The Role of KdmB, a Lysine- dependent Histone Demethylase in Secondary Metabolism of *Aspergillus nidulans*

Master Thesis

to obtain the academic degree Master of Science

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II. ABSTRACT

Filamentous fungi have the genetic capacity to produce a huge array of secondary metabolites (SMs). SMs comprise a structurally highly diverse class of compounds that are not directly involved in basic metabolism and growth but are advantageous in some way for the producing organism, e.g. for combating competitors (antibiotics), protection against UV irradiation (especially pigments like melanin) and desiccation. Some of them have pharmaceutical relevance, e.g. penicillin, whereas others are detrimental for livestock and mankind upon consumption, e.g. aflatoxin. Sequencing of fungal genomes has shown that SM genes are generally organized in gene clusters which are often located in subtelomeric regions. It has previously been shown that specific posttranslational histone modifications involved in chromatin remodelling modulate the expression pattern of these gene clusters. One such modification is methylation of lysine residues in histone H3 and depending on which lysine is targeted, this can lead to gene activation (e.g. H3K4me, H3K36me) or repression (H3K9me, H3K27me). The necessary flexibility in gene expression is hence mediated by different methyl-transferases, which methylate each position with a specific mark and by the counteracting demethylases which are able to remove one or more of these modifications again.

This work describes the further characterization of a recently discovered JmjC domain containing demethylase, KdmB, encoded by the gene *AN8211*, which was shown to act on the Histone 3 Lysin 4 trimethyl mark (H3K4me3), and is not only a general repressor of gene transcription but also necessary for induction of many SM gene clusters in *Aspergillus nidulans.* (Gacek-Matthews et al., 2016). Here, the crucial jumonji-type demethylase domain (JmjN-JmjC) s been analysed in more detail.

Therefore, two amino acid substitutions were introduced in the highly conserved catalytic JmjC domain, responsible for Fe^{2+} binding, rendering the demethylase domain non-functional ($KdmB^{H642G,\ E644Q}$). As a control, the *kmdB*Δ deletion strain was complemented by *in loco* integration of *kdmB* driven by its native promoter ($KdmB^{Cil}$).

Subsequently, we analysed the expression of sterigmatocystin and penicillin in the strains carrying these mutated constructs. Surprisingly, while expression and production levels were rescued to wild type level in $KdmB^{Cil}$, this was not the case for the $KdmB^{H642G,\ E644Q}$ strains. Thus, demethylation by KdmB is required for the activation/repression of at least these two SM genes in *A. nidulans*. To see the correlation between expression and chromatin modification, we next analysed relevant histone modification, i.e. H3K4me2 and H3K4me3, at the respective SM cluster genes, thereby gaining insight

into the function of the demethylase KdmB and its role in secondary metabolism in *Aspergillus nidulans.*

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1 INTRODUCTION

1.1 *Aspergillus nidulans* as a model organism

Aspergilli belong to an ubiquitous group of filamentous fungi spanning over 200 million years of evolution (Galagan et al., 2005), and perhaps no other fungal genus contains species that have so diverse characteristics, either beneficial or very detrimental to mankind.

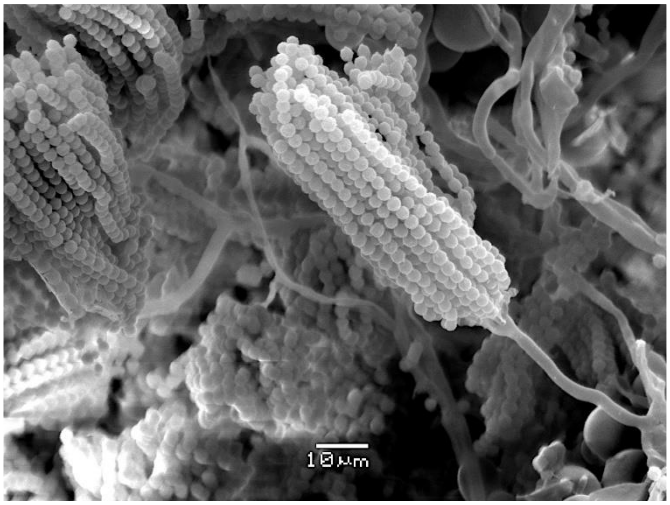


Figure 1 Electron Microscope (SEM) image of *Aspergillus nidulans* conidiophores. (picture kindly provided by David Canovas (University of Sevilla)

For instance, *Aspergillus niger* is widely exploited by industry for production of citric acid, similar as *Aspergillus oryzea* which is widely used in China to ferment soybeans for making soy sauce and other Japanese beverages (Goldman & Osmani, 2007). Beside beneficial species the genus *Aspergillus* contains also 20 human pathogens including *Aspergillus fumigatus* that causes aspergillosis in immunocompromised patients, and *Aspergillus flavus,* a plant and animal pathogen producing the potent carcinogen aflatoxin (Goldman & Osmani, 2007).

1.1.1 Why study *Aspergillus nidulans*?

A. *nidulans* has its importance in scientific research and has established as a great model organism for genetics and cell biology. Now after half a century of *A. nidulans* research the study of eukaryotic cellular physiology has contributed to our understanding of metabolic regulation development, cell cycle control, chromatin structure, cytoskeletal function, DNA repair, pH control, morphogenesis, mitochondrial DNA structure and human genetic diseases (Galagan et al., 2005).

A. nidulans can reproduce asexually, by producing vegetative and haploid conidiospores, or sexually, by undergoing sexual life cycle and producing ascospores. In contrast to most Aspergilli, *A. nidulans* possesses not only well-characterized sexual cycle (Han, 2009), but the availability of the genome sequence that has been completed in 2005 by the Broad Institute makes this fungus an important and well accepted model organism to study . *A. nidulans* contains approx. 9,500 genes distributed over eight chromosomes.(Galagan et al., 2005)

1.2 Secondary metabolism of *Aspergillus nidulans*

Aspergillus and other filamentous fungi are known for their ability to produce secondary metabolites (SMs). Formally, the term 'secondary metabolite' describes low molecular mass molecules which are usually not necessary for growth, development and reproduction of the organism and are often synthesized subsequent to primary metabolism (Galagan et al., 2005). However, many SMs are necessary for the fungal fitness by providing an advantage for the producing organism, e.g. against UV irradiation, desiccation or competing organisms (Fox & Howlett, 2008; Reverberi, Ricelli, Zjalic, Fabbri, & Fanelli, 2010; Rohlfs & Churchill, 2011).

Such potential benefits can act in several ways. For instance, the production of pigments such as melanin (Geib et al., 2016) confers resistance against UV light and hence protects the fungus from environmental stress. The most prominent example is given by the production of mycotoxins (aflatoxin, sterigmatocystin, …) and antibiotics (penicillin) counteracting with the defence mechanism of a competitor and thus resulting in a growth advantage of the fungus. Other SMs are involved in the development of fruiting bodies and therefore are important for sexual development. This broad range of bioactivities results from a great structural diversity of SMs (Brakhage, 2013; Brakhage & Schroeckh, 2011). In addition, to the ecological role of SMs for the fungus, they also have a considerable impact on mankind. The most famous example for a beneficial SM is penicillin (Then Bergh & Brakhage, 1998). It was the first discovered antibiotic from *Penicillium notatum* by Sir Alexander Fleming in 1928 (Fleming, 1929) and thus led to the beginning of the 'antibiotic era' but with it the sooner or later aroused resistance to it (Aminov, 2010). With that the need of new antibacterial substances emerged and until now there is a rising focus on fungi as a promising source of antimicrobial substances due to their ability to produce a great variety of SMs (Chavez, Fierro, Garcia-Rico, & Vaca, 2015).

Aspergilli are capable to produce a huge variety of SMs, including beneficial SMs such as penicillin but also others serving as food additives, cholesterol- lowering drugs, immunosuppressants, antibiotics or anticancer agents (Klejnstrup et al., 2012). Moreover, Aspergilli and filamentous fungi in general exhibit a huge potential of producing SMs and recent sequencing efforts predict still a great number of silenced SM gene clusters and their unknown metabolites.

1.2.1 Diversity and classes of fungal SMs

Generally, the SMs can be classified in four distinct chemical groups: polyketides (PK), non-ribosomal peptides (NRP), terpenoids and shikimic acid derived compounds. However, hybrid metabolites composed of moieties from different classes are common (Brakhage, 2013). In an *Aspergillus nidulans* screen 27 polyketide synthases and 14 non-ribosomal peptidases have been found so far (Goldman & Osmani, 2007). Since polyketides and no- ribosomal peptides belong to the majority of fungal secondary metabolites, those two types of SMs are described below.

1.2.1.1 Polyketides (PKs)

PKs are the main group of SMs in fungi. They are synthesized by polyketide synthases (PKSs) which use acetyl-CoA units as general substrates. PKSs are large multi-domain proteins with similarity to fatty acid synthases. The minimal domain structures consist of the following domains: a keto-synthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP) (Klejnstrup et al., 2012).

Fungal PKs are generally synthesized by type I PKSs. Few examples exist in which a type III PKS is involved in PK generation, *e.g.* the PKS involved in biosynthesis of resorcyclic acid (pentaketide) in *Neurospora crassa* (Funa *et al*., 2007). In *A. nidulans* only the iterative type I PKS was reported which can be further classified in non-reducing PKS, partially reducing and highly reducing PKS. PKs produced by fungi have drawn much attention due to their important role in human health since these compounds include toxins such as sterigmatocystin, aflatoxin and the virulence factor melanin (Chiang, Oakley, Keller, & Wang, 2010).

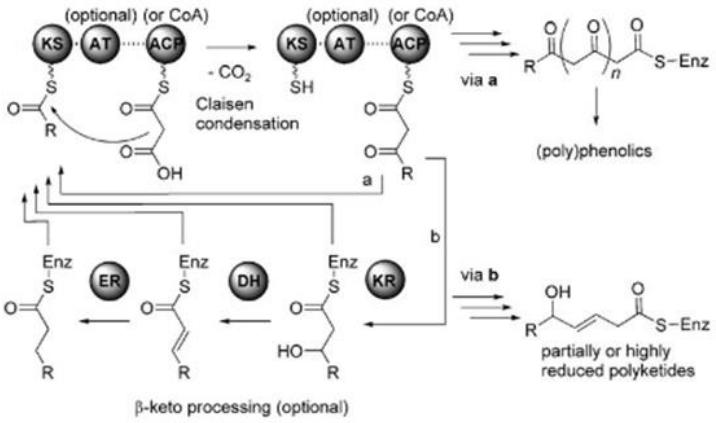


Figure 2 Schematic presentation of polyketide biosynthesis. Polyketide biosynthesis is quite similar as fatty acid biosynthesis using simple precursors, such as acetyl-coA as building blocks. Through repetitive decarboxylative Claisen thioester condensation the polyketides are constructed. Typically, this process involves a keto-acylsynthase (KS), an acyl transferase (AT) and a phosphopantethienylated acyl carrier protein (ACP) (Hertweck, 2009)

Sterigmatocystin

Sterigmatocystin (ST) belongs to the PK-family and is a precursor of aflatoxin, one of the most carcinogenic mycotoxins. This mycotoxin has been shown to cause cytotoxic effects, hepatocarcinoma and genetic abnormalities (Sugihara et al., 2016).

However, *A. nidulans* does not synthesize aflatoxin since the biosynthesis stops at the pathway intermediate sterigmatocystin. For aflatoxin biosynthesis the sterigmatocystin needs to be converted into O-methylsterigmatocystin, requiring the gene *aflP*, which is responsible for the conversion. The absence of the *aflP* orthologue in *A. nidulans* is the missing link between sterigmatocysin and aflatoxin biosynthesis(Yu, 2012). None the less sterigmatocystin remains a powerful mycotoxin and is a common contaminant of food and feed (Klejnstrup et al., 2012).

The genes responsible for sterigmatocystin synthesis are organized in a cluster on chromosome IV in *A. nidulans*. The cluster contains 27 genes named *stcA-X* and has a size of about 60 kb. It is thought that the cluster contains two transcription factor-encoding genes (*aflR* and *aflJ)*, six monooxygenases (s*tc B, stcC, stcF, stcL, stcM, stcS, stcW*), two dehydrogenases (*stcE, stcU*), a VERB synthase (*stcN*), an oxidase (*stcO*), a monooxydase/oxidase (*stcQ*), a Bayer-Villiger oxidase (*stcR*) and a fatty acid synthase (*stcJ, stcK*). Still some genes are unassigned and are thought to be part of the cluster without knowing their function (Klejnstrup et al., 2012).

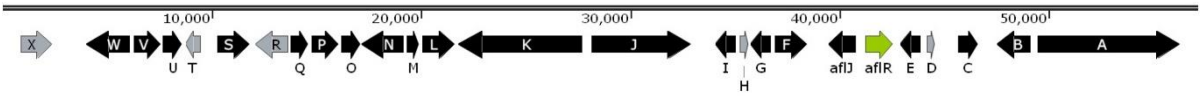


Figure 3 Sterigmatocystin gene cluster of *A.nidulans* containing 27 genes named *stcA-stcX*

Orsellinic acid

Orsellinic acid synthase is another fungal PKS and orsellinic acid as well as F-9775A/B, two yellow polyketides and cathepsin K inhibitors, have been isolated from *A. nidulans* which was co-incubated with an actinomycete (Sanchez et al., 2010). Still very little is known about the metabolites produced by the *ors* locus (Klejnstrup et al., 2012). However recent studies have shown that *orsA,* encoding a non-reduced PKS alone is required for the formation of orsellinic acid plus two additional genes for F-9775A and B biosynthesis (Klejnstrup et al., 2012; Sanchez et al., 2010)

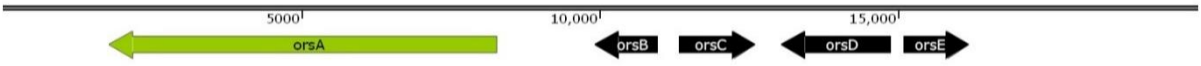


Figure 4 Orsellinic acid gene cluster of *A.nidulans*

Monodictyphenone

Monodictyphenone is another SM produced by *Aspergillus nidulans* (Bok et al., 2009) and recently it has been shown that the monodictyphenone gene cluster produces not only monodictyphenone but also emodine and emodine derivates. Emodine has been under extensive research since this substance displays anti-mutagenic, anti-cancerogenic and immunosuppressive anti-inflammatory effects (Dong et al., 2016).

The monodictyphenone gene cluster, located near the telomere of chromosome VIII (*Figure 8*), includes 12 ORFs encoding one non-reducing polyketide syhthase (*mdpG*). Since monodictyphenone, emodine and emodine derivates share the same aromatic PK structure this single non-reducing PKS is considered to be the main key enzyme, creating the backbone of the SMs (Bok et al., 2009). Furthermore, the cluster contains two transcriptional activators, i.e. *mdpE* and *mdpA* (Klejnstrup et al., 2012).

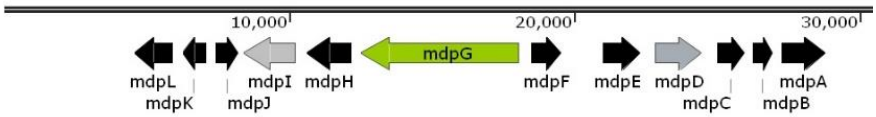


Figure 5 Monodictyphenone gene cluster in *A.nidulans* spanning 12 ORFs, i.e. *mdpA-mdpL*

1.2.1.2 Non ribosomal peptides (NRPs)

Another relevant chemical group of SMs are the non-ribosomal peptides (NRPs). They are synthesized similar to the PKs by multi-domain enzymes called non-ribosomal peptide synthetases (NRPSs) (Soukup, Keller, & Wiemann, 2016). This chemical group is especially of pharmaceutical importance since some of these compounds display a high antibacterial effect. Penicillin, cephalosporin and cyclosporine are only a few examples. NRPs display a great chemical diversity as they can be linear or cyclic with additional modifications. Moreover, this structural diversity also reflects in their broad spectrum of biological activities (Soukup et al., 2016). NRPSs work independent of mRNA, they use proteogenic and non-proteogenic amino acids as starting subunits synthesizing small oligopeptides *sans* ribosomes in the cytoplasm. A NRPS needs three domains to be functional: an adenylation (A) or thiolation domain, a peptidyl carrier protein (PCP) domain (equivalent to ACP) required for the amino acid attachment and a condensation (C) domain. Occasionally other domains like epimerase (convert L to D-amino acids) and N-methyltransferase domains (N-methylate peptide bonds) can be present within the enzyme complex (Soukup et al., 2016).

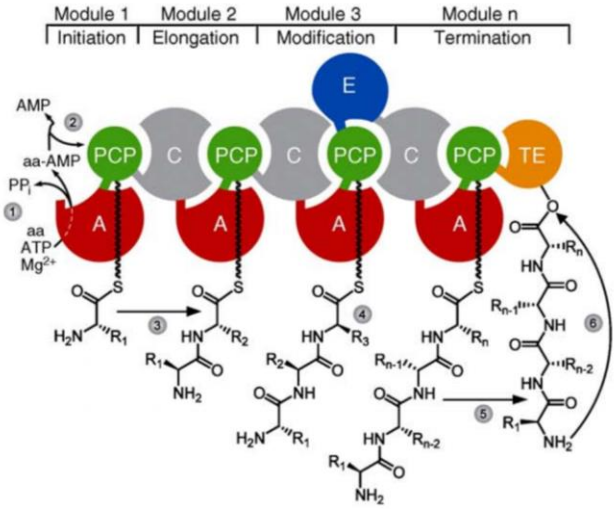


Figure 6 Simplified non-ribosomal peptide biosynthesis. (1) The Adenylation domain (A) activates the amino acid (2) which is transferred onto the peptidyl carrier protein (PCP) domain. (3) Condensation (C) of PCP bound amino acids. (4) other domains like epimerase (E) modify certain amino acids. (5) Transesterification (TE) is required for release either by hydrolysis or macro cyclisation (Strieker, Tanovic, & Marahiel, 2010).

In order to initiate biosynthesis, PKSs and NRPSs require post-translational modification of the ACP and PCP domain, respectively, by a phosphopantetheinyl transferase (PPTase) that adds a phosphopantetheinyl group to the conserved serine residue thereby activating the respective key enzyme (Crawford, Vagstad, Ehrlich, Udwary, & Townsend, 2008).

Penicillin

Aspergilli are known to have the genetic capacity for penicillin production. Only three enzymes are required to form the β-lactam tripeptide by enzymatic formation of the precursors L-cysteine, L-valine and L-aminoadipic acid (Herr & Fischer, 2014). Those three enzymes are encoded by the genes *acvA, ipnA* and *aat* and are clustered in chromosome VI (3.0 Mb) of *A. nidulans* (Martin et al., 1994).

