of small RNAs in gene regulation, germ cell maintenance, and transposon silencing are widespread and are active areas of research. In *Neurospora*, the products of the *qde-1*, *qde-2*, and *qde-3* genes encode, respectively, an RNA/DNA-dependent RNA polymerase, an “Argonaute”-like protein, and a RecQ-like DNA helicase. Together, they have been implicated in the production of a new class of small RNAs called Qde-2-associated RNAs (or qiRNAs) (Lee et al. 2009). Unlike other *Neurospora* siRNAs, which are ~25 nucleotides long, qiRNAs are ~20–21 nucleotides long and have a strong preference for uridine at the 5′ end. In addition, they have been reported to originate mostly from the ribosomal DNA locus in response to DNA damage (Lee et al. 2009). The investigators hypothesized that the observed burst of

Figure 7. Quelling. For simplicity, only two of the seven chromosomes are diagrammed (straight line segments in gray circles representing nuclei). The native *albino* gene (*al*) is indicated by the dark orange rectangle on the top chromosome; rectangles on the lower chromosome (dark orange or yellow) represent ectopic *al* sequences introduced by transformation. Because transformed cells are often multinucleate, transformants are often heterokaryotic, as illustrated. Whether or not the transforming DNA includes the entire coding region, in some transformants it silences (“quells”) the native *al*⁺ gene in both transformed and nontransformed nuclei through an undefined *trans*-acting molecule (red lines emanating from the transforming DNA indicated by the yellow rectangle). This results in poorly pigmented or albino (*Al*[−]) tissue in some transformants, as shown.

rDNA-related qiRNAs inhibits protein translation as a response to DNA damage. MicroRNA-like RNAs (miRNAs) and Dicer-independent small interfering RNAs (disiRNAs) have also been reported (Lee et al. 2010b). miRNAs are produced by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE-2, the exonuclease QIP, and an RNase III-like domain-containing protein, MRPL3. In contrast, disiRNAs do not require the known RNAi components as they originate from loci producing overlapping sense and antisense transcripts (Lee et al. 2010b). Notably, it was observed that the product of *qde-1*, QDE-1, has DNA- and RNA-dependent biochemical activities (Lee et al. 2010a). QDE-1 seems to play a central role generating the aberrant RNA required for RNAi. In vitro, QDE-1 produces dsRNA from ssDNA, a process that is strongly promoted by replication protein A (RPA). In vivo, this interaction probably occurs during DNA replication, as suggested by the observed interaction

of QDE-1 with RPA and DNA helicases (Lee et al. 2010a). Importantly, although DNA methylation is frequently associated with transforming DNA, neither the DNA methyltransferase, DIM-2, nor the H3K9 methyltransferase, DIM-5, are required for quelling (Cogoni et al. 1996; Chicas et al. 2005).

Neurospora is proving to be a rich system for the study of the genesis and characterization of diverse pathways involved in the generation of small RNAs, thus shedding light on the diversity and evolutionary origins of eukaryotic small RNAs.

7 MEIOTIC SILENCING

The most recent addition to the list of known silencing mechanisms is meiotic silencing, which was originally called “meiotic transvection” and later referred to as “meiotic silencing by unpaired DNA” (MSUD; Aramayo and Metzenberg 1996; Aramayo et al. 1996; Shiu et al. 2001; Kelly and Aramayo 2007). As implied by its name, meiotic silencing operates only in meiosis, in which it evaluates the identity of the homologous chromosomes in two stages. First, regions located at equivalent locations on homologous chromosomes are evaluated by a process called *trans*-sensing. Second, regions identified as nonhomologous are silenced by a mechanism related to RNAi.

