The RNA Polymerase II-associated factor 1 complex represses small-RNA-mediated heterochromatin formation and gene silencing

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1 Summary

RNAi interference (RNAi) is a highly conserved regulatory mechanism employed by almost all Eukaryotes. With RNAi organisms can modulate the expression of endogenous genes and protect the integrity and identity of their genomes. All RNAi-based processes depend on a complex containing small non-coding RNAs (sRNA) associated with Argonaute proteins. In this sRNA-Argonaute complex, sRNA recognizes its sequence-specific target messenger RNA (mRNA) via a base-pairing interaction, and directs the Argonaute protein to it. Upon binding, the Argonaute protein can repress target gene expression at different stages.

In the case of the most studied class of sRNAs, the microRNAs, the repression of gene expression occurs at the post-transcriptional level. MicroRNAs inhibit the translation of target mRNAs and promote their degradation in the cytoplasm of a cell. In contrast, nuclear RNAi-based processes have been implicated in directing chromatin modifications and repressing gene activity at the transcriptional level. RNAi-mediated chromatin modifications have been linked to epigenetic gene silencing across kingdoms but the mechanistic details of the small RNA-dependent transgenerational silencing remain uncovered. One of the obstacles in the way to understanding these regulatory processes is the fact that attempts to stably silence genes by ectopic small RNA mediated, locus-independent heterochromatin formation, have proven to be inherently difficult.

By performing a mutagenesis screen we identified the highly conserved RNA Polymerase II-associated factor 1 (Paf1) complex as a repressor of sRNA-directed heterochromatin formation in the fission yeast *Schizosaccharomyces pombe*. We showed that small RNAs produced from a hairpin construct effectively silenced the expression of the target gene *in trans,* if the function of Paf1 complex was impaired. The induced repression was locus- and sequence-independent, and involved *de novo* formation of a functional heterochromatic domain. Importantly, we observed that the silent state could be transmitted through meiosis and was subsequently inherited through tens of generations, even in the absence of the primary siRNAs source. Thus, the Paf1 complex represses sRNA-induced heterochromatin formation in an epigenetic fashion [1]. By performing a genetic analysis, we found that the Paf1 complex represses sRNA-mediated heterochromatin formation by contributing to efficient transcription termination and nascent transcript release. Thereby, we demonstrate that defective transcription termination exposes genes to sRNA-mediated repression.

The findings described in this dissertation are not only an advancement to the mechanistic research on sRNA-directed transgenerational gene silencing. The ability to stably repress gene activity without changing the underlying DNA sequence may also provide important technological implications, in particular in plant biotechnology.

2 Contributions

Results presented in this dissertation were obtained in a collaborative effort and published in the following article:

Katarzyna Maria Kowalik*, Yukiko Shimada*, Valentin Flury, Michael Beda Stadler, Julia Batki, Marc Bühler *The Paf1 complex represses small-RNA-mediated epigenetic gene silencing*. *Nature* 2015 Apr 9;520(7546):248-52.

Highlited in: Mikel Zaratiegui *Molecular Biology: RNAi hangs by a thread. Nature* 2015 Apr 9;520(7546): 162-4.

This work was published as a co-authorship with Yukiko Shimada, to whom I am immensely grateful for sharing the project with me. The manuscript and the comment article [2], [3] can be found in the Section 10.

Experiments described in the section 7 were performed by me, Yukiko Shimada and Valentin Flury, with the help of Julia Batki.

I reconstituted identified point mutations and created strains for validation of the screen results, as well as the control strains (Figures: 10A, 10B); performed the H3K9me2, H3K36me3 and RNA PolII ChIP experiments (Figures: 12, 19 22B, 22C); prepared and analyzed genome-wide data sets for RNA expression profiling and small-RNA expression profiling (Figures: 6A, 6B, 10C, 15, 16A, 17, 18); prepared the ribozyme strains and performed the silencing assays presented in the Figure 23; made the cross from Figure 21B.

Yukiko Shimada performed the screen together with all the control experiments and analyzed the hits (Figures: 5B, 6C, 6D, 8); performed crosses and tetrad dissections presented in Figures 20C, 20D, 21A; prepared and performed the silencing assays presented in the Figures 11, 13, 16B.

Yukiko Shimada and Valentin Flury prepared the set of deletions and mutations presented in the Figure 22D. Valentin Flury performed the establishment and maintenance scoring assay (Figure 22E). Yukiko Shimada, with the help of Julia Batki, prepared the set of deletions of RNAi and heterochromatin factors (Figure 14). I, with the help of Julia Batki, performed the establishment and maintenance scoring assays (Figures: 20A, 20B).

Michael Stadler designed and performed the computational analysis of the mutant genome resequencing data.

I wrote all the sections of this dissertation independently, with the exception of the description of the Figure 9. Figures were prepared by Marc Bühler, me, and Michael Stadler (Figure 9). Marc Bühler critically read the manuscript of this dissertation.

3 Introduction

3.1 RNAi-mediated regulatory mechanisms

3.1.1 RNAi phenomenon

RNAi interference (RNAi) is a highly conserved regulatory phenomenon employed by almost all eukaryotic organisms to modulate expression of endogenous genes and protect integrity or identity of their genomes. RNAi was first described as a gene silencing mechanism mediated by double stranded RNA (dsRNA) in the nematode *C. elegans* [4]. Soon after this finding had been published, it became evident that other known gene silencing effects, like co-suppression in plants or quelling in the fungus *Neurospora crassa*, were also examples of the RNAi-based regulatory processes [5], [6].

As it was first shown in plants, RNAi-mediated silencing occurs through small interfering RNAs [7]. In the cell, siRNAs form effector complexes with small-RNA-binding proteins of the Argonaute family. What is common to all RNAi-based processes is that recognition of RNAi target sequences occurs *via* complementarity-based interaction with siRNAs [8], [9]. Thereby, these relatively short (20-30 nucleotides) RNA molecules serve as an address tag for the Argonaute-containing effector complexes and can be specifically directed to virtually any target RNA sequence. Importantly, since it is a variable siRNA sequence that directs the recruitment of universal proteins, RNAi-based mechanisms can always adapt to novel, exogenous targets. Bacteria also adapted the mechanism of RNA-based target recognition in the CRISPR-Cas9 system, which is an independently evolved concept similar to RNAi, where an RNA-guided DNA nuclease protects the bacterial genome from exogenous genetic elements [10].

3.1.2 RNAi-mediated Post-Transcriptional Gene Silencing

RNAi-based regulatory mechanisms are traditionally categorized into post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). During PTGS, recognition of a target sequence by the siRNA leads to a direct cleavage and degradation or

destabilization of the target RNA molecule by the effector complex called RNA-induced Silencing Complex (RISC) [11]. Thus, in a classic PTGS, small RNA-mediated repression affects cellular levels of a target RNA without affecting its transcription. Micro RNAs (miRNAs) and small interfering RNAs (siRNAs) are two examples of small RNA groups mediating PTGS.

miRNA-mediated gene silencing

miRNAs constitute a class of small RNAs, approximately 22 nt long, which are commonly produced in plants and animals. Generally, miRNAs originate from precursor miRNA transcripts (pri-miRNAs), which are encoded in the genome in a form of microRNA genes and are transcribed by RNA Polymerase II (PolII) [12], [13]. Alternatively, many miRNA precursors can be also encoded within introns of protein coding genes [14], [15]. Maturation of miRNAs involves recognition of the pri-miRNA transcripts by the RNA binding protein DGCR8 and subsequent cleavage by the ribonuclease (RNAse) III-type endonuclease Drosha [16]. The cleavage event takes place in the nucleus and produces ~70 nt long hairpin structures termed pre-miRNAs. Pre-miRNAs are then transported to the cytoplasm with the help of the shuttling protein Exportin-5 [17]. In the cytoplasm, pre-miRNAs are further processed by another RNAse III-type endonuclease, Dicer, into 22-nucleotide long miRNA duplexes [18]. In the plant miRNA biogenesis pathway both of these processing steps are performed in the nucleus by one enzyme - Dicer homolog DCL1 (Dicer-like 1) [19]. Dicer processing is coupled to loading of one of the strands of the miRNA duplex onto the RISC effector complex [20].

The key factor of the RISC complex is the aforementioned Argonaute protein, which is directly binding the single stranded miRNA [18]. In animals, miRNAs commonly show a limited complementarity to their targets, which is the highest in the so-called ‘seed region’ in the 5’ end of the small RNA sequence [21], [22]. This imperfect binding implies that one miRNA can regulate many different cellular transcripts. It is generally believed that imperfect binding between the target RNA and the miRNA-loaded RISC induces translational repression of the target mRNA, followed by the transcript deadenylation and degradation [23], [24].

In plants, miRNAs exhibit almost perfect complementarity to the target mRNAs and therefore can induce a direct cleavage of their targets, like typical siRNAs (see below). However, regulation by translational repression is also frequently observed in plants [25].

siRNA-mediated silencing

Small interfering RNAs (siRNAs) constitute another class of small RNAs. siRNAs are 21-24 nt long and they are usually produced from exogenous, long, linear dsRNAs by the endonuclease Dicer [9]. Commonly, in the natural environment such dsRNA arrives to the cell for instance through a viral infection or it is transcribed in the cell after transposon invasion or derepression. Upon Dicer cleavage, the siRNA duplex is loaded onto the Argonaute protein, but only one siRNA strand is retained while the other is destroyed [26], [27]. Such a minimal siRNA-Ago complex is capable of recognition, binding and repression of a target RNA molecule. Importantly, siRNAs bind their targets with full complementarity. Such binding results in destruction of the target RNA through a direct endonucleolytic cleavage by the 'slicer' activity of the Argonaute protein [28]. Mismatches in the siRNA-target RNA duplex around the cleavage site prevent the 'slicer' activity and abolish direct degradation of the target [26]. Importantly, siRNA-triggered PTGS can be very potent due to the siRNA amplification mechanism that exists in some organisms. In such an amplification loop, dsRNA induces the generation of secondary siRNAs through the activity of RNA-dependent RNA polymerase (RdRP) [29].

In addition to the RNAi triggered by exogenous dsRNA, multiple cases of siRNA production from endogenous dsRNA were reported in animals [30]. Probably the most striking example of endogenous siRNA function is the siRNA-dependent silencing of endogenous transposable elements (TE) in *Drosophila* somatic cells that lack the piRNA pathway [31] (see below). Other reported sources of endogenous siRNAs include overlapping antisense transcripts, inverted repeats or bi-directionally transcribed pseudogenes [30].

3.1.3 RNAi-mediated CDGS and heterochromatin assembly in fission yeast

In *S. pombe*, the RNAi pathway is involved in formation of heterochromatin. Classically, the term heterochromatin refers to the more compact, inaccessible and transcriptionally inactive form of DNA in the nucleus [32]. Several chromatin modifications like histone marks or DNA methylation play a cooperative role in order to convey this silent state. Most notably for the study presented in this dissertation, in *S. pombe* heterochromatin is identified as the chromatin regions rich in methylation of histone H3 lysine 9 (H3K9) [32]. Di- and trimethylated H3K9 is recognized by chromodomains of the conserved Heterochromatin Protein 1 (HP1) proteins [33]. By binding to the methylated H3K9, HP1 proteins contribute to maintaining the silent chromatin.

Heterochromatin assembly in fission yeast was the most extensively studied example of small RNA-mediated Transcriptional Gene Silencing (TGS). In the classical understanding of siRNA-mediated TGS, siRNA-directed chromatin modifications repress target genes by drastically reducing their transcriptional activity and making the chromatin inaccessible to the transcriptional machinery. An important feature of siRNA-mediated TGS emerges here - it should occur on chromatin, as the TGS-associated chromatin modifications must be localized and directed towards the right loci that are ‘addressed’ by siRNAs. Therefore, it is believed that siRNAs recruit the effector complexes to their target genes by interactions with nascent transcripts that are still associated with chromatin and RNA Polymerase [34].

Contrary to the traditional understanding of RNAi-mediated chromatin repression by TGS, it quickly became evident that heterochromatin is not completely devoid of active transcription [34] (See below). It was proposed that the RNAi machinery confers the silent chromatin state by directing transcriptional silencing, as well as by mediating on-chromatin degradation of nascent heterochromatic transcripts [35], [36]. Thus, RNAi-mediated repression of heterochromatin is a combined effect of TGS and co-transcriptional gene silencing (CTGS), which can be together referred to as chromatin-dependent gene silencing (CDGS).

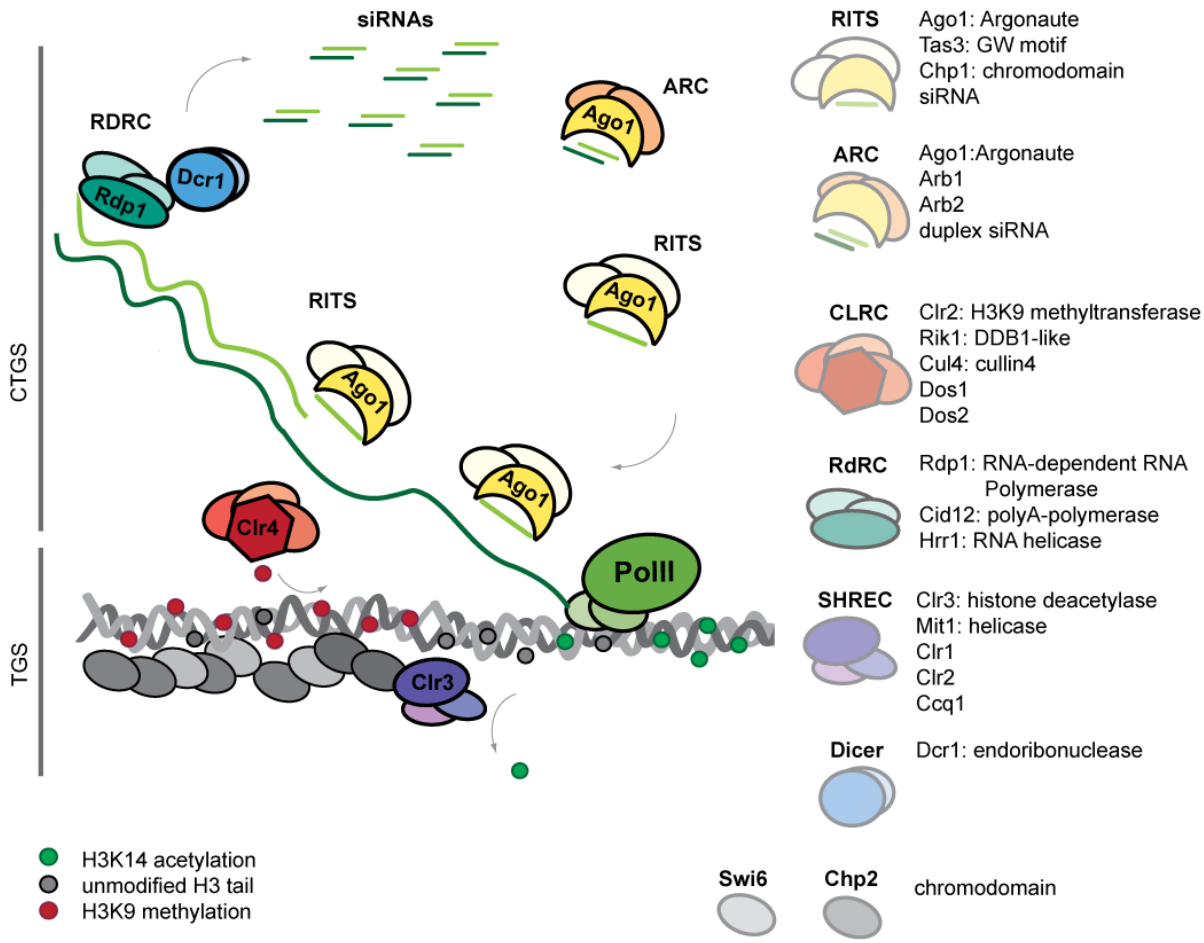

Figure 1. Model for RNAi-mediated heterochromatin formation at centromeric repeats in *S. pombe*. In *S. pombe* repression of centromeric repeats is an interplay between CTGS and TGS mechanisms and involves a self-reinforcing positive feedback loop coupling H3K9 methylation to small RNA production. The RITS complex is guided to the centromeric repeats by a base-pairing interaction with nascent centromeric transcripts. Upon binding of RITS, the nascent transcript becomes a template for dsRNA synthesis, which is performed by RNA-directed RNA polymerase-containing RDRC. Recruitment of RDRC is facilitated by a direct interaction with RITS. Dcr1 processes dsRNA into siRNA duplexes, which get loaded onto the Ago1 protein within the ARC complex. The Ago1-bound siRNA from the ARC complex undergoes transition into the RITS complex and can target nascent transcripts again. RITS complex recruits the H3K9 methyltransferase Clr4 associated within the CLRC complex. Clr4 methylates H3K9, which creates a binding platform for the HP1 proteins Swi6 and Chp2. The HP1 proteins promote recruitment of the Clr3 histone deacetylase present within the SHREC complex and deacetylation of histones restricts access to chromatin for RNA PolII. RITS binding to chromatin is enhanced by direct interaction with methylated H3K9 via Chp1. Recruitment of the CLRC complex to the chromatin is also enforced by direct binding of the H3K9-methylated nucleosomes by the chromodomain of the Clr4 methyltransferase.

RNAi-mediated heterochromatin formation in S. pombe

In the *S. pombe* genome one can distinguish three regions of constitutive heterochromatin: centromeres, telomeres and the mating type locus [32]. Although in each of them the mechanism of silencing is slightly different, they all constitute repetitive regions and share homologous sequences called *dg* and *dh* repeats that can serve as heterochromatin nucleation centers. RNAi-mediated heterochromatin formation at *S. pombe* centromeres was extensively studied over the past years and the role of individual RNAi components in this process, in particular Dcr1, Ago1 and Rdp1, is very well established [37] (Figure 1).

Fission yeast centromeres consists of innermost repeats (*imr*), followed by outermost repeats (*otr*) located in a mirrored orientation on both sides of the chromosome center [32]. *otr* comprise the aforementioned *dg* and *dh* repeats, which contain regions homologous to the other constitutive heterochromatin loci. *otr* are bi-directionally transcribed by the RNA PolII [38], [39]. The product, long non-coding double-stranded transcripts are processed into siRNAs by the RNA endonuclease Dicer (Dcr1) [40]. Single stranded siRNAs are then loaded onto the RNA-Induced Transcriptional Silencing complex (RITS; consisting of the Argonaute protein Ago1, the chromodomain-containing protein Chp1 and Tas3, [41] through the intermediate Argonaute siRNA Chaperone complex (ARC; consisting of Ago1, Arb1 and Arb2 [42]). RITS is subsequently guided to chromatin by a base-pairing interaction between the Ago1-bound siRNA and the nascent transcript transcribed from the centromeric repeats [35], [41]. Upon establishment of this interaction, RITS recruits CLRC (Clr4-Rik1-Cul4 Complex, including the Cullin-4 ubiquitin ligase), which brings the sole *S. pombe* H3K9 methyltransferase Clr4 to the centromeric repeats [43]. Methylation of H3K9 by Clr4 provides in turn a binding site for the HP1 homologues Swi6 and Chp2 [32].

HP1 proteins bound to H3K9me serve as recruiting platforms for Histone Deacetylases (HDACs), and by this contribute to TGS. It was shown that Swi6 and Chp2 recruit two important complexes: the Snf2-HDAC-containg complex (SHREC; which consists of the Clr3 histone deacetylase and the Mit1 helicase) and the Clr6 deacetylase complex to the centromeric repeats [44]. Clr3 is responsible for deacetylation of histone H3 lysine 14 (H3K14), whereas Clr6 is a histone deacetylase with broader specificity. These HDACs not only promote histone hypoacetylation, but also contribute to chromatin remodeling into a

more compact, repressive form [44], [45]. As a result, the access of RNA PolII to chromatin is restricted and transcription of centromeric repeats is limited.

Recruitment of CLRC to heterochromatin is also reinforced by H3K9 methylation, as Clr4 itself contains an H3K9me-binding chromodomain. The whole process is amplified through the activity of the RNA-directed RNA polymerase (Rdp1). Rdp1 mediates the synthesis of dsRNA that gets further processed by Dcr1 to enrich the pool of centromeric siRNAs [46]. Rdp1 is a part of the RNA-dependent RNA polymerase complex (RDRC), which consists of Rdp1, the polyA-polymerase Cid12 and the RNA helicase Hrr1. Importantly, not only deletions of the genes encoding for RNAi factors cause a decrease in H3K9 methylation at the centromeric repeats [47], but also loss of the H3K9 methyl mark causes a decrease in the small RNA pool [46], [48]. This interdependence can be explained by the fact that recruitment of RDRC is promoted by the RITS complex [49]. In turn, interaction of the RITS complex with heterochromatin is strengthened by the chromodomain-containing Chp1. In this way, the whole process is potentiated through a self-reinforcing loop, which couples siRNA biogenesis to high levels of histone methylation, and in consequence to CDGS.

As mentioned above, centromeric silencing in *S. pombe* is a result of the cooperative action of CTGS and TGS, rather than a simple consequence of transcriptional shutdown. It was observed that loss of the SHREC complex results only in partial derepression of centromeric repeats, as compared to the complete loss of H3K9 methylation in the *clr4Δ* strain. However, the increase in PolII occupancy was comparable in both mutant situations [46]. This can be explained by the contribution of the RNAi machinery to degradation of a portion of centromeric transcripts directly on chromatin. In addition to the RNAi machinery, other factors are supposedly involved in the degradation of the nascent heterochromatic transcripts. In particular, the non-canonical polyA polymerase Cid14 was suggested to target the centromeric transcripts for degradation by the nuclear RNA surveillance machinery and the RNAi pathway as well [36].

3.1.4 Nuclear RNAi pathways and CDGS in other eukaryotes

Nuclear RNAi mediates chromatin and DNA modifications also in higher eukaryotic organisms [37]. RNAi-mediated CDGS was first described in *Arabidopsis thaliana*, [50], [51], yet the role of nuclear RNAi in chromatin silencing was quickly expanded by examples from *C. elegans*, *Drosophila* and mammals [37].

RNA-directed DNA methylation in plants

In *A. thaliana*, siRNAs direct establishment and maintenance of DNA methylation [52], [53]. This process, called RNA-directed DNA methylation (RdDM) resembles in many aspects the RNAi-mediated TGS pathway from *S. pombe*. In *Arabidopsis*, 24nt long siRNAs that mediate RdDM usually derive from repetitive elements of viral or centromeric origin [52]. The silencing cycle is initiated by exogenous dsRNA molecules, or when the plant specific RNA PolII homologue RNA PolIV transcribes precursor RNAs, which are used as templates by the RdRP RDR2 and turned into dsRNA molecules [54]. dsRNAs are subsequently processed into siRNAs by Dicer-like 3 protein (DCL3) [55]. These siRNAs are then loaded onto the plant Argonaute proteins, including the Ago4 [56]. siRNA-loaded Ago4 is guided via base-pairing interactions to nascent transcripts produced by another homologue of PolII – RNA PolV [57]. The target recognition and binding is supported by a direct interaction between Ago4 and the GW repeats of the C-terminal domain of RNA PolV [58]. Ago4, through an interaction with the RDM1 protein recruits the *de novo* DNA methyltransferase DRM2 to the loci transcribed by RNA PolV, which leads to establishment of DNA methylation and CDGS [59].

It is believed that RdDM and TGS are also potentiated through a self-reinforcing loop that involves siRNAs, DNA methylation and H3K9 methylation [60]. Genomic regions undergoing RdDM in *A. thaliana* are also enriched with H3K9me marks [60]. siRNA-directed DNA methylation is required for recruitment of the plant H3K9 methyltransferase SUVH4 (also known as KYP), and small RNAs were shown directly to be necessary for high levels of H3K9 methylation [61]. In turn, it was also suggested that SUVH4 and its homologues contribute to the maintenance of RdDM [60]. These findings suggest a complex interplay between the components of RdDM pathway, but also point

towards the importance of epigenetic feedback loops in maintenance of a heritable chromatin state.

RNAi-mediated H3K9 methylation in somatic cells of C. elegans

In somatic cells of *C. elegans*, exogenous dsRNA triggers a classical RNAi response, in which dsRNA is processed into primary siRNAs by the Dicer homologue Dcr-1 [62], [63]. Primary siRNA are bound by the Argonaute homolog RDE-1 and elicit siRNA-mediated PTGS (outlined in section 3.1.2). However, some of the RDE-1-bound siRNAs, with the help of the RdRP homologue RRF-1 induce generation of secondary siRNAs called 22G-RNAs [64]. In the cytoplasm, 22G-RNAs are then loaded on several Argonaute proteins and can further support PTGS initiated by the primary siRNAs. However, when a 22G-RNA gets loaded on the specific Argonaute homologue NRDE-3, it guides it to nascent transcripts, where they recruit the silencing factor NRDE-2 that promotes H3K9 methylation and inhibits transcription elongation [65]. This silencing pathway is known as the NRDE pathway. It was reported that transcriptional silencing by the NRDE pathway could be inherited through generations, yet the silent state was later described as not very stable [66], [67]. Much more evidence for stable epigenetic silencing mediated by small RNAs comes from the germline of *C. elegans*, where another class of small RNAs called piRNAs confers heritable TGS, sharing some nuclear components of the aforementioned NRDE pathway [67]. I will discuss germ line nuclear RNAi in *C. elegans* further in the Discussion.

piRNA-mediated control of transposable elements

piRNAs constitute a distinct class of small RNAs that play a key role in suppression of transposable elements (TE) during animal germline development. piRNAs are longer than other small RNAs, ~24-32 nt long. They are bound by a clade of Argonaute proteins called the PIWI proteins, and most importantly, they do not depend on the activity of Dicer nucleases for their biogenesis [68]. Our current understanding of the piRNA pathway comes from studies in *Drosophila melanogaster* and mice [69]. piRNA biogenesis begins with the production of primary piRNAs from long precursors that are transcribed from genomic loci

called piRNA clusters [70]. Primary piRNAs are antisense to the transcripts of TE and, upon loading on the PIWI proteins Aubergine (Aub) or Piwi, they can mediate cleavage of the transposons in a classic PTGS event. The initial cleavage event is not only destroying the active transposon, but also providing a sense transposon transcript for the so-called piRNA ping-pong cycle, in which the sense and antisense piRNAs can be amplified to potentiate the silencing response. The ping-pong cycle was first proposed based on extensive small RNA deep-sequencing experiments performed with *Drosophila* ovaries [70], [71]. In the *Drosophila* germline, the PIWI proteins Piwi and Aub associate predominantly with piRNAs antisense to TE, whereas the third PIWI protein, Ago3, is mainly loaded with sense piRNAs [70]. It was observed that sense and antisense piRNAs originating from the same TE overlap precisely by 10 nt. This position corresponds to the distance at which the target RNA molecules are cleaved by piRNA-loaded PIWI proteins. In the ping-pong cycle model, antisense piRNAs direct Aub to cleave the sense TE transcripts and the remaining cleavage products serve as the source of secondary, sense piRNAs. Sense piRNAs associate with Ago3 and mediate the subsequent cleavage of antisense transcripts derived from piRNA clusters. Importantly, only piRNAs against actively transcribed transposons will trigger the ping-pong cycle to amplify the response [70]. However, since primary piRNAs are produced from piRNA clusters, the basic defense line is always present in the cell. Importantly, it was shown in *Drosophila* that, in addition to conferring the classical PTGS towards active transposons, piRNAs also target TE at the transcriptional level [72]. The piRNA-loaded Piwi protein directs the deposition of H3K9 trimethylation at the transposons in the genome. In turn, the HP1 homologue Rhino recognizes the H3K9 trimethylated loci and, together with the Cutoff protein, marks them as templates for the production of piRNA precursors [73]. The existence of the piRNA pathway has also been confirmed in zebrafish, *Xenopus* and mammals. Importantly, the role of piRNAs in transcriptional silencing of transposons via de novo DNA methylation was also confirmed in mice [71], [74]. Taken together, the piRNA pathway provides another great example of a positive feedback loop, which combines interdependent chromatin modifications and small RNA production to elicit efficient transposon silencing through a combination of TGS, CTGS and PTGS.