The enzymatic formation of the tripeptide *sans* ribosomes is done by the ACV-synthetase (ACVS) encoded by *acvA*, followed by oxidative ring closure and therefore leading to the formation of the β-lactam isopenicillin N. This step is catalyzed by the isopenicillin N synthetase (IPNS), encoded by *ipnA.* Those two processes are located in the cytoplasm. In the final step the α-aminoapidic acid side chain is processed by an acyltransferase (ACT) (encoded by *aat*) in the peroxisomes of the cell (Brakhage, Browne, & Turner, 1992). The last enzyme has a broad substrate specificity and therefore different penicillins can be synthesized (Sprote et al., 2008).

Figure 7 Penicillin gene cluster in *A. nidulans*. The gene cluster consists of the three genes *acvA, ipnA* and *aatA* located on chromosome VI.

1.2.2 Organization of SM genes in clusters?

Recently fungal genome projects have shown that fungi have a surprisingly large number of SM gene clusters but still the products are currently unknown (Chiang et al., 2010). This is due to the fact that a lot of SM genes remain silent under standard laboratory conditions. A common feature in SM biosynthesis is the fact that the genes involved in SM biosynthesis are often physically linked and organized in clusters. Generally, such a cluster contains one or more central biosynthetic genes encoding one or more of the aforementioned key enzyme-encoding genes for the formation of the SM backbone, as well as genes encoding further decorating enzymes, regulatory proteins or transporters (Brakhage, 2013).

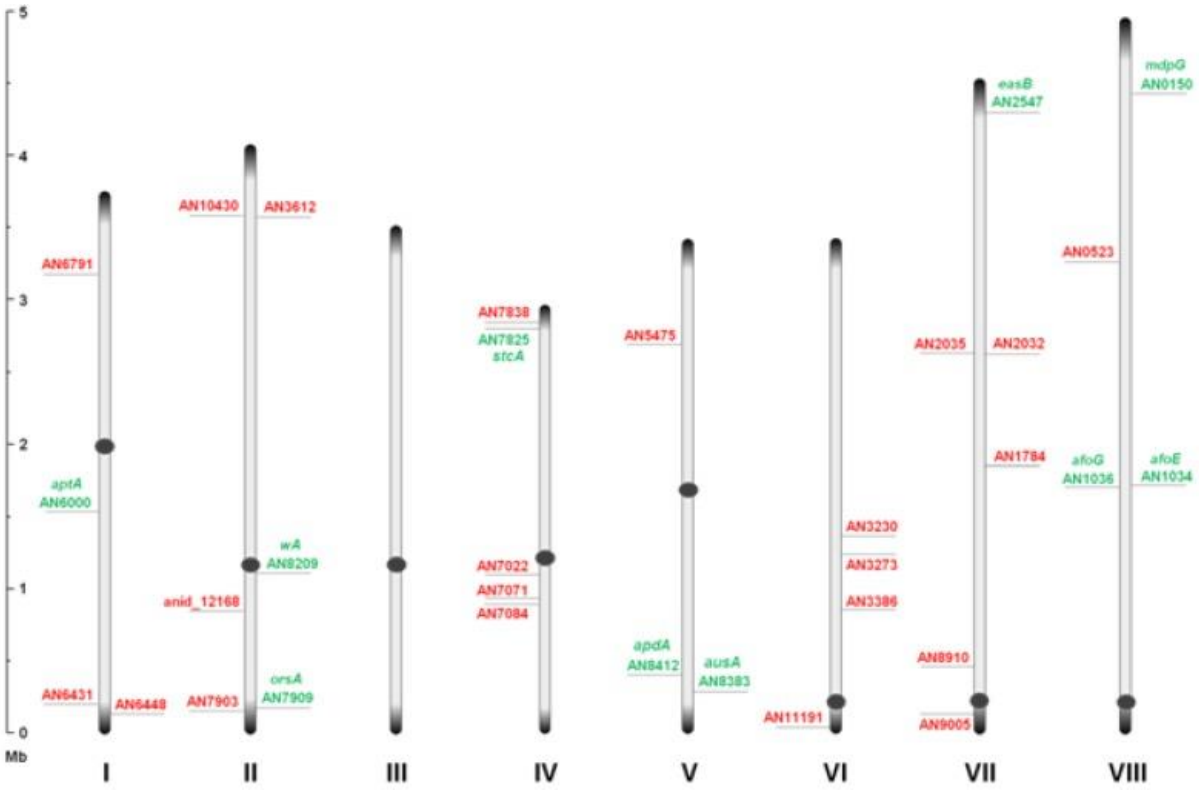


Figure 8: Distribution of PKS gene clusters on the eight chromosomes of *A. nidulans*: 32 putative PKS open reading frames (ORFs) are distributed on the eight chromosomes of *A. nidulans*. AN gene numbers highlighted in green are annotated whereas AN numbers in red are still unannotated. This figure also indicates that some SM gene clusters are located subtelomeric and some centromeric which are known as (facultative) heterochromatic regions (Klejnstrup et al., 2012).

This physical linkage of genes which are involved in the same biosynthetic pathway minimizes the amount of regulatory steps necessary to regulate the biosynthetic machinery and thus helps conserving energy. All these characteristics make SM gene clusters a good target for the regulation by chromatin- based mechanism (Gacek & Strauss, 2012).

1.3 Regulation of SM clusters

The regulation of SM gene clusters is a complex network responding to various signals including environmental stimuli, such as light, carbon and nitrogen sources, the ambient pH as well as temperature. Several regulatory layers exist, which can be distinguished between narrow and broad domain regulation. Shortly pathway specific regulation encodes transcription factors commonly located within the gene cluster whereas globally regulation is mediated by global transcription factors encoded by genes which do not belong to specific SM clusters. Moreover, cross talk between SM gene clusters is quite common in gene regulation. In recent years chromatin-mediated regulation became a highly interesting topic to scientists hence it provides a new strategy for SM gene cluster activation (by manipulation of chromatin modifications) and thus identification of new SMs (Brakhage, 2013; Soukup et al., 2012; Strauss & Reyes-Dominguez, 2011; Zutz et al., 2016; Zutz et al., 2013).

1.3.1 Chromatin mediated regulation of secondary metabolism genes

Chromatin is a highly dynamic structure and regulates DNA in many ways. So packaging of the DNA is just a small part of its function. In recent years a regulatory component of chromatin caught more and more the attention of scientists: Modification of histones. Through posttranslational modifications (PTMs - like histone acetylation, phosphorylation, methylation…) of histones biological processes can be regulated by influencing transcription (through regulating the accessibility to chromatin) but also DNA repair, replication and recombination (Bannister & Kouzarides, 2011; Gacek & Strauss, 2012). And since Shwab et al. (Shwab et al., 2007) could have shown that histone modifying enzymes alter SM production, intense research focuses more and more on chromatin modifying factors regulating SM production in filamentous fungi (Gacek-Matthews et al., 2015; Gacek & Strauss, 2012; Strauss & Reyes-Dominguez, 2011).

In the following chapter a short briefing of chromatin and its functions is given. Furthermore, this chapter focuses on the histone modifying enzyme KdmB and its demethylation mechanism as well as its epigenetic effects on SM metabolite regulation.

1.3.2 Chromatin Organization

Generally, a main aim of chromatin is the packaging of the DNA into a smaller volume. In most eukaryotic cells the DNA macromolecule would extend for approximately two meters if stretched out and fitting this huge molecule into the nucleus of a cell of few hundred micrometres is quite a

challenge (Armstrong, 2013). To solve this space problem nature has evolved a very elegant way of packaging: the DNA is wrapped into tight spheres called nucleosomes. Each nucleosome organizes 147 bp of DNA in a 1.7 left-handed superhelical turn around an octamer of four core histones: H2A, H2B, H3 and H4. H1 is associated with small sections of DNA, the so called linker DNA, and joins the nucleosomes together (Armstrong, 2013). These beads on a string can further condensate. An overview of the packaging of DNA is shown in *Figure 9*.

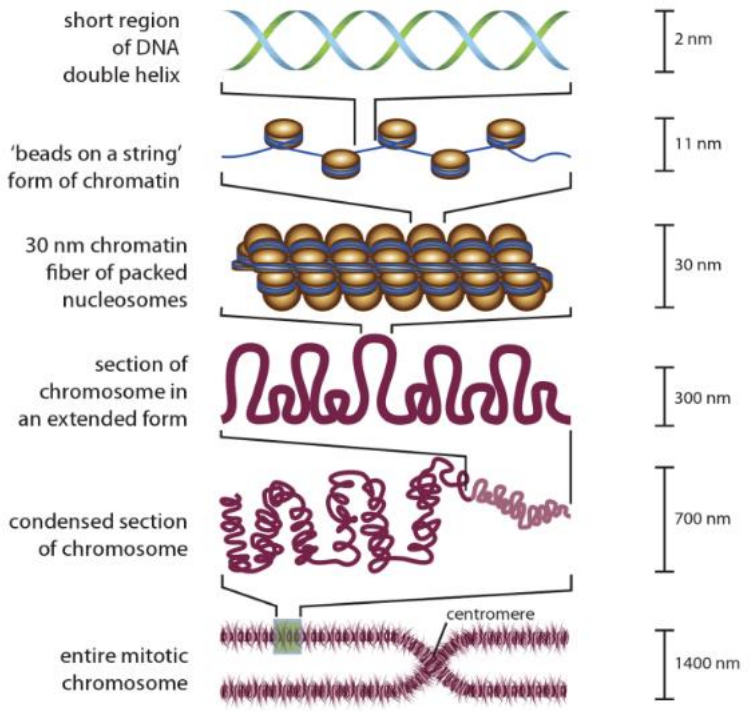


Figure 9 Overview of the packaging of DNA. DNA is wrapped around histones forming chromatin. Through further condensation of the 'beads on a string' a higher compaction level is reached (30 nm fibre). Further condensation results in different states of chromatin: euchromatin is looser packed that heterochromatin. The last compaction step results in an entire mitotic chromosome, which is ready for duplication (Armstrong, 2013).

1.3.3 Heterochromatin and Euchromatin

Furthermore, these chromatin fibres can generate different types of chromatin compaction. Simply speaking euchromatin represents DNA material which is less dense packed and contains most of the transcriptionally active genes while heterochromatin is very densely packed and remains transcriptionally silent. However, heterochromatin can be further divided into facultative and constitutive heterochromatin. While the latter is includes telomeres and centromeric regions but also repetitious DNA sequences such as satellite DNA, facultative heterochromatin can gain euchromatic characteristics and thus become transcriptionally active (Armstrong, 2013).

Irrespective from packaging of DNA, chromatin has other important functions as well. Chromatin alterations induce genome wide and local changes in the expression patterns of genes (Brosch, Loidl, & Graessle, 2008). This changes result as an answer to external and internal signals during growth, differentiation, development, metabolic processes but also diseases, abiotic and biotic stresses. These chromatin alterations and changes in gene expressions require a highly dynamic chromatin structure and until now several mechanisms could have been identified. Chromatin can undergo ATP-dependent remodelling, which can lead to the exchange of histone primary variants. Furthermore, histones are subjected to PTMs (Turner, 2007) and DNA itself can be modified by methylation. All these processes occur dependently from each other (Brosch et al., 2008).

1.3.4 Histone posttranslational modifications

As already discussed above, histones present the core proteins of nucleosomes. In assembling a nucleosome, a histone octamer is formed consisting of two dimers of H2A-H2B and a tetramer of H3-H4, around which the DNA is wound. In addition, each core histone has an N-terminal amino acid tail extending from the nucleosome which can be subjected to many histone modifications influencing chromatin structure and function (Alberts et al., 2010).

However, not only the chromatin structure is affected by these PTMs, chromatin remodelling complexes are recruited to specific PTMs. Since the discovery that histones are posttranslational modified the list of these modifications is ever growing (Bannister & Kouzarides, 2011). So far many modifications have been identified including acetylation, methylation of lysine and arginine, phosphorylation, sumoylation, deamination and ubiquitination. This variety of different modifications offers enormous possibilities of gene regulation.

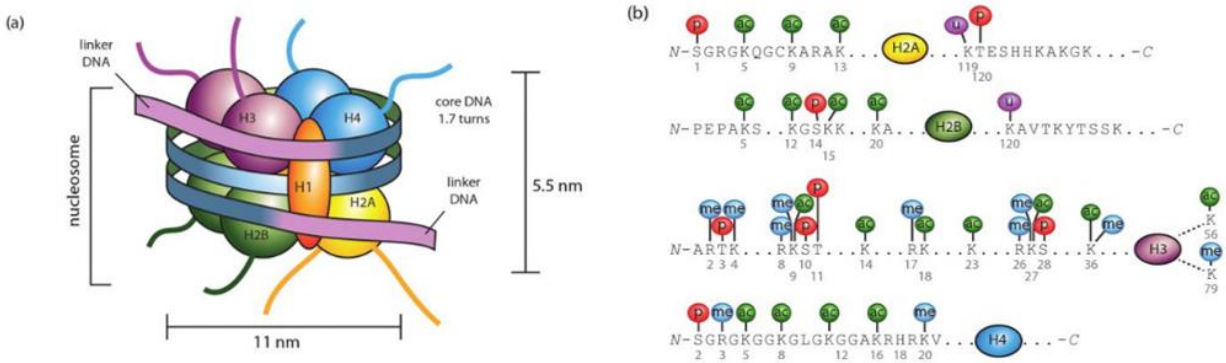


Figure 10 Composition of a nucleosome core particle and N-terminal modifications. (a) Structure of the histone octamer and (b) location of the amino acid residue sites where histone modifications can occur. These modifications include acetylation (ac), methylation (me), phosphorylation (p) and ubiquitination (u) (Armstrong, 2013).

1.3.5 Histone Methylation and Demethylation

Contrary to histone acetylation which results mainly in activation of chromatin, histone methylation is ambivalent. Dependent on the modified lysine residue, histone methylation can be associated with transcriptional activation (H3K4, H3K36) but also transcriptional repression (H3K9, H3K27 and H4K20). In addition, the degree of methylation, the chromosomal location, histone site (promotor region, coding sequence) and the organism strongly influence the functional output of (Bannister, Schneider, & Kouzarides, 2002; Brosch et al., 2008; Gacek & Strauss, 2012).

Figure 11 shows a simplified image of histone methylation and demethylation mediated by the enzymes histone methyltransferases and histone demethylases.

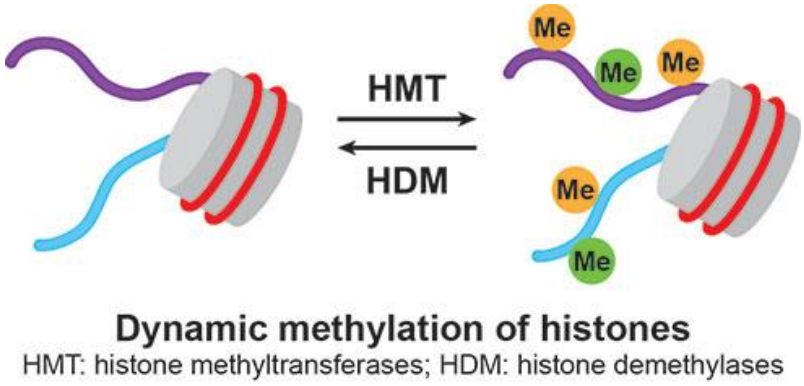


Figure 11 Histone methylation and demethylation accomplished by histone methyltransferases (HTMs) and histone demethylases (HDMs) (https://pharmacy.wisc.edu/jiang-lab/research/)

1.3.5.1 Histone Methylation

Methylation of histones is considered to increase or decrease transcription of genes by transferring one or more methyl groups from S-adenosyl-L-methionine to the substrate proteins. Principally, the change in gene expression depends on the amount of transferred methyl groups and the methylated amino acid. Either lysine or arginine residues can be methylated. Methylated lysines can be found in a mono, -di and tri-methylated state whereas arginines can be either mono or di-methylated, asymmetrically or symmetrically (Bannister et al., 2002). The chemical structure of lysine and its methylated derivates is shown in Figure 12.

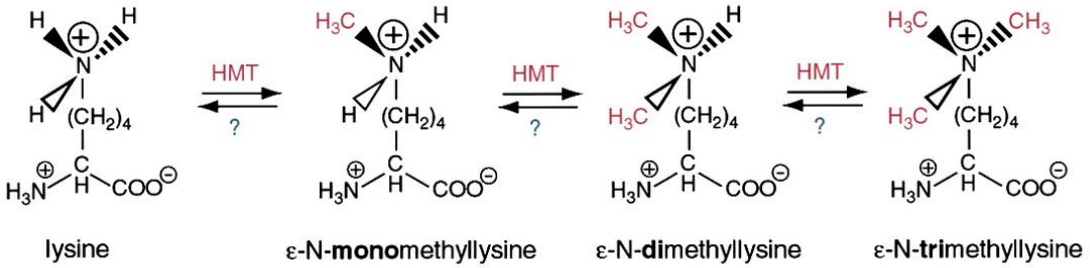


Figure 12 Methylation of lysine by Histone Methyltransferases (HTMs). (Bannister et al., 2002)

In comparison to other prominent modifications like acetylation or phosphorylation, methylation does not alter the charge of the histones. However, it changes the sterical structure of the histone protein, making the DNA more accessible for chromatin remodelling proteins or the transcription machinery.

1.3.5.2 Histone Demethylation

For a long time, histone methylation was conferred to be a stable and irreversible epigenetic mark that committed chromatin to a specific state. This dogma lasted until the discovery of the first histone demethylase, i.e. Lsd1 , suggesting histone methylation as a dynamically regulated process (Dimitrova, Turberfield, & Klose, 2015).

1.3.5.3 Lysine Demethylation

The lysine-specific demethylase KDM1A/LSD1 was shown to actively remove mono and di-methyl groups on Histone 3 Lysine 4 (H3K4) using its amine oxidase domain via a Flavin Adenin dinucleotide (FAD) dependent manner. Shortly afterwards it has been shown that KDM2A, another histone demethylase, removes methyl groups from H3K36 via its Jumonji C domain, using Fe^{2+} and α-ketoglutarate (2-OG) as co-factors (Dimitrova et al., 2015).

The family of histone demethylases has strongly extended and until now 20 demethylases have been identified and characterized (Shi & Tsukada, 2013). They can be grouped either into the LSD family or the Jumonji domain-containing family. LSD demethylases can only demethylate mono- and di-methylated states of histone lysine residues, whereas enzymes belonging to the Jumonji domain family can demethylate all three states of lysine residues. The reaction mechanism of both families is shown in *Figure 13*.