The discovery of meiotic *trans*-sensing and silencing was the result of a thorough characterization of an *Ascospore maturation-1* (*Asm-1*) deletion mutant of *Neurospora* generated by gene replacement (Aramayo et al. 1996). *ASM-1* has a putative DNA binding domain, consistent with it being a transcription factor required for the expression of genes involved in ascospore maturation. The deletion mutant was unable to form aerial hyphae, and protoperithecia (see Fig. 2). Deletion strains carrying ectopically integrated DNA copies of the gene were normal. The ectopic copy could complement the vegetative defects. Interestingly, it could not rescue defects in the sexual phase. In contrast, strains carrying frameshift alleles of the gene (*asm-1*^{fs}) had the same mutant phenotype in vegetative cultures as those carrying the *Asm-1*^Δ deletion but showed different properties in sexual development (Fig. 8). Crosses between strains carrying functional (*asm-1*⁺) and nonfunctional (*asm-1*^{fs}) alleles resulted in 4:4 segregation of mature and immature spores (Fig. 8; compare Panel A *asm-1*⁺ × *asm-1*⁺, with Panel B, *asm-1*⁺ × *asm-1*^{fs}), suggesting that the product of *asm-1*⁺ plays a critical role in ascospore development/maturation and indicating that the *asm-1*^{fs} allele is recessive. Surprisingly, crosses heterozygous for a deletion allele of *Asm-1* (Fig. 8C; *asm-1*⁺ × *Asm-1*^Δ), produced only white (inviable) spores, i.e., all spores within the ascus failed to develop, including the ones carrying the *asm-1*⁺

allele. This ascus-dominance of the *Asm-1 Δ* deletion allele contrasted with its recessive behavior in vegetative tissue.

One explanation for the observed dominance of the deletion allele was that a single functional allele of the gene was insufficient to produce adequate product in the diploid and/or meiotic cells. The possibility of such “haploinsufficiency” was tested by crossing wild-type strains with deletion strains carrying ectopic functional copies of the gene, that is, $asm-1^+ \times Asm-1^{\Delta}$, $asm-1^+$ (Fig. 8D). The fully functional ectopic gene failed to correct the spore maturation defects (Aramayo and Metzenberg 1996). It was conceivable that the ectopic $asm-1^+$ failed to rescue the defect because of an unknown requirement for interactions between alleles at homologous chromosomal positions, reminiscent of the transvection phenomenon described in *D. melanogaster* (Wu and Morris 1999). This hypothesis was tested by crossing strains both carrying copies of the gene located at ectopic positions in an *Asm-1 Δ* background, i.e., $Asm-1^{\Delta}$, $asm-1^+$ (ectopic) × $Asm-1^{\Delta}$, $asm-1^+$ (ectopic), (Fig. 8E). Indeed, the two ectopic alleles rescued the ascus-dominant defect of *Asm-1* deletion alleles, supporting the idea that some form of *trans*-sensing was occurring, involving pairing.

To distinguish between the possibility that meiotic silencing is due to absence of pairing or due to an unpaired allele, crosses in which the meiotic nucleus had three copies of a gene: two wild-type alleles (which should pair) and an ectopic copy (which should be unpaired) were analyzed. Silencing was observed, implying that meiotic silencing results from the presence of unpaired alleles rather than from absence of paired ones [Fig. 8F; $asm-1^+ \times asm-1^+$, $asm-1^+$ (ectopic)] (Shiu et al. 2001; Kutil et al. 2003; Lee et al. 2003; Lee et al. 2004).

Figure 8. Discovery and characterization of meiotic silencing. Key genetic experiments are illustrated using the *Ascospore maturation-1* (*Asm-1*) gene, as a reporter. For each cross, the relevant genotype of the haploid parents of mating type A (red boxes) or mating type a (blue boxes) is shown on the left, and cartoons showing the predicted chromosome pairing in the diploid cell (violet boxes) is shown on the right. The phenotypes of resulting asci are presented on the far right. Black represents mature (typically viable) ascospores and white represents immature (inviable) ascospores. (A) Wild-type cross. (B) 4:4 segregation of ascospores from a heterozygous cross of wild type and a frameshift mutant in which alleles can pair and no meiotic silencing occurs. (C) Cross of strains with wild-type and deletion alleles triggers meiotic silencing. (D) Meiotic silencing is not rescued by ectopic wild-type allele, indicating that the developmental defect is not due to haploinsufficiency. (E) Allelic (pairable) ectopic copies $asm-1^+$ in crossing partners rescue *Asm-1 Δ* defect. (F) Presence of an unpaired allele triggers silencing of all $asm-1^+$ alleles (paired and unpaired) in meiosis. (G) Silencing of the suppressor of ascospore dominance (*Sad-1*), because of a *Sad-1* deletion in one parent, suppresses meiotic silencing.

