The histone code of the fungal genus *Aspergillus* uncovered by evolutionary and proteomic analyses

Xin Zhang[1,2], Roberta Noberini[3], Tiziana Bonaldi[3,4], Jérôme Collemare[2,*,+], Michael F. Seidl[1,*,+]

Author affiliations: [1]Theoretical Biology & Bioinformatics Group, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands; [2]Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands; [3]Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Via Adamello 16, 20139 Milan, Italy; [4]Department of Oncology and Haematology-Oncology, University of Milano, Via Santa Sofia 9/1, 20122 Milano, Italy

[+]These authors contributed equally

[*]Correspondence: J. Collemare (telephone: (+31) 030 21 22 600; email: j.collemare@wi.knaw.nl) and M. F. Seidl (telephone: (+31) 030 253 36 94; email: m.f.seidl@uu.nl)

Abstract

Chemical modifications of DNA and histone proteins impact the organization of chromatin within the nucleus. Changes in these modifications, catalyzed by different chromatin-modifying enzymes, influence chromatin organization, which in turn is thought to impact the spatial and temporal regulation of gene expression. While combinations of different histone modifications, the histone code, have been studied in several model species, we know very little about histone modifications in the fungal genus *Aspergillus*, whose members are generally well-studied due to their importance as models in cell and molecular biology as well as their medical and biotechnological relevance. Here, we used phylogenetic analyses in 94 Aspergilli as well as other fungi to uncover the occurrence and evolutionary trajectories of enzymes and protein complexes with roles in chromatin modifications or regulation. We found that these enzymes and complexes are highly conserved in Aspergilli, pointing towards a complex repertoire of chromatin modifications. Nevertheless, we also observed few recent gene duplications or losses, highlighting *Aspergillus* species to further study the roles of specific chromatin modifications. SET7 and other components of PRC2 (Polycomb Repressive Complex 2), which is responsible for trimethylation on histone H3 at lysine 27 in many eukaryotes including fungi, are absent in Aspergilli as well as in closely related *Penicillium* species, suggesting that these lost the capacity for this histone modification. We corroborated our computational predictions by performing untargeted mass spectrometry analysis of histone post-translational modifications in *Aspergillus nidulans*. This systematic analysis will pave the way for future research into the complexity of the histone code and its functional implications on genome architecture and gene regulation in fungi.

Keywords

Aspergilli, chromatin, DNA methylation, histone modification, H3K27methylation, mass spectrometry

Data Summary

The predicted proteomes used in this study are publicly available at the JGI (Joint Genome Institute) MycoCosm repository (1); the species names and abbreviations are listed in Table S1. To evaluate the completeness of the predicted proteomes and to obtain a species phylogeny, 758 fungal BUSCO (Benchmarking Universal Single-Copy Ortholog) genes were used, and their names are listed in Table S2. The fasta, trimmed alignment, and maximum-likelihood phylogenetic tree files can be found in Supplementary Data 1 and 2 deposited at Zenodo (doi:10.5281/zenodo.5907090), as well as the mass spectrometry results are mentioned in Table S4.

Impact Statement

Uncovering how fungi regulate gene expression and genome organization is an important step to understand how they control biological processes such as growth or biosynthesis of bioactive molecules. Despite the known importance of chromatin modifications in controlling a plethora of biological processes across eukaryotes, they remain particularly poorly understood in filamentous fungi, even in model organisms like Aspergilli. Our systematic evolutionary analysis provides a robust framework for the functional analysis of chromatin modifications in *Aspergillus* species and other fungi. Our results do not only implicate candidate enzymes to play a role in new chromatin modifications, but they also point at species that have experienced duplications or losses of genes encoding enzymes for specific chromatin modifications. We therefore expect that this work will set the stage for future research into the complexity of the histone code and its functional implications on gene regulation and genome organization in fungi.

Introduction

Inside eukaryotic cells, the membrane-bound nucleus facilitates the storage and replication of the genetic material as well as the regulation of gene expression (2). Within the nucleus, DNA is arranged as a chromatin fiber formed by repeated nucleosome units consisting of histone protein octamers (two copies of each H2A, H2B, H3, and H4 histones) that are wrapped by 145-147 bp of DNA (3–5). The chromatin structure is regulated by chemical modifications such as methylation of DNA on cytosines (5mC) and adenines (6mA), and different post-translational modifications (PTMs) on the histone proteins, including methylation and acetylation (6–8). The combination of these different modifications, the histone code, influences the recruitment of diverse nucleosome-associated proteins (e.g., DNA binding proteins), which can cause dynamic transitions between the condensed and transcriptionally silent heterochromatin and the accessible and transcriptionally active euchromatin (9–11). Chromatin dynamics is therefore key for organisms to adapt gene expression patterns in response to a variety of developmental and environmental clues.

In the past decades, several enzymes involved in orchestrating chromatin dynamics have been identified and functionally characterized in diverse eukaryotes (3,12,13). These enzymes can be broadly classified into three distinct types: (i) writers that place modifications on nucleosomes or linker DNA; (ii) erasers that remove these modifications; and (iii) readers that first recognize specific modifications and subsequently recruit writers, erasers, or other proteins to the correct genomic location (14–16). Chromatin modifiers that catalyze the same type of chemical modification often harbor functionally and evolutionarily conserved catalytic domains. For instance, DNA methylation (5mC) is catalyzed by different DNA methyltransferases (DNMTs) which contain a DNA methyltransferase domain, while histone mono-, di-, and tri-methylation are nearly exclusively catalyzed by proteins containing a SET (Su (var) 3-9, E(z) and Trithorax) domain (13). Next to these catalytic domains, the enzymes often contain accessory domains, which can contribute to the overall stability of the enzyme or establish interactions with other proteins (17). Furthermore, these multi-domain proteins,

101 together with other accessory protein subunits, can form multi-protein complexes to find the
102 correct target region witin the genome and modify the exact amino acid residue, collectively
103 contributing to the specific functions of these complexes (18,19). For example, the histone
104 methyltransferase complex PRC2 (Polycomb Repressive Complex 2) is responsible for the
105 mono-, di-, and tri-methylation of histone protein H3 on the lysine at position 27 (abbreviated
106 as H3K27me1/2/3; the same abbreviation principle will be applied below for other histone
107 PTMs) (20–23). Evolutionary analyses have suggested that the key enzymes responsible for
108 chromatin modifications have evolved and siginificantly expanded early in eukaryotic evolution
109 (24). An analysis of 94 gene families with roles in chromatin modifications reveals that 87 out
110 of 94 gene families emerged more than one billion years ago, and 48 were already present in
111 the last eukaryotic common ancestor (24).
112
113 Among eukaryotes, the fungal kingdom is remarkably large and diverse, comprising
114 an estimated 1,5-5 million species with various morphologies, life cycles, and ecological
115 niches (25). The most well-studied model fungal species in respect of chromatin modifications
116 is the unicellular yeast *Saccharomyces cerevisiae*, in which 75 histone PTMs on 43 distinct
117 amino acid positions have been reported with histone methylation and acetylation being most
118 abundant (16). In addition to ancient chromatin modifiers, also lineage-specific innovations
119 such as duplications and losses were found in fungi. For example, Dim-2 and RID (Repeat-
120 Induced Point Mutation (RIP)-Defective) are considered to be fungal-specific DNMTs, while
121 DNMT3 that is typically found in animals and plants seems to be absent in fungi (26–28).
122 However, *S. cerevisiae* is not representative of the complete fungal kingdom, and detailed
123 studies on PTMs are needed for other, less studied fungal species.
124
125 The fungal genus *Aspergillus* comprises approximately 350 species, including
126 pathogens of humans, animals, and crops, food contaminants, as well as important cell
127 factories for industrial and medical applications (29). Chromatin dynamics influences gene
128 expression in several Aspergilli and regulates growth, sexual development, secondary

129 metabolite biosynthesis, and virulence (16,30–32). Thus far, very few well-known chromatin
130 modifications and their catalytic protein complexes have been identified and studied in few
131 *Aspergillus* species (16). For example, gene knockout of the H3K9 methyltransferase *Clr4*
132 homolog in *Aspergillus fumigatus* reduced histone H3K9me1/3 results in reduction of radial
133 growth and conidia production, as well as delayed conidiation (31). Similarly, CclA, a subunit
134 of the COMPASS (COMplex of Proteins ASsociated with SET1) complex, is essential for
135 H3K4me2/3 in *A. fumigatus*, and the deletion strain showed increased secondary metabolite
136 production and decreased growth (33). The SAGA (Spt-Ada-Gcn5 Acetyltransferase) complex,
137 which is responsible for histone acetylation, has been functionally characterized in *Aspergillus*
138 *nidulans* (34). Notably, this complex in *A. nidulans* lacks the Sgf11 and Sus1 subunits that
139 have been described in *S. cerevisiae*, suggesting that this complexe evolves in distinct
140 trajectories in different fungi (34). Besides these few examples, we so far lack a complete view
141 of the occurrence and evolution of chromatin modifiers in the *Aspergillus* genus. Here, we
142 report the phylogenetic analyses of core enzymes involved in the deposition and removal of
143 histone PTMs, and assess the conservation of all the subunits belonging to chromatin
144 regulator complexes in 94 diverse Aspergilli. We also performed proteomics analyses to
145 corroborate our computational predictions and reveal a broader spectrum of histone
146 modifications.

147 **Material and Methods**

148 **Acquisition of predicted proteomes for 109 fungal species**

149 The predicted proteomes of 109 fungal species were retrieved from JGI (Joint Genome
150 Institute) MycoCosm (https://mycocosm.jgi.doe.gov/mycocosm/home) (1) on 24th January
151 2020. These species comprise 94 different Aspergilli species, 13 Ascomycota species from
152 other genera, as well as two Basidiomycota species (*Coprinopsis cinerea* and *Dendrothele*
153 *bispora*) (Supplementary Table 1).

154

155 **Phylogenetic analyses of chromatin modifiers**

156 Phylogenetic trees for five major chromatin modifiers were generated using their conserved
157 catalytic domains. Catalytic domain HMMs (Hidden Markov Model) of DNA methyltransferase
158 domain (PF00145), histone methylation SET domain (PF00856), acetyltransferase domain
159 (PF00583), histone acetyltransferase MOZ/SAS FAMILY domain (PF01853), and histone
160 deacetylase domain (PF00850) were downloaded from the Pfam database
161 (https://pfam.xfam.org). Hmmsearch, which is part of the HMMER package (v3.1b2) (35), with
162 the Gathering Cut-Off threshold (-cut_ga) was used to identify occurrences of these domains
163 in the predicted proteomes of all 109 fungal species considered here. To filter out short and
164 incomplete fragments, we applied a cutoff calculated by 50% length coverage of the query
165 sequence (domain) or the hit. Subsequently, the identified domain sequences were aligned
166 using MAFFT v7.271 (36). TrimAl v1.2 (-gt 0.1) was used to remove positions in the alignments
167 with gaps in more than 90% of the sequences (37). Maximum-likelihood phylogenetic trees
168 were constructed using IQ-TREE v1.6.10 (38,39) using ultrafast bootstrap and Shimodaira–
169 Hasegawa approximate likelihood ratio test (SH-aLRT) (40) with 1,000 pseudo-replicates, and
170 standard model selection to automatically determine the best-fit model. We predicted
171 additional domains in the analyzed proteins using Hmmsearch with all Pfam domain profiles
172 that are present in the Pfam database (v28.0) and visualized them along the phylogenetic
173 trees using iTOL (41).

174

175 **Species tree construction**

176 To construct a species phylogeny of the here analyzed fungi, we used BUSCO v4.0.1
177 (Benchmarking Universal Single-Copy Ortholog assessment tool) (42) to identify the
178 occurrence of 758 fungal single-copy genes (Supplementary Table 2) within the 109 predicted
179 proteomes (Supplementary Table 1). The identified homologs of single-copy BUSCO genes
180 were subsequently concatenated into a single super-alignment. Then, the maximum-likelihood
181 species phylogeny was estimated using IQ-TREE v1.6.10 (38,39) by employing partitioned
182 model selection, which allows selecting a substitution model for each BUSCO gene. Branch
183 supports were obtained using ultrafast bootstrap and SH-aLRT (40) with 1,000 pseudo-
184 replicates separately.

