REVIEW

DNA methylation and the formation of heterochromatin in *Neurospora crassa*

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Studies of the control and function of DNA methylation in *Neurospora crassa* have led to a greater understanding of heterochromatin formation. DNA methylation in *Neurospora* is dependent on trimethylation of histone H3 lysine 9 (H3K9me3) by the histone methyltransferase, DIM-5. The linkage between these two methyl marks is facilitated by heterochromatin protein 1 (HP1), which serves as an adapter protein. HP1 binds to the H3K9me3

and recruits the DNA methyltransferase, DIM-2. Although HP1 links H3K9me3 to DNA methylation, it also serves to recruit the DNA methylation modifier complex to the edges of heterochromatin regions, where it serves to limit the spreading of the heterochromatin by countering H3K9me3.

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Introduction

Constitutive heterochromatin, the densely staining fraction of the eukaryotic genome, has been historically regarded as an unimportant component of the genome. Compared with euchromatin, the fraction of the genome containing most of the genes, heterochromatin is regarded as a more 'closed' or 'condensed' chromatin state, which is refractory to DNA-based processes such as recombination and transcription. Recently, however, heterochromatin has attracted attention, in part because it has been implicated in human diseases, most notably cancer (Sharma *et al.*, 2010). Moreover, there is increasing evidence that heterochromatin has roles in normal cells, for example promoting genome stability by preventing illegitimate recombination between repeats, serving to silence invasive elements and participating in centromere function (Grewal and Jia, 2007; Peng and Karpen, 2008; Rountree and Selker, 2009). Thus, there is considerable interest in how regions of heterochromatin are formed and maintained. Recent research implicates an intricate interplay of covalent modifications of histones and a variety of nuclear proteins that are positively or negatively influenced by these modifications. Covalent modifications of histones include methylation, acetylation, phosphorylation, and/or ubiquitination of various residues. In addition to histone modifications, in many eukaryotes chromatin can be modified by methylation of cytosines in the DNA. Modifications of histones and DNA should provide a virtually limitless combination of chromatin modifications that could be used for regulatory control. Unfortunately, our understanding of these modifications and their interplay is still in its infancy.

The use of simple model organisms, such as yeasts, filamentous fungi, flies, and *Arabidopsis*, has greatly aided our understanding of chromatin modifications and will continue to be invaluable in deciphering the chromatin modification 'code'. However, some powerful model systems lack chromatin modifications that are present in higher eukaryotes, such as DNA methylation. *Neurospora crassa* is the simplest model organism to sport most of the chromatin modifications seen in higher eukaryotes and offers many advantages for their study. Some of the heterochromatin markers, such as DNA methylation, are not essential in *Neurospora*, facilitating dissection of the processes involved in the establishment and maintenance of heterochromatin.

DNA methylation and repeat-induced point mutation in *Neurospora*

Much of what has been learned about heterochromatin in *Neurospora* resulted from studies on DNA methylation. Approximately 1.5% of cytosines are methylated in the *Neurospora* genome (Selker and Stevens, 1987; Foss *et al.*, 1993). None of this DNA methylation has been found in protein-coding genes; rather, nearly all is found within transposon relics found in the centromere regions, near the telomeres and elsewhere. Essentially all of these sequences are relics of a genome defense system called repeat-induced point mutation (RIP) (Foss *et al.*, 1993; Selker *et al.*, 2003; Lewis *et al.*, 2009). RIP apparently protects the genome from the assault of invasive DNAs (transposons, retrotransposons, viruses, and so on) (Rountree and Selker, 2009). RIP is a potent genome defense system, leaving duplicated sequences both mutated and methylated. The effectiveness of RIP is illustrated by the fact that no recognizable transposon is found within the wild-type *Neurospora* genome (Galagan *et al.*, 2003). Early genetic studies showed that RIP senses duplicated sequences and subjects both sequences to numerous polarized transition mutations