A hunt for mutants defective in meiotic silencing resulted in the identification of a telling member of the silencing machinery. The *Sad-1* gene, encoding an RdRP was identified by selection for mutants that were able to pass through a cross in which *Asm-1* is not paired [Fig. 8G; *Sad-1*^Δ, *asm-1*⁺ × *sad-1*⁺, *Asm-1*^Δ]. This suggested that meiotic silencing is related to quelling in *Neurospora*, and to RNAi, generally. Further screens for meiotic silencing factors identified two RNAi-related genes in addition to *Sad-1*: an Argonaute-like protein, *suppressor of meiotic silencing-2* (*Sms-2*; Lee et al. 2003); and a Dicer-like protein, *suppressor of meiotic silencing-3* (*Dcl-1/Sms-3*; Alexander et al. 2008). In addition, the involvement of a putative helicase, *Sad-3*, has been reported (Hammond et al. 2011). Several other *Sms* mutants have also been identified (DW Lee and R Aramayo, pers. comm.). Although all of the genes shown to be required for meiotic silencing in *Neurospora* are also required for fertility, strains carrying loss-of-function mutations in the meiotic silencing pathway do not have a discernible vegetative phenotype, and as noted above, do not affect heterochromatin formation and DNA methylation. This contrasts with the situation in the fission yeast *S. pombe*, in which orthologs of *Sad-1*, *Sms-2*, and *Dcl-1/Sms-3* (Rdp1, Ago1, and Dcr1, respectively) are essential for normal chromosome biology, including heterochromatin formation (e.g., histone H3K9 methylation), and normal centromere and telomere functions (Martienssen et al. 2005).

The PTGS nature of meiotic silencing was confirmed using transgene reporters. Only regions containing homology with the reporter transcript result in silencing when unpaired (Lee et al. 2004). Intriguingly, all reported components of the meiotic silencing machinery identified so far have a perinuclear localization (Fig. 9) (Shiu et al. 2006). Our working model (Fig. 9) for the mechanism of meiotic silencing postulates two steps: (1) “sensing,” in which pairing of homologous chromosomes reveals unpaired DNA, which then gives rise to an aberrant RNA (aRNA); and (2) “processing” of the aberrant RNA by perinuclear RNA silencing machinery (*SAD-1*, *SAD-2*, *SMS-2*, *DCL-1/SMS-3*, etc.). Silencing presumably results from degradation of normal RNAs targeted by siRNAs, generated in the cascade initiated by the aberrant RNA.

Exactly what constitutes “unpaired” DNA is an area of active investigation. Some of the quantitative and qualitative aspects of the “sensing” threshold have been addressed, however (Lee et al. 2004). The findings can be summarized as follows: (1) Given one small and one large loop of unpaired DNA, both carrying the same length of DNA homologous to a set of paired reporter genes, the large loop will silence more efficiently than the smaller one; (2) given two loops of identical size, but one carrying twice as much

Figure 9. A model for meiotic silencing. An image of a developing ascus from a cross between parents engineered to contain paired copies of *sad-1*⁺ fused to a reporter gene *gfp*⁺ (i.e., *sad-1*⁺::*gfp*⁺) at the Pachytene stage of meiosis I (left; DW Lee and R Aramayo, unpubl.). Inside this cell, the meiotic nucleus, delineated by its nuclear membrane, is surrounded by a perinuclear structure that supports the attachment of components of the meiotic silencing apparatus. Predicted nuclear and perinuclear steps in meiotic silencing are diagrammed (right). It is hypothesized that *trans*-sensing, a mechanism preceding silencing, identifies heterologous regions of interacting chromosomes. The degree of heterology determines the strength of the induction step, which presumably involves the synthesis of aRNA and its conversion to double-stranded RNA (dsRNA) by the *SAD-1* RdRP, a perinuclear event. The presence of dsRNA triggers the initiation of the silencing process, which involves the conversion of the dsRNA trigger into siRNAs via the *DCL-1/SMS-3* Dicer (initiation step), and use of these siRNAs primers and normal RNAs as templates, by *SAD-1* RdRP to generate dsRNA (amplification cycle). The incorporation of the siRNAs, generated by both the initiation step and the amplification cycles, into the RNA-inducing silencing complex (RISC) directs the endonucleolytic cleavage of mRNA or ssRNA (single-stranded RNA).