185

186 **Chromatin modifier subunits homology search and gene tree construction**

187 We obtained previously studied chromatin modifier complexes from literature, and the
188 associated protein sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov)
189 (Supplementary Table 3). For each subunit of the chromatin modifier complexes, homologs in
190 the analyzed 109 predicted proteomes were detected based on sequence similarity searches.
191 First, BLASTP (v2.2.31+) (43) searches were performed, and an e-value threshold of 10^{-2} was
192 applied. Multiple BLAST matches (High-Scoring Pairs: HSPs) of the same query were
193 concatenated if they were aligned with different non-overlapping regions. Only the non-
194 redundant region was retained when HSPs of the same query were overlapping. For these
195 two conditions, the highest e-value of the different matches was used to represent the merged
196 match. Only matches with the e-value lower than 10^{-5} and covering more than 50% of query
197 or subject were retained for the following analyses. If we observed less than ten hits for the
198 BLASTP search after filtering in Aspergilli, we used PSI-BLAST (Position-Specific Initiated
199 BLAST v2.2.31+) (43) with five iterations (-num_iterations 5) using the best Aspergilli hit from
200 the BLASTP as the query; if no Aspergilli match was retained, we used the same query as for
201 the BLASTP searches. The same filter criterion as for the BLASTP selection was applied to

202 the PSI-BLAST hits, and the results of these two homology search strategies were
203 subsequently jointed.
204
205 To account for apparent protein absences due to erroneous gene annotation, we
206 tested these potential losses by using TBLASTN (BLAST v2.2.31+) (43) to query protein
207 sequences against the genome assemblies. Matches with e-value lower than 10^{-5} together
208 with 4,500 nucleotides upstream and downstream were extracted, and Exonerate v2.2.0 (44)
209 was used to predict the gene structure (options: protein2genome, extensive search,
210 showtargetgff, showcigar, and showquerygff options were set as true, and ">%ti(%tcb
211 - %tce)\n%tcs\n" option was used to output the complete aligned coding sequence). Transeq
212 v6.6.0.0 (45) was used to translate the coding sequence into protein sequences.
213
214 Together with the protein sequences identified from BLASTP and PSI-BLAST, all
215 protein sequences from each subunit were retrieved to build a phylogenetic tree (MAFFT,
216 TrimAl, and IQ-TREE settings were the same as for the tree reconstruction using the catalytic
217 domains above).
218
219 For each sequence included in the phylogenic trees, the domain organization was
220 identified and visualized as described above. When the domain organization of a protein
221 differed from its orthologues, the corresponding gene model was manually checked in the
222 MycoCosm genome browser (1) and corrected. After manual curation, the presence and
223 absence patterns of 86 subunits from 16 different chromatin modifier complexes were
224 visualized using the Seaborn library in Python (46,47).
225
226 **Histone enrichment and mass spectrometry**
227 Spores of 4- or 5-day old sporulating *A. nidulans* (AnWT pabaA1 veA1, kindly provided by
228 Prof. Joseph Strauss) growing on ME (Malt Extract) agar medium were harvested with 2 mL

229 sterile water and cultured in 50 mL ME liquid medium at 28 ℃, 200 rpm for 16 hours. The
230 sample was centrifuged at 8,000 g for 10 minutes to discard supernatant, and germinating
231 spores were resuspended in 25 mL 0.8 M NaCl and centrifuged again. Ten mL protoplasting
232 solution (20 mg/mL *Trichoderma* lysing enzyme and 5 mg/mL driselease in 0.8 M NaCl) were
233 used to resuspend spores, which were then incubated at 30 ℃, 100 rpm for 2-3 hours.
234 Protoplasts were retrieved by filtering through sterile Miracloth and were concentrated by
235 centrifugation at 3,000 rpm, 4 ℃ for 5 minutes. Histone proteins were extracted according to
236 an adapted histone protein extraction protocol (48). Briefly, 10^8 protoplasts were resuspended
237 in 1 mL Nuclei Isolation Buffer (1×PBS, 0.5 mM PMSF, 5 μM Leupeptin, 5 μM Aprotinin, and
238 5mM Na-butyrate) with 1% Triton X-100, followed by vigorous pipetting through a 200 μL
239 pipette several times. Then, centrifuged at 2,300 g for 15 minutes at 4 ℃, discarded the
240 supernatant, and resuspended the nuclear pellet in 200 μL Nuclei Isolation Buffer with 0.1%
241 SDS and 250 U Pierce™ Universal Nuclease and incubated at 37 ℃ for 30 minutes to digest
242 nucleic acids. The protein concentration was measured using the Bradford Protein Assay (49).
243
244 The samples were separated on a 17% SDS-PAGE gel and histone bands were
245 excised, chemically acylated with propionic anhydride and in-gel digested with trypsin,
246 followed by peptide N-terminal derivatization with phenyl isocyanate (PIC) (50). The samples
247 were then desalted on handmade StageTips columns (51). Peptide mixtures were separated
248 by reversed-phase chromatography on an EASY-Spray column (Thermo Fisher Scientic), 25-
249 cm long (inner diameter 75 μm, PepMap C18, 2 μm particles), which was connected online to
250 a Q Exactive Plus instrument (Thermo Fisher Scientific) through an EASY-Spray™ Ion Source
251 (Thermo Fisher Scientific), as described (50).
252
253 The acquired RAW data were analyzed using the integrated MaxQuant software
254 v.1.6.10. The Uniprot UP000000560 database was used for identification of *A. nidulans*
255 histone peptides. Enzyme specificity was set to Arg-C. The estimated false discovery rate

256 (FDR) was set at a maximum of 1%. The mass tolerance was set to 6 ppm for precursor and
257 fragment ions. Two missed cleavages were allowed, and the minimum peptide length was set
258 to 4 amino acids. Min. score for modified peptides and min. delta score for modified peptides
259 were set to 1. Variable modifications included lysine propionylation, monomethylation +
260 propionylation, dimethylation, trimethylation and acetylation. N-terminal PIC labeling was set
261 as a fixed modification (50). To reduce the search time and the rate of false positives, with
262 increasing the number of variable modifications included in the database search, the raw data
263 were analyzed through multiple parallel MaxQuant jobs (52), setting different combinations of
264 variable modifications. Peptides identified by MaxQuant with Andromeda score higher than 50
265 and localization probability score higher than 0.75 were quantitated, either manually or by
266 using a version of the EpiProfile 2.0 software (53) adapted to the analysis of histones from *A.*
267 *nidulans*. Identifications and retention times were used to guide the manual quantification of
268 each modified peptide using QualBrowser version 2.0.7 (Thermo Fisher Scientific). Site
269 assignment was evaluated from MS2 spectra using QualBrowser and MaxQuant Viewer.
270 Extracted ion chromatograms (XICs) were constructed for each doubly charged precursor,
271 based on its *m/z* value, using a mass tolerance of 10 ppm. For each histone modified peptide,
272 the % relative abundance (%RA) was estimated by dividing the area under the curve (AUC)
273 of each modified peptide for the sum of the areas corresponding to all the observed forms of
274 that peptide (54). The AUC values are reported (Supplementary Table 4) and visualized using
275 GraphPad Prism.

276

277

278 **Results and discussion**

279 DNA methylation, histone methylation, and histone acetylation are among the most studied
280 chromatin PTMs in animals, plants, and fungi (55–57). Protein complexes involved in writing,
281 reading, and erasing these modifications have been reported in very few model fungal species
282 to date (16,22), and the evolution and conservation of these complexes in diverse fungi has
283 not been systematically explored. To start uncovering the occurrence and evolutionary
284 histories of chromatin modifiers involved in these major modifications in the fungal genus
285 *Aspergillus,* we first focused on five conserved catalytic domains reported to be involved in
286 histone PTMs: DNA methyltransferase domain for DNMTs, SET domain for histone
287 methyltransferases (HMTs), acetyltransferase domain and MOZ/SAS FAMILY domain for
288 histone acetyltransferases (HATs), and histone deacetylase domain for histone deacetylases
289 (HDACs). We identified homologs harboring these conserved domains in 109 predicted
290 proteomes; the completeness of the predicted proteomes was generally high as 97 had more
291 than 97% complete BUSCO predicted proteins (Supplementary Table 1). The 94 Aspergilli
292 species cover 18 recognized sections: *Polypaecilum*, *Cremei*, *Restricti*, *Aspergillus*, *Cervini*,
293 *Clavati*, *Fumigati*, *Candidi*, *Circumdati*, *Janorum*, *Terrei*, *Flavi*, *Ochraceorosei*, *Bispori*,
294 *Cavernicolarum*, *Usti*, *Nidulantes*, and *Nigri* (58–64), and 15 other species spanning the
295 Dikarya were used as outgroups to the *Aspergillus* genus (Figure S1). We used the identified
296 homologs for each catalytic domain to determine their evolutionary history using maximum-
297 likelihood phylogenies (Figure 1-4).

298

299 **Aspergilli harbor conserved DNA methyltransferases for genome defense and**
300 **methylation maintenance**

301 The conserved DNA methyltransferase domain is the only domain known to catalyze the
302 addition of methyl groups to DNA specifically at cytosines (5mC) (26). Based on previous
303 domain architecture analyses, phylogeny reconstructions, and functional experiments, five
304 distinct protein families containing a DNA methyltransferase domain have been recognized in
305 fungi: DNMT1, DNMT2, and DNMT5 are commonly found in eukaryotes, while Dim-2 and RID

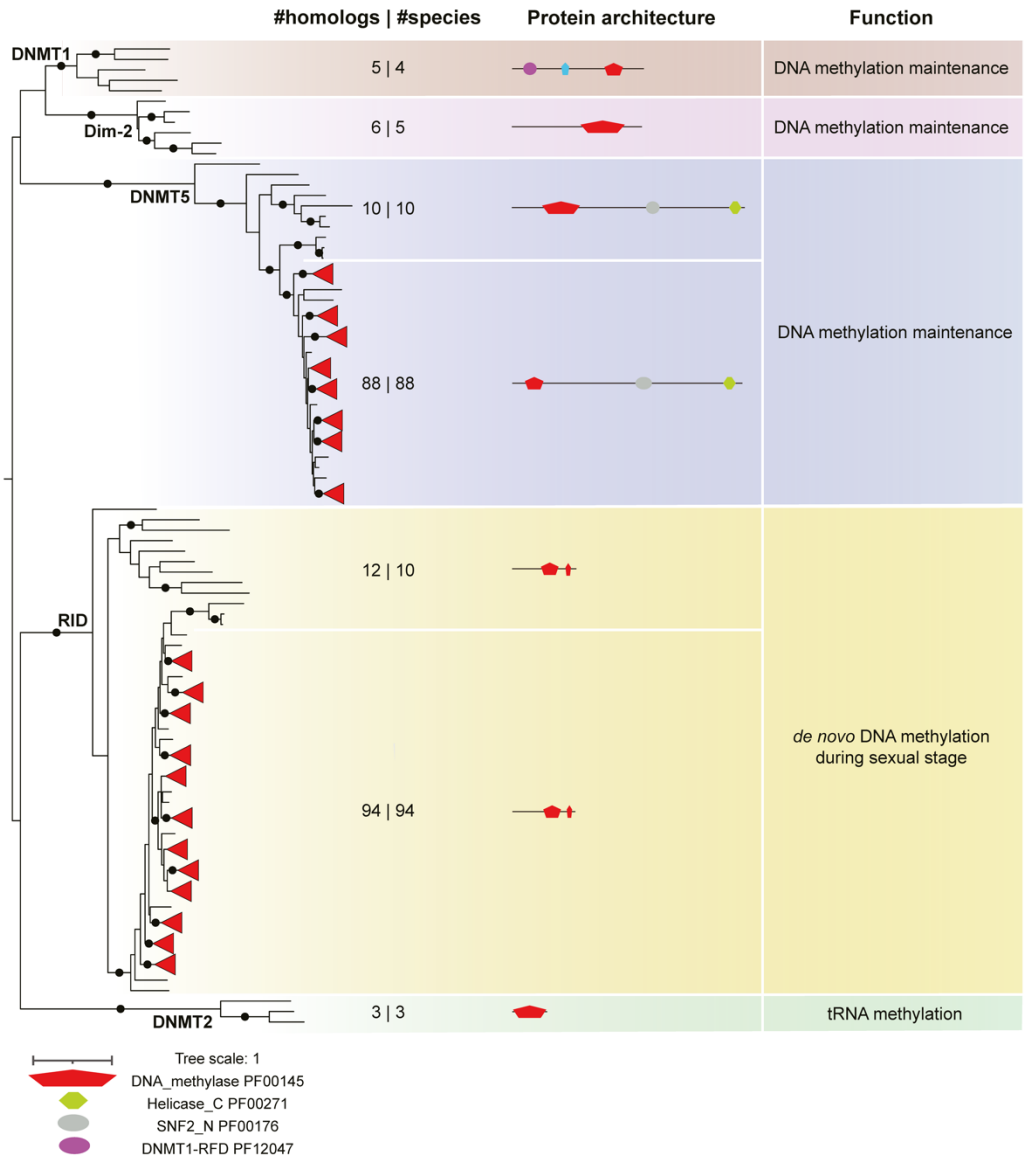


Figure 1. Conservation of DNA methyltransferase families in the Aspergillus genus. The maximum-likelihood phylogeny of DNA methyltransferase domains (PF00145) found in 94 Aspergilli and 15 outgroup species was determined using IQ-TREE (37,38). The black dot on the branch indicates ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with trustworthy support are collapsed and shown as red triangles. Additional conserved domains in DNA methyltransferase domain-containing proteins were identified using the PFAM database (https://pfam.xfam.org).

are considered to be fungal specific (26). Among these five families, DNMT2 functions as a tRNA methyltransferase rather than DNA methyltransferase (65), and it only occurs in our study in two Basidiomycota and one Ascomycota (*Schizosaccharomyces pombe*), which is in line with previous observation suggesting that it has been lost in most Ascomycota (26,66).

