Loss of EZH2-like or SU(VAR)3–9-like proteins causes simultaneous perturbations in H3K27 and H3K9 tri-methylation and associated developmental defects in the fungus Podospora anserina

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Abstract

Background: Selective gene silencing is key to development. It is generally accepted that H3K27me3-enriched heterochromatin maintains transcriptional repression established during early development and regulates cell fate. Conversely, H3K9me3-enriched heterochromatin prevents differentiation but constitutes protection against transposable elements. We exploited the fungus Podospora anserina, a valuable alternative to higher eukaryote models, to question the biological relevance and functional interplay of these two distinct heterochromatin conformations.

Results: We established genome-wide patterns of H3K27me3 and H3K9me3 modifications, and found these marks mutually exclusive within gene-rich regions but not within repeats. We generated the corresponding histone methyltransferase null mutants and showed an interdependence of H3K9me3 and H3K27me3 marks. Indeed, removal of the PaKmt6 EZH2-like enzyme resulted not only in loss of H3K27me3 but also in significant H3K9me3 reduction. Similarly, removal of PaKmt1 SU(VAR)3–9-like enzyme caused loss of H3K9me3 and substantial decrease of H3K27me3. Removal of the H3K9me binding protein PaHP1 provided further support to the notion that each type of heterochromatin requires the presence of the other. We also established that P. anserina developmental programs require H3K27me3-mediated silencing, since loss of the PaKmt6 EZH2-like enzyme caused severe defects in most aspects of the life cycle including growth, differentiation processes and sexual reproduction, whereas loss of the PaKmt1 SU(VAR)3–9-like enzyme resulted only in marginal defects, similar to loss of PaHP1.

Conclusions: Our findings support a conserved function of the PRC2 complex in fungal development. However, we uncovered an intriguing evolutionary fluidity in the repressive histone deposition machinery, which challenges canonical definitions of constitutive and facultative heterochromatin.

Keywords: Histone methylation, Sexual development, ChIP-seq, Podospora anserina

Background

Histones are subjected to a variety of post-translational covalent modifications [1] that may impact the overall degree of packing of the genome. In most organisms, the opened euchromatin is enriched in tri-methylation of lysine 4 and lysine 36 (H3K4me3 and H3K36me3),
two concomitant modifications associated with active transcription [2]. In contrast, histones present in the compacted heterochromatin are enriched in either tri-methylated H3K27 (H3K27me3) or tri-methylated H3K9 (H3K9me3) [3]. The compact architecture of heterochromatin limits the accessibility of the transcription machinery to the embedded DNA, thereby silencing gene expression. Repeat-rich genomic regions enriched in H3K9me3 are referred to as ‘constitutive’ heterochromatin because the subsequent silencing may be constant across development [4]. In contrast, the ‘facultative’ heterochromatin corresponds to the deposition of H3K27me3 on gene-rich regions, whose silencing is transient and dynamic across developmental processes, allowing cell type-specific differentiation and rapid adaptation of gene expression [5].

H3K9me3 is catalyzed by the SET-domain SU(VAR)3–9 enzymes [6–9] and bound by the chromodomain of Heterochromatin Protein 1 (HP1) [10–12]. Subsequent HP1 oligomerization, which results in nucleosome binding [13] and phase separation [14–16], further enhances chromatin compaction and spreads this structure over large genomic compartments. When present, high levels of DNA cytosine methylation are found in H3K9me3-enriched regions. This modification is associated with genome stability by preventing either expression of transposable elements or mitotic recombination. As demonstrated in mouse and drosophila models, when H3K9me3 enzymatic activity is absent or reduced, the embryos die because of diverse developmental defects [17]. Accordingly, H3K9me3-dependent heterochromatin is considered as a barrier to cell fate changes, by preventing some transcription factors to bind DNA [18].

First identified in Drosophila, the conserved Polycomb group (Pc-G) protein complexes were shown to be both writers (Polycomb Repressive Complex 2, PRC2) and readers (Polycomb Repressive Complex 1, PRC1) of H3K27me3 [19–21]. The catalytic subunit of PRC2 is made of the SET-domain protein enhancer of zest homolog 2 (EZH2), which tri-methylates H3K27. The H3K27me3-polycomb modification is instrumental for maintaining transcription repression established during early development and, therefore, has been linked to biological functions related to cell fate, as diverse as X-inactivation [22] and hematopoiesis [23]. Conversely, when PCR2 functions are impaired, development of plants [24], insects [25] and mammals [26, 27] is disrupted, presumably because lineage-specific programs are no longer accurate. Mutations affecting EZH2 lead to cell proliferation in cancer [13], while others are associated with congenital disorders such as the Weaver syndrome [28] or Ataxia-telangiectasia disease [29].

In animals and flowering plants, H3K9me3 and H3K27me3 marks are almost always mutually exclusive [30], but the molecular basis for this exclusion is unknown. To further investigate the genomic distribution and functional interplay of these two marks, filamentous fungi of the Pezizomycotina clade are a valuable alternative to higher eukaryotic models. Unlike ascomycete yeasts [8], most of them display activity of both H3K9 and H3K27 histone methyltransferase enzymes [31]. The role of the H3K27 histone methyltransferase was investigated in the model fungus Neurospora crassa [32–40], the human pathogen Cryptococcus neoformans [41], the plant endophyte Epichloë festucae [42] and the plant pathogens Fusarium graminearum [43], Fusarium fujikuroi [44], Zymoseptoria tritici [45] and Magnaporthe oryzae [46]. As for metazoa and plants, H3K27me3 is essential for proper development of F. graminearum [43], F. fujikuroi [44] and M. oryzae [46] (including growth, sporulation, fertility, pathogenicity, and expression of mycotoxins, pigments, and other secondary metabolites). Paradoxically, deletion of the PRC2 enzymatic subunit of N. crassa, C. neoformans or Z. tritici does not result in obvious development defects. However, H3K9me3 methylation is required for N. crassa normal growth and full fertility [47, 48] and in the wheat pathogen Z. tritici, lack of H3K9me3 induces genomic rearrangements. H3K9me3 and H3K27me3 modifications do not overlap in these fungal genomes, a pattern also found in animals and plants. Interestingly, depletion of H3K9me3 in either N. crassa or Z. tritici leads to a massive redistribution of H3K27me3 in genomic compartments that were previously embedded in constitutive H3K9me3 heterochromatin [35, 45]. Altogether, these reports present a contrasted picture, suggesting that connections between heterochromatin features and regulation of gene expression need to be further investigated in fungi.

In this study, we used the model system P. anserina to establish the genome-wide distribution of H3K4me3, H3K9me3 and H3K27me3. Most of the H3K9me3 is found in repeat-rich regions also enriched in H3K27me3, while both the H3K9me3 and H3K27me3 are co-exclusive with the H3K4me3 marks. We demonstrated that absence of PaKmt1, the H3K9me3 methyltransferase, resulted in loss of H3K9me3 and in significant reduction of H3K27me3, but had a limited impact on P. anserina growth, differentiation and sexual reproduction. Likewise, removal of PaHP1 diminished H3K9me3 and H3K27me3 modifications, with impact on P. anserina physiology similar to that of the PaKmt1 mutation. Conversely, we observed that absence of PaKmt6, the H3K27me3 methyltransferase, resulted in drastic depletion of both H3K27me3 and H3K9me3 and caused severe defects in most aspects of the life cycle including growth,
differentiation processes and sexual reproduction. These findings point towards an interdependence of the two types of heterochromatin within the repeated regions of the genome as well as a conserved function of Polycomb silencing in *P. anserina* development.

**Results**

Chromatin-modifying enzymes are present in the *P. anserina* genome and expressed throughout its life cycle

*P. anserina* genome contains a single gene (*Pa_6_990*) encoding a putative H3K9me3 methyltransferase, named PaKmt1 (342 aa) and a single gene (*Pa_1_6940*) encoding a putative H3K27me3 methyltransferase, named PaKmt6 (1090 aa). Both of these highly conserved proteins show a canonical SET domain typical of *bona fide* histone methyltransferases (Fig. 1a and Additional file 1: Fig. S1A–C). PaKmt1 displays pre- and post-SET domains (PS50867 and PS50868) (Additional file 1: Fig. S1D). PaKmt6 contains a CXC domain (PS51633), a cysteine-rich motif frequently found in polycomb group (Pc-G) proteins, but no chromodomain. PaKmt1 and PaKmt6 cluster in two different clades of histone methyltransferase (Fig. 1b). Their evolutionary history is canonical since the topology of the two branches of the tree is consistent with the species tree.

RNA-seq experiments detected *PaKmt6* transcripts of predicted structure in both vegetative mycelium and fruiting bodies [49]. However, while annotation of the *PaKmt1* gene predicted that its ORF was made of two exons, RNA-seq experiments showed ambiguous profiles for *PaKmt1* transcripts [49]. Dedicated reverse-transcription polymerase chain reaction (RT-PCR) experiments revealed two distinct transcripts (Additional file 2: Fig. S2). From 1-day-old mycelium cDNA, we amplified a single 1108-bp fragment corresponding to the *PaKmt1* spliced transcript. However, from either 4-day-old mycelium RNA extracts or fruiting body RNA extracts (2-day and 4-day post-fertilization fruiting bodies), we amplified an additional 1170-bp fragment, which corresponds to a *PaKmt1* unspliced transcript. The translation of the 1170-bp fragment results in a truncated protein of 76 amino acids lacking the conserved functional motifs (pre-SET and SET domains). This observation suggests that, in addition to the functional full length PaKmt1 H3K9 methyltransferase, *P. anserina* may produce a truncated version of this protein at specific developmental stages of its life cycle.

**Genome-wide distribution of H3K4me3, H3K9me3 and H3K27me3 histone modifications in the *P. anserina* genome**

Using western-blotting, we first showed that H3K4me3, H3K9me3 and H3K27me3 marks were present in the *P. anserina* genome (Fig. 1c, WT). Then, to further characterize the genomic patterns of these three histone modifications, we performed ChIP-seq experiments on chromatin samples collected from vegetative mycelia grown 60 h at 27 °C. For mapping genome-wide patterns of histone modifications, we used two distinct methodologies (i) domainogram analysis [50] based on enrichment within multiscale windows and (ii) peak calling analysis using the MACS2 algorithm (Fig. 2a and Additional file 19: Table S1). Clustering analysis of histone marks in the wild-type background established that the biological replicates clustered together with high correlation coefficients (Additional file 3: Fig. S3A).