DNA homologous to a set of paired reporter genes, the loop carrying more homologous DNA will silence more efficiently than the one carrying less homologous DNA; (3) the silencing signal produced by an unpaired loop is confined to the unpaired region and does not “spread” to neighboring regions (e.g., paired reporter genes can be located next to a region of unpaired DNA without being significantly perturbed); (4) the canonical promoter of a gene need not be present in the loop of unpaired DNA for a gene to be silenced; and (5) meiotic silencing does not affect the ability of a promoter to direct transcription at a later developmental time (Kutil et al. 2003; Lee et al. 2003, 2004; Pratt et al. 2004).

In general, our understanding of homology sensing mechanisms—even those giving rise to homologous

recombination (ectopic and standard) and RIP, as well as those behind meiotic silencing—is incomplete. Although details of the mechanism that detects unpaired DNA in meiotic silencing remain to be discovered, interesting features of this sensing mechanism have come to light. For example, it was found that quasi-homologous sequences (i.e., homeologous sequences), like those produced by RIP, can induce meiotic silencing. The degree of identity that is required to escape meiotic silencing was assessed by crossing strains carrying wild-type alleles of *Rsp* (*Round spore*) with strains carrying various alleles generated by RIP that differed in their density of mutations (Pratt et al. 2004). Some alleles (e.g., *RspRIP93*) conferred a dominant phenotype, comparable to that shown by a deletion of *Rsp*. As little as 6% sequence divergence (94% identity) could trigger silencing; 3% divergence (97% identity) did not. Interestingly, the methylation status of the RIP-mutated alleles shifted the sequence identity threshold—that is, methylated alleles triggered silencing more effectively than when methylation was prevented by a mutation in the DNA methyltransferase gene, *dim-2* (Pratt et al. 2004). This observation provided the first evidence that DIM-2 is functional in the sexual phase of *Neurospora* and suggested that either 5mCs contribute to heterology as a “fifth base,” and/or that an indirect effect of DNA methylation (e.g., recruitment of a methylated DNA binding protein or an unknown effect on chromatin structure) impacts homology recognition.

In retrospect, it is not surprising that meiotic silencing escaped detection for many years of genetic studies with *Neurospora*. To be detected, a reporter gene must fulfill a series of strict requirements. Most unpaired genes (i.e., those whose gene product are involved in vegetative processes) would probably not impact meiotic and/or spore development. Thus, lack of pairing might only be evident for genes whose products are required for the completion of meiosis, the reestablishment of mitosis, cellularization, or maturation of ascospores and/or whose gene products are essential structural components of the ascus.

8 PROBABLE FUNCTIONS AND PRACTICAL USES OF RIP, QUELLING, AND MEIOTIC SILENCING

RIP seems custom-made to limit the expression of “selfish DNA” such as transposons that direct the production of copies of themselves in a genome. Consistent with this possibility, the vast majority of relics of RIP are recognizably similar to transposons known from other organisms, and most strains of *Neurospora* lack active transposons (Galagan et al. 2003; Selker et al. 2003; Galagan and Selker 2004). Nevertheless, because RIP is limited to the premeiotic dikaryotic cells, this process should neither prevent the spread

of a new (e.g., horizontally acquired) transposon in vegetative cells nor prevent the duplication of a single-copy transposon in meiotic cells. Quelling and meiotic silencing should deal with such eventualities, however. Although quelling does not completely suppress the spread of transposons in vegetative cells, as evidenced by the proliferation of an introduced copy of the LINE (long interspersed element)-like transposon, *Tad*, it does appear to partially silence such transposons (Nolan et al. 2005). Information about the action of meiotic silencing suggests that this process will silence any transposed sequence in meiotic cells, even if it is only present as a single copy in the genome (Shiu et al. 2001; Kelly and Aramayo 2007). In addition to dealing with errant transposons in meiosis, some of the genes involved in meiotic silencing also appear to play an important role in the process of speciation, as shown by the observation that mutants defective in meiotic silencing relieve the sterility of strains bearing large duplications of chromosome segments and allow closely related species to mate with *N. crassa* (Shiu et al. 2001).

Although RIP, quelling, and meiotic silencing can all be a nuisance for some genetic experiments, all have been exploited for research purposes. RIP provided the first simple method to knock out genes in *Neurospora* and is still the preferred method for generating partial-function mutants. Quelling has also been used to reduce, if not eliminate, gene function, much as RNAi is exploited in a variety of organisms. And meiotic silencing provides a simple assay to test whether particular genes are required to function in (or immediately after) meiosis; if a gene is found to cause sterility when duplicated, or when at an ectopic location, and the sterility is rescued by a mutation blocking meiotic silencing, it is safe to assume that it plays an important function in meiosis.

In addition to the postulated evolutionary roles of RIP, quelling, and meiotic silencing, and to their utility in the laboratory, it is worth considering the possibility that these processes serve in other ways. For example, the fact that *Sad-1* function is required for full fertility suggests that meiotic silencing is directly or indirectly required for meiosis (Shiu et al. 2001). Surprisingly, however, not all genes shown to be required for meiotic silencing in *Neurospora* are required for fertility (R Pratt, DW Lee, and R Aramayo, unpubl.), indicating that despite their temporal and spatial colocalization, the connection(s) and/or interdependencies between the meiotic silencing pathway and meiosis are still poorly understood. In the case of RIP, although this process is nonessential, the distribution of products of RIP, which are concentrated in the genetically mapped centromeres of *Neurospora* (Fig. 5), suggest that junked transposons can serve the organism as substrates for kinetochore formation, much as repeated sequences do in *S. pombe* and other

organisms. Indeed, *Neurospora* centromere sequences consist primarily of relics of transposons heavily mutated by RIP and the normal distribution of kinetochore proteins depends on DIM-5 and HP1 (Smith et al. 2011). Relics of RIP are also found adjacent to telomere sequences of *Neurospora* (Smith et al. 2008). Interestingly, transposons and relics of transposons are also commonly found in heterochromatic sequences of other organisms, such as *Drosophila*, mammals, plants, and other fungi.

9 CONCLUDING REMARKS

The fungus *N. crassa* has emerged as a powerful system to discover and elucidate epigenetic phenomena. Because epigenetics is still a young field and studies of epigenetic processes have for the most part arisen from discoveries stemming from a variety of research programs, it is not surprising that our breadth and depth of understanding of epigenetic processes in *Neurospora*, yeasts, and other systems vary substantially. It is too early to know how general the various epigenetic mechanisms are. Nevertheless, it is already clear that various model eukaryotes have both important differences and striking similarities. For example, whereas *Neurospora* sports DNA methylation and H3K27 methylation and *S. pombe* does not, both of these fungi show histone H3K9 methylation and RNAi processes, neither of which are found in the budding yeast, *S. cerevisiae*. It is also noteworthy that a given process may be functionally distinct in different organisms. For instance, in *Neurospora*, RNAi components have been implicated in quelling and meiotic silencing, but not heterochromatin formation, whereas in fission yeast, RNAi components have been implicated in heterochromatin formation but nothing more. Finally it is worth noting that even shared features, such as the association of heterochromatin with centromeres of both fission yeast and *Neurospora*, may have important differences. An important goal for the future is to discover the extent to which information gleaned from one organism is applicable to others. Continued exploitation of various model organisms, including *Neurospora*, should both provide this information and reveal features of epigenetic processes that are still unknown today. We anticipate that the richly diverse fungi will serve as useful systems for epigenetic research for many years.

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WWW RESOURCES

- <http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html> A website housed by the Broad Institute that serves as the official repository of the information generated by, and associated with, the *Neurospora crassa* genome
- <http://www.broadinstitute.org/igv> A website housed by the Broad Institute that serves as the official repository of the Integrative Genomics Viewer, a powerful Java-based high-performance visualization tool for interactive exploration of large, integrated genomic data sets
- <http://www.repeatmasker.org> A website housed by the Institute for Systems Biology that serves as the official repository for Repeat-Masker, an industry-standard program used for the screening of DNA sequences for interspersed repeats and low-complexity DNA sequences

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