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(G:C to A:T) in the stage of the life cycle between fertilization and nuclear fusion (Selker *et al.*, 1987; Selker and Garrett, 1988). Up to ~30% of the G:C pairs of duplicated sequences are mutated in a single passage through the sexual cycle (Cambareri *et al.*, 1989; Cambareri *et al.*, 1991). Although the mechanism of RIP remains to be elucidated, one of *Neurospora*'s two putative DNA methyltransferases (DMT), RIP defective (RID), is essential for the process (Table 1) (Freitag *et al.*, 2002). Sequences altered by RIP are typically methylated at the remaining cytosines and this methylation is not restricted to symmetrical sequences (for example, CpG), unlike the case of DNA methylation in most vertebrate cells (Selker and Stevens, 1985). Indeed, it appears that any cytosine residue in a methylated region can be modified and this methylation is typically dense but heterogeneous within a population of nuclei (Selker *et al.*, 1993). Importantly, the DNA methylation does not depend on the sequence duplication that originally triggered RIP and is relatively independent of chromosomal position (Selker, 2004).

Treating *Neurospora* with the histone deacetylase inhibitor, Trichostatin-A, affects DNA methylation (Selker, 1998). Although this observation was the first indication that DNA methylation is closely linked to histone modifications in this organism, genetic studies have been most important in defining a sequence of chromatin events that lead to DNA methylation. Two mutants *defective in DNA methylation* (*dim*), *dim-2* and *dim-5*, completely abolish DNA methylation in vegetative tissue were most informative (Foss *et al.*, 1993; Tamaru and Selker, 2001). Unlike DNA methylation patterns in animals and plants, which are established by multiple DMTs in different pathways, one DMT, encoded by the *dim-2* gene, is responsible for all DNA methylation found in vegetative tissue of *Neurospora* (Table 1) (Kouzminova and Selker, 2001). Although the role of *dim-2* in DNA methylation was obvious, the role of *dim-5*, which encodes a histone methyltransferase, was less so, but provided a concrete link between histone modification and DNA methylation (Table 1) (Tamaru and Selker, 2001). The identification of these and other *dim* mutants, coupled with proteomic, bioinformatics, and high-throughput approaches have served to unlock the pathway that leads to heterochromatin formation in *Neurospora*.

Characteristics of heterochromatin in *Neurospora*

Heterochromatin is typically enriched for hypoacetylated histones, methylated H3K9, heterochromatin protein 1

(HP1) and DNA methylation (Bhaumik *et al.*, 2007). To achieve a more thorough understanding of the relationship between such heterochromatin markers in *Neurospora*, Lewis *et al.* (2009) used a ChIP-chip approach to generate a high-resolution map of these heterochromatin markers along *N. crassa* Linkage Group VII. This analysis demonstrated that DNA methylation, H3K9me3, and HP1 are nearly perfectly co-localized at 44 discrete heterochromatic domains on this chromosome and showed that they do not overlap with H3K4me2, a mark of euchromatin (Lewis *et al.*, 2009). All 44 of these domains appear to be relics of RIP, consistent with earlier studies showing that virtually all DNA methylation is associated with RIP'd sequences in *Neurospora* (Selker *et al.*, 2003; Lewis *et al.*, 2009).

Establishment of heterochromatin domains

In a number of organisms, most notably *Schizosaccharomyces pombe* (Cam and Grewal, 2004; Allshire and Selker, 2007), RNAi-dependent mechanisms have been implicated in the establishment of heterochromatin. In contrast, known elements of *Neurospora*'s RNAi machinery (two dicer proteins, three RNA-dependent RNA polymerases, and two argonaut proteins) are not required for the initiation or maintenance of heterochromatin (Freitag *et al.*, 2004a).

Methylation is at the core of heterochromatin formation in *Neurospora* as both *dim-2* and *dim-5* encode methyltransferases, the former for DNA methylation and the latter for H3K9 methylation. We will use a bottom up approach, starting with the DNA sequence characteristics that trigger heterochromatin formation, to discuss what has been learned and what remains to be discovered about how heterochromatin domains are established and maintained in this relatively simple organism.