320 Three (DNMT1, DNMT5, and Dim-2) of the four remaining families catalyze the
321 maintenance of DNA methylation pattern from parental DNA strand onto synthesized daughter
322 DNA strands (67,68). Based on our species selection and phylogenetic analyses, we here
323 found *DNMT1* and *Dim-2* to be absent in all Aspergilli and only present in four or five out of 15
324 outgroup species, respectively, while *DNMT5* is found in most Aspergilli and outgroup species
325 (Figure 1). Only four outgroup species harboring *DNMT1*, the two Basidiomycota species,
326 *Botrytis cinerea*, and *Plectania melastoma*, which corroborates previous findings (26,69); and
327 *Dim-2* was identified in only five Ascomycota outgroup species. Recently, it was found that
328 Dim-2 is inactivated in some *Zymoseptoria tritici* isolates only, which uncovered that Dim-2
329 plays a role in genome defense and genome evolution (70). In contrast to the *DNMT1* and
330 *Dim-2* losses in Aspergilli, *DNMT5* is identified in 88 out of 94 Aspergilli and 10 out of 15
331 outgroup species (Figure 1), with a conserved domain architecture consisting of a DNA
332 methyltransferase domain, an SNF2 family domain (PF00176), and a Helicase conserved C-
333 terminal domain (PF00271), in which the latter two domains are involved in transcription
334 regulation (71). The five outgroup species without DNMT5 are *S. cerevisiae*, *S pombe*,
335 *Neurospora crassa*, *Fusarium graminearum*, and *Cladonia grayi*, among which the two yeast
336 species also lack the other two DNA methylation maintaining enzymes, and thus accordingly
337 are considered to be incapable of DNA methylation (72–74). Five out of the six *Aspergillus*
338 species that lost *DNMT5* belong to a single clade within the *Nidulantes* section (*A. undulatus*,
339 *A. olivicola*, *A. filifera*, *A. venezuelensis*, and *A. stella-maris*), and the sixth one is *A. robustus*
340 which is assigned to the *Circumdati* section (Supplementary Figure 1). In the Basidiomycota
341 fungus *Cryptococcus neoformans*, DNMT5 maintains DNA methylation using the same
342 cofactors as DNMT1 (73,75). Thus, it is most likely that DNMT5 is similarly involved in DNA
343 methylation maintenance in Aspergilli as in *C. neoformans*. Moreover, DNA methylation
344 maintenance does not appear to be required for the survival of fungi as shown with the
345 absence of all three *DNMT1*, *DNMT5*, and *Dim-2* not only within the *Nidulantes* section but
346 also in *S. cerevisiae* and *S. pombe* (Figure 1). However, the absence of these genes could
347 have an impact on the regulation of certain genes like secondary metabolite biosynthetic gene

348 clusters and it would be interesting to compare species within the *Nidulantes* section in more
349 detail to identify how such loss may have affected gene expression and genome organization.
350
351 RID is a more recently acquired single domain DNMT that specifically occurs in fungi
352 and is involved in a fungal-specific genome defense mechanism called RIP (Repeat-Induced
353 Point mutation) (26). RID is required for *de novo* DNA methylation of transposable elements
354 (TEs) in both CG and non-CG context relating to RIP during the sexual stage in *N. crassa*, but
355 its *de novo* DNA methylation capacity during the vegetative growth has not been reported (76).
356 Here, we identified RID in all analyzed Aspergilli species as well as in ten outgroup species.
357 A RID-like homolog knock-out mutant in the model filamentous Ascomycota *Podospora*
358 *anserina* shows blocked sexual development, as gametes formation and fertilization can
359 happen but dikaryotic cells cannot develop normally (77). Interestingly, the knock-out mutant
360 of *DmtA* (homolog of *RID* in *A. flavus*) shows altered conidiation, sclerotial production,
361 aflatoxin biosynthesis, and virulence (32), suggesting that RID might have additional functions
362 next to genome defense. Additionally, fungi lost the *de novo* DNA methyltransferase *DNMT3*
363 around 50–150 million years ago (75,78), and only *DNMT5* and *RID* were consistently found
364 in Aspergilli (Figure 1). Thus, we hypothesize that Aspergilli can only maintain DNA
365 methylation *via* DNMT5 and may generate *de novo* DNA methylation *via* RID during the
366 transient sexual stages, but do not spur genome-wide *de novo* DNA methylation. This
367 hypothesis seems to be corroborated as bisulfite sequencing in *A. flavus* has uncovered very
368 little genome-wide DNA methylation (79). However, systematic analyses of DNA methylation
369 patterns and experimental evidence for the roles of DNMTs in Aspergilli are still largely missing.
370
371 **Aspergilli lack *SET7*, but SET-domain proteins are abundant and histone**
372 **methyltransferases are conserved**
373 Histone methylation is typically catalyzed by SET domain-containing proteins (80). We
374 identified a total of 1,548 SET domain-containing proteins and classified them into 15 groups
375 based on their domain architecture and phylogenetic support that contain species both from

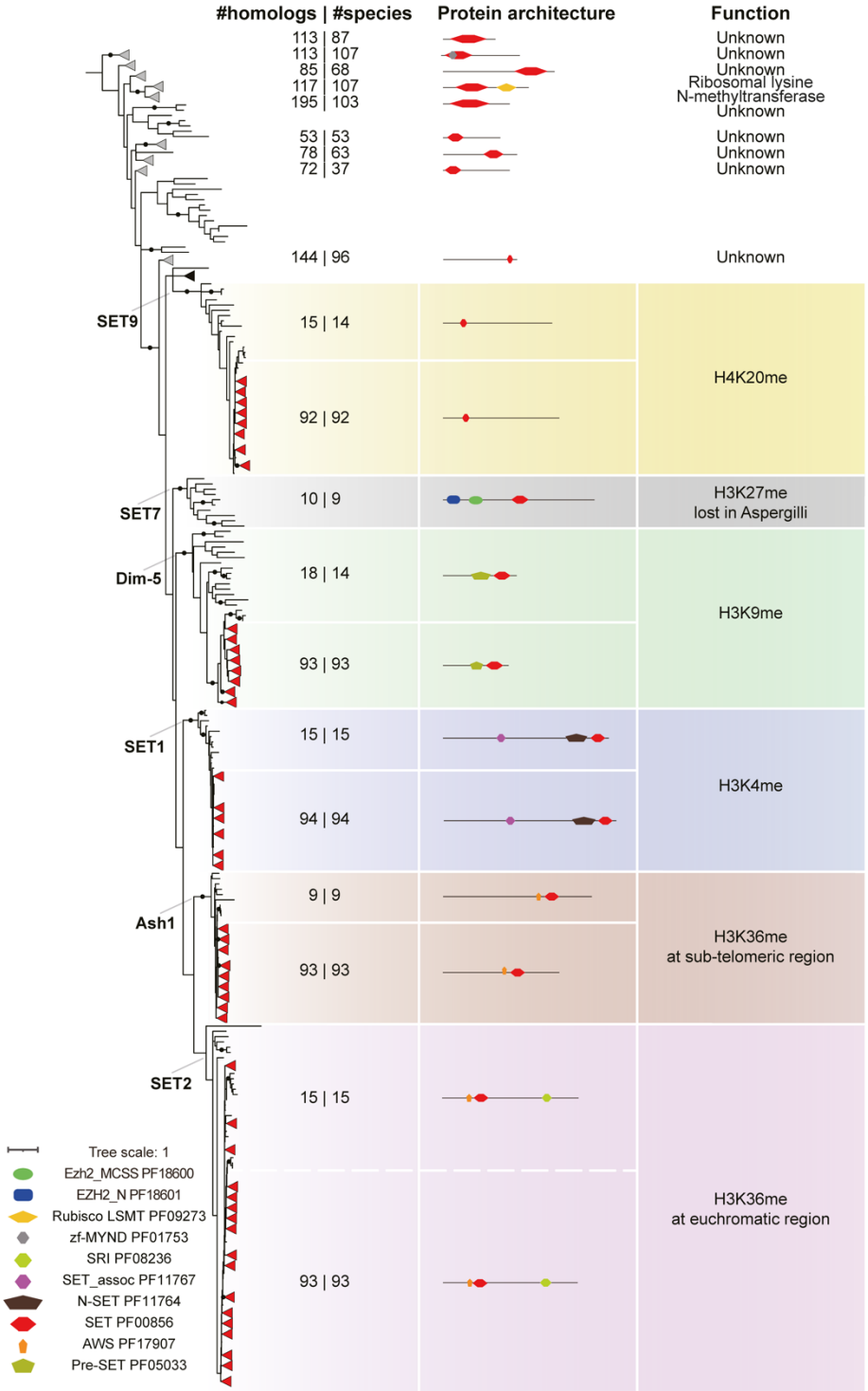


Figure 2. Conservation of histone methyltransferase families in the Aspergillus genus. The maximum-likelihood phylogeny of SET domains (PF00856) found in 94 Aspergilli and 15 outgroup species was determined using IQ-TREE (37,38). The black dot on the branch indicates ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with trustworthy support are collapsed and shown as red triangles and leaves with similar protein architecture with unknown functions covering both Aspergilli and outgroup species are collapsed and shown as grey triangles. Additional conserved domains in SET domain-containing proteins were identified using the PFAM database (https://pfam.xfam.org).

385 Aspergilli as well as from the outgroup. Among these groups, 107, 111, 109, 102, and 108
386 sequences represent homologs of the known histone methyltransferases (HMTs) SET9, Dim-
387 5, SET1, Ash1, and SET2, respectively (Figure 2). The remaining homologs were divided into
388 nine groups that we broadly classified as SET domain-containing proteins. Among them, eight
389 groups contain a SET domain but lack experimentally determined functions, and 117
390 sequences could be grouped as Ribosomal lysine N-methyltransferases (Figure 2). Enzymes
391 in this group contain a SET domain together with a Rubisco LSMT substrate-binding domain
392 (PF09273). While the homolog in plant transfers a methyl group to the large subunit of the
393 Rubisco holoenzyme complex (81,82), the homolog in *S. cerevisiae* has been characterized
394 as a ribosomal lysine N-methyltransferase (83).
395
396 The well-described HMTs SET1, Dim-5, SET2, Ash1, and SET9 all contain distinct
397 accessory domains and catalyze methylation on lysine residues at different positions of H3 or
398 H4 tails (22). SET1 is composed of the SET domain, the N-SET domain (PF11764), as well
399 as the SET_assoc domain (PF11767), and is responsible for H3K4me1/2/3 in *S. cerevisiae*
400 (84). SET1 occurs in a single copy in all species (Figure 2), indicating that this enzyme and its
401 catalyzed PTM play fundamental roles in fungal biology. Dim-5 contains a SET domain and
402 Pre-SET domain (PF05033) and acts as an H3K9 methyltransferase inducing heterochromatin
403 formation in *N. crassa* (85). Dim-5 was found in all species included in this study except *S.*
404 *cerevisiae* and *A. calidoustus*, among which *S. cerevisiae* is reported to lack H3K9me (85,86).
405 How such a loss has impacted gene expression in *A. calidoustus* in comparison to its closest
406 relative *A. carlsbadensis* deserves further investigation. Both SET2 and Ash1 catalyze
407 H3K36me in *N. crassa* and share the SET domain and the AWS domain (PF17907) (Figure
408 2), yet SET2 harbor one additional SRI domain (PF08236) that interacts with RNA polymerase
409 II and links H3K36me with transcript elongation (87–89). In *N. crassa,* Ash1 was shown to
410 contribute to H3K36me at sub-telomeric regions, while SET2 acts primarily at euchromatic
411 regions (90,91). Considering the common origin of SET2 and Ash1 as well as their similar
412 activities, an ancient duplication followed by the loss or gain of the SRI domain is likely to have

413 contributed to their functional diversification (Figure 2). SET2 was found in all outgroup
414 species, while *Ash1* was lost in both Basidiomycota, both yeasts, *B. cinerea*, and *F.*
415 *graminearum*. Both enzymes are conserved in all *Aspergillus* species except in *A. carbonarius*
416 from the *Nigri* section, suggesting that either other SET domain-containing proteins exhibit
417 functional redundancy or that H3K36 methylation is not fully required in this species. In
418 *Fusarium fujikuroi*, deletion of *SET2* and *ASH1* strongly affected vegetative growth and
419 conidiation (91). SET9 homologs harbor only a SET domain, and were shown to catalyze
420 H4K20me in *S. pombe* (92). This group is well conserved in most of the species except in *S.*
421 *cerevisiae* (93) and in the two closely related Aspergilli, *A. novoparasiticus* and *A. arachidicola*
422 that both belong to section *Flavi* (Figure 2 and Figure S1).
423
424 SET7 is the catalytic subunit of PRC2, and is highly conserved in mammals, plants,
425 and *N. crassa*, in which it has been well-characterized to catalyze methylation on H3K27, a
426 PTM that is deposited at facultative heterochromatin (21). Even though PRC2 is considered
427 to be a key chromatin modifier complex, its catalytic subunit *SET7* was lost in many fungal
428 species (22). *SET7* has previously been reported to be absent in *A. nidulans* and *A. fumigatus*
429 (20–22), and we here demonstrate that *SET7* is absent from all analyzed Aspergilli as well as
430 from *Penicillium* (Figure 2), suggesting that this enzyme was most likely lost at or before the
431 divergence of the last common ancestor of these two fungal genera. Thus, in line with previous
432 reports (22,30,94,95), our results indicate that methylation on H3K4, H3K9, H3K36, and
433 H4K20 are likely catalyzed by conserved enzymes in all Aspergilli, while H3K27 methylation
434 is absent. Considering that loss of function of these enzymes are usually reported to trigger
435 deleterious phenotypes (32,77), investigating how some species are able to cope with such
436 losses will likely provide new insights on the histone code and its conservation in fungi.
437 Importantly, the occurrence of a high number of uncharacterized SET domain-containing
438 proteins opens the possibility that these contribute to not yet characterized histone
439 modifications. Especially, four uncharacterized SET domain-containing groups with 144, 195,