The H3K4me3 modification (Fig. 2a, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1, Additional file 20: Table S2 and Additional file 21: Table S3) covered nearly 11% of the *P. anserina* genome, with 3050 detected peaks. This mark appeared to be present in blocks of approximately 1.3 kb (Fig. 2b) in gene-rich regions, mostly located in coding sequences (CDS, Figs. 2c and 3a and Additional file 20: Table S2) and in 5′ UTR (Figs. 2c and 3a). As in other eukaryotes, H3K4me3 showed a positive correlation with active transcriptional activity (Fig. 3b). On rare instances, when H3K4me3 peaks appeared to be localized in repeats (Figs. 2c and 3a, Additional file 5: Fig. S5A, B and Additional file 21: Table S3), it mainly corresponded to transposable element (TE) relics or transposase genes inserted into coding sequences (49 out of the 63 H3K4me3 peaks found in repeats).

Conversely, H3K9me3 (Fig. 2a, Additional file 3: Fig. S3B–D and Additional file 19: Table S1, Additional file 20: Table S2 and Additional file 21: Table S3) was the least abundant mark found in *P. anserina* genome (1090 detected peaks, 2.94% of the genome), forming approximately 0.9-kb-long blocks (Fig. 2b). This mark is mostly found at repeat-rich regions, i.e., TE (728 peaks, Additional file 21: Table S3), centromeric regions and telomeric regions (Figs. 2c and 3a), so that the abundance of H3K9me3 modification in the *P. anserina* genome reflected its repeat content, omitting the rDNA cluster [51]. In accordance with its genomic distribution, the H3K9me3 modification showed a negative correlation with active transcriptional activity (Fig. 3b). Annotated TE families (i.e., Copia, Gypsy TEs, Tc1 mariner TEs and soloLTR relics) were marked with H3K9me3, and transcriptionally silenced, with the exception of MITEs (Additional file 5: Fig. S5A–C, Additional file 6: Fig. S6 and Additional file 21: Table S3). In the *P. anserina* genome, nearly all copies of TEs differ by polymorphisms generated by the repeat-induced point mutation (RIP) mechanism [51]. Originally described in *N. crassa* [52], RIP is a mutagenic process that occurs
during the sexual dikaryotic stage of many filamentous fungi (for review see [53]). In addition of C–G to T–A transition accumulations, the RIPed repeats are embedded in H3K9me3-rich heterochromatin, which leads to transcriptional gene silencing. MITEs are an exception to this pattern because their short length (< 500 pb, Additional file 21: Table S3) makes them less prone to RIP.

The second most common mark found in the P. anserina genome in terms of peak numbers (1941 detected peaks, Additional file 19: Table S1), H3K27me3 nevertheless covered 18% of the genome (Fig. 2a, Additional
file 3: Fig. S3B–D. Additional file 4: Fig. S4 and Additional file 21: Table S3), showing large blocks approximately 3.5 kb long (Fig. 2b). This mark was found enriched at sub-telomeric regions (Figs. 2c and 3a, Additional file 3: Fig. S3C, D and Additional file 4: Fig. S4), but also on about one third of the annotated coding sequences (2992 detected peaks, Additional file 20: Table S2). Most of these H3K27me3-marked coding sequences were likely not expressed during the vegetative growth phase (Fig. 3b). Importantly, H3K27me3 modification was also found on all TE families, including members of MITEs, soloLTR relics and unclassified TEs (Additional file 5: Fig. S5A, B, Additional file 6: Fig. S6 and Additional file 21: Table S3).

Zooming in on the P. anserina genomic patterns, it appeared that distribution of H3K4me3 was found to be mutually exclusive with the distributions of both H3K9me3 and H3K27me3 (Fig. 3c, Additional file 3: Fig. S3C, D, and Additional file 4: Fig. S4). This observation was further confirmed by both the domainograms, where domains with most significant enrichment of the H3K9me3 and H3K27me3 marks, but not the H3K4me3 mark, showed a large degree of overlap (Fig. 2a and Additional file 20: Table S2) and the Spearman correlation test of individual bins, where the H3K9me3 and H3K27me3 IPs were grouped in the same cluster and were negatively correlated with the H3K4me3 cluster (Additional file 3: Fig. S3A). On the same note, we noticed that
chromosome five is significantly enriched in H3K9me3 and depleted in H3K4me3 (chi² test, \( p \)-value < \( 2.2 \times 10^{-16} \)), which is in line with its high TE and repeat content. However, we did find a few genes that harbored both H3K4me3 and H3K27me3 marks in these experimental conditions (Additional file 20: Table S2). Remarkably, this set was significantly enriched (\( p \)-value < 0.001) in genes encoding proteins involved in non-allelic heterokaryon incompatibility (HI), transcription and chromatin remodeling (Additional file 20: Table S2). More importantly, patterns of H3K27me3 and H3K9me3 modifications, determined either as peaks or by significant genomic coverage, were often found overlapping in gene-poor compartments scattered across the genome (Fig. 3c,
Additional file 3: Fig. S3C, D, Additional file 4: Fig. S4 and Additional file 6: Fig. S6), especially on repeats, which included most of the TE families (Fig. 3a: cluster_1, and Additional file 21: Table S3). Indeed, we identified 954 H3K27me3 and H3K9me3 common peaks, representing 90% of the H3K9me3 peaks (Additional file 19: Table S1 and Additional file 21: Table S3). This observation was consistent with the Spearman clustering analysis showing that H3K9me3 and H3K27me3 IP samples belong to the same cluster (Additional file 3: Fig. S3A).

PaKmt1 and PaKmt6 are histone methyltransferases and deletion of their genes impacts both H3K9me3 and H3K27me3 distribution

To determine the histone methyltransferase(s) required for the deposition of the heterochromatin marks, we constructed ΔPaKmt1 and the ΔPaKmt6 deletion mutants (Additional file 8: Fig. S8). Double ΔPaKmt1ΔPaKmt6 mutant strains were obtained by crossing the corresponding single mutants. All three mutants were viable, demonstrating that neither PaKmt1 nor PaKmt6 are essential genes. Western-blotting showed that H3K9me3 was almost absent in proteins extracted from the ΔPaKmt1 mutant (Fig. 1c), whereas the H3K27me3 was absent from the ΔPaKmt6 sample. The faint remaining signal seen in the ΔPaKmt1 sample could be due to the cross-reactivity between the anti-H3K9me3 antibody and the H3K27me3 modification, which was evaluated at 3% of the specific anti-H3K9me3/H3K9me3 immunogenic reaction (Additional file 7: Fig. S7). Importantly, neither mark was present in the double ΔPaKmt1ΔPaKmt6 mutant, showing that no additional H3K9 or H3K27 methylation exists in *P. anserina*. As expected, H3K4me3 was detected in all samples. We concluded that PaKmt1 is required for H3K9me3, while PaKmt6 is required for H3K27me3.

ChIP-seq experiments performed with either ΔPaKmt1 mutants or ΔPaKmt6 mutants showed that the H3K4me3 patterns remained unchanged in all the mutant genetic contexts, suggesting that loss of H3K27me3 or H3K9me3 has no impact on the H3K4 methyltransferase activity (Fig. 4a, b, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1 and Additional file 20: Table S2). According to domainogram analysis, ChIP-seq experiments performed in the ΔPaKmt1 background revealed a nearly comparable span of H3K9me3 domains, as compared to WT (Fig. 4a, b, Additional file 3: Fig. S3B–D, Additional file 20: Table S2), while MACS2 analysis did not detect any significant peak enrichment (Fig. 4a, b, Additional file 19: Table S1, Additional file 20: Table S2 and Additional file 21: Table S3). First, the average length of the H3K27me3 blocks was reduced by two thirds (Fig. 4b). Second, about 30% of the H3K27me3 peaks were missing, especially those located in the gene-rich blocks (Fig. 4a, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 20: Table S2). Third, some the remaining H3K27me3 peaks were re-localized within TEs, where they replaced the missing H3K9me3 marks (Additional file 21: Table S3).

ChIP-seq experiments performed using the ΔPaKmt6 mutant strains revealed that this deletion drastically reduced the overall H3K27me3 content (significant genome coverage dropped from 18.95 to 0.04%, (Fig. 4a, b, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1, Additional file 20: Table S2 and Additional file 21: Table S3). To our surprise, we noticed that the loss of PaKmt6 severely impacted the amount of H3K9me3 marks, since in the ΔPaKmt6 background, H3K9me3 genome coverage dropped from 2.94

(See figure on next page.)
to 0.18% (Fig. 4a, b, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1 and Additional file 21: Table S3). This means that the diminution in H3K9me3 peaks was as strong as the diminution in H3K27me3 peaks in this mutant background. Nevertheless, the remaining H3K9me3 peaks in the ΔPaKmt6 background were not displaced, nor were the missing H3K9me3 blocks replaced by H3K27me3 ones.
Loss of H3K27me3 in ΔPaKmt6 background modifies gene expression

To assay the impact of H3K27me3 and/or H3K9me3 on gene expression, we performed RT-qPCR experiments on vegetative mycelia grown 60 h at 27 °C, for either the wild-type genotype (5 independent samples) or mutant genotypes (ΔPaKmt1 and ΔPaKmt6, 5 independent samples each). We first focused on six genes that harbor H3K27me3 marks in wild-type background (Fig. 5a, Additional file 10: Fig. S10 and Additional file 20: Table S2). In the ΔPaKmt6 background (Fig. 5a, Additional file 20: Table S2), all of them lost their H3K27me3 marks and were over-expressed (fold change ≥ 2). This result is consistent with H3K27me3 being involved in regulation of gene expression during development programs since Pa_1_6263, which showed the most dramatic up-regulation effect (fold change > 25, p-value = 0.001 Fig. 5a), is normally expressed only during the sexual phase [49]. Expression of genes encoding secondary metabolites (SM) is known to be sensitive to the sexual phase. Indeed, when we measured expression of two such genes (Pa_5_10 and Pa_6_7370) in the ΔPaKmt6 background, we found them both up-regulated (Fig. 5a, Additional file 20: Table S2). Notably, loss of PaKmt1 had no significant impact (fold change ≤ 2) on the expression of this set of genes, even if some of the H3K27me3 marks were lost in this mutant background (Additional file 10: Fig. S10 and Additional file 20: Table S2).

We also tested the expression of three TE families (Fig. 5b) in the wild-type background. The Copia_Ty1_nephelobates and the Tc1_mariner-like_pelobates are heavily RIPed and harbor both H3K9me3 and H3K27me3 marks (Additional file 21: Table S3), and ΔPaKmt6 has stronger impact on vegetative growth than loss of PaKmt1 and results in male gamete over-production

We then assessed the impact of loss of either H3K27me3 or H3K9me3, or both, on the P. anserina life cycle. Pigmentation, branching and the overall aspect of ΔPaKmt1 mycelia were similar to those of wild-type strains, except for reduced production of aerial hyphae (Fig. 5c) and slight growth defects (Fig. 5d and Table 1), including an unusual failure to resume growth after it has been stopped for a few days (Additional file 11: Fig. S11A). We ruled out the possibility that these phenotypes result from activation of Crippled Growth (CG), an epigenetically triggered cell degeneration [57], by showing that the ΔPaKmt1 mutation did not promote CG...
(Additional file 11: Fig. S11B) but behaved like the wild-type strain in that respect. This suggests that the reduced capability to resume growth may be indicative of less plasticity to perform an adaptive developmental program under changing environmental conditions.

