Minireview

Post-translational modifications drive secondary metabolite biosynthesis in Aspergillus: a review

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Summary

Post-translational modifications (PTMs) are important for protein function and regulate multiple cellular processes and secondary metabolites (SMs) in fungi. Aspergillus species belong to a genus renown for an abundance of bioactive secondary metabolites, many important as toxins, pharmaceuticals and in industrial production. The genes required for secondary metabolites are typically co-localized in biosynthetic gene clusters (BGCs), which often localize in heterochromatic regions of genome and are ‘turned off’ under laboratory condition. Efforts have been made to ‘turn on’ these BGCs by genetic manipulation of histone modifications, which could convert the heterochromatic structure to euchromatin. Additionally, non-histone PTMs also play critical roles in the regulation of secondary metabolism. In this review, we collate the known roles of epigenetic and PTMs on Aspergillus SM production. We also summarize the proteomics approaches and bioinformatics tools for PTM identification and prediction and provide future perspectives on the emerging roles of PTM on regulation of SM biosynthesis in Aspergillus and other fungi.

Introduction

The genus Aspergillus is among the most common fungal taxon, which consists of a few hundred species (Samson et al., 2014). These saprophytic filamentous fungi are typically found in soil or organic debris and are adaptive and resilient to fluctuating environment conditions, growing under a wide range of temperature and at relatively low humidity. Most Aspergillus spp. produce abundant secondary metabolites (SMs) and fermentative enzymes, leading to a close relationship with human health, daily life and industry production. On the detrimental side are harmful mycotoxins produced by A. flavus, A. parasiticus, A. nidulans (Keller et al., 2005) and the human pathogens – primarily A. fumigatus and A. flavus – causing invasive aspergillosis (Patterson et al., 2000). On the other hand, several industrial spp. such as A. oryzae (Abe et al., 2006) and A. niger (Baker, 2006) produce useful enzymes and/or fermented foods. A. cvjetkovicii, dominated at asymptomatic rice phyllosphere microenvironment, produces such SMs as 2-(3H)-benzofuranone and azulene to suppress the invasion of the rice blast Magnaporthe oryzae during different growth stages (Fan et al., 2019). Dozens of Aspergillus genomes have been sequenced and functionally annotated, which shows a large number of biosynthetic gene clusters (BGCs) in these fungi (Kjaerbolling et al., 2020; Drott et al., 2021), which are responsible for the biosynthesis of secondary metabolites, including mycotoxins. Secondary metabolites play critical roles in fungal development and combatting other organisms and can serve as virulence factors when invading plant or animal hosts.

The great impact of SMs produced by Aspergillus on human activities has driven intense focus on characterizing the Aspergillus secondary metabolome. The production of fungal SMs is limited to very specific ecological conditions (Collemare and Seidl, 2019) and most of the BGCs are silent in laboratory conditions. Epigenetic and post-translational modifications (PTMs) have been suggested to regulate transcription of genes and are considered to be involved not only in secondary metabolism but general environmental responses in most eukaryotes (Shwab et al., 2007; Cichewicz, 2010; Yan et al., 2015). In this review, we first make a brief introduction of secondary metabolite cluster architecture and regulation in...
Aspergillus and then focus on post-translational regulation on the Aspergillus secondary metabolism. Finally, we also explore the connection between different PTMs on SM biosynthesis and highlight some questions that still remain to be answered.

Overview of secondary metabolite cluster organization and regulation in Aspergillus

In Aspergillus spp. or other filamentous fungi, the synthesis of secondary metabolites is preliminarily generated from precursors catalysed by the backbone (also called core) enzymes (e.g. polyketides, non-ribosomal peptides, terpenes, PKS-NRPS hybrids and indole alkaloids) that use primary metabolites (acyl-CoA, amino acids) to generate a SM carbon backbone (Lim and Keller, 2014; Keller, 2018) (Fig. 1A). Further modification of the carbon backbone is modulated by the tailoring enzymes to form the final BGC metabolite(s). The gene number of BGCs can range from two (such as the valactamide gene cluster) (Keller, 2018) to more than 20 (such as the aflatoxin gene cluster, Fig. 6B). Genes encoding both backbone enzymes and tailoring enzymes are necessary for all canonical BGCs. Additionally, the BGCs may contain genes encoding transporters that are found to transfer the SM or its precursor, a cluster-specific transcription factor that positively regulates the other genes within the BGC (Wang et al., 2021b), and some genes encoding hypothetical or protective functions (Keller, 2018) (Fig. 1B).

Biocatalytic algorithms, such as antiSMASH and SMURF, are based on the presence of typical conserved backbone genes (e.g. PKSs and NRPSs) in SMs cluster, have been utilized to identify BGCs in Aspergillus and other filamentous fungi. However, these algorithms are merely predictive and may underestimate or overestimate inclusion of genes in a BGC (Keller, 2018). To identify more SMs or BGCs in fungi, a range of research approaches including molecular, biochemical or bioinformatics tools have been developed. The genomic sequence of five genetic model Aspergillus spp. (A. fumigatus, A. nidulans, A. niger, A. flavus and A. oryzae) has been functionally annotated, and their SMs have been identified. BGCs of A. nidulans and A. fumigatus in particular have been systematically studied with recent reviews presenting a full picture of their BGCs and transcription factor regulation of these BGCs (Caesar et al., 2020; Wang et al., 2021b). In antiSMASH online programmes, the number of individual PKS, NRPS, DMAT/Terpenes and other backbone genes are shown to vary considerably from an estimated 37 in A. fumigatus to 89 in A. niger (Table 1). A recent study examining 94 sequenced isolates of A. flavus showed a median of 82 BGCs with a range from 78 and 85 BGCs thus demonstrating variation within a species (Drott et al., 2021). To date, up to 50 specific SM gene clusters have been experimentally isolated in the Aspergillus (de Vries et al., 2017). The fluctuation of BGC number with genome size may indicate a dependence on the lifestyle.

Fig. 1. The general schematization of secondary metabolites and their biosynthetic gene cluster.

A. Most secondary metabolites (SMs) are generated from precursors catalysed by backbone enzymes (e.g. polyketide synthase, non-ribosomal synthetase and terpene synthase and/or cyclase) that use primary metabolites (e.g. acyl-CoA, amino acids, isoprenoid) to synthesize the SM precursor (carbon backbones) (Lim and Keller, 2014). These carbon backbones are further modified by the tailoring enzymes to form the final active SMs.

B. Fungal biosynthetic gene cluster basically consists of a backbone enzyme and tailoring enzymes (e.g. methyltransferases and P450 monooxygenases) (indicated with dotted lines) (Keller, 2018; Collemare and Seidl, 2019). The transporters are also found in most of the BGCs to secrete/move pathway precursors or finished product. Additionally, BGCs may contain a cluster-specific transcription factor that activates the other genes within the BGC; resistance genes to product the producing fungus from its own BGC product (e.g. many are antifungal) and often genes with hypothetical functions (indicated with grey).
and the specific requirements of each *Aspergillus* spp. (de Vries et al., 2017).

**Initial studies connecting secondary metabolite in Aspergillus to epigenetic processes**

The biosynthesis of fungal secondary metabolism is a complicated process that is subjected to a complex system of multi-tier regulation, such as signal transduction pathways (e.g. cAMP and MAPK signalling), environmental factors, and regulation at transcriptional, translational and epigenetic levels. A pioneering study in 2007 was the first to demonstrate a role for histone modifications in regulating fungal SMs where deletion of a HdaA, histone deacetylase (HDAC), led to induced expression of two telomere-proximal BGCs in *A. nidulans* (Shwab et al., 2007). This study further went on to show that treatment of fungal cultures HDAC inhibitors led to the overexpression of several metabolites in other fungal genera. Additional seminal studies involved demonstrations that LaeA, a global regulator of SM in all filamentous fungi studied to date (Perrin et al., 2007; Zhang et al., 2016b) was involved in chromatin level activation of BGCs (Bok et al., 2006), in part by counteracting the establishment of heterochromatic marks histone H3 lysine 9 trimethylation (Reyes-Dominguez et al., 2010). Later, additional studies further established a role of LaeA in a histone modification manner (Bok and Keller, 2004; Bok et al., 2006; Lind et al., 2018) that is important for histone H3 trimethyl K4. A recent study also showed that LaeA controls citric acid metabolism by regulating the expression of the citrate exporter-encoding *cexA* gene via methylation levels of the histones H3K4 and H3K9 (Kadooka et al., 2020). These initial studies set the stage for a huge expansion of BGC product mining through PTM strategies.

**Strategies to activate fungal BGCs**

Although thousands of BGCs have been predicted in *Aspergillus* genomes, the majority have not been characterized and are often not expressed during fungus growth on laboratory conditions. A major challenge for researchers is to develop strategies to activate the expression of BGCs and allow isolation of their associated products. One strategy is the use of genetic manipulation at BGC transcriptional level, inducing the expression of pathway specific transcription factor (PSTF) by replacing its native promoter with a constitutive promoter (e.g. *gpdA* promoter) or an inducible promoter (e.g. alcohol dehydrogenase promoter *alcAp* or nitrate inducible promoter). Serial promoter exchanges strategy and CRISPR-mediated activation system (*CRISPRa*) (Roux et al., 2020) are both promising approaches for activating cryptic BGCs (Fig. 2), for those fail to express the whole cluster by single promoter replacement of PSTF. Roux et al. used CRISPR/
dLbCas12a-based activation system, by concurrently expressing multiple gRNAs (guide RNAs), to activate three mic cluster genes, leading to the discovery of the final product, dehydromicroperfuранone (Roux et al., 2020).

Another strategy that has significantly increased SM output in many fungal species is the use of epigenetic strategies that convert heterochromatic regions of the genome to euchromatin (reviewed in the study by Pfannenstiel and Keller, 2019), by which overexpression or knock-down of genes encoding global regulatory complexes (e.g. OE::læA) or histone modifiers (e.g. ΔHDAC) helps to turn on the biosynthetic clusters in fungal species with accessible genetic systems (Fig. 2). Alternatively, histone remodelling agents, such as DNA methyltransferases inhibitor 5-azacytidine, and HDAC inhibitors (Pfannenstiel and Keller, 2019) (e.g. trichostatin A, sodium valproate, vorinostat) could be utilized to identify novel metabolites by activation of silent BGCs in fungi recalcitrant to genetic manipulation.

Below we will cover the current literature that describes epigenetic and other PTMs that have been used to express BGC products in Aspergilli.

### Chromatin-dependent regulation of secondary metabolite biosynthesis

PTMs target all histone proteins (H2A, H2B, H3 and H4) that dominate the chromatin-dependent regulation of BGCs and non-histone proteins that can also impact expression or activity of BGCs and proteins encoded by these clusters. The chromatin is organized by a complex of DNA and histone proteins, where DNA wraps around histones to form the chromatin basic unit, the nucleosome (Armeev et al., 2019). A transition from heterochromatin to euchromatin could lead to loosely packed and open nucleosomes, which allows RNA polymerase to bind to DNA and activate gene expression (Fig. 3). Efforts to identify global PTMs have revealed the particular roles of non-histone proteins in fungal development and SM (Ren et al., 2018; Li et al., 2019; Yang et al., 2019). Several PTMs occurring both in histone and non-histone proteins that are the focus of recent work interest in Aspergillus are listed in Table 2.

### Chromatin reader protein

The chromatin readers are important for epigenetic modification that could direct the PTM writers and erasers to the correct chromatin region (Yun et al., 2011). One of the most studied chromatin reader is SntB in Aspergillus, which was first found as a regulator of ST biosynthesis from a forward genetic screen in A. nidulans (Pfannenstiel et al., 2017). The A. nidulans SntB is a homologue of yeast E3 ubiquitin ligase, SNT2, which has five conserved domains for recognizing specific histone PTMs, including one bromo-adjacent homology (BAH) domain, one SANT domain, and three PHD (plant homeodomain) fingers. Two mutations were found in the BAH and led to the dysfunction of SntB in A. nidulans (Pfannenstiel et al., 2017). The deletion of A. nidulans sntB led to the decrease (e.g. chiorine, alternariol, microperfuранone) or increased (such as F-9775A/B) production of several SMs. The function of SntB was conserved in A. flavus (AFLA_029990), where the ΔsntB mutant fails to produce many SMs (AFB1, aflavarin, asparasone A, aflatem and kojic acid) but shows increase synthesis of other SMs such as leporine B and the cryptic BGC products aspergillicin A and aspergillicin F (Pfannenstiel et al., 2018; Greco et al., 2019). As a

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Fig. 2. Strategies to mine fungal secondary metabolites. The epigenetic induction methods include overexpression, deletion of the histone modifiers, treatment with histone remodelling agents, and co-cultures. The transcriptional manipulation, including overexpression of the pathway specific transcription factor (PSTF), global regulators, or deletion of negative regulators, serial promoter exchanges of the BGC genes promoters with inducible promoter, and CRISPR-mediated activation system (CRISPRa) can be used for activation of the cryptic BGCs. IP, inducible promoter; M, selective marker.© 2022 The Authors. Environmental Microbiology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Environmental Microbiology, 24, 2857–2881

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chromatin reader, SntB was involved in global histone modifications in the *A. flavus*, where the sntB deficient mutant was enhanced in both H3 acetylation and H3K4me3 (Pfannenstiel et al., 2018), indicating the hybrid roles of SntB in recognizing these two epigenetic PTMs in *Aspergillus*. A recent study of *Penicillium expansum* also revealed the conserved function of SntB (PEX2_110610) in filamentous fungi (Tannous et al., 2020), where deletion of PesntB led to several phenotype defects and decreased production of both patulin and citrinin. *P. expansum* SntB was found to positively regulate activation of three global regulators of SM and virulence (LaeA, CreA, and PacC), which could potentially explain the phenotype changes in the ΔsntB mutant.

Another two readers, heterochromatin protein 1 (HP1) and SppA are also reported in *Aspergillus* and other fungi. In *A. nidulans*, the deletion of the HP1 homologue (HepA) led to decreased levels of H3K9me3 in several BGCs (ST, isopenicillin A and terraquinone A), and, as a result, upregulated the expression levels of these BGC genes (Reyes-Dominguez et al., 2010). SppA, as a member of the COMPASS complex, is also a chromatin reader, and deletion of SppA (AO090003001570) in *A. oryzae* induced astellolide production by affecting the trimethylation status of H3K4 (Shinohara et al., 2016).

**DNA methylation**

DNA methylation at selected cytosine (5-mC) is an important epigenetic process that has been extensively studied in animals, plants and several fungi (Suzuki and Bird, 2008; Jeon et al., 2015). In animals and plants, DNA methylation is associated with the formation of heterochromatin and plays an important role in gene transcription regulation, transposable element silencing, genome imprinting, X-chromosome inactivation and development (Law and Jacobsen, 2010). DNA methylation has been reported in several model fungi, such as *Neurospora crassa* (Hosseini et al., 2020), *Candida albicans* (Russell et al., 1987), *M. oryzae* (Jeon et al., 2015) and *Uncinocarpus reesii* (Zemach...
et al., 2010), but it is absent in two model yeasts, Saccharomyces cerevisiae and Schizosaccharomyces pombe (Antequera et al., 1994; Profitt et al., 1984).