440 113, and 113 members are conserved in Aspergilli and thus likely play important roles in
441 different cellular processes.
442
443 **Histone acetyltransferases are conserved in Aspergilli**
444 Histone acetyltransferases (HATs) have been classified into five families: MYST ('MOZ,
445 Ybf2/Sas3, Sas2, and Tip60)-related HATs, Gcn5-related acetyltransferases (GNATs),
446 p300/CBP HATs, general transcription factor HATs, and nuclear hormone-related HATs (96–
447 98). We here only focused on the first two HAT families because these are known to be
448 involved in the regulation of histone proteins and appear largely conserved in fungi (96).
449
450 The MOZ/SAS FAMILY domain is the catalytic domain of MYST-related HATs (99). As
451 previously reported (100), the three characterized MYST-related HATs Sas3, Sas2, and Esa1
452 belong to distinct monophyletic clades, and these paralogues are nearly fully conserved in the
453 here analyzed fungi (Figure 3). MOZ/SAS FAMILY domain and zf-MYST domain (PF17772)
454 are present in Sas3, which can on its own acetylate histone H3 and H4, and H2A weakly *in*
455 *vitro* (101). Within the NuA3 (Nucleosomal Acetyltransferase of histone H3) complex, Sas3
456 histone acetylation activity is *in vitro* restricted on H3K14 and to a less extent on H3K23, while
457 in *S. cerevisiae*, only H3K14ac can be detected (102). *Sas3* occurs in most of the species but
458 is lost in *S. pombe*, as previously observed (103,104), and is duplicated in *A. carbonarius*
459 (Figure 3). In addition to the two domains found in Sas3, Sas2 and Esa1 carry a PHD_4
460 domain (PF16866) or Tudor-knot domain (PF11717), respectively (Figure 3). *Esa*1 is present
461 as a single copy in all species included in this study, and its functions is known to contribute
462 to the acetylation of K5, K8, K12, K16 on histone H4, as well as on H2AZK14 in *S. cerevisiae*
463 (105–108). *Sas2* is similarly found in most of the species except for two Basidiomycota as well
464 as for *A. carbonarius*. Sas2 is involved in chromatin-mediated gene regulation and H4K16
465 acetylation in both *S. cerevisiae* and *Candida albicans* (105,109,110). Although Esa1 and
466 Sas2 catalyze H4K16ac, it was shown in *C. albicans* that they are differentially recruited at
467 different stages of development (105). Because both Esa1 and Sas2 are well-conserved in

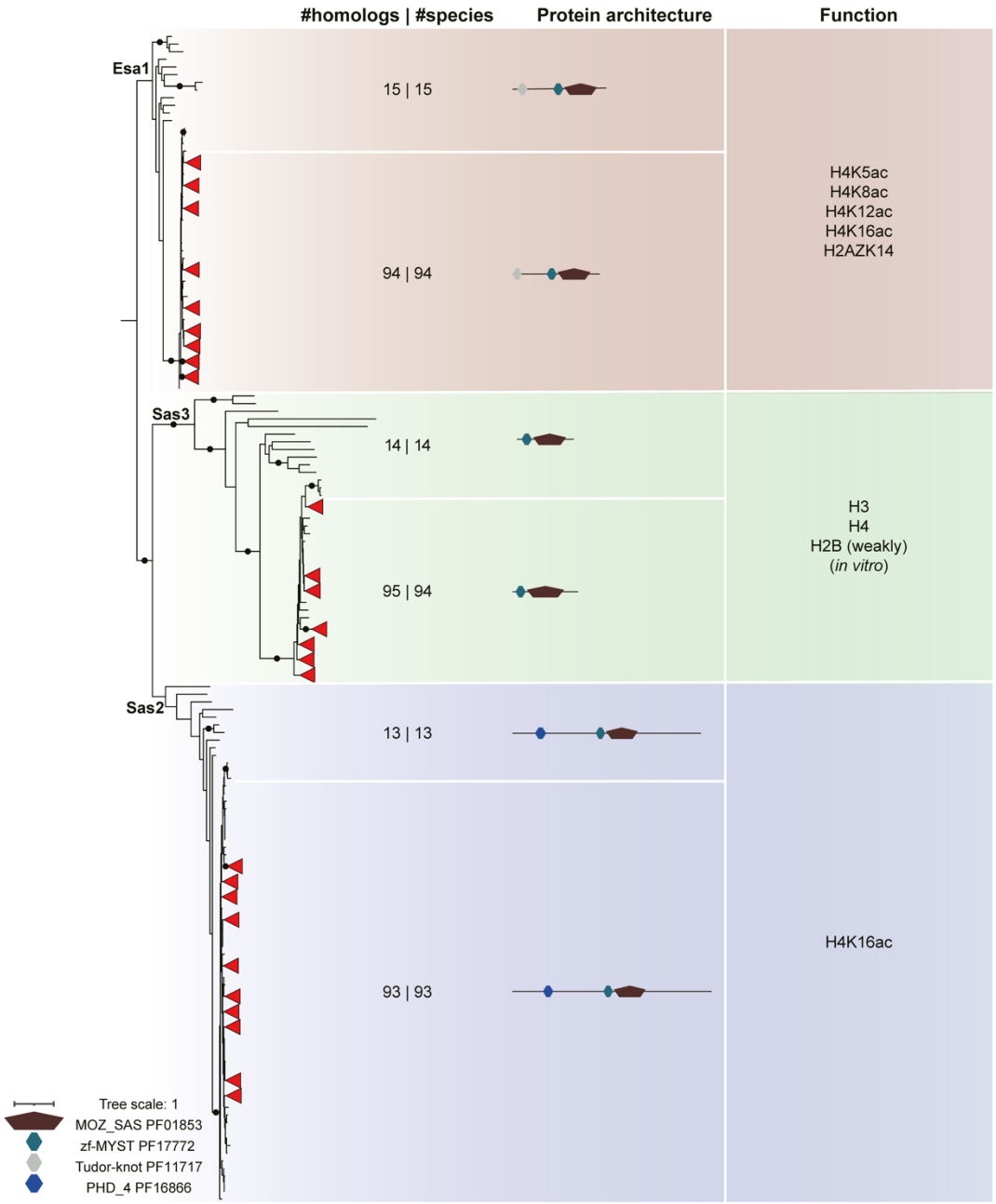


Figure 3. Conservation of MOZ/SAS FAMILY domain-containing histone acetyltransferases in the Aspergillus genus. The maximum-likelihood phylogeny of MOZ/SAS FAMILY domains (PF01853) found in 94 Aspergilli and 15 outgroup species was determined using IQ-TREE (37,38). The black dot on the branch indicates ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with trustworthy support are collapsed and shown as red triangles. Additional conserved domains in MOZ/SAS FAMILY domain-containing proteins were identified using the PFAM database (https://pfam.xfam.org).

the *Aspergillus* genus, we hypothesize that these two HATs may also have distinct roles during development.

479 GNATs have a acetyltransferase domain as its catalytic core, and 2,604
480 acetyltransferase domain-containing proteins were found in our search (Supplementary
481 Figure 2). The two well-characterized GNATs, *Gcn5* and *Elp3*, occur in a single copy in every
482 species included in our study (Supplementary Figure 2). Gcn5 harbors a Bromodomain
483 (PF00439) in addition to the Acetyltransferase domain and is a crucial subunit of distinct
484 histone acetyltransferase complexes such as the HAT-A2 complex, the SAGA complex, and
485 the ADA complex in *S. cerevisiae* (96,98). All these complexes contain two coactivator
486 proteins, Ada2 and Ada3, that directly bind to Gcn5 (111) and the recruitment of different other
487 subunits determines the location where they catalyze acetylation (96). Elp3 is characterized
488 by the Acetyltransferase domain, Radical SAM (PF04055), and Radical SAM C-terminal
489 domain (PF16199), and has been shown to catalyze H3K14ac, H4K8ac, and H3K56ac in *S.*
490 *cerevisiae* (112–114). Elp3 also exhibits a dual enzymatic activity as it catalyzes
491 demethylation in mouse zygotes (115), which is due to the Radical SAM domain that catalyzes
492 the demethylation of methylated lysyl residues on histones (116).
493
494 Next to Gcn5 and Elp3, we have identified 2,386 additional acetyltransferase domain-
495 containing proteins. Because their functions remain unknown due to the lack of homology to
496 sequences with characterized functions, they are valuable candidates that may acetylate
497 histones or other proteins. Interestingly, it has been shown that Gcn5 also exhibits
498 succinyltransferase activity in humans (117) and can also function as histone
499 crotonyltransferase to regulate gene transcription *in vitro* (118). It is expected that other less
500 common histone modifications, *i.e.* histone lysine butyrylation, propionylation, or malonylation,
501 may also be catalyzed by histone acetyltransferases because these histone modifications all
502 use acyl-Coenzyme A to complete the reactions (119–121). Further research is needed to
503 determine whether these less common histone modifications are catalyzed by the known
504 histone acetyltransferases Gcn5 and Elp3, or as of yet unstudied HATs. In conclusion, all five
505 characterized fungal HATs described above are highly conserved in the *Aspergillus* genus,
506 showing their importance in fungal biology. Many other acetyl-transferase domain-containing

507 proteins are encoded in *Aspergillus* genomes and may be responsible for histone acetylation
508 or additional less common histone modifications like butyrylation, succinylation, and
509 malonylation.
510
511 **Duplication of *RpdA* histone deacetylase gene in the *Flavi* and *Janorum* sections**
512 Five families of histone deacetylases (HDACs) can be grouped into two classes based on
513 sequence similarity: class I (HOS1, HOS2, and Rpd3/RpdA) and class II (HdaA and HOS3)
514 (122). They all harbor the Histone deacetylase catalytic domain, and our phylogenetic tree
515 corroborates the HDAC classification into two distinct classes (Figure 4). *HdaA* is found as a
516 single copy in each species included in this study (Figure 4). It harbors the Histone
517 deacetylase domain and C-terminal Arb2 domain (PF09757) that serves as an anchor to target
518 centromeric heterochromatin region (123,124). HdaA deacetylates lysine in histones H3 and
519 H2B, but not H4 nor H2A in *S. cerevisiae* (125,126), and the Δ*hdaA* knock-out strains show
520 increased or reduced production of different secondary metabolites in *A. nidulans* (127), *A.*
521 *niger* (127), and *A. fumigatus* (128). By contrast, *HOS3* is found in only 80 *Aspergillus* species,
522 as it is lost in nine species (*A. nomiae*, *A. pseudocaelatus*, *A. tamarii*, *A. pseudotamarii*, *A.*
523 *caelatus*, *A. sergii*, *A. novoparasiticus*, *A. minisclerotigenes*, *A. arachidicola*) in the *Flavi*
524 section, three species (*A. desertorum*, *A. stercorarius*, and *A. multicolor*) in the *Nidulantes*
525 section, and two species (*A. heteromorphus* and *A. carbonarius*) in the *Nigri* section. *HOS3* is
526 well conserved in all outgroup species except *S. pombe* (Figure 4) (129). HOS3 has been
527 shown to catalyze deacetylation with distinct specificity for histone H2AK7, H2BK11, H3K14
528 and H3K23, as well as H4K5 and H4K8 in *S. cerevisiae* cell extracts (130). Notably, *HOS1*
529 was only detected in three outgroup species, *S. cerevisiae* and both Basidiomycota (Figure
530 4). The specificity of Hos1 in catalyzing deacetylation on histone proteins is not known yet
531 (131), but it can catalyze the deacetylation of the Smc3 subunit of Cohesin and influence the
532 regulation of chromosome segregation during mitosis in *S. cerevisiae* (132). *HOS2* is found in
533 15 outgroup species and most of Aspergilli except *A. oryzae* (Figure 4). It is known to
534 deacetylate lysine residues in H3 and H4 histone tails (133). The conservation of *HOS2* in

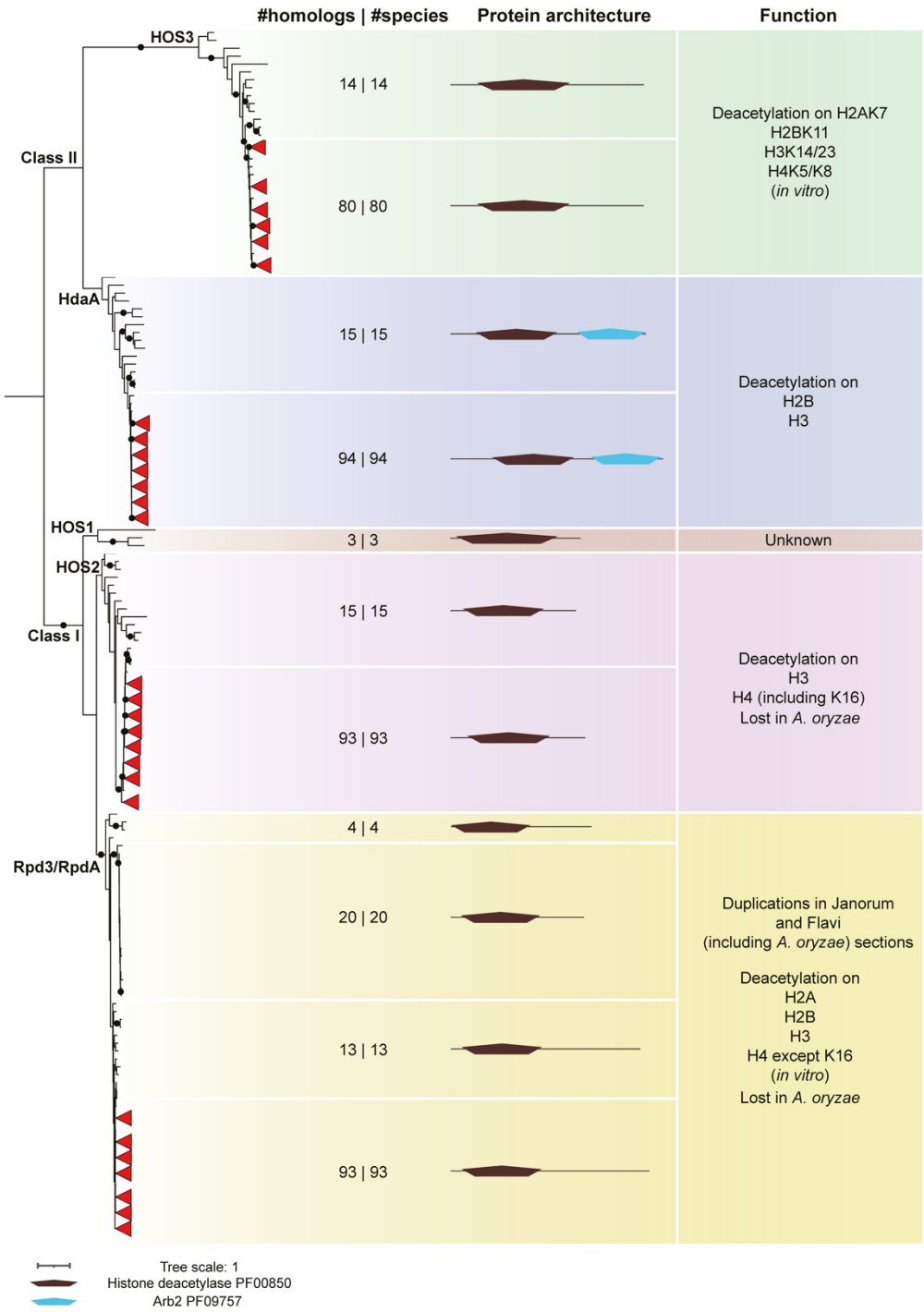


Figure 4. Conservation of histone deacetylase domain-containing proteins in the Aspergillus genus. The maximum-likelihood phylogeny of Histone deacetylase domains (PF00850) found in 94 Aspergilli and 15 outgroup species was determined using IQ-TREE (37,38). The black dot on the branch indicates ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with trustworthy support are collapsed and shown as red triangles. Additional conserved domains in histone deacetylase domain-containing proteins were identified using the PFAM database (https://pfam.xfam.org).

543 Aspergilli suggests that it plays a key role, which is supported by the deletion of *HOS2* in *A.*
544 *niger* where the deletion strains displays severe reduction in growth, sporulation, SM
545 biosynthesis, and stress resistance (127).
546
547 Within class I HDACs, the Rpd3/RpdA clade is divided into two sub-clades (Figure 4).
548 One is nearly fully conserved (except in *A. oryzae*) and comprises the well-characterized
549 Rpd3/RpdA HDACs, while the other one only comprises sequences from the *Flavi* and
550 *Janorum* sections (Figure 4). Our phylogenetic reconstruction suggests a duplication event
551 that has occurred in the ancestor of all Aspergilli giving rise to a paralog that was retained in
552 two sections only and could explain the *A. oryzae* loss in the conserved sub-clade. Rpd3 is a
553 crucial deacetylase in eukaryotes, as it is required for deacetylation in most locations on core
554 histone proteins, except H4K16 *in vitro* (134) that is catalyzed by HOS2 (133). *S. cerevisiae*
555 lacking *Rpd3* is sensitive to high osmolarity and shows compromised expression of osmotic
556 stress genes (135). Deletion of *RpdA* in *A. nidulans* leads to the reduction of its growth and
557 sporulation (136), while only heterokaryon disruptants could be obtained in *A. oryzae* (137),
558 indicating its crucial role in the fungal genus *Aspergillus*. In summary, class I and class II
559 deacetylases are well conserved in Aspergilli, and duplication events like the one observed
560 for *RpdA* may confer new activities. While only one paralog was retained in *A. oryzae*, the
561 presence of two paralogs in sections *Flavi* and *Janorum* may provide functional redundancy
562 or diversification, which remains to be determined.
563
564 **Presence-absence patterns of subunits that compose histone modifier complexes**
565 Histone PTMs are catalyzed by the conserved domains found in DNMTs, HMTs, HATs, and
566 HDACs (Figure 1-4), but these chromatin modifying enzymes are often not acting alone.
567 Instead, they are part of protein complexes that comprise accessory subunits, which are
568 involved in the recognition and induction of specific histone modifications at the correct
569 location (18,19). To further investigate the presence-absence patterns (PAPs) of known
570 chromatin modifier complexes in Aspergilli, we focused on 16 chromatin modifier complexes

571 with 86 subunits previously identified and studied in *S. cerevisiae* or *N. crassa* (Supplementary
572 Table 3). Apart from the Velvet complex, whenever a catalytic subunit is absent in one species,
573 there seems to be a trend for the absence of additional accessory subunits, which is
574 particularly observed in outgroup species (Figure 5). Only a few PAPs are observed, and most
575 accessory subunits are either fully conserved or absent in Aspergilli. Nine subunits appear
576 specific to *S. cerevisiae* as they are not found in any other species and are likely to play a
577 specific role in this species. Seven subunits are absent in Aspergilli and show PAP in the
578 outgroup species.
579
580 Five histone methyltransferase complexes, COMPASS, DCDC (Dim-5/-7/-
581 9/CUL4/DDB1 Complex), RNAP2, PRC2, and Dot1 (disruptor of telomeric silencing 1), as well
582 as the putative VelB/VeA/LaeA methyltransferase complex (Velvet), were included in our
583 analyses (Figure 5). Five COMPASS subunits (*SET1*, *CclA*, *Swd1*, *Swd2*, and *Swd3*) are fully
584 conserved, suggesting an important function for H3K4 methylation. Consistent with a PAP in
585 most outgroup species and the loss in Aspergilli, Spp1p is not essential for H3K4 methylation
586 (138). As *Sdc1* shows a PAP in Aspergilli and outgroup species, it is likely that this subunit
587 also does not play a key role in H3K4 methylation. In the DCDC complex, while Dim-7 and
588 Dim-9 seem to be dispensable for H3K9 methylation, *Dim-5*, *Cul4*, and *Dim-8* are highly
589 conserved, and we thus expect these enzymes to play key roles in the activity of this complex
590 (Figure 5 and Table S3). However, it was shown that Dim-7 and Dim-9 are responsible for
591 heterochromatin recognition, which leads to subsequent recruitment of the complete DCDC
592 to induce H3K9 methylation in *N. crassa* (139). Thus, it is very likely that other accessory
593 subunits are involved in recruiting DCDC at specific chromatin locations in Aspergilli.
594 Furthermore, the unique absence of *Dim-8* in *A. indicus* may suggest altered H3K9
595 methylation in this species. Consistent with the absence of *SET7* (Figure 2), all components
596 of the PRC2 complex are lost in Aspergilli (Figure 5). *PRC2* is found in different filamentous
597 fungi such as *N. crassa* (21), *Z. tritici*, *B. cinerea*, and *F. graminearum* (22). The apparent loss
598

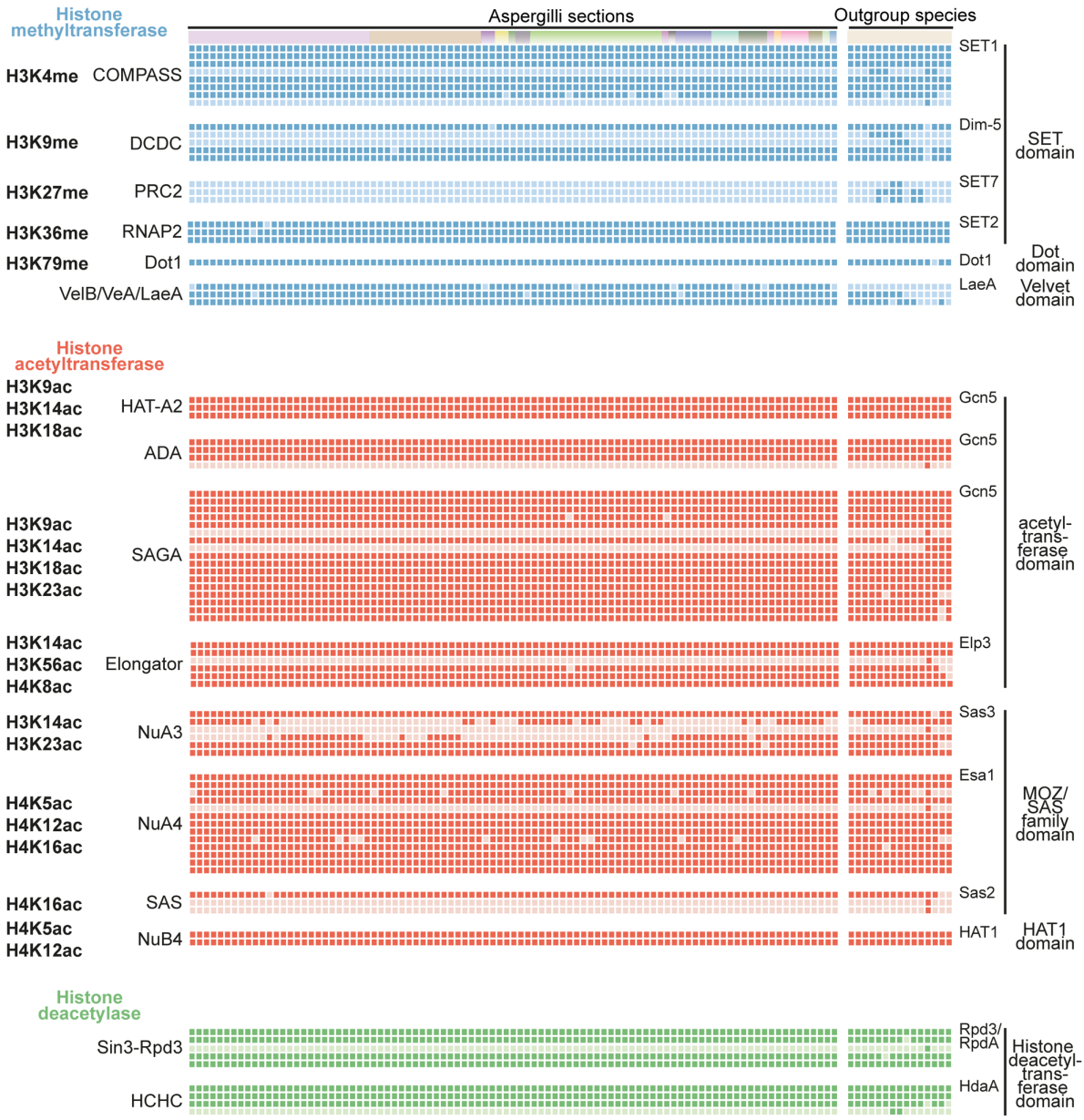


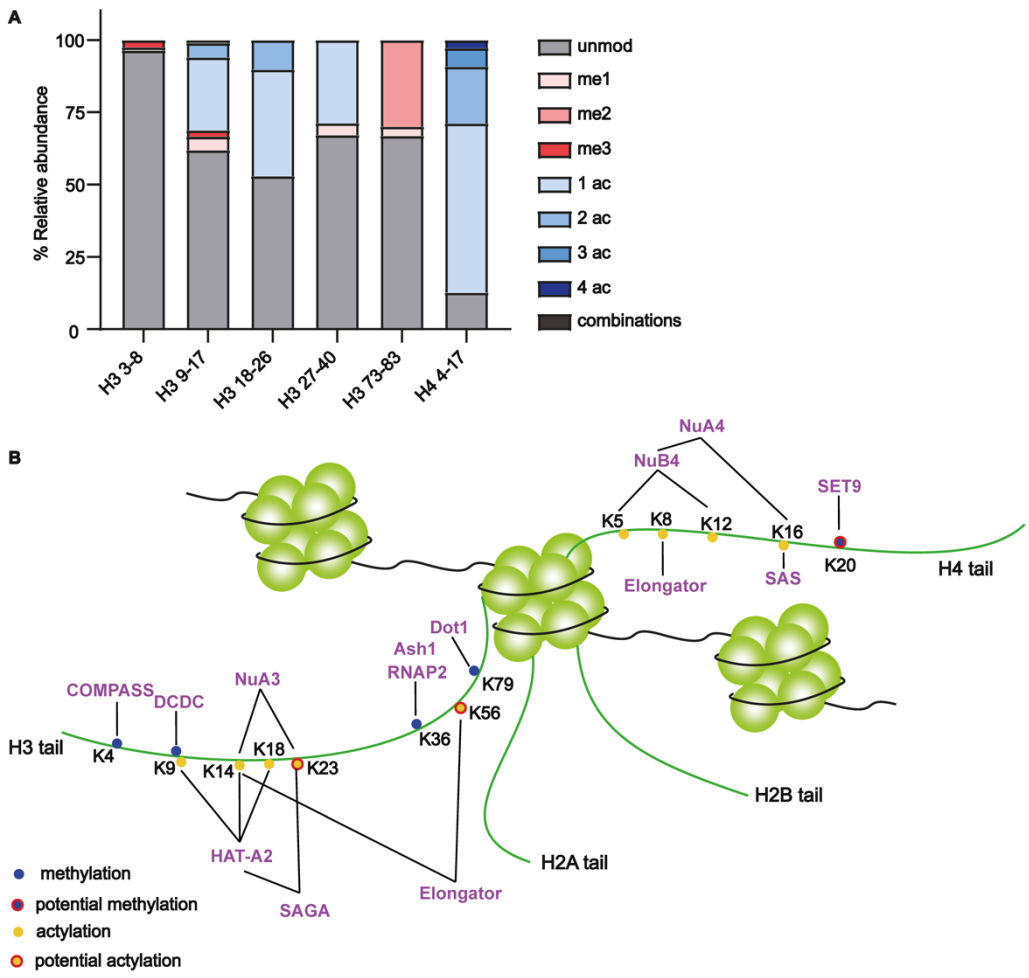
Figure 5. Presence-absence pattern of subunits that constitute characterized histone modification complexes. The top row with distinct color boxes indicates outgroup species and Aspergilli sections, the order of the species from right to left is the same as in the species tree from top to bottom (Supplementary Figure 1) without Fusarium oxysporum and Aspergillus kawachii since they were excluded in the later analysis. Each row represents a subunit in the histone modification complexes, the order agrees with the summary list (Supplementary Table 3). Each box in the matrix indicates the presence (dark color) or absence (light color) of a subunit (Supplementary Table 3). Each row shows the presence/absence of a specific complex subunit. For each complex, the first row represents the catalytic subunit.

of *SET7* in *B. cinerea* and *Z. tritici* in our study (Figure 5) is due to their length that was too short to pass the inclusion threshold compared to the *N. crassa SET7* query.