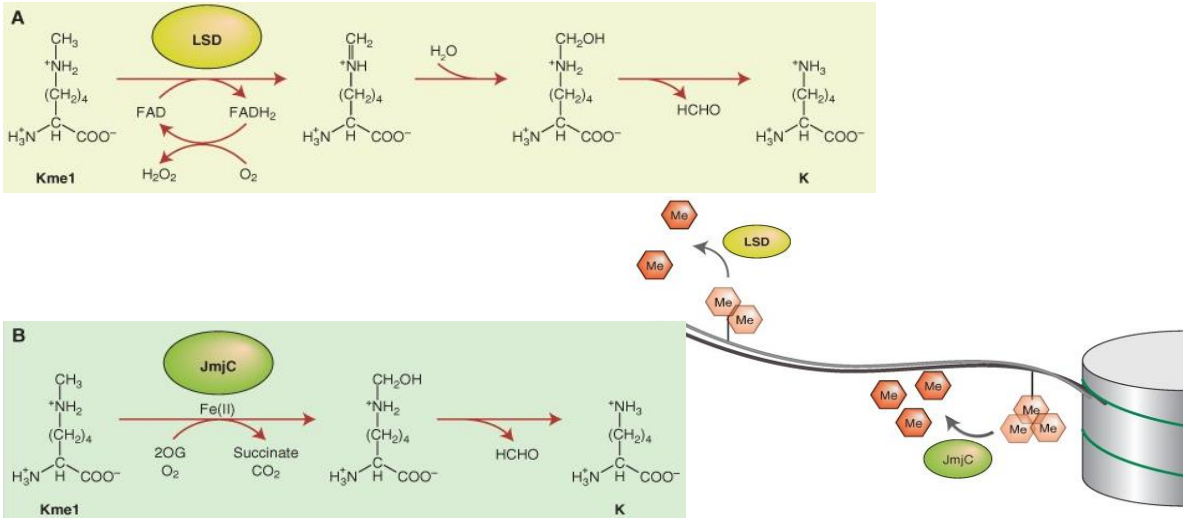


Figure 13 Histone demethylation mediated by (A) an LSD family demethylase through an FAD-dependent amine oxidase reaction, and (B) a JmjC domain family demethylase through 2OG-Fe(II)-dependent dioxygenase reaction (Shi & Tsukada, 2013)

1.3.6 Jumonji domain family demethylases

The Jumonji domain is a widely used protein module which has been identified in a mouse transcription factor needed for neuronal development. Mutants of this gene developed an abnormal neural-plate phenotype, giving the gene its name *Jumonji* (jumonji stands for cruciform in Japanese) (Armstrong, 2013). Further sequence analysis revealed several subvariants of the Jumonji. The original gene discovered from the neural malformation experiments was dedicated as Jumonji C (JmjC). However, this protein module is present in a large number of proteins. This protein building block consist of a double stranded β-helix (DSBH) which is built by eight antiparallel β-strands containing binding sites for Fe^{2+} and 2-OG. In studied histone demethylases this JmjC domain has histone demethylase catalytic activity (Accari & Fisher, 2015). In Humans about 20 demethylases have been identified and subdivided into seven distinct families: JHDM1, JHDM2, JMJD2, PHF2, PHF8, Jumonji (A+T) rich interactive domain (JARID) and JmjC domain only (Armstrong, 2013).

As already mentioned Jumonji histone demethylases are specific to individual methylation states and lysine residues. For instance, the JARID 1 group is active against H3K4me3 but inactive against K9 residues. In following table some Jumonji domain containing histone demethylases and their targets are listed.

Table 1 Histone demethylases containing Jumonji domains ((Armstrong, 2013)

Demethylase	Site specificity	demethylates
(FAD dependent) LSD1	H3K4	Mono, di
(Fe(II) 2-OG dependent) JHDM1A	H3K36	Mono, di
JMJD1A	H3K9	Mono, di
JMJD2A	H3K9, H3K36	Tri, di
JMJD2B	H3K9	Tri,di
JMJD2C	H3K9, H3K36	Tri,di
JMJD2D	H3K9	Tri, di, mono
JARD1A	H3K4	Tri,di
JARID1B	H3K4	Tri,di
JARID1C	H3K4	Tri,di
JARID1D	H3K4	Tri,di

1.3.7 Histone demethylases in *Aspergillus nidulans*

Gacek-Matthews *et al*. has recently discovered and characterized a new Jumonji histone demethylase, KdmA, targeting H3K36 also in *A. nidulans.* The authors showed that KdmA plays an important role in gene expression, including secondary metabolism (Gacek-Matthews et al., 2015). After the recent discovery of KdmA a second jumonji histone H3 demethylase was discovered, designated as KdmB.

KdmB, encoded by *AN8211*, belongs to the JARID group of enzymes and as shown in Table 1 JARID subfamily demethylases target di- and tri methylated H3K4. Since high levels of H3K4 methylation are detected in promotor regions of active genes (Barski et al., 2007) KdmB is thought to be a repressor of gene transcription by removing the active mark H3K4me3 leading to downregulation of transcription.

A comprehensive characterization of KdmB has been done in *A. nidulans* by Strauss and co-workers (Gacek-Matthews et al., 2016). This functional characterization revealed while several genes were up-regulated by upon deletion of *kdmB* (kdmBΔ) as expected, a large group of genes (40%) related to secondary metabolism, including genes involved in penicillin and sterigmatoycstin biosynthesis were found to be down-regulated albout lack of KdmB (Fig 14).

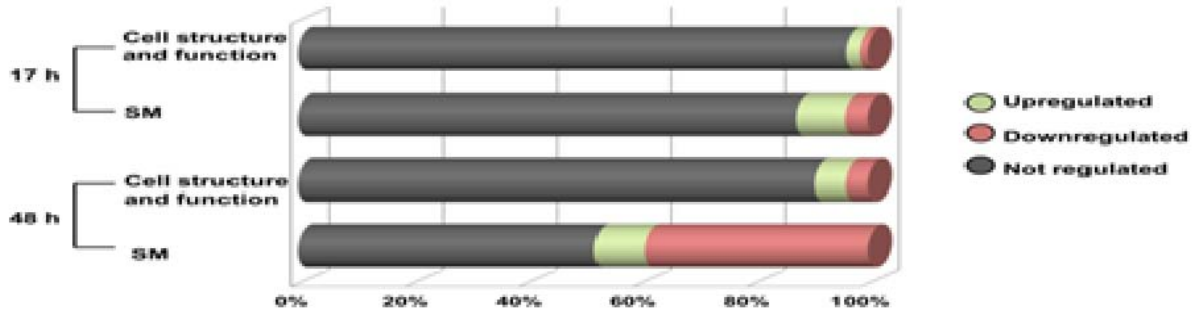


Figure 14 Percentage of de-regulated genes in *kdmB*Δ during primary metabolism PM (17 h) and secondary metabolism SM (48 h) with the division into SM cluster genes and basic metabolism genes (cell structure and function) (Gacek-Matthews et al., 2016)

These finding suggest that KdmB does not only function in transcriptional repression, through demethylation of the active histone mark H3K4me, but also as positive regulator in some way. Furthermore, it indicates that KdmB is required for full expression of several genes involved in SM biosynthesis.

KdmB is a multi-domain protein harbouring the following domains: JmjC, JmjN, ARID/BRIGHT, PHD and a C5HC2 ZF (*Figure 15*). The JmjC domain contains binding sites for Fe(II) and 2-OG and is required for the catalytic demethylase activity. The role of JmjN domain is still less clear but it is thought to support the catalytic function of JmjC (Huang et al., 2010; Quan, Oliver, & Zhang, 2011). Further domains such as an ARID/BRIGHT DNA binding domain, two plant homeodomain (PHD) fingers and a C5HC2 zinc finger exist. Still the complete functionality of each domain is quite unclear. For instance, it is known that the ARID/BRIGHT DNA-binding domain is required for the demethylation activities of JARID1 proteins, however, it is undiscovered whether the ARID/BRIGHT domain is essential for their association with chromatin (Huang et al., 2010).

Figure 15 Domain composition of KdmB. KdmB is a multi- domain protein and consists of JmjN, Arid/Bright (AT rich interactive domain), two PHD (plant homeodomain) fingers, JmjC domain and a C5HC2 zinc finger. JmjC-domain, which functions as a demethylase, is often found together with JmjN. Thus JmjN is thought to support JmjC in its catalytical function. The Arid Bright domain and the C5HC2-ZF are capable of DNA binding but little is known (Gacek-Matthews et al., 2016). The PHD finger is a nuclear protein interaction domain and new evidence suggests chromatin as a possible nuclear ligand of PHD fingers (Bienz, 2006).

1.4 Aim of work

Fungal SMs have attracted our interest due to their wide range of bioactivities. Some of them are highly beneficial (antibiotics) to us, whereas others can be detrimental upon consumption or exposure (mycotoxins). Nevertheless, most of the SM gene clusters remain silent under standard laboratory conditions thereby hampering the discovery of the respective putatively novel SMs. Several elegant approaches have been applied in the past that resulted in the activation of otherwise silent gene clusters, including manipulation of chromatin-mediated regulation.

Previous work showed that histone demethylases are involved in secondary metabolism to a great extent. Deletion of *kdmA* as well as *kdmB* resulted in an altered SM profile ((Gacek-Matthews et al., 2016; Gacek-Matthews et al., 2015)). Interestingly, KdmB seems to be not only involved in transcriptional repression, but also in activation of gene expression which is illustrated by down-regulation of 40 % of SM-related genes in strains lacking KdmB. The methylation mark H3K4 is usually associated with gene activation thus it was surprising to find about 40 % of SM genes downregulated upon deletion of KdmB. Next to the demethylase domain JmjC, KdmB possess additional domains, which functions are not totally understood in respect to lysine methylation. Thus, another domain apart of JmjC might be involved in transcriptional activation of SM genes in *A. nidulans*.

In the current work, mutation of the demethylase domain has been performed to dissect the demethylase function of KdmB from the additional functions of the protein. This was accomplished by introducing two amino acid substitutions that resulted in a non-functional JmjC-domain of KdmB. Furthermore, the phenotype of KdmB is verified by homologous in loco complementation of the deletion strain ($kdmB^{CiL}$).

The generated strains were subsequently analysed for differences in their SM production as well as for relevant histone modifications at respective SM cluster genes, thereby gaining further valuable insight into the function of the demethylase KdmB and its role in secondary metabolism in *A. nidulans.*

2 MATERIALS AND METHODS

2.1 DNA methods

2.1.1 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is a very simple, effective and convenient method for separation of DNA, RNA or protein samples according to their size, charge or conformation in a very short time.

Agarose is usually isolated from seaweed *Gelidium* and *Gracilaria* and consist of repeated agarbiose (L- and D-galactose) subunits. Upon gelation agarose polymers form a non-covalently network, which pore size determine the molecular sieving property of the gel. By simply increasing the agarose concentration the stiffness of the gel gets higher. For separation the DNA molecules are loaded into the gel and a current is applied. The phosphate backbone of nucleic acids is negatively charged and therefore when placed in an electric field the DNA fragments will migrate to the positively charged anode (Lee, Costumbrado, Hsu, & Kim, 2012).

In this work a 1% agarose gel was used for separation of DNA fragments. The agarose was molten in 1x TAE buffer [40 mM TRIS, 5.71% (v/v) glacial acetic acid, 50nM EDTA pH 8.0] by heating in a microwave. The same buffer was also used as a running buffer. To the molten gel four to six µL Midori Green Advanced DNA stain (Nippon Genetics) were added per 100 mL agarose for staining RNA or DNA fragments in the agarose gel.

If needed the samples were mixed with a 6x loading dye (Thermo Scientific) before loading into the gel. Depending on the DNA size of the fragments a DNA standard 1 kb ladder or a 100 bp ladder (Fermentas) was used.

2.1.2 Polymerase Chain Reaction

Polymerase Chain Reaction is one of the most abundant methods in molecular biology simply by using the ability of a DNA Polymerase to synthesize a new complementary strand by the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs designed primes to which it can add the first nucleotide. This requirement makes it possible to amplify specific regions of interest by designing primers for the target DNA. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies.

2.1.2.1 Standard PCR

For a routine PCR the DreamTaq Green PCR Master Mix (Thermo Scientific) was used. This PCR mixture contains already all necessary compounds essential for a PCR reaction. Only primers and DNA template need to be added. Pipetting Instructions are summarized in Table 2 and cycling instructions are shown in Table 3.

Table 2Pipetting Instructions for standard PCR reaction

Component	**15 µL Reaction**
DreamTaq Green PCR Master Mix	7.5 µL
Primer F	1 µL
Primer R	1 µL
DNA template	1 µL
H_2O	Up to 15 µL

Table 3 Cycling Instructions for standard PCR reactions

Cycle Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	95	1-3 min	1
Denaturation Annealing Extension	95 55-63 72	30 s 30 s 1 min/ 1 kb	25-40
Final Extension	72	5-15 min	1

2.1.2.2 PCR for the Construction of Deletion Cassettes Fragments

For the amplification of deletion cassettes fragments a high-fidelity Taq polymerase should be used to minimize errors in the final knockout cassettes. For this work the Thermo Scientific™ Phusion™ Flash High-Fidelity PCR Master Mix (F548L) based on a modified Phusion Hot Start II DNA Polymerase was used. This used Phusion Flash II DNA Polymerase is a proofreading polymerase containing an unique processivity- enhancing domain, making this polymerase accurate and rapid. Again this Master Mix contains all the reagents required for PCR except for the DNA template and primers. The Pipetting instructions and Cycling Instructions are shown below.

Table 4 Pipetting instructions Phusion Plash High Fidelity PCR MM

Component	**50 µL Reaction**	**Concentration**
Phusion Flash MM	25 µL	1 X

Primer F	X	0.5 µM
Primer R	X	0.5 µM
DNA template	X	
H_2O	Add to 50 µL	

Table 5 Cycling Instructions Phusion Flash High Fidelity PCR

Cycle Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	98	10 s	1
Denaturation Annealing Extension	98 55-63 72	1 s 5 s 15 s/ 1 kb	30
Final Extension	72 4	1 min hold	1

2.1.2.3 qPCR

Real time PCR is one of the most abundant methods for analysing mRNA expression. In this case the polymerase chain reaction is used to amplify cDNA, which has been reverse transcribed from mRNA first and therefore is a nice detection method for Expression analysis.

The most current detection method uses SYBR Green 1 fluorescence dye, which binds specifically to the minor groove of the double-stranded DNA. The detection is monitored by the increase of the fluorescence throughout the cycle, which of course is proportional to the amount of targeted amplicon.

In this study iTaq™ Universal SYBR® Green Supermix (Bio-Rad) was used and the thermal cycling protocol was followed by the manufacturer. For standard curve series 10 fold dilution of genomic DNA was prepared (10^{-3} up to 10^{-6})

Table 6 Pipetting instructions for Real Time PCR

Component	**15 µL Reaction**	**Concentration**
SYBR Green MM	10 µL	1 X
Primer F	0.8 µL	0.05 µM
Primer R	0.8 µL	0.05 µM
cDNA template	5 µL	
H_2O	Add to 15 µL	

Table 7 Cycling Instructions for Real Time PCR

RT- PCR System	**Denaturation at 95°C**	**Annealing/Extension and Plate Read at 66°C**	**Cycles**	**Melt Curve Analysis**	**Polymerase Activation and DNA Denaturation at 95°C**
MyiQ™	10-15 s	15-30	35-40	65-95°C	20-30 sec for cDNA

2.1.2.4 Colony PCR

Colony PCR is a convenient and simple method for determining the presence or absence of insert DNA in plasmid constructs. In this case individual *E.coli* transformants were added directly to the PCR reaction and lysed during the initial heating step.

The pipetting instruction is the same as for a standard PCR. The Cycling Constructions are shown below.

Table 8 Cycling Instructions for Colony PCR

Cycle Step	**Temperature °C**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation Annealing Extension	95 54 72	1 min 1 min 2 min	30
Final Extension	72 4	1 min hold	1

2.1.3 DNA Purification

For the purification of DNA fragments from PCR reactions or agarose gels the MinElute PCR Purification Kit from QUIAGEN was used. This MinElute DNA Cleanup system is specially designed for fast and easy isolation of DNA fragments from PCR reactions, agarose gels, or enzymatic reactions with an extremely small elution volume. The purification was done according to the MinElute® Handbook from QUIAGEN which can be found at www.quiagen.com/handbooks.

2.2 RNA Methods

Working with RNA is more demanding than working with DNA since RNA is a very unstable molecule getting easily degraded by ubiquitous RNases. These facts lead to a very short half-life of the molecule. Unlike DNases, RNases do not need metal ion co-factors and can maintain activity even after prolonged boiling or autoclaving. Therefore, special precautions need to be taken when working with RNA. First of all RNA samples need to be kept on ice (4°C) to avoid RNA degradation. Furthermore, all lab equipment needed for RNA experiments were treated with 0.1% DEPC (Diethyl pyrocarbonate) which inhibits RNases. Also the surface of the working bench was treated with RNase-inhibiting agents.

2.2.1 Fungal RNA Preparation and Isolation

2.2.1.1 Standard Fungal RNA Preparation

In order to investigate the RNA Expression level of certain secondary metabolite genes in *Aspergillus nidulans* fungal RNA had to be isolated from mycelia in liquid culture.

The mycelia was filtrated (by using Miracloth from EMD Millipore), squeezed between two papers to get rid of remaining culture medium and transferred into petri dishes. Liquid nitrogen was immediately poured into the dishes to shock-freeze the fungal mycelia. Afterwards the frozen mycelia was dried by lyophilisation overnight.

2.2.1.2 Fungal Preparation for Chromatin Immunoprecipitation

Another approach for fungal preparation, which is also used for Chromatin Immunoprecipitation is to filtrate the crosslinked mycelia by using Miracloth and squeeze it between two papers. Afterwards the mycelia was immediately frozen in liquid nitrogen and grinded in mortars. The grinded mycelium powder is then transferred in 2 mL Eppendorf Tubes and stored at 80°C.

2.2.1.3 RNA Isolation

For RNA Isolation approximately 200 mg of mycelium were transferred in 2 mL eppendorf tubes and grinded in liquid nitrogen with the help of toothpicks (Another option is to grind the mycelia in liquid nitrogen in mortars). The powder was suspended in 800 µL Trizol (Invitrogen), vortexed vigorously and 200 µl Chloroform were added. After vortexing the sample was centrifuged at 10,000 rpm for 10 minutes. The Supernatant was transferred into fresh eppendorf tubes and 500 µL isopropanol were added to precipitate RNA. For effective precipitation the mixture was incubated on ice for 15

minutes. After the incubation the precipitated RNA was spun down for 15 minutes at 10,000 rpm at 4°C, the supernatant discarded and the RNA pellet washed in 200 µL ethanol (70%). Again the mixture was spun down, the supernatant discarded and the pellet was dissolved in 80 µL of RNase free H_2O.

To get rid of the phenol residues (which might disturb further measurments) the RNA was once again precipitated. Therefore 8 µL 3M NaAc and 240 µL Ethanol (100%) were added and incubated overnight. After incubation the samples were spun down, washed with Ethanol (75%) and the pellet was dried in a centrifugal evaporator (SpeedVac) for 10-15min. Afterwards the dried RNA pellet is dissolved in 80 µL RNase free H_2O. After dissolving RNA in H_2O the amount of RNA was measured by Nanodrop™ Spectrophotometer.

The samples were always kept on ice and the centrifuge steps were performed at 4 °C.

2.2.2 DNase treatment of RNA

After RNA Isolation a DNase treatment is recommended to purify and concentrate RNA and to get rid of DNA contaminations as well. For this purpose, Thermo Scientific DNase I (EN0521) was used. Thermo Scientific DNase I is an endonuclease that digests single- and double-stranded DNA. It hydrolyzes phosphodiester bonds producing mono- and oligodeoxyribonucleotides with 5'-phosphate and 3'-OH groups.

For the removal of genomic DNA from RNA preparations following mixture shown in Table 9 is prepared. The mixture was incubated for 30 minutes at 37°C. After the incubation 2 µL EDTA were added and incubated for 10 minutes at 65°C in order to inactivate the DNase I. After inactivation the DNA free RNA was used as a template for reverse transcriptase. (As a control a standard PCR was performed with 5 µL of the DNA free RNA reaction volume with housekeeping primers, genomic DNA was used as a positive control)

Table 9 Pipetting instructions for DNase treatment

Compounds	**Concentration**
RNA	2 µg
10X reaction buffer with MgCl2	2 µL
DNase I, RNase free	2 µL
DEPC- treated H2O	Up to 20 µL

2.2.3 cDNA synthesis

In order to investigate RNA with the same molecular approaches used for DNA investigations RNA samples have to be transcribed into DNA via reverse transcription. cDNA generated by reverse transcription can be amplified as any other DNA sample using PCR or qPCR. For cDNA synthesis the iScript™ cDNA Synthesis Kit (BioRAD; #170-8890) was used and the procedure was done according to the iScript™ cDNA Synthesis Kit – Manual. The Pipetting instructions and the reaction protocol for cDNA synthesis are shown in Table 10 and Table 11.

Table 10 Pipetting instructions cDNA synthesis

Components	**Volume per Reaction**
5x iScript reaction mix	4 µL
iScript reverse transcriptase	1 µL
Nuclease free water	X µl
RNA template (1 µg)	X µL
Total volume	**20 µL**

Table 11 Reaction Protocol for cDNA synthesis

Incubation Time	**Incubation Temperature**
5 minutes	25°C
30 minutes	42°C
5 minutes	85°C
Hold at	4°C

https://www.neb.com/applications/rna-analysis/cdna-synthesis-and-rt-pcr

2.2.4 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation provides one way of empirically determining the sites of DNA that a given gene regulatory protein occupies under a particular set of conditions. In this approach proteins are covalently crosslinked to DNA in living cells *in vivo*, afterwards the cells are broken open and the DNA is mechanically sheared into small fragments. Antibodies directed against a given gene regulatory protein are then used to purify the DNA that became covalently crosslinked to the protein in the cell (Alberts et al., 2010).

In this approach Formaldehyde was used for crosslinking proteins to DNA. Among various crosslinking agents, formaldehyde (HCHO) is the most commonly used. It efficiently crosslinks protein– DNA, protein–RNA, and protein–protein in vivo by interacting between the amino and imino groups of lysine, arginine, and histidine and those of DNA. Furthermore, the chromatin structure is

faithfully preserved by HCHO treatment, and the crosslinks can be readily reversed under mild conditions (Tollefsbol).

Before ChIP was performed, *Aspergillus nidulans* strains were grown in liquid cultures and incubated at 37°C at 180 rpm for 48 hrs. To fix *Aspergillus nidulans* cells the liquid cultures were incubated with formaldehyde (1 %) for 15 minutes at RT. Accordingly 125 mM of Glycine was added and incubated for another five minutes. After crosslinking the mycelia was filtered and prepared as described in 2.2.1.2.

For DNA shearing approximately 200 mg of powder were transferred to fresh eppendorf tubes and mixed with 1 mL of Sonication buffer (50 mM Hepes KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Sodiumdeoxycholate, protease inhibitors 1:200). The Fragmentation of the *Aspergillus nidulans* chromatin was done by Sonication on ice for 15 minutes (2 minutes on, 1 minute off).

After Sonication the fragmented chromatin was spun down at at 4°C, 13,000 rpm. The upper phase was recovered to a new eppendorf tube and 40 µL proteinA agarose slurry was added and kept on a rotary shaker for one hour at 4°C. Finally, the samples were diluted with Dilution buffer (0.1 % Triton X-100, 2 mM EDTA pH 8, 20 mM Tris- HCl pH 8, 150 mM NaCl, Protease Inhibitors 1:200) to a concentration 1:10 (900 µL Dilution buffer and 100 µL of the sample) and frozen in liquid nitrogen.

Antibody precipitation

For Antibody precipitation the samples were thawed at RT and put on ice after complete thawing.

100 µL of sonicated chromatin were used as an input control (non precipitated control). The Input control-samples were de-crosslinked over night by adding 4 µL NaCl (final concentration 200 mM) and heated to 65°C.

The remaining 900 µL of diluted sample were incubated over night with the desired antibody at 4°C on a rotary shaker.

Precipitation of the protein-antibody conjugate

In order to precipitate the protein- antibody conjugate the samples were incubated with 40 µL dynabeads for 40 minutes at 4°C on a rotary shaker. After incubation the samples were put in a magnetic rack and after the dynabeads settled the liquid phase was removed. The Dynabead were washed this way three times with a Low Salt buffer (0.1 % SDS, 1 % Trition X-100, 2 mM EDTA ph 8,

20 mM Tris-HCl pH 8, 150 mM NaCl) and one time with a High Salt buffer (0.1 % SDS, 1 % Trition X-100, 2 mM EDTA ph 8, 20 mM Tris-HCl pH 8, 500 mM NaCl)

Reverse Crosslinking

In order to de-crosslink the protein-DNA conjugate the dynabeads were resuspended in 125 µL TES buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1 % SDS) at 65°C, 600-700 rpm overnight.

After reverse crosslinking the remaining proteins are digested of a proteinase K in order to receive the protein binding DNA –fragments. Therefore 100 µL of the samples were incubated with 2 µL EDTA (0.5 M), 4 µL Tris- HCl and 2 µL proteinase K (1 mg/ mL MBI) at 45°C for one hour.

Finally, the specific DNA fragments were purified with the MinElute PCR Purification Kit (QUIAGEN) and the samples were eluted in 100 µL 1:10 dilution of Elution buffer. In case of the input samples the samples were diluted in 500 µL 1:10 Elution buffer.

Quantification of the DNA

The concentration of the specific DNA fragments was determined by qPCR myIQ cycler Biorad using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) with 2 pmol of the specified primers in each reaction.

2.3 *E.coli* genetics

2.3.1 Media and solutions used in *E.coli* genetics

Table 12 E.coli Media and solutions

Luria Broth (LB) medium	Yeast extract	5 g/L
	Sodium chloride	10 g/L
	Peptone	10 g/L
	Agar- Agar	15 g/L
	Bidestilled water	
	Autoclaved	
	If necessary: dominant selection markers like kanamycin and ampicillin to a final concentration of 50 µG/mL	
***E.coli* kryo culture**	60% (v/v) sterile glycerol mix with 1:1 (v/v) overnight grown culture store at -80 C	

2.3.2 Heat shock transformation of chemical- competent *E.coli* cells

For transformation and Cloning Purposes Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were used. The transformation procedure was done according to the manufacturer's manual.

2.3.3 Plasmid Miniprep

QIAprep Spin Miniprep Kit from QUIAGEN was used for plasmid purification. Therefore overnight cultures of transformed *E.coli* incubated at 37°C, 180 rpm were prepared. The plasmid purification procedure was done according to the manufacturer's manual

2.3.4 Plasmid Control Digest

A Plasmid Control Digest is done to make sure the plasmid contains the expected size insert sequence. Therefore, the plasmid is cut into specific size pieces by restriction enzymes and the resulting fragments are analysed by gel electrophoresis.

For the plasmid control digest of the *kdmB* complementation cassette pRS_kdmB_comp_pyrG_fum Eco32I (#ER0301) and EcoRI (#ER0271) from Thermo Scientific were used as restriction enzymes. The digest was done according to the recommended procedure of the manufacturer's manual.

2.4 Yeast genetics

2.4.1 Media and solutions used in *E.coli* genetics

Table 13 Yeast Media and solutions

YPD broth pH 5.8	Yeast extract	10 g/L
	Difco-Bacto-Trypton	20 g/L
	D-glucose	20 g/L
	Agar (optional)	17.5 g/L
	Bidestilled water	
	Autoclaved	
SD – uracil pH5.8	D-glucose	20 g/L
	Difco® yeast nitrogen base	
	without amino acids	6.7 g/L
	Clontech- Ura DO supplement	0.77 g/L
	Agar (optional)	16 g/L
	Bidestilled water	
	Autoclaved	
1 M litthiumacetate LiOAc	1 M lithiumacetate	
	pH 7.5 with diluted acetic acid	
50 % PEG 3350 (W/V)	25 g PEG3350 in 50 mL bidestilled water	

2.4.2 Yeast recombinational cloning for plasmid construction

- Yeast strain **FGSC 9721 (FY834)**, uracil-auxotrophic
- Plasmid PRS426

For yeast transformation 5 mL of YPD liquid medium were inoculated with *S.cerevisiae* and incubated over night at 200 rpm and 30°C. Accordingly 50 mL YPD liquid medium was inoculated with 1-5 mL from the overnight culture and incubated for further four to six hours at 200 rpm and 30°C until an OD_{600} around 1 was measured. The 50 mL culture was centrifuged for 10 min at 4000 rpm, the supernatant discarded and the cell pellet resuspended in 1 mL 100 mM LiOAc. The culture was again spun down another time (4000rpm, 10min) and the pellet resuspended in 400 µL 100 mM LiOAc. Denaturate carrier DNA (salmon sperm DNA) was treated in the thermocycler for 5 min at 95°C and cooled on ice afterwards. In the meantime, fresh tubes containing 240 µL 50% PEG3350, 36 µL 1 M LiOAc, 1.5 µL digested shuttle vector pRS246, 5 µL of each PCR fragment (gene flanks, auxotrophy

cassette) and ddH2O (adjust volume to 360 µL) were prepared. To this tubes 10 µL salmon sperm and 50 µL aliquots of cell suspensions were added. The whole mixture was incubated 30 min at 30°C in the heating block, following another 30 min of 42°C. Then the mixture was centrifuged shortly, the supernatant discarded and the cells resuspended in the remaining fluid. Finally, the cell suspension was plated on SD-ura agar and incubated for 3-4 days at 30°C.

However, for yeast recombinational cloning a positive (yeast strain transformed only with digested vector) and a negative control (yeast strain transformed with the undigested vector) should be performed as well.

2.4.3 Isolation of plasmid DNA from yeast cells

For isolation of the plasmid DNA from yeast cells the solutions of GENEJET^{TM} Plasmid Miniprep Kit (Fermentas) were used.

5 mL selection medium (SD-xxx) were inoculated with the prototrophic yeast transformants at 30°C for 4 hours. The cell suspension is centrifuged for 10 min at 4000 rpm, the supernatant discarded and in 250 µL of resuspension solution resuspended. Accordingly, the solution was transferred in a fresh 2 mL tube and 100 µL glass beads were added. 250 µL of the lysis solution was added and inverted for 10 times. 350 µL of the neutralization solution was added and inverted 4-6 times. The mixture was spun down for 10 min to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a spin column and centrifuged for another minute, the flow-through was discarded and the column placed back in the same tube. Two washing steps followed 500 µL of washing solution. Finally, the spin column was transferred into a fresh tube and the plasmid was eluted by 25 µL of bidestilled water. The solution containing now isolated plasmid was further used for the transformation of competent *E.coli* cells.

2.5 *Aspergillus nidulans* genetics

2.5.1 Media and Solutions used in *Aspergillus nidulans* genetics

Table 14 Aspergillus nidulans- Media and Solutions

Minimal Medium MM	ASPI salt(20x)	50 mL/L
	$MgSO_4$ (200x)	5 mL/L
	Trace elements	1 mL/L
	Glucose	10 g/L (1 % final)
	Agar (optional)	15 g/L
	ddH_2O	to one litre
	Adjust pH 6.8, autoclaved (15 min, 121°C)	
ASPI-salt solution	KCl	10.4 g/L
	KH_2PO_4	16.3 g/L
	K_2HPO_4	20.9 g/L
	ddH_2O	to one litre
$MgSO_4$ (200x)	$MgSO_4$	10.4 g/ 100 mL
	ddH_2O	to 100 mL
Trace elements 100 mL	$ZnSO_4.7H_2O$	2.2 g
	H_3BO_3	1.1 g
	$MnCl_2.4H_2O$	0.5 g
	$FeSO_4.7H_2O$	0.5 g
	$CoCl_2.5H_2O$	0.16 g
	$CuSO_4.5H_2O$	0.16 g
	$(NH_4)6Mo_7O_{24}.4H_2O$	0.11 g
	Na_4EDTA	5.0 g
0.1 % (v/v) Tween®20 (Sigma)	Tween	1mL
	ddH_2O	to one litre
Top Agar for Transformation 100 mL	ASPI- salt	2 mL
	Trace elements	0.1 mL
	Glucose	1 g
	Sucrose	34.2 g
	Agarose	0.25 g
Bottom Agar for Transformation 100 mL	ASPI- salt	2 mL
	Trace elements	0.1 mL
	Glucose	1 g
	Sucrose	34.2 g

	Agarose	1.5 g
Supplements 100 mL All supplements were used in 1:100 dilutions		
Ammonium tartrate	NH4- tartrate ddH_2O	9.4g to 100 mL
NaNO3 (Nitrogen source for secondary metabolite experiments)	NaNO3 ddH_2O	8.5 g to 100 mL
p-Aminobenzoic acid (PABA)	p- amino benzoic acid ddH_2O	20 mg to 100 mL
Riboflavin (ribo)	Riboflavin ddH_2O	25 mg to 100 mL
Uridin- Uracil (UU) 50x stock 50 mL	Uridine Uracil ddH_2O	6.1 g 3.9 g to 50 mL
Arginine	Arginine ddH_2O	5.3 g to 100 mL

2.5.2 Strain list

Investigated *Aspergillus nidulans* strains are described in Table 15.

Table 15 Aspergillus nidulans strains

Strain	**Genotype**	**Characteristics**
WT	pabaA1	
*kdmB*Δ (AN8211)	Δ8211::ribo_fum, ΔnkuA::argB2, pabaB22	The gene *kdmB* is deleted
$kdmB^{H642G,\ E644Q}$≡ $kdmB^{Mut}$	ΔnkuA::argB2, pabaB22 pyrG89	Two amino acid substitutions in the JmJC domain of *kdmB*, responsible for Fe^{2+}binding
$kdmB^{CiL}$	ΔnkuA::argB2, pabaB22 riboB2	*Kdmb* gene plus ribo marker reintegrated in genome, for complementation studies

2.5.3 Marker genes in *Aspergillus nidulans*

Table 16 Marker genes in Aspergillus nidulans

Name	**Description**
argB	Arginine requiring, ornitine carbamoyl transferase gene *(Aspergillus nidulans*)
pyrG	Pyrimidine requiring orotidine 5'- monophosphate decarboxylase gene *(Aspergillus fumigatus)*
Paba	P- amino benzoic acid requiring gene *(Aspergillus nidulans)*
riboB	Riboflavin requiring gene *(Aspergillus nidulans)*

2.5.4 Cultivation of *Aspergillus nidulans*

For growth the strains were maintained on Aspergillus Minimal Media (MM) and when appropriate the media was *supplemented* with 10 mM of Uracil and Uridine for pyrG strains, 15 µM 4-Aminobenzoicacid for paba strains and 2,5 µg/mL Riboflavin for ribo strains. For normal growth Ammonium tartrate (10 mM) was added as Nitrogen source whereas for the secondary metabolite cultures Sodiumnitrate $NaNO_3$ (10 mM) was added. In case of liquid cultures, the *Aspergillus nidulans* strains were grown in 200 mL liquid MM at 37°C at 180 rpm. An inoculum density of $4X10^6$ spores/ mL was applied.

All strains were stored also as glycerol stocks at -80°C.

2.5.5 *Aspergillus nidulans* transformation

Experimental strains were obtained by transformation into an *nkuA*Δ strain which reduces the frequency by non-homologous integration due to missing nkuA gene, whicht is essential for nonhomologous end joining of DNA in double-strand break repair (Nayak et al., 2006).

The cellophane method:

For *Aspergillus nidulans* transformation minimum medium (MM) plates containing appropriate supplements were covered with a sterile cellophane sheet. For each transformed plasmid 3-4 cellophane plates per strain need to be prepared. 100 µL of a fresh spore solution of the appropriate strain was dispensed on each plate. Incubation was achieved at 37°C for 10 to 11 hours (the mycelia should not start to sporulate). Afterwards the cellophanes were transferred into a petri dish

containing a Glucanex solution (0.4 g lysing enzymes/ 10 mL 0.7 M KCl). Incubation was performed at 30°C and 50 rpm for 45 minutes in order to get protoplasts. The Lysing solution was filtrated through a sterile blutex and spun at 4000 rpm and 4°C for 20 minutes. The supernatant was discarded and the remaining protoplast pellet was dissolved in an appropriate volume of 0.7 M KCl (100 µL per plasmid and control plate). Optionally the protoplasts may be counted under the microscope to control the effectivity of the protoplast digestion (10^8 protoplasts should be used for the transformation of one plasmid). Furthermore, the protoplasts aliquots were washed in 1 mL 0.7 M KCl and centrifuged at 3000 rpm at RT for five minutes. After discarding the supernatant, the protoplast pellet was resuspended in 200 µL TN1 buffer (0.7 M KCl; 50 mM $CaCl_2$) and 1- 10 µg DNA (plasmid) were added. For the control one aliquot was performed without DNA. After addition of 50 µL TN2 buffer 60 % (w/v) PEG 6000, 100 mM Tris/ HCl pH 8, 50 mM $CaCl_2$) and gently mixing the transformations were leaved on ice for 30 minutes. Afterwards another mL of TN2 was added to each transformation mixture and incubated for further five minutes at RT. Each transformation mix was transferred into 50 mL pre-warmed top agar (40°C) and poured onto three bottom agar plates containing all necessary supplements (excepting the one used as marker). Also a negative and a positive control were performed. For the positive control all necessary supplements were added. The plates were incubated overnight at RT. The Regeneration of protoplast should be visible after 2-3 days, transformants take up to 4 days to appear.

3 RESULTS

To gain more insight into the function of the histone demethylase KdmB in secondary metabolism of *A. nidulans,* differences in SM production as well as relevant histone modifications (H3K4me3, H3K4me2) at respective SM genes were analysed in WT, *kdmB*Δ and *kdmB*$^{H642G,\ E644Q}$ strains. Furthermore, to verify the observed phenotype of *kdmB*, the mutant was complemented by re-introducing the native gene *in loco*.

3.1 Generation of *kdmB* complementation strain (*kdmb*$^{Cil)}$

3.1.1 Construction of complementation cassette

In order to re-introduce the gene *kdmB* into the *kdmB*Δ strain, a complementation cassette was constructed. Therefore, the following PCR fragments were amplified: a 1,884 bp upstream and a 1,034 bp downstream fragment of *kdmB* using the primer pairs kdmB_comp_F1//kdmB_comp_R1 and kdmB_comp_F5//kdmB_comp_R5, respectively. *A. nidulans* WT genomic DNA was used as a template. The *kmdB* gene itself was amplified with the primer pairs kdmB_comp_F2//kdmB_comp_R2 and kdmB_comp_F3//kdmB_comp_R3 from the plasmid RS_kdmB_213. As a selectable marker the *Aspergillus fumigatus pyrG* gene was used, that was amplified with the primers kdmB_comp_F4 and kdmB_comp_R4 from the plasmid 3RD_KD_pyrG.

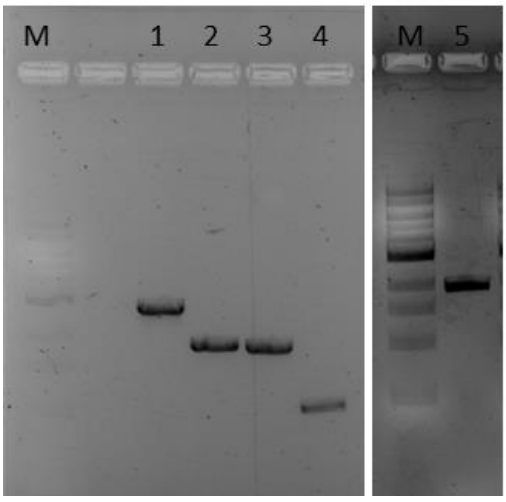


Figure 16 Amplified fragments for *kdmB* complementation cassette. (M) 1kbp Marker, (1) *kdmB* fragment (2833 bp) amplified with kdmB_comp_F2 & kdmB_comp_R2 from pRS_kdmB_213, (2) *kdmB* fragment (1947 bp) amplified with kdmB_comp_F3 and kdmB_comp_R3 from pRS_kdmB_213, (3) amplified *pyrG* marker with kdmB_comp_F4 and kdmB_comp_R4 from 3RD_KD_pyrG, (4) *kdmB* downstream fragment (1034 bp) using kdmB_comp_F5 and kdmB_comp_R5 as primers from *A. nidulans* WT, (5) *kdmB* upstream fragment using kdmB_comp_F1 and kdmB_comp_R1 as primes from *A. nidulans* WT.

3.1.2 Yeast recombinational cloning strategy

The amplified PCR fragments were assembled in the uracil- auxotrophic yeast strain FGSC 9721 (FY 834 using the endogenous homologous recombination system. Notably, kdmB_comp_F1 and kdmB_comp_R5 have overlapping ends with the *Saccharomyces cerevisiae* shuttle vector RS_426. Furthermore, the amplified fragments have homologous tails to the adjacent fragment (*Figure 17*). The yeast shuttle vector RS_426 contains the *URA3* gene for selection on medium without uracil. Moreover, the plasmid contains the *ampR* gene for bacterial selection.

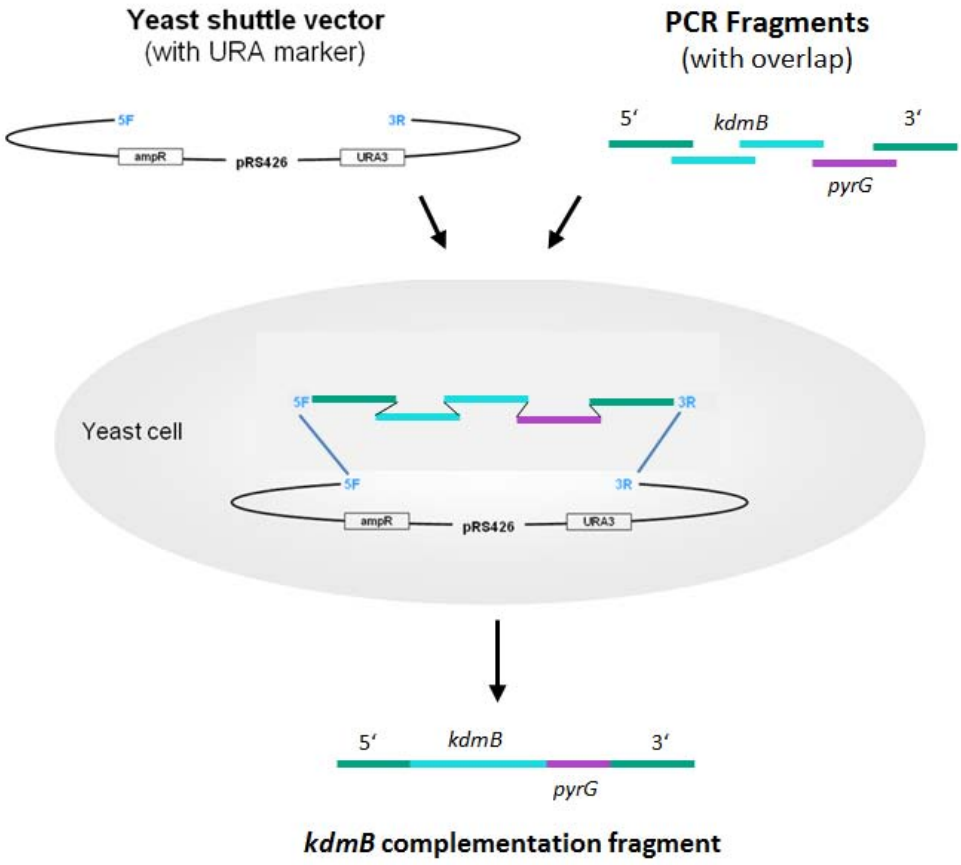


Figure 17 Schematic representation of the yeast recombinational cloning strategy. The uracil-auxotrophic yeast strain FGSC 9721 (FY 834) is transformed with the amplified fragments and the XhoI/EcoRI-restricted pRS_426 carrying overlapping ends. The yeast endogenous recombination system assembles the fragments with the vector yielding in the desired complementation cassette.

After yeast recombinational cloning, the final plasmid with a size of 15,072 bp was isolated with GeneJet™ Plasmid Miniprep Kit (Fermentas) from the yeast cells and further cloned in *E.coli* (Methods). Correct assembly of the plasmid was verified by sequencing (data not shown) and digestion.

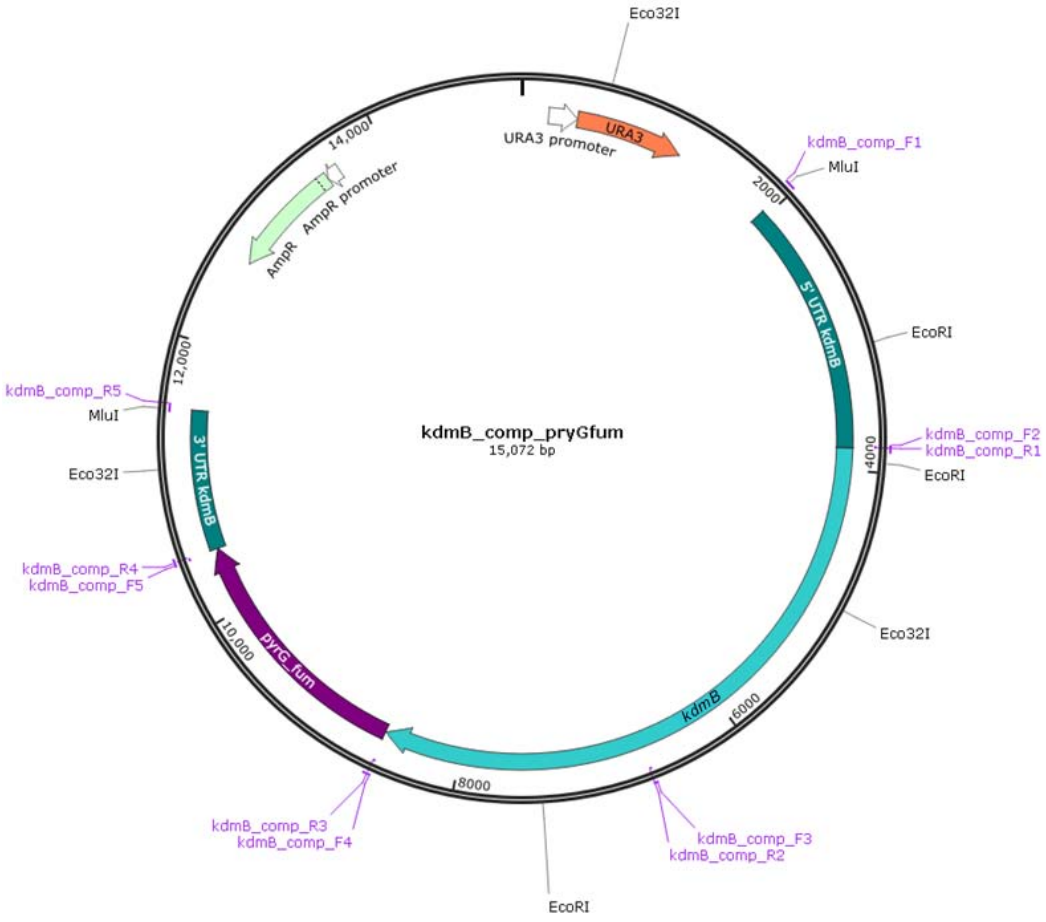


Figure 18 pRS426_kdmB_comp_pyrGfum plasmid map. The whole plasmid has a size of about 15,072 bp and the primers for yeast recombinational cloning are indicated in purple: kdmB_comp_F1/R1 were used for 5'UTR amplification, kdmB_comp_F2/R2 and kdmB_comp_F3/R3 were used for amplification of the gene *kdmB*, kdmb_compF4/R4 were used for the amplification of the selection gene *pyrG*, and kdmB_comp_F5/R5 were used for the amplification of the 3'UTR. The plasmid contains the *URA3* gene as well as the *AmpR* gene for selection. The restriction sites for Eco32I, EcoRI and MluI are indicated as well.

In order to check the correct assembly of the complementation cassette, pRS426_kdmB_comp_pyrGfum was restricted with *Eco*RI and *Eco*32I resulting each in three fragments of different sizes. The restriction with *Eco*RI resulted in a 800 bp, a 3,4 kb and a 10 kb fragment whereas the restriction with *Eco*31I resulted in a 4.3 kb, 4.6 kb and a 6 kb fragment (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Unfortunately, the 800 bp fragment resulted from *Eco*RI restriction is very weak and almost not seen on the gel, whereas the 4.3 and 4.6 kb fragments were not separated properly and resulted in one band. For transformation the plasmid was cut with *Mlu*I (restriction sites indicated in **Fehler! Verweisquelle konnte nicht gefunden werden.**) resulting in a 5.5 kb backbone fragment and a 10 kb insert fragment (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This probe was used directly for transformation of the *kdmB*Δ strain (Δ8211::ribo_fum, ΔnkuA::argB2, pabaB22) without further purification steps. Out of this, several

pyrG prototrophic transformants were gained. The correct integration of the *kdmB in loco* complementation construct was subsequently verified by PCR.

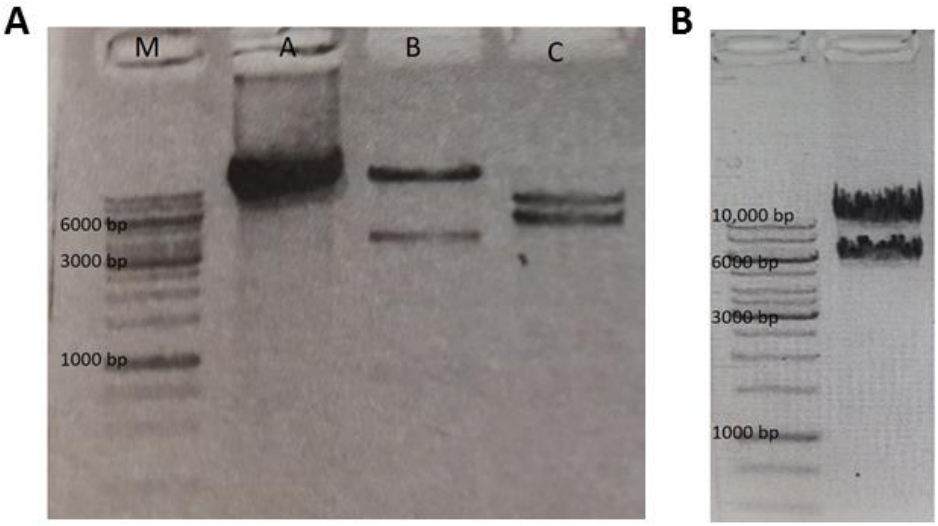


Figure 19 (A) Control digest of pRS426_kdmB_comp_pyrGfum. (M) 2-Log DNA ladder (A) undigested plasmid; (B) plasmid restricted with EcoRI resulting in three fragments of 800 bp, 3.4 kb and 10 kb. Notably, the band at 800 bp is very weak and almost not seen.; and (C) restricted with Eco32I resulting in three fragments of 4.3 kb, 4.6 kb and 6 kb. Unfortunately, the 4.3 kb and 4.6 kb fragment were not separated properly and result in one band. (B) Digest of pRS426_kdmB_comp_pyrGfum with MluI. The gel image displays two bands, one at 10 kb (insert) and one at 5.5 kb (backbone). The digest was used directly for transformation.

3.1.3 Transformation of *A.nidulans kdmB*Δ strain with *kdmB* complementation fragment

For complementation studies the *kdmBΔ* strain, which harbours a *ribo* marker instead of the original *kdmB* gene, was used. Via homologous recombination the *kdmB* gene was exchanged for the auxotrophic marker *ribo*, and thus, integrated *in loco*. To pick correct transformants the new generated strains were grown on AMM with different sets of supplements (Table 17).

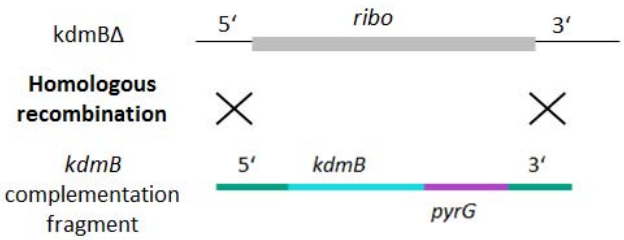


Figure 20 Complementation strategy. Integration of *kdmB* via homologous recombination resulting in knock out of the auxotrophic selection marker ribo (riboflavin).

Table 17 Transformants: Combination of supplements

Combination of supplements	***Growth***

NH_4 tartrate, p-amino benzoic acid (paba)	No
NH_4 tartrate, riboflavin (ribo)	No
NH_4 tartrate, paba, ribo	**Yes**
Negative control: NH_4 tartrate	No

The newly generated complementation strains *kdmB*^{Cil} are auxotroph for p-amino benzoic acid (paba) and riboflavin (ribo), due to the absence of the respective genes responsible for their biosynthesis. Thus, only transformants that are able to grow on medium supplemented with paba and ribo show correct *in loco* integration of the *kdmB* insert. Furthermore, an *nkuA*Δ (Nayak et al., 2006) strain was used for transformation which minimizes the frequency of non-homologous integration events. Correct integration of the *kdmB* complementation construct was subsequently verified by diagnostic PCR.

Therefore, primers were designed upstream and downstream of the predicted complementation construct as well as within the gene *kdmB* (Figure 18). Transformants that showed homologous integration events were picked and used for further analysis.

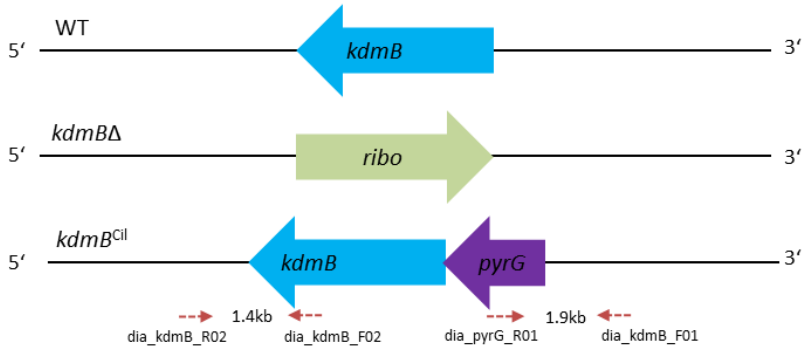


Figure 21 *KdmB* locus of WT: *kdmB*Δ deletion and complementation strain *kdmB*^{Cil}. The WT strain has still its original gene *kdmB* (blue arrow) whereas *kdmB* was replaced with the marker gene *ribo* (green arrow)) in the deletion strain *kdmB*Δ. In the complementation strain *kdmB*^{Cil} the gene *kdmB* is re-integrated with the auxotroph selection gene *pyrG* (violet arrow). For verifying the correct integration of the complementation cassette the primers dia_kdmB_F01/F02/R02 and dia_pyrG_R01 were used (red arrows).

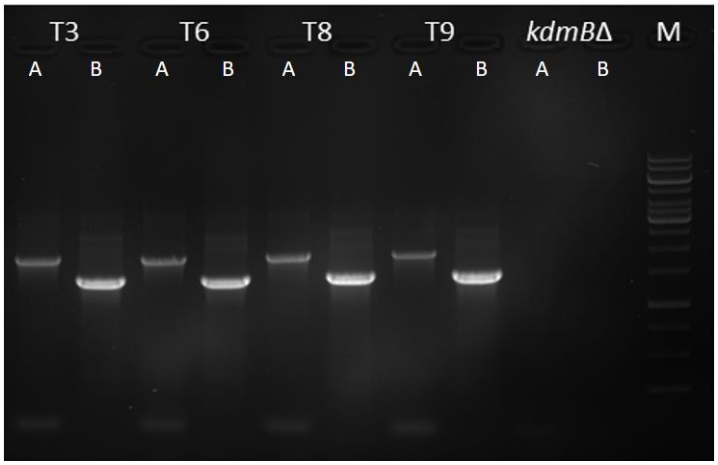


Figure 22 Verification of in loco integration of kdmB. Used primers have their binding sites upstream and downstream of the complementation construct as well as within the gene *kdmB* to check the correct in loco integration. Picked transformants T3, T6, T8 and T9 of the complementation strain display both bands at 1.4 and 1.9 kb. (M) 1 kb Ladder (A) 1.9 kb Fragment (B) 1.4 kb Fragment.

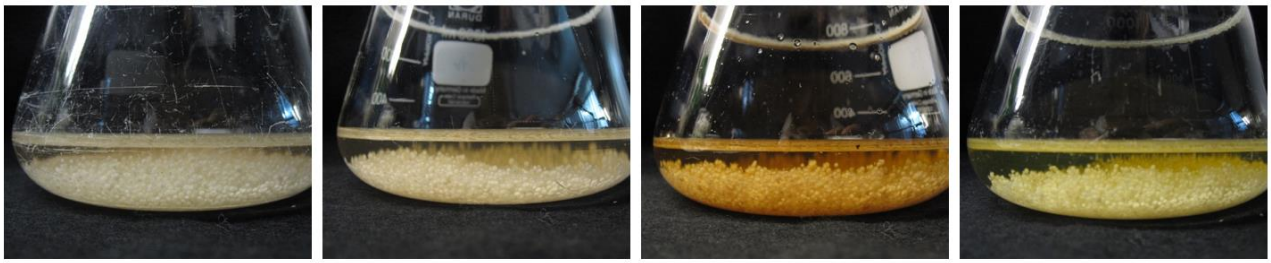
3.2 Altered Regulation of Secondary Metabolite gene clusters

In general, the H3K4me3 mark is associated as an active mark, although it is still not 100 % clear why H3K4me3 marks promote activation. It is certain that specific histone methylation states regulate transcription by promoting the binding of transcription factors. For instance, in human cells H3K4me3 recruits the chromatin remodelling factor CHD1, which is associated with active chromatin (Flanagan et al., 2005). Therefore, deletion of *kdmB* is thought as a transcriptional activator, as the active H3K4 methylation mark cannot be erased. Previously, Strauss and co-workers showed that, deletion of *kdmB*, results in a strong deregulation of SM genes. Unexpectedly, 40% of the SM genes were found to be downregulated, while only a few SM genes are indeed up-regulated due to the loss of KdmB (Gacek-Matthews et al., 2016). In order to gain further insights into the role KdmB plays in SM regulation, we now performed expression analysis of selected SM genes, including the key enzyme-encoding genes (*ipnA, orsA, mdpG*) as well as genes encoding transcriptional activators (*aflR).* In addition, production of the corresponding SMs was analysed. To link the observed trend of increased or decreased transcript of SM key-enzyme encoding genes to the Δ*kdmB* and kdmB$^{H642G, E644Q}$ strain, relevant histone modification, i.e. H3K4me2 and H3K4me3, at respective SM cluster genes were analysed, thereby gaining further insight into the function of the demethylase KdmB.

3.2.1 Procedure for SM quantification, gene expression analysis and ChIP

SM metabolite and gene expression analysis

For metabolite and gene expression analysis, all obtained strains (WT, *kdmb*Δ, *kdmB* $^{H642G, E644Q}$ and *kdmB*^{Cil}) were grown for 48 hours in liquid minimal medium at 37°C and 180 rpm in the dark. Figure 19 shows each of the different strains after 48 hrs. Already at this stage one can assume that different metabolites are produced due to the different colours of the supernatant (*kdmB*^{Cil} is excluded from this consideration as the supplement riboflavin colours the supernatant yellowish). Notably, the colour of the supernatant of *kdmB*Δ is lighter than of *kdmB*$^{H642G, E644Q}$. The liquid cultures were filtered over miracloth. While the harvested mycelia were quick-frozen in liquid nitrogen, lyophilized and subsequently applied for RNA extraction, the culture filtrate was directly used for chemical analysis.



WT *paba* | **kdmBΔ** *paba* | **kdmB** $^{H642G, E644Q}$ *paba uu* | **kdmB**^{Cil} *paba ribo*

Figure 23 *A.nidulans* liquid SM (48 hrs) cultures (Aspergillus Minimal Media) supplemented either with paba (p-amino benzoic acid), uu (uracil-uridine) and ribo (riboflavin). The strains are differently coloured due to the SM production. *KdmB*^{Cil} is excluded from this consideration as the supplement ribo colours the supernatant yellowish.

RNA was extracted (see Materials and Methods) and subsequently reverse transcribed into cDNA using the iScript^{TM} cDNA Synthesis Kit (BioRAD). Successful DNase treatment and cDNA synthesis was verified by PCR using the actin-encoding gene, *AN7077*, as a target gene (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

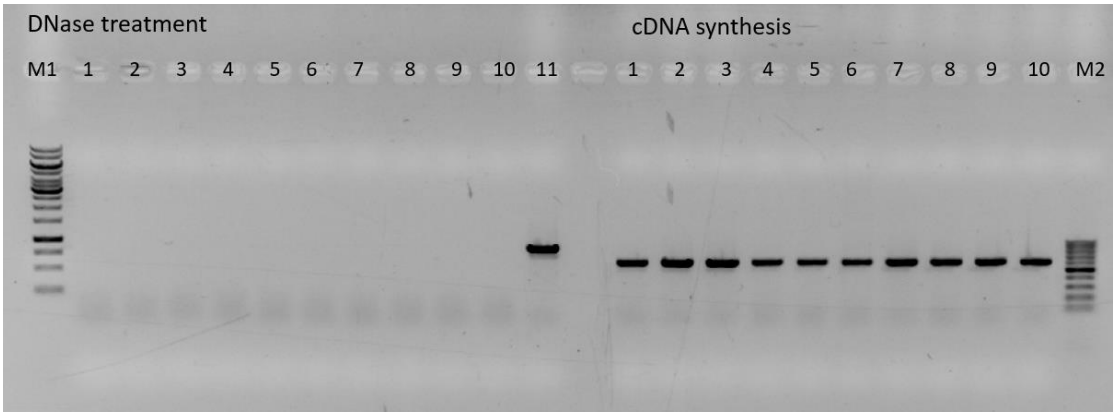


Figure 24 DNase treatment and cDNA synthesis control. (M1) 1 kbp Marker, (M2) 100 bp Marker, (1, 2) WT; (3, 4) kdmBΔ; (5, 6, 7, 8) kdmB $^{H642G, E644Q}$; (9, 10) kdmB ^{Cil}; (11) positive control: gDNA WT. All fragments have a size of approximately 870 bp. This figure shows the complete absence of DNA in the RNA samples after DNase treatment and the effective and consistent cDNA synthesis. The samples were done in duplicates.

Expression of the target genes was normalised to the expression of the actin- and the ß-tubulin-encoding genes (*AN7077, AN1182*). The relative expression levels were calculated according to the ddCT method (Pfaffl, 2001). The primer efficiencies were between 89-110% (Table 19).

First, expression analysis of *kdmB* itself was analysed in the respective strains WT, *kdmB*Δ, *kdmB*$^{H642G, E644Q}$ and *kdmB*^{Cil} and as expected *kdmB* is expressed in the complementation strain again.

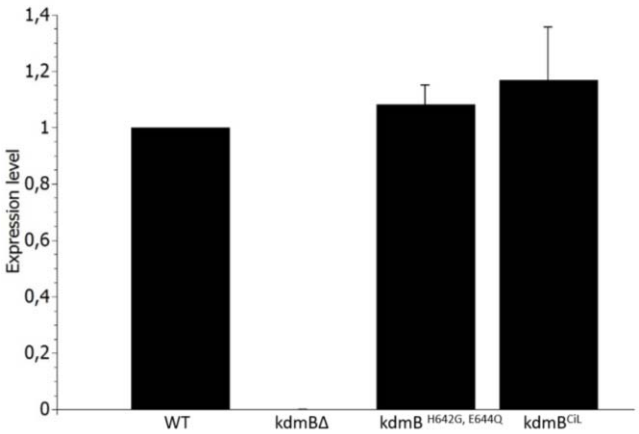


Figure 25 *KdmB* expression in all four strains. As expected there is no expression of *kdmB* in the deletion strain whereas *kdmB* expression is restored in the mutant as well as in the complementation. For expression analysis RNA was isolated from a 48 h SM culture. Mean values and standard deviations are shown; RT-qPCR Experiments were done in biological triplicates giving the same results; only technical replicates are shown here

Next, we performed RT-qPCR with selected SM candidate genes.

Chromatin Immoprecipitaion experiments

For ChIP experiments WT, *kdmB*Δ and *kdmB*$^{H642G,\ E644Q}$ strains were grown in liquid for 48 hrs (37 °C), and subsequently, the histones were crosslinked to the DNA with formaldehyde. In order to obtain chromatin for the ChIP, the mycelia were quick-frozen and powdered in liquid nitrogen, resuspended in ChIP-lysis buffer and directly sonicated as described in material and methods. For ChIP a rabbit polyclonal anti-H3K4me3 and anti-H3K4me2 antibody from Abcam was used. In addition to samples treated with the antibody, a control sample that was not treated with the primary antibody (no-antibody control). This control sample was used for normalization, as these samples contained total isolated chromatin (Fold Enrichments). Samples were related to the input control. The *Input sample*s were neither treated with the primary nor the secondary antibody. These samples represent controls for non-specific precipitation.

The amount of precipitated DNA was quantified with qPCR using specific primes (Table 18). Furthermore, successful shearing of DNA and ChIP (Fold Enrichment) was checked (Appendix).

3.2.2 Negatively regulated SM clusters

Originally KdmB was thought to have a repressing effect on SM gene clusters since H3K4me2 and -me3 abundance was generally associated with active transcription. But upon deletion of *kdmB* downregulated SM gene clusters have been found as well, suggesting that KdmB has a role in gene activation as well. Notably, most of the SM gene clusters are almost empty of H3K4 tri-methylation. Only two genes in the *ors* (AN7913) and *stc* (AN7822) locus show a H3K4me3 mark. AN7913 is part of the ors cluster, annotated as *orsD,* and is required for the biosynthesis of F-9775A and F-9775B (Schroeckh et al., 2009). Unfortunately, AN7822 is still uncharacterized and the function remains unclear.

Orsellinic acid and emodine production increased during *kdmB* deletion, therefore, those two secondary metabolites are regulated in a classical repressive manner of KdmB. Furthermore, we have also found two SM gene clusters behaving the opposite.

3.2.2.1 Orsellinc acid gene cluster (ors)

The gene *orsA,* encoding a non-reducing PKS, is required for the formation of the first pathway-specific intermediate, orsellinic acid. Next to *orsA*, the *ors* cluster contains further genes encoding for decorating enzymes leading to the formation of the two yellow polyketides F-9775A and F-9775B.

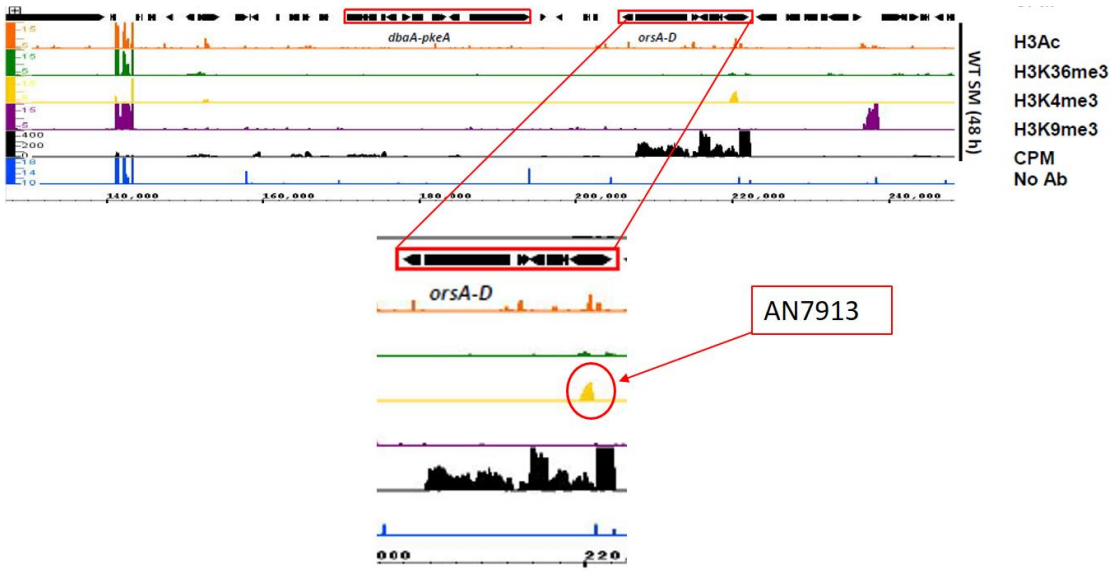


Figure 26 Chromatin landscape of the *ors* locus. The *ors* locus is indicated within the red box and is strongly expressed in the wild type during SM (48 h) growth; Just one gene (*AN7913-orsD*) exhibits a high level of H3K4me3 (Gacek-Matthews et al., 2016). To the left of the orsA-D cluster is the boxed DBA (derivate of benzylaldehyde) gene cluster. Distribution of the analysed histone marks and RNA-seq reads (CPM- transcript counts per million) in and around the clusters are shown for wild type cells in the secondary (48 h) metabolism phase (Gacek-Matthews et al., 2016).

Here, we show that orsellinic acid biosynthesis is significantly increased in both the *kdmB*Δ and the *kmdB*$^{H642G\ E644Q}$ strain (B). Consistent with this finding, F-9775A and F-9775B production is also

increased (*Figure 28*). It is noteworthy, that production of F-9775A and F-9775B was significantly higher in the *kmdB*$^{H642G\ E644Q}$ strain compared to *kdmB*Δ. Unfortunately, the expression level of *orsA* (A) does not reflect the detected metabolite level. Going in line with the ChIP-seq analysis investigation of the methylation status (H3K4me3 and H3K4me2) of *orsA* has shown no significant differences compared to the WT (C, D), whereas the gene *AN7913 (orsD)*, that showed H3K4me3 in the ChIP-seq, shows significantly higher tri-methylation of H3K4 in both the *kdmB*Δ and the *kmdB*$^{H642G\ E644}$ strains (E), thereby indicating that KdmB is normally involved in demethylating H3K4 associated with this gene. However, the transcription of this gene is not increased in the deletion mutant but reduced (Gacek-Matthews et al., 2016) indicating that higher H3K4 methylation is not sufficient to promote transcription.

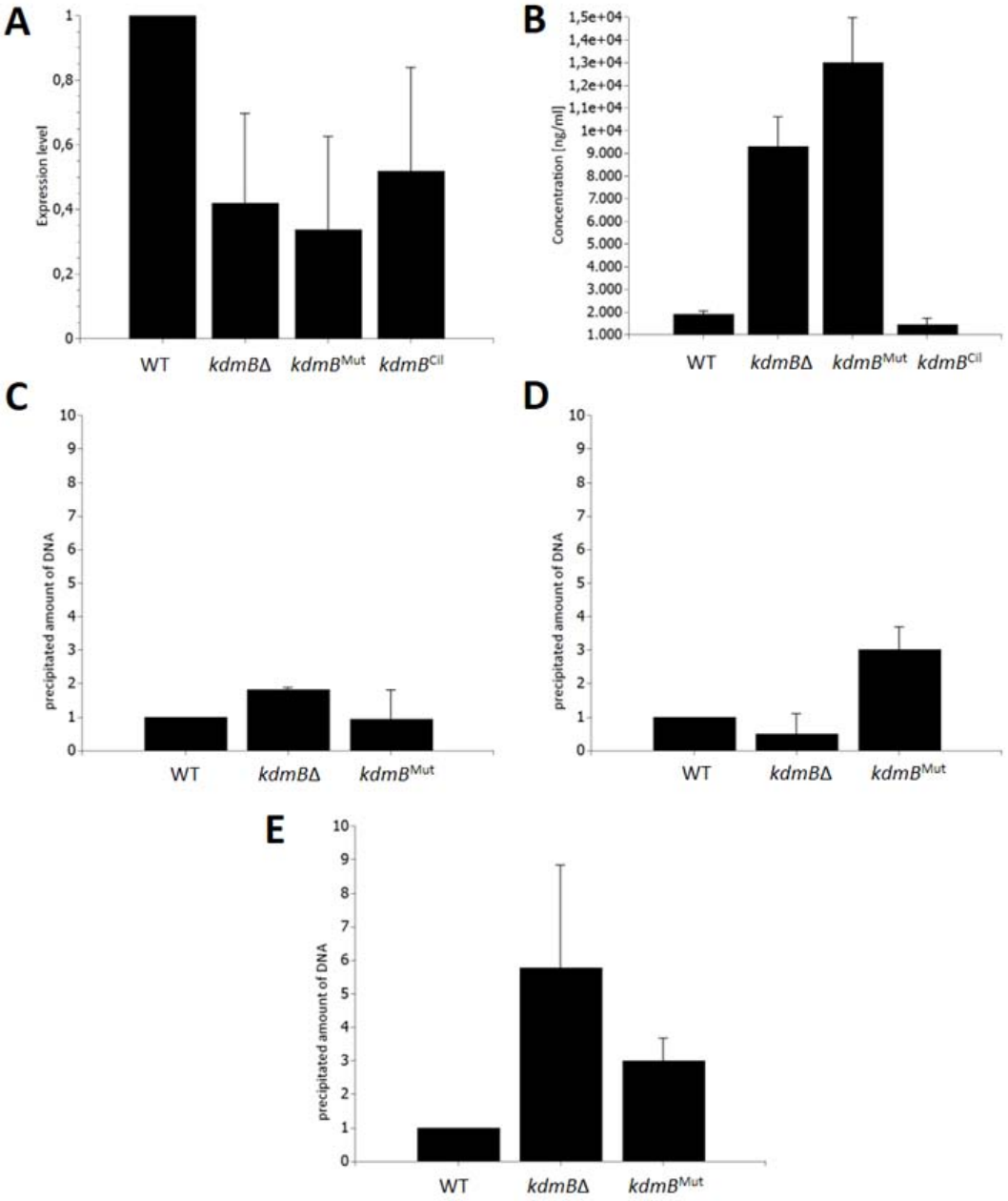


Figure 27 Orsellinic acid gene cluster. (A) RNA Expression analysis of the SM gene *orsA* encoding a PKS responsible for the formation of orsellinic acid. The expression of the gene is a little lower in the deletion, mutant and complementation strain whereas the formation of the metabolite orsellinic acid (B) is strongly upregulated in the deletion and mutant strain. The tri-methylation level (C) as well as the di-methylation level (D) of the gene *orsA* fluctuate around the WT level, supporting the fact, that no H3K4me3 mark exists at this gene. The H3K4me3 mark of the *orsD* gene *AN7913* (E), which is still uncharacterized, shows as expected a higher tri-methylation in the deletion and mutant strain. Obviously, this mark is targeted by KdmB in the WT.

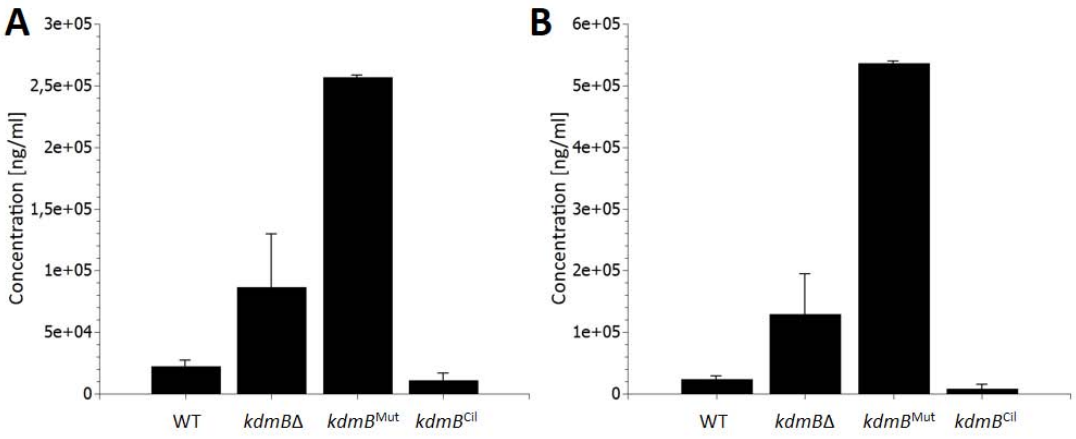


Figure 28 Metabolite levels of F-9775A (A) and F-9775B (B). The two yellow polyketides are slightly upregulated in *kdmB*Δ and strongly upregulated in the mutant strain *kdmB*^{Mut}.

3.2.2.2 Monodictyphenone gene cluster (mdp)

The monodictyphenone gene cluster displays a similar trend as the *ors* gene cluster, and the increased production of emodine is consistent with the up-regulation of *mdpG*, the PKS-encoding gene involved in the formation of monodictyphenone (A, B) in *kdmB* deletion background. The mutant strain *KdmB*$^{H642G,\ E644Q}$ displays almost WT-like features despite the lack of demethylase function. This expression pattern indicates that some other domain(s) but not the JmjC-demathylase domain are involved in repression of *mdpG*. Again, *KdmB*^{CiL} rescues the WT phenotype.

In accordance with the ChIP-seq data (see appendix), analysis of the H3K4 methylation status of *mdpG* (H3K4me3, H3K4me2) reveals no significant difference in H3K4 methylation (C; D).

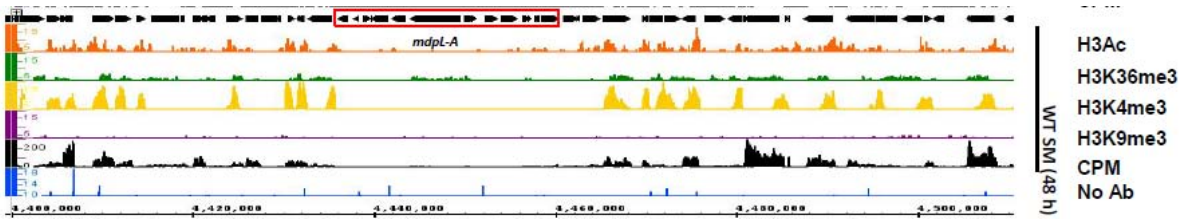


Figure 29 Chromatin landscape of the monodictyphenone gene cluster. The monodictyphenone gene cluster (mdpL-A) is indicated within the red box. Distribution of the analysed histone marks and RNA-seq reads (CPM- transcript counts per million) in and around the clusters are shown for wild type cells in the secondary (48 h) metabolism phase. The transcript level for the mdp gene cluster remains very low. Furthermore, the cluster is free of any H3K4me3 marks (Gacek-Matthews, 2016 #3471}

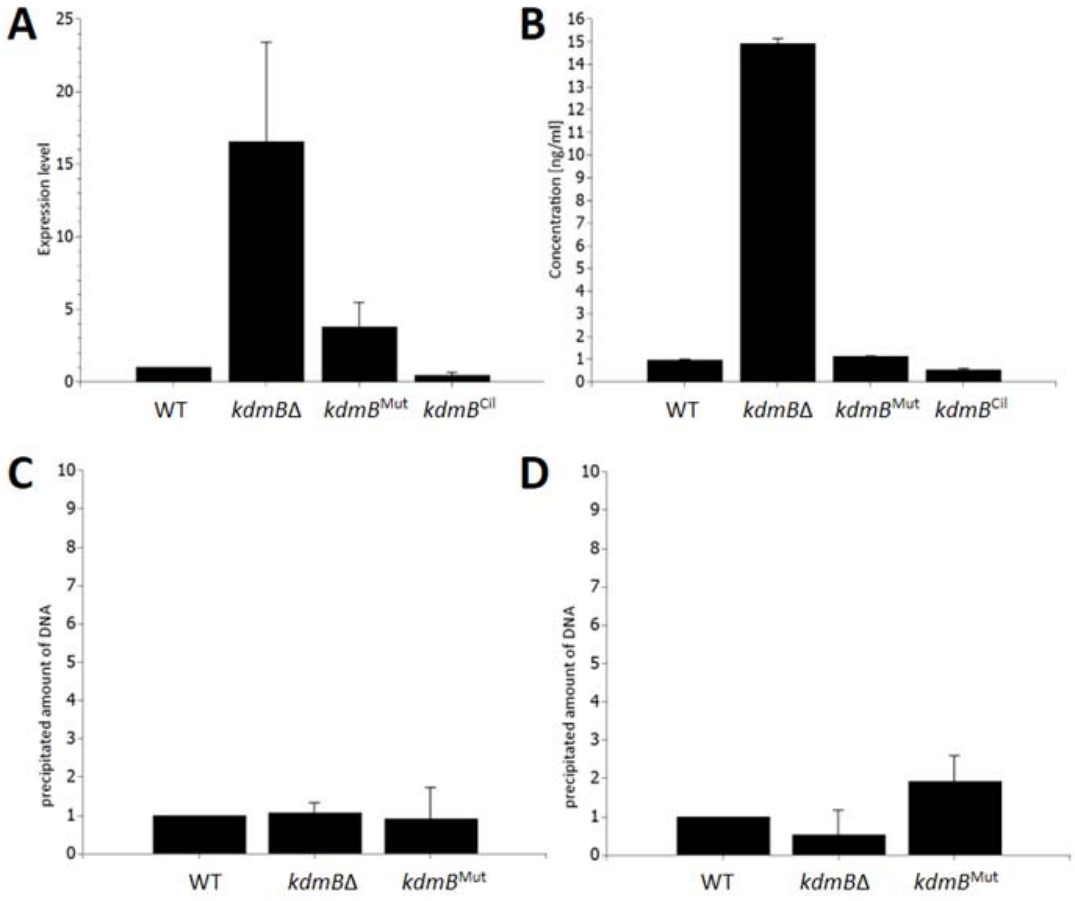


Figure 30 Monodictyphenone gene cluster. Expression of the gene *mdpG* is strongly upregulated in the deletion strain (A). The same trend is verified by the measurement of the metabolite emodine. Interestingly the mutant strain *KdmB*^{Mut} displays WT-like features. The H3K4me3 (C) as well as the H3K4me2 (D) levels in the mutants fluctuate around the WT background level, confirming the generally low H3K4 methylation mark found by ChIP-seq in the cluster.

3.2.3 Positively regulated SM clusters

To investigate the involvement of KdmB in gene activation of several SM clusters we have taken a closer look at two affected SM genes clusters: sterigmatocystin and penicillin gene cluster.

3.2.3.1 Sterigmatocystin gene cluster (stc)

The *stc* cluster is a huge cluster, containing 27 genes all involved in sterigmatocystin biosynthesis either by regulating gene expression of the remaining cluster genes or by decorating the polyketide-derived backbone. Notably, only one out of these 27 genes, that is *AN7822,* is enriched for H3K4me3. Unfortunately, the function of this gene is still unknown.

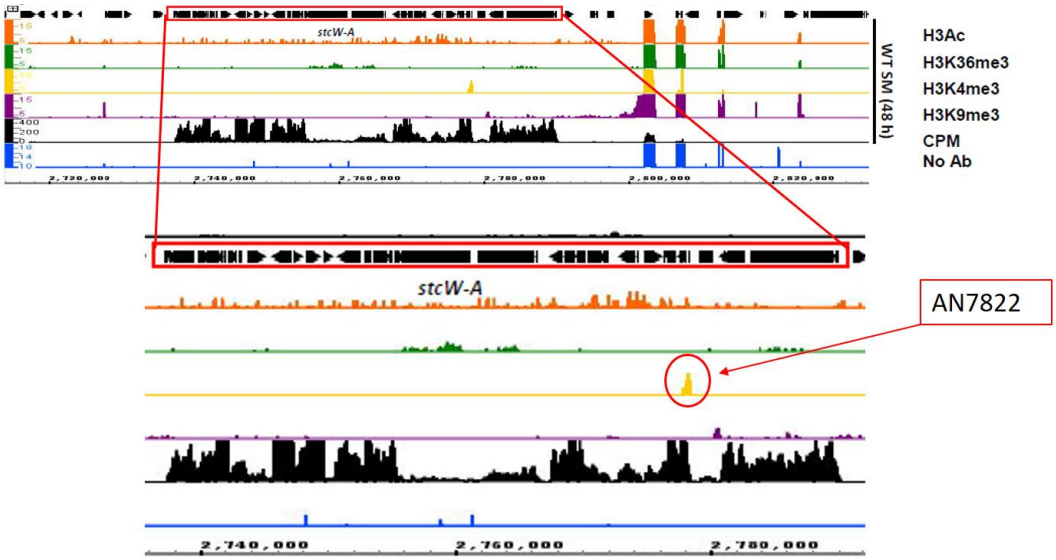


Figure 31 Chromatin landscape of the sterigmatocystin cluster. The *stc* cluster is indicated within the red box. The *stc* cluster is strongly induced in the wild type during SM (48 h) growth. Distribution of the analysed histone marks and RNA-seq reads (CPM- transcript counts per million) in and around the clusters are shown for wild type cells in the secondary (48 h) metabolism phase. The sterigmatocystin gene cluster is stronger expressed during secondary metabolism phase but just one gene (*AN7822*) exhibits a high level of H3K4me3 (Gacek-Matthews et al., 2016)

Here, we show that lack of KdmB as well as demethylase deficiency results in reduced to no production of sterigmatocystin (*Figure 31*). Expression analysis of the gene *aflR*, encoding a transcriptional activator for this cluster, is strongly downregulated in the deletion as well as the demethylase-deficient strain (A). Again, expression analysis of *aflR* is consistent with the production levels of sterigmatocystin (B). Production of sterigmatocystin is absent in the *kdmB*Δ and the *kdmB*$^{H642G, E644Q}$ strains compared to the WT. Complementation of the *kdmB*Δ mutant rescues the WT phenotype.

Further analysis of the H3K4 methylation status of the gene *aflR* support Gacek et al. ChIP Sequence data indicating that *aflR* has no trimethylation mark. The analysed gene *AN7822*, which was shown to

be enriched for H3K4me3, has no higher H3K4m3 level in the *kdmB*Δ and *kdmB*^{H642G,E644Q}compared to the WT. Thus might indicate that this gene is no target of KdmB.

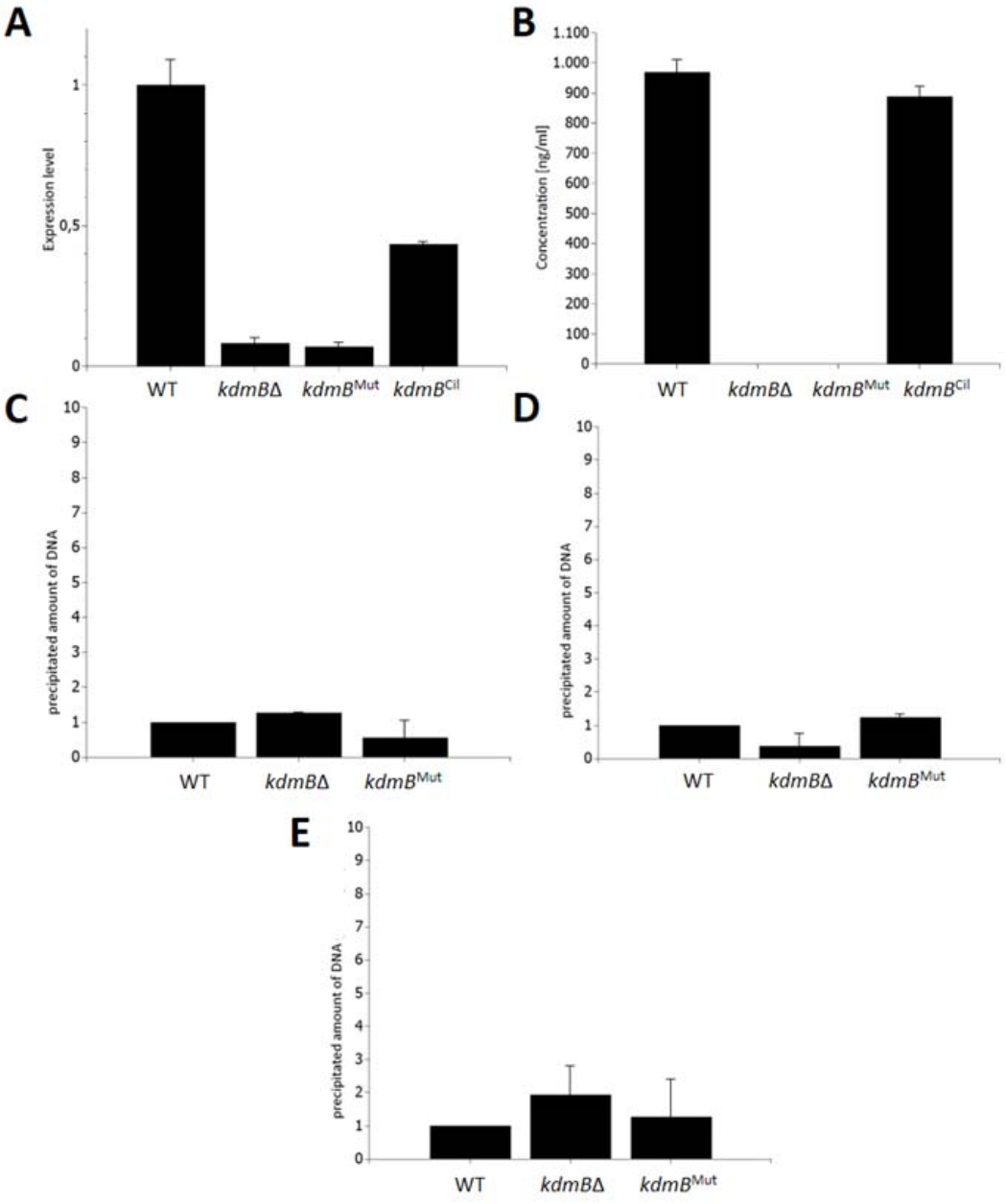


Figure 32 Sterigmatocystin gene cluster. (A) Expression analysis of the transcriptional activator *aflR* exhibits the same trend as measurement of the metabolite sterigmatocystin. The expression of the gene and the formation of the metabolite is strongly downregulated in *kdmB*Δ and *kdmB*^{Mut} in both experiments. Analysis of the H3K4me3 (C) and H3K4me2 (D) fluctuate around WT level whereas the H3K4me3 level of *AN7822* (E), which is known to have a -me3 mark, is higher in the deletion and mutant strain. Due to strong standard deviations this result is not significant.

3.2.3.2 Penicillin gene cluster (pen)

The second SM gene cluster which undergoes a downregulation is the penicillin gene cluster. Unfortunately, the metabolite level was not analysed due to the strong instability of the metabolite. However, the expression analysis of the gene *ipnA*, encoding the N-isopenicillin synthase, is almost silent in the deletion and mutant strain of *kdmB*. *KdmB*^{CiL} complements the WT just to 10%. Furthermore, H3K4-me3 (B) and -me2 (C) levels of *ipnA* were analysed but no significant conclusions can be made. This is also consistent with Gacek's ChIP Sequence data, showing the penicillin gene cluster empty of any H3K4 methylation marks.

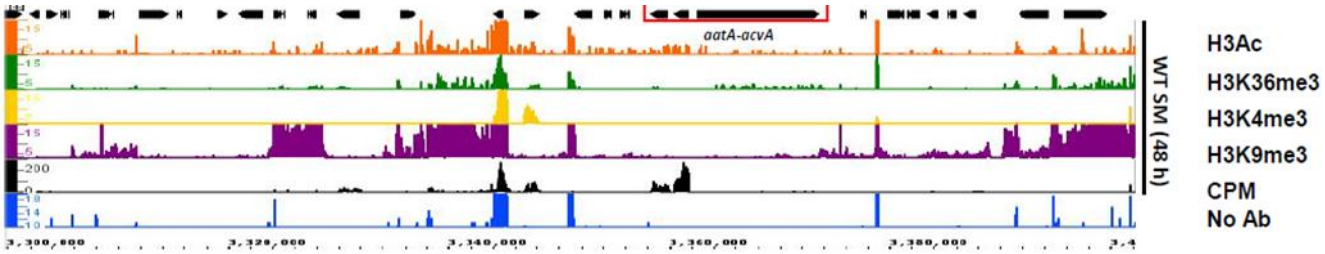


Figure 33 Chromatin landscape of the penicillin (*aatA-acvA*) gene cluster. The pen gene cluster is indicated within the red box. Distribution of the analysed histone marks and RNA-seq reads (CPM- transcript counts per million) in and around the clusters are shown for wild type cells in the secondary (48 h) metabolism phase. The penicillin gene cluster is stronger expressed during secondary metabolism phase but the cluster is empty of any H3K4 trimethylation marks (Gacek-Matthews et al., 2016)

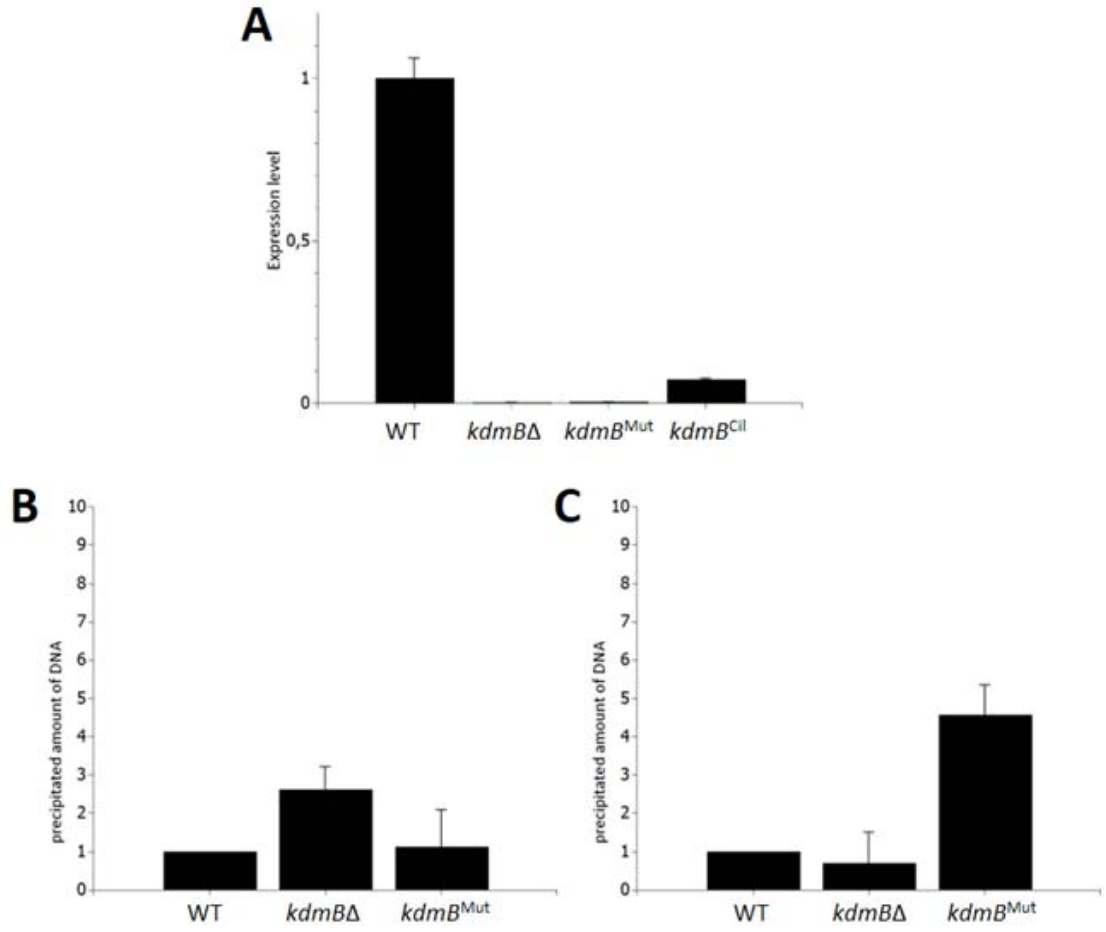


Figure 34 Penicillin gene cluster. The expression of *ipnA* is strongly downregulated in the deletion and mutant strain, whereas the complementation strain expresses the gene again to 10% compared to the WT. ChIP experiments revealed no higher H3K4me3 level of *ipnA* (B) whereas the H3K4me2 level is significantly higher in the mutant strain (C). But there are no differences between WT and deletion strain.

4 DISCUSSION AND PERSPECTIVES

4.1 The role of KdmB in the regulation of secondary metabolite genes

The production of secondary metabolites underlies a complex regulatory network, involving not only environmental and genetic factors but also epigenetic. This is supported by the physical linkage of the genes in clusters, thus minimizing the amount of necessary steps for regulation of the biosynthetic machinery (Brakhage, 2013; Gacek & Strauss, 2012). It becomes more and more evident that secondary metabolism is controlled by the chromatin structure: For example, activation of SM clusters is generally accompanied by increased acetylation of histones. In contrast to this, histone methylation is more complex. While methylation of H3K9, H3K27 and H4K20 are associated with transcriptional silence, H3K4, H3K36 and H3K79 methylation marks are often read as activating signals (Strauss & Reyes-Dominguez, 2011). Methylation marks are erased by histone- demethylases, thereby inducing or repressing the underlying targeted gene, depending on the modified amino acid. In the current study, lack of KdmB would result in overall higher H3K4me3 levels. Thus, the finding that the demethylase KdmB does not only mediate transcriptional downregulation but also activation of a high number of SM genes, lead us to the assumption that the protein somehow functions as a transcriptional activator. The aim of this study was to start looking into the function of the different domains in this unexpected feature of the protein and to determine the role of the Jumonji domain in the two opposing transcriptional processes.

4.2 Transcriptional repressor or activator and correlation to H3K4 methlytion?

We analysed in detail four *kdmB*-dependent genes involved in SM: *mdpG* and *orsA* are repressed by KdmB and thus up-regulated in the mutant. In contrast*, ipnA* and *aflR* are activated by KdmB and only basal low levels are consequently detected in the mutant. In parallel, H3K4 methylation was tested and the situation was clarified to some extent. All four investigated secondary metabolite gene clusters (*ors*, *mdp*, *stc* and *pen*) show no or only very little H3K4me3 throughout the whole cluster region. Only two genes have been identified that harbour H3K4me3, that is *AN7913* (*orsD*) which is part of the *ors* locus and involved in the formation of the two yellow polyketides F9775A and F9775B, and *AN7822* which is part of the *stc* locus. Unfortunately, the function of this gene has yet to be determined.

4.2.1 Dissection of the demethylase enzyme activity

In this study, we set out to dissect the demethylase function from other, potentially activating or repressing functions of KdmB. However, although the ambivalent role of KdmB appeared to be independent of the demethylase enzyme activity at first sight, our detailed investigations showed that upon mutation of the demethylase domain different responses were obtained in different gene clusters.

In case of *orsA*, *ipnA* and *aflR,* the gene expression is strongly downregulated in the deletion strain as well as in the demethylase-deficient mutant strain. This is accompanied by a complete loss of sterigmatocystin production (and probably penicillin production which we unfortunately were not able to determine in this study) in both the *kdmB*Δ and *kdmB*^{H642G,E644Q} strain. Interestingly, the metabolite of the *ors*-gene cluster orsellinic acid and the yellow polyketides were not reduced despite low expression of the biosynthetic genes pointing towards an indirect involvement of KdmB in precursor supply for these SMs.

On the KdmB protein level, these results suggest that the integrity of the JmjC-demethylase domain is required for the activating function of KdmB. This is double-surprising as (i) demethylation would have been expected to act as negative signal and (ii) the functional domain carrying out this "repressing" reaction would not be expected to take part in the activation process. However, we have no structural information on this protein so it could well be that the disruption of the JmjC domain by replacing the iron-coordinating amino acids has detrimental effects on the overall protein folding and structural features. Therefore, it is advisable to first obtain structural data for KdmB before we can speculate more on the necessity of JmjC for the activating function.

Only for the monodictyphenone gene cluster the situation seems to be different. In this cluster, KdmB acts as repressor and the deletion mutant shows strong up-regulation of the key enzyme gene *mdpG*. Concomitantly, the amount of product (emodine) is also increased. In this case, however, mutantion of the JmjC domain produced a different effect compared to the full gene deletion: this strain displays an almost WT-like phenotype indicating that the integrity of the demethylase domain is not necessary for the repressing function. Again this is double-surprising because the "canonical" role of KdmB as H3K4 de-methlyase would be to remove the activating histone mark and thereby act as mediator of repression. This mediation would consequently be dependent on the enzymatic domain necessary for removal of the methylation mark. So, in the case of the *mdp* cluster, KdmB seems to act as repressor by using one of the other domains to either directly block transcription or to act as recruitment platform for other repressors. One can probably regard this latter case as more

likely because the ChIP analysis of histones associated with *mdpG* showed only background decoration with H3K4me3 in all strains. This makes an important role of the methylation-demethlyation cycle unlikely and enforces the view that KdmB acts via other partners to mediate gene repression. Which partners are recruited by KdmB remains to be studied, a suitable method for this would be co-immunoprecipitation.

In some cases, like in the *ors*-cluster we see a disagreement between transcription – which is down in both mutants – and metabolite production –which is up in the mutants. One explanation might be that the amount of transcript is strongly time-dependent, compared to the accumulation of the respective secondary metabolite over the whole time. Moreover, we have to consider, that fungal strains adapt to environmental and cultural changes very quickly, thus each observation and analysis is just a glimpse of the ongoing development of the strains. Hence, gene expression is very flexible changing possibly within minutes of a received stimulus. This was also reported for genes involved in trichothecene biosynthesis in *Fusarium graminearum*, which were analysed in a time course study, revealing that expression of these genes is initiated after two days *post* infection of wheat, has a peak after four days and then steadily decreases (Amarasinghe & Fernando, 2016). Alternatively, or additionally, in the case of KdmB, an indirect effect of the mutation via increased production of orsellinic acid precursor molecules must also be considered. One could distinguish between the direct and indirect effects by tagging KdmB with an epitope and performing ChIP analysis on the expected target genes to see if KdmB is present at all at the analysed genes and at which time points it can be detected. This type of work will certainly be subject to future investigations in our lab.

Interestingly, orthologues of KdmB, which are usually associated as repressors by demethylation of H3K4Me3, have been shown to act as activators as well under certain circumstances. For instance, the human orthologue RBP2 is classically considered as a repressor, unless it binds to the retinoblastoma protein pRB, resulting in gene activation of certain mammalian genes. The pRB is reported as a cell-cylce regulator acting through the E2F transcription factor family (Gutierrez, Kong, & Hinds, 2005). Thus it is thought that association of pRB with RPB2 can either prevent the repressor function of the demethylase or by modulates RBP2 in an activating manner. Quite a similar case is found for the *Drosophila melanogaster* ortholog Lid (Secombe, Li, Carlos, & Eisenman, 2007). Normally, it functions as a repressor through H3K4me3 demethylation as well but upon binding to the MYC transcription factor certain genes can be activated. The MYC transcription factor is important for normal cell function and deregulation can result in activation of potent oncogenes. The binding of MYC to Lid might have a similar effect as the association between RBP2/pRB. It is thought that upon binding of the transcription factor Myc to the JmjC domain, the enzymatic activity of Lid is

inhibited (Secombe & Eisenman, 2007). Therefore, it is possible that KdmB might function in a similar way by associating with a transcriptional regulatory protein that activates certain gene clusters. But still, evidence is needed for prove.

4.3 Future Perspectives

The most striking finding of this work is the requirement of a functional demethylase domain for gene regulation. Our experiments point out that there is in three out of four cases no difference in gene regulation between the *kdmB*Δ and *kdmB*$^{H642G,\ E644Q}$ strain. Only for the *mpdG* gene KdmB-mediated repression must be carried out by some other domains because in the JmjC-demethylase domain mutant repression still functions. How the exact molecular mechanism works is not clear and future experiments are needed for further conclusions.

Surprisingly the demethylase KdmB does not only have repressing effects but also plays a role in gene activation. Furthermore, gene clusters that are free of any histone marks are deregulated upon *kdmB* deletion as well. These findings let us assume that KdmB regulation happens rather indirectly. To gain a deeper understanding of the regulation mechanism it would be important to know where KdmB binds to and to which interaction partners. This would be achieved via Chromatin Immunoprecipitation against the histone demethylase KdmB itself. Furthermore, a Co-Precipitation would be another option to screen for a binding partner, eg. a transcriptional regulator, which might inactivate the demethylase activity from KdmB upon binding.

Another approach could be the dissection of the other domains of the protein to gain a deeper insight into the functionalities of each domain. Similar experiments have been done in Rph1 – a histone demethylase in *Saccharomyces cerevisiae* targeting tri- and di-methylated Lys^{36} on H3, reporting that a deletion of the JmjN domain leads to the loss of demethylase enzyme activity (Chang, Wu, Tong, Zhou, & Ding, 2011). Recently, it also was shown that the ARID and PHD1 domains are dispensable for enzymatic activity of KDM5 family members (Horton et al., 2016).

Taken together, this study provides evidence that KdmB needs a functional demethylase domain to carry out gene repression and activation in most clusters but further studies are needed to provide insight into the molecular basis of the histone demethylase activity and SM gene regulation.

5 Appendix

5.1 Primer List

Table 18 List of oligonucleotides used in this study

kdmB complementation cassette	
kdmB_comp_F1	gtaacgccagggttttcccagtcacgacgACGCGTTTGCTTCAGACTGTGTTGCTCATGTAGTCG
kdmB_comp_R1	TTCTTGGGCATAGGCGAATG
kdmB_comp_F2	CATTCGCCTATGCCCAAGAATCCCACCCCTCTAGAGGCTGAGA
kdmB_comp_R2	ATATTGCATCTACAGCTGCG
kdmB_comp_F3	CGCAGCTGTAGATGCAATAT
kdmB_comp_R3	CAATCACGAAGCCACACCTAC
kdmB_comp_F4	GTAGGTGTGGCTTCGTGATTGGCCTCAAACAATGCTCTTCAC
kdmB_comp_R4	ATTCTGTCTGAGAGGAGGCAC
kdmB_comp_F5	GTGCCTCCTCTCAGACAGAATTTCGGTGTATGAGGGTTCGGATTG
kdmB_comp_R5	gcggataacaatttcacacaggaaacagcACGCGTGAAGTGAGGATGTTTGAGAAGCCTGTGG
kdmB complementation check	
dia_kdmB_F01	CGATTCGTCATCGCCTTCAGC
dia_kdmB_F02	AGTCAATGGTCAACCTGAGC
dia_ribo_R	GGGTTATTCGTGAGTAGTCG
dia_kdmB_comp_R2	CAGTGCGAAGGACAAAGCAGC
Quantative PCR	
aflR_F	AGCCCAGCTGGTGCTGAGCGAGCTATAC
aflR_R	CCAGGGTGGTCGACGACAAGGGGGT
ipna_F	GGAGACGACCAAGCAGCCAAA
ipna_R	TTTTCCCGGGGATGGACAGG
orsA_F	GCACTGCTGTTCTATTGCC
orsA_R	CAGCTTCCAGCCATGATTAAG
mdpG_F	GCACCGGCGTCAGTTACTCCAG
mdpG_R	CCAAAGCCCAGCGCAGC
Tubq_F	GATGGCTGCCTCTGACTTCCG
Tubq_R	GCGCATCTGGTCCTCAACCTC
ACN_F	CACCGGTATCGTCCTTGACT
ACN_R	CTCAGCGGTAGTGGAGAAGG

ChIP	
aflR_F	CCCCCAGCGATCAGCCAGC
aflR_R	GCGTCCAGTGCGCCTG
ipna_F	GGGCAAGCAGCAGTTGAGACTGAT
ipna_R	TGGTCGGCTCGATGTCCAAG
orsA_F	GCACTGCTGTTCTATTGCC
orsA_R	CAGCTTCCAGCCATGATTAAG
mdpG_F	GCTCACTGCCGGAGTACTCCAA
mdpG_R	AGCCGTCGGTAACTGCCG
CYP450	GAGCAGGGCGCTCCTCAG
CYP450	CCTCGCCGGCTTCCTGC
tubqF	GATGGCTGCCTCTGACTTCCG
tubqR	GCGCATCTGGTCCTCAACCTC
acnA_F	CTGGGCTGAACCATCATTAC
acnA_R	ACTAGATAAAGACTGCTACG
DNase treatment and cDNA synthesis control	
acnA_F	TGTGCAAGGCCGGTTTCG
acnA_R	GGCGACGTAGCAGAGGCTTC

5.2 Primer Efficiency

Table 19 Primer Efficiency

Primer	***RT-qPCR Efficiency***
α-Actin (act)	106%
β- Tubulin (tub)	107,7%
kdmB	89,1%
aflR	100,6%
ipnA	102,4%
mdpG	89,2%
orsA	90,1%
Cyp450	89,5%

5.3 ChIP-DNA Shear Control

For an optimal Chromatin Immunoprecipitation, *A.nidulans* chromatin was fragmented via sonication to an average fragment size of 200-1000 bp.

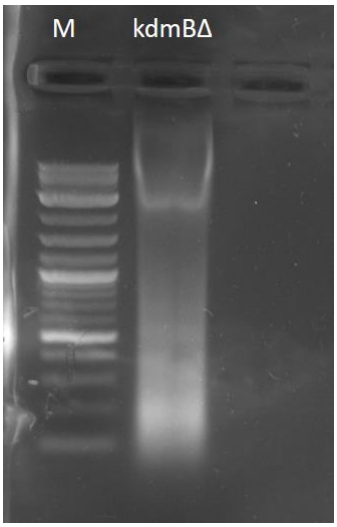


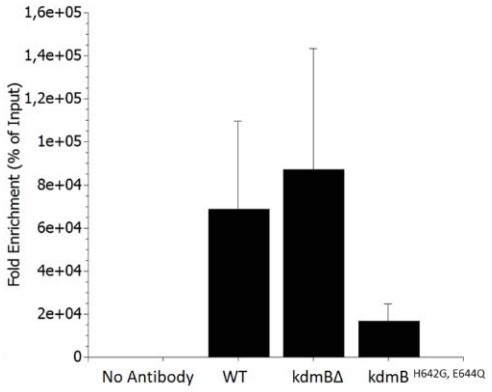
Figure 35 ChiP DNA shear control. Sonication on ice for 15 minutes (2 minutes ON, 1 minute OFF)

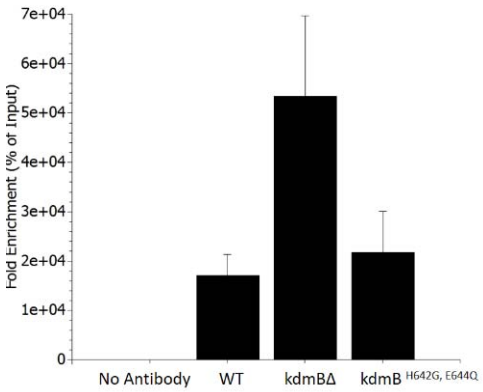
5.4 Fold Enrichment -ChIP

The fold enrichment, also known as signal over background method, is used for normalization and confirmation that the ChIP was successful. In this method the ChIP signals are divided by a no-antibody signal, representing the ChIP signal as fold increase (Haring et al., 2007).

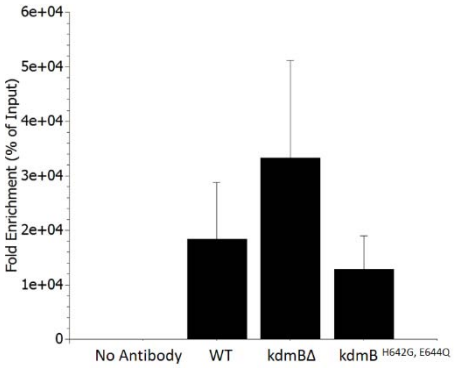
Table 20 Fold Enrichment for H3K4me3

aflR ***ipnA***

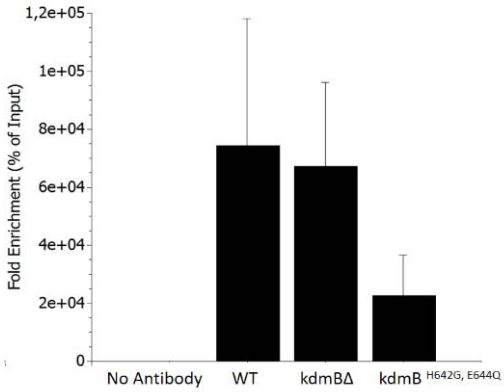




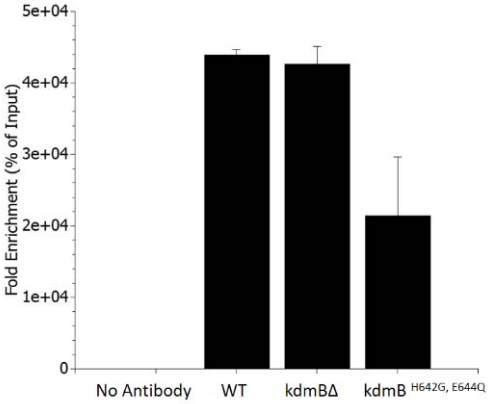
orsA



mdpG



acn



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