RIP creates the signal

The majority of sequences altered by RIP trigger *de novo* methylation in vegetative tissue (Singer *et al.*, 1995). This was first shown by removing the existing methylation, either by treatment with the methylation inhibitor 5-azacytidine or by molecular cloning followed by transformation back into *Neurospora* (Selker *et al.*, 1987; Singer *et al.*, 1995). More recently, the distribution of DNA methylation was analyzed on all of Linkage Group VII after passage through the DNA methylation mutant, *dim-2*, followed by re-introduction back into a DNA

Table 1 *Neurospora*'s heterochromatin machinery

Protein	Domain(s)	Mutant phenotype	Principal activity
RID	DMT	RIP defect	DMT and/or deaminase in ascogenous cells
DIM-5	HMT	No H3K9me3; no 5mC ^a	Trimethylation of H3K9
HP1	Chromo and Chromo-shadow	No 5mC	Adapter protein that binds H3K9me3 and recruits DIM-2 and DMM-1
DIM-2	DMT and 2 PXVXL-related	No 5mC	5mC in vegetative cells
DMM-1	JmjC; Cys-rich and 3 A:T-hooks	5mC patterns altered	?
DMM-2	Zn(II) ₂ Cys6	5mC patterns altered	?
PP1	Serine/threonine phosphatase	H3K9me3 and 5mC deficits	H3S10p phosphatase

Abbreviations: DMM, DNA methylation modifier; DMT, DNA methyltransferases; HMT, histone methyltransferase; HP1, heterochromatin protein 1; RID, RIP defective; RIP repeat-induced point mutation.

^a5mC, 5-methylcytosine.

methylation-proficient background. Strikingly, DNA methylation was reestablished at all of the 44 heterochromatic regions identified on this chromosome (Lewis *et al.*, 2009). As pointed out above, all DNA methylation in vegetative cells is dependent on DIM-2; RID is not required to establish DNA methylation in vegetative tissue (Freitag *et al.*, 2002). Notably, although the majority of RIP'd sequences can trigger *de novo* DNA methylation in vegetative tissue, there are some examples of RIP'd sequences that cannot. These sequences, which are relatively lightly RIP'd, provided the first evidence of maintenance methylation in *Neurospora* (Singer *et al.*, 1995). It is attractive to suppose that DNA methylation was established by RID during the sexual phase and was then maintained through vegetative growth by DIM-2.

What are the characteristics of a RIP'd sequence that cause it to trigger *de novo* DNA methylation? Although any cytosine in a duplicated sequence can be mutated, RIP has sequence preference for C residues preceding A's (that is, the dinucleotide CpA) (Margolin *et al.*, 1998). This produces a recognizable signature, namely the resulting high density of TpA dinucleotides, and correspondingly low density of CpA dinucleotides (and the complementary GpT dinucleotides), rendering identification of RIP'd sequences straightforward (Margolin *et al.*, 1998). Studies of the cis-acting signals of RIP'd sequences concluded that the higher A:T richness and TpA density are not essential features of the DNA methylation signal but are attributes of additive, positive signals that promote *de novo* DNA methylation (Miao *et al.*, 2000). Further refinement of the *de novo* methylation signal revealed that both A and T residues are required on both strands and suggested that multiples of the sequence (TAAA)_n or (TTAA)_n constitute the most potent methylation signals (Tamaru and Selker, 2003). In addition, weak signals could be strengthened by extending their length, whereas the presence of G:C base pairs suppressed DNA methylation with Cs 5' of ApT dinucleotides being the most inhibitory (Tamaru and Selker, 2003).