3.1.5 Inducible RNAi-mediated gene silencing *in trans*

The phenomenon of RNAi is not only an interesting regulatory mechanism, but it has proven itself as a very powerful technology that advanced modern genetics. Shortly after the RNAi phenomenon was described, scientists learned how to use small RNAs to induce gene silencing at a post-transcriptional level with synthetic siRNAs [75]. However, in order to obtain a stable and heritable effect, this method requires constant supply of the small RNAs. To circumvent this problem, hairpin-expressing constructs were developed and became a tool, which until recently was the method of choice for long-term gene silencing [76], [77]. However, when using hairpin constructs, maintenance of the silent state relies on integration of the construct into the genome. Lately, more efficient alternatives have become available [78]. Genome-editing technology based on bacterial-origin TALENs (transcription activator-like effectors nucleases) turned out to be a very feasible method to interfere with gene expression. TALENs can be engineered to bind almost any genomic sequence and mediate its cleavage, which can be subsequently repaired using a provided template DNA. As a result, one can generate gene knockouts, conditional alleles and tagged genes of choice in a relatively fast and efficient way. Most recently, a new genome-editing approach using the CRISPR-Cas9 system has been developed. CRISPR-Cas9 uses RNA molecules to target the desired sequence, so it is much easier to adapt for editing different genomic sequences and circumvents the need for designing and cloning new TALENs for every experiment. However, both of the approaches involve induction of permanent and irreversible changes in the sequence of the gene [78].

Better understanding of the nuclear RNAi pathways in fission yeast and plants prompted the idea to use small RNAs to induce transcriptional gene silencing of desired genes. The obvious advantages of this system would be the heritability and reversibility of the repression, which could not be achieved by the approaches mentioned above. In *S. pombe*, the first attempts to use hairpins to induce gene silencing led to a conclusion that ectopic siRNAs can induce RNAi-mediated silencing of a euchromatic reporter, but at the post-transcriptional level [79]. Importantly, despite all the factors being present, the provided hairpin did not induce CDGS. The induced effects were not very strong, but it was an interesting observation as it showed that the yeast RNAi machinery can be programmed with any sequence to silence gene expression. However, the observations made by Zamore and

colleagues [79] could not be reproduced by other groups including ours (see the Results section and Figure 5 and 6).

Later on it was shown that siRNA-mediated TGS of chosen genes is highly dependent on several conditions. At first, the *ura4*+-targeting hairpin was shown to induce silencing of the *ura4*+ gene, but only upon overexpression of the HP1 protein Swi6 [80]. Furthermore, it was observed that maintenance of the silent state required constant presence of the small RNA source. It was subsequently suggested that siRNA-mediated gene silencing *in trans* was under negative control of the small RNA nuclease Eri1, and could be enhanced when the *eri1*+ gene had been deleted [35]. Finally, a study by Allshire and colleagues suggested that siRNAs could induce gene silencing by H3K9 methylation *in trans*, but only at a small number of genomic loci and the silencing effect was weak and unstable [81]. The authors observed stronger silencing when the *ura4*+ gene was inserted in close proximity to constitutive heterochromatin and no silencing at all at the endogenous *ura4*+ locus. Importantly, all the studies mentioned above use the *ura4*+ gene as a reporter, and test the expression of this reporter with a silencing assay on medium containing 5-Fluoroorotic acid (5-FOA). 5-FOA is toxic to cells when the *ura4*+ gene is active, so performing a silencing assay by growing cells on the 5-FOA-containg plates may create selective pressure and give a false idea of the efficiency of the process.

The potential of siRNAs to direct heritable heterochromatin states has also been tested in plants. With pathways executing siRNA-mediated DNA methylation and DNA methylation-dependent chromatin modifications, *Arabidopsis* seems to be a very promising model system to induce heritable TGS. Initial experiments confirmed that, by providing a promoter-targeting hairpin as a source of dsRNA, it was possible to induce DNA methylation of a provided transgene [82]. Subsequent studies provided more evidence for siRNA-directed DNA-methylation, yet it was shown that susceptibility of individual transgenes to such modifications depended on their pre-existing chromatin state [83]. The authors suggested that transgenes containing tandem repeats recruit the siRNA-producing machinery, but TGS might be only established once the target locus had already some pre-existing DNA methylation marks.

Reports of siRNA-mediated TGS in mammalian cells have been rather ambiguous. Several groups tested the idea of siRNA-mediated programmable TGS by providing synthetic siRNAs targeting promoters of chosen genes, but the results they obtained were sometimes contradictory. It was shown that transient transfection with synthetic siRNAs can lead to

induction of low levels of H3K27 methylation and H3K9 methylation at the *RASSF1A* gene promoter [84]. The enrichment of histone marks correlated with an increased occupancy of Ago1 and the EZH2 histone methyltransferase, but no evidence was provided for the role of these chromatin modifications in gene repression. On the contrary, the authors observed that the siRNA-mediated repression was dependent on the TRBP2 protein, a component of the RISC complex from the canonical siRNA pathway (See section 3.1.2). The authors reported also an accumulation of low levels of DNA methylation, but only if the transient transfection was replaced by a stable integration of a hairpin construct expressing promoter siRNAs. Therefore, neither the contribution of TGS to the observed repression of the *RASSF1A* gene, nor the heritability of the induced state could be concluded from this study.

On the other hand, other groups who also provided examples of siRNA-mediated repression of gene promoters observed opposite effects on DNA methylation [85]. Finally, the most recent studies support a different model of siRNA-mediated TGS, in which promoter-directed siRNAs induce gene repression by interfering with the assembly of the pre-initiation complex [86], [87].

Taken together, despite our good understanding of siRNA-mediated regulation of gene expression, the efforts to apply this knowledge to induce stable and heritable repression of desired genes encountered some inherent difficulties.

3.2 The Paf1 complex

3.2.1 Discovery, composition and conservation of the Paf1 complex

The Polymerase-Associated Factor 1 (Paf1) complex is a highly conserved protein complex associated with RNA PolII, which is believed to regulate multiple aspects of the PolII transcription cycle. The Paf1 complex was initially found in *Saccharomyces cerevisiae* through an affinity purification experiment that aimed at identification of general transcription factors associated with RNA PolII [88]. Initially, two subunits of the complex had been found – Paf1 and Cdc73. Subsequently, several groups showed that Paf1 forms a nuclear complex with four other proteins: Ctr9, Rtf1, Leo1 and Cdc73, and confirmed the association of the complex with PolII by biochemical and proteomic approaches [89–91]. Importantly, a vast majority of the current knowledge about Paf1 complex composition and

function has been obtained from studies performed in budding yeast. These findings serve us as a starting point for our work on the Paf1 complex in *S. pombe*.

The amino acid sequence of the Paf1 complex components, as well as the interactions between the Paf1 complex subunits are generally well conserved from yeast to higher eukaryotes, including humans [92], [93] However, some small differences in the composition of the complex were reported. The Rtf1 homolog was found to be rather loosely associated with the complex in higher eukaryotes including human, therefore it is not considered as the core component in these organisms [92–94]. Furthermore, an additional protein – Ski8 was reported to form the core human Paf1 (hPaf1) complex and to associate with Paf1 on chromatin [92]. Ski8 is also a component of the human SKI complex, which is involved in 3' to 5' end mRNA degradation events [95]. Identification of a stable association between the conserved Paf1 components and hSki8 implicated a role for the hPaf1 complex in RNA surveillance mechanism in the cellular nucleus [92].

In fission yeast, the core Paf1 complex comprises Paf1, Leo1, Cdc73 and Tpr1 (homolog of *S. cerevisiae* Ctr9) [96]. Unlike in *S. cerevisiae*, the Rtf1 homolog (which in the fission yeast is called Prf1) does not form a stable association with the other four subunits [96]. This finding is consistent with the composition of the Paf1 complex in metazoans, arguing for *S. pombe* as a more suitable model organism to study functions of the Paf1 complex, if one wants to extend the findings and conclusions to the higher organisms.

3.2.2 Interactions of Paf1 complex with RNA PolII and chromatin

The Paf1 complex is physically associated with RNA PolII. It has been shown that the yeast Paf1 complex interacts with PolII through two subunits, Cdc73 and Rtf1 [90], [97]. Cdc73 is believed to serve as a direct contact point with the polymerase, whereas the interaction with Rtf1 probably occurs through Spt5, a component of the DSIF (DRB sensitivity inducing factor) transcription elongation factor [97]. Mutations of any of these two subunits result in dissociation of the complex from chromatin, however the remaining subunits are believed to remain in association with each other [89], [98]. For the hPaf1 complex, direct interactions with RNA PolII were reported for Paf1 and Leo1 [99] (See Figure 2).

Studies on single gene examples showed that the *S. cerevisiae* Paf1 complex associates together with PolII at the promoters, transcription start sites (TSS) and along the open reading frames (ORF) of active genes [100]. However, a genome-wide meta gene analysis revealed that the Paf1 distribution along the gene body resembles a typical elongation factor pattern – with high enrichment starting over 100 base pairs downstream to the TSS and dropping abruptly at the polyadenylation (polyA) site [101]. In contrast, RNA PolII continues downstream and remains associated with chromatin until the transcription termination signal. Importantly, as demonstrated by the same study, Paf1 chromatin occupancy correlates with profiles obtained for general transcription elongation factors on active genes, and the Paf1 complex enrichment on chromatin is proportional to the activity of the gene [101]. In our lab, we also assayed genome-wide binding of the Paf1 complex to chromatin in fission yeast and mouse cells (Alex Tuck, personal communication) and obtained a similar profile of Paf1 distribution along the genes. However, we still know little about recruitment of the Paf1 complex in the fission yeast.

3.2.3 Recruitment of the Paf1 complex

In the budding yeast, recruitment of the Paf1 complex to chromatin is believed to occur through the yeast homolog of the pTEFb complex, comprising of the cyclin-dependent kinase Bur1 and its partner cyclin - Bur2 [102]. The Bur1 kinase has multiple substrates, including, most notably, the Rpb1 subunit of RNA PolII [103]. Bur1 is well known to be responsible for phosphorylation of Serine 2 (Ser-2) of the Rpb1 C-terminal domain (CTD), which is an important step in licensing RNA PolII for transition from transcription initiation to elongation [103]. Bur1-mediated phosphorylation of another important substrate – Spt5 – is necessary for recruitment of the Paf1 complex to chromatin [104], [105]. Spt5 together with Spt4 form the DSIF transcription elongation factor, which associates with PolII at the beginning of ORFs [104], [105]. The phosphorylated Spt5 is believed to mediate the interaction between RNA PolII and the Paf1 complex through its subunit Rtf1 [106]. This model is also consistent with observations made in higher eukaryotes.

A second, possibly redundant mechanism of recruitment was recently postulated in *S. pombe* [96]. As mentioned above, in fission yeast the Prf1 (Rtf1) subunit is not stably associated with the rest of the complex. It was observed that phosphorylation of Spt5 by the Bur1 kinase

homolog Cdk9 is required for Prf1 recruitment to chromatin, but recruitment of the remaining components of the Paf1 complex is dependent on some other substrate of Cdk9, likely the CTD of Rpb1. Thus, according to this model, recruitment of the Paf1 complex to chromatin would occur in two steps. Different mechanistic requirements for the recruitment of Prf1 and the rest of the complex could have implications in distinct functions of this subunit in *S. pombe*.

Several other recruitment models have been proposed for budding yeast and higher eukaryotes. Single locus studies and *in vitro* assays point at the role of phosphorylation status of Ser-2 and Ser-5 in the CTD of Rpb1 [107], as well as the Spt6 kinase [108], the components of the FACT chromatin remodeling complex [109], and the Not4 component of the Ccr4-Not complex [110].

Taken together, these and other evidence show that the exact recruitment mechanism of the Paf1 complex remains unclear and most likely, is a combinatorial effect of multiple steps and contact points between the Paf1 complex, transcription factors and the RNA PolII itself.

3.2.4 Genetic properties of the Paf1 complex and intracomplex interactions

Mutations causing deletions of individual components of the Paf1 complex are viable in both *S. cerevisiae* and *S. pombe* [96], [111]. In contrast, in higher eukaryotes Paf1 complex is essential for viability, as homozygous mutations of any of the Paf1 complex genes are lethal at an early embryonic stage [93], [112].

Although mutations of the individual components are viable, different mutant strains exhibit phenotypes of different strength in both fission and budding yeast. Notably, it was reported that deletions of Paf1 and Ctr9 in the budding yeast show the most severe growth phenotype under various growth conditions [111]. Curiously, certain double combinations of the severe *paf1*Δ or *ctr9*Δ mutations with the milder *leo1*Δ or *rtf1*Δ lead to partial rescue of growth sensitivity in stress conditions like DNA damage, heat shock, osmotic stress or caffeine-induced stress [89].

In *S. cerevisiae* it was shown that loss of individual components of the complex had a consequence on the cellular levels of the other subunits[113]. The authors performed a comprehensive analysis of these interdependencies at the protein level. Most notably, the

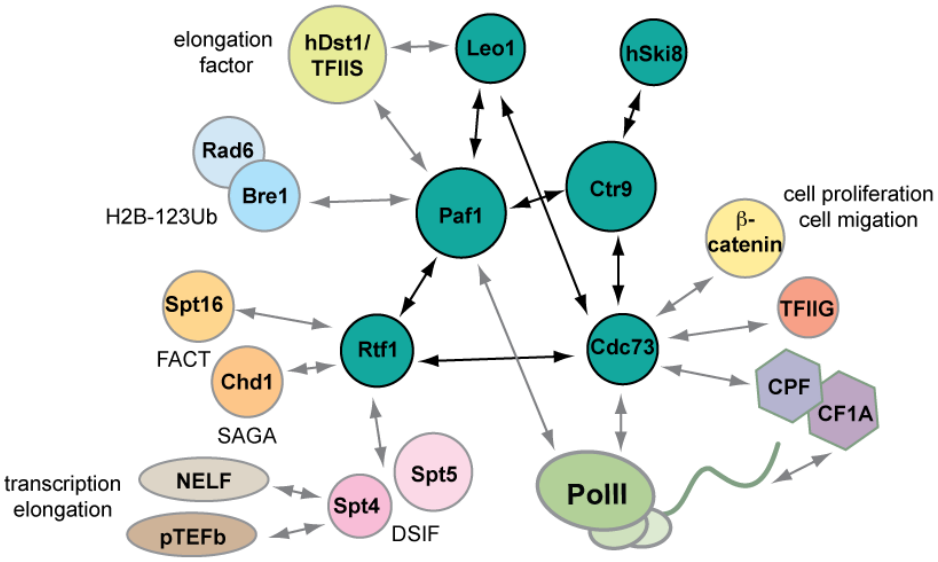

Figure 2. Summary of inter- and intracomplex interaction partners of the Paf1 complex identified in budding yeast, Drosophila and human. Recreated based on [112].

presence of Ctr9 is necessary for normal expression levels of all the other subunits, and the loss of Paf1 affects the levels of all other components except for the Leo1. These observations are consistent with the fact that the Ctr9 and Paf1 mutants exhibit the most severe growth phenotypes [111]. Presumably, in these mutants, the integrity of the complex is the most affected, whereas deletions of the other subunits still allow at least partial activity of the complex.

Figure 2 illustrates a Paf1 complex interaction map created based on the data obtain for the yeast, *Drosophila* and human Paf1 complex [114]. Many of the depicted interactions are conserved in eukaryotes. Paf1 appears to be the most central subunit of the complex, as it interacts with PolII and all remaining subunits of the complex except for Ski8. Unfortunately, such a comprehensive study for the fission yeast Paf1 complex is not available.

The phenotypic differences described above imply that the contribution of the individual components to the Paf1 complex activity might be different, or they may elicit additional roles in the cell. Although not much functional evidence has been acquired so far, both in the budding and fission yeast the Rtf1 homolog has been suggested to play a Paf1-independent role in chromatin remodeling and histone modifications [96], [115].

3.2.5 Main molecular functions of the Paf1 complex

The Paf1 complex serves multiple functions in contributing to the efficiency of transcription by Pol II and formation of the 3'end of cellular mRNAs. Many of these roles are conserved from yeast to human. In particular, gathered evidence strongly supports the role of the Paf1 complex in facilitating transcription elongation, recruitment of chromatin modifiers responsible for transcription-coupled chromatin modifications, as well as efficient transcription termination and production of functional, stable mRNAs. Although connections of Paf1 complex to other cellular processes have been suggested [116], in this introduction I will focus only on the conserved molecular functions of the Paf1 complex that are related to transcription and general RNA expression.

Role of the Paf1 complex in promoting CTD phosphorylation

The C-terminal domain (CTD) of the large subunit of RNA Polymerase II – Rpb1 contains tandem repeats of a sequence of seven amino acids, which are modified in a specific order throughput the transcription cycle. When transcription begins, the CTD of a newly recruited Rpb2 is not phosphorylated. Upon transcription initiation, CTD repeats are phosphorylated on serine 5 (Ser-5) and serine 7 (Ser-7) and subsequent phosphorylation of serine 2 (Ser-2) residues contributes to regulation of the transcription cycle, and transition from the initiation to the elongation phase. A modified CTD serves as a platform for the recruitment of transcriptional regulators specific for different phases of the transcription cycle, thanks to the different specificities towards the CTD phosphorylation pattern. Paf1 complex has been shown to contribute to the proper levels of Ser-2 phosphorylation within the ORFs of actively transcribed genes [98], [113]. This, in turn, has consequences in recruitment of downstream factors that shape the proactive transcription environment, like in the case of the Set2 methyltransferase, which governs methylation of histone H3 lysine 36 (H3K36) in the ORF of actively transcribed genes [117].

Contribution of the Paf1 complex to co-transcriptional chromatin modifications

In the genomic context histone proteins that form nucleosomes are frequently modified on the post-translational level in patterns reflecting the transcriptional activity of the underlying genes [118]. Multiple studies linked the activity of the Paf1 complex to several of these histone modifications. Importantly, roles of the Paf1 complex in transcription-coupled chromatin modifications have been well established both in yeast and human cells, pointing towards high conservation of these molecular functions. In both budding yeast and human, the Paf1 complex has been shown to promote histone H2B lysine 123 monoubiquitination through recruitment of the ubiquitin-conjugating enzyme Rad6 and the ubiquitin ligase Bre1 [119]. Importantly, monoubiquitination of this residue is necessary for another chromatin modification – Set1-mediated methylation of H3K4, which is enriched at gene promoters and 5' regions of CDS [120]. Stimulation of the H2BK123 ubiquitination by the Paf1 complex is also a prerequisite for H3K79 methylation [121]. The role of the Paf1 complex in deposition of both of these modifications is well described [122] and conservation of all of its molecular functions in governing these processes was shown in human cells [99], [109].

Histone H3 K36 trimethylation, a chromatin mark located in the bodies of actively transcribed genes was also shown to be dependent on the Paf1 complex components Ctr9, Cdc73 and Paf1 in yeast [123]. However, the role of the Paf1 complex in this process is indirect, as it was shown that recruitment of the Set2 methyltransferase, which methylates H3K36, depends on Ser-2 phosphorylation [117] of the RNA Pol II CTD and the Paf1 complex is necessary for the high levels of CTD phosphorylation at the actively transcribed genes [113].

Taken together, the Paf1 complex seems to play a highly conserved role by having a direct and indirect influence on the status of chromatin at actively transcribed genes. Paf1 shapes the local chromatin landscape by supporting proper distribution of histone modifications. Histone marks contribute to an open chromatin structure by themselves, but they are also read by chromatin factors that recognize modification patterns and further facilitate gene transcription. Therefore, Paf1 plays an important role in creating a right transcription environment and ensuring the proper gene expression. Contribution of the Paf1 complex to correct patterns of histone modifications has also been confirmed in plants, where Paf1 was shown to be crucial for correct distribution of the H3K4 trimethylation and H3K36

dimethylation [124]. Effects of the Paf1 deficiency were especially prominent for highly transcribed genes like the family of FLC, whose expression is highly dependent on the Paf1 complex.

Role of the Paf1 complex in regulation of transcription elongation

Although the Paf1 complex was initially identified as an elongation factor [125], its connection to transcription elongation is often based on co-purification studies, rather than on direct evidence. For example, the Paf1 complex was shown to associate with the FACT complex (Facilitates Chromatin Transcription), which is a highly conserved factor acting as a histone chaperone promoting transcription through nucleosomes in the chromatin environment [126]. It was suggested that the Paf1 complex contributed to the interaction between RNA PolII and FACT [93]. Furthermore, cooperative interaction between Paf1 complex, PolII and DSIF (DRB Sensitivity Inducing Factor) was also reported [127]. DSIF is another conserved complex involved in regulation of the RNA PolII pausing at the transition from transcription initiation to elongation [103]. Finally, in the human cells a cooperative effect of Paf1 complex and the TFIIS elongation factor on transcription elongation was described.

The reported physical interactions with the important elongation regulators like FACT in *Drosophila*, DSIF and TFIIS in human strongly suggest the involvement of the Paf1 complex in the regulation of transcription elongation *in vivo*. However, it is often difficult to distinguish between direct and indirect effects. For instance, it was suggested that Paf1 contributed to transcription facilitation through the activity of FACT [93]. However, this function was dependent on the Paf1-mediated H2B ubiquitination which stimulates FACT (see above), rather than on the direct Paf1 activity [109].

Direct evidence for the involvement of the Paf1 complex in transcription elongation came from elegant *in vitro* studies. It was shown that the recombinant human Paf1 complex alone and in cooperation with another elongation factor Dst1 facilitate transcription in the *in vitro* transcription reconstitution assays with artificial templates [99]. Importantly, these experiments showed the activity of the Paf1 complex independently of any chromatin modifying activity and thanks to the use of the recombinant transcription complex with a

pre-initiated nascent transcript – that the Paf1 function is specific to the elongation phase of transcription.

Role of the Paf1 complex in transcription termination and RNA 3'end formation

Besides contribution to the transcription process itself, the Paf1 complex conveys interesting functions in transcription termination and processing of the 3' end of the nascent transcript. In yeast, it was observed that deletion of the components of the Paf1 complex results in global reduction of the length of polyA tails of cellular mRNAs [113]. This is an interesting observation since it provides an alternative explanation for the curious fact, that despite the that Paf1 complex is a general transcription regulator, the Paf1 mutant yeast cells exhibit changes in RNA expression levels only for a relatively small subset of genes [128]. In turn, changes in the length of polyA tails could affect the stability for only some mRNAs and explain this modest effect. In addition to shortening of the polyA tails, alternative usage of the polyadenylation signals for several mRNAs was also reported [128]. The authors of this study show that, despite the established role in regulation of transcription, Paf1 complex contributes to the control of the RNA expression through ensuring the proper transcription termination independently of the RNA PolII. On the molecular level, this effect could be explained by the fact that the Paf1 complex mutants show decreased recruitment of the cleavage and polyadenylation factors Pcf1 and Ctf1 [98], [113]. Less evidence for the involvement of the human Paf1 complex in transcription termination is available. However, most importantly, interactions between the Paf1 complex and several components of the Cleavage and Polyadenylation Specificity Factors (CPSF) and Cleavage and stimulation Factor (CstF) complexes were reported in human cells [129]. In agreement with the studies done in yeast, upon depletion of the Paf1 complex components, decrease in chromatin association of the CPSF and CstF, as well as read-through transcription were also observed in human.

Finally, in addition to the involvement in mRNA processing outlined above, the Paf1 complex was also implicated in transcription termination and processing of the newly synthesized small nucleolar RNAs (snoRNAs), possibly through the recruitment of a known factor involved in snoRNA 3'-end formation, an RNA-binding protein Nrd1 [130]. As this

process is mechanistically distinct from 3'end formation of the mRNAs, this study provides an example of another molecular function of the Paf1 complex.

3.2.6 Connections of the Paf1 complex to cancer

Multiple mutations in the genes encoding for the Paf1 complex subunits were found to be associated with cancer. Interestingly, individual subunits of the Paf1 complex were shown to act as both tumor suppressors and oncogenes. This suggests that the role of Paf1 complex in human is quite complex, since the opposite effects of mutations of the Paf1 genes cannot always be explained simply by a loss the Paf1 complex activity in regulation of the transcriptional events.