Pinkish pigmentation, crooked branching pattern and the overall aspects of ΔPaKmt6 mycelia were clearly different from those of wild-type strains (Fig. 5c). First of all, ΔPaKmt6 mutant growth rate was 43% lower than that of wild-type strains during the first 60 h of growth.
on M2 at 27 °C (Fig. 5d). After this time, progression of the ΔPaKmt6 colony margins became so erratic and irregular that the standard assay consisting in thallus radius measurements was no longer accurate. Indeed, ΔPaKmt6 mycelia showed “stop-restart” growth resulting in patchy mycelium sectors, which literally sprouted out of the colony margins like cauliflower inflorescences (Fig. 5c). These outgrowths are manifest in the increasingly large error bars along the corresponding growth curve (Fig. 5d). However, we were able to grow the ΔPaKmt6 mutants in race tubes to determine that this mutation had no effect on senescence (Additional file 12: Fig. S12).

In addition to growth defects, the ΔPaKmt6 mutant mycelium showed a powdery phenotype. At the microscopic scale, we observed that this corresponds to an over-production of male gametes (spermatia) (app. 130 times more than in the wild-type strains, Fig. 6a). We tested the ability of these ΔPaKmt6 male gametes to fertilize wild-type female gametes (ascogonia). To do so, we sprayed a defined quantity of either mutant or wild-type male gametes onto wild-type female gametes of compatible mating type. Given that one fruiting body originates from one fertilization event, we established that the ΔPaKmt6 male gametes were as efficient as the wild-type ones, since a similar number of fruiting bodies was obtained in each condition (4 independent experiments). This functional test indicates that the ΔPaKmt6 mutant male gamete over-production had no obvious impact on their biological properties.

Finally, when we constructed the ΔPaKmt1ΔPaKmt6 double mutant, the phenotypes generated by the ΔPaKmt6 null allele were epistatic upon those generated by the ΔPaKmt1 allele.

Table 1 Mycelium growth at different temperatures

|                  | 11 °C | 27 °C | 35 °C |
|------------------|-------|-------|-------|
| **WT**           | 100   | 100   | 100   |
| ΔPaKmt1          | 72.4  | 94.2  | 91.8  |
| ΔPaKmt1 PaKmt1+  | 104   | 100   | 99.7  |
| ΔPaKmt6          | ND    | Irregular slow growth | Irregular slow growth |
| ΔPaKmt6 PaKmt6+  | 98.3  | 99.0  | 99.3  |
| ΔPaKmt1 ΔPaKmt6  | ND    | ΔPaKmt6 aggravated | ΔPaKmt6 |
| ΔPaHP1           | 65.7  | 76.2  | 74.9  |
| ΔPaHP1 PaHP1+    | 99.1  | 101   | 98.5  |
| ΔPaKmt1 ΔPaHP1   | 60.7  | 89.9  | 80.7  |
| ΔPaKmt1 ΔPaKmt6  | ND    | ΔPaKmt6 aggravated | ΔPaKmt6 |
| ΔPaKmt1 ΔPaHP1 ΔPaKmt6 | ND | ΔPaKmt6 aggravated | ΔPaKmt6 |

Relative growth rates (WT %) at optimal temperature (27 °C) and sub-optimal temperature (11 °C and 35 °C) on M2 minimal medium. ΔPaKmt6 mutant strains display irregular and slow growth at 27 °C and 35 °C, which cannot be quantified. At 11 °C, growth is too weak to be assayed (ND). At 27 °C, double and triple mutants containing the ΔPaKmt6 allele display aggravated ΔPaKmt6-like growth defects.

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Fig. 6 Sexual features of the ΔPaKmt6 strain. a Spermatium production in wild-type and mutant backgrounds. See “Methods” section for details. b ΔPaKmt6 homozygote crosses result in non-homogeneous distribution of late appearing fruiting bodies showing multiple morphological defects. These crippled structures could be due to lack of neck, lack of ostiols, lack of setae, or non-canonical pear-shape envelopes, supernumerary necks, misdirected orientation, upside down growth in agar, fusions of fruiting bodies, etc.
The ΔPaKmt6 mutant formed crippled fruiting bodies yielding a reduced number of ascospores

We then investigated the ability of the histone methyltransferase mutant strains to perform sexual reproduction. Sexual development and ascospore production of crosses involving two ΔPaKmt1 parental strains were indistinguishable from those of wild-type strains. By contrast, when we performed homozygous ΔPaKmt6 crosses, fruiting bodies were formed, which means that fertilization occurred, but their developmental time frame was delayed. They also displayed a large palette of morphological defects (e.g., lack of necks or extra necks, lack of ostioles, upward/downward mis-orientation, development into agar, fusion) and their ascospore production was significantly reduced (Fig. 6b).

Heterozygous crosses showed that when the ΔPaKmt6 null allele was present in the female gamete genome and the wild-type allele was present in the male gamete genome, the fruiting bodies were crippled and the ascospore production reduced, as in the homozygous ΔPaKmt6 crosses (Additional file 13: Fig. S13A). Conversely, heterozygous crosses involving female wild-type strains and male mutant strains produced well-formed and fully fertile fruiting bodies, indicating a ΔPaKmt6 maternal effect.

To determine whether the ΔPaKmt6 mutant’s reduced fertility resulted from either a female body envelope defect or a zygotic tissue defect, we set up tricaryon crosses involving the Δmat strain [58, 59]. The Δmat strain lacks the genes required for fertilization, so it does not participate either as male or female in the sexual reproduction. However, the Δmat mycelium is able to provide maternal hyphae required to build fruiting bodies. Consequently, the Δmat strain can complement mutants defective for fruiting body formation but not for zygotic tissue defect. In this study, we observed that the Δmat; ΔPaKmt6 mat+; ΔPaKmt6 mat– tricaryon yielded few but fully matured and well-formed fruiting bodies that produced normal ascospores (Additional file 13: Fig. S13B). This experiment demonstrates that ΔPaKmt6 mutation compromised the fruiting body envelope formation but had no impact on zygotic development. This result is in line with the observed ΔPaKmt6 maternal effect (see the above paragraph).

Finally, crosses involving the double ΔPaKmt1ΔPaKmt6 mutant strains displayed a typical ΔPaKmt6 phenotype with respect to the perithecium number, morphology and ascospore production, demonstrating that the ΔPaKmt6 null allele was epistatic. In addition, one third of the asci recovered from homozygous ΔPaKmt1ΔPaKmt6 crosses showed fewer pigmented ascospores than asci from wild-type crosses (with a color panel spanning from white to green). In general, deficit in pigmentation is indicative of incomplete maturation during the ascospore formation process [60].

The PaKmt6 null allele reduces the ascospore germination efficiency

Homokaryotic ascospores originating from homozygous ΔPaKmt1 crosses germinated like those of wild-type crosses (>96% germination rate, N=100, Table 2). However, about two thirds of normal looking ascospores produced by the homozygous ΔPaKmt6 crosses did not germinate (3 independent experiments, N=100 for each experiment, Table 2). The reduced germination efficiency was further enhanced in ΔPaKmt1ΔPaKmt6 double mutants (Table 2). On germination medium, wild-type ascospore germination occurs at a germ pore located at the anterior side of the ascospore, by the extrusion of a spherical structure. This transient structure, called a germination peg, gives rise to a polarized hypha that develops into mycelium. In the ΔPaKmt6 mutants, the ascospore germination process was stopped at an early stage, since germination pegs were not formed. However, the ΔPaKmt6 defect was ascospore autonomous since ascospores from wild-type progeny obtained in WT × ΔPaKmt6 crosses germinated normally, whereas those from ΔPaKmt6 progeny did not. In addition, PaKmt6+/ΔPaKmt6 dikaryotic ascospores had a wild-type germination rate showing that the ΔPaKmt6 defect was recessive (2 independent experiments, N=80 dikaryotic ascospores).

It was previously shown that unmelanized ascospores carrying the pks1-193 mutant allele germinate spontaneously at high frequency (over 95%), even on non-inducing media [60, 61]. Crosses involving pks1-193 ΔPaKmt6 mat- and pks1+ ΔPaKmt6 mat+ strains yielded asci made of two unmelanized (pks1-193 ΔPaKmt6) and two melanized (pks1+ ΔPaKmt6) ascospores. As expected,

### Table 2  Ascospore germination efficiency

|                | Germination efficiency |
|----------------|-----------------------|
| WT             | 92.8±4.26             |
| ΔPaKmt1        | WT                    |
| ΔPaKmt6        | 293±0.44              |
| ΔPaKmt6 ΔPaKmt6+ | WT                   |
| ΔPaKmt1 ΔPaKmt6 | 11.4±3.54            |
| ΔPahP1         | WT                    |
| ΔPaKmt1 ΔPahP1 | WT                    |
| ΔPahP1 ΔPaKmt6 | 25.2±2.31             |
| ΔPaKmt1 ΔPahP1 ΔPaKmt6 | 21.1±11.2 |

Number of germinated ascospores out of 100 issued from homozygote ΔPaKmt6 crosses and transferred to appropriate germination medium. WT means wild-type germination efficiency (>95%).
about one third of the ΔPaKmt6 melanized ascospores germinated on inducing medium. By contrast, almost all of the ΔPaKmt6 unmelanized spores germinated, even on non-inducing medium. This result shows that the absence of melanin suppressed the ΔPaKmt6 ascospore germination defect, as found for ΔPaPls1 and ΔPaNox2 mutants [60].

Loss of PaHP1 leads to overall reduction and partial relocation of H3K9me3 modifications

We searched the *P. anserina* genome for a homolog of heterochromatin protein 1 (HP1, [62]). This protein is known not only to bind the H3K9me3 marks of the constitutive heterochromatin, but also to perform a wide variety of functions ranging from cohesion of sister chromatids for the maintenance of telomeres, DNA repair and replication, cell cycle control and even RNA splicing [63]. We identified one gene (*Pa_4_7200*) encoding the protein PaHP1 (252 aa, 57% identity, e-value = 4e⁻⁷⁸) (Additional file 14: Fig. S14A). It displays conserved chromo (PS00598) and chromo-shadow (PS50013) domains (Additional file 14: Fig. S14A, B and Additional file 15: Fig. S15A). By self-aggregation, chromo-shadow domain-containing proteins can bring together nucleosomes and, thus, condense the corresponding chromatin region [13].