Recognition of the signal

As no single sequence motif serves as the '*de novo* DNA methylation signal' in RIP'd sequences, it seems likely the factor(s) that are involved in recognizing RIP'd sequences show relatively indiscriminate recognition of the sequence alterations produced by RIP. A protein activity dubbed methyl/RIP-binding protein 1 has been detected by gel mobility shift experiments, which shows preference for RIP'd sequences, relative to its nonRIP'd counterpart; however, this activity has the strongest affinity for RIP'd sequences that are also methylated (Selker *et al.*, 2002; Kothe and Selker, unpublished). Considering that the A:T-hook motif of proteins (for example, high mobility group proteins) is known to bind in the minor groove of A:T-rich sequences (Reeves, 2001), A:T-hook proteins are candidates for recognition of RIP'd sequences. Supporting this hypothesis, distamycin A, a chemical analog of the A:T-hook motif, was found to interfere with DNA methylation in *Neurospora* (Tamaru and Selker, 2003). *Neurospora* has at least 30 genes encoding proteins with an A:T-hook domain. Study of one of these A:T-hook motif proteins, led to the discovery of a putative histone demethylase (DNA Methylation

Modifier (DMM-1); see below) that does have a role in DNA methylation, but did not turn out to be the elusive RIP recognition factor (Honda *et al.*, 2010).

Histone H3K9 methylation marks the way

Neurospora's *dim-5* encodes a histone methyltransferase that is responsible for the tri-methylation of the lysine 9 residue on histone H3 (H3K9me3) (Table 1) (Tamaru and Selker, 2001). *Neurospora* strains lacking functional DIM-5 show a complete loss of DNA methylation, indicating that H3K9me3 is essential for DNA methylation (Figure 1). Although the situation appears more complicated in higher organisms, there is evidence that methylation of lysine 9 of histone H3 (H3K9me) also has roles in DNA methylation at some sites in both plants and animals (Jackson *et al.*, 2002; Lehnertz *et al.*, 2003).

As mentioned earlier, H3K9me3 shows extensive co-localization with DNA methylation at the 44 heterochromatin domains along *Neurospora* Linkage Group VII (Lewis *et al.*, 2009). In the absence of DNA methylation, in a *dim-2* mutant, H3K9me3 remained at these domains, confirming that DIM-5 is upstream of *dim-2*. How DIM-5 is targeted to RIP'd sequences is unclear, but presumably there is a factor or histone modification that is responsible for recruiting DIM-5. As pointed out above, unlike the situation in *S. pombe*, where the DIM-5 homologue, Ctr4, is targeted by the RNA-induced transcriptional silencing complex (Zhang *et al.*, 2008), the RNAi pathway does not appear to be involved in establishing or maintaining heterochromatin in *Neurospora* (Freitag *et al.*, 2004a). Furthermore, unlike DIM-5, the H3K9 histone methyltransferases in *S. pombe*, *Drosophila*, and mammals contain a chromo-domain that binds to H3K9me3, which apparently helps to maintain and spread this modification in these systems (Zhang *et al.*, 2008).

HP1 recruits DIM-2

Once H3K9me3 is established, how does this lead to DNA methylation by DIM-2? Proteins with a chromo-domain are known to bind to methylated lysine residues in histones. Most notably, HP1, first described in *Drosophila*, has been shown to bind to methylated H3K9 (Bannister *et al.*, 2001; Lachner *et al.*, 2001). HP1 has been implicated in the spreading and maintenance of H3K9 methylation (Hall *et al.*, 2002; Schotta *et al.*, 2002). *Neurospora* has several chromo-domain proteins, one of which also has a chromo-shadow domain, characteristic of HP1 homologues. In contrast to the results in fission yeast and flies, inactivation of the *hpo* gene, which encodes the HP1 homologue of *Neurospora*, showed very little alteration in H3K9me3 (Lewis *et al.*, 2009). However, *hpo* mutants show a complete loss of DNA methylation in vegetative tissue, supporting the idea that this protein is required for reading the H3K9me3 mark and relaying this information to the DNA methylation machinery (Table 1) (Freitag *et al.*, 2004b). Consistent with this, HP1 was found to generally co-localize with H3K9me3 along Linkage Group V11 in *Neurospora* (Lewis *et al.*, 2009). In addition, HP1 was found localized to discrete heterochromatic foci in nuclei and this localization was lost when H3K9me3 was eliminated by mutation of *dim-5* (Freitag *et al.*, 2004b). More recent