In N. crassa, genome-wide DNA methylation is regulated by a DNA methyltransferase (DMT) called Dim-2 (defective in methylation) (Kouzminova and Selker, 2001), which belongs to the Dmnt1 family (Zemach and Zilberman, 2010). A single DMT, DmtA, had been characterized in A. flavus and A. nidulans genomes (Lee et al., 2008; Yang et al., 2016b). However, although the A. flavus DmtA was reported to be involved in asexual development and AF production (Yang et al., 2016b; Zhi et al., 2017), DNA methylation levels are low or negligible in A. flavus and other Aspergillus spp. (Montiel et al., 2006; Liu et al., 2012). Studies using DNA methyltransferase inhibitors like 5-azacytidine (5 AC) reveal that treatment with this chemical alters the transcriptional regulation target to activate the SM production (Liu et al., 2020). Disruption of another COMPASS component Bre2 (CclA) in A. nidulans activated several silent BGCs (e.g. F9775A/F9775B) and showed a significant decrease of H3K4me2/3 (Bok et al., 2009). Deletion of cclA led to activation of the gliotoxin BGC and subsequent product in A. fumigatus (Palmer et al., 2013). Similar deletions also affected SM synthesis in other fungal genera (Studt et al., 2016a; Dallery et al., 2019).

H3K9 methylation

In A. fumigatus, the H3K4 methyltransferase AflSet1 also affects di-methylation of H3K9. In A. nidulans, the TF BrlA, required for asexual sporulation, was found to regulate production of developmental and vegetative SMs in a concordant fashion to LaeA (Lind et al., 2018). The deletion of laeA leads to a decrease of a modification related with euchromatin (H3K4me3) on the brlA promoter, while the heterochromatic mark H3K9me3 is greatly enriched (Lind et al., 2018). As mentioned earlier, LaeA activity has also been associated with decreased levels of H3K9me3 (Reyes-Dominguez et al., 2010). In general, H3K9 methylation can be considered an epigenetic mark for SM gene repression production in Aspergillus spp. and other fungi (Fig. 3).

H3K27 methylation

H3K27 methylation is another repressive mark for gene expression, which is catalysed by KMT6, a part of the polycomb repressive complex 2 (PRC2) in fungi (Erlendson et al., 2017). In Fusarium graminearum, about 30% of all expressed genes in the genome are covered by H3K27me3, many of them located in SM gene clusters and are turned on or upregulated when FgKMT6 is absent (Connolly et al., 2013). The deletion of kmt6 is lethal and causes metabolic dysfunction in Fusarium fujikuroi (Studt et al., 2016b). Although the repressive role of H3K27me3 in gene expression is established in many fungi, no H3K27 Methyltransferases or Kmt6 homologues or even the PRC2 complex have been identified in the genus Aspergillus.

H3K36 methylation

H3K36 methylation is most likely involved in transcriptional activation. Two methyltransferases Set2 and Ash1 in F. fujikuroi have been reported to be responsible for H3K36 methylation, each functioning specifically at euchromatic and subtelomeric regions of the genome, respectively (Janovska et al., 2018). Deletions of set2 and ash1 resulted in decreased production of gibberellins and increased production of fusarins and fusaric acid (Janovska et al., 2018).
Although the roles of H3K36 methyltransferases have not been studied in Aspergillus, H3K36me3 was found to accumulate at multiple active SM gene clusters in A. nidulans (Gacek-Matthews et al., 2015).

**H3K79 methylation**

Dot1 encodes a H3K79 methyltransferase in yeast, and its function is not clear in filamentous fungi yet. A dot1 homologue (AFLA_093140) in A. flavus functions as a...
regulator of pathogenicity and aflatoxin (AF) synthesis in A. flavus (Liang et al., 2017). However, it remains to be shown if Afldot1 possesses H3K79 methyltransferase activity, and how it regulates the AF BGC in A. flavus.

H4R3 methylation

Arginine residues are alternative targets for methylation, which can lead to gene activation or repression. Three histone 4 arginine methyltransferases have been identified and defined as RmtA, RmtB, and RmtC in A. nidulans (Trojer et al., 2004; Bauer et al., 2010). The homologue of RmtA in A. flavus was found to play a positive role in AF biosynthesis and sclerotia formation (Satterlee et al., 2016; Li et al., 2017).

Histone demethylase

Histone demethylases are responsible for removing the reversible methylation mark on histone tail lysines. In A.
nidulans, two Jumonji histone H3 demethylases KdmA (homologue of yeast Rph1) and KdmB (homologue of yeast Jhd2) have been identified, which de-methylate H3K36me3 and H3K4me3 (Gacek-Matthews et al., 2015; Gacek-Matthews and Berger, 2016), respectively. KdmA is a transcriptional regulator for primary and secondary metabolism genes that regulates methylation at specific loci in the genome. In kdmA mutants, levels of H3K36me3 were found to accumulate at regions in the penicillin and sterigmatocystin (ST) BGCs (Gacek-Matthews et al., 2015).

Unlike KdmA, KdmB plays a global role in regulation of SM gene expression. The deletion of kdmB in A. nidulans led to the dysregulation of 50% of known SM genes, that most of which showed decreasing expression levels (Gacek-Matthews and Berger, 2016). These findings suggest that KdmB is necessary for the normal activation of the majority of SM gene clusters (e.g. ST, emericellamides, and orsellinic acid) in A. nidulans (Gacek-Matthews and Berger, 2016). A study in A. flavus showed that the KdmB homologue, Rum1 (AFLA_006240) is required for AF biosynthesis (Hu et al., 2018).

Histone acetylation

Histone acetylation was first characterized in A. nidulans as playing an important role in regulating activation of BGCs (Shwab et al., 2007). In A. parasiticus, the transcriptional activation of AF biosynthetic pathway genes is positively correlated with the initiation and spread of histone H4 acetylation (Roze et al., 2007). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) add or remove acetyl groups on histones respectively, often on lysine residues (Fig. 4). Both activities are involved in gene activation/repression and are involved in multiple processes in filamentous fungi (Dubey and Jeon, 2017).

Histone acetyltransferases

GcnE is one of the most well-known histone acetyltransferases that acetylates many residues including H3K14, H3K18 and H3K27 in fungi (Kong et al., 2018) and belongs to the GCN5-related N-acetyltransferase (GNAT)-type family. Interestingly, several studies have shown that Streptomyces species can impact fungal growth and secondary metabolism via activating (GcnE in A. nidulans) or degrading (Gcn5 in Fusarium) this acetyltransferase (Nutzmann et al., 2011; Nützmann et al., 2013; Wang et al., 2021a). The GcnE homologue (AFLA_051420) in A. flavus is also required for the global acetylation of histone 3 and H3K14 acetylation (Lan et al., 2016). Deletion of gcnE leads to the loss of production AF by repressing the transcriptional activation of AF cluster genes (Lan et al., 2016).

The MYST-type (named after the founding members MOZ, YBF2/SAS3, SAS2, and Tip60) acetyltransferase

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family is another important HAT in fungi. Esa1 belongs to the MYST-type HAT and is a component of the NuA4 (nucleosomal acetytransferase of histone H4) complex, that acetylates several H4 lysine residues, including H4K5, H4K8, and H4K12 (Suka et al., 2001). In A. nidulans, overexpression of the esaA increases the production of ST, penicillin, terrequinone and orsellinic acid, coupled with enhanced acetylation of H4K12 at these BGCs loci (Soukup et al., 2012).

Rtt109 is a member of the P300/CBP acetyltransferase family, which is responsible for the acetylation of histone 3 lysine 56 (H3K56) (Han et al., 2007). In many plant-pathogenic fungi, Rtt109 is reported to play vital roles in DNA damage repair, fungal development and pathogenicity by acetylating H3K56 (Han et al., 2007; Cai et al., 2018). A Rtt109 homologue (AFLA_098450) has been identified in A. flavus and shown to be required for AF biosynthesis (Sun et al., 2021). The deletion of rtt109 in A. flavus shows a significant reduction in total acetylation of histone 3 and H3K56 acetylation (Sun et al., 2021). Intriguingly, H3K9 is also an acetylated target of Rtt109.

Histone deacetylases

Histone acetylation is generally involved in the activation of BGCs, but the loss of acetylation does not necessarily result in silencing of BGCs and in fact can also activate some BGCs (Albright et al., 2015). Deacetylation is conferred by HDACs, which can be grouped into three classes in fungi (Pfannenstiel and Keller, 2019).

Class I-Rpd3 HDACs. Shwab et al. was the first study to show HDAC inhibitors (trichostatin A, TSA) can induce production of fungal natural products (Shwab et al., 2007). Since then, several studies have used TSA to increase production of fungal SMs (Zutz et al., 2013; Albright et al., 2015; Aldholm et al., 2020). However, in addition to activating some BGCs, HDAC inhibitors and even HDAC gene suppression can inhibit expression of other BGCs (Albright et al., 2015). Class I-Rpd3 HDACs are TSA targets and are well conserved in eukaryotes. The yeast Rpd3 homologue in A. nidulans (rpdA, AN4493) is essential for growth and development (Tribus et al., 2010). The metabolome of an A. nidulans rpdA knockdown mutant showed great similarities with the treatment by the HDAC chemical inhibitor suberylanilide hydroxamic acid (SAHA) where some SMs were upregulated and some down regulated (Albright et al., 2015; Henke et al., 2016). Three Class I HDACs, RpdA (AFLA_092360), Rpd3 (RpdA-like, AFLA_025120) and HosA (AFLA_087410) have been characterized in A. flavus where HosA plays a crucial role in AFB1 biosynthesis (Lan et al., 2019). An in vitro analysis revealed that HDAC activity of HosA was considerably inhibited by TSA in A. flavus (Lan et al., 2019). The deletion of hosA leads to the significant increase in acetylation levels of H4 and, particularly, H4K16. The homologue of HosA in A. oryzae, HdaD/AoHos2, is important for fungal development and kojic acid production in this species (Kawauchi et al., 2013; Kawauchi and Iwashita, 2014).

Class II-Hda1 HDACs. HdaA belongs to the HDA1-Class II HDAC family, which contributes to the majority of HDAC activity in A. nidulans (Tribus et al., 2005). The inactivation of hdaA led to the accumulation of the production of some metabolites (ST, penicillin) and the upregulation of BGC expression in A. nidulans (Shwab et al., 2007). The homologue of HdaA (Afu5g01980) in A. fumigatus is also involved in regulation of several secondary metabolites including gliotoxin and some other unknown SMs (Lee et al., 2009). Although HdaA is required for the majority of HDAC activity in A. nidulans, the global acetylation of histone 3 did not show a difference when hdaA was deleted in A. fumigatus. The function of HdaA (AFLA_006160) was also studied in A. flavus, which, like many other histone modifying enzymes was found to play a positive role in AF production and sclerotia formation (Lan et al., 2019). The function of the other Class II HDACs, HosB (AFLA_114920) was also studied in A. flavus, which indicates that HosB is dispensable for fungal development and AF production in this fungus (Lan et al., 2019). It seems that HosB plays little role in HDAC activity in fungi (Studt et al., 2013).

Class III-Sirtuin HDACs. Sirtuins are the last class of HDACs (or called Class III HDACs) and are NAD⁺ dependent. HstA (AN11067) was the first NAD⁺-dependent sirtuin studied in A. nidulans. Deletion of the gene had minimal effects on the production of norsolorinic acid and penicillin (Shwab et al., 2007). A. nidulans SirA (AN10449) is the homologue of S. cerevisiae sirtuin Hst1 and uses NAD⁺ as a cosubstrate to deacetylate H4K16 in the promoter regions of several BGCs (e.g. penicillin and ST). Deletion of sirA increases the production of these metabolites (Shimizu et al., 2012). SirE (AN1226) is another sirtuin HDAC that has been studied in A. nidulans, which functions in regulation of both primary and secondary metabolism by deacetylating the H3K9ac, H3K18ac and H3K56ac during different growth phases (Itoh et al., 2017). The function of the A. nidulans SirE homologue in A. oryzae, HstD (AO090038000370), was found to regulate development and SM biosynthesis via the regulation of the global regulator of secondary metabolism LaeA (Kawauchi et al., 2013).
Post-translational regulation of secondary metabolite biosynthesis beyond histone modification

Acetylation of nonhistone proteins

Lysine acetylation is also widely distributed in many nonhistone proteins in both prokaryotes and eukaryotes where acetylation can impact transcriptional regulation, enzymatic activity and cellular metabolism (Zhang et al., 2013; Li et al., 2019). Recently, a global comparative acetyome was studied in three human fungal pathogens, *A. fumigatus*, *Cryptococcus neoformans* and *C. albicans*, of which 2312 lysine acetylated (Kac) proteins were identified in *A. fumigatus* (Li et al., 2019). The conservative acetylated orthologues in these three fungal pathogens were found enriched in core biological processes (e.g. TCA, ribosome and histone modifications), while the distribution of acetylation on the secondary metabolites pathways was not mentioned in this study. In *A. flavus*, the acetylome has been performed in two studies (Lv, 2017; Yang et al., 2019). More than 1300 lysine acetylation sites were identified in hundreds of *A. flavus* proteins. Lv (2017) reported the acetylation of AF biosynthetic pathway enzymes AfIA (AFLA_139380) and AfIB (AFLA_139370) in *A. flavus* (Lv, 2017), while Yang et al. found that AfIK (AFLA_139190), AfIM (AFLA_139300), AfIO (AFLA_139220, formerly OmtB) and AfIP (AFLA_139210) were also acetylated in the AF biosynthetic pathway (Yang et al., 2019) (Fig. 6). To discern a role for Kac of AfIO (a methyltransferase involved in conversion of demethylsterigmatocystin to sterigmatocysin (Yu et al., 2000), a $afIO^{K384C}$ mutant that mimics the non-acetylated state failed to produce AF, indicating that acetylation of AfIO is important for AF biosynthesis. Additionally, 42 Kac proteins from 30 different BGCs were identified including those involved in synthesis of cyclopiazonic acid and imizoxquin (Fig. 5). These data suggest the potential global regulatory role of non-histone acetylation regulation on secondary metabolism.

Protein phosphorylation

Phosphorylation is one of the most studied PTMs that usually occurs at serine (S), threonine (T), and tyrosine (Y) residues. The reversible protein phosphorylation is catalyzed by a kinase and a phosphatase. The two best known phosphorylation pathways are the cAMP-dependent protein kinase A (cAMP-PKA) signalling pathway and mitogen-activated protein kinase (MAPK) cascades, both of which have been reported to be involved in multiple cellular processes and secondary metabolism in Aspergillus.

CAMP-protein kinase A signalling pathway

The cAMP-PKA pathway is a conserved signalling cascade in eukaryotic organisms, which utilizes cAMP as a second messenger to regulate a wide variety of biological processes. In *Aspergillus*, the cAMP-PKA pathway controls the responses to multi extracellular stimuli (e.g. oxidative agents, osmotic stress, pH changes and thermal stress), fungal development and infection (Liebmann et al., 2003; Grosse et al., 2008; Yang et al., 2016a). This pathway is also important for the regulation of mycotoxin biosynthesis in *Aspergillus*, which negatively regulates ST/AF biosynthesis in *A. parasiticus* and *A. nidulans* (Shimizu and Keller, 2001; Roze et al., 2004) (Fig. 7). In the ST BGC gene cluster, the activity of AfIR is inhibited by PkaA via phosphorylation in *A. nidulans* (Shimizu et al., 2003). We also found that the deletion of genes involved in cAMP/PKA pathway, such as the G protein alpha-subunit-encoding gene fadA (Yang et al., 2022) or adenylate cyclase encoding gene acyA (Yang et al., 2016a), leads to an alteration in AF production by regulating cAMP synthesis and PKA activity in *A. flavus*. There are 15 putative GPCRs identified in *A. flavus*, among which the gpra and gprP deletion mutants produce much more AF compared to the wild type (Affeldt et al., 2014). In *A. nidulans*, GprH, GprM and Gprl are found to regulate fungal development and mycotoxin production by functioning upstream of cAMP-PKA signalling (Dos Reis et al., 2019). In *A. fumigatus*, the deletion of a regulator of G protein signalling (RGS) domain protein, RgsC, exhibits defective gliotoxin production and decreased virulence in *Galleria mellonella* (Kim et al., 2017), while $\Delta$rgsA and $\Delta$rgsD mutants are increased in gliotoxin production, both of which show elevated PKA catalytic activity in this fungus (Kim et al., 2019; Lwin et al., 2019).

The cAMP-PKA pathway is also reported to be involved in regulation of epigenetic processes in fungi (Kang et al., 2015; Bao et al., 2022). The sirtuin HDAC Sir2 is phosphorylated through cAMP-PKA signalling and casein kinase 2, downstream of cAMP-PKA signalling, leading to loss of activity of Sir2 and increased acetylation of H4K16 in the promoter regions of its target genes (Kang et al., 2015). A recent study in yeast demonstrated that mutations in genes of cAMP signalling inhibit the heterochromatin defects linked to overexpression of Epe1, an enzyme that removes histone modifications associated with heterochromatin (Bao et al., 2022). In *M. oryzae*, the addition of cAMP could restore the defects of infection-related morphogenesis in the deletion mutant of H3K4 methyltransferase Moset1 (Pham et al., 2015). In *A. flavus*, the cAMP signalling-induced AFB1 biosynthesis is found to have a closed relationship with the HDAC, HosA (Lan et al., 2019).

MAPK pathways are composed of three conserved kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK)
kinase kinase (MAPKK) and MAP kinase (MAPK) that function in environmental signal transduction in eukaryotes. The three main MAPK-mediated signalling pathways, known as Fus3, Hog1 and Slt2-MAPK in yeast, have been identified and characterized in filamentous fungi as well, among which Slt2-MAPK regulates cell wall integrity, Fus3-MAPK involves in the pheromone response and Hog1-MAPK regulates the high osmolarity response (Turrà et al., 2014). These MAPK signal transduction pathways, Slt2, Fus3 and Hog1, have been well studied in Aspergillus, orthologous to MpkA, MpkB and HogA, respectively. Intriguingly, the MAPK protein of Hog-MAPK cascade is duplicated in A. fumigatus and A. nidulans, both of which possess HogA/SakA and MpkC, and triplicated in A. oryzae and A. niger, that all have HogA/SakA, MpkC, and MpkD (Hagiwara et al., 2016). In A. fumigatus, the disruption of pheromone module-MpkB pathway led to decreased levels of several secondary metabolites (e.g. gliotoxin, pseudotrin A, pseudotrin D, pyripyropene A) (Frawley et al., 2020). The MpkB pathway is also shown to negatively regulate the activation of secondary metabolite gene clusters, including ST genes in A. nidulans, and the state of MpkB phosphorylation is positively regulated by HamE, a ham (hyphal anastomosis) protein characterized as a scaffold for the MAK-2 cascade (Frawley et al., 2018). An early study of A. nidulans showed that AnFus3/MpkB is involved in the regulation of secondary metabolite production by phosphorylating VeA (Bayram et al., 2012), a member of the conserved fungal velvet complex linking secondary metabolism and fungal development with light. Four phosphorylation sites are identified in A. nidulans VeA that affect the interaction between VeA and VelB or FphA (Rauscher et al., 2016). In turn, mutation of these sites impacted ST synthesis. The studies in A. flavus demonstrated that MpkA and HogA pathways act as negative regulators of AF biosynthesis. The A. flavus sakA/hogA mutant produces more AF than WT (Tumukunde et al., 2019). The deletion of the MAPK kinase, Slt2/MpkA (AFLA_052570), or loss of phosphorylation of Slt2 lead to a severe growth defect, but a significant increase in AF production(Zhang et al., 2020a,b) (Fig. 7). However, it remains unclear how these MAPK pathways regulate AF biosynthesis. One of the explanations for the regulation of Slt2 on AF production is that the Slt2 protein might regulate the activity of a sirtuin HDAC such as Sir2 by phosphorylation (Ray et al., 2003; Zhang et al., 2020b) (see ‘cAMP-protein kinase A signalling pathway’ section). The MpkB/Fus3 pathway is also required for AF production (Qin et al., 2020; Yang et al., 2020).
Studies in yeast show that MAPK pathways are related to chromatin remodelling as well (Mas et al., 2009). An early study reported by De Nadal et al. (2004) showed that MAPK Hog1 recruits histone deacetylase Rpd3 to activate osmo-responsive genes (De Nadal et al., 2004). Mas et al. showed that MAPK Hog1 directs recruitment of a chromatin-remodelling complex to the ORF of osmo-responsive genes during stress in yeast (Mas et al., 2009). Hog1 phosphorylates the nucleolar protein Net1, altering its affinity for the phosphatase Cdc14, whose activity is essential for mitotic exit and completion of the cell cycle (Tognetti et al., 2020). A genetic analysis in yeast reveals that phosphorylation at histone residues H4-S47 and H4-T30, catalysed by the kinases Cla4 and Ste20, PAK2, and the Ste11 MAPK are important for response to osmotic and heat stress, respectively (Viéitez et al., 2020).

Two-component systems

The TCS (also known as His-Asp phosphorelay signalling) system is also a common signal transduction cascade in both prokaryotes and eukaryotes, which consists of two components in the former, a histidine kinase (HK) and a response regulator (RR) in bacteria, while a histidine-containing phosphor-transmitter (HPt) is required additionally in eukaryotes (Fig. 7). The His-Asp phosphorelay systems are mainly involved in regulation of fungicidal susceptibility, conidial development and virulence in fungal pathogens (Viaud et al., 2006; Rispail and Di Pietro, 2010). A total of 15 HK genes are identified both in A. nidulans and A. oryzae by genomic analyses (Hagiwara et al., 2016), indicating that the TCS regulation networks are more complex in Aspergillus or other filamentous fungi. In yeast, the TCS system functions in the upstream of HOG MAPK cascade. A study of histidine kinases in A. nidulans showed that the HK gene fphA is required for the phosphorylation of SakA/HogA during heat sensing (Yu et al., 2019). A recently study in A. nidulans demonstrated that a putative sensor HK VadJ coordinated the balance between sexual and asexual development and negatively regulated ST production (Zhao et al., 2021). In A. flavus, the response regulators Skn7 are also showed to play roles in regulating oxidative stress response and AF biosynthesis (Zhang et al., 2016a). The deletion of a phytochrome that functions as a hybrid histidine kinase in the Monascus pigments, monacolin K and citrinin production (Xiong et al., 2021).

Target of rapamycin kinase

The serine–threonine kinase is the target of rapamycin (TOR) and regulates cell growth and stress signalling in eukaryotes. A single TOR kinase TorA was characterized in A. nidulans, which contained four phosphosites during growth and three of them were dephosphorylated under oxidative stress (Carrasco-Navarro and Aguirre, 2021). The TOR signalling pathway regulates phosphorylation of the Ser/Thr kinase Sch9, a substrate of TOR, to control fungal development and mediate multiple stresses responses as well as mycotoxin synthesis in some fungal pathogens (Gu et al., 2015; Alves de Castro et al., 2016). An early study in Fusarium fujikuroi demonstrated that the TOR kinase was involved in nitrogen regulation of secondary metabolism such as gibberellins and bikaverin (Teichert et al., 2006). The TOR pathway can also cooperate with cAMP/PKA signalling and the MAPK cascades and function in fungal growth and virulence of A. fumigatus. The deletion of schA in A. fumigatus increase the phosphorylation of SakA that responses to hyperosmotic stress (Alves de Castro et al., 2016). In A. flavus, the inhibition of TOR kinase by rapamycin suppresses the glutamine inducing effect on AFs production (Wang et al., 2017). However, it remains unclear whether TOR could regulate AF biosynthesis directly or cooperates with other signalling cascades like cAMP/PKA signalling and the MAPK cascades in Aspergillus.

Protein phosphorylation involved in metabolic pathways

Reversible protein phosphorylation also regulates many important metabolic pathways. A phosphoproteome study of the A. fumigatus wild-type strain and mpkA and sakA deletion mutants showed a dynamic changes of phosphopeptides when exposed to the antifungal drug caspofungin, which are mainly enriched in transcription factors, protein kinases, and cytoskeleton proteins (Mattos et al., 2020). Proteomic analysis of these two MAPK mutants suggested proteins involved in secondary metabolism (e.g. AFUA_1G10320, AFUA_1G10330, AFUA_1G10350 and AFUA_1G10360 in the nidulanin-like BGC, and AFUA_8G00410 in the fumagillin/pseurotin BGC) were also impacted. Another global phosphoproteomic analysis was also studied in A. flavus, of which 283 phosphoproteins were identified in this fungus (Ren et al., 2016). The identified phosphoproteins were found involved in signal transduction and metabolic pathways known to impact AF biosynthesis. The enzymes that are involved in primary metabolites, e.g. glycolysis/gluconeogenesis and pentose phosphate pathway were phosphorylated. These pathways are important for the synthesis of acetyl-CoA that is the primary metabolite used for AF biosynthesis. The peroxisome protein Pex19 was also found phosphorylated in A. flavus. The first few precursors of AF and ST appear to be synthesized in the peroxisome (Kistler and Broz, 2015). These results indicate the association between the reversible phosphorylation and mycotoxin biosynthesis. Although the
phosphopeptides were not identified among the AF synthetic enzymes, 16 phosphorylated proteins were distributed in 14 different BGCs, including some known SMs such as leporin B and the sclerotia related-secondary metabolite, aflavarin (Fig. 5).

In A. nidulans, over 1964 proteins were characterized phosphorylated, among which the reversible phosphorylated state of 131 proteins were influenced by the oxidative stress agent hydrogen peroxide (H2O2) (Carrasco-Navarro and Aguirre, 2021). Additionally, the phosphorylation of critical components of MAPK, and TOR signalling as well as the proteins that involved in the regulation of primary and secondary metabolism were found affected by H2O2 in A. nidulans. A system-wide characterization of protein phosphatases (PPs) in Trichoderma reesei showed that some of these PPs were involved in regulation of specific cellulase activity and protease production (Rodriguez-Iglesias and Schmoll, 2019). The differences in the secreted SM patterns between light and darkness were observed in the Δpp mutants. However, it remains unknown how PPs regulate SM biosynthesis or other metabolites production.

Succinylation
Protein succinylation (su) is the addition of a succinyl group to a specific lysine residue of a protein occurring both in prokaryotes and eukaryotes. Succinylation has been found in both non-histone and histone proteins, which is involved in controlling various cellular processes (Xie et al., 2012; Weinert et al., 2013). Recently, a global analysis of lysine succinylome was studied in A. flavus, and a total number of 349 succinylated proteins were identified in this fungus, which were found involved in diverse biological processes, especially for the AF biosynthesis-related process (Ren et al., 2018). In the enzymatic cascade of AF synthesis, AfIE (AFLA_139310), AfIK (AFLA_139190), and AfIM (AFLA_139300) were found succinylated (Fig. 6). The role of the lysine succinylation at the norsolorinic acid reductase NorA (AfIE) was analysed and showed that succinylation at K370 is important for the activity of AfIE. From the published data, another 24 Ksu proteins were distributed in 20 different BGCs, such as cyclopiazonic acid (Fig. 5). Accumulating evidence suggests that succinylation on lysine overlaps with other PTMs, such as acetylation and malonylation, to function in metabolic pathways and other cellular processes (Xie et al., 2012; Weinert et al., 2013). Intriguingly, 20 of the 26 identified succinylated proteins involved in BGCs synthesis pathway are all acetylated (Fig. 5), and they do share the same modification site at lysine. In the model medicinal mushroom Ganoderma lucidum, lysine succinylation is also related to many secondary metabolites, such as pharmacologically bioactive compounds (Wang et al., 2019). A total of 47 succinylated enzymes were found to be involved in biosynthesis of triterpenoids and polysaccharides in G. lucidum. These data suggest that PTMs at the SM synthetic enzymes could alternatively regulate the SMs biosynthesis when fungi response to different environmental stimulus.

Ubiquitin and ubiquitin-like modification

Ubiquitin
Ubiquitination is known for its role in proteasome-dependent protein degradation. The process involves three enzymes including an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). An ubiquitinated protein is the target of ubiquitin, a small conserved protein with 76 amino acids that attaches to the lysine residues of target proteins. In addition to degradation, ubiquitination is also found involved in the regulation of protein activity and subcellular localization. The function of a HECT (homologous to E6AP C-terminus) ubiquitin ligase called HulA has been studied in several Aspergilli. In A. oryzae, HulA regulates the endocytosis of a maltose permease, MalP, which is important for alpha-amylase production (Hiramoto et al., 2015). The cullin-RING ligases (CRLs) comprise the largest family of E3 ligases, among which, the SCF (Skp1/Cullin-1/F-box) ligases are the best-studied examples (Frawley and Bayram, 2020a). In SCF complex, Skp1 (S-phase kinase-associated protein 1) functions as the adaptor between the cullin scaffold and the F-box substrate receptor. There are approximately 70 F-box protein-encoding genes predicted in A. nidulans genome (von Zeska Kress et al., 2012). One of the F-box protein GrrA (Fbx29) is involved in meiosis and sexual spore formation of ascospores in A. nidulans (Krappmann et al., 2006). Other F-box proteins like Fbx15 and Fbx23 are found required for sexual development in this fungus (Krappmann et al., 2006).

The reversible ubiquitination also regulates carbon catabolite repression (CCR) of Aspergillus and other filamentous fungi. In CCR, the activity of the transcription factor CreA is regulated by ubiquitination modification to some extent, which is ubiquitinated by CreD-HulA ubiquitination ligase complex, and deubiquinated by the CreB-CreC deubiquitination (DUB) complex, leading to its activation (Alam et al., 2017; Nie et al., 2018). In A. nidulans, 22 putative deubiquitinating enzymes were identified, and a defect of one of the DUB members, UspA, leads to the delayed formation of developmental structures and altered secondary metabolism (Meister et al., 2019). The presence of UspA, together with Fbx23, leads to degradation of VeA, which is a key regulator of

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fungal development and SMs in *Aspergillus*. To identify some new SMs in *A. nidulans*, the disruption mutant of a gene that encodes the small ubiquitin-like protein SUMO was generated and led to the characterization of asperthecin (Szewczyk *et al.* 2008).

**Ubiquitin-like modification**

In addition to ubiquitination, several ubiquitin-like modifications are found as well, which share similar mechanism to attach to the lysine residue of target proteins. These ubiquitin-like modifications, which include sumoylation and neddylation, regulate various cellular processes, including normal growth, signal transduction, and secondary metabolism.

**Sumoylation**. Sumoylation is the attachment of the small ubiquitin-like modifier protein SUMO to target proteins, which regulates protein interactions and activity and involves in many key cellular processes. As mentioned in ‘Ubiquitin’ section, the ΔsumO mutant in *A. nidulans* dramatically increased in the production of asperthecin, it was also shown to be important for the expression of the austino/dehydroaustinol and sterigmatocystin BGCs (Szewczyk *et al.*, 2008). Recently, a SUMOylome study of *A. nidulans* showed that a total number of 149 SUMOylated proteins were identified in this fungus, which are predicted to be involved in transcription, RNA processing and DNA replication (Horio *et al.*, 2019). In *Aspergillus*, a key sumoylation pathway is catalysed subsequently by three enzymes, E1 (activating enzyme; UbaB/AosA), E2 (conjugation enzyme; UbcN), and E3 (ligases; SizA, SizB, and MmsU), which are indicated in Table 3. The SumO isopeptidases UlpA/UlpB are responsible for removing SumO from the sumoylated proteins. In *A. nidulans*, The E1 enzymes AosA and UbaB and the sole E2 enzyme UbcN are required for multicellular development (Harting *et al.*, 2013). The mmsU deletion mutant exhibited defects of growth and conidiation, while the other two E3 ligases SizA and SizB are dispensable in this fungus (Horio *et al.*, 2019). The SumO isopeptidase UlpA and SumO processing enzyme UlpB are involved in asexual and sexual development as well in *A. nidulans*. The function of sumoylation is also reported in *A. flavus*. The deletion of *AfsumO* leads to defects of growth and conidiation and decreased production of AF (Nie *et al.*, 2016).

**Neddylation.** Neddylation is another ubiquitin-like modification that attaches the NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) polypeptide to lysine residues of target proteins. Neddylation is important for fungal growth and secondary metabolism (Köhler *et al.*, 2019) and has a closed relationship with ubiquitination that could regulate the activity of the SCF E3 ligase complex by neddylyating the cullin-1 protein at a lysine residue (Deshaies *et al.*, 2010). The neddylylated cullin proteins can be deneddylylated by the COP9 signalosome (CSN), a conserved complex with eight subunits. The deneddylyated cullin proteins are then blocked by Cand1 (Cullin-associated-Nedd8-dissociated protein 1) that attaches to the Neddb binding site with the N-terminal domain. The Cand1 homologue CandA was a trimeric complex in *A. nidulans* and a dimeric complex in *A. fumigatus*. In *ΔcandA-C1* mutant, *A. nidulans* accumulated more production of many SMs such as austino, dehydroaustinol, asperthecin and emericellin (Köhler *et al.*, 2019). The deletion mutants of the genes encoding CSN subunits CsnA, CsnB, CsnD, or CsnE in *A. nidulans* produce aberrant red pigment and can initiate the sexual cycle and develop primordia but are blocked in maturation to sexual fruit bodies (Busch *et al.*, 2003; Busch *et al.*, 2007). The CSN complex of *A. nidulans* also plays a dual role during development, which functions early for protection against oxidative stress and hormone regulation and is critical for regulation of secondary metabolism in the later growth stage (Nahlik *et al.*, 2010). CSN can interact with the deneddylyase A/1 (DenA/Den1) in fungi and humans, which targets DEN1/DenA for protein degradation (Christmann *et al.*, 2013). The activity of DenA in *A. nidulans* is required for asexual development and reduces sexual fruiting body formation in light.

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**Table 3. SUMO and SUMO pathway genes in *A. nidulans* and *A. flavus***

| Pathway component | *A. nidulans* | Gene name | *A. flavus* | Gene name |
|-------------------|---------------|-----------|-------------|-----------|
| SUMO              | AN1191        | sumO      | AFLA_068730 |           |
| SumO activating enzyme E1 | AN2450        | ubaB      | AFLA_106510 |           |
| SumO conjugating enzyme E2 | AN2298        | aosA      | AFLA_048570 |           |
| SumO E3 ligases  | AN4399        | ubcN      | AFLA_113060 |           |
| SumO Isoproteidases | AN10822        | sizA      | AFLA_054730 |           |
|                   | AN4497        | sizB      | AFLA_092420 |           |
|                   | AN10240       | mmsU      | AFLA_036350 |           |
|                   | AN2689        | ulpA      | AFLA_005900 |           |
|                   | AN8192        | ulpB      | AFLA_005920 |           |

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however, the deletion of both deneddylases (DenA and CSN) impairs multicellular fungal development (Christmann et al., 2013).

**Palmitoylation**

Palmitoylation is a reversible lipid modification on the side chain of cysteine residues of target proteins by protein S-acetyltransferases (PATs). Palmitoylation plays important roles in various cell functions, physiology and pathophysiology (Zhang et al., 2016c). In A. nidulans, the function of a palmitoyl transferase AkrA was studied, which regulates Ca\(^{2+}\) homeostasis by palmitoylating its protein substrates (Zhang et al., 2016c). A total amount of 234 palmitoylated proteins are identified in A. fumigatus conidia, which includes several G-protein subunits and Rho GTPases that are found palmitoylated in other organisms. In this fungus, four enzymes involved in early melanin synthesis were found strongly palmitoylated during asexual development, and this lipid modification links with the specific subcellular compartments of these palmitoylated melanin enzymes (Upadhyay et al., 2016).

**Other PTMs**

Besides the modifications mentioned above, there are still some other PTMs, including protein glycosylation, farnesylation, prenylation, S-nitrosylation and some other ubiquitin-like modifications (e.g. urmylation, and pupylation), and N-myristoylation, that have been identified and shown to play roles in membrane targeting, fungal growth, development, cell wall synthesis and virulence in Aspergillus and other fungi (Fang et al., 2015; Liu et al., 2021). S-nitrosylation has been shown to regulate styrylpyrene biosynthesis in Inonotus obliquus (Zhao et al., 2016) and prenylated secondary metabolites are found in many fungi (Yu et al., 2011; Genovese et al., 2015).

**Crosstalk between different PTMs on SM expression**

Fungal secondary metabolism is, not surprisingly, regulated by combinations and interactions (e.g. crosstalk) of different modifications, especially between histone modifications. An interplay between H3K4 methylation and H3K9 methylation in A. nidulans, both of which are all decreased in the deletion mutant of deletion of the COMPASS component Bre2/CclA, leads to the activation of cryptic secondary metabolite clusters in this fungus (Bok et al., 2009). In A. flavus, the acetylation of H3K56 and H3K9 is found co-regulated by a member of the P300/CBP acetyltransferase family, Rtt109, which is required for AF biosynthesis (Sun et al., 2021). The crosstalk between methylation and acetylation at H3K4 and H3K36 for transcriptional activation (Martin et al., 2006; Ginsburg et al., 2014) is another example to provide the valuable information of the interaction between different histone modification. A recent study in F. graminearum showed that TOR inactivation promotes the degradation of Gcn5 by ubiquitination, which reduces acetylation level of Atg8, leading to the translocation of deacetylated Atg8 into the cytoplasm and subsequent induction of fungal autophagy (Wang et al., 2021a).

There is also interplay between different PTMs at non-histone proteins. For example, as mentioned earlier, acetylation and malonylation or succinylation of the same lysine residues is involved in the regulation of metabolic pathways and other cellular processes (Xie et al., 2012; Weinert et al., 2013). In A. flavus, we found that 20 of the 26 identified succinylated proteins associated with SMs biosynthesis are all acetylated (Fig. 5). In the AF biosynthetic pathway, AflK and AflM are both succinylated and acetylated (Fig. 6A). The modification on the specific lysine residues on these AF related enzymes is important for their activities and subsequent AF synthesis (Ren et al., 2018; Yang et al., 2019). Although it remains unclear whether these overlapping PTMs on the enzymes could affect each other, the dynamic changes of different modifications at these sites could alternatively regulate the SMs biosynthesis when fungi response to different environmental stimulus.

**Identification and prediction of PTM**

To identify the specific PTM sites, many experimental methods have been performed, among which immunoaffinity enrichment combined with MS-based proteomics approach is often used to measure the PTM-modified proteins (Liu et al., 2021). By combining use of high-sensitivity mass spectrometry analysis with the gel-based or gel-free approaches, like chemical labeling or immunoaffinity enrichment approaches, the current catalogue of observed PTMs is expanding in fungi (Fig. 8). These PTM detection approaches have been successfully used for identifying sites of phosphorylation, acetylation, and succinylation of many fungi. For instance, high accuracy nano-LC–MS/MS allowed for analysis of global lysine succinylation, contributing to the knowledge of regulation of various metabolic processes including AF biosynthesis in A. flavus (Ren et al., 2018).

Since the motif sequence of the PTM site is conserved in most PTMs, many algorithms have been applied to predict the PTM sites. Based on the published phosphorylation proteomics of several fungi including A. flavus,
A. nidulans, F. graminearum, Bai et al. established the Fungi Phosphorylation Database (FPD) that can serve as a comprehensive protein phosphorylation data resource for further fungal studies (Bai et al., 2017). Some other bioinformatics programmes, such as NetPhos, GPS and Scansite, are also used to predict phosphorylation sites (Table 4). Methylated sites can be predicted by GPS-MSP, acetylation sites and proteins can be predicted by NetAcet and GPS-PAIL, succinylation sites can be predicted by HybridSucc, sumoylated sites can be predicted by GPS-SUMO and SUMOplot (Table 4). Although the bioinformatics prediction is useful for identification of specific PTM sites, it is not enough for understanding their global roles in biological mechanisms. Other approaches, such as the information of protein–protein interaction (PPI) and protein structure, could help to identify PTM site and their roles in biological processes.

Future prospects

It has been more than 10 years since the first published research on epigenetic (histone acetylation) regulation of secondary metabolism in fungi (Shwab et al., 2007). Rapid progress in mining new fungal SMs has been made with the applications of PTMs on chromatin remodelling, which provides powerful tools to activate silent BGCs in fungal genome. Thus far, the research of regulation fungal SMs is mainly focused on transcriptional regulation and chromatin modifications (histone

**Table 4.** The available tools for specific PTM and site prediction.

| PTM type        | Tool              | Website                                      | Function                                                                 |
|-----------------|-------------------|----------------------------------------------|--------------------------------------------------------------------------|
| Methylation     | GPS-MSP           | [http://msp.biocuckoo.org/online.php](http://msp.biocuckoo.org/online.php) | Prediction of general or type-specific methylline and methylarginine residues in proteins |
| Acetylation     | NetAcet-1.0       | [https://services.healthtech.dtu.dk/service.php?NetAcet-1.0](https://services.healthtech.dtu.dk/service.php?NetAcet-1.0) | Prediction of N-terminal acetylation in eukaryotic proteins                |
|                 | GPS-PAIL          | [http://pail.biocuckoo.org](http://pail.biocuckoo.org) | Prediction of acetylation substrates and sites of HATs                    |
| Phosphorylation | FPD               | [http://bis.zju.edu.cn/FPD/](http://bis.zju.edu.cn/FPD/) | Fungi phosphorylation database                                            |
|                 | NetPhos-3.1       | [https://services.healthtech.dtu.dk/service.php?NetPhos-3.1](https://services.healthtech.dtu.dk/service.php?NetPhos-3.1) | Prediction of Generic phosphorylation sites in eukaryotic proteins         |
|                 | GPS               | [http://gps.biocuckoo.cn](http://gps.biocuckoo.cn) | Prediction of kinase-specific phosphorylation sites                      |
|                 | Scansite          | [http://scansite.mit.edu](http://scansite.mit.edu) | Prediction of phosphorylation sites                                       |
| Succinylation   | HybridSucc        | [http://hybridsucc.biocuckoo.org](http://hybridsucc.biocuckoo.org) | Prediction of lysine succinylation site                                  |
| Ubiquitination  | iUbiquin-Lys      | [http://www.jci-bioinfo.cn/iUbiquin-Lys](http://www.jci-bioinfo.cn/iUbiquin-Lys) | Prediction of lysine ubiquitination sites in proteins                    |
|                 | UbiNet            | [http://140.138.144.145/~ubinet/index.php](http://140.138.144.145/~ubinet/index.php) | Prediction of E3 ligase-mediated ubiquitination networks                |
| Sumoylation     | GPS-SUMO          | [http://sumosp.biocuckoo.org/online.php](http://sumosp.biocuckoo.org/online.php) | Prediction of sumoylated sites                                            |
|                 | SUMOplot          | [https://www.abcepta.com/sumonlot](https://www.abcepta.com/sumonlot) | Prediction of sumoylated sites                                            |
| Palmitoylation  | CSS-Palm          | [http://csspalm.biocuckoo.org/online.php](http://csspalm.biocuckoo.org/online.php) | Prediction of S-palmitoylation proteins                                  |
|                 | TermiNator        | [https://bioweb.i2bc.paris-saclay.fr/terminator3/](https://bioweb.i2bc.paris-saclay.fr/terminator3/) | Prediction of N-terminal acetylation and S-palmitoylation of proteins    |
|                 | SwissPalm         | [http://swisspalm.epfl.ch](http://swisspalm.epfl.ch) | Prediction of S-palmitoylation proteins                                  |
|                 | SeqPalm           | [http://lishuyan.lzu.edu.cn/seqpalm/index](http://lishuyan.lzu.edu.cn/seqpalm/index) | Prediction of S-palmitoylation proteins                                  |
|                 | GPS-SNO           | [http://sno.biocuckoo.org](http://sno.biocuckoo.org) | Prediction of S-nitrosylation site                                       |
methylation and histone acetylation). However, recent studies on non-histone protein modifications have indicated the potential roles of PTMs on regulation of fungal secondary metabolism. Understanding the important roles of the non-histone PTMs, such as succinylation, ubiquitin-like modifications, palmitoylation, and others, need more extensive studies in *Aspergillus* and other fungi.

Additionally, some other PTMs, such as S-nitrosylation, glycosylation, or lysine lactylation are found involved in biological processes including SMs synthesis, but they are largely unknown in the context of regulation of SM or other metabolism processes and need further investigation. Efforts on the dynamic balance between different PTMs and the regulatory networks mediated by these PTMs will help to characterize new SMs or antibiotics in *Aspergillus* and other fungi. Also some SMs produced by fungi are toxic or act as virulence factors. Because many PTMs are important for regulating activities of the enzymes involved in SM biosynthesis, the application of chemical inhibitors of PTM enzymes may be useful in controlling synthesis of harmful secondary metabolites.

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