RNA-seq data showed that the *PaHP1* gene is expressed in both vegetative mycelium and fruiting body tissues [49]. We also explored *PaHP1*-GFP cellular localization in 2-day-old growing mycelium. In wild-type *PaHP1*-GFP-expressing strains, the fluorescence signal was nuclear and punctate (Fig. 7a). However, when the *PaHP1*-GFP allele was expressed in a Δ*PaKmt1* background, the GFP fluorescence was still nuclear but no longer punctate (Fig. 7a), as already observed in *N. crassa* [48]. On the contrary, when expressed in a Δ*PaKmt6* background, the *PaHP1*-GFP signal appeared punctate as in wild-type nuclei.

To explore potential role(s) of PaHP1, we deleted the corresponding *Pa_4_7200* gene (Additional file 15: Fig. S15B, C). ChIP-seq experiments performed in the Δ*PaHP1* background showed that the H3K4me3 pattern was similar to that of wild-type strains (Fig. 7b, c, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1 and Additional file 20: Table S2), while both H3K9me3 and H3K27me3 marks were reduced. The H3K9me3 genome coverage was reduced by half (Fig. 7c, Additional file 3: Fig. S3B–D, Additional file 4: Fig. S4 and Additional file 19: Table S1, Additional file 20: Table S2 and Additional file 21: Table S3), while the H3K27me3 loss of coverage was similar to that of Δ*PaKmt1* strains. Interestingly, on TEs, we detected replacement of some of the missing H3K27me3 by H3K9me3 modifications. This was especially noticeable for the soloLTR relics, although this was also the case for all the annotated TE families, albeit to a lesser extent (Additional file 21: Table S3). In addition, we observed both focalized spreads of H3K9me3 marks on TEs (Additional file 3: Fig. S3C, D, Additional file 21: Table S3) and massive H3K9 methylation on the rDNA cluster (Additional file 16: Fig. S16). These observations may account for the H3K9me3 block widening in the Δ*PaKmt1* genetic context. Altogether these results suggest that, in the absence of the recruitment platform mediated by the binding of PaHP1 to the constitutive heterochromatin, the compaction may be partly relaxed and the histone modifications lost, but also that the boundaries between heterochromatin and euchromatin may be less clear-cut. However, in this mutant context, the H3K9me3 reduction has no impact on gene expression (Additional file 17: Fig. S17A).

Loss of PaHP1 has no more impact than that of PaKmt1 on the *P. anserina* life cycle

Throughout all the *P. anserina* life cycle, the Δ*PaHP1* mutants behave similarly to the Δ*PaKmt1* ones (Table 1, Additional file 17: Fig. S17B–D). Importantly, the double Δ*PaKmt1ΔPaHP1* mutants did not show any additional or more severe phenotypes (Table 1, Additional file 17: Fig. S17A). When the Δ*PaHP1* allele was associated with the Δ*PaKmt6* one, all the phenotypes displayed by the single Δ*PaKmt6* mutants were epistatic upon those of the Δ*PaHP1* mutants. The only exception is related to the germination efficiency of the triple Δ*PaKmt1ΔPaHP1ΔPaKmt6*, which was similar to that of the double Δ*PaHP1ΔPaKmt6* rather than the double Δ*PaKmt1ΔPaKmt6* mutant (Table 2). This may be due to a slightly reduced pigmentation of the ascospores produced by the homozygous Δ*PaKmt1ΔPaHP1ΔPaKmt6* crosses. Indeed, even if only the darker wild-type looking ones were used in this test, slightly reduced pigmentation facilitates germination.

Discussion

In eukaryotes, the deposition of histone modifications is essential to chromatin features, which impact cell fate through regulation of gene expression. In this study, we aimed at understanding how heterochromatic territories are assembled within the *P. anserina* genome and how this genome organization influences the *P. anserina* life cycle.

H3K27me3 and H3K9me3 modifications are not mutually exclusive

To get the first hints of *P. anserina*’s genome organization, we took advantage of ChIP-seq experiments to
uncover genome-wide chromatin landscapes in a wild-type background. Our results highlighted two uncommon features of both the constitutive and the facultative heterochromatin. Firstly, in most animals and plants, the H3K9me3 modification covers fairly large domains, while the H3K27me3 modification forms shorter ones. But for the *P. anserina* genome, we observed the opposite, since H3K27me3 blocks were on average three times longer than H3K9me3 blocks. Second, if as expected the H3K9me3 modification was restricted to repeat-rich regions, mostly located near telomeres and centromeres [64], the H3K27me3 modification encompasses both annotated CDS and H3K9me3 marked repeats. Co-occurrence of H3K9me3 and H3K27me3 at same genomic loci is of particular interest because these two heterochromatic marks were long considered mutually

Fig. 7 Chromatin modifications in the ΔPaHP1 strain. **a** Subcellular localization of PaHP1. Fluorescence microscopy pictures show PaHP1-GFP chimeric protein during vegetative growth in mycelium. Nuclei are marked using the PaH1-mCherry allele that encodes histone H1 protein tagged with mCherry fluorophore (red). Indicated scale is the same for all the pictures (5 µm). PaHP1-GFP displays nuclear localization and forms foci that likely correspond to heterochromatin domains (arrows). This specific localization is disturbed only in ΔPaKmt1 mutants. **b** Visualization of histone mark localization across the genome in the ΔPaHP1 strain. Domainograms (top) show significance of enrichment of H3K4me3, H3K9me3 and H3K27me3 marks in windows of varying size, according to the indicated p-value (right). ChIP-seq patterns (bottom) display histone modification coverage and MACS2 detected peaks. Both patterns are represented with the same scale on chromosomes one and five of *P. anserina*. Both centromeres are depicted (pink). The "Mat region" (orange) corresponds to the 800 kbp region devoid of recombination and containing the mating-type locus [93]. Repeated sequences are depicted in light blue. **c** Peak size distribution in the ΔPaHP1 mutant strain. Box plots showing sizes in base pair of the MACS2-predicted peak of H3K4, H3K9 and H3K27 trimethylation are shown in green, red and blue, respectively. The box is delimited, bottom to top, by the first quartile, the median and the third quartile. Numbers on top of each box represents the number of peaks detected. Outliers are not shown.
exclusive in flowering plants, mammals, and fungi [30, 65]. Yet recently, this dogma has been challenged. Mass spectrometry experiments demonstrated that H3K27me3 and H3K9me3 can coexist within the same histone octamer in embryonic stem cells [35, 66]. Co-occurrence of H3K9me3 and H3K27me3 was found at TE loci of the ciliate Paramecium tetraurelia [67], the nematode Caenorhabditis elegans [68] and the bryophyte Marchantia polymorpha [69]. In mammals, these two marks were found together in rare instances, along with DNA methylation, either at a subset of developmentally regulated genes in mouse extra-embryonic early embryo lineages [70] or in some human cancer cells [71–73]. Likewise, such H3K9me3 and H3K27me3 overlap, exclusively associated with DNA methylation, has also been described for A. thaliana TE loci [74].

In the basidiomycete yeast C. neoformans, removal of the Enhancer-of-zeste-like protein EZH eliminates H3K27me3, while loss of its reader, the chromodomain Ccc1 protein, redistributes H3K27me3 to new genomic locations that coincide with H3K9me2 heterochromatin domains [41]. Further disruption of C. neoformans H3K9 methyltransferase Clr4 causes loss of both H3K9me2 and redistributed H3K27me3 modifications [41]. These findings suggest that co-factors, such as readers, are responsible for anchoring the H3K27 methyltransferase to its specific facultative heterochromatin compartments, that otherwise can be recruited to H3K9me constitutive heterochromatin, resulting in H3K9me/H3K27me co-occurrence. P. anserina has neither Ccc1 homologs nor a PRC1 complex. If such recognition modules were lost, it would explain why PaKmt6 is recruited by both facultative and constitutive heterochromatin. Interestingly, H3K9me3 and H3K27me3 modifications also overlap in the sub-telomeric regions of some other filamentous fungi, such as Z. tritici, Fusarium oxysporum and Leptosphaeria maculans 'brassicae' [32, 33, 38, 75–77].

**Are bivalent-like genes present in fungi?**

In this study, we showed that H3K4me3 modification does not overlap with either of the H3 repressive marks at a genome-wide scale. However, we identified 50 genes (Additional file 20: Table S2) that make an exception to this rule by harboring both H3K4me3 and H3K27me3 marks. In mammals, this pattern is typical of bivalent genes found in totipotent embryonic stem cells [78], which play a central role in developmental and lineage specification processes. Unlike those of animals, fungal nuclei remain totipotent throughout the entire life cycle [79, 80]. Yet the molecular basis of this peculiar feature is not understood and it will be of interest to explore the function of the H3K4me3 and H3K27me3 dual-marked genes identified in the P. anserina genome (Additional file 20: Table S2). So far, we noted that a sizeable portion of them belongs to the *het* gene family. In filamentous fungi, the HET proteins trigger a cell death reaction as a consequence of a non-self-recognition process known as heterokaryon incompatibility [81]. It has been proposed that these *het* genes could be components of a fungal innate immune system. Since immune response must be quick and massive upon non-self-contacts, marking these genes with both repressive and activator modifications may be a way to fine tune their expression. Recently, a large number of genes associated with both H3K27me3 and H3K4me2 were identified in *L. maculans* 'brassicae' [77], indicating that bivalent domains occur in other fungi.

**PaKmt1 and PaKmt6 are not independent chromatin-modifying enzymes**

ChiP-seq experiments carried out on mutants allowed us to test for potential cross-talk between constitutive and facultative heterochromatin. Our data established that *P. anserina*’s constitutive and facultative heterochromatin are inter-dependent since removal of either PaKmt1 or PaKmt6 impacted both marks although not to the same extent. This is in contrast with the corresponding mutant phenotypes reported so far in *N. crassa* [33, 35], *Z. tritici* [45] and *C. neoformans* [41] where H3K9me3 content is not affected by loss of H3K27me3 and conversely (Table 3). In addition, H3K27me3 reduction in ΔPaKmt1 was not associated with any substantial genomic redistribution in contrast to what has been described in the corresponding *N. crassa* and *Z. tritici* mutants [33, 35, 45] (Table 3). Lack of PaHP1 further supported the observation that either type of heterochromatin requires the presence of the other, since in this mutant background, we saw fewer H3K9me3 and H3K27me3 marks but also reduced size of facultative heterochromatin domains. These concomitant observations suggest that initiation and propagation of the deposition of chromatin marks by the PRC2 complex are inter-dependent processes in *P. anserina*. Alternatively, if, as shown in *A. thaliana* for the FLC locus [89], that initiation and propagation of the deposition of chromatin marks by the PRC2 complex were independent in *P. anserina*, both of them would be impaired when the H3K9me3–heterochromatin pathway is damaged.

In general, two distinct enzymes catalyze methylation of either H3K9 or H3K27 residues. However, the ciliate *P. tetraurelia* is endowed with only one class of histone methyltransferase, the Enhancer-of-zeste-like protein Ezl1, which methylates both H3K9 and H3K27 residues [67]. This observation suggests that eukaryotes initially acquired a single, versatile, methyltransferase that duplicated and became sub-functionalized in the course of...
| Species                      | KMT1 mutant                                                                 | KMT6 mutant                                                                 | HP1 mutant                                                                 | References                                                                                     |
|------------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| *Podospora anserina*         | Growth and development: Reduced production of aerial hyphae, slight growth defect, delay in resuming vegetative growth | Very poor growth, less pigmentation, overproduction of conidia, misshapped fructification, defect in spore germination | Reduced production of aerial hyphae, slight growth defect | This study                                                                                     |
| Chromatin and regulation    | ChIP-seq: Loss of H3K9me3                                                  | Loss of H3K27me3 and H3K9me3                                              | Broader H3K9me3 domains                                                   |                                                                                               |
| *Neurospora crassa*          | Growth and development: Reduced growth, affected in sexual development    | None                                                                      | Reduced growth, affected in sexual development                            | Basenko et al. [34], Freitag et al. [48], Jamieson et al. [33, 35], Klocko et al. [36] |
| Chromatin and regulation    | ChIP-seq and Hi-C: Redefinition of H3K27me3, relocation and diminution       | Loss of H3K27me2/3                                                       | Affected in DNA methylation relocalization with H3K9me3                  |                                                                                               |
| *Fusarium verticilloides*    | Growth and development: Defects in conidiation, perithecium production and fungal virulence, overpigmentation | N/A                                                                      | Affected in DNA methylation relocalization with H3K9me3                  | Gu et al. [82]                                                                                 |
| Chromatin and regulation    | N/A                                                                        | N/A                                                                      | N/A                                                                       |                                                                                               |
| *Fusarium graminearum*       | N/A                                                                        | Growth defects, sterility, overpigmentation                               | Overpigmentation                                                          | Connolly et al. [43], Reyes-Dominguez et al. [83]                                           |
| Chromatin and regulation    | ChIP-seq: Loss of H3K27me3, constitutive expression of genes encoding mycotoxins, pigments-producing enzymes and other SM | Loss of H3K27me3                                                        | Enhanced activity of activity of biosynthetic cluster genes               |                                                                                               |
| *Epichloë festucae*          | Growth and development: Reduced vegetative growth, deregulation of symbiosis | Deregulation of symbiosis                                                | Deregulation of symbiosis                                                | Chujo and Scott [42], Chujo et al. [84]                                                    |
| Chromatin and regulation    | ChIP-qPCR: Derepression of symbiosis-specific alkaloid genes clusters    | Derepression of symbiosis-specific alkaloid genes clusters               | Derepression of symbiosis-specific alkaloid genes clusters               |                                                                                               |
| *Magnaporthe oryzae*         | Growth and development: Reduced vegetative growth, reduced virulence       | Reduced vegetative growth, severe reduction in conidiation, slight defects in appressorium formation, reduction in pathogenicity | N/A                                                                       | Pham et al. [46]                                                                               |
| Chromatin and regulation    | WB: Loss of H3K9me3                                                        | Loss of H3K27me3                                                        | N/A                                                                       |                                                                                               |
| Species             | KMT1 mutant                                      | KMT6 mutant                                      | HP1 mutant | References          |
|---------------------|--------------------------------------------------|--------------------------------------------------|------------|---------------------|
| Zymoseptoria tritici| Reduced vegetative growth                        | Reduced virulence                               | N/A        | Möller et al. [45]  |
| Growth and development | Reduced virulence                                | Loss of H3K9me3                                 | Loss of H3K27me3 | N/A                |
| Chromatin and regulation | Loss of H3K9me3                                 | TE activation and genomic rearrangements        | Increased stability of accessory chromosomes  |        |
| ChIP-seq            | Loss of H3K27me3還有 TE activation and genomic rearrangements | H3K27me3 relocation                           | Reduction of H3K27me3 |        |
| Aspergillus nidulans| No developmental phenotype                       | N/A                                              | No developmental phenotype                      | Reyes-Dominguez et al. [85] |
| Growth and development | Enhanced expression of a subset of secondary metabolite (SM) clusters | N/A                                              | Enhanced expression of a subset of secondary metabolite (SM) clusters |        |
| Chromatin and regulation | Enhanced expression of a subset of secondary metabolite (SM) clusters | N/A                                              | N/A        |        |
| Aspergillus fumigatus| Reduced vegetative growth delayed conidiation    | N/A                                              | Reduced vegetative growth delayed conidiation  | Palmer et al. [86] |
| Growth and development | N/A                                              | N/A                                              | N/A        |        |
| Chromatin and regulation | N/A                                              | N/A                                              | N/A        |        |
| Leptosphaeria maculans| Reduced vegetative growth                        | Reduced virulence                               | Reduced vegetative growth                       | Soyer et al. [87] |
| Growth and development | Reduced virulence                                | Up-regulation of pathogenicity-related genes    | Up-regulation of pathogenicity-related genes   |        |
| Chromatin and regulation | Up-regulation of pathogenicity-related genes     | N/A                                              | N/A        |        |
| Penicillium oxalicum| N/A                                              | N/A                                              | Reduced vegetative growth Delayed conidiation  | Zhang et al. [88] |
| Growth and development | N/A                                              | N/A                                              | N/A        |        |
| Chromatin and regulation | N/A                                              | N/A                                              | N/A        |        |

Growth and development: macroscopic major defects caused by the specified mutant allele. Chromatin and regulation: H3K9me3 or H3K27me3 modification status in the mutant background and major impact on regulation of gene expression. Techniques used to characterize chromatin status are listed. ChIP-seq: chromatin immune-precipitation followed by sequencing, Hi-C: genome-wide chromosome conformation capture followed by sequencing, WB: Western blot.
Conclusions

Recent studies (Table 3) suggest that the canonical definitions of facultative and constitutive heterochromatin may be challenged in fungi. Further contributing to this complex picture, in this study we showed that besides the H3K9me3 modifications that are likely installed by RIP [90], P. anserina repeats also harbor H3K27me3 modifications. It has already been postulated that the ancestral role of Enhancer-of-zeste enzymes, along with the SET-domain SU(VAR)3–9 enzymes, may be to silence TEs, through the concerted action of both H3K27me3 and H3K9me3 [67, 69, 91]. The H3K9me3/H3K27me3 overlap we have observed could therefore be a relic of the concerted action of these two classes of histone methyltransferases. Recently, a BAH-PHD protein mediating Polycomb silencing [40] and an ISWI chromatin remodeling factor were identified in N. crassa [92]. Interestingly, the phylogenetic distribution of this BAH-PHD protein suggests that it belongs to an ancestral Polycomb repression system which still exists in Fungi and early emerging eukaryotes but no longer in animals. The search for P. anserina PRC2 associated proteins will help us to understand Polycomb silencing in the absence of canonical PRC1, as well as functional cross-talk between facultative and constitutive heterochromatin.

Methods

Strains and culture conditions

The strains used in this study derived from the “S” wild-type strain of P. anserina that was used for sequencing [91]. Standard culture conditions, media and genetic methods for P. anserina have been described [94, 95]. Most recent protocols can be accessed at either [96] or http://podospora.i2bc.paris-saclay.fr. Mycelium growth is performed on M2 minimal medium, in which carbon is supplied as dextrin and nitrogen as urea. Ascospores germinate on a specific germination medium (G medium) containing ammonium acetate. The methods used for nucleic acid extraction and manipulation have been described [97, 98]. Transformations of P. anserina protoplasts were carried out as described previously [99].

Identification and deletions of the PaKmt1, PaKmt6 and PaHP1 genes

PaKmt1, PaKmt6 and PaHP1 genes were identified by searching the complete genome of P. anserina with tblastn [100], using the N. crassa proteins DIM-5 (NCU04402), SET-7 (NCU07496) and HP1 (NCU04017). We identified three genes: Pa_6_990, Pa_1_6940 and Pa_4_7200 renamed PaKmt1, PaKmt6 and PaHP1, respectively. To confirm gene annotation, PaKmt1 transcripts were amplified by RT-PCR experiments performed on total RNA extracted from growing mycelia.
(1-day- and 4-day-old mycelium) and developing fruiting bodies (2 and 4 days post-fertilization) using primers 5s-DIM5 and 3s-DIM5 (Additional file 23: Table S5). Functional annotation was performed using InterProScan analysis (http://www.ebi.ac.uk/interpro/search/sequence-search), Panther Classification System (http://www.pantherdb.org/panther), PFAM (http://pfam.xfam.org/) and Prosite (http://prosite.expasy.org/).

Deletions were performed on a Δmus51::bleoR strain lacking the mus-51 subunit of the complex involved in end-joining of DNA double strand breaks as described in [60]. In this strain, DNA integration mainly proceeds through homologous recombination. Replacement of the PaKmt1 wild-type allele with hygromycin-B resistance marker generated viable mutants carrying the ΔPaKmt1 null allele. Replacement of the PaKmt6 wild-type allele with a nourseothricin resistance marker generated viable but severely impaired mutants. Replacement of the PaHP1 wild-type allele with a hygromycin-B resistance marker also generated viable mutants carrying the ΔPaHP1 null allele. All deletions were verified by Southern blot (Additional file 8: Fig. S8 and Additional file 12: Fig. S12A).

Construction of fluorescent-HA-tagged chimeric proteins PaHP1-GFP-HA, PaKmt1-mCherry-HA, PaKmt6-GFP-HA

The pAKS106 and pAKS120 plasmids were described in [101]. All the cloned inserts were sequenced before transformation.

To gain insight into PaHP1 localization, we constructed a PaHP1-GFP-HA fusion chimeric protein. To this end, the PaHP1 gene was PCR amplified from the S strain genomic DNA using primers FC3-BamH1 and FC4-GFP (Additional file 23: Table S5). The resulting amplicon harbors 520 bp of promoter sequence followed by PaHP1 CDS cleared from its stop codon. In parallel, eGFP CDS was amplified from peGFP plasmid using FC5-HP1 and FC6-HindIII primers. The two DNA segments were fused by PCR using primers FC3-BamH1 and FC6-HindIII. This chimeric DNA fragment was digested with BamH1 and HindIII and cloned into the pAKS106 plasmid linearized with the same enzymes. When transformed into ΔPaHP1 mutant strain, the PaHP1-GFP-HA allele is able to restore a wild-type phenotype (growth rate and aerial mycelium), showing that the tagged version of PaHP1 protein is functional.