Figure 1 Model for *de novo* heterochromatin formation of RIP'd sequences. Mutations by RIP typically create an AT-rich cis-acting signal for *de novo* heterochromatin formation. In the upper section, DNA is shown wrapped around green nucleosomes with a region of RIP'd DNA represented in red and flanking unRIP'd DNA in black. The double arrow and associated question mark (?) represent the recognition of the RIP'd DNA by one or more unknown factors important for the initial step(s) in the establishment of heterochromatin. In the middle section, a sequence of events is depicted on a representative nucleosome from the RIP'd region. The cell cycle regulated H3S10p (yellow lollipops) is a posttranslational modification that is inhibitory to both the action of the DIM-5 complex (red oval with unidentified members of the complex represented by the '?') and to the binding of the chromo-domain protein HP1 (green oval). Removal of H3S10p by the phosphatase, PP1 (orange oval), allows the DIM-5 complex to trimethylate H3K9 (red triple-headed lollipops) and initiate heterochromatin formation. HP1 binds to the H3K9me3 through its chromo-domain and recruits the DNA methyltransferase DIM-2 (blue oval) through a direct interaction of its chromo-shadow domain with two PXVXL-related motifs in DIM-2. DIM-2 methylates any cytosine residue (white circles) within the associated RIP'd DNA. In the bottom section, the fully modified RIP'd region is depicted in a more condensed state with bound HP1 concentrated at the flanks where it recruits the DMM complex (light blue oval). The DMM complex prevents the heterochromatin from spreading into neighboring unRIP'd DNA by inhibiting H3K9me3.

work showed that the chromo-shadow domain of HP1 interacts directly with the two PXVXL-related domains of DIM-2 (Table 1) (Honda and Selker, 2008). Thus, HP1 provides the link between H3K9me3 established by DIM-5 and DNA methylation established by DIM-2 (Figure 1). Although these studies of HP1 in *Neurospora* have provided a direct link between HP1 and DNA methylation, HP1 proteins have also been shown to interact and/or colocalize with DMTs and methyl-binding proteins in mammals (Bachman *et al.*, 2001; Fujita *et al.*, 2003; Fuks *et al.*, 2003; Reese *et al.*, 2003). On the other hand, the Arabidopsis HP1 homologue, TFL2/LHP1, is dispensable for DNA methylation (Malagnac *et al.*, 2002).

Containing heterochromatin domains: role of a putative histone demethylase complex

Although silencing of transposons by DNA methylation may be beneficial to an organism, presumably it is important to prevent the DNA methylation from spreading into, and potentially silencing, neighboring genes (Cambareri *et al.*, 1996; Slotkin and Martienssen, 2007). Recent findings suggest that *Neurospora* uses a

protein complex that prevents spreading of DNA methylation and the underlying H3K9me3. The protein DMM-1 (DNA Methylation Modulator; Honda *et al.*, 2010) is the central player. DMM-1 is a 1458 amino-acid protein with three A:T-hook motifs near its C-terminus, making it a candidate to bind to the AT-rich RIP'd sequences, a JmjC domain near its N-terminus, and two cysteine-rich domains (Table 1). Interestingly, JmjC domains are frequently implicated in histone demethylation (Klose *et al.*, 2006). A null mutation of *dmm-1* resulted in severe growth defects and a disruption of normal DNA methylation patterns; many RIP'd transposons showed spreading of DNA methylation into neighboring sequences. Honda *et al.* (2010) hypothesized that the growth defects in *dmm-1* mutants result from the inappropriate silencing of critical genes because of the spreading of DNA methylation from RIP'd transposons. Consistent with this hypothesis, the *dmm-1* growth defects were complemented when DNA methylation was eliminated in a *dim-2* background. Furthermore, the spreading of methylation into a neighboring gene (NCU02414) in a *dmm-1* mutant resulted in reduced gene expression that was relieved in a *dmm-1 dim-2* double mutant.

A second DMM protein, DMM-2, was found to co-immunoprecipitate with DMM-1 (Honda *et al.*, 2010). DMM-2 is a 1133 amino-acid protein containing a fungal-specific Zn(II)₂Cys₆ DNA-binding domain (Table 1). Although *dmm-2* strains show DNA methylation defects congruous with *dmm-1* strains, the hypermethylation in *dmm-2* strains is less extreme. Consistent with this observation, *dmm-2* strains do not have the severe growth defects seen in *dmm-1* strains. Presumably, DNA methylation spreads into and silences important endogenous genes in *dmm-1* strains whereas the less pronounced hypermethylation defect of *dmm-2* strains results in less aberrant silencing and thus less growth defects.

Although histone demethylase activity has not been detected *in vitro* for DMM-1, it is attractive to speculate that the JmjC domain of DMM-1 has histone demethylase activity that prevents the spreading of DNA methylation by removing H3K9me3 deposited by DIM-5 (Figure 1). Such a role would be consistent with the observed localization of DMM-1 at the edges of methylated regions (Honda *et al.*, 2010). Interestingly, this localization is dependent on both the presence of H3K9me3 and on interaction with HP1, the location of which (Table 1; Figure 1) is found preferentially at the edges of methylated regions (Lewis *et al.*, 2009). Thus, HP1 not only serves as an adapter to promote DNA methylation by recruiting the DMT, DIM-2, but also to recruit the DMM-1/2 complex that prevents the spreading of DNA methylation (Figure 1). Somewhat similar anti-silencing mechanisms that use a JmjC domain protein have been identified in *Arabidopsis* and fission yeast (Zofall and Grewal, 2006; Saze *et al.*, 2008; Miura *et al.*, 2009).

Cell cycle propagation of heterochromatin

Proper propagation of chromatin domains during passage through the cell cycle is essential to insure that regulatory controls are maintained. Interplay of histone modifications is critical for this propagation. Notably, phosphorylation of the serine 10 residue of histone H3 (H3S10p) is required for the faithful propagation of heterochromatin through the cell cycle. Increased H3S10p is required in fission yeast during S phase to drive the transcription of repeats in heterochromatin regions to generate small RNAs that nucleate heterochromatin formation by recruiting the RNA-induced transcriptional silencing and ClrC complexes (Chen *et al.*, 2008; Kloc *et al.*, 2008; Zhang *et al.*, 2008). Such an RNAi-based mechanism may not be necessary for heterochromatin in *Neurospora*, however (Freitag *et al.*, 2004a). The H3S10 residue is known to be phosphorylated by a number of kinases in response to environmental signals to activate genes, or in response to internal signals that initiate chromosome condensation and segregation during mitosis and meiosis (Prigent and Dimitrov, 2003; Nowak and Corces, 2004; Johansen and Johansen, 2006). Phosphorylation of H3S10 occurs at the onset of mitosis, disrupting the binding of HP1 to methylated H3K9 at pericentric heterochromatin (Fischle *et al.*, 2005; Hirota *et al.*, 2005; Chen *et al.*, 2008; Kloc *et al.*, 2008). Re-association of HP1 cannot occur until H3S10p is dephosphorylated (Fischle *et al.*, 2005; Hirota *et al.*, 2005; Chen *et al.*, 2008). Structural studies have shown that DIM-5 directly interacts with S10 of H3, the residue next to its

site of action, H3K9, and that this interaction is critical for its activity (Zhang *et al.*, 2003; Rathert *et al.*, 2008; Adhvaryu *et al.*, unpublished results). *In vitro* assays with H3 peptide substrates have shown that phosphorylation of S10 inhibits the activity of mammalian SUV39H1, fission yeast Clr4, and *Neurospora* DIM-5 (Rea *et al.*, 2000; Nakayama *et al.*, 2001; Adhvaryu *et al.*, unpublished results). Consistent with the idea that H3S10p is inhibitory to DIM-5 binding and activity, a partial loss-of-function mutant defective in the gene encoding the H3S10 protein phosphatase, PP1, results in growth and developmental defects as well as deficits of H3K9me3 and DNA methylation (Adhvaryu and Selker, 2008). Hence, removal of the H3S10p modification by PP1 during the cell cycle is required to allow DIM-5 and HP1 binding, resulting in the propagation of heterochromatin (Figure 1).

Function of heterochromatin

As mentioned above, virtually all heterochromatin domains in *Neurospora* appear to be based on sequences altered by RIP (Lewis *et al.*, 2009); the only currently known exception being the tandemly arranged rRNA repeats. Most RIP'd sequences are relics of transposons and it is presumably in the best interest of an organism to protect its genome by silencing these invasive elements (Galagan *et al.*, 2003). The mutations that occur by RIP would seem to be an effective barrier against these transposons. However, as a result of these transition mutations, expression of RIP'd sequences could lead to the production of dominant negative proteins, or RNAs with deleterious effects. Thus, relics of RIP may be packaged into heterochromatin to silence their expression.

Removal of DNA methylation, either with the drug 5-azacytidine or with a *dim-2* mutation, can lead to activation of transcription (Irelan and Selker, 1997; Rountree and Selker, 1997). Furthermore, DNA methylation appears to exert its suppressive effect downstream of transcription initiation in *Neurospora* (Rountree and Selker, 1997). These *Neurospora* studies showed that once DNA methylation is removed, expression is restored suggesting that DNA methylation is the critical modification that 'locks down' the heterochromatin, making it refractory to transcription. Interestingly, although H3K9 methylation is largely upstream of DNA methylation, H3K9me3 was lost from a RIP'd allele of the *Neurospora am* gene (*am*^{RIPs}) in a *dim-2* mutant (Tamaru *et al.*, 2003). This *am*^{RIPs} allele is known to have a functional promoter (Singer *et al.*, 1995; Rountree and Selker, 1997). In contrast, H3K9me3 was not lost from any of the natural heterochromatic regions along Linkage Group VII in a *dim-2* mutant (Lewis *et al.*, 2009). Lewis *et al.* (2009) suggested that the extensive RIP of the heterochromatic sequences on Linkage Group VII probably inactivated promoter sequences. Thus, in the absence of DNA methylation, transcriptional activity may be capable of overcoming and preventing the formation of heterochromatin.

In animals, DNA methylation induced transcriptional repression has been attributed to a series of methylated DNA-binding proteins that complex with chromatin modifiers to establish, or at least perpetuate, a silenced chromatin state (Fuks, 2005; Sasai and Defossez, 2009).

A number of methylated DNA-binding proteins have been identified in plants based on homology to the mammalian methyl-binding domain motif, but *Neurospora* lacks proteins with homology to higher eukaryotic methylated DNA-binding proteins (Zemach and Grafi, 2007). However, as mentioned above, a methylated DNA-binding activity (methyl/RIP-binding protein 1) has been detected that has the strongest affinity for RIP'd methylated sequences (Selker *et al.*, 2002). The role of this factor in the establishment and perpetuation of heterochromatin requires further work.

Conclusion and perspectives

Although our understanding of how heterochromatin domains are established and maintained in *Neurospora* has grown considerably, many questions remain to be answered regarding these processes, including but certainly not limited to: What factor(s) recognizes RIP'd sequences and recruit the DIM-5 complex to these sequences? Where in the process of heterochromatin formation is histone deacetylation critical? How does the DMM complex inhibit H3K9me3 at the boundaries of heterochromatin regions? What other histone modifications have a role in heterochromatin formation and/or maintenance? Why do *hpo-1* and *dim-5* mutants display growth defects, unlike *dim-2*? The answer to this latter question most likely has to do with the role of HP1 and DIM-5 in additional cellular processes, such as chromosomal segregation. DNA methylation can be regarded as the 'final layer' of heterochromatin, which though not essential for the cell, 'locks' down the heterochromatin to prevent transcription of invasive elements.

Conflict of interest

The authors declare no conflict of interest.

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