612 We included eight histone acetyltransferase complexes that differ by their distinct
613 catalytic subunits (Figure 5). The HAT-A2, ADA, SAGA, and Elongator complexes use an
614 acetyltransferase domain-containing protein as catalytic subunit (34,140). The SAS
615 (Something About Silencing), NuA4 (Nucleosomal Acetyltransferase of histone H4), and NuA3
616 complexes use a MOZ/SAS FAMILY domain-containing protein (141). The NuB4 (Nuclear
617 Hat1p-containing type B histone acetyltransferase) complex uses the histone
618 acetyltransferase HAT1 domain-containing protein (142). Gcn5, Ada2, and Ada3 are fully
619 conserved in all fungi and constitute the core enzymatic module of the HAT-A2, ADA, and
620 SAGA complexes. This catalytic module contributes to catalyzing histone acetylation, and
621 other subunits in these complexes guide them to specific locations and link them to the
622 transcriptional machinery (143). Besides the catalytic module, the ADA complex also harbors
623 the Ahc1 subunit that is found in *S. cerevisiae* only (140,144). Twelve additional subunits of
624 the SAGA complex are well conserved in Aspergilli and outgroup species (Figure 5). The
625 SAGA complex also contains a deubiquitylase (DUB) module, for which the composition
626 differs between Aspergilli and other species. *Sgf11* is unique to *S. cerevisiae* and *Sus1* was
627 likely lost in the ancestor of Pezizomycotina because we can only identify *Sus1* orthologs in
628 both Basidiomycota and the yeasts (Figure 5). The Elongator complex appears more variable
629 as four subunits are mostly conserved, but *Elp6* is specific to *S. cerevisiae*, and *Hpa2* and
630 *Hpa3* show PAPs in both Aspergilli and outgroup species.
631
632 The NuA4, SAS, and NuB4 complexes are well-conserved in Aspergilli as well as in
633 the outgroup species (Figure 5). These complexes appear slightly different in *S. cerevisiae*
634 with the presence of specific subunits (*Eaf5* in the NuA4 complex; *Sas4* and *Sas5* in the SAS
635 complex). Both SAS and NuA4 complexes can catalyze H4K16ac with opposite effects during
636 cell growth and morphogenesis in *C. albicans* (105). It would be interesting to assess whether
637 these two complexes exhibit similar activities in Aspergilli. For the NuA3 complex, two
638 accessory subunits are well conserved among Aspergilli while two others, *Pdp3*, and *Nto1*,
639 show different PAPs, and *Yng1* is specific to *S. cerevisiae*. *Pdp3* and *Nto1* seem to be lost in

640 a complementary manner, suggesting a possible functional redundancy (Figure 5). Finally,
641 two histone deacetylase complexes, Sin3-Rpd3 and HCHC, are fully conserved (Figure 5).
642 The Sin3-Rpd3 complex is slightly different in *S. cerevisiae* with the specific of the *Ume1*
643 subunit.
644
645 Overall, our analysis showed that the core catalytic modules of complexes involved in
646 histone PTMs are mostly conserved in all fungi, including Aspergilli, but variability in accessory
647 subunits could contribute to differences in the localization of histone PTMs along the genome.
648
649 **Unbiased mass spectrometry captures the diverse histone modifications in *A. nidulans***
650 **and verifies the absence of H3K27 methylation**
651 Our genomic and phylogenetic analyses have identified the presence of multiple chromatin
652 modifiers in Aspergilli, suggesting that these species have the ability to establish a wide variety
653 of histone modifications. To assess the presence of histone PTMs predicted in our
654 evolutionary analyses (Fig 1-5), we extracted histone proteins from *A. nidulans* and performed
655 unbiased mass spectrometry analyses. H3 and H4 peptides with PTMs corresponding to
656 H3K4me, H3K9me, H3K36me, and H3K79me were detected (Figure 6), which we anticipate
657 being catalyzed by SET1, Dim-5, Ash1 and SET2, and Dot1, respectively. Interestingly,
658 H4K20me, which is catalyzed by SET9 in *S. pombe* (92), was not detected (Figure 6).
659 However, *SET9* is well conserved in Aspergilli (Figure 2), and thus it is possible that Aspergilli
660 lost subunits that are needed to induce this specific PTM or that H4K20me is not detectable
661 under the here tested conditions. Additionally, acetylation of H3K9, H3K14, H3K18, H4K5,
662 H4K8, H4K12, and H4K16 were detected (Figure 6). Acetylation on H3K23 and H3K56 were
663 not detected in this study even though the core catalytic enzymes of SAGA, NuA3, and
664 Elongator complexes involved in these modifications are conserved (Figure 5). These
665 modifications either do not occur in Aspergilli, maybe due to the absence of the necessary
666 accessory subunits or occur below our detection threshold. As expected, we did not observe
667 any peptide that would indicate the presence of H3K27me in *A nidulans* (Figure 6A;

668 Supplementary Table 4), thereby corroborating our computational analysis that the PRC2
669 complex is absent in *A. nidulans* as well as in all other Aspergilli. Thus, the mass spectrometry
670 results verify the occurrence of histone modifications predicted to be present based on our
671 comparative analyses in the fungal genus *Aspergillus*. Moreover, the differences between the
672 relative abundance of distinct histone modifications provide interesting information to
673 investigate these abundances and identify specific conditions under which these modifications
674 increase or decrease in abundance.



675

676 **Figure 6.** Schematic representation of the histone code in Aspergilli. (A) Relative abundance
677 of post-translational modifications detected on peptides of histone H3 and H4 from Aspergillus
678 nidulans using unbiased mass spectrometry. The term 'combinations' refers to multiple
679 modifications present on the same peptide (Supplementary Table 4). (B) Histone
680 modifications are predicted by genomic and phylogenetic analyses, and/or proved by mass
681 spectrometry in *A. nidulans*. Histone modifications verified by mass spectrometry are labeled
682 as solid dots, while the ones that are only predicted by phylogenic analysis are labeled as
683 solid dots with a red outline.
684

685 **Conclusion**

686 Changes in chromatin organization play an important role in regulating the expression of
687 genes (129). The fungal genus *Aspergillus* is well known for its ability to thrive in a broad range
688 of different environmental niches and the huge potential of secondary metabolite biosynthesis
689 (16,145). However, a detailed and complete overview of occurrence and evolution of
690 chromatin modifiers in Aspergilli is a prerequisite to be able to better understand how these
691 fungi regulate gene expression. In the future, more research is needed to uncover how the
692 absence of *de novo* DNA methylation impacts genome evolution in Aspergilli. We demonstrate
693 that PRC2 is lost throughout Aspergilli, and the absence of PRC2 is responsible for the lack
694 of H3K27me. Although histone acetyltransferases and deacetylases are overall conserved,
695 our study also revealed specific duplication and loss events, which require further investigation
696 to understand their impact on chromatin and gene regulation in certain *Aspergillus* species.
697 Moreover, other less common histone modifications like histone lysine succinylation,
698 crotonylation, butyrylation, propionylation, and malonylation, are all likely catalyzed by HATs
699 and HDACs as they all use Coenzyme A (CoA) to complete the reactions (119–121), but may
700 require specific yet to be discovered subunits. In conclusion, our study provides a detailed
701 overview of the evolutionary routes of chromatin modifier complex in the fungal genus
702 *Aspergillus*, and therefore generates the necessary framework to identify targets for functional
703 studies to understand how chromatin regulates gene expression in Aspergilli.

704

705

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Authors and contribution

X.Z. formal analysis, investigation, methodology, visualization, writing – original draft; R.N. formal analysis, investigation, methodology, visualization; T.B. funding acquisition, supervision, resources; J.C. Conceptualization, funding acquisition, methodology, project administrations, supervision, visualization, writing original draft; M.F.S. Conceptualization, funding acquisition, methodology, project administrations, supervision, visualization, writing original draft.

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1106 among biosynthetic gene clusters, secondary metabolite profiles, and cards of
1107 virulence across *Aspergillus* species. Genetics. 2020;216(2):481–97.
1108
1109

1110 **Figure captions**

1111 **Figure 1. Conservation of DNA methyltransferase families in the *Aspergillus* genus.** The
1112 maximum-likelihood phylogeny of DNA methyltransferase domains (PF00145) found in 94
1113 Aspergilli and 15 outgroup species was determined using IQ-TREE (38,39). The black dot on the
1114 branch indicates ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from
1115 Aspergilli with trustworthy support are collapsed and shown as red triangles. Additional conserved
1116 domains in DNA methyltransferase domain-containing proteins were identified using the PFAM
1117 database (https://pfam.xfam.org).

1118

1119 **Figure 2. Conservation of histone methyltransferase families in the *Aspergillus* genus.**
1120 The maximum-likelihood phylogeny of SET domains (PF00856) found in 94 Aspergilli and 15
1121 outgroup species was determined using IQ-TREE (38,39). The black dot on the branch indicates
1122 ultrafast bootstrap values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with
1123 trustworthy support are collapsed and shown as red triangles and leaves with similar protein
1124 architecture with unknown functions covering both Aspergilli and outgroup species are collapsed
1125 and shown as grey triangles. Additional conserved domains in SET domain-containing proteins
1126 were identified using the PFAM database (https://pfam.xfam.org).

1127

1128 **Figure 3. Conservation of MOZ/SAS FAMILY domain-containing histone**
1129 **acetyltransferases in the *Aspergillus* genus.** The maximum-likelihood phylogeny of
1130 MOZ/SAS FAMILY domains (PF01853) found in 94 Aspergilli and 15 outgroup species was
1131 determined using IQ-TREE (38,39). The black dot on the branch indicates ultrafast bootstrap
1132 values over 95 and SH-aLRT values over 80. Leaves from Aspergilli with trustworthy support are
1133 collapsed and shown as red triangles. Additional conserved domains in MOZ/SAS FAMILY
1134 domain-containing proteins were identified using the PFAM database (https://pfam.xfam.org).

1135

1136 **Figure 4. Conservation of histone deacetylase domain-containing proteins in the**
1137 ***Aspergillus* genus.** The maximum-likelihood phylogeny of Histone deacetylase domains

1138 (PF00850) found in 94 Aspergilli and 15 outgroup species was determined using IQ-TREE (38,39).
1139 The black dot on the branch indicates ultrafast bootstrap values over 95 and SH-aLRT values
1140 over 80. Leaves from Aspergilli with trustworthy support are collapsed and shown as red triangles.
1141 Additional conserved domains in histone deacetylase domain-containing proteins were identified
1142 using the PFAM database (https://pfam.xfam.org).
1143
1144 **Figure 5. Presence-absence pattern of subunits that constitute characterized histone**
1145 **modification complexes.** The top row with distinct color boxes indicates outgroup species and
1146 Aspergilli sections, the order of the species from right to left is the same as in the species tree
1147 from top to bottom (Supplementary Figure 1) without *Fusarium oxysporum* and *Aspergillus*
1148 *kawachii* since they were excluded in the later analysis. Each row represents a subunit in the
1149 histone modification complexes, the order agrees with the summary list (Supplementary Table
1150 3). Each box in the matrix indicates the presence (dark color) or absence (light color) of a
1151 subunit (Supplementary Table 3). Each row shows the presence/absence of a specific
1152 complex subunit. For each complex, the first row represents the catalytic subunit.
1153
1154 **Figure 6. Schematic representation of the histone code in Aspergilli. (A)** Relative abundance
1155 of post-translational modifications detected on peptides of histone H3 and H4 from *Aspergillus*
1156 *nidulans* using unbiased mass spectrometry analysis. The term “combinations” refers to multiple
1157 modifications occurring on the same peptide (Supplementary Table 4). **(B)** Histone modifications
1158 are predicted by genomic and phylogenetic analyses, and/or proved by mass spectrometry in
1159 *A. nidulans*. Histone modifications verified by mass spectrometry are labeled as solid dots,
1160 while the ones that are only predicted by phylogenic analysis are labeled as solid dots with a
1161 red outline.
1162
1163

1164 **Supplementary material**

1165 **Supplementary Table S1. List of species included in this study.**

1166 **Supplementary Table S2. List of BUSCO genes used to build the species tree.**

1167 **Supplementary Table S3. List of chromatin modifier complexes reported in literature.**

1168 **Supplementary Table S4. Mass spectrometry result for *A. nidulans*.**

1169

1170 **Supplementary Figure 1. A robust species phylogeny for the fungal genus *Aspergillus*.**

1171 **Supplementary Figure 2. Two known histone acyltransferase-domain containing protein**

1172 **groups are conserved in the *Aspergillus* genus together with many unknown proteins.**

1173

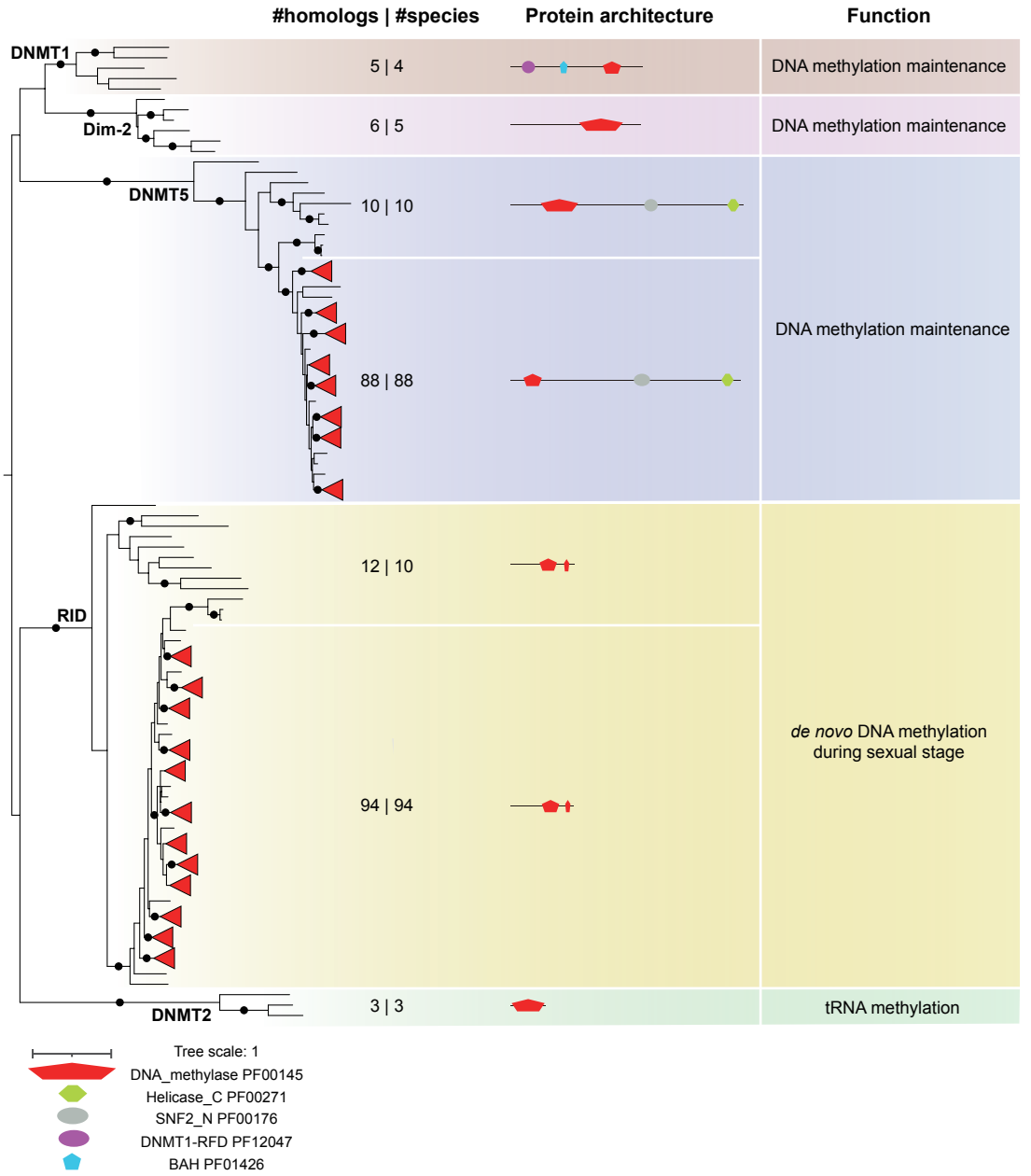
1174 **Supplementary Data 1. Fasta, trimmed alignment, and maximum-likelihood phylogenetic**

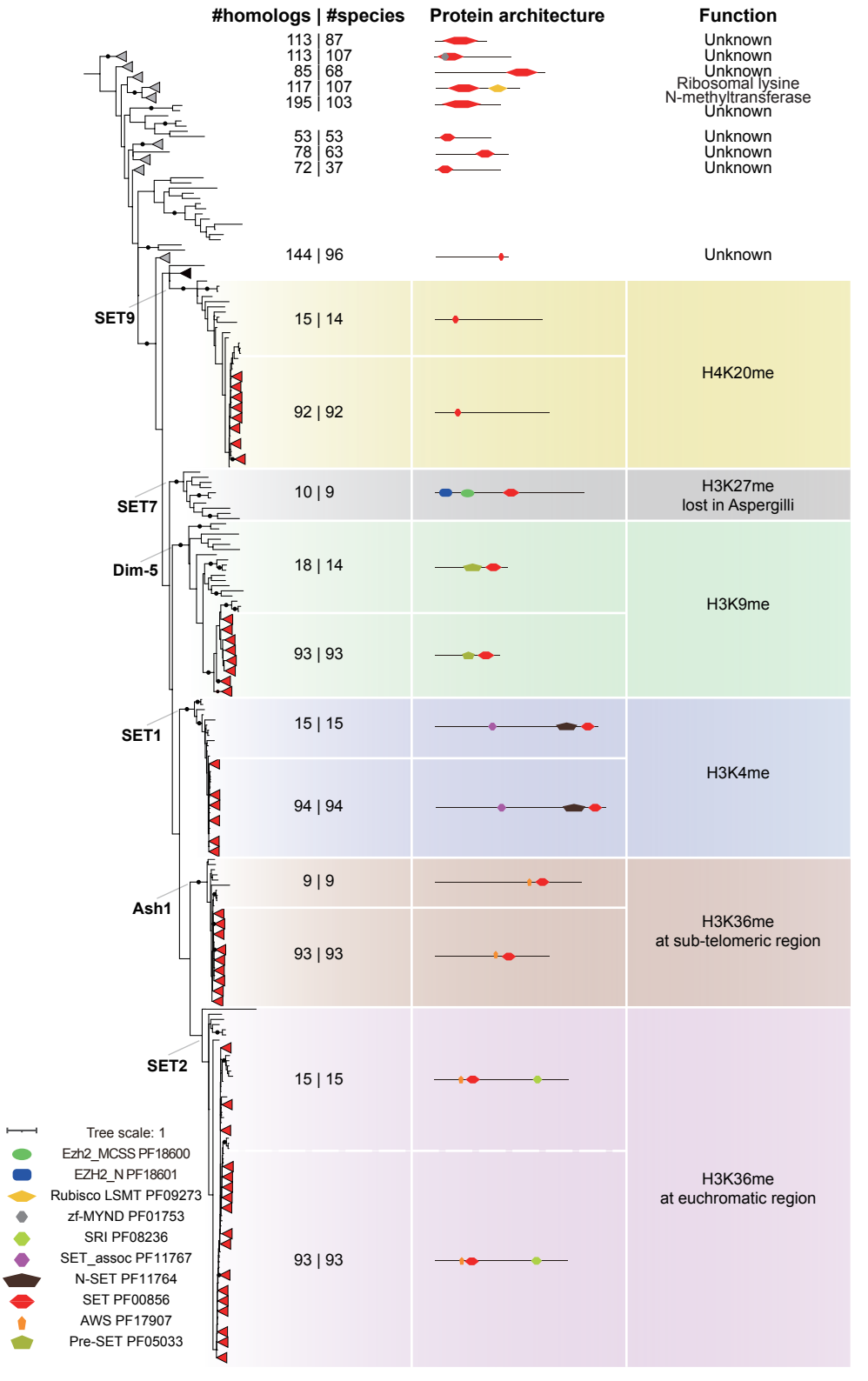
1175 **tree files associated with Figure 1-4.**

1176 **Supplementary Data 2. Fasta, trimmed alignment, and maximum-likelihood phylogenetic**

1177 **tree files associated with Figure 5.**

1178


#homologs | #species
Protein architecture
Function
DNMT1
5 | 4
DNA methylation maintenance
Dim-2
6 | 5
DNA methylation maintenance
DNMT5
10 | 10
88 | 88
DNA methylation maintenance
RID
12 | 10
94 | 94
de novo DNA methylation during sexual stage
DNMT2
3 | 3
tRNA methylation
Tree scale: 1
DNA_methylase PF00145
Helicase_C PF00271
SNF2_N PF00176
DNMT1-RFD PF12047
BAH PF01426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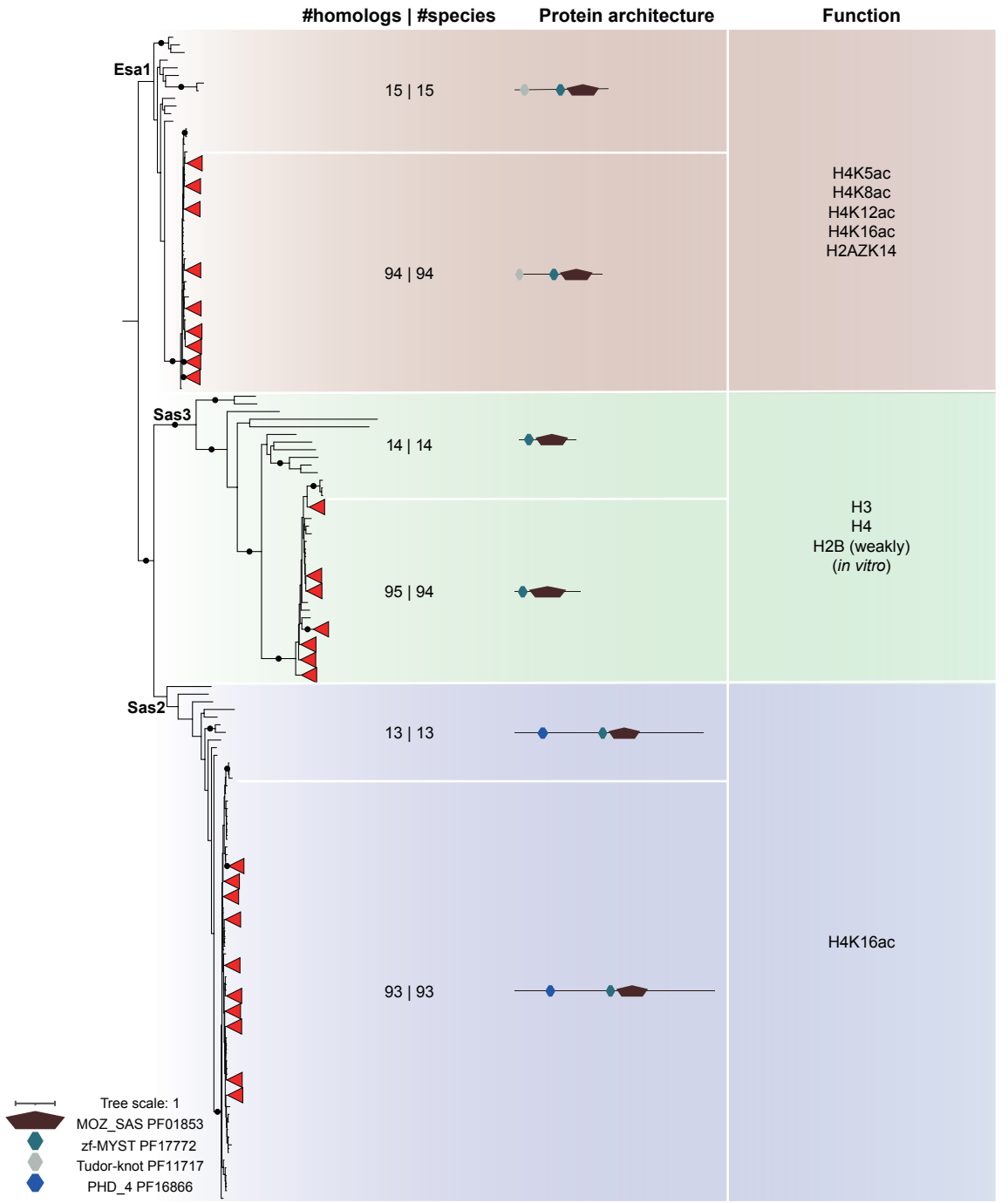
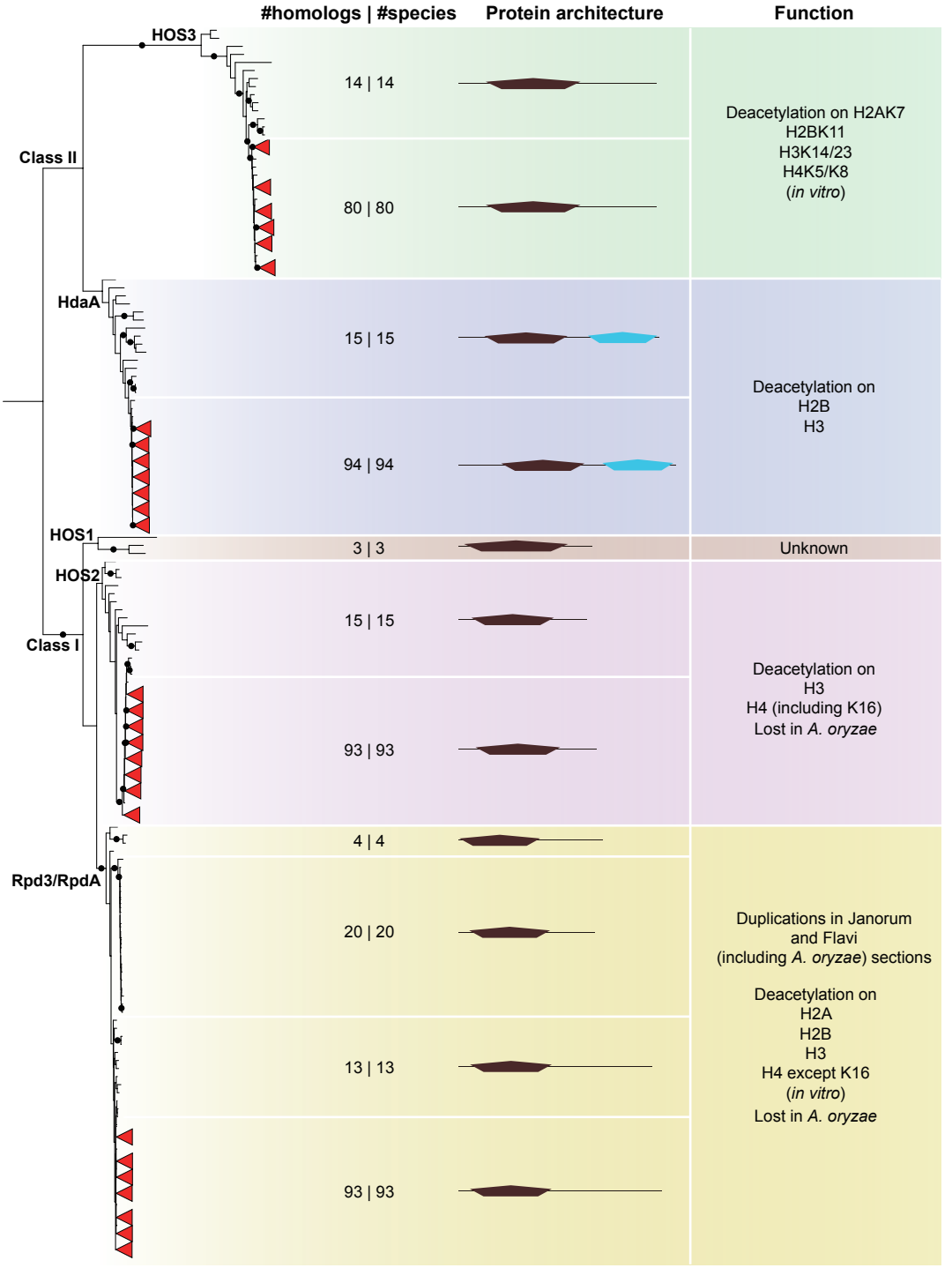
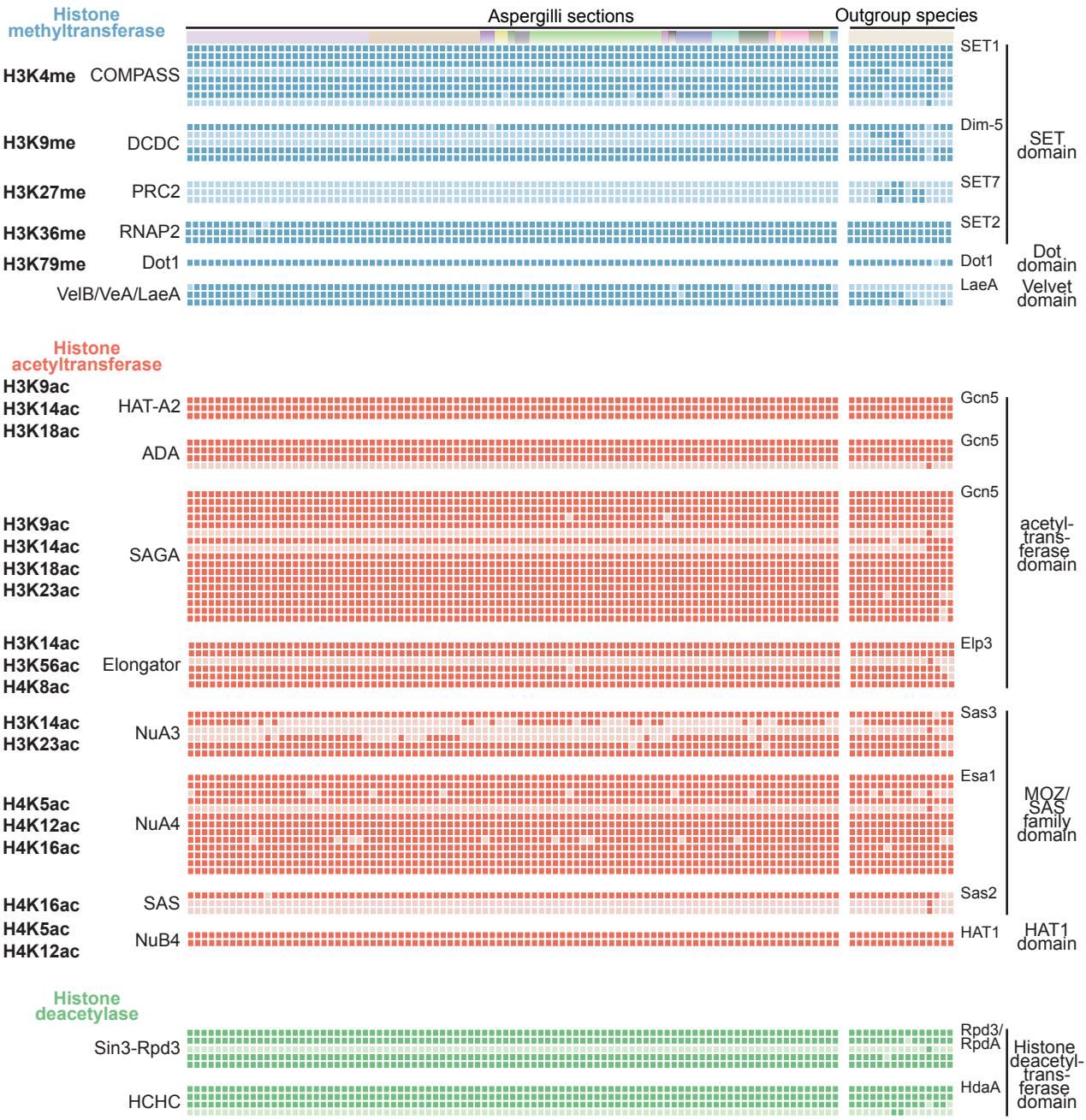
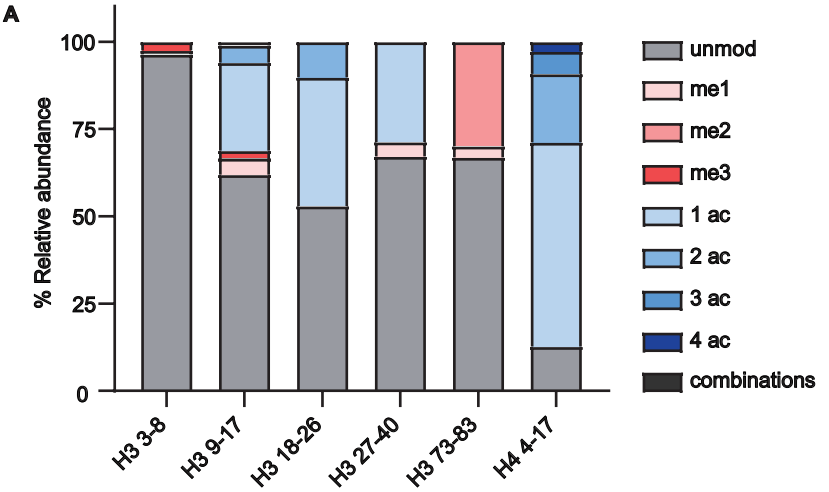
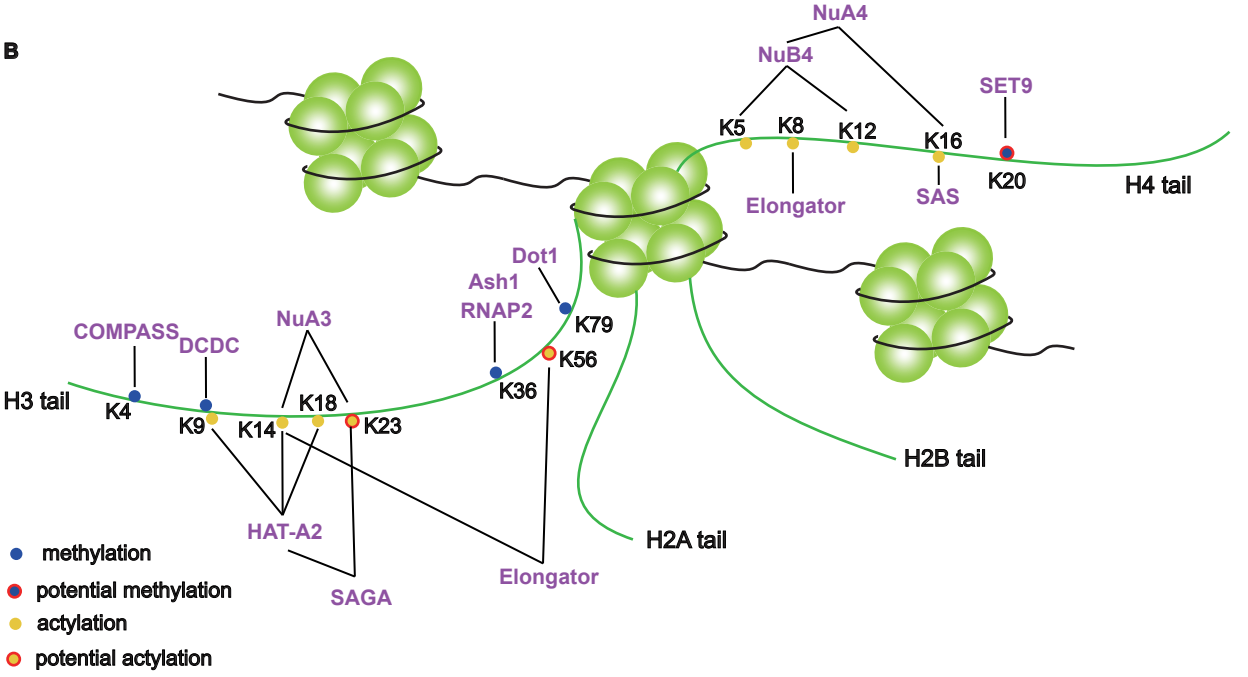
Protein architecture
Function
113 | 87
Unknown
113 | 107
Unknown
85 | 68
Unknown
117 | 107
Ribosomal lysine
195 | 103
N-methyltransferase
Unknown
53 | 53
Unknown
78 | 63
Unknown
72 | 37
Unknown
144 | 96
Unknown
SET9
15 | 14
92 | 92
H4K20me
SET7
10 | 9
H3K27me
lost in Aspergilli
Dim-5
18 | 14
93 | 93
H3K9me
SET1
15 | 15
94 | 94
H3K4me
Ash1
9 | 9
93 | 93
H3K36me
at sub-telomeric region
SET2
15 | 15
93 | 93
H3K36me
at euchromatic region
Tree scale: 1
Ezh2_MCSS PF18600
EZH2_N PF18601
Rubisco LSMT PF09273
zf-MYND PF01753
SRI PF08236
SET_assoc PF11767
N-SET PF11764
SET PF00856
AWS PF17907
Pre-SET PF05033


#homologs | #species
Protein architecture
Function
Esa1
15 | 15
94 | 94
H4K5ac
H4K8ac
H4K12ac
H4K16ac
H2AZK14
Sas3
14 | 14
95 | 94
H3
H4
H2B (weakly)
(in vitro)
Sas2
13 | 13
93 | 93
H4K16ac
Tree scale: 1
MOZ_SAS PF01853
zf-MYST PF17772
Tudor-knot PF11717
PHD_4 PF16866


#homologs | #species
Protein architecture
Function
HOS3
Class II
14 | 14
80 | 80
Deacetylation on H2AK7
H2BK11
H3K14/23
H4K5/K8
(in vitro)
HdaA
15 | 15
94 | 94
Deacetylation on
H2B
H3
HOS1
3 | 3
Unknown
HOS2
Class I
15 | 15
93 | 93
Deacetylation on
H3
H4 (including K16)
Lost in A. oryzae
Rpd3/RpdA
4 | 4
20 | 20
13 | 13
93 | 93
Duplications in Janorum
and Flavi
(including A. oryzae) sections
Deacetylation on
H2A
H2B
H3
H4 except K16
(in vitro)
Lost in A. oryzae
Tree scale: 1
Histone deacetylase PF00850
Arb2 PF09757


Histone methyltransferase
Aspergilli sections
Outgroup species
H3K4me
COMPASS
SET1
H3K9me
DCDC
Dim-5
SET domain
H3K27me
PRC2
SET7
H3K36me
RNAP2
SET2
H3K79me
Dot1
Dot1
Dot domain
VelB/VeA/LaeA
LaeA
Velvet domain
Histone acetyltransferase
H3K9ac
H3K14ac
H3K18ac
HAT-A2
Gcn5
ADA
Gcn5
Gcn5
H3K9ac
H3K14ac
H3K18ac
H3K23ac
SAGA
acetyl-trans-ferase domain
H3K14ac
H3K56ac
H4K8ac
Elongator
Elp3
H3K14ac
H3K23ac
NuA3
Sas3
H4K5ac
H4K12ac
H4K16ac
NuA4
Esa1
MOZ/SAS family domain
H4K16ac
SAS
Sas2
H4K5ac
H4K12ac
NuB4
HAT1
HAT1 domain
Histone deacetylase
Sin3-Rpd3
Rpd3/RpdA
HCHC
HdaA
Histone deacetyl-trans-ferase domain


A
% Relative abundance
100
75
50
25
0
H3 3-8
H3 9-17
H3 18-26
H3 27-40
H3 73-83
H4 4-17
unmod
me1
me2
me3
1 ac
2 ac
3 ac
4 ac
combinations


B
NuA4
NuB4
SET9
K5
K8
K12
K16
K20
Elongator
SAS
H4 tail
Dot1
Ash1
RNAP2
K79
K56
K36
COMPASS
DCDC
NuA3
H3 tail
K4
K9
K14
K18
K23
H2B tail
HAT-A2
H2A tail
SAGA
Elongator
methylation
potential methylation
actylation
potential actylation