The subunit of the Paf1 complex most often associated with tumorigenesis is the homologue of the yeast Cdc73 protein called parafibromin in human [131]. Parafibromin, which is encoded by the *CDC73* gene (also known as *HRPT2*), was described as a tumor suppressor

Table 1. Mutations in the *CDC73* gene found in the germline of cancer patients. Table was adapted from [132]

Mutation[a]	Codon[b]	Predicted effect[c]	Clinical manifestation[d]	Reference
3G>C	1	Met1	HPT-JT	[133]
12_31dup	11	Tyr11Cys, fs STOP 17	HPT-JT	[133]
20_24del, insCCCT	7	Val7Ala fs STOP15	HPT-JT	[134]
22delC	8	Leu8Arg fs STOP13	HPT-JT	[135]
25C>T*	9	Arg9STOP	HPT-JT	[132], [133], [136]
30delG	10	Gln10His fs STOP11	HPT-JT	[133]
34_40del	12	Asn12Arg fs STOP7	HPT-JT	[133]
40delC*	14	Gln14Arg fs STOP7	HPT-JT	[133]
62_66del	21	Lys21Arg fs STOP43	FIHP	[137]
76delA*	26	Ile26Ser fs STOP11	HPT-JT	[138]
96G>A	32	Trp32STOP	HPT-JT	[139]
127_128 insC	43	Trp43Ser fs STOP23	sporadic PTC	[140]
131+1G>A	NA	Splice [d]	FIHP	[141], [142]
140_144del	47	Lys47Arg fs STOP17	FIHP	[143], [144]
162C>G*	54	Tyr54STOP	sporadic PTA	[145]
165C>G	55	Tyr55STOP	HPT-JT	[133]
176C>T	59	Ser59Phe #	Sporadic PTC	[146]
188T>C	63	Leu63Pro	FIHP, HPT-JT	[132], [147]
191T>C	64	Leu64Pro	FIHP	[138], [148]
226C>T*	76	Arg76STOP	HPT-JT	[132]
237+1G>C	NA	Splice [d]	FIHP	[148]
238_1G>A	NA	Splice [a]	HPT-JT	[149]
272G>C	91	Arg91Pro #	sporadic PTA	[145]

206_307 del	103	Ser103Asn fs Stop 5	HPT-JT	[133]
343G>T	115	Glu115STOP	sporadic PTC, PTA	[143], [150]
356delA	119	Gln119Arg fs STOP 14	HPT-JT	[133]
375dupA	126	Arg126Thr fs STOP5	sporadic PTA	[151]
374_375 dup	126	Arg126Asn fs STOP8	FIHP	[147]
406A>T	136	Lys136STOP	HPT-JT	[133]
415C>T	139	Arg139STOP	HPT-JT	[152]
518_521del	174	Ser174Lys fs STOP27	sporadic PTC, FIHP	[137], [150]
639delT	213	Phe213Leu fs STOP6	HPT-JT	[133]
668_669delinsG	223	Asp223Gly fs STOP34	HPT-JT	[153]
679_680insAG	227	Asp227Lys fs STOP31	HPT-JT, sporadic PTC, FIHP	[133], [151], [153], [154]
685_688del	229	Arg229Tyr fs STOP27	FIHP	[155]
687_688del	229	Arg229Ser fs STOP37	HPT-JT	[132], [138], [139], [156]
692-693insT	231	Trp231Cys fs STOP36	sporadic PTC	[146]
700C>T	234	Arg234STOP	HPT-JT, sporadic PTC	[132], [142], [157]
745dupA	249	Ile249Asn fs STOP18	FIHP HPT-JT	[132], [157]
765_766del	255	Val255Lys fs STOP10	HPT-JT	[158]
815A>G	272	Asn272Ser #	sporadic PTA	[159]
1124-1125dup	376	Asn376Leu fs STOP10	sporadic OF	[160]
1135G>A	379	Asp379Asn	HPT-JT	[157]
1239delA	413	Gln413His fs STOP15	HPT-JT	[133]

[a]Location of mutations are annotated based on the cDNA of CDC73, assuming that +1 nucleotide corresponds to the A from the ATG translation initiation codon. dup, duplication; del, deletion; ins, insertion; > substitution mutation. * indicate mutations identified also in somatic cells.

[b]+1 codon is the ATG translation initiation codon.

[c] predicted effect of the identified gene mutation at the protein level. Splice [d], donor splice site; Splice [a], acceptor splice site; fs, frame shift; number after STOP annotation indicates at which codon position after the mutation comes the premature stop codon. # marks missense mutations.

[d] Clinical manifestation of the disease. HPT-JT, hyperparathyroidism-jaw tumor syndrome; PTC, parathyroid carcinoma; PTA, parathyroid adenoma; OF, ossifying fibroma; FIHP, familial isolated primary hyperparathyroidism.

that is frequently linked to both sporadic and familial incidents of parathyroid cancer (PTC) [133]. Hereditary forms of PTC are often connected to the hyperparathyroidism-jaw tumor (HPT-JT) syndrome. Multiple mutations in the *CDC73* gene were identified in the families of the patients with HPT-JT syndrome, and these mutations were found to predispose the carriers to parathyroid cancer [151]. Until the year 2010, the total number of 77 different mutations in the *CDC73* gene was described in both sporadic and familial disease incidents [132]. I summarized mutations of the *CDC73* gene that were found in the germline of affected patients in the table below (Table 1). Identified gene variations were most frequently mapped as point mutations or frame-shift insertion and deletions that created a premature stop codon in one of the first exons of the gene [132]. The mutations were frequently present in the N-terminal domain of the protein and resulted either in loss of the protein due to

nonsense-mediated decay of the aberrant mRNA, or in expression of a C-terminal truncated forms of Cdc73 [132]. Interestingly, the missing C-terminal part shares the highest homology to the yeast Cdc73 and was found to be responsible for interaction of the Cdc73 homologue with RNA PolII and the Paf1 complex [131][161]. Such truncation leads to reduction of binding of the entire complex to PolII and its recruitment to chromatin [131], [161].

Table 2. Mutations in the *CTR9* gene found in the Wilms tumor families. Table was prepared based on [162].

Mutation[a]	Codon[b]	Predicted effect[c]	Clinical manifestation[d]	Reference
106C>T	36	Q36STOP	Wilms tumor	[162]
1194+2T>C	Splice [d]	320-398del	Wilms tumor	[162]
1194+3A>C	Splice [d]	320-398del	Wilms tumor	[162]

[a]Location of mutations are annotated assuming that +1 nucleotide corresponds to the A from the ATG translation initiation codon. > substitution mutation; 1194+2 and 1194+3 indicate intronic sequences +2 and +3 nt after the end of the previous exon.

[b]+1 codon is the ATG translation initiation codon.

[c] predicted effect of the identified gene mutation at the protein level. Splice [d], donor splice site; 1194+2T>C and 1194+3A>C result in an in-frame deletion.

[d] Clinical manifestation of the disease. All patients developed Wilms Tumor.

An alternative explanation of the deleterious consequences of the mutation was that the C-terminal truncated form of parafibromin lost its nuclear localization signal (NLS) [161]. Loss of recruitment of parafibromin to chromatin was suggested to cause activation of genes encoding cell cycle regulators and pro-survival factors, what explains the tumor suppressor function of the Cdc73 homologue [162]. In line with the findings presented above, overexpression of the parafibromin gene, but not the mutant allele found in the cancer patients leads to repression of the gene encoding a cell cycle regulator Cyclin D1 [162]. In addition to mutations resulting in truncations of the protein, 11 missense mutations were also identified [132]. Most of them are present in the N-terminal region of the protein and their molecular consequences are unknown. Mutations in CDC73 are not only limited to parathyroid tumors. Patients with HPT-JT syndrome, in addition to parathyroid cancer, develop tumors in other tissues including uterus and kidneys [163].

The gene encoding for a human homologue of the Ctr9 subunit has been also identified as a tumor suppressor gene [164]. Mutations in the *CTR9* gene were found in selected Wilms tumors – a form of pediatric kidney cancer. The Wilms tumor develops usually in very young children from embryonic cells that undergo uncontrolled proliferation and differentiation due

to defects in regulation of cellular development. The authors of this comparative study identified three nonsense mutations that were associated with development of the tumor (see Table 2). More interestingly, they also found two splice site mutations that lead to the exon skipping, resulting in loss of two out of sixteen tetratricopeptide repeat domains that build the Ctr9 protein (see Table 2). Finally, upon comparison of the genetic material from the tumor and another patient's tissue, the authors concluded that most likely both of the CTR9 alleles need to be mutated to result in carcinogenesis and inheritance of only one of them did not cause tumor development [164]. I addition to the Wilms tumor, several reports linked loss of the locus encoding for the human Ctr9 protein in breast cancer, neuroblastoma, and pancreatic cancer [165].

In turn, other subunits of the Paf1 complex were reported to play an oncogenic role. Duplications of the human *PAF1* gene were found in patients with pancreatic and ovarian cancer [165]. Intriguingly, this observation points towards the opposite role of two components of the same complex - Ctr9 and Paf1 in the pancreatic tumors. The oncogenic potential of the *PAF1* gene was also directly confirmed, since the overexpression of the human Paf1 leads to cellular transformation and tumor formation in vitro and in vivo [166]. Duplications of the human *LEO1* locus were also found to be associated with several types of cancer [165]. On the molecular level, human Leo1 was suggested to interact with components of the Wnt signaling pathway, which regulates expression of genes controlling cell fate and homeostasis [167].

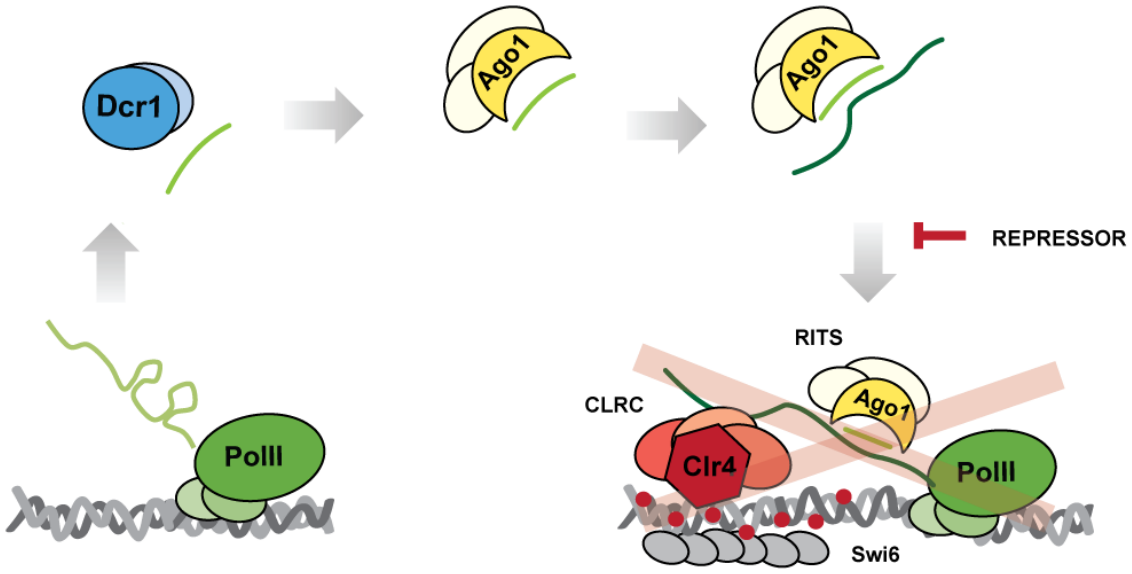

Figure 3. We hypothesized that small RNA-mediated heterochromatin formation in fission yeast is under control of a repressive factor. We aimed at identifying this repressor by a mutagenesis screen approach. See section 4 for detailed description.

4 Rationale

In fission yeast, the majority of small RNAs associated with the Ago1 protein constitute siRNAs mapping to non-coding centromeric transcripts, and RNAs derived from the ribosomal RNA. However, a small portion of endogenous siRNAs that map to protein coding genes was also shown to be loaded onto the Ago1 protein [168]. The authors of this study suggest that 2% of the Ago1-bound small RNAs comes from degradation products of cellular mRNAs and named this class of Dcr1-independent small RNAs primal RNAs (priRNAs). PriRNAs are believed to associate with Ago1 and drive the initial step of the siRNA amplification loop, leading to deposition of H3K9 methylation. Additionally, convergent genes or overlapping transcripts can also serve as a source of dsRNA and undergo processing into primary small RNAs. It was observed that upon overexpression of Dcr1, the number of small RNAs mapping to such genomic loci heavily increased [169].

From the cellular perspective, small RNAs mapping to protein coding genes and constitutive heterochromatic siRNAs differ from each other only in terms of abundance. Hypothetically, with all the RNAi factors in place, such mRNA-targeting siRNAs could induce spontaneous, unregulated formation of heterochromatin throughout the genome and impose a danger on cellular gene expression levels.

Since no compelling and consistent evidence for such nucleation events to happen in wild type cells had been available, we hypothesized that yeast employed a repressive mechanism or factor in order to protect the gene expression from the consequences of endogenous small RNAs (Figure 3). We reasoned that by designing an appropriate experimental setup testing for the siRNA-mediated *in trans* gene silencing, we could perform a mutagenesis screen and identify the putative repressor.

5 Results

5.1 Forward genetic screen - design and results

In order to identify the suppressors of siRNA-mediated heterochromatin formation *in trans,* we designed a forward genetic screen that we named *sms* for small-RNA-mediated silencing. In the screening approach, we used a reporter strain *sms0,* which carried a functional *ade6+* gene at the *trp1+* locus on chromosome II (Figure 4A). At the endogenous *nmt1+* locus on chromosome I, we inserted a construct encoding an RNA hairpin targeting 250 nucleotides of the *ade6+* gene (*ade6-hp*), which was expressed from the promoter of the *adh1+* gene and terminated by the terminator of the endogenous *nmt1+* gene (Figure 4B). Additionally, the *sms0* strain carried at the endogenous *ade6+* locus a mutant *ade6-704* allele that encoded a non-functional form of the Ade6 protein. We chose to insert the *ade6+* reporter at the *tpr1+* locus, because it had been shown before that under conditions when the HP1 protein Swi6 is overexpressed, siRNA-mediated heterochromatin silencing of a *ura4+* reporter gene can be induced in this genomic location [80].

Use of the *ade6+* gene as a reporter provides a great advantage for performing a silencing screen, because the activity of this gene can be easily assayed based on the color of the yeast colonies grown on Yeast Extract (YE) indicator plates that contain limiting amounts of adenine. If the *ade6+* gene is silent or mutated, cells grown on such plates are red; in contrast, when the *ade6+* gene is fully active – cells appear white (Figure 5A, Figure 5B).

By deep sequencing of small RNAs isolated from the *sms0* strain we confirmed that the *ade6-hp* construct produced small RNAs complementary to the *ade6+* mRNA in high quantities (Figure 6A). These small RNAs showed characteristic length and preference for the U base at the starting position, which is known to be a signature for siRNAs loaded onto Ago1 (Figure 6B). Despite the evidence for siRNA production, colonies of the *sms0* strain were white on adenine-limiting plates (Figure 5B, Figure 6D) and the *ade6+* mRNA levels in the *sms0* strain remained unchanged, as compared to the strain without the *ade6-hp* (Figure 6C*)*. The aforementioned observations confirmed the fact that small RNAs cannot induce repression of the *ade6+* reporter *in trans* in the wild type cells. Furthermore, overexpression of the HP1 protein Swi6 in the *sms0* strain did not lead to induction of *ade6+* reporter silencing (Figure 6D). In order to identify mutations that would enable siRNA-mediated repression of the *ade6+* gene *in trans,* we subjected the *sms0* strain to ethylmethansulfonate (EMS) treatment

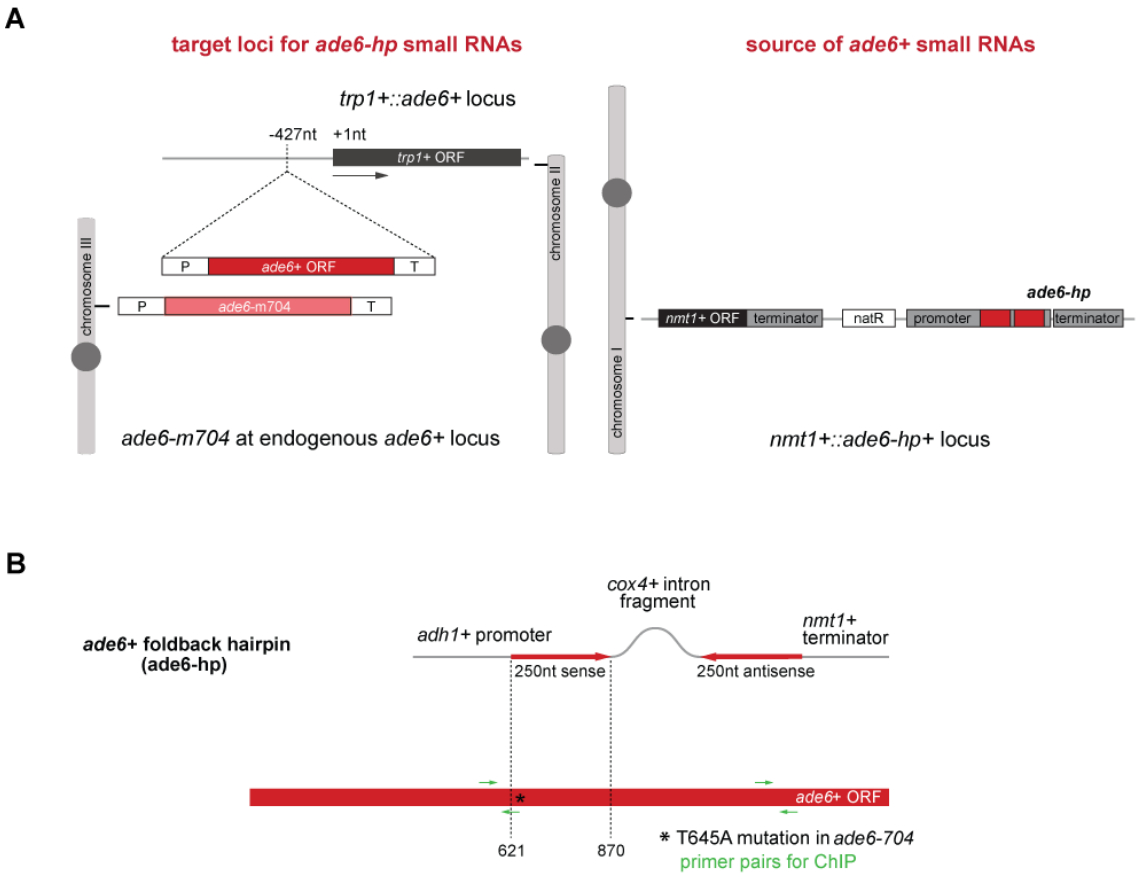

Figure 4. Scheme representing genomic locations of the source of hairpin-derived small RNAs and their target loci in the *sms0* strain. A wild type *ade6+* gene was inserted upstream of the *trp1+* gene on chromosome II to create a reporter locus. Endogenous *ade6* locus on chromosome III carries a mutant *ade6-m704* allele, which gives rise to non-functional product due to single point mutation: Thr645Ala. *ade6-hp* construct linked to noureseothricin resistance cassette (Nat) was inserted into *nmt1+* locus on chromosome I **B** *Ade6-hp* cassette encodes for a hairpin encompassing 250nt long fragment of the *ade6+* gene from the base pair 621 to the base pair 870. The hairpin loop sequence comes from the full-length intron of the *cox4+* gene. The expression of the hairpin is driven from the *adh1+* gene promoter and terminated with *nmt1+* terminator sequence. Asterisk denotes the site of point mutation in the *ade6-m704* allele. Green arrows mark locations of the primers used for PCR in ChIP experiments (see Figure 12.). ORF, open reading frame.

(Figure 7). EMS is a chemical mutagen which alkylates guanine residues in DNA, leading predominantly to transition mutations from G:C to A:T pairs. Upon EMS treatment, we selected colonies that demonstrated red color. In a screen based on the *ade6+* silencing assay, loss-of-function mutations in the adenine synthesis pathway would also result in a red coloration of the cells, and therefore constitute false-positive hits. In order to eliminate false-positive results, red colonies isolated upon EMS treatment were tested for growth in the absence of adenine. As a result of this secondary screen, which was followed by four rounds

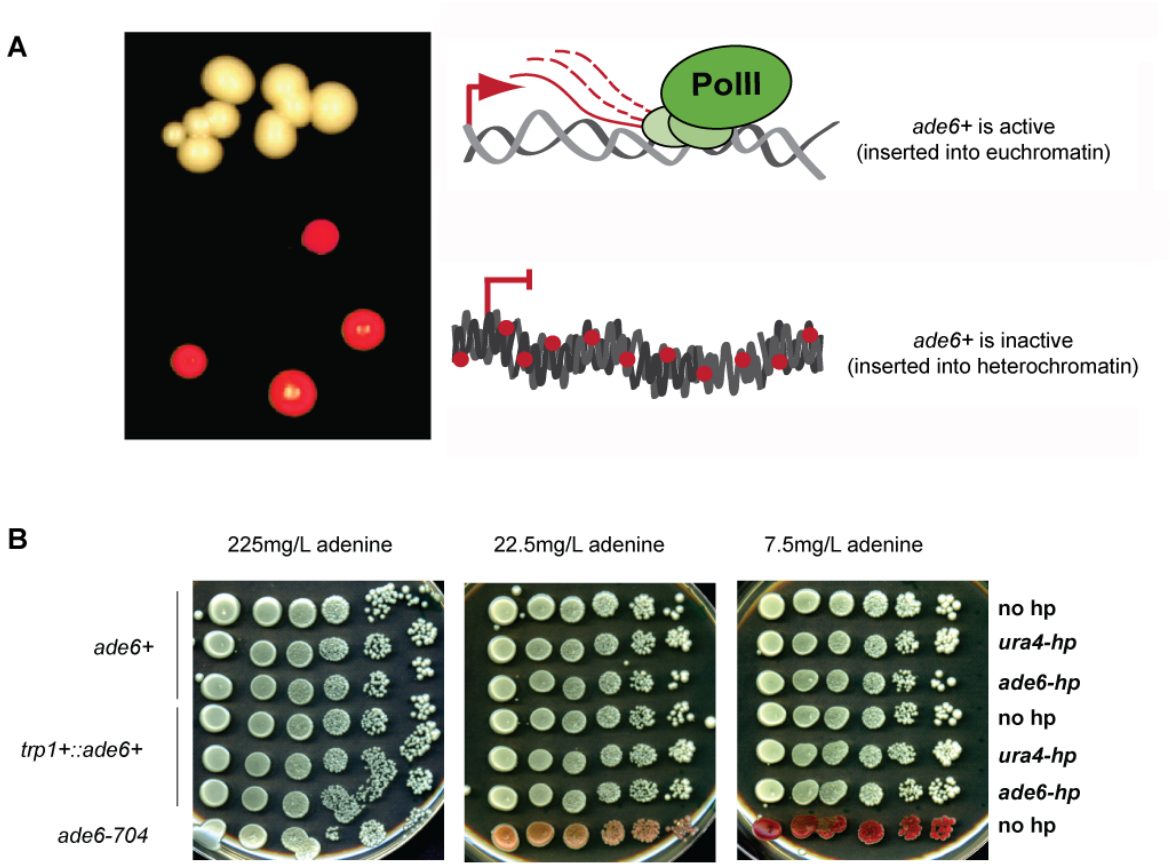

Figure 5. *ade6+* silencing assay. A If the *ade6+* gene is active, cells grow white on Yeast Extract (YE) medium. If *ade6+* gene is mutated or silenced – cells appear red. **B** The ability of *ade6+* hairpin-derived small RNAs to silence the *trp1+::ade6+* reporter gene was tested by the *ade6+* silencing assay using plates with decreasing adenine concentrations. Cells carrying the *ade6+* gene at the endogenous location, as well as the *trp1+::ade6+* reporter locus were spotted in ten-fold serial dilutions on YE (See Figure 4). As a negative control cells expressing *ura4+*-targeting hairpin were used. As a positive control cells carrying the mutant *ade6-m704* allele were also spotted.

of backcrossing, we obtained ten independent mutants that we named *sms1* to *sms10* (Figure 8A). Five of the identified mutants (*sms1*, *sms3*, *sms4*, *sms6*, *sms8*) showed *ade6+* silencing that was dependent on the presence of Dcr1, as the red cell phenotype disappeared when the *dcr1+* gene was deleted (Figure 8B). The Dcr1-dependent phenotype was consistent with the fact, that the same five mutants showed H3K9me2 accumulation over the *ade6+* gene (Figure 8C).

In order to identify the mutations responsible for the observed silencing phenotype, we subjected genomic DNA isolated from the *sms1* to *sms10* strains to whole-genome next-generation sequencing (Figure 9A). In the Dcr1-dependant mutants we identified missense and nonsense mutations in the following genes: SPBC651.09c, SPAC664.03,

SPBC13E7.08c, and SPBC17G9.02c (Figure 9B, Table. 3). These genes encoded for homologues of four out of five protein subunits of the *S. cerevisiae* RNA polymerase-associated factor 1 (Paf1) complex. Since the genes SPAC664.03, SPBC13E7.08c, and SPBC17G9.02c had not been described in *S. pombe* so far, we named them after their *S. cerevisiae* homologues *paf1+*, *leo1+* and *cdc73+*, respectively. The SPBC651.09c gene, which encodes for the homolog of *S. cerevisiae* Rtf1 had already been named as *prf1+* [96]. In the remaining five mutants showing the Dcr1-independent red phenotype, mutations were mapped to *ade6+* and *ade7+* genes. These false positive hits were weak loss-of-function alleles that were able to survive the secondary screen in the absence of adenine.

In summary, we have identified five mutations that enabled *in trans* siRNA-mediated heterochromatic silencing in Dcr1-dependent manner in *S. pombe*. We obtained the mutants through an EMS mutagenesis screen approach followed by whole-genome sequencing. All of the identified mutations were mapped to genes encoding homologues of subunits of the Paf1 complex, implicating a repressive role of the Paf1 complex in *in trans* silencing.

5.2 Validation of the screen results

After identifying the mutant alleles potentially responsible for the silencing phenotype, we went on to validate our results by reconstituting the point mutations in the *paf1+*, *leo1+*, *cdc73+* and *prf1+* genes in the *sms0* strain. All the mutants confirmed the red colony phenotype in the silencing assay when the *ade6-hp* construct was present, and no silencing was observed in the absence of the *ade6-hp* construct (Figure 10A). The *ade6+* repression was more stable and potent once we picked red colonies for each mutant and propagated them in a second silencing assay experiment (Figure 10B). As expected, we could also confirm reduced *ade6+* mRNA levels in the reconstituted mutants by an RNA expression The fifth subunit of the Paf1 complex is called Tpr1. Although in our screen we had not recovered any mutations in the *tpr1+* gene, we observed that in the strain where the analysis endogenous *tpr1+* gene had been tagged with 3xFLAG tag on the C-terminus, siRNA-mediated repression of the *ade6+* reporter was also occurring (Figure 11).
Tagging of the *cdc73+*gene with the 3xFLAG also caused the red cell phenotype. We concluded that *tpr1::3xFLAG and cdc73::3xFLAG* act as hypomorphic alleles, possibly affecting the function of the Paf1 complex.

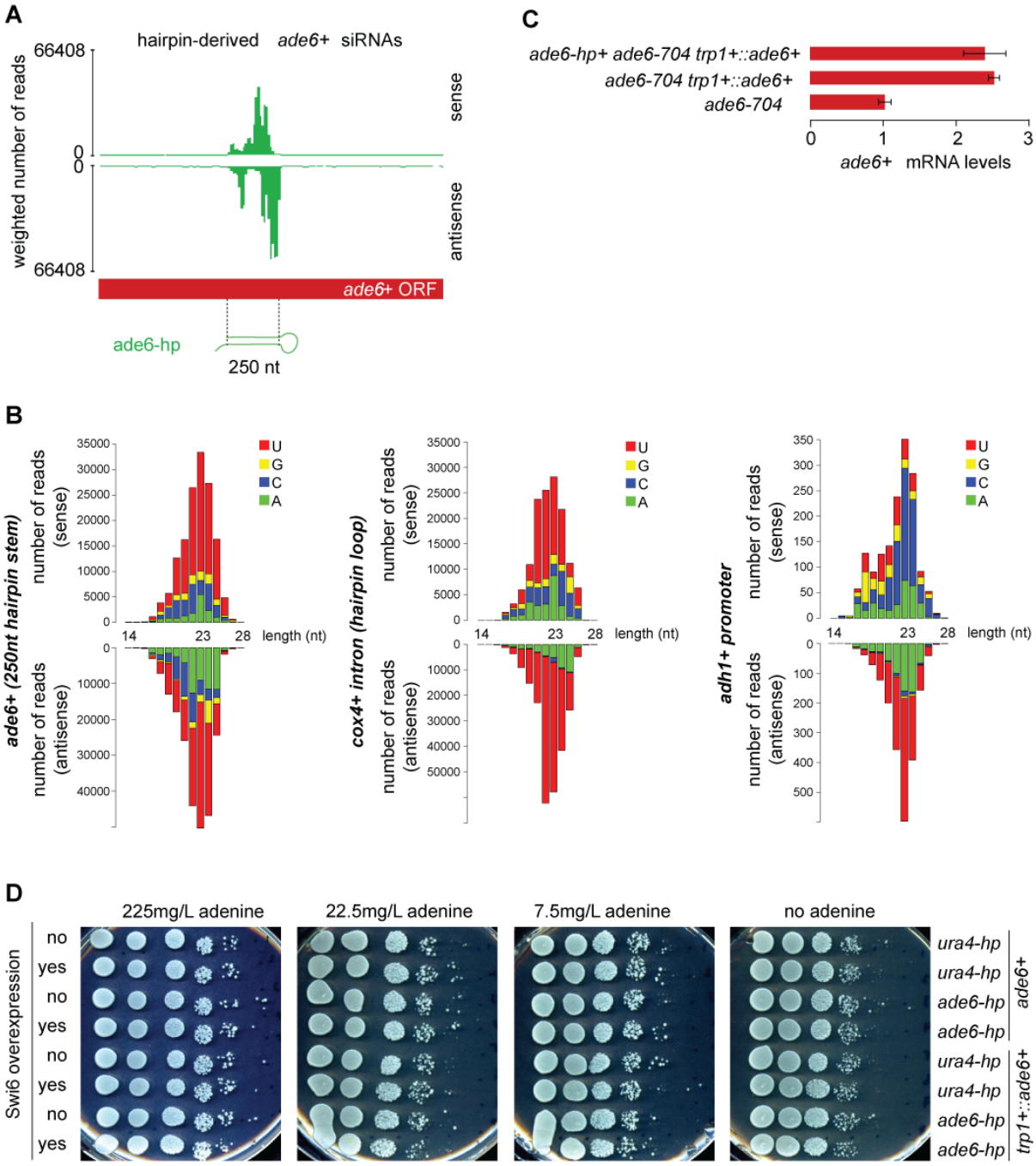

Figure 6. *ade6-hp*-derived siRNAs cannot silence the expression of the *ade6+* gene. A Production of small RNAs from the stem of the *ade6-hp* in the *sms0* strain was confirmed by next-generation deep sequencing. **B** Histograms illustrating size and the first nucleotide distribution for the sense and antisense small RNAs originating from the *ade6-hp* construct. siRNAs targeting the *ade6+* ORF are produced efficiently in the wild type strain. siRNAs mapping to the sense and antisense strand of the *adh1+* promoter and *cox4+* intron were also detected. Note that *cox4+* siRNAs are almost as abundant as *ade6+* siRNAs. **C** *ade6+* mRNA levels were measured by RT-qPCR and normalized to *act1+* mRNA. Expression of *ade6+*-targeting small RNAs did not lead to repression of the *trp1+::ade6+* reporter. Note that *ade6+-m704 trp1::ade6+* strain carries two alleles of the *ade6* gene and therefore exhibits two fold higher *ade6+* mRNA levels. One representative biological replicate is shown. **D** The inability of *ade6+*-targeting small RNAs to induce silencing of the *ade6+* gene was confirmed by the silencing assay at various adenine concentrations. Cells carrying the *ade6+* gene at the endogenous location, as well as at the *trp1+* reporter locus were spotted in ten-fold serial dilutions on YE in ten-fold serial dilutions on YE plates. As a negative control cells

expressing a *ura4+*-targeting hairpin were used. As a positive control, cells carrying the mutant *ade6-m704* allele were spotted. Note that overexpression of Swi6 did not lead to establishment of silencing.

These results further supported our hypothesis that the Paf1 complex acts as a suppressor of the small-RNA-mediated repression of gene activity *in trans* in fission yeast.

5.3 siRNA-mediated gene repression is due to heterochromatin formation

On centromeric repeats, siRNA-directed gene silencing leads to formation of silent chromatin, which is associated with the accumulation of histone H3 lysine 9 methylation (H3K9) and leads to reduced transcriptional activity. By performing a chromatin immunoprecipitation (ChIP) experiment in the Paf1 complex mutant strains, we detected high levels of H3K9 methylation at the *trp1+::ade6+* locus in the presence of the *ade6-hp* (Figure 12A). We also observed reduced transcriptional activity at the *trp1+::ade6+* locus, which was demonstrated by lowered H3K36 tri-methylation levels along the *ade6+* gene body (Figure 12B), as well as reduced PolII occupancy at the end of the gene (Figure 12C). Therefore, we could further confirm that the reduction in *ade6+* expression in all the Paf1 complex mutant strains is due to *de novo* formation of heterochromatin.

As mentioned above in Section 5.1, the *sms0* strain that we used for the screen carries, in addition to the functional *ade6+* allele at the *trp1+* locus, a mutated *ade6-m704* allele at its endogenous location (Figure 4). Since the mutant allele differs only by a point mutation from the wild type *ade6+* alele, small RNAs produced from the *ade6-hp* are also complementary to the *ade6-m704* transcript. We therefore speculated that siRNA-mediated *de novo* heterochromatin formation would also occur at the *ade6-m704* genomic location. As expected, we observed high enrichment of the H3K9me2 mark over the *ade6-m704* allele in all the reconstituted mutants (Figure12D). This further confirmed the *in trans* nature of the heterochromatin formation phenomenon we identified. Importantly, we also detected high levels of H3K9 methylation at the *ade6-hp* locus, indicating that the *ade6+*-targeting small RNAs direct heterochromatin formation in the Paf1 complex mutants also *in cis* (Figure 12E). Expression of the *nmt1+::ade6-sh+* construct is driven from the *adh1+* promoter, and it encodes for a hairpin containing a *cox4+* intron sequence in the place of the loop (Figure 4B).

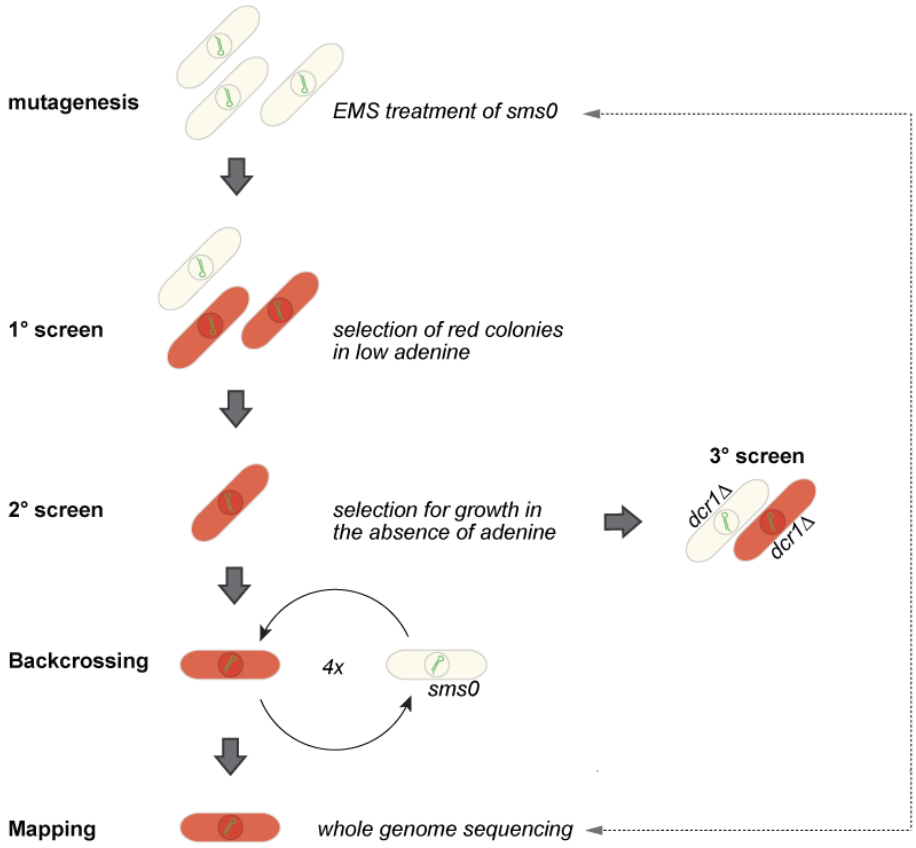

Figure 7. *sms* forward genetic screen designed to identify putative repressor of the siRNA-mediated transcriptional gene silencing. Scheme depicting the workflow of EMS mutagenesis screen performed in this study. Parental strain *sms0* was treated with EMS and plated on the YE plates. In the secondary screen, red colonies were picked and tested for growth on adenine-depleted medium to eliminate false positive hits with mutations in the adenine synthesis pathway. In order to identify clones in which *trp1+::ade6+* silencing is dependent on siRNAs we deleted *dcr1+* in a tertiary screen. Subsequently, upon four rounds of backcrossing, positive clones were subjected to whole-genome sequencing in order to map the mutations causing the red phenotype.

Interestingly, despite the fact that functional siRNAs targeting both strands of the *cox4+* intron were detected in high quantities in the *sms* strains (Figure3B), we did not observe accumulation of H3K9 methylation at the *cox4+* coding sequence (Figure 12F). Next, we wanted to confirm that *in trans* silencing was a sequence-independent phenomenon that could be used to silence other euchromatic genes. To this end, we created a strain expressing small RNAs targeting the endogenous *ura4+* gene from the *ura4+* hairpin (*ura4-hp*) construct integrated at the *nmt1+* locus, and then introduced the *paf1-Q264Stop* mutation (Figure 13A). To test the expression of the *ura4+* gene we performed a silencing assay on medium containing 5-FOA, which is toxic to yeast cells when the *ura4+* gene is active. Paf1 mutant cells expressing small RNAs targeting the *ura4+* gene grew on the plates with 5-FOA

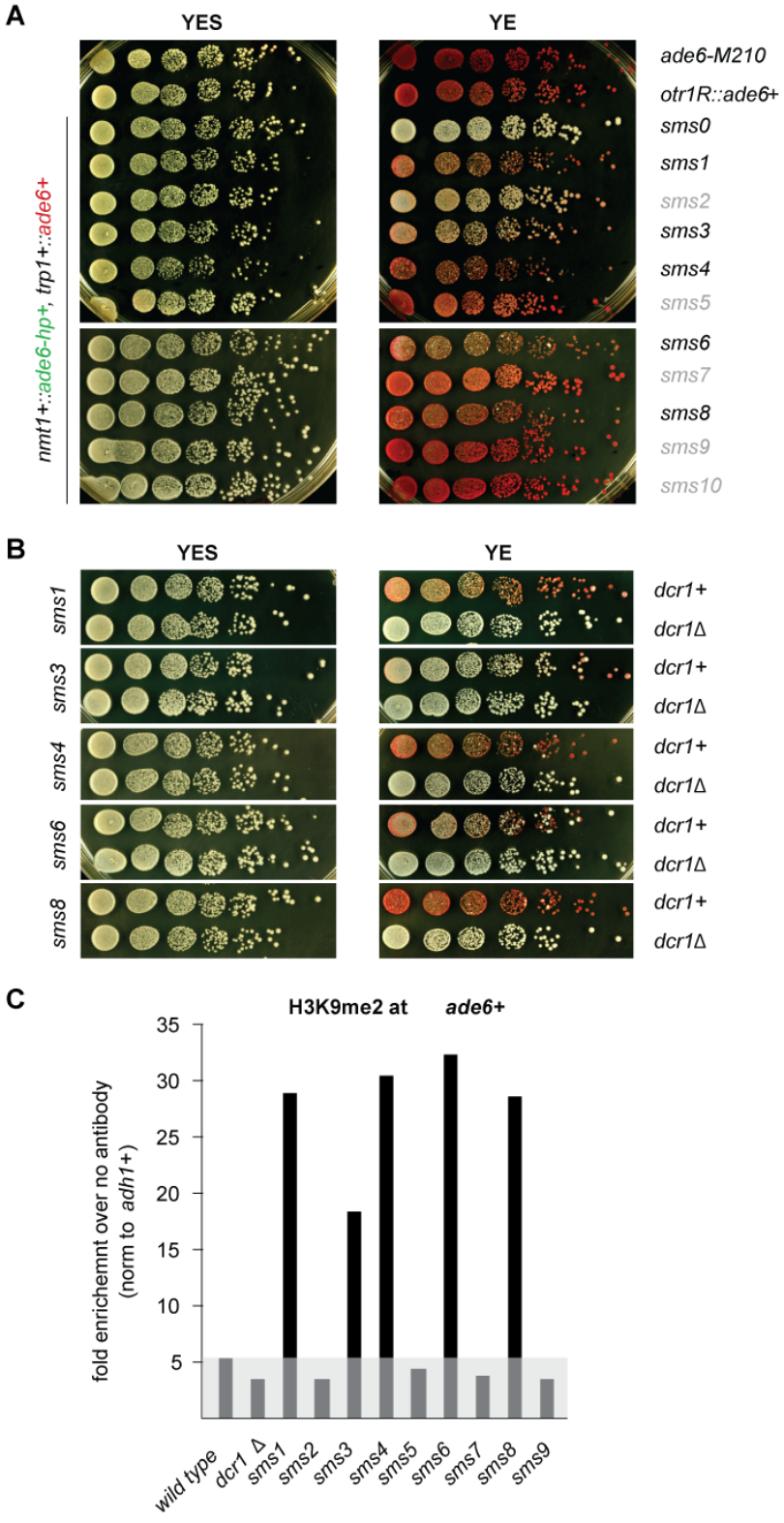

Figure 8. *sms* screen identifies five independent mutants showing siRNA-dependent repression of the *ade6+* gene by accumulation of H3K9 methylation. A sms screen gave rise to 10 independent clones showing red phenotype on YE plates. Cells carrying an *ade6-M210* allele and a heterochromatic copy of the *ade6+* reporter (*otr1R::ade6+)* were used as a positive control **B** Five mutants showed silencing phenotype that was dependent on the presence of Dcr1. **C** Accumulation of the H3K9 dimethyl mark at the *trp1+::ade6+* gene was tested by ChIP. Enrichments were calculated relative to the 'no antibody' control. To control for background signal and for ChIP efficiency, scores were normalized to enrichments over the *adh1+* gene.

whereas *paf1+* cells did not, suggesting that in the presence of the *paf-Q264Stop* mutant allele, siRNAs induced silencing of the endogenous *ura4+* gene, as anticipated (Figure 13 A). We could also observe silencing of the *ade6+* gene at its endogenous location, when *ade6-hp*-derived small RNAs were expressed in the Paf1 mutant strain (Figure 10C).

We subsequently confirmed that siRNAs generated from a source different than a hairpin construct could also effectively silence genes in trans in a Paf1 mutant strain. Using a tethering system described before [35] we induced heterochromatin formation and small RNA production at the *ura4+::5BoxB* locus. *ura4+*-targeting siRNAs produced from this locus are necessary to establish heterochromatin formation *in cis,* but they cannot mediate heterochromatin nucleation at the second, euchromatic copy of the *ura4+* allele *in trans* in wild type cells [35]. In *a paf1-Q264Stop* strain, siRNAs generated from the *ura4+::5BoxB* locus could initiate repression of the second, euchromatic *ura4+* copy. Notably, siRNA-mediated *ura4+* repression *in trans* was more pronounced, when the gene encoding for the Eri1 nuclease was deleted. This observation is in agreement with the fact that the number of siRNA produced from heterochromatic loci was enhanced upon deletion of the *eri1+* gene.

Taken together, we showed that the Paf1 complex controls small RNA-mediated gene repression and heterochromatin formation both *in cis* and *in trans,* and that this repression can occur on different target genes independent of their sequence or chromosomal location.

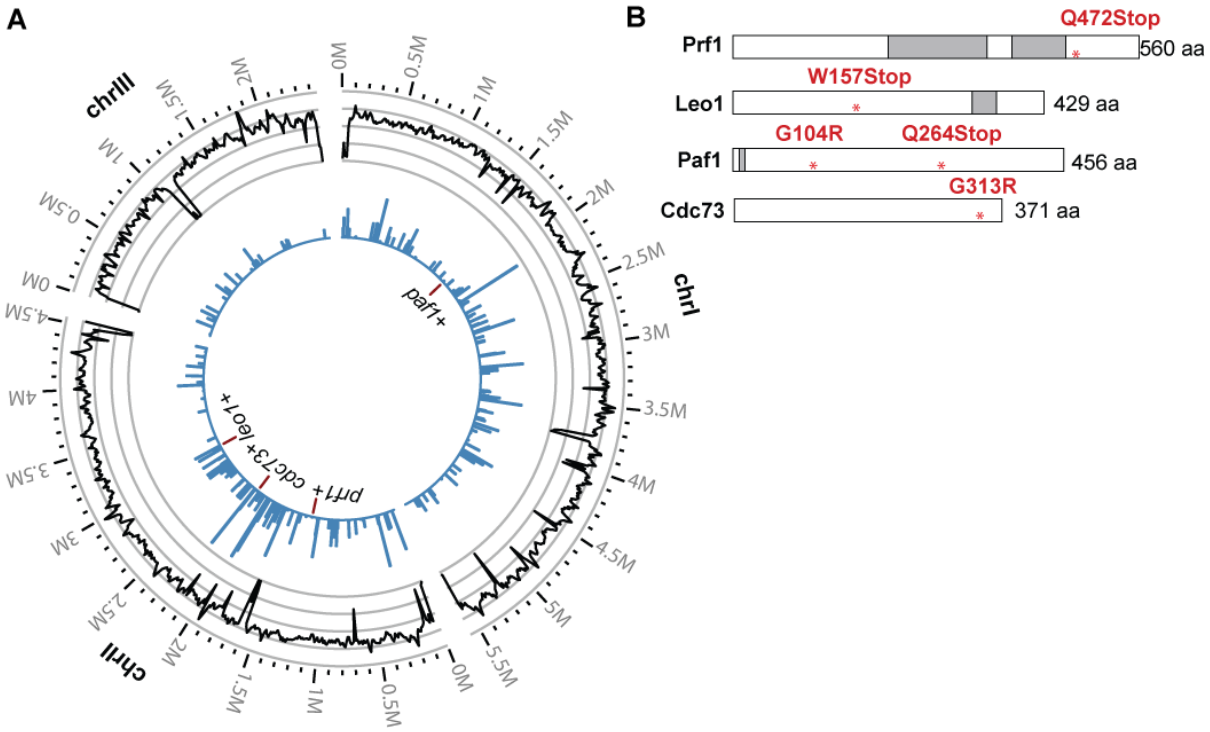

Figure 9. **Causative mutations in Paf1 complex were identified using whole-genome next-generation sequencing. A** Resequencing of EMS-mutagenized *S. pombe* strains. From outside to inside, the tracks show the genomic location, the average coverage per window of 10kb (black line, scale from zero to 30), the number of sequence variations identified prior to filtering in all strains per window of 10kb (blue bars, scale from zero to 90) and the five mutations that passed the filtering and overlapped with Paf1C genes (red lines, the two mutations in Paf1 are too close to be resolved individually) **B** Mutations from Dcr1-dependent clones were mapped to genes encoding homologues of the *S. cerevisiae* Paf1 complex. Scheme representing location of the missense and nonsense mutations is shown. Domains of the highest conservation are marked in gray.

5.4 Genetic requirements for silencing

In order to further characterize heterochromatin formation that is repressed by Paf1 complex, we performed a series of gene deletions in a strain carrying the *ade6+::trp1* reporter, the *nmt1+::ade6-hp* construct and the *paf1-Q264Stop* allele to test the genetic requirements for maintenance of the *ade6+* silent state. We observed that, in addition to being Dcr1-dependent (Figure 8B), the *ade6+* repression was dependent on Rdp1 and other components of the RDRC complex, as well as on Ago1 and members of the RITS and ARC complexes (Figure11). Since the silencing was lost in all these canonical RNAi pathway mutants, we concluded that continuous production of small RNAs is necessary for maintenance of the *ade6+* repression. Triman (Tri) is a 3' exonuclease that was suggested to take part in

Table 3 Mutations in the *sms* hits mapped by whole genome sequencing.

Clone	Dcr1-dependency	H3K9me2 accumulation	Mapped mutations
sms1	+	+	SPBC651.09c (472 Gln to UAA); Prf1
sms2	-	-	*ade6+*
sms3	+	+	SPAC664.03 (104 Gly to Arg); Paf1
sms4	+	+	SPBC13E7.08c (157 Trp to UGA); Leo1
sms5	-	-	*ade7+*
sms6	+	+	SPBC17G9.02c (313 Gly to Arg); Cdc73
sms7	-	-	*ade6+*
sms8	+	+	SPAC664.03 (264 Gln to STOP); Paf1
sms9	-	-	*ade7+*
sms12	-	-	*ade7+*

Column 2 indicates whether the silencing phenotype was Dcr1-dependent. Column 3 indicates whether the H3K9me2 mark was detected at the *trp1+::ade6+* reporter locus. In Dcr1-dependent mutants, we mapped mutations in the genes SPBC651.09c, SPAC664.03, SPBC13E7.08c, and SPBC17G9.02c, which encode for homologues of the budding yeast Paf1 complex.

processing of Ago1-bound precursors of siRNAs and pri-RNAs, in order to shorten them to the length suitable for Ago1 binding [170]. Deletion of the Tri gene (*tri+*) resulted only in a mild derepression of the *ade6+* gene. This result indicates that Tri does not contribute strongly to siRNA-mediated *de novo* formation of heterochromatin by the hairpin-derived small RNAs.

In addition, we could confirm that siRNA-directed silencing in the *paf1-Q264Stop* strain was also dependent on the components of the SHREC complex, on the Clr4 methylthranseferase and other subunits of the CLRC complex (Figure 14). Finally, the HP1 proteins Swi6 and Chp1, which are vital for formation of constitutive yeast heterochromatin, were also required for the silencing. These observations further confirm that siRNA-mediated gene silencing that we observe when Paf1 complex function is impaired occurs through establishment of heterochromatin.

5.5 Impact of the Paf1 complex mutations on global gene expression.

A number of studies performed mainly in budding yeast and mammalian cells suggested multiple roles of the Paf1 complex in regulation of transcription by RNA Polymerase II (Section 3.7). To assess the consequences of interfering with such a broad-acting complex,

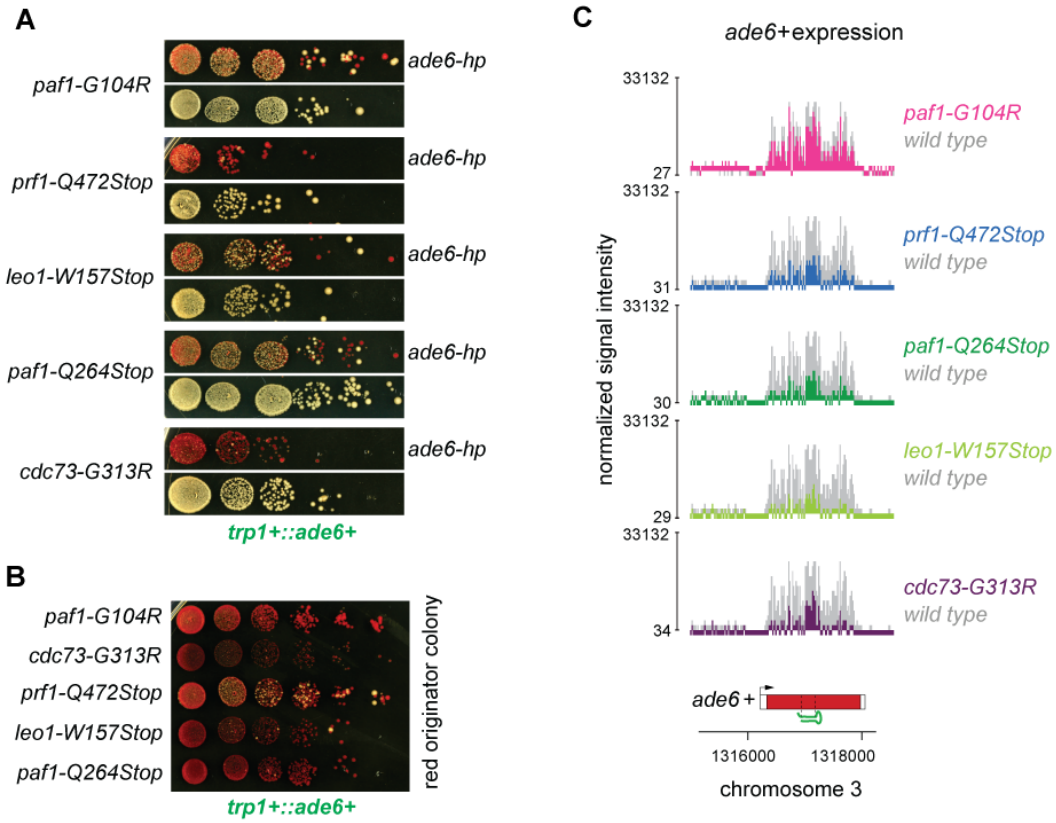

Figure 10. Mutations in the subunits of the Paf complex cause siRNA-dependent repression of the *ade6+* gene. A Validation of the sms screen results. Individual point mutations in Paf1 complex subunits were reconstituted in the *sms0* parental strain. Obtained mutants were tested by the *ade6+* silencing assay. As a control, strains without the *nmt1+::ade6-hp* construct were generated. **B** *ade6+* silencing assay repeated with red colonies picked from A. Note that induced silencing is very stable. C Whole genome expression profiling with tiling microarrays was used to confirm the repression of the *trp1+::ade6+* gene caused by siRNAs in the identified mutants of the Paf1 complex. Normalized signal intensity is shown in the linear scale.

we looked globally at changes in gene expression by genome-wide RNA expression profiling with tiling microarrays. In our analysis, we included the *paf1+* strain *sms0*, all five point mutations identified in the screen, as well as full deletions of *paf1+* and *leo1+* genes. We obtained genome-wide expression profiles for two biological replicates for each strain, and compared them by performing a Principal Component Analysis (PCA). Principal Component (PC) 1 and 2 explained 41.5% and 16.4 % of the variance between samples, and the remaining PCs represented 57.9 % of the variance. An analysis of the loadings indicated that the remaining PCs all captured at least in part non-reproducible effects in the experiment, such as the differences between replicate sets. Therefore, we decided to focus in our analysis on the first two PCs and choose them for the visualization (Figure 15A). Comparison of samples based on PC1 and PC2 (Figure 15A) revealed that the *cdc73-G313R* and *paf1Δ* cells are most different from the *paf1+* cells. All the remaining mutants clustered together with the

wild type samples, generally suggesting that these mutations of the Paf1 complex have very little consequence on gene expression. Indeed, comparisons of mean steady state RNA expression values between wild type and mutant samples further support the conclusion that transcription was not strongly affected in these mutants (Figure 15B).

Based on the comparison with the data obtained for the *paf1Δ* mutant, we concluded that *paf1-G104R*, *paf1-Q264Stop*, *rot1-Q472Stop* and *leo1-W157Stop* might represent separation-of-function alleles that do not cause severe alterations on the gene expression level, and presumably, do not impair the function of Paf1 complex in regulation of transcription by RNA PolII significantly.

5.6 Leo1 as a *bona-fide* repressor of siRNA-directed heterochromatin formation

It drew our attention that in the visualization of the PCA, *leo1*Δ strain clusters together with Paf1 complex point mutants, rather than with the other full deletion strain – *paf1Δ* (Figure15 A). Furthermore, the *leo1+* deletion caused only mild changes in gene expression at the global level (Figure 16A). This suggests that the Leo1 subunit of the complex may elicit a special, separate function. Such a function could, for instance, involve direct suppression of siRNA-mediated heterochromatin formation by preventing the interaction of the RNAi machinery with RNA PolII. Consistent with the result obtained by PCA, we noticed that siRNAs were able to induce repression of *ade6+* reporter in the *leo1*Δ strain, whereas in the *paf1Δ* strain the *ade6+* gene remained desilenced (Figure 16B). In support of our hypothesis, we also noticed that the *prf1Δ, paf1Δ, tpr1Δ,* and *cdc73Δ* mutants showed very severe growth defects, but the *leo1Δ* deletion did not cause retarded colony growth (Figure 16B).

In summary, computational analysis of the genome wide expression profiles and the small RNA expression profiles pointed towards a separate role that Leo1 could play in the Paf1 complex. This observation was reflected by the phenotypic differences between the individual Paf1 mutants. Therefore, we concluded that Leo1 could function as the actual repressor of small RNA-mediated formation of heterochromatin *in trans*.

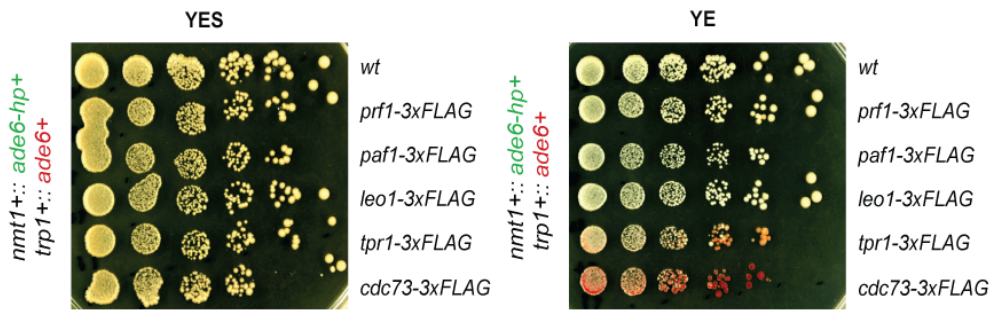

Figure 11. C-terminal tagging of the Paf1 complex subunits results in creation of hypomorphic alleles and causes *ade6+* silencing. Silencing assay was performed with strains where subunits of the Paf1 complex were C-terminally tagged with 3xFLAG-Tag. Tagging renders Cdc73 and Tpr1 partially non-functional and results in siRNA-mediated silencing of the *trp1+::ade6+* reporter. Note that full deletions of *cdc73* and *trp1* are deleterious to cell growth (Fig. 16.), whereas cells carrying the tagged alleles grow normally.

5.7 Formation of facultative heterochromatin

As described in details in Section 4, the reasoning behind performing our screen for a repressor of small-RNA-mediated silencing was that such repressors could function in the cell in order to prevent priRNAs and primary siRNAs from inducing heterochromatin formation and gene silencing in a random, uncontrolled manner.

To test our initial hypothesis and to identify loci that would be susceptible to priRNA-mediated heterochromatinization, we compared the aforementioned gene expression profile for the *leo1*Δ mutant strain with a genome-wide small RNA expression profile obtained by deep sequencing (Figure 17A). We expected such facultative heterochromatin loci to show reduced gene expression combined with an increase in siRNAs that would originate from the small RNA amplification loop triggered by the priRNAs or the endogenous primary siRNAs.

Being a proof of principle, all the three loci that are homologous to the *ade6-hp*: *nmt1+::ade6-hp+, trp1+::ade6+,* and *ade6-704* fulfilled our criteria (Figure 17A). Additionally, we observed the same effect on the genes neighboring the *ade6+* siRNA targets, suggesting spreading of heterochromatin over the adjacent regions for a distance up to 6 kbp (Figure 18).

Despite the fact that the *ade6-sh* construct contained only 250 bp of the *ade6+* gene, in Paf1 mutants, we consequently observed accumulation of siRNA targeting both the plus and the

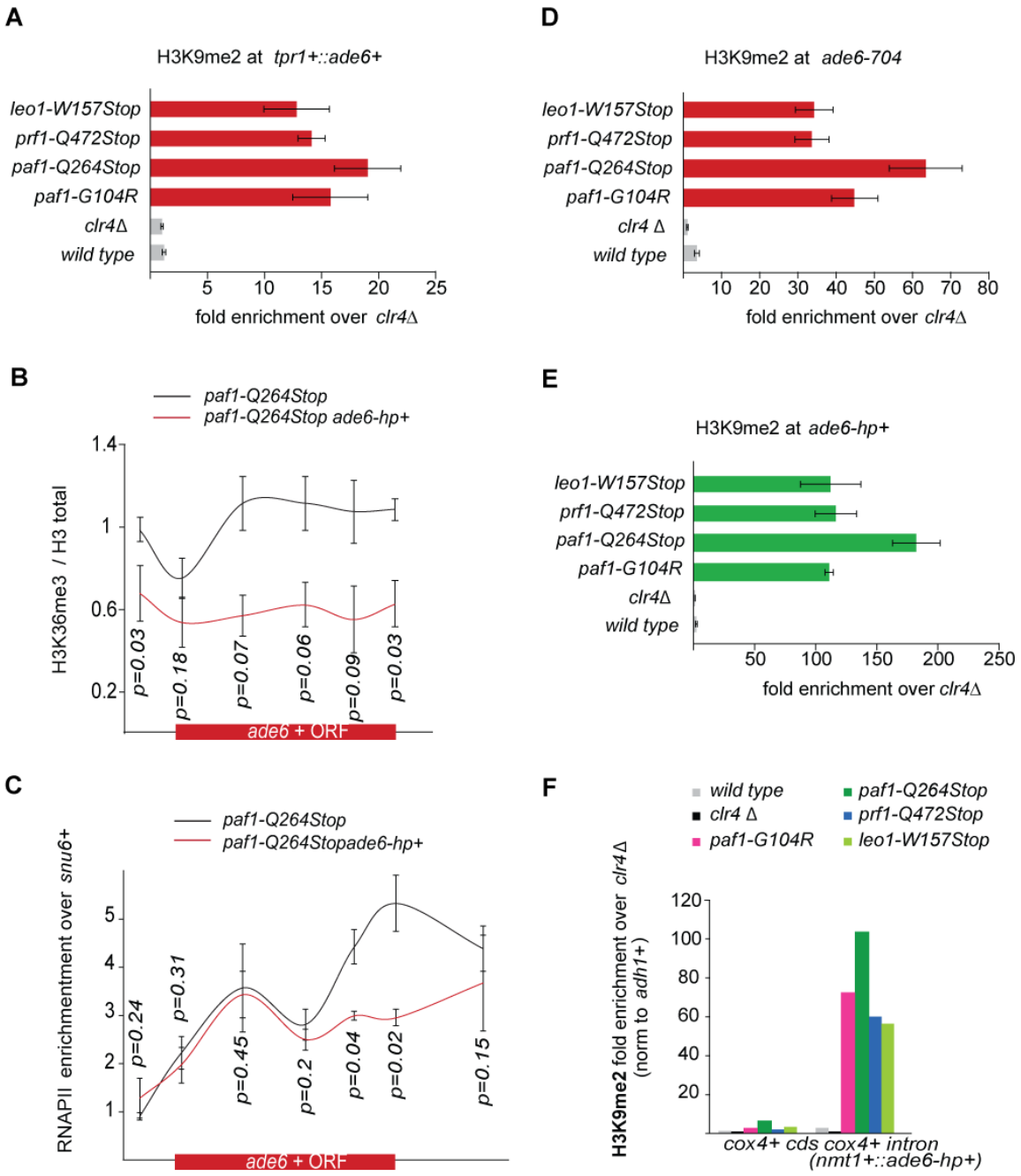

Figure 12. Mutations in the Paf1 complex cause siRNA-directed H3K9 methylation and transcriptional repression of the *ade6*+ gene. A ChIP experiment illustrating the H3K9me2 enrichment at the *trp1+::ade6+* reporter locus in the indicated mutants. Enrichments were calculated relative to the values for the *clr4*Δ mutant strain and normalized to the values for the *adh1*+ gene. Mean of 3 biological replicates is shown. Error bars represent standard error of mean (s.e.m.). **B** and **C** Results of ChIP experiments for H3K36me3 and RNA PolII occupancy at the *ade6*+. To control for background signal and for differences in ChIP efficiency. Values were normalized to the enrichments over the *snu6*+ gene. For the H3K36me3 ChIP, scores were normalized to the total histone H3 enrichments. Mean of 3 independent biological replicates is shown. P-values were calculated using the one-tailed Student's t-test. **D** and **E** Same as (A), but the H3K9me2 enrichment was measured at the *ade6-m704* locus and *nmt1+::ade6-hp+* locus. **F** ChIP experiment illustrating the H3K9me2 enrichment at the *cox4*+ ORF and *cox4*+ intron sequence in the indicated mutants of the Paf1 complex. Enrichments were calculated relative to the values for the *clr4*Δ

minus strand of the entire *ade6+* ORF (Figure 17B). This strongly indicated that the primary siRNAs encoded within the hairpin triggered the RNAi amplification loop, in which highly abundant secondary siRNAs were being produced. We also observed spreading of secondary siRNA production into adjacent genes, which was accompanied by the spreading of the H3K9me2 mark over these regions, as measured in a ChIP experiment in the *leo1Δ* strain in comparison to the *leo1+* strain (Figure 19). Interestingly, in the pool of the small RNAs originating from these genes, we detected also siRNAs that were mapping to intronic sequences (please note the *rpl2302+* gene), suggesting that secondary siRNA production could possibly be dependent on transcription of nascent transcripts by Rdp1 (Figure 18). Taken together, we observed that the siRNA-induced gene silencing in the Paf1 mutants leads to formation of a *bona fide* heterochromatic domain by spreading from the targets of the *ade6-hp*. The assembled domain is several kb long and exhibits all the hallmarks of heterochromatin, i.e. reduction in gene expression, accumulation of H3K9 methylation mark and production secondary small RNAs.

We also detected a small number of loci independent from *ade6-hp* targets that showed weak signature of siRNA-mediated silencing (Figure 17A, Tab. 7), yet when we compared the data obtained for different mutants, we were unable to recover the same loci in different Paf1 mutant strains. This suggests that, if the observed decrease in gene expression was indeed caused by the priRNAs and facultative heterochromatin formation, these target genes were silenced in a random manner. Therefore, we concluded that during mitotic growth in standard conditions there are no specific sites strongly primed for silencing by small RNAs.

5.8 Establishment and maintenance of silencing

During reconstitution of the Paf1 mutant strains, we observed that in a population of freshly generated mutants not all of the clones initially exhibited full repression of the *ade6+* reporter (full red phenotype), and therefore, remained white or pink on the low adenine indicator plates. However, when we repeated the *ade6+* silencing assays with red colonies selected for each of the obtained mutants, we observed that once the silent state was established through a number of mitotic divisions, repression was very stable in every case (Figure 20A, 20B, Figure 10B). We quantified this observation by scoring the red, white and pink colonies originating from a parental red clone for all the mutants. This analysis confirmed that descendants of the red colony maintained the silencing of *ade6+* state of the reporter with a frequency of at least 90% (Figure 20A). Notably, the *paf1-Q264Stop* mutant

showed the highest maintenance rate from all the strains. In order to quantify the frequency of initiation of heterochromatin formation in mitotic cells we repeated the experiment with descendants of white colonies for each strain (Figure 20B). This time, the frequency varied substantially between the strains. For instance, we observed that establishment of the silent state was the most frequent in the *leo1-W157Stop* mutant (Figure 20B). In contrast, the *paf1-Q264Stop* mutant strain, which showed the highest heterochromatin maintenance rate was not very efficient in establishing of the silent state, with only approximately 10% of colonies that showed the *ade6+* repression (Figure 20B).

5.9 Propagation of silencing through meiosis

We then went on to investigate rates of establishment and maintenance of the silent ate at the *trp1+::ade6+* reporter locus during meiosis. We analyzed this by performing a series of crosses. First, we crossed a red clone of the *paf1-Q264Stop* strain with the wild type strain (both of the strains were carrying the *ade6-hp* hairpin construct). We confirmed that the silent state was very efficiently maintained over the meiotic division, and we observed as well that only the progeny cells carrying the mutant Paf1 allele were able to keep the reporter gene silent (Figure 20C). Thus, we concluded that, just like in the case of mitotic cells, heterochromatic silencing can be efficiently transmitted through meiosis, but the mutation in the Paf1 is necessary for its maintenance. (Figure 20B). We then repeated this cross, but this time we used a white clone of the *paf1-Q264Stop* strain and crossed it with the *paf1+* strain (both of the strains were carrying the *ade6-hp* hairpin construct). Interestingly, we observed that initiation of the siRNA-mediated repression was more efficient in the meiotic cells than during the mitotic growth (Figure 20B). We analyzed progeny of several tetrads coming from this cross and saw that that in 70% of all crosses, at least one of the two progeny cells that carried the mutant Paf1 allele turned on the silencing of the *ade6+* reporter (Figure 20D). Besides, we noticed again that only the progeny cells that inherited the Paf1 mutant allele were able to keep the ade6+ gene silent. Therefore, we concluded that mutation in the Paf1 complex is a prerequisite for both establishment and maintenance of the siRNA-directed heterochromatin. As described in the section 5.7, hairpin-derived small RNAs triggered the production of highly abundant secondary siRNAs targeting the entire *ade6+* locus in the Paf1 mutants (Figure 17B, Figure 18). We went on to test whether these *ade6+* secondary siRNAs

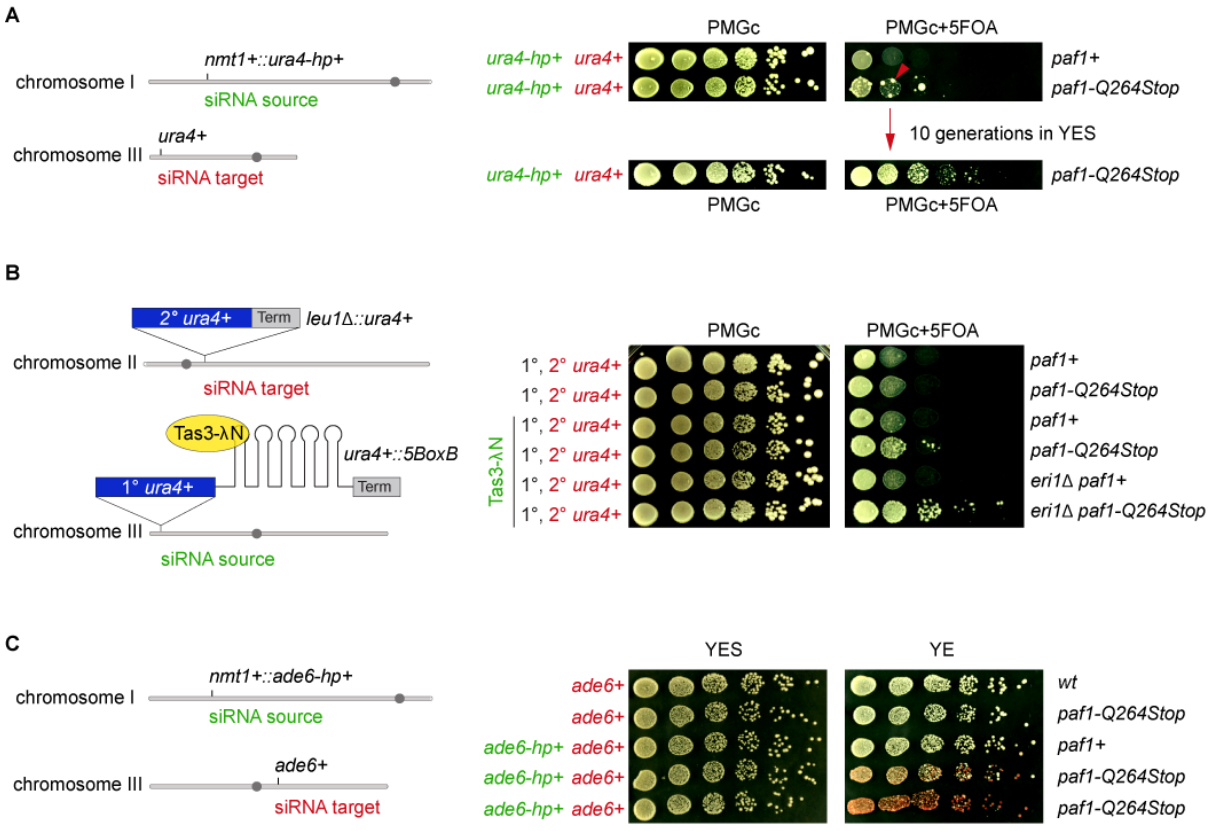

Figure 13. siRNA-mediated silencing in Paf1 complex mutants is independent of the location and sequence of the target gene. A A construct encoding for a *ura4+*-targeting hairpin (*ura4-hp*) was introduced at the *trp1+* locus on chromosome I. Synthetic siRNAs expressed from the hairpin construct target the endogenous *ura4+* gene located on chromosome III. On the right: *paf1-Q264Stop* cells and *paf1+* cells carrying the *nmt1+::ura4-hp* construct were spotted on plates containing 5-FOA for a *ura4+* silencing assay. 5-FOA is toxic to cells expressing the *ura4+* gene. Note that in the *paf1-Q264Stop* strain the silent state was maintained for several generations on non-selective medium. **B** Tethering of the RITS complex to mRNA expressed from the endogenous *ura4+* locus induces *de novo* synthesis of *ura4+* siRNAs (Bühler, Verdel, & Moazed, 2006). These *ura4+* siRNAs can establish heterochromatin at the *ura4+* locus *in cis*, but not *in trans* on the second *ura4+* allele (*leu1D::ura4+*, chromosome II). *paf1+* was mutated and *ura4+* repression was assessed by FOA silencing assays. Hairpin symbols downstream of the *ura4+* ORF denote BoxB sequences. Upon transcription BoxB RNA forms stem loop structure when that is bound by the λN protein. **C** *ade6+* silencing assay was performed on YE medium. It demonstrates that also the endogenous *ade6+* gene is repressed, if ade6-hp siRNAs are expressed in *paf1-Q264Stop* cells. Two biological replicates are shown.

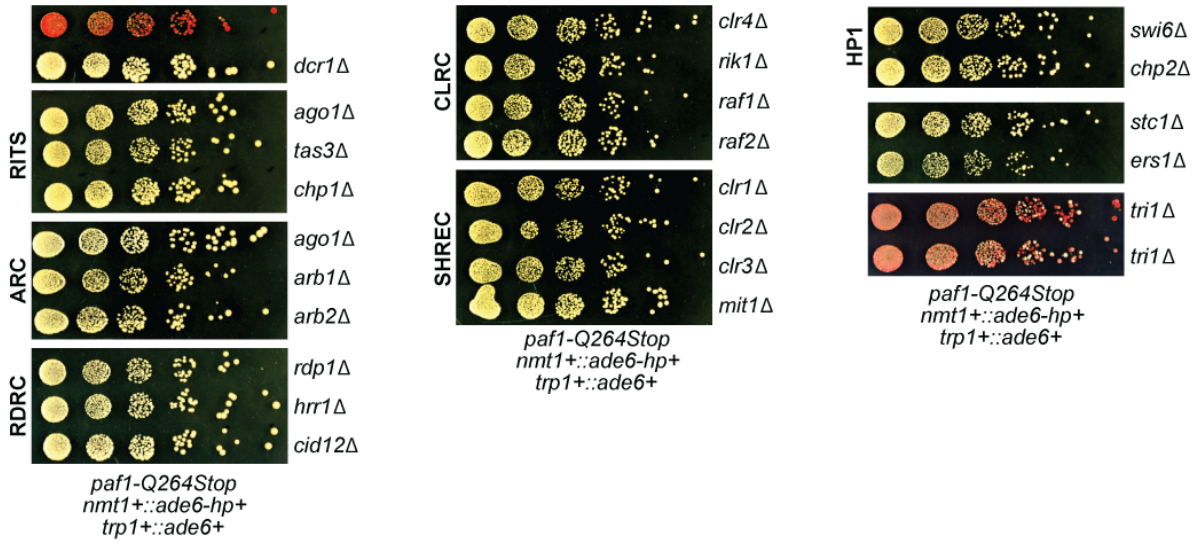

Figure 14. siRNA-directed silencing of the ade6 gene depends on the same factors that are required for centromeric heterochromatin formation. *ade6+* silencing assay on YE medium performed with *paf1-Q264Stop nmt1+::ade6-hp+ trp1::ade6+* strain in which indicated factors had been deleted. *dcr1Δ* strain was used as a control. RITS, RNA-induced transcriptional silencing complex; ARC, Argonaute siRNA Chaperone Complex; RDRC, RNA-Dependent RNA Polymerase Complex; CLRC, Clr4-methylatransferase Complex; SHREC, Snf2/Hdac-containing Repressor Complex; HP1, Heterochromatin Protein 1; Tri, Triman

were also sufficient for the maintenance of the silent state. To this end, we crossed the *trp1+::ade6+ paf1-Q264Stop ade6-hp+* strain (red) with the *trp1+::ade6+ paf1-Q264Stop* (white) strain. To our surprise, we observed that most of such crosses gave red progeny and this silencing phenotype was maintained for hundreds of subsequent mitotic divisions, even though the *ade6-hp* was not present (Figure 21A). These results show that silencing of the *ade6+* gene was stably maintained in the absence of the source of primary siRNAs, likely due to the secondary siRNAs produced form the entire *ade6+* ORF in the siRNA-amplification loop. In summary, our results showed that under conditions when the Paf1 repressive function was impaired, siRNA-directed heterochromatin was maintained very efficiently through meiotic division. Additionally, establishment of the silent state happened much more frequently when cells underwent a meiotic division than in mitotic cells. Importantly, the mutations in the Paf1 complex were necessary for both establishment of the silencing and for its subsequent maintenance. Finally and most importantly, secondary siRNAs produced from the repressed loci were sufficient to maintain the heterochromatic silencing through generations. This conclusion strongly argues for the fact that siRNA-mediated heterochromatin formation *in trans* that I described here is an epigenetic phenomenon triggered by the hairpin-encoded siRNAs. The silent state can be maintained

independently of the hairpin-derived siRNAs, with the pool of secondary siRNAs serving as the carrier of epigenetic information.

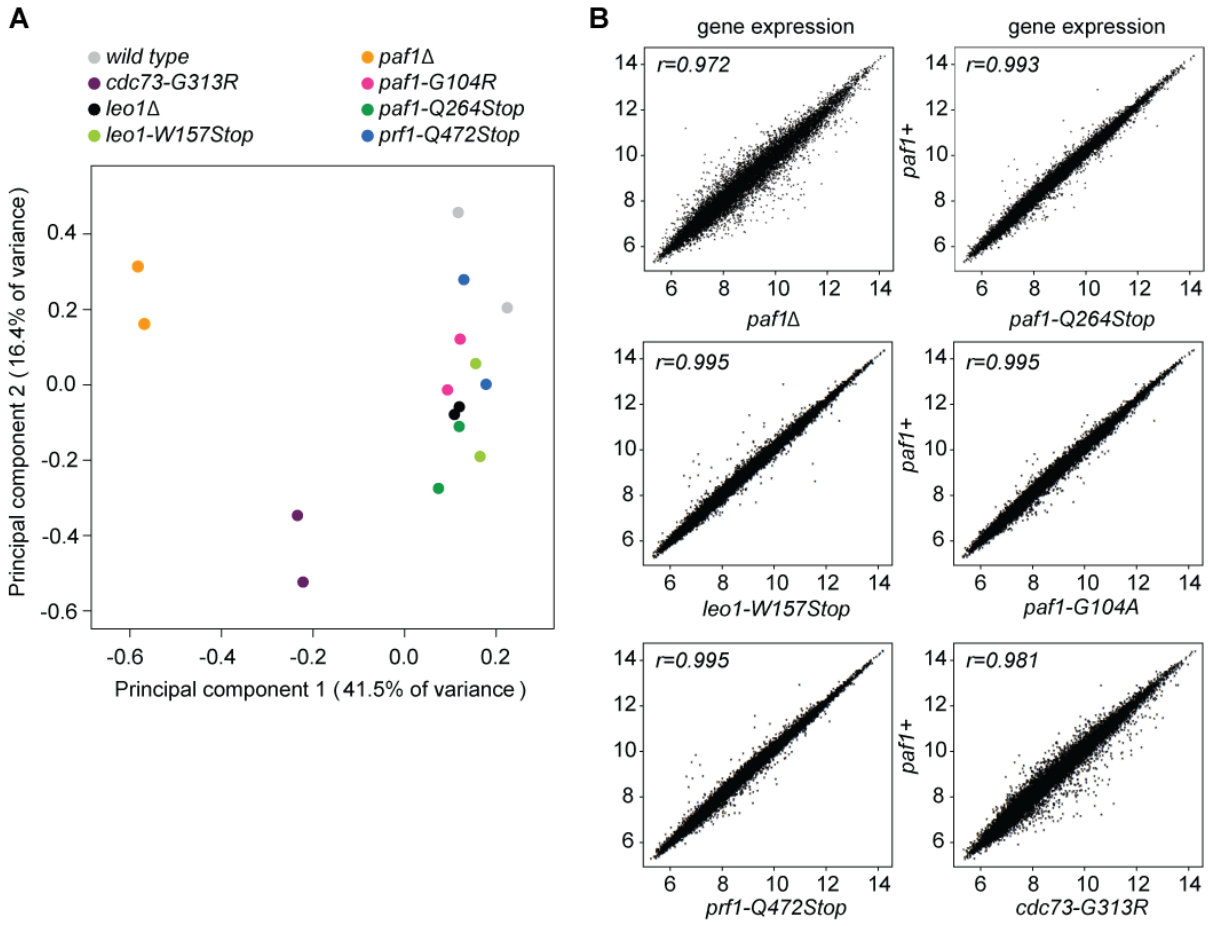

Figure 15. Point mutations in the subunits of the Paf1 complex have little effect on gene expression globally. A Genome-wide expression profiling using tiling microarrays was performed for two biological replicates of the indicated strains. Obtained expression profiles were used to conduct Principal Component Analysis (PCA). Principal component 1 and 2 were chosen for visualization. Note that the *wild type* strain clusters together with the mutant strains, whereas the *paf1Δ* and *cdc73-G313R* are the most distant from the other point mutation strains. Note as well that *leo1Δ* strain clusters together with other point mutations rather than with the *paf1Δ* strain. **B** Scatter plots illustrating pairwise comparisons of mean gene expression values for the Paf1 complex mutants and the *wild type* strain. Note that the *paf1Δ* strain shows the most pronounced changes in RNA steady state levels. Mean gene expression values were calculated for two biological replicates.

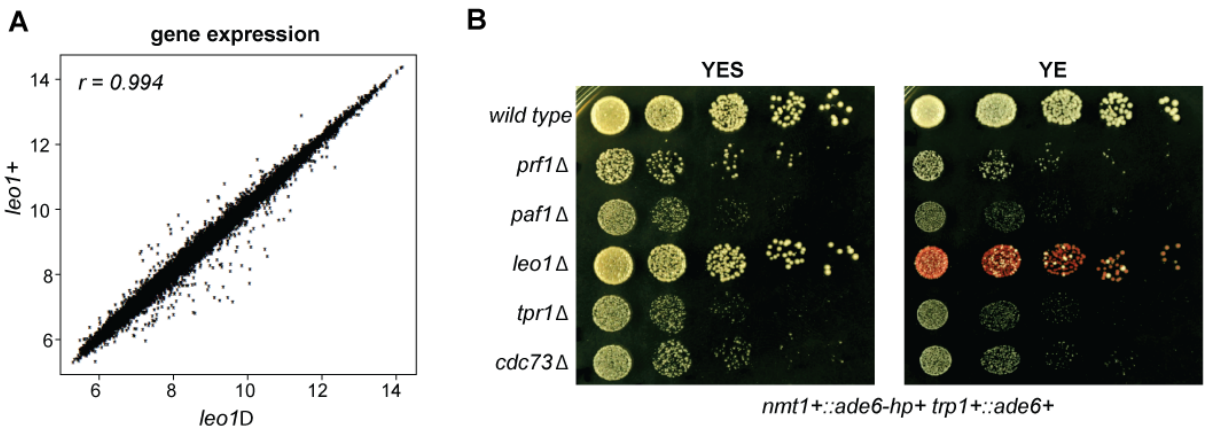

Figure 16. Leo1 subunit of the Paf1 complex is a *bone fide* repressor of siRNA-mediated heterochromatin formation. **A** Scatter plot illustrating a comparison of the mean gene expression values for *leo1Δ* and *leo1+* strains. Mean gene expression values were calculated for two biological replicates. **B** *ade6+* silencing assay performed on YE medium with strains in which genes coding for the subunits of the Paf1 complex were fully deleted. Note that siRNA-mediated silencing of the *trp1+::ade6+* reporter gene was established only in the *leo1Δ* strain. Note as well that all strains except for the *leo1Δ* exhibit severe growth defects on rich YES medium.

5.10 Identification of putative endogenous targets through meiotic division

Prompted by the interesting observation that the establishment of the *ade6+* silencing in naïve *paf1-Q264Stop* cells occurs very efficiently when the cells are propagated through meiosis (Figure 20 D), we hypothesized that putative targets of the endogenous siRNAs would be also more prone to silencing during or after the meiotic division. We reasoned that we could look at the colonies derived from single clones obtained by spore dissection in order to identify any genes that were primed for silencing. We crossed the *leo1 Δ* strain with the *leo1+* strain and looked at the progeny of this cross after few days of growth standard laboratory conditions. Interestingly, we observed that some of the tetrads gave rise to small *leo1Δ* progeny colonies and for some others the *leo1+* and the *leo1Δ* progeny were of the same size (Figure 21B).

5.11 Mechanism of repression

Next, we sought after identifying the mechanistic details of the Paf1-mediated repression of heterochromatin assembly in order to learn about its mechanism. In *S. cerevisiae*, the Paf1 complex has been implicated in regulation of the transcription process on multiple levels,

including promoting transcription elongation, termination, and RNA 3'-end processing (See section 5.2.5, Figure 22A). In the *paf1-Q264Stop* mutant, we detected a small reduction in H3K36 tri-methylation and an increase in PolII occupancy over the reporter gene, pointing towards the involvement of the complex in regulation of transcription in *S. pombe* (Figure 22B, 22C). To dissect which of the Paf1 functions are most critical to prevent RNAi-mediated heterochromatin assembly, we took a genetic approach and performed a set of deletions. By deleting and mutating the genes encoding for the elongation factors Tfs1 and Spt4, termination factors Ctf1 and Res2, as well as histone methyltransferases Set1 and Set2 [103], [171], [172], we interfered with transcription elongation, termination, or co-transcriptional histone modifications, respectively (Figure 22A).

We observed siRNA-mediated silencing of the *ade6+* reporter in the *ctf1-70* and to a smaller degree in the *res2Δ* mutant cells, but no repression was detected in the other mutants (Figure 22D, 22E). Importantly, silencing in these two strains was not as potent, and the propagation of the silent state was not as stable as in the *paf1-Q264Stop* mutant. Instead, the *ctf1-7*0 and *res2Δ* cells showed variegation of the red phenotype, indicating a bi-stable silencing state that could spontaneously undergo de-silencing in the progeny cells during colony growth.

The *ctf1-70* gene encodes for a truncated form of the mRNA cleavage and polyadenylation specificity factor Ctf1. In a mutant strain harboring this allele, RNA PolII fails to terminate properly at the polyadenylation signal, yet the nascent transcript is still properly processed downstream of it, and released from the site of transcription [173]. We then went on to test the importance of transcript release for the siRNA-directed silencing specifically. In order to uncouple release of the transcript from cleavage by the endogenous factors, we inserted a 52-nucleotide hammerhead ribozyme (Rz), preceded by a 75-nucleotide long polyA tail template downstream of the endogenous *ade6+* ORF (*ade6-Rz*) (Figure 23A). A newly transcribed RNA containing such ribozyme undergoes self-cleavage and can be released from chromatin independent of the subsequent transcription termination signal. In a control strain we used an identical *ade6-Rz* construct, but the catalytic site of the ribozyme was mutated by a single nucleotide substitution (*ade6-Rzm*). Such mutation abolishes the self-cleavage event and as a result, the *ade6-Rzm* mRNA is dependent on the endogenous transcription termination machinery for transcript release. We observed that silencing of *ade6-Rz* was less efficient and the repression was rather poorly propagated through generations in the *paf1-Q264Stop* strain, whereas the *ade6-Rzm* allele was very effectively silenced (Figure 23B). This indicates that the retention of the nascent transcript on chromatin contributes to subjecting a gene for siRNA-mediated repression. Taken together, we conclude that

impairment of transcription termination and nascent transcript release is the underlying cause for siRNA-directed heterochromatin assembly in the Paf1 mutant cells. The fact that improper transcription termination, observed in *ctf1-70* mutant cells, was not enough to induce efficient and stable silencing, is further confirmed by the identification of impaired nascent transcript release as a contributing factor. We, therefore, postulate that identified Paf1 mutations lead to defects in both transcription termination and transcript release and the combinatorial effect of these two roles is a cause for observed strong repression. This allowed us to identify Paf1 complex mutants in our screen, in contrary to factors like Ctf1 that regulate only one of these events (Figure 22).

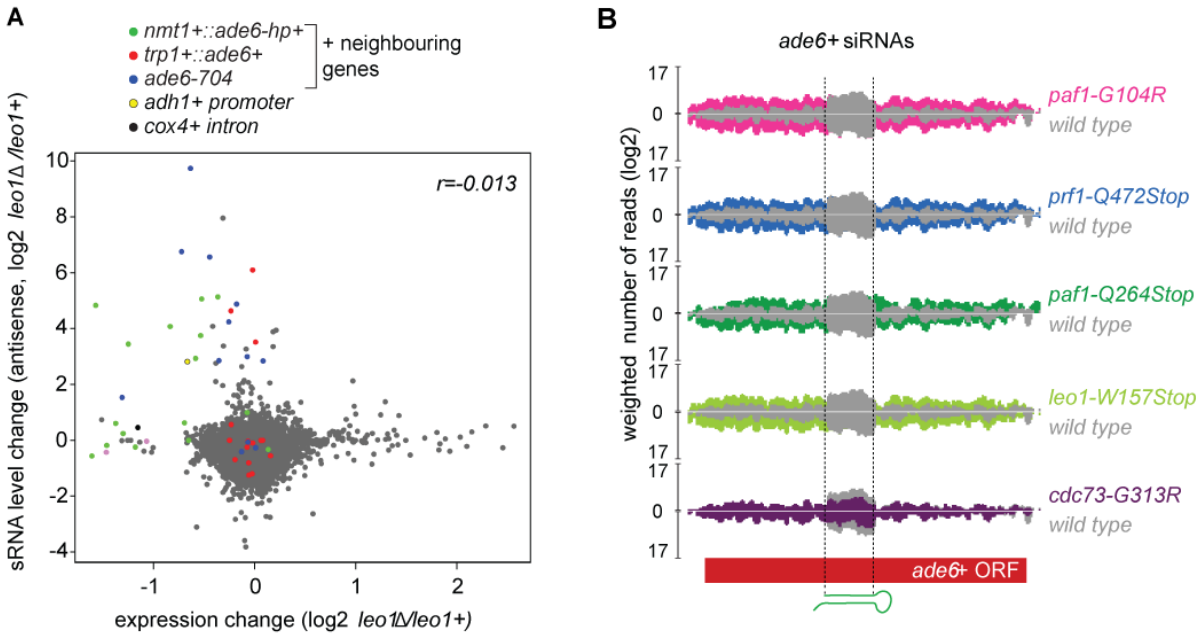

Figure 17. Nucleation of ectopic heterochromatin by endogenous siRNAs. A Comparison of differential gene expression and differential expression of small RNAs for the *leo1Δ/leo1+* pair of strains. Differential gene expression was obtained from genome-wide expression profiling data generated with tiling microarrays. Small-RNA-expression profiling was performed by next-generation deep sequencing, and differential small RNA expression was calculated for siRNAs mapping to the antisense strand. Gene neighboring the siRNA target loci: *trp1+::ade6+*, *ade6-m704*, *nmt1+::ade6-hp+* are marked in red, blue and green, respectively. Elements of the hairpin construct: *cox4+* intron sequence and *adh1+* promoter are marked in black and yellow, respectively. Log2 scale was used. **B** Profiles of small RNAs mapping to the *ade6+* gene. Small RNA profiles for all point mutants were obtained by next-generation deep sequencing. Read counts were normalized to the size of the individual libraries and depicted in log2 scale. Profile for the *wild type* strain is shown in gray, profiles for the mutants are shown in indicated colors. The region marked with dashed lines refers to the hairpin-encoded ectopic small RNAs. Note that secondary small RNAs mapping to the entire ORF on both sense and antisense strands were detected.

6 Discussion and outlook

The results presented in this dissertation demonstrate that in fission yeast, under conditions when the Paf1 complex function is impaired, siRNAs can efficiently mediate transcriptional gene silencing *in trans*. The siRNA-induced repression can be directed against euchromatic genes independent of their genomic location, and involves *de novo* formation of functional heterochromatin. The silent state fulfills all the criteria of a *bona fide* heterochromatic domain, like the accumulation of H3K9 methylation, generation of target-derived secondary small RNAs, and reduction of the transcriptional activity at the target gene (Figure 10, Figure 12, Figure 18). We showed that mutation of the Paf1 complex is required for both the establishment and the maintenance of the repression (Figure 20). Importantly, the silent state was transmitted through meiosis and subsequently inherited through several hundred mitotic divisions in the absence of the primary siRNA source (Figure 21). This observation demonstrates the epigenetic nature of the phenomenon and raises the possibility of stable transgenerational silencing of selected target genes 'at will', providing an exciting scientific finding and novel technological implications at the same time.

6.1 The Paf1 complex represses epigenetic gene silencing

One of the most exciting results presented in my thesis is that under the condition when the Paf1 function is impaired, the silent chromatin state of the *ade6+* locus can be efficiently transmitted through meiosis and hundreds of mitotic cell divisions, even if the hairpin-derived primary small RNAs are not provided any longer (Figure 21). The heterochromatic state is maintained in the progeny cells due to secondary siRNAs originating from the siRNA amplification cycle established at the target locus (Figure 3, Figure17). This self-reinforcing positive feedback loop comprises of small RNA production and deposition of H3K9 methylation. Thus, RNAi-directed transcriptional gene silencing induced in the Paf1 mutant cells complies even with the strictest definitions of epigenetic gene silencing [1], [174].

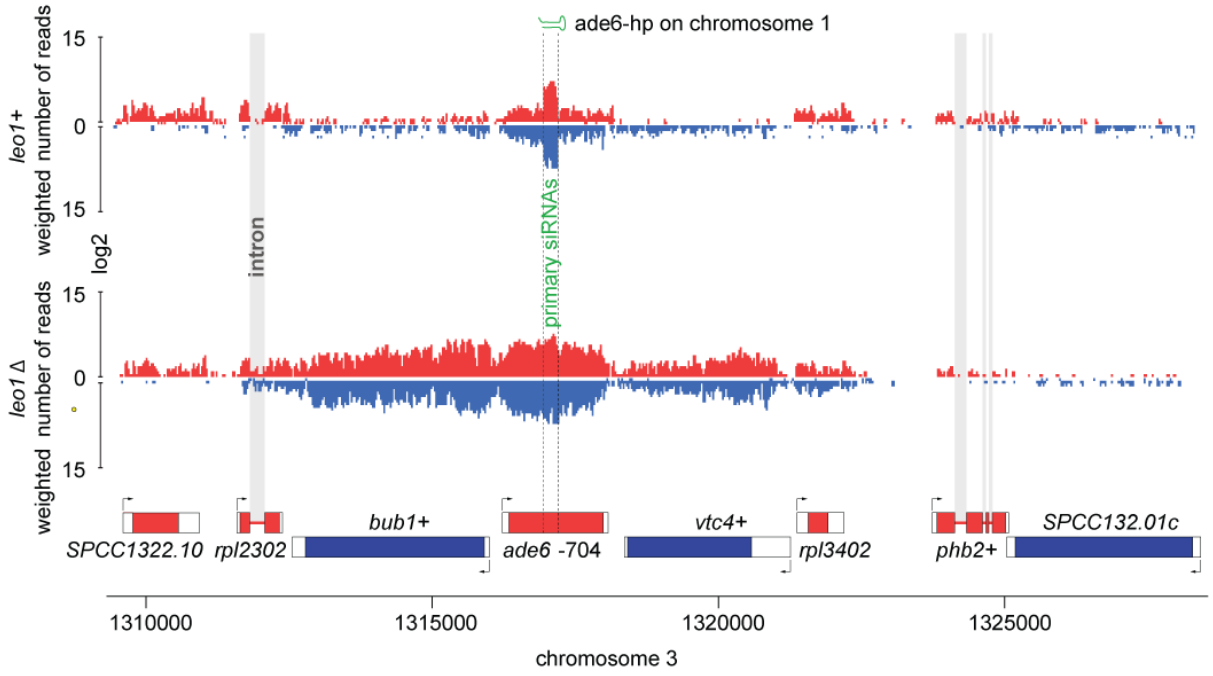

Figure 18. The *ade6-m704* locus produces secondary siRNAs when the function of the Paf1 complex is impaired. Small RNA profile at the *ade6-m704* locus was obtained by next-generation deep sequencing for the *leo1+* and *leo1Δ* strains. Read counts were normalized for the size of the sequencing libraries and represented in log2 scale. Sense reads are depicted in red, antisense reads in blue. Gray bar indicates ectopic small RNAs produced from the *ade6-hp*. Note that siRNAs mapping to the intron of the *rpl2302+* gene were also detected.

6.2 Potential conservation of the repressive role of the Paf1 complex

Despite the high evolutionary conservation of the RNAi pathways and their components, the function of small RNAs in stable transcriptional silencing of protein coding genes has not been unanimously confirmed. However, several biological phenomena show intriguing resemblance to the small RNA-mediated transgenerational gene silencing that we observe when the Paf1 complex function is impaired.

One of most notable examples is the phenomenon of paramutation observed first in plants [175]. In maize, expression of the B locus responsible for plant pigmentation is regulated by the chromatin state of its enhancer. Interestingly, upon crossing two maize plants, one carrying the silent copy of the gene and one carrying the active copy of the gene, the active allele becomes repressed *in trans* by the presence of the silent allele during the embryonic development of the heterozygous progeny. This leads to the non-mendelian inheritance of the pigmentation phenotype. It is unclear why the active enhancer undergoes hetero-chromatinization, but it is known that this process depends on small RNAs and the RNAi

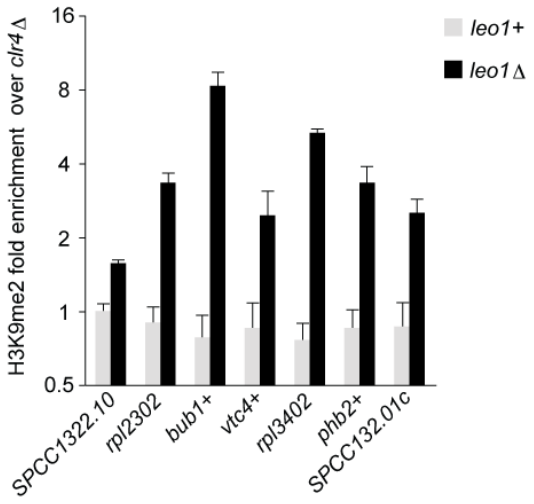

Figure 19. Heterochromatin spreads from the *ade6-m704* locus when the function of the Paf1 complex is impaired. ChIP experiment illustrating the H3K9me2 mark enrichment at the genes neighboring the *ade6-m704* locus in *leo1+* and *leo1Δ* strains (see Fig. 18. for the scheme of locus architecture). Enrichments were calculated relative to the values for *clr4*Δ mutant strain and normalized to the values for the *adh1+* gene. Error bars represent standard deviation. Mean of two biological replicates is shown.

machinery [175]. Most intriguingly, the enhancer sequence functions as a source of 24 nt long small RNAs, regardless of whether it is present in active or silent chromatin form. It is tempting to speculate that the difference between the two alleles lies in the occupancy of active or functional Paf1 complex, which, when present, represses formation of heterochromatin on the enhancer locus and keeps the gene active. Examples of mechanisms resembling the paramutation phenomenon have been also described in *Drosophila* and mice [176]. In a recent study Baulcombe and colleagues identified a mutant plant background, in which *de novo* induced RNA-directed DNA methylation and TGS can be epigenetically propagated to the progeny plants with an increased stability [177]. Interestingly, if the mutant plants are propagated for several generations, the induced silent state also shows an effect similar to paramutation. The authors propose that their finding can be applied in the future to generate epigenetic transgenic plants without the need for constant modifications of their genome.

Transgenerational gene silencing induced by small RNAs has also been reported in *C. elegans* [67], [178]. In a phenomenon named RNAe, for RNA-induced epigenetic silencing, transcriptional silencing of a reporter gene is induced by the PIWI protein PRG-1 and piRNAs, or by the RDE-1-bound 21-U RNAs (Described in section 3.1.4). Both of these pathways lead to the generation of secondary 22G-RNAs and involve the RdRP activity.

22G-RNAs are bound by the worm specific Argonaute protein WAGO-9 (also known as HRDE-1), and the worm nuclear Argonaute protein NRDE-3 [179]. Upon loading with small RNAs, WAGO-9 translocates to the nucleus where it targets the nascent transcripts of RNA PolII and mediates transcriptional gene silencing through H3K9 methylation. Members of the NRDE pathways (See section 3.1.4): NRDE-1, NRDE-2 and NRDE-4, as well as the HP1 homolog HPL-2, are required for the establishment and maintenance of this repression [179], [180]. The silent state can be efficiently transmitted through multiple generations [67], however, it is not dependent on PRG1 or on the primary piRNAs that maintain the silencing. It was also suggested that to some extent RNAe shows the properties of paramutation, however, unlike in plants, the paramutagenic properties of the silent allele depend on the gender of the parent through which it was inherited [67]. This remarkable stability of the silent state and dependence on production of secondary siRNAs resemble the repression induced by *ade6-hp*-derived siRNAs when the Paf1 complex function is impaired (Figure 18, Figure 21). No mechanistic details of RNAe were reported so far, so the putative involvement of the Paf1 complex and the role of transcription kinetics in this small RNA-mediated transcriptional silencing remain to be uncovered.

Finally, in line with our hypothesis on the role of Paf1 complex in preventing small RNA mediated gene silencing, the Paf1 complex was suggested to act as a protector of the pluripotent state of mouse Embryonic Stem Cell (ESC) [181], [182]. The authors showed that Paf1 complex associates with the promoters of key pluripotency genes, including Oct4, Sox2 and Klf4. This association contributes to maintenance of the active chromatic state at these loci. Depletion of the Paf1 complex leads to decreased expression of pluripotency genes, formation of repressive chromatin state, and cellular differentiation. We speculate that this process might also be under control of small RNAs and RNA-directed heterochromatin formation. Intriguingly, in this scenario the Paf1 complex would selectively dissociate from some, but not all the PolII transcribed genes. Conservation of the Paf1 repressive function and the existence of small RNAs targeting the pluripotency genes remain to be confirmed in the future. An inherent difficulty of these experiments comes from the fact that Paf1 complex is essential for the viability of mouse cells.

6.3 The Leo1 subunit is a *bona fide* repressor of silencing.

The genome-wide expression profiling analysis (Figure 17A) and the *ade6+* silencing assay in the *leo1Δ* mutant both point towards the distinct role for the Leo1 subunit in the Paf1 complex. In fact, several studies performed in budding yeast described slightly distinct phenotypes of the mutants of individual Paf1 complex subunits. These differences indicate that the individual subunits might play separate roles in the cell. (See section 3.2.4). For instance, the budding yeast Leo1 was found to be dispensable for proper H3K36 and H3K4 methylation levels, which is likely to contribute to the less severe growth phenotype of *leo1Δ* mutants. Whether this is the case for the *S. pombe* Paf1 complex remains unknown.

We found striking differences in silencing and growth phenotypes between the strains harboring full deletions of the *leo1* and the *paf1* genes (Figure 16B). We postulated that the Leo1 subunit acts as the actual repressor of the siRNA-directed heterochromatin formation and this hypothesis made us speculate that maybe the point mutations within the other subunits result in disruption of the Leo1 binding to the complex. Intriguingly, the Leo1 subunit of the hPAf11 complex was implied to function as a direct contact point to the PolII [99]. Assuming that the complex architecture is conserved between fission yeast and human, mutations causing loss of the Leo1 subunit could result in detachment of the complex and its dissociation from chromatin. These hypotheses remain to be tested by *in vitro* biochemical analysis of the complex integrity, as well as by *in vivo* functional studies.

The budding yeast Leo1 was also suggested to contribute to the RNA binding properties of the Paf1 complex, which in turn was crucial for stable association with chromatin and with the transcription elongation machinery [183]. Therefore, mutations of the putative Leo1 RNA binding site could result in dissociation of the remaining subunits from chromatin as well. The special function of the Leo1 subunit in the context of siRNA-mediated silencing remains to be determined.

A very recent study found Leo1 in a screen for suppressors of heterochromatin spreading in fission yeast [184]. Subsequently, the authors confirmed that the remaining components of the Paf1 complex, but not other factors regulating transcription elongation and known Paf1-related chromatin modifications, also function as negative regulators of the spreading.

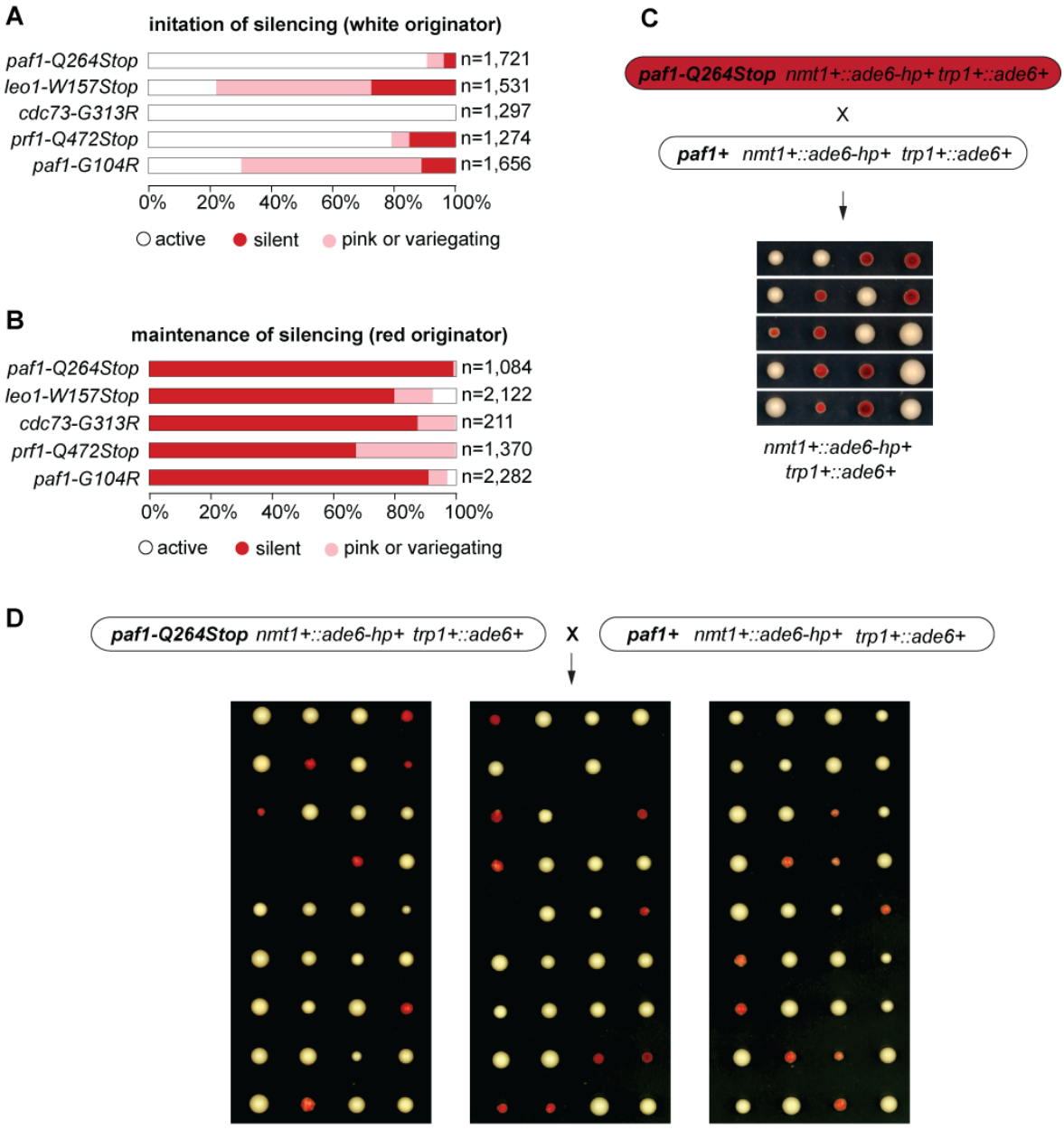

Figure 20. Establishment and maintenance of the siRNA-directed heterochromatin through mitotic and meiotic divisions. A and **B** Establishment and maintenance of heterochromatin at the *trp1+::ade6+* locus in the Paf1 complex mutants was assessed upon approx. 30 mitotic divisions, based on the phenotype in the silencing assay on YE plates. White and red parental colonies were used for establishment (A) and maintenance (B) assay, respectively. **C** Maintenance of the silent state at the *trp1+::ade6+* locus upon meiotic division was assessed by crossing of the indicated strains. Progenies of five tetrad dissections are shown. Red *paf1-Q264Stop* cells were used for the cross. White progeny colonies are *paf1+*. **D** Establishment of the silent state at the *trp1+::ade6+* locus upon meiotic division was assessed by crossing the indicated strains. Progenies of 24 tetrad dissections are shown. White (naïve) *paf1-Q264Stop* cells were used for the cross. Note that silencing of the *trp1+::ade6+* gene was frequently established upon meiosis.

The study proposes a model, in which Leo1 recruits the histone H4 lysine 16 (H4K16) acetyltransferase Mst1 to actively transcribed chromatin, and by this antagonizes the spreading of heterochromatin onto neighboring regions. The model is supported by the observation that deletion of the *leo1+* gene results in a decrease in H4K16 acetylation and loss of the Mst1 binding to chromatin. Although this mechanism is generally in agreement with our findings, the aforementioned study does not provided compelling evidence for the role of H4K14 acetylation mark in negative regulation of silent chromatin. To strengthen their hypothesis, the authors could check whether using a temperature-sensitive allele of Mst1 would cause a similar spreading effect in the presence of the Paf1 complex [185]. Since Mst1 is an essential protein, creating a catalytically-dead mutant likely would have similar deleterious consequences. However, two mutant alleles of the histone H4 were reported, H4K16R which abolishes the acetylation and H4K16Q, which mimics the acetylated state [186]. By combining these mutations with the *leo1+* deletion, one could test more directly whether the H4K16 acetyl mark is a cause, or a consequence of heterochromatinization of boundary regions. Finally, the authors do not exclude the possibility that the spreading of heterochromatin is actually due to the activity of the RNAi machinery. In fact they observed that the spreading phenotype is suppressed when the *leo1Δ* mutation is combined with deletions of genes encoding for the RITS complex components Chp1 and Tas3. Although mutations of the RITS complex cause defects in centromeric silencing, which could have an effect on the spreading by itself, this result points towards a putative role of the RNAi machinery in the spreading of heterochromatin repressed by Leo1.

6.4 Inefficient transcription termination and silencing

We proposed a model according to which the Paf1 complex represses formation of siRNA-mediated facultative heterochromatin by ensuring proper transcription elongation, and transcript termination and release (Figure 22, Figure 23, Figure 24). Contribution of the Paf1 complex to multiple stages of transcription seems to be a key feature of the repressor, since deletion of factors affecting only individual stages gave rise to a much weaker and unstable silencing phenotype (Figure 22). This multilevel effect was probably what contributed to the strongest phenotype we were selecting for in our screen, and allowed us to identify the Paf1 complex, but not other factors like Ctf1, which regulate only one for these events.

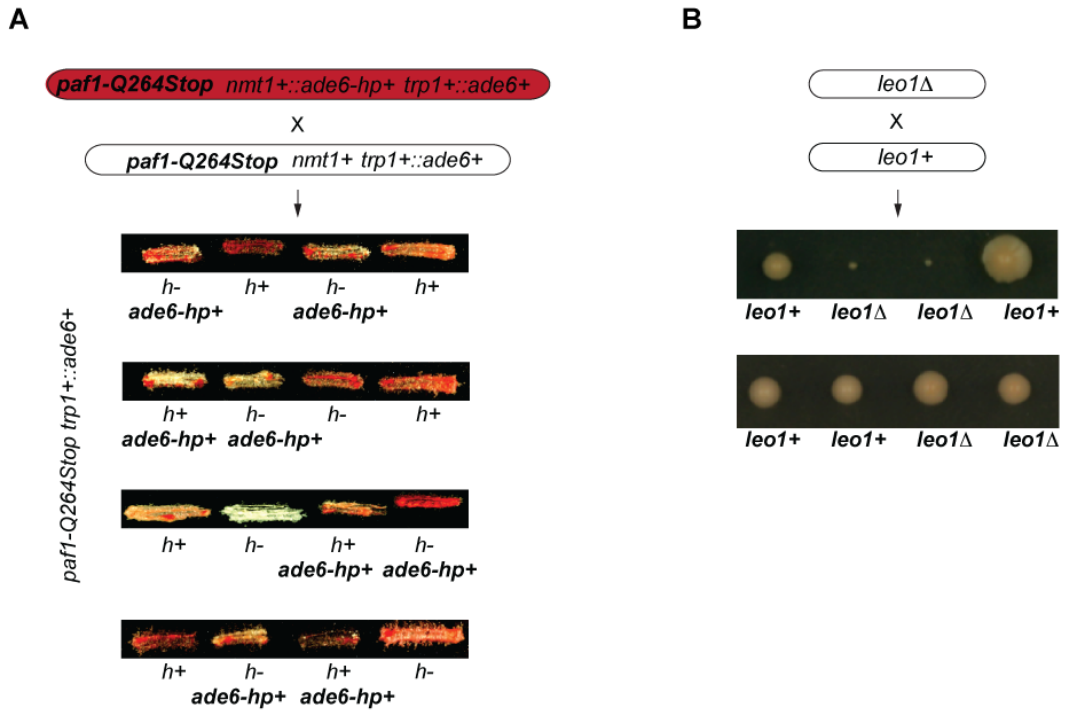

Figure 21. siRNA-induced silencing of the *trp1+::ade6+* gene can be transmitted through meiosis in an epigenetic manner. A Maintenance of heterochromatin at the *trp1+::ade6+* locus in the absence of the *ade6-hp*-derived ectopic small RNAs was assessed by crossing of the indicated strains. Progenies of four tetrad dissections were struck on YE plates. Note that progenies show red phenotype despite the fact that source of initial siRNAs is missing. *ade6-hp+* annotations marks colonies carrying the hairpin construct. *h+* and *h-* denote mating types. **B** Progenies of tetrad dissection obtained by crossing of a *leo1Δ* strain with a *leo1+* strain. *leo1Δ* and *leo1+* annotation indicate respective genotypes of the progeny colonies. Note that, despite being isogenic, *leo1Δ* progenies exhibit different growth phenotype.

There is strong evidence for the link between inefficient transcription termination and licensing of genes for siRNA-mediated repression. Most importantly, it was shown that if the termination signal of a euchromatic *ura4+* gene was impaired, siRNA-directed nucleation of heterochromatin could be efficiently induced at this locus [169]. The authors propose that the presence of termination and polyadenylation signals close to the coding sequence causes efficient transcript release and makes genes refractory to siRNA-directed repression. In a study conducted in plants, several mutations in the cleavage and polyadenylation factors were identified in a screen for factors enhancing small RNA-mediated silencing. These mutations subject target genes to RNA-directed repression, possibly by causing impaired 3' end formation and read-through transcription of the transgene [187]. Intriguingly, the authors point out that both mis-terminated and mis-spliced transcripts could be recognized as aberrant and become sources and targets for small RNAs. Their hypothesis supports well our model, as problems with RNA splicing and termination would increase the time nascent transcripts spend on chromatin and expose them as a platform for the RITS complex binding.

We observed that secondary small RNAs matching the *cox4+* intron sequence were produced from the hairpin transcript, but they did not cause accumulation of the H3K9 methylation over the *cox4+* gene (Figure 12). It would be therefore interesting to test whether these small RNAs could induce ectopic heterochromatin formation at the *cox4+*locus in a strain where the splicing machinery was impaired. Intriguingly, in the yeast *Cryptococcus neoformans,* inefficient splicing and stalling of the spliceosome results in the production of small RNAs from the defective transcripts and subjects them to transcriptional silencing [188]. In this manner, foreign genetic elements with poor or inadequate splicing signals can be repressed by *C. neoformans*.

Deficiency of some splicing factors was suggested to impair RNAi-mediated heterochromatin assembly at the centromeric repeats *S. pombe* [189]. Later studies contradicted this result and explained that the silencing defects might be due to inefficient splicing of mRNA encoding for the RNAi pathway components [190]. In fact, several of the RNAi pathway genes, including *ago1+*, *arb1+* and *arb2+* contain introns. It was shown that the silencing defects observed upon impairment of splicing could be alleviated by replacing the RNAi pathway genes with their cDNA equivalents [190]. Requirement for splicing factors in heterochromatin formation implies that it might be more difficult than anticipated to understand the role of efficient splicing in protecting the euchromatic and will require more careful experimental design.

The link between silencing and inefficient transcription termination provides a hint on why centromeric non-coding repeats can serve as targets for transcriptional gene silencing by small RNAs, despite the fact that they are also transcribed by RNA PolII [39]. It was suggested that centromeric transcripts frequently undergo aberrant and inefficient transcription termination due to collision of the transcribing polymerase with the replication machinery [191]. Interestingly, the authors of this study also point towards the role of the RNAi machinery in actual transcription termination and release of PolII from mis-terminated genes. This hypothesis is in agreement with our general model, implicating the role of slowed down transcription kinetics and transcript retention ‘on chromatin’. However, it does not address the repressive function of the Paf1 complex. An alternative explanation would be that the Paf1 complex interacting with PolII on centromeric repeats is post-transcriptionally modified, and this modification affects its activity or causes dissociation from chromatin. There is no direct evidence for this hypothesis, yet several observations point to this direction. First of all, the components of the Paf1 complex were shown to be post-

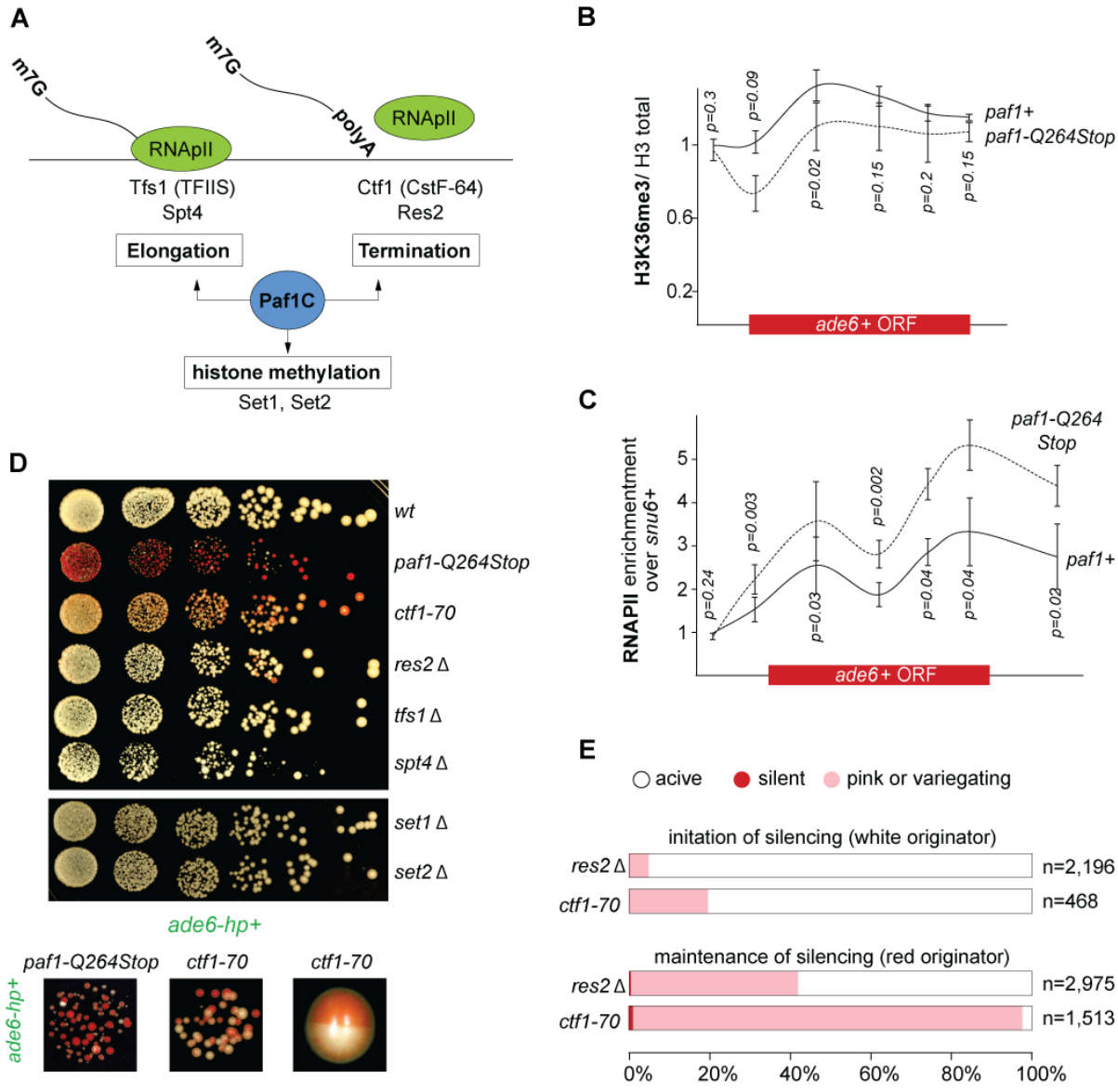

Figure 22. Repression occurs due to inefficient transcription termination. A Schematic representation of the stages of transcription process in which the Paf1 complex was suggested to play an important role. **B** and **C** Results of ChIP experiments to assess accumulation of the H3K36me3 mark and RNA PolII occupancy at the *ade6+* locus in *paf1+* and *paf1-Q264Stop* strains, in the absence of *ade6-hp* construct. In order to control for signal background and for differences in ChIP efficiency, scores were normalized to the enrichments over *snu6+* gene. For the H3K36me3 ChIP, scores were normalized to the total histone H3 enrichments. Mean of three biological replicates is shown. Error bars represent standard error of mean (s.e.m.). P-values were calculated using one-tailed Student's t-test. **D** *ade6+*-silencing assay on YE plates using the *nmt1+::ade6-hp+ tpr1+::ade6+* strain, in which indicated factors had been deleted or mutated. Lower panel shows close-up pictures of *ctf1-70* mutant. Note the variegating colonies indicating the bi-stable silencing state. *paf1-Q264Stop* strain was used as a positive control. **E** Establishment (top) and maintenance (bottom) of heterochromatin at the *trp1+::ade6+* locus in the ctf1-70 and res2D mutants were assessed upon approx. 30 mitotic divisions White and red parental colonies were used to assess establishment and maintenance, respectively. *n*, number of scored colonies.

transcriptionally modified both in the fission and the budding yeast [192–194]. Furthermore, many connections link the Paf1 complex to regulation of the cell cycle as well. Most notably, the Cdc73 subunit was identified in a screen for regulators of the yeast cell cycle in the budding yeast, and the fission yeast Cdc73 was found in a global analysis of proteins that acquire cell-cycle dependent modifications [192]. It was proposed in the past that siRNA production and the subsequent reinforcement of silencing at centromeric repeats occurs in a cell cycle-dependent manner [195]. Thus, one could imagine that a crosstalk between these two processes exists. Paf1 binding to centromeric DNA could be regulated by cell cycle and have a direct consequence in making the centromeric chromatin permissive for the RNAi factors, small RNA production and transcriptional silencing.

An alternative model of siRNA-mediated transcriptional silencing could also be drawn. A study using a mass spectrometry approach detected an interaction between the Cdc73 subunit of the Paf1 complex and the putative histone H3 lysine 9 demethylase Epe1 [186]. Thus, the Paf1 complex could protect the active chromatin state of protein coding genes by continuous recruitment of Epe1, which would remove any H3K9 methylation induced by the endogenous primary siRNAs. Mutations in the Paf1 complex would disrupt this interaction and prevent Epe1 recruitment, allowing siRNA-mediated nucleation of heterochromatin and establishment of the self-reinforcing loop. In line with this idea, it has been recently shown that in the *epe1Δ* strain, heterochromatin can be stably maintained and inherited [196], [197]. However, contrary to our model, in the *epe1Δ* mutant, heterochromatin can be stably maintained in the absence of the RNAi machinery. This discrepancy speaks against this alternative mechanistic interpretation. Furthermore, a molecular mechanism in which the Paf1 mutations cause loss of the Epe1 demethylase does not explain the fact that mutations in the factors mediating transcription termination give rise to the similar silencing phenotype as the Paf1 complex mutants (Figure 22). Therefore, is more likely that putative recruitment of Epe1 would rather contribute to the role of the Paf1 complex as a repressor of siRNA-mediated heterochromatin formation, than be the main cause of it.

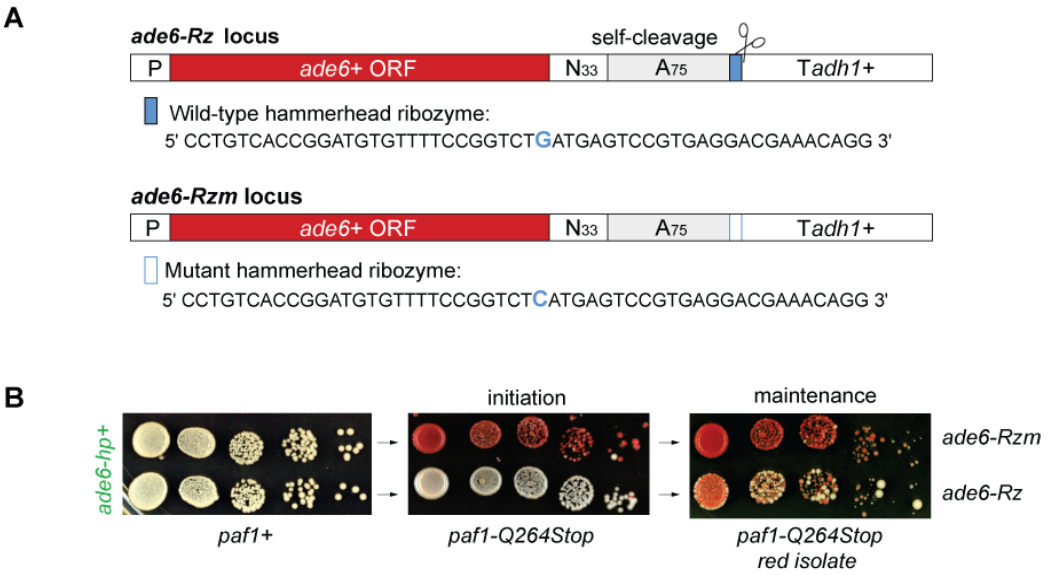

Figure 23. Efficient transcript release contributes to escape from siRNA-mediated silencing. A Schematic representation of the ribozyme constructs inserted at the endogenous *ade6+* locus. *ade6+* ORF is followed by a sequence encoding for a synthetic polyA signal. A 52nt-long sequence encoding for a *wild type* (*ade6-Rz*) or mutant (*ade6-Rzm*) self-cleaving hammerhead ribozyme was inserted downstream. *ade6-Rzm* differs from *ade6-Rz* by one nucleotide, which introduces a mutation in the active site and renders the ribozyme inactive. Ribozyme-encoding sequence is followed by a terminator sequence from the *adh1+* gene. **B** *paf1-Q264Stop* strain carrying the *ade6-Rz and ade6-Rzm* alleles was spotted in ten-fold serial dilutions on YE plates for the *ade6+* silencing assay. Red colonies from the middle panel were selected for second silencing assay to assess the efficiency of heterochromatin maintenance (most right panel). Note that both *ade6-Rz* constructs produce functional *ade6+* mRNA (white color of the cells in the *paf1+* cells).

6.5 Possible approaches to test conservation of Paf1 complex function

As mentioned in the Section 8.2, studies of putative conservation of small-RNA-mediated transcriptional gene silencing upon depletion of the Paf1 complex are inherently difficult in higher eukaryotes, because all of the components of the complex are essential for cell viability. Therefore, in order to elucidate the role of hPaf1 in transcription and epigenome regulation, one needs to employ approaches more complicated than complete gene deletions.

hPaf1 complex has been implicated in maintenance of the pluripotent state of ESC. To test the positive effect of hPaf1 complex on expression of the pluripotency genes, we could generate various conditional knockout alleles in mouse ESC lines and assess the pluripotency and differentiation potential upon depletion of the complex. To this end, one can employ the genome editing technologies aforementioned in the Section 3.1.5. Importantly, as complete ablation of the hPaf1 complex is likely to be deleterious for cell survival, it will be crucial to find a time window when we could assay gene expression and ideally small RNA expression

as well. This would allow us to test the model proposed in this study directly. Alternatively, we could also try to perform the experiments in heterozygous cell lines, assuming that depletion of one allele will have a sufficient effect on the protein levels of the Paf1 complex without causing cell death.

Mutations in genes encoding the Paf1 complex have been linked to tumorigenesis. Several mutations that correlated with the occurrence of the disease (See. Table 1 and Table 2) were identified in the *CDC73* and *CTR9* genes. In case of the hereditary tumors, it was suggested that the patients were carriers of one mutated allele (See Table 1), and inactivation or loss of the second one caused the development of cancer [163]. In some cases development of the tumors was caused by a second mutation event but in a different tumor suppressor gene [163]. Although this approach would be highly speculative, we could assume that the expression of such mutated alleles lead to production of a defective form of the Paf1 complex. The expression of this allele would still allow cell survival, causing only a partial loss-of-function phenotype. Thus, we could reconstitute some of these mutations in order to impair the function of the Paf1 complex.

Finally, we could also test whether impaired transcription termination and transcription kinetics would subject genes to small-RNA-mediated repression also in human cells without interfering with the Paf1 complex expression levels. During influenza virus infection in human cells, the viral protein NS1 interacts with the cleavage and polyadenylation factor of the host and causes inefficient release of the nascent transcript by inhibiting the transcript cleavage event [198]. By this mechanism, the virus can promote transcription of its own genome. In a separate study, the NS1 protein has been suggested to interact with the Paf1 complex, affecting efficient transcription elongation and by this suppressing the expression of antiviral response genes [199]. If both of these functions were correctly described, overexpression of the NS1 protein should affect the kinetics of transcription termination and elongation. Thus, we could provide the cells with a potent source of small RNAs and combine it with the overexpression of NS1 and by this we would likely circumvent the problem of the lethality caused by knockouts or knockdowns of the Paf1 complex genes. Overexpressing NS1 could be relatively easily used to test the model we proposed for the fission yeast in the human cells in the future.

6.6 Implications for mechanistic studies

The results obtained in our study provide a novel experimental setup that can be used to address some fundamental mechanistic questions in an unprecedented way. We show that a potent source of siRNAs is required to establish the silencing (Figure 10, Figure 14). Afterwards, the function of primary siRNAs can be taken over by the secondary siRNAs, but the silencing is still highly dependent on Dicer and other RNAi machinery components (Figure 14). By performing a series of crosses, we showed that the heterochromatin maintenance could be uncoupled from the constant expression of the primary siRNAs from the hairpin. We believe that this clean and elegant experimental design can be used to determine other requirements for heterochromatin establishment and maintenance. Until now, these experiments were performed in a laborious way, involving deletion and reconstitution of the *clr4*+ methylatransferase gene, or relied on treatment of the cells with a histone deacetylase inhibitor that could be insufficient or elicit pleiotropic effects [200], [201]. Furthermore, by using gene specific hairpins, with our new approach we can study heterochromatin independently of the repetitive nature of the centromeric repeats and their partial complementarity to other heterochromatic loci. We can also test the requirements for the length and sequence of a minimal siRNA source, or avoid the redundancy of multiple maintenance pathways at the mating type locus [32].

As mentioned above, expression of the *ade6-sh*+ construct is driven from the *adh1*+ promoter, and it encodes for a hairpin containing the *cox4*+ intron sequence in the place of the loop (Figure 4B). Despite the fact that small RNAs targeting both strands of the *cox4*+ intron and the *adh1*+ promoter were made, they did not induce accumulation of the H3K9 methylation mark on either of these genes (Figure 12). Production of *cox4*+ intron-derived secondary siRNAs, but not siRNAs mapping to the rest of the transcript were also observed by another group, which used a similar hairpin construct [169]. This finding seems to be the closest indication of the interaction between siRNAs and the nascent RNA that is required for silencing and heterochromatin formation, rather than an interaction with the underlying DNA sequence or the mature transcripts, To prove this hypothesis, careful experiments will be required; for instance, starting with designing a hairpin targeting the intronic sequence directly or designing splice site mutants that would retain the intron in the *cox4*+ transcript without affecting the stability of the mRNA. In addition, this observation indicates that the small RNAs mapping to the hairpin locus are also secondary siRNAs produced by Rdp1, as

the *cox4*+ intron sequence is only present in one orientation, but sequenced small RNAs are mapping to both strands and are present in equally high amounts.

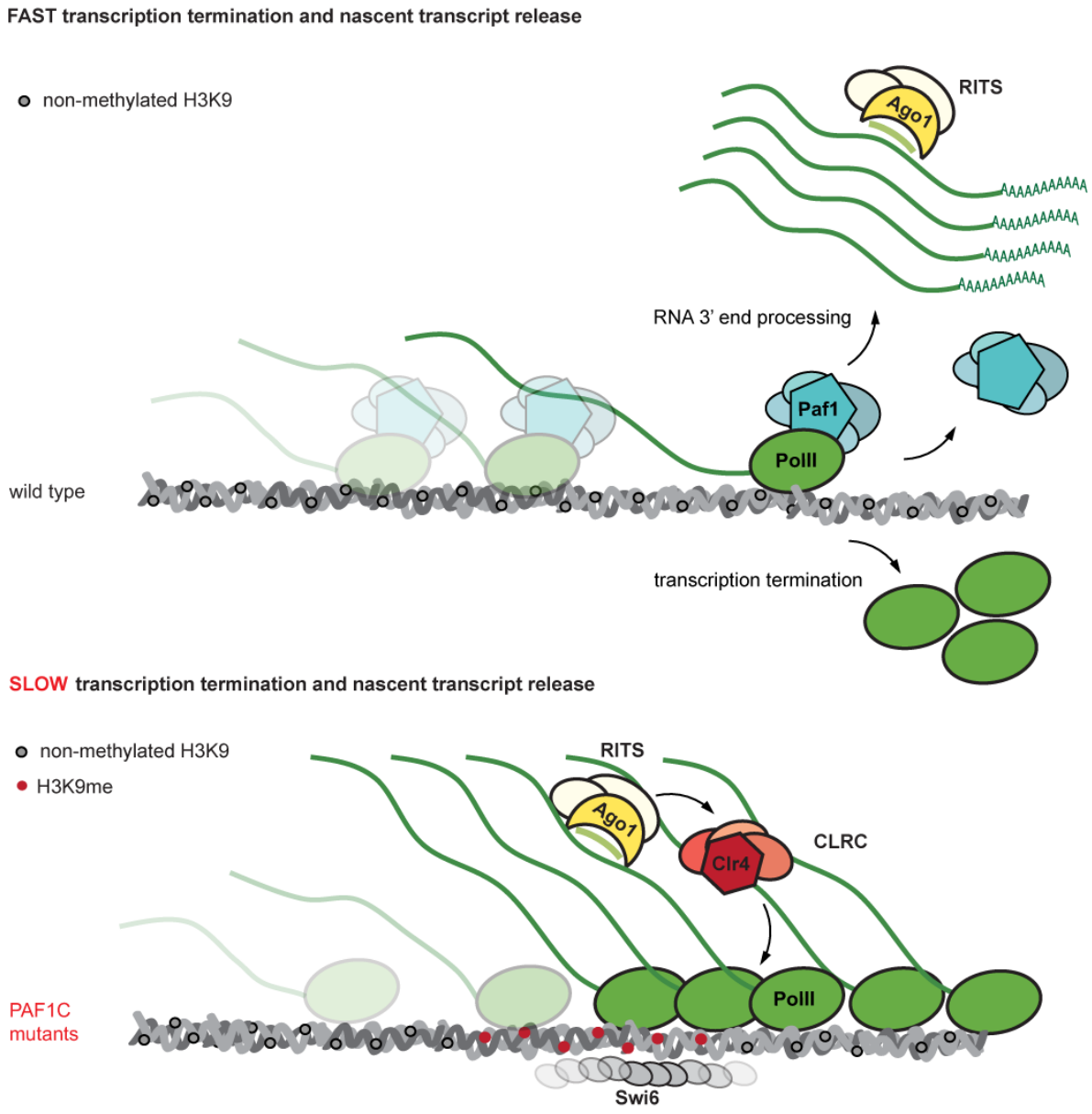

Figure 24. Proposed model for Paf1-mediated repression of siRNA-directed heterochromatin formation. **A** Paf1C facilitates rapid transcription and release of the nascent transcript from the DNA template. Because the kinetics of transcription termination and transcript release is faster than siRNA-mediated binding of the RNAi machinery and recruitment of the H3K9 methyltarsferase Clr4, heterochromatin formation cannot be induced. **B** In the Paf1C mutants, transcription elongation, termination, and the release of the nascent transcript from chromatin are slowed down. This results in an accumulation of RNA polymerases associated with nascent transcripts, providing a time window in which siRNA-guided RITS complex can bind nascent transcripts and recruit the CLRC complex. Consequently, highly stable and repressive heterochromatic domain that produced secondary siRNAs is assembled.

6.7 Formation of facultative heterochromatin

As outlined in Section 4.3, we initiated this study to identify putative repressors of siRNA-mediated transcriptional silencing *in trans*. We reasoned that a yeast cell would employ such repressive mechanism to protect gene expression from the consequences of endogenous, low abundant small RNAs. We identified the Paf1 complex as the sought-after repressor and we hypothesized that under the condition when the Paf1 complex function is impaired, priRNAs and other dsRNA-derived primary RNAs should induce heterochromatin formation on protein coding genes even more efficiently.

Although we identified a number of loci that underwent mild repression in different Paf1 complex mutants, we failed to detect silencing repeatedly at the same genomic location in different strains (Table 7, Figure 17A). This result is in agreement with our observation that a stable source of small RNAs is required for establishment of heterochromatin (Figure 11). Since priRNAs were derived from degradation products of cellular mRNAs, they would make a low abundant source of siRNAs that is not potent enough to establish heterochromatin at corresponding euchromatic locations. Alternatively, it could be that priRNAs and endogenous small RNAs were able to induce silencing but in a random manner and the strong silencing effect could not be observed because we performed the experiment in a mixed population of cells. Dcr1 was suggested to be a limiting factor for siRNA generation and it was reported that overexpression of Dcr1 protein increases drastically the levels of primary siRNAs [80], [169]. Thus, combining the Dcr1 overexpression with Paf1 complex mutation could increase the population of endogenous small RNAs potentially required to initiate heterochromatin formation.

Several genes have been reported to be targets of RNAi-mediated facultative heterochromatin formation in cells grown at varied growth conditions [202]. We suspected that these loci could be inherently primed for repression already under normal conditions and that we could induce heterochromatin formation at these loci in the Paf1 mutant strains. Since that was not the case, we concluded that no genes were specifically primed for silencing by siRNA-mediated formation of heterochromatin in *S. pombe* under standard laboratory growth conditions. Consistently, we have not observed the signature of heterochromatin establishment at the convergent gene pairs, as was previously suggested [203], [204]

Another likely explanation for the fact that we did not identify any genes that had undergone silencing by endogenous small RNAs is that such small RNAs were able to mediate heterochromatin formation; but this heterochromatic repression was weak or could not be propagated for long. If this was the case, repeating the expression profiling in the conditions where maintenance of heterochromatin was more potent could increase the chance of success. Recently, two groups reported that upon removal of the putative histone demethylase Epe1, epigenetic inheritance of the silent chromatin state independent of small RNAs was enhanced [196], [197]. Combining the *epe1+* deletion with the impairment of the Paf1 complex could lead to a more stable propagation of heterochromatin and allow us to identify possible targets of endogenous primal RNAs. Following this logic, deletion of other factors that negatively regulate heterochromatin formation like Mst2 [205] or Eri1[35], [206] could also cause more potent maintenance. Finally, as it was reported that *in trans* silencing of homologues sequences could be enhanced by overexpression of the Swi6 protein [80], we could also try overexpressing Swi6 in the Paf1 complex mutant strain.

Results presented in the section 5.9 demonstrate that if the naïve cells with an active copy of the *ade6+* gene undergo meiosis, the efficiency of the RNAi-mediated nucleation of heterochromatin at the reporter gene is notably increased (Figure 20). Although this observation serves more as indirect evidence, we noticed that the mutant progeny from a cross of the *leo1+* strain with the *leo1*Δ strain differ in size (Figure 21B), even though all crosses should have produced an isogenic set of four progeny colonies. We speculate that these phenotypic differences are a consequence of the fact that upon meiosis, endogenous ectopic heterochromatin formation in the *leo1*Δ progenies is more potent than during mitotic growth. However, the euchromatic genes become targets of the RNAi in a random fashion, which causes different growth phenotypes in genetically identical cells. This observation might serve as a starting point to further analyze the progeny of the *leo1+*and *leo1*Δ crosses, for instance, by RNA-sequencing or small RNA profiling. In order to identify such endogenously silenced genes, RNA isolation and sequencing might need to be performed directly from the colonies obtained from the crosses, under conditions resembling the ‘single cell’ RNA sequencing experiment. This approach could help to avoid dilution of the effect induced upon meiosis during long-propagation of the mutant cells.

6.8 Potential applications

In our analysis we did not identify genes that would be silenced by endogenous siRNAs in the Paf1 complex mutants. This speaks in favor of using the mutation of the Paf1 complex as a tool to induce targeted transgenerational gene expression changes in various organisms. In case of creating knockout transgenic plants, applying our finding would, in theory, alleviate the need to edit the genome every time when a new gene needs to be removed. Instead, since the mutations in the Paf1 complex seem to have very minor effects on the global gene expression and cellular fitness (Figure 15, Figure 16B), one could use a Paf1 mutant plant and provide a potent source of small RNAs to induce gene silencing. Most importantly, the silent state could then be propagated to the next generations without the need for constant supply of the primary siRNA source. Furthermore, the gene silencing is also reversible, as it does not imply any changes in the sequence of the target gene.

In our model system, the silent state is maintained thanks to the secondary small RNAs, which are produced in the efficient amplification loop by Rdp1 at the target locus. Thus, it might be that effective transgenerational silencing could only be applied in organisms with RDRP activity. In worms and plants, RDRPs have been well described [207]. Especially in plants there is a great potential for applying this approach as there is strong evidence for the existence of the self-reinforcing loop that involves siRNA production, DNA methylation and H3K9 methylation and for its role in the maintenance of silencing [60]. Evolutionary studies did not reveal the presence of genes encoding for RdRP homologues in vertebrates and it remains uncertain whether they carry any non-viral genes encoding RdRP activity. Furthermore, it remains unclear whether mechanisms coupling the small RNA-mediated target recognition with establishment of a heterochromatic domain are also present in mammals. If so, one could speculate that in mammalian cells, transient presence of small RNAs could induce heterochromatin formation and establish transcriptional silencing, which could then be reinforced by more classic heterochromatin factors and propagated through mitotic divisions independent of the small RNAs, with the help of DNA methylation, histone modifications, chromatin readers and HP1s[1].

7 Bibliography

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8 Abbreviations

Ago	Argonaute
ARC	Argonaute siRNA chaperone complex
Aub	Aubergine
bp	base pair
ChIP	chromatin immunoprecipitation
CLRC	Clr4-Rik1-Cul4 complex
CPSF	cleavage/polyadenylation specificity
CstF	cleavage stimulation factor
CTD	C-terminal domain
CTGS	co-transcriptional gene silencing
DCL	Dicer-like
Dcr1	*S. pombe* Dicer
DRB	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB sensitivity-inducing factor
dsRNA	double-stranded RNA
EMS	ethyl methanesulfonate
ESC	embryonic stem cells
EZH2	Enhancer of Zeste 2
FACT	facilitates chromatin transcription
FIHP	familial isolated primary hyperparahthyroidism
GW	glycine-tryptophane
H2BK123	histone H2B lysine 123
H3K27	histone H3 lysine 27
H3K36	histone H3 lysine 36
H3K4	histone H3 lysine 4
H3K79	histone H3 lysine 79
H3K9	histone H3 lysine 9
H3K9me	histone H3 lysine 9 methylation
H3K9me2	histone H3 lysine 9 dimethylation
H3K9me3	histone H3 lysine 9 trimethylation
H4K16	histone H4 lysine 16
H4K16Q	histone H4 lysine 16 substitution with glutamine
H4K16R	histone H4 lysine 16 substitution with arginine
HDAC	histone deacetylase
hp	hairpin
HP1	heterochromatin protein 1
HPT-JT	hyperparathyroidism-jaw tumor syndrome
imr	innermost repeats
kb	kilo base
miRNA	micro RNA
NLS	nuclear localization signal
NRDE	nuclear RNAi-defective
NS1	non-structural protein 1

nt	nucleotide
OF	ossifying fibroma
ORF	open reading frame
otr	outermost repeats
Paf1	RNA Polymerase II-associated factor 1
piRNA	Piwi-interacting RNA
PIWI	P-element induced wimpy testis
PolII	RNA Polymerase II
PolIV	RNA Polymerase IV
PolV	RNA Polymerase V
polyA	polyadenine tail
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PTA	parathyroid adenoma
PTC	parathyroid carcinoma
pTEFb	positive transcription elongation factor
PTGS	post-transcriptional gene silencing
RdDM	RNA-directed DNA methylation
RDE	RNAi-defective
RDRC	RNA-directed RNA polymerase complex
RdRP	RNA-directed RNA polymerase
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing complex
RNAe	RNA-induced epigenetic silencing
RNAi	RNA interference
RNASe III	ribonuclease III
RT-qPCR	real-time quantitative PCR
Ser-2	serine 2
Ser-5	serine 5
Ser-7	serine 7
SHREC	Snf2/HDAC-containing repressor complex
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
splice [a]	splice acceptor site
splice [d]	splice donor site
TALEN	transcription activator-like effector nuclease
TE	transposable element
TGS	transcriptional gene silencing
Tri	Triman
TSS	transcription start site
UTR	untranslated region
WAGO	worm-specific Ago
WT	wild type
YE	yeast extract

9 Experimental Procedures

9.1 Methods

All the experiments and analysis were conducted as described in the *Methods* section of the published manuscript that can be found in the Section 10 [2].

9.2 Strains

Table 4 Yeast strains used in this study.

Name	Genotype	Ref
SPB155	*h- leu1-32 ura4-D18 ade6-704*	b
SPB389	*h- leu1-32 ura4-D18*	a
SPB462	*h- leu1-32, ura4-D18 nmt1+::ade6-hp+*	a
SPB426	*h- leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB464	*h- leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2163	*h- sms1 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2164	*h- sms2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2165	*h- sms3 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2166	*h- sms4 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2167	*h- sms5 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2168	*h- sms6 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2169	*h- sms7 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2170	*h- sms8 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2171	*h- sms9 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2172	*h- sms10 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2173	*h- sms1 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2174	*h- sms2 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2175	*h- sms3 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2176	*h- sms4 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2177	*h- sms5 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2178	*h- sms6 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2179	*h- sms7 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2180	*h- sms8 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2181	*h- sms9 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2182	*h- sms10 dcr1D::hph leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2047	*h- paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2127	*h- dcr1D::hph paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2128	*h- ago1D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2129	*h- tas3D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2130	*h- chp1D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2131	*h- arb1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2132	*h- arb2D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2133	*h- rdp1D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a

	nmt1+::ade6-hp+	
SPB2134	*h- hrr1D::TAP?-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2135	*h- cid12D::TAP-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2136	*h- clr4D::hph paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2137	*h- rik1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-M210 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2138	*h- raf1D::TAP?-kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2139	*h- raf2D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2141	*h- clr1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2142	*h- clr2D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2143	*h- clr3D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2144	*h- mit1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2145	*h- swi6D::hph paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2147	*h- chp2D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2146	*h- stc1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2148	*h- ers1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-M210 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2150	*h- tri1D::kan paf1-Q264stop::LEU2 leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1788	*h+ leu1-32 ura4D18 ade6-M210 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2063	*h+ paf1-Q264stop::kan leu1-32 ura4-D18 ade6-m210 trp1+::ade6+*	a
SPB2016	*h- prf1D::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1953	*h- paf1D::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1955	*h- leo1D::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1956	*h- tpr1D::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1957	*h- cdc73D::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB2020	*h- paf1+::kan leu1-32 ade6-M210 nmt1+::ura4-hp+*	a
SPB2021	*h- paf1-Q264stop::kan leu1-32 ade6-M210 nmt1+::ura4-hp+*	a
SPB2002	*h- paf1+::LEU2 ura4+::5BoxB-hphR leu1D::ura4-intron(StuI)-natR*	a
SPB2003	*h- paf1-Q264Stop::LEU2 ura4+::5BoxB-hphR leu1D::ura4-intron(StuI)-natR*	a
SPB2004	*h- paf1+::LEU2 ura4+::5BoxB-hphR tas3+::lN-kanR leu1D::ura4-intron(StuI)-natR*	a
SPB2005	*h- paf1-Q264Stop::LEU2 ura4+::5BoxB-hphR tas3+::lN-kanR leu1D::ura4-intron(StuI)-natR*	a
SPB2029	*h- paf1+::LEU2 eri1D::ble ura4+::5BoxB-hphR tas3+::lN-kanR leu1D::ura4-intron(StuI)-natR*	a
SPB2030	*h- paf1-Q264Stop::LEU2 eri1D::ble ura4+::5BoxB-hphR tas3+::lN-kanR leu1D::ura4-intron(StuI)-natR*	a
SPB2022	*h- paf1+::kan leu1-32 ura4-D18*	a
SPB2023	*h- paf1-Q264Stop::kan leu1-32 ura4-D18*	a
SPB2024	*h- paf1+::kan leu1-32 ura4-D18 nmt1+::ade6-hp+*	a
SPB2025	*h- paf1-Q264Stop::kan leu1-32 ura4-D18 nmt1+::ade6-hp+*	a
SPB2039	*h- paf1-Q264Stop::kan leu1-32 ura4-D18 nmt1+::ade6-hp+*	a
SPB1995	*h- prf1-3xFLAG::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1996	*h- paf1-3xFLAG::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1997	*h- leo1-3xFLAG::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1998	*h- tpr1-3xFLAG::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a

SPB1999	*h- cdc73-3xFLAG::kan leu1-32 ura4D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1960	*h- paf1-G104R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1961	*h- paf1+::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1962	*h- paf1-G104R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB1963	*h- cdc73-G313R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1965	*h- cdc73-G313R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB1966	*h- prf1-Q472Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1967	*h- prf1+::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1968	*h- prf1-Q472Stop:: kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB1969	*h- leo1-W157Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1970	*h- leo1+::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1971	*h- leo1-W157Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB1972	*h- paf1-Q264Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+*	a
SPB1973	*h- paf1-Q264Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+*	a
SPB2009	*h- paf1-G104R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+ (heterochromatic)*	a
SPB2010	*h- cdc73-G313R::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+ (heterochromatic)*	a
SPB2011	*h- prf1-Q472Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+ (heterochromatic)*	a
SPB2012	*h- leo1-W157Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+ (heterochromatic)*	a
SPB2013	*h- paf1-Q264Stop::kan leu1-32 ura4-D18 ade6-704 trp1+::ade6+ nmt1+::ade6-hp+ (heterochromatic)*	a
SPB2276	*h- leu1-32 ura4-D18 shade6-250/natMX paf1-Q264Stop::kan ade6+::ade6-polyA(75)-Rzm-Tadh1*	a
SPB2277	*h- leu1-32 ura4-D18 shade6-250/natMX paf1-Q264Stop::kan ade6+::ade6-polyA(75)-Rz-Tadh1*	a
SPB2318	*h+ leu1-32 ura4-D18 shade6-250/natMX ade6-M210 trp1+::ade6+ set2D::kan*	a
SPB2353	*h+ leu1-32 ura4-D18 shade6-250/natMX ade6-M210 trp1+::ade6+ set1D::kan*	a
SPB2355	*h- leu1-32 ura4-D18 shade6-250/natMX ade6-704 trp1+::ade6+ ctf1-70::hph*	a
SPB2357	*h- leu1-32 ura4-D18 shade6-250/natMX ade6-704 trp1+::ade6+ res2D::hph*	a
SPB2358	*h+ leu1-32 ura4-D18 shade6-250/natMX ade6-M210 trp1+::ade6+ spt4D::kan*	a
SPB2359	*h+ leu1-32 ura4-D18 shade6-250/natMX ade6-M210 trp1+::ade6+ tfs1D::kan*	a
SPB2286	*h- leu1-32 ura4-D18 shade6-250/natMX ade6+::ade6-polyA(75)-Rzm-Tadh1*	a
SPB2287	*h- leu1-32 ura4-D18 shade6-250/natMX ade6+::ade6-polyA(75)-Rz-Tadh1*	a

a this study
b obtained from Katja Ludin/Jürg Kohli

9.3 Plasmids

Table 5 Plasmids used in this study.

Name	Description	Ref
pMB417	pNatMXARTade6-hp	a
pMB1344	pJET1.2-*paf1-Q264stop*-LEU2(s.c)	b
pMB1313	pFA6a-*paf1-G104R*-kanMX	b
pMB1314	pFA6a-*cdc73-G313R*-kanMX	b
pMB1318	pFA6a-*prf1-Q472Stop*-kanMX	b
pMB1319	pFA6a-*leo1-W157Stop*-kanMX	b
pMB1320	pFA6a-*paf1-Q264Stop*-kanMX	b

a kind gift from Tetsushi Iida
b generated in this study

9.4 Oligonucleotides used for RT-qPCR

Table 6 Oligonucleotides used in this study as primers for RT- qPCR analysis

Name	Sequence	Target	Comment
mb6582	ATAAGGTATAACGACAACAAACG	*ade6+* promoter	forward
mb6583	GCATACGCTAAAATCAATATAGC	*ade6+* promoter	reverse
mb6584	CGAAAAACAGGTTGTAGGGATCC	*ade6+* CDS	forward
mb6585	GAATTTGCTGCATCCAAGATGATGC	*ade6+* CDS	reverse
mb6586	AAACATTGGCTTACGACGGTCG	*ade6+* CDS	forward
mb6587	GAACGAACTTTTCAACATAAAGCG	*ade6+* CDS	reverse
mb6588	TTCCACAACTCATGCGTTGATGG	*ade6+* CDS	forward
mb6589	GATGCAAAGTTGCACCGGGAATGG	*ade6+* CDS	reverse
mb6590	TCACCGCACACCAGATCGCATGG	*ade6+* CDS	forward
mb6591	ATACCAGGCAAATGAGCGGCACC	*ade6+* CDS	reverse
mb6592	CCCTTTTGGCTGCTATGGAGAGC	*ade6+* CDS	forward
mb6593	CTATGCAGAATAATTTTTCCAACC	*ade6+* CDS	reverse
mb6730	GCATTGAAGTTTAAGATAACATTGG	*ade6+* terminator	forward
mb6731	TAACATAGCCAAACATAATGCGG	*ade6+* terminator	reverse
mb555	TCCTCATGCTATCATGCGTCTT	*act1+* CDS	forward
mb556	CCACGCTCCATGAGAATCTTC	*act1+* CDS	reverse
mb557	CTGGCCAGCTTATTCAACTTCAT	*fbp+* CDS	forward
mb558	GATTTCGTCGAGATCTTTTTTCATG	*fbp1+* CDS	reverse
mb2202	CATGGAAATTGCAGTGATGGTAGT	*ade6+* CDS, wt and mutant allele	forward
mb2203	TGAATGGTCTCAGTTGTAGGATAAGC	*ade6+* CDS, wt and mutant allele	reverse
mb6373	GCTTGCCCAACTTCTCAGT	hairpin, binds the targeting seq.	forward
mb6374	AGTTTGTCATAGCAGCTTAATGGT	hairpin, binds the loop seq.	reverse
mb6416	CCATTCAAAAGGATAATGTTTGA	*ade6-m704* CDS only	forward
mb6417	CATGGCAAGGGTTTGAGCACGCTG	*ade6+* CDS, wt and mutant allele	reverse
mb6415	CCATTCAAAAGGATAATGTTTGT	*ade6+* CDS, wt allele only	forward

10 Manuscripts

LETTER

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The Paf1 complex represses small-RNA-mediated epigenetic gene silencing

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RNA interference (RNAi) refers to the ability of exogenously introduced double-stranded RNA to silence expression of homologous sequences. Silencing is initiated when the enzyme Dicer processes the double-stranded RNA into small interfering RNAs (siRNAs). Small RNA molecules are incorporated into Argonaute-protein-containing effector complexes, which they guide to complementary targets to mediate different types of gene silencing, specifically post-transcriptional gene silencing and chromatin-dependent gene silencing[1]. Although endogenous small RNAs have crucial roles in chromatin-mediated processes across kingdoms, efforts to initiate chromatin modifications *in trans* by using siRNAs have been inherently difficult to achieve in all eukaryotic cells. Using fission yeast, here we show that RNAi-directed heterochromatin formation is negatively controlled by the highly conserved RNA polymerase-associated factor 1 complex (Paf1C). Temporary expression of a synthetic hairpin RNA in Paf1C mutants triggers stable heterochromatin formation at homologous loci, effectively silencing genes *in trans*. This repressed state is propagated across generations by the continual production of secondary siRNAs, independently of the synthetic hairpin RNA. Our data support a model in which Paf1C prevents targeting of nascent transcripts by the siRNA-containing RNA-induced transcriptional silencing complex and thereby epigenetic gene silencing, by promoting efficient transcription termination and rapid release of the RNA from the site of transcription. We show that although compromised transcription termination is sufficient to initiate the formation of bi-stable heterochromatin by *trans*-acting siRNAs, impairment of both transcription termination and nascent transcript release is imperative to confer stability to the repressed state. Our work uncovers a novel mechanism for small-RNA-mediated epigenome regulation and highlights fundamental roles for Paf1C and the RNAi machinery in building epigenetic memory.

In the fission yeast *Schizosaccharomyces pombe*, a functional RNAi pathway is required for the formation and stable propagation of constitutive heterochromatin found at pericentromeric repeat sequences. *S. pombe* contains single genes encoding for an Argonaute and a Dicer protein, called *ago1*+ and *dcr1*+, respectively. Centromeres of *ago1*Δ or *dcr1*Δ cells have markedly reduced histone 3 lysine 9 (H3K9) methylation, which is a hallmark of heterochromatin, and defective chromosome segregation and heterochromatic gene silencing[2]. Ago1 is loaded with endogenous small RNAs corresponding to heterochromatic repeats, and interacts with Chp1 and Tas3 to form the RNA-induced transcriptional silencing (RITS) complex[3]. Current models propose that Ago1-bound small RNAs target RITS to centromeres via base-pairing interactions with nascent, chromatin-associated non-coding transcripts. Consequently, RITS recruits the RNA-dependent RNA polymerase complex (RDRC) to initiate double-stranded RNA synthesis and siRNA amplification, as well as the cryptic loci regulator complex (CLRC) to facilitate methylation of histone H3K9 (ref. 4). Chp1 reinforces the heterochromatