Chapter 3
Epigenetics of Fungal Secondary Metabolism Related Genes

Ming-Yueh Wu and Jae-Hyuk Yu

Fungal Secondary Metabolism and Epigenetics

Fungi produce a diverse array of low molecular weight, bioactive secondary metabolites are not essential for their survival. Secondary metabolism (SM) is defined as "the production of ancillary metabolites and 'useful' compounds, initiated after using preferred carbon and nitrogen sources" [1, 2]. Secondary metabolites are not necessary for normal growth, but are considered important for the producing fungus to flourishing in its niche [3–5], stress tolerance [6, 7], or defense against hostile and/or competing organisms [1, 8]. They are important for day-to-day human life as beneficial antibiotics, pharmaceuticals, and/or harmful mycotoxins [9]. However, the true biological functions of many fungal secondary metabolites in producing fungi are largely cryptic.

Fungal SM is a complex process, which is often tightly related with morphological development [10]. Due to the importance of fungal secondary metabolites, an increasing number of genes associated with SM have been identified and characterized. Furthermore, the availability of fungal genomes accelerates the identification of biosynthetic genes for secondary metabolites. However, the role and regulatory mechanisms of many of the newly defined genes remain to be investigated [11]. In fungi, secondary metabolite biosynthetic and regulatory genes are usually clustered and not evenly distributed across the genomes [12–14]. Many of the clusters are silent under the standard laboratory culture conditions, which makes it difficult to

J.-H. Yu (✉) · M.-Y. Wu
Department of Bacteriology and Genetics, University of Wisconsin-Madison,
1550 Linden Drive, Madison, WI 53706, USA
e-mail: Jyu1@wisc.edu

M.-Y. Wu
e-mail: mwu33@wisc.edu

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elucidate their functions and regulatory mechanisms. It is both time and resource consuming to find the appropriate conditions to express the gene clusters of interest. A promising strategy to investigate unknown SM cluster(s) is via modifying global epigenetic regulators to activate the silenced SM clusters [15, 16].

Epigenetic phenomena are defined as reversible and heritable changes in gene expression levels without altering the DNA sequences. Epigenetic phenomena can derive from DNA-, chromatin-, and RNA-based effects, and include DNA methylation, position effects, RNA silencing systems, centromere/telomere location, and chromatin structure changes. Many of the aforementioned phenomena occur in fungi throughout the life cycle [17] which makes fungi an excellent model system to understand the fundamental principles of epigenetics. During the life cycle, fungi regulate development by several epigenetic mechanisms. Most steps or cell types are known to be under control by DNA methylation, which is regulated by changes in the chromatin state. Methylation induced premeiotically (MIP) and repeat-induced point mutation (RIP) occur during dikaryon formation and conjugated nuclear division [18–25]. MIP is regulated by DNA methylation, and RIP may also be regulated by it. Moreover, filamentous fungi share conserved silencing systems with higher eukaryotes, such as RNA interference (RNAi) and DNA methylation [26–31]. However, it is uncertain whether RNAi, which regulates the parasexual cycle and germination, is related to DNA- or chromatin-mediated epigenetic phenomena [32]. In addition, meiotic silencing by unpaired DNA, also known as meiotic silencing (MSUD), is another RNA silencing mechanism, that occurs throughout meiosis [33, 34].

As mentioned, fungal secondary metabolite synthetic and regulatory genes tend to be clustered. Gene clusters may originate from the horizontal transfer of genes from bacteria to fungi [35–40]. However, some SM gene clusters—e.g., gibberellin (GB) gene cluster—are unlikely a result of horizontal transfer [41]. The clustered SM genes are likely subject to co-regulation by epigenetic changes. An emerging field, chemical epigenetics, has been evolving to stimulate expression of secondary metabolite gene clusters by altering epigenetic status such as DNA and/or histone modifications [1, 42].

Epigenetic Modifications that Affect Secondary Metabolism

The epigenetic regulation of fungal SM is mainly through histone modifications: methylation, acetylation, and sumoylation (Fig. 3.1) [43–47]. Histone proteins are the primary protein components of chromatin and function as a scaffold for the nucleosome formation. Histone octamer consisting of two each of H2A, H2B, H3, and H4 is wrapped by DNA and forms a nucleosome [48].