PaKmt1-mCherry-HA allele construction followed the same experimental design used for PaHP1-GFP-HA construction. First, PaKmt1 was PCR amplified using Dim5mChFBamH1 and Dim5mChR and resulted in a DNA amplicon composed of 454 bp of promoter followed by the PaKmt1 CDS. The mCherry CDS was amplified from the pmCherry plasmid using mChDim5F and mChDim5R HindIII primers. Fragments were fused by PCR, digested and cloned in pAKS106. The Kmt1-mCherry-HA allele was able to restore wild-type growth phenotype when transformed in ΔPaKmt1 mutant strains, indicating that this chimeric protein is functional.

PaKmt6 (Pa_1_6940) was PCR amplified from S genomic DNA using FC73-GA1KMT6 and FC74-GA1KMT6 primers, resulting in 4232 bp DNA amplicons composed of 912 bp of promoter followed by PaKmt6 CDS cleared from its stop codon. In parallel, the GFP allele was amplified from peGFP plasmid using FC75-GA1GFP and FC76-GA1GFP. Both fragments were cloned together in one step in the pAKS-106 plasmid previously digested with NotI and BamH1 restriction enzymes. This cloning step was performed using Gibson Assembly kit (New England Biolabs). This yielded the chimeric PaKmt6-GFP-HA allele. An additional PaKmt6-HA allele was generated by PCR amplification from the wild-type strain DNA of PaKmt6 CDS along with its own promoter, using FC65-NotI and FC72-BamH1 primers. These amplicons were digested and cloned in pAKS106 plasmid using NotI and BamH1 restriction enzymes. When transformed into ΔPaKmt6 mutant strain, both PaKmt6-GFP-HA and PaKmt6-HA alleles were able to restore wild-type growth and sexual development, suggesting that this chimeric version of the PaKmt6 protein is functional. However, no GFP fluorescence signal was detected in the ΔPaKmt6, PaKmt6-GFP-HA complemented strains.

To overexpress PaHP1, the endogenous promoter were replaced by the AS4 promoter that drives high and constitutive expression throughout the life cycle [102]. PaHP1-GFP allele was PCR amplified from pAKS106-HP1-GFP (this study) using primers FC21-BamH1 and FC6-HindIII primers. The two DNA segments were fused by PCR using primers FC3-BamH1 and FC6-BamH1. This chimeric DNA fragment was digested with BamH1 and HindIII and cloned into the pAKS106 plasmid linearized with the same enzymes. When transformed into ΔPaHP1 mutant strain, the PaHP1-GFP-HA allele is able to restore a wild-type phenotype (growth rate and aerial mycelium), showing that the tagged version of PaHP1 protein is functional.

PaKmt1-mCherry-HA over-expressed allele construction followed the same experimental procedures as the one used for pAS4-PaHP1-GFP-HA construction. PaKmt1-mCherry CDS was PCR amplified using FC22-BamH1 and mChDim5R-HindIII using pAKS106-PaKmt1-mCherry-HA plasmid as DNA template. Amplified DNA fragments were then digested and cloned in pAKS120 plasmid using HindIII and BamH1 restriction enzymes. This allele was transformed in ΔPaKmt1 strain.
Complementation experiments with GFP-tagged proteins

To perform complementation experiments, the PaKmt1-mCherry allele was transformed into the ΔPaKmt1 strain. Because the PaKmt1-mCherry allele was linked to a phleomycin marker, 55 phleomycin-resistant transformants were recovered. Among those, five wild-type looking independent transgenic strains were selected and crossed to a wild-type strain. In the progeny, all the phleomycin- and hygromycin-resistant haploid strains behaved similarly to the wild-type strain. Two of these independent transformants were selected for further phenotypic characterization. To ask whether a constitutively over-expressed PaKmt1 allele could have an impact on the physiology of P. anserina, we introduced the AS4-PaKmt1-mCherry allele into the ΔPaKmt1 strain. All the phleomycin-resistant transformants looked like a wild-type strain (N=30 phleomycin resistant). Two of them were crossed to a wild-type strain. In their progeny, all the phleomycin-resistant strains carrying either the PaKmt1⁺ allele or the ΔPaKmt1 were indistinguishable from wild-type strains.

Similarly, we transformed a PaHP1-GFP-HA allele, linked to a phleomycin marker, into a mutant ΔPaHP1 strain. Among the 20 phleomycin-resistant transformants that were recovered, five independent transformants displaying a wild-type phenotype were selected and crossed to the wild-type strain. In the progeny, all the phleomycin- and hygromycin-resistant haploid strains behaved similarly to the wild-type strain. Transformation of the AS4-PaHP1-GFP-HA allele into the ΔPaHP1 strain generated phleomycin-resistant transformants (N=19) showing intermediate to full complementation but no extra phenotypes. Two transformants presenting full complementation were crossed to a wild-type strain. In their progeny, all the phleomycin-resistant strains carrying either the PaHP1⁺ allele or the ΔPaHP1 behaved like the wild-type strains.

Finally, the PaKmt6-GFP-HA allele and the PaKmt6-GFP-HA allele were independently transformed into a ΔPaKmt6 strain. For the two transformation experiments, among the phleomycin-resistant transformants that were recovered (N=20 and N=32, respectively), two independent transgenic strains displaying a wild-type phenotype were selected and crossed to a wild-type strain. In the progeny of these four crosses, all the phleomycin- and nourseothricin-resistant haploid strains behaved similarly to wild-type strain.

Phylogenetic analysis

Orthologous genes were identified using the MycoCosm portal [103] and manually verified by reciprocal Best Hit BLAST analysis. Sequences were aligned using Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/) and trimmed using Jalview (version 2.9.0b2) to remove non-informative regions (i.e., poorly aligned regions and/or gap containing regions). Trees were constructed with PhyML 3.0 software with default parameters and 100 bootstrapped data sets [104]. The trees were visualized with the iTOL server (http://itol.embl.de/).

Phenotypic analyses

Spermatium counting was performed as follows: each strain was grown on M2 medium with two cellophane layers at 18 °C for 14 days (P. Silar’s personal communication). To collect spermatia, cultures were washed with 1.5 mL of sterile water. A Malassez counting chamber was used for enumeration.

Cytological analysis

Light microscopy was performed on 2-day-old mycelium. Explants taken from complemented strains expressing PaHP1-GFP-HA were analyzed by fluorescence microscopy. Pictures were taken with a Leica DMIRE 2 microscope coupled with a 10-MHz Cool SNAPHQ charge-coupled device camera (Roper Instruments), with a z-optical spacing of 0.5 mm. The GFP filter was the GFP-3035B from Semrock (Ex: 472 nm/30, dichroïc: 495 nm, Em: 520 nm/35). The Metamorph software (Universal Imaging Corp.) was used to acquire z-series. Images were processed using the ImageJ software (NIH, Bethesda).

Western blot analysis

Western blots were performed on nuclear extracts made from 200 mg of ground mycelia resuspended in 3 mL of New Cell Lysis Buffer [Tris–HCL pH 7.5 50 mM, NaCl 150 mM, EDTA 5 mM, NP-40 0.5%, Triton 1% and protease inhibitor (Roche-04693132001)]. The nuclear extracts were collected after centrifugation (300×g, 5 min) and resuspended in 300 µL of Nuclei Lysis Buffer (Tris–HCl pH 8 50 mM, EDTA 10 mM, SDS 0.5% and protease inhibitor (Roche-04693132001)). Nuclei were then sonicated (Diagenode bioruptor plus sonicator, 20 min at high level). Protein contents of nuclear extracts were quantified using the Qubit Protein assay kit (Invitrogen). Then, 35 µg of nuclear extract were diluted in Laemmli sample buffer and loaded on a 10% SDS polyacrylamide gel. Membranes were probed using the following high affinity ChIP-grade monoclonal antibodies: anti-Histone 3 (Abcam ab12079), anti-H3K9me3 (Abcam ab8898), anti-H3K27me3 (Millipore 17-622) and anti-H3K4me3 (Active Motif 39159).

Chromatin immuno-precipitation

Each ChIP-seq experiment was performed on two independent biological replicates for each genotype (mat+...
strains only). Chromatin extractions were made from mycelium grown for 60 h at 27 °C in liquid culture (KH₂PO₄ 0.25 g/L, K₂HPO₄ 0.3 g/L, MgSO₄ 0.25 g/L, urea 0.5 g/L, biotin 0.05 mg/L, thiamine 0.05 mg/L, oligo-element 0.1%, yeast extract 5 g/L and dextrin 5.5 g/L). Mycelium was filtered and quickly washed with phosphate-buffered saline (PBS), resuspended and then cross-linked in 100 mL of PBS with 1% paraformaldehyde for 15 min at 27 °C. Reaction was stopped by addition of glycine (60 mM). Cross-linked mycelium was filtered, washed with cold PBS buffer and dried on Whatman paper. Dried mycelium was frozen in liquid nitrogen and ground to powder in a mortar. Aliquots of mycelium powder (200 mg) were resuspended in 1 mL of Lysis Buffer [Hepes pH 7.5 50 mM, NaCl 0.5 M, EDTA pH 8 1 mM, Triton X-100 1%, Sodium deoxycholate 0.1%, CaCl₂ 2 mM and protease inhibitor (Roche)]. Chromatin was digested to 0.15–0.6-kb fragments using Micrococcal nuclease (NEB #M0247) for 30 min at 37 °C (10,000 gels units/mL). The enzymatic digestion was stopped by addition of EGTA (15 mM). After centrifugation (15,000×g, 15 min, twice) of the digested samples, pelleted genomic DNA fragments were discarded. Concentration of the soluble chromatin fractions was assayed using the fluorescence system Qubit dsDNA HS assay kit (Invitrogen). Prior to immune-precipitation, 5 μg of soluble chromatin was washed in 1 mL of Lysis buffer containing 30 μL of magnetic beads (Invitrogen 10001D, 4 h at 4 °C). From the 1.1-mL soluble chromatin fractions was assayed for correct 260/280 and 260/230 ratios, and RNA quality was checked by gel electrophoresis. Reverse transcription was performed with the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) and oligo-dT. A non-reverse transcribed (NRT) control was systematically performed on a pool of NRT controls to ensure that the Cq was above the Cq obtained from corresponding reverse transcribed RNAs. For genes without introns, NRT control was performed for each replicate sample to ensure that the Cq was above the Cq obtained from corresponding reverse transcribed RNAs. When possible, primers were designed on two consecutive exons (Additional file 23: Table S5). Normalization genes were selected among a set of 8 housekeeping genes using geNorm (see Additional file 21: Table S3 for details of analysis) [105]. Selected normalization genes were ASI (Pa_1_16650), GPD (Pa_3_5110) and PDF2 (Pa_7_6690), with an average expression stability M = 0.23 indicating a homogeneous set of samples and a V3/4 < 0.15 (see Additional file 22: Table S4 for details of
analysis). RT-qPCR normalization according to the ΔΔCt method [106], standard error and 95% confidence interval calculations, and statistical analyses were performed using REST 2009 software (Qiagen, Hilden, Germany). Genes were defined as down-regulated in the mutant strain if the ratio of their transcript level in the mutant strain compared with that in the wild-type strain showed a fold change > 0 and < 1, with a p-value < 0.05. On the other hand, genes were defined as up-regulated in the mutant strain if the ratio of their transcript level in the mutant strain compared with that in the wild-type strain showed a > onefold change, with a p-value < 0.05. Ratios with a 95% confidence interval, including 1, were not considered significant [107]. RT-qPCR experiments were MIQE compliant [108].

RT-qPCR experiments for detection of TE

Primers for detection of TE were designed to detect as many members as possible for each family (Additional file 23: Table S5). These primer couples detect genomic DNA as well as cDNA. As described above, NRT-qPCR controls were performed with these primer couples on each biological replicate and revealed for some of them NRT-qPCR signals were too close to the RT-qPCR signals to allow a reliable analysis of the data. Biological replicates that displayed NRT-qPCR signals less than 2.9 cycles above the RT-qPCR signal were discarded and analyses were performed if three biological replicates at least were available (see Additional file 22: Table S4 for details of analysis).

qPCR analysis to test ChIP efficiency

Quantitative PCR experiments were performed with dsDNA fluorescent detection method using the FastStart Universal SYBR Green Master from Roche (04913850001).

IP enrichments were assayed with the following protocol: 5 μL of IPed DNA samples and non-IPed input were diluted 10 times in nuclease free water. qPCR experiments were done on 2 μL of the diluted samples using the pairs of primers (0.5 μM) FC77-Actin/FC78-Actin and FC125-580/FC126-580 (see Additional file 23: Table S5). For each IP sample, relative concentrations were determined as an “Input percentage” with the following equation:

\[
\% \text{Input} = \left( e^{C_{\text{q Input}} - \left( \frac{\ln(10)}{\text{EF}} \right) - C_{\text{q Sample}}} \right) \times 100.
\]

Seqeuncing

ChIP-seq libraries were built using the NEB Next Ultra II DNA library Prep kit for Illumina (New England Biolabs #E7645S/L) and Agencourt Ampure XP beads (Beckman Coulter, #A63880) according to the manufacturer’s protocols. PCR amplification was made of 12 cycles. ChIP-seq sequencing was performed by the I2BC High-throughput sequencing facility [NextSeq 500/550 High Output Kit v2 (75 cycles), SR 75 bp]. Reads were trimmed with Cutadapt 1.15 and filtered for control quality by FastQC v0.11.5.

ChIP-seq sequencing data analysis and visualization

Sequencing generated between 8 and 45 million reads depending on samples. The ChIP-seq analysis was performed on sets of data that contained 8 to 10 million reads at most. If needed, the number of reads was downsized randomly. Reads were mapped on the S mat+ P. anserina genome [51] using Bowtie 2 software (version 2.3.0, see annex for results). For visualization, data were normalized using Deeptools 2.0 [109] bamcoverage with the BPM method. Spearman correlation factors were calculated on normalized data using Deeptools 2.0. For each condition, we made sure that the two biological replicates correlate (Additional file 14: Fig. S14 and Additional file 25: Table S7 and Additional file 26: Table S8) and then merge them for peak calling. Peak calling was performed using MACS2 software (version 2.1.1) using the mock sample as control. Further peak annotations and comparisons were done using Bedtools (version 2.26.0). Genome-wide visualization of peaks was also generated with Circos software [110]. All graphs have been drawn using ggplot2 [111]. Heatmap and metaplot figures have been generated using Deeptools2. Genomic regions have been extracted from the current genome annotation. Promoters have been arbitrarily defined as 1 kb 5′ of the start codon. Scores of the heatmap represent the mapped reads after normalization (see above), hence not only the MACS2-predicted peaks. The Euler diagram has been generated by the eulerR package (v 6.0.0, https://cran.r-project.org/package=eulerR). The domainogram analysis [50] has been performed using scripts described in [112] and downloaded from the BBCF Github (EPFL, Lausanne, https://github.com/bbcf/). The domainograms were calculated on binned data sets (100 bp binning), using windows sizes ranging from 1 to 500 bins. Individual BRICKs (Blocks of Regulators In Chromosomal Kon-text), which represent regions with significant clustering of ChIP-seq signal, were limited to 50 bins each.

To assess whether any of the biological functions observed in H3K4me3+H3K27me3 gene lists (Additional file 20: Table S2) were present at a frequency greater than that expected by chance, p-values were calculated using hypergeometric distribution as described in [113].
detected peaks. D Domainogram representations for all marks on the seven *P. anserina* chromosomes for all conditions. Domainograms show significance of enrichment of H3K4me3, H3K27me3, H3K36me3, H3K72me3 marks in windows of varying size. Color-coding of p-value is indicated (top).

**Additional file 4: Figure S4.** Combined epigenetic landscapes in wild-type and heterochromatin mutant strains of *P. anserina*. Panorama of genome-wide peak localization for each genotype, wild-type, Δ*PaKmt1*, Δ*PaKmt6* and Δ*PaHPI* strains. Telomeres sequences were arbitrarily defined as the segment going from the end of each arm of the chromosomes to the first annotated gene (with the exception of the tRNA cluster localized on chromosome 3) and centromeres are indicated. Mat region = Non-recombining region containing the mating-type locus as defined in [93]. A segment overlapping portions of chromosomes 3 and 4 is expanded to show a zoom of the combined epigenetic landscapes.

**Additional file 5: Figure S5.** H3K4me3, H3K27me3 and H3K9me3 modifications of *P. anserina* TE families. Top panel: Plots of normalized ChIP signal. H3K4me3 (green), H3K9me3 (red) and H3K27me3 (dark blue) signals in the wild-type strain for five TE families, i.e., *Copia*, *Gypsy*, *MITE*, *Tc*1 *Mariner*, solo *LTR* and unclassified TEs [51] (Additional file 21: Table S3). Because MITE TE are shorter than the other categories (< 500 bp in length), the genomic window was narrowed on the graph. Aligned sequences correspond to TE bodies ±0.5 kb surrounding region (see Additional file 21: Table S3 for TE's numbers). Bottom panel: K-means built clusters representing the association versus non-association of the indicated histone modifications within a specific TE family. Histone modification levels in the heatmaps were calculated for non-overlapping 10-bp windows within the specific genomic regions and sorted by average value of each row. B Number of TEs, classified by family, marked with H3K4me3, H3K27me3 or H3K9me3, according to genetic backgrounds (wild-type strain, Δ*PaKmt1*, Δ*PaKmt6* and Δ*PaHPI* mutant strains). C Violin plots of expression of TEs classified by family. Gene expression was inferred from the TPM (Transcripts Per Kilobase Million) values calculated in [49]. Gene expression of non-repeated CDS (i.e., *genes*) was added for comparison.

**Additional file 6: Figure S6.** Snapshots of a set of TEs representing all the annotated TE families in *P. anserina* genome. ChIP-seq signals were normalized as described in “Methods” and visualized using the Integrated Genomics Viewer (IGV) [115]. H3K4me3 (green), H3K9me3 (red) and H3K27me3 (blue).

**Additional file 7: Figure S7.** Antibody specificity analysis. Dot blot results using H3K9me3 and H3K27me3 peptides (left) at different concentration (top). Membranes were inoculated with the corresponding antibody (right). Signal intensity comparison shows a cross-reactivity between anti-H3K9me3 antibody and H3K27me3 evaluated at 3% compared to the immunogen reaction.

**Additional file 8: Figure S8.** Molecular characterization of knockout mutants by Southern blot hybridization. Schematic representations of the endogenous and disrupted loci are given (Left). Replacement by homologous recombination of the wild-type *PaKmt1* allele by the disrupted Δ*PaKmt1* allele results in the substitution of 2.4 kbp and 5.7 kbp EcoRI fragments by a unique 1.1 kbp PstI fragment as revealed by hybridization of the 5' and 3' digoxigenin-labeled probes (dashed rectangles *PaKmt1* locus). Replacement by homologous recombination of the wild-type *PaKmt6* allele by the disrupted Δ*PaKmt6* allele results in the substitution of a unique 6.5 kbp KpnI fragment by two 1.8 and 2.4 kbp KpnI fragments as revealed by hybridization of the 5' and 3' digoxigenin-labeled probes (dashed rectangles *PaKmt6* locus). A second verification has been made with the HindIII enzymes and the same probes shows the substitution of two 1.9 kbp and 4.6 kbp HindIII fragments by a 3.7 kbp HindIII fragment as revealed by hybridization of the 5' and 3' digoxigenin-labeled probes.

**Additional file 9: Figure S9.** Localization of histone marks on specific genomic regions in the Δ*PaKmt1* and Δ*PaKmt6* mutant strains. Top panel: Plots of normalized ChIP-seq signal. Bottom panel: Heatmaps divided in K-means built clusters representing the association versus non-association of the indicated histone modifications with the specific genomic regions.
Coding sequences or CDS were aligned by their two ends (indicated by START and STOP) ± 1 kbp of surrounding sequence (N = 10,839; Additional file 20: Table S20). Table S20 presents: were duplicated in TE bodies and the tRNA array ±0.2 kbp surrounding regions (N = 1,680; Additional file 21: Table S3). Histone modification levels in the heatmaps were calculated for non-overlapping 10 bp windows within the specific genomic regions and sorted by average value of each row.

Additional file 10: Figure S10. Relative expression of selected genes in the ΔPaKmt1 mutants. Caption as in Fig. 5a. The error bars represent the 95% confidence interval. No significant fold change was detected, except for Pa_1_6263 and Pa_6_7370, which both lost the H3K27me3 mark and were up-regulated and down-regulated, respectively. Pa_4_1170 expression ratio = 1.535, p-value = 0.049; Pa_1_63300 expression ratio = 1.393, p-value = 0.092; Pa_6_7270 expression ratio = 1.397, p-value = 0.035; Pa_6_7370 expression ratio = 0.696, p-value = 0.006; Pa_5_10 expression ratio = 0.759, p-value = 0.017; Pa_1_1880 expression ratio = 1.422, p-value = 0.034; Pa_7_9210 expression ratio = 1.082, p-value = 0.252; TC1mrl represent quantification of the CDNAs from the members of the TC1_mariner-like_rainette family: expression ratio = 1.127, p-value = 0.339; TC1mpl represent quantification of the CDNAs from the members of the TC1_mariner-like_pebolates family: expression ratio = 0.851, p-value = 0.308. Copia_1y1_rephelobates transcripts could not be quantified as NRT-qPCR controls were too close to RT-qPCR (see Additional file 21: Table S3 for details of analysis).

Additional file 11: Figure S11. Growth features of the ΔPaKmt1 strain. A Experimental procedure to test ability to resume growth of the ΔPaKmt1 strain. To set up this restart test, mycelium implants issued from germination thalli were inoculated onto fresh M2 medium and incubated at 27 °C for 8 days (step 1). Two independent plugs from each location (purple cross), 8-day stationary phase (plug#3), pink cross, 4-day stationary phase (plug#2), and green cross, growing phase (plug#1) were then transferred to fresh M2 solid medium and incubated for 3 days at 27 °C (step 2). The growing phase of each thallus originating from step 2 (green margins) were then transferred to fresh M2 medium solid medium and incubated at 27 °C for 3 days (step 3). Growth restart from stationary phase was impaired for ΔPaKmt1 mutants, which resulted in smaller and thinner colonies than the wild-type ones (white arrows), whereas continuous growth (plug#1) was not altered. Complemented ΔPaKmt1-PaKmt1+ strains behaved as wild-type strains. As control experiments (step 3), we then transferred mycelia from growing margins (marked in green, step 2) of thalli deriving from Plug#1, Plug#2 and Plug#3. In this case, ΔPaKmt1 mutants did not show any delay to resume growth (orange arrows), confirming that this defect was not permanent but rather linked to the disability of the ΔPaKmt1 strains to resume growth properly. B Crippled growth test for ΔPaKmt1 strain. Crippled growth (CG) process can be shown with the “in-band test”. Strains were incubated on M2 medium for 7 days at 27 °C. Two 1-mm-wide slices of agar were then inoculated onto fresh M2 media with or without yeast extract (YE) in the following method for 3 days. Picture shows actively growing apical hyphae (right) and resting stationary phase hyphae (left). The surface side of the slice on plate has been orientated at the top. CG is a degenerative process caused by C. element production in the stationary phase. When inoculated on yeast extract medium, it displays slow growth, alteration of pigmentation, inability to differentiate aerial hyphae, and female sterility. The ΔPaKm1p1 mutant strain is impaired for CG development while the PDC2208 strain is supposed to display CG on M2 medium without yeast extract [116]. The single ΔPaKmt1 mutants are not impaired for CG.

Additional file 12: Figure S12. Longevity tests for ΔPaKmt1 and ΔPaKmt6 strains. Graphs show the maximal growth length on M2 medium at 27 °C in race tubes for each genotype (WT). ΔPaKmt1 and ΔPaKmt6 mutant strains for both mating types. Data correspond to the mean of three technical replicates from five biological samples.

Additional file 13: Figure S13. Role of PaKmt6 during sexual development. A Heterozygote oriented crosses between wild-type strains (WT) and ΔPaKmt6 mutants. Fertilization of wild-type ascogonia with ΔPaKmt6 male gametes results in normal fruiting body development displaying a single neck (black arrow), while fertilization of ΔPaKmt6 ascogonia with wild-type spermatia results in fewer crippled ΔPaKmt6-like fruiting bodies (two necks are indicated by two black arrows). These features indicate that Patkmt6 is a maternal gene. B Mosaic analyses using the Δmat mutant strain. Wild-type or ΔPaKmt6 mat+ and mat− strains were mixed with or without Δmat mycelium and inoculated onto fresh M2 medium. After a week of incubation on M2 medium, ΔPaKmt6 dikaryon displayed crippled and mis-oriented perithecia, resulting in scattered and reduced ascospore production. The tricaryon ΔPaKmt6 mat+/- ΔPaKmt6 mat−/− Δmat showed nearly wild-type restoration of mycelium growth, as well as perithecia and ascospore production. These features confirm that PaKmt6 is a gene expressed in the maternal tissues of the perithegium and not in its zygotic tissues (centrum).

Additional file 14: Figure S14. Structure and evolutionary relationships of Hp1 orthologs. A Domain structure comparison of heterochromatin protein 1 homologs. Size in amino acids (aa) is given (right). PaH1 displays the evolutionary conserved and topologically connected chromodomain (turquoise) and chromoshadowdomain (dark green), along with three disordered elements: the N-terminal extension (NTE), the Hinge region (HR) and the C-terminal extension (CTE). Chromodomain recognizes H3K9me2/3 histone modification, while chromoshadowdomain is a protein–protein interaction domain. B Alignment of the chromodomains of Hp1 homologs for a set of selected species. The aromatic cage residues (three aromatic amino acids shaded in yellow, i.e. Y and WxxY) form the binding cavity for H3K9me. Positions of the sequence evolutionarily conserved/homologous residues in the Hp1 chromodomain are highlighted in blue. Residues of chromo-shadow domain involved in dimerization are shown in green, those involved in folding are shown in lavender [63].

Additional file 15: Figure S15. A Tree showing evolution of some Hp1 orthologs from fungi, plant and metazoans. The P. anserina heterochromatin protein-1 homolog is marked with a rectangle. Bootstraps are given. Filamentous fungi: Podospora anserina (Pa), Neurospora crassa (Nc), Fusarium graminearum (Fg), Trichoderma reesei (Tr), Epichloë festucae (Et) Botrytis cinerea (Bc), Magnaporthe oryzae (Mo), Zymoseptoria tritici (Zt), Leptosphaeria maculans (Lm), Aspergillus nidulans (An), Aspergillus fumigatus (Af), Penicillium oxalicum (P0), Ascobulus immersus (Ai), Puccinia graminis (Pg), Pneumocystis jirovecii (Pj), Conidiobolus coronatus (Cc), Mucor circinelloides (Mc); yeasts: Schizosaccharomyces pombe (Sp) and Cryptococcus neoformans (Cn); the worm Caenorhabditis elegans (Ce), the fruit-fly Drosophila melanogaster (Dm), the mouse Mus musculus (Mm) and the model plant Arabidopsis thaliana (At). Accession numbers for proteins used in alignments are listed in Additional file 24: Table S6. B Molecular characterization of knockout ΔPaH1P1 mutants by Southern blot hybridization. Replacement by homologous recombination of the wild-type PaH1P1 allele by the disrupted ΔPaH1P1 allele results in the substitution of a unique 3.4 kbp EcoRI/EcoRV fragment by two 1.4 and 2.5 kbp EcoRI/EcoRV fragments as revealed by hybridization with the wild-type and the mutant-C probes. C In situ hybridization with the wild-type and the mutant-C probes. D The presence of EcoRI and EcoRV restriction-site labeled probes (dashed rectangles PaH1P1 locus). E Vegetative growth of ΔPaH1P1 single mutants and ΔPaKmt1ΔPaH1P1 double mutants compared to wild-type strains. ΔPaKmt1 and ΔPaH1P1 single mutants are impaired in aerial mycelium production. This defect was more pronounced for the single ΔPaH1P1 mutants. ΔPaKmt1ΔPaH1P1 double mutants show ΔPaKmt1 morphologic phenotype. The strains have been grown on M2 medium 3 days.

Additional file 16: Figure S16. Localization of histone marks on specific genomic regions in the ΔPaH1P1 mutant strains. Top panel: Plots of normalized ChIP-seq signal. Bottom panel: heatmaps divided in K-means built clusters representing the association versus non-association of the indicated histone modifications with the specific genomic regions. Coding sequences or CDS were aligned by their two ends (indicated by START and STOP) ± 1 kbp of surrounding sequence (N = 10,839; Additional file 20: Table S20). The ΔPaKmt6 mutants were defined as ΔPaKmt6 mut− strains. Cis-regulatory DNA elements were identified based on the tRNA array ±0.2 kbp surrounding regions (N = 1,680; Additional file 20: Table S3). Histone modification levels in the heatmaps were calculated for non-overlapping 10 bp windows within the specific genomic regions and sorted by average value of each row.

Additional file 17: Figure S17. A Relative expression of selected genes in the ΔPaH1P1 strain. Caption as in Fig. 5a. The error bars represent the 95% confidence interval. No significant fold change was assayed, except
for Pa_1_6263 and Pa_5_10, which both lost the H3K27me3 mark and were up-regulated and down-regulated, respectively. Pa_1_6263 expression ratio = 2.463, p-value = 0.004; Pa_4_1170 expression ratio = 1.911, p-value = 0.032; Pa_1_16300 expression ratio = 0.931, p-value = 0.693; Pa_6_7270 expression ratio = 1.527, p-value = 0.004; Pa_6_7370 expression ratio = 0.766, p-value = 0.009; Pa_5_10 expression ratio = 0.490, p-value = 0.006; Pa_1_1880 expression ratio = 1.313, p-value = 0.013; Pa_7_9210 expression ratio = 0.951, p-value = 0.547. T1-mRNA represents quantification of the CNAs from the members of the Tc1_mariner-like_class_haematobium family expression ratio = 1.264, p-value = 0.008. Tc1_mariner-like_peolobates and copia_Ty1_nephelobates transcripts could not be quantified as NRT-qPCR controls were too close to RT-qPCR (see Additional file 21: Table S5 for details). All experiments were performed in triplicate for each batch of experiments.

### Additional file 21: Table S5. Heatmap of Spearman correlation coefficient comparison between ChIP-seq samples. Samples are labeled as follow: Genotype_strain_marks: e.g., KMT6-11d_K9 = ΔPaKmt6 strain, from an RNA sample. The ChIP-seq correlation coefficient confirms the reproducibility of the experiments since biological replicates cluster together with high correlation coefficients. Moreover, H3K1me3 and H3K27me3 IP share a same cluster that negatively correlates with the H3K4me3 cluster. Both display no correlation with non-IP input and negative sample (Mock and H3K27me3 in the ΔPaKmt6), which means that IP results are significant and specific.

### Additional file 26: Table S8. Raw data from the heatmap of Spearman's correlation coefficient comparison in Additional file 18: Fig. S18.

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### Authors' contributions

Conceived and designed the experiments: FM. Performed the experiments: FC, ML, LM, RD, PG, FM. Analyzed the data: FC, RD, DN, PG, FM. Contributed reagents/materials/analysis tools/expertise sharing: PG, CS, DN. Wrote the paper: FC, DN, PG, FM. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) (BioProject: PRJNA574032, https://www.ncbi.nlm.nih.gov/bioproject?term=PRJNA574032).

### Declarations

#### Competing interests

The authors declare that they have no competing interests.

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