Histone modifications can affect chromatin conformation and recruited proteins that cause epigenetic changes by interacting with histones [49]. Most histone modifications involve histones H3 and H4 [44, 45, 50]. The N-terminus of H3 and H4 are

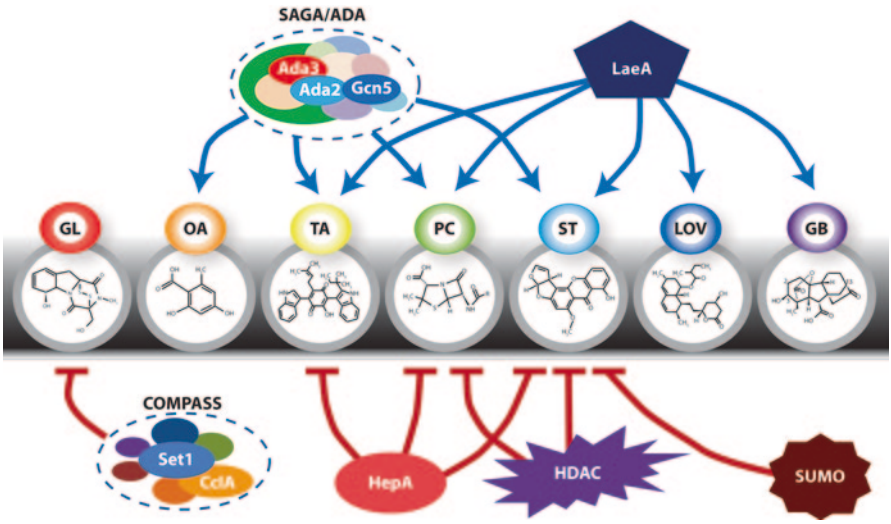

Fig. 3.1 Examples of fungal secondary metabolites and their epigenetic regulators. Certain fungal secondary metabolites regulated by one or more epigenetic regulators are shown. *PC* Penicillin, *LOV* Lovastatin, *GB* Gibberellin, *AT* Asperthecin, *GL* Gliotoxin, *OA* Orsellinic Acid, *TA* Terrequinone A, *ST* Sterigmatocystin

crucial to generate heterochromatin or euchromatin. In euchromatin, lysines in the H3 and H4 tails are hyperacetylated and H3K4 is trimethylated. In heterochromatin, in comparison, lysines in H3 and H4 are hypoacetylated and H3K9 is trimethylated [51]. By histone modifications, only a group of specific target genes inside of distinct regions of the chromosomes can be regulated, further supporting the advantage of SM genes being clustered [44, 45, 50].

Genes Affecting Histone Methylation

HepA HepA is the *Aspergillus nidulans* homolog of HP1 (the heterochromatin protein-1, SWI6 in *Schizosaccharomyces pombe*) [52–54]. Heterochromatin domains are silenced and have hypoacetylation of lysines in H3 and H4 [55] with different degrees of methylation of H3K9 (H3K9me) by a histone methyltransferase (Clr4 in *S. pombe*) [56, 57]. As a transcriptional repressor, HP1 recognizes H3K9me and directly binds to it, achieving both targeting and transcriptional repression by maintaining the heterochromatin structure [58–63]. Artificial recruitment of HP1 to a gene promoter region leads to gene repression, supporting that HP1 is essential in gene silencing [64, 65].

HepA acts as an epigenetic repressor in expression of secondary metabolite genes [52]. The deletion of *HepA* leads to derepression of secondary metabolite biosynthetic genes, including sterigmatocystin (ST), penicillin (PC), and terrequinone A (TA).

Biochemical analysis shows that the silent ST gene cluster is marked by H3K9me3 and recruits high levels of HepA, leading to repression of ST production during growth phase. Upon growth arrest and activation of SM, HepA, and H3K9me3 levels decrease while the acetylated histone H3 increases [52]. HepA occupancy and H3K9me3 levels are counteracted by the global SM regulator (LaeA) (Fig. 3.2).

LaeA (loss of aflR expression-A) is a global regulator of SM and development in filamentous fungi. This nuclear protein was first reported in *Aspergillus* spp. [44]. The lack of *laeA* blocks expression of several metabolic gene clusters, including ST, PC, and lovastatin (LOV). The overexpression of *laeA* contrarily increases expression of ST and LOV gene clusters and subsequent ST and LOV production [44]. In *Penicillium chrysogenum,* the overexpression of *laeA* increases PC production (~125%) and the lack of *laeA* dramatically reduces PC gene expression levels and PC production [50]. Similarly, LaeA serves as a positive regulator of GB production in *Fusarium fujikuroi* [66]. In addition, microarray analysis indicates that LaeA regulates up to 9.5% of the *Aspergillus fumigatus* transcriptome and up to 13 of its

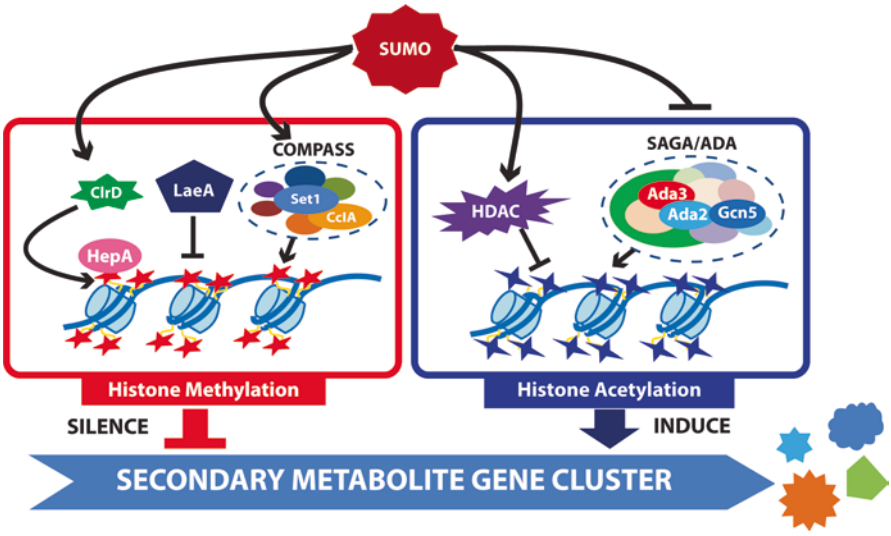

Fig. 3.2 Overview and the roles of the epigenetic regulators in fungal secondary metabolism. Many epigenetic regulators participate in fungal secondary metabolism. HepA, Clr4, COMPASS, and LaeA are involved in histone methylation (*red box, red stars indicate histone methylation*). Clr4 leads to H3K9 methylation, which enables HepA binding to histone. HepA binding stabilizes the heterochromatin structure and thus leads to silencing the secondary metabolic gene clusters. COMPASS methylates H3K4 and H3K9, and silences SM, while LaeA removes the histone methylation and HepA binding and induces SM. HDACs and SAGA/ADA complex play a role in controlling histone acetylation (*blue box, blue stars indicate histone acetylation*), which induces fungal SM. HDACs deacetylate the lysines of H3 and/or H4, while the SAGA/ADA complex acetylates them. SUMO (*the scarlet decagon*) conducts sumoylation of several epigenetic regulators, including Clr4, COMPASS, HDACs, and SAGA/ADA, and silences SM

22 secondary metabolite gene clusters, containing NRPS, PKS, and P450 monooxygenase genes [67].

LaeA forms a key heterotrimeric complex with the two *velvet* proteins, VelB and VeA. The VelB/VeA/LaeA trimeric complex coordinates light signals with fungal development and SM [68]. VeA physically interacts with VelB, and bridges it to LaeA. All three components in this complex are essential for sexual development and ST production in *A. nidulans*. Previous studies showed that LaeA and VeA interact in *P. chrysogenum* and *F. fujikuroi*, too [50, 66]. The successful cross-genus complementation between *Fusarium, Aspergillus*, and *Penicillium* indicates that the VelB/VeA/LaeA complex has undergone a divergence in specific functions mediating SM [66].

LaeA-mediated SM regulation primarily depends on histone methylation. LaeA contains a predicted and functionally necessary S-adenosyl-methionine (SAM) binding domain [68–70], which is present in all members of the methylase superfamily [71], and has sequence similarity to histone and arginine methyltransferase [44, 72]. The *laeA* gene is negatively regulated by AflR, a Zn_2/Cys_6 transcription factor located in the aflatoxin and ST gene clusters, in a feedback loop [44]. In *A. nidulans*, the ST gene cluster expression analysis shows that LaeA-mediated regulation of the cluster is location specific. The placement of *argB* in the ST cluster results in *argB* silencing in the *laeA* deletion background, whereas the genes bordering the ST cluster are unaffected [69]. Similar location-specific effects on SM gene regulation have been reported in other *Aspergillus* species as well [73–75]. Notably, the location specific effect is only reported in *Aspergillus* and *Neurospora* [13, 76],

These findings indicate that LaeA may differentially affect histone protein methylation, which in turn allows the cluster region to be more accessible to gene transcription [69]. Biochemical analyses of *laeA* and heterochromatin mutants (e.g., histone deacetylase and histone methyltransferase mutants) in *A. nidulans* demonstrate that LaeA activates SM gene expression by being involved in the removal of heteromatin marks like H3K9 methylation and HepA binding [13, 52, 77]; i.e., the LaeA-involved machinery reverses the heterochromatic signature and activates the gene expression inside the SM cluster.

COMPASS COMPASS (complex proteins associated with Set1) is a multi-subunit complex consisting of Set1, Bre2, Sdc1, Spp1, Swd1, Swd2, and Swd3 [78–80]. COMPASS is involved in H3K4 mono-, di-, and tri-methylation [77, 79–82], which is necessary for RNA Pol II binding and transcriptional activity in development and differentiation [79, 80, 83] in *Saccharomyces cerevesiae*. Three core components, Set1, Swd1, and Swd3 are essential for COMPASS [78]. Swd2, Bre2, Sdc1, and Spp1 affect the degree of Set1 methylation [84–86]. Set1 has the SET domain, which possesses histone or lysine methyltransferase (HMTase or KMTase) activity [87].

CclA (Bre2 in *S. cerevisiae*) is one of the eight members of COMPASS in *A. nidulans*. The lack of CclA leads to reduced levels of H3K4 and H3K9 di- and tri-methylation, as well as reduced H3 acetylation [88]. H3K4 di- and tri-methylation is associated with actively expressed genes and are required for telomere silencing in *S. cerevesiae* [79, 80, 89–91], and activating *A. nidulans* SMs, e.g.,

monodictyphenone, emodins, and the polyketides F9775A and F9775B [52, 77]. In *A. fumigatus*, loss of CclA results in slow fungal growth and increased SM production like gliotoxin [92]. Based on 6-azauracil (6AU) sensitivity test result, CclA plays a role in transcription elongation [92].

Genes Influencing Histone Acetylation

Histone Deacetylases (HDAC) Histone deacetylases (HDACs) and histone acetyltransferases (HATs) play critical roles in fungal epigenetic regulatory mechanism. Histone acetylation is reversible and controlled by HDACs and HATs [51]. HDACs are classified into three main groups based on their homology to yeast proteins: Class I HDACs have homology to yeast Rpd3; Class II HDACs have homology to yeast Hda1; Class III HDACs have homology to yeast Sir2. Both Classes I and II HDACs contain zinc in their catalytic site, and are known as epigenetic regulators in fungal SM. Class III HDACs do not have zinc in the catalytic site but require NAD^+ instead [93].

A. nidulans RpdA is a Class I HDAC and the homolog of the global repressor Rpd3 in *S. cerevisiae*. RpdA is necessary for growth, conidiation, and gene regulation. The lack of Rpd3 leads to increased acetylation of H4K5, H4K12, and H3K18 in derepressed genes [94]. The absence of RpdA is lethal in *A. nidulans* and *Neurospora crassa* [95]. Silencing of RpdA in *A. nidulans* reveals that RpdA is involved in normal growth and H3 and H4 deacetylation [96].

Histone deacetylase A (HdaA) is a Class II HDAC playing a counter role to LaeA in SM regulation in *A. nidulans*. Loss of *hdaA* causes precocious and increased expression of ST and PC biosynthetic genes. The deletion of *hdaA* causes derepression of SM gene clusters that are located close to the telomeres in *A. nidulans* [97]. In *A. fumigatus*, HdaA plays a similar role in SM regulation [98]. Inhibition of most HDACs induces the production of unknown SMs in Penicillium expansum [97]. Treating the fungus with HDAC inhibitors leads to overproduction of several secondary metabolites, suggesting that HDAC-mediated repression of certain SM gene clusters is conserved in fungi [97].

SAGA/ADA Complex The Spt-Ada-Gcn5-acetyltransferase (SAGA/ADA) coactivator complex regulates numerous cellular processes by posttranslational modifications of histones [99]. SAGA/ADA contains a HAT, Gcn5, and acetylates multiple lysine residues at the N-terminal tails of H3 and H2B. In *A. nidulans*, GcnE (Gcn5 homolog in *A. nidulans*) regulates PC biosynthesis gene cluster located on chromosome VI by histone acetylation [45, 100]. The Ada1–5 proteins (Alteration/deficiency in activation) are components of SAGA/ADA in *S. cerevisiae* [101]. Ada2/Ada3/Gcn5 complex is sufficient for robust histone and nucleosomal HAT activity in yeast [102].

Both GcnE and AdaB are required for induction of the orsellinic acid gene cluster in *A. nidulans*. Similarly, SAGA/ADA plays a major role in specific induction of

other SM gene clusters, such as ST, PC, and terrequinone [45]. Chromatin immunoprecipitation (ChIP) data shows that SAGA/ADA increases acetylation at H3K9 and H3K14 in *A. nidulans*. Interestingly, the increase of H3K14 acetylation is a global phenomenon of the whole genome, while the increase of H3K9 acetylation can be only observed within SM gene clusters [45].

Genes Impacting Sumoylation

*SUMO S*mall *u*biquitin-like *m*odifier (SUMO) is a small protein that has high structural similarity to ubiquitin, despite its low similarity at the level of the amino acid sequence [103–106]. SUMO covalently attaches to other proteins through the activities of an enzyme cascade (E1-E2-E3) similar to that of ubiquitination, and is known to play a role in histone modification like ubiquitin [105, 107–111]. Histone sumoylation mediates gene silencing through recruitment of HDAC and Hp1 both in vitro and in vivo in human cells [112, 113]. SUMO also modifies Gcn5, a member of the SAGA/ADA complex, and results in gene silencing in yeast [114].

In *A. nidulans*, SUMO represses sexual development and is involved in accurate induction and light stimulation of asexual development [104, 115]. CclA and SetA, two members of COMPASS, connects the SUMO network to histone modification. The interplay of the fungal sumoylation network controls temporal and spatial steps in cell differentiation [104].

SUMO is also essential for sexual fruiting body formation and SM in *A. nidulans* [47, 116]. Deleting *sumo* causes about 200-fold increase of asperthecin production but decreases production of austinol/dehydroaustinol and ST [47]. The effect of sumoylation on SM may occur at several levels, such as silencing the secondary metabolite gene clusters at the chromatin level or regulating TFs involved in the SM regulation [47]. Additional work needs to be done to elucidate how SUMO regulates specific secondary metabolite production.

Application of Epigenetic Regulators of Fungal Secondary Metabolites

Understanding the SM epigenetic regulators can accelerate fungal SM studies by activating certain SM gene clusters that are often silent and cryptic in lab culture conditions. Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, has been used to stimulate the production of new cladochromes and calphostin B, a known protein kinase C inhibitor [117], in *Cladosporium cladosporioides* [118]. Treatment with SAHA can boost nygerone A production in *Aspergillus niger* [119, 120] and orsellinic acid production in *A. nidulans* without coculturing with *Streptomyces rapamycincus* [45]. In addition, using a global SM regulator is a new approach to identify new secondary metabolic genes. For example, LaeA is an excellent ge-

nomic mining tool and has successfully been manipulated to uncover several novel secondary metabolites such as terrequinone A [15, 16]. Another way to alter expression of SM gene clusters is to manipulate histone modification, for example, by the deletion of *hepA* [52] or *hdaA* [97].

Conclusion

Fungi produce a wide range of secondary metabolites. These low molecular weight compounds are diverse in structure and perform important yet often cryptic biological functions. The scientific community shows great interest in fungal secondary metabolites due to their importance to humankind. However, sequencing data of the fungal genomes indicate that a large number of fungal secondary metabolites are yet to be uncovered and characterized. As most fungal secondary metabolic gene clusters are silent under standard laboratory conditions, the importance of global regulators and epigenetic regulatory mechanism has been increasingly recognized. Various proteins and their complexes play a role in the regulation of fungal SM gene clusters through histone modification. Some of these epigenetic regulators mediate modification at distinct sites, such as methylation, acetylation, and sumoylation, whereas others inhibit such alterations (Fig. 3.2).

In this chapter, we have reviewed several known epigenetic regulators that are involved in regulating fungal SM. Epigenetics is an emerging area for investigating fungal SM, and a better understanding of SM epigenetic regulation would lead to the discovery of new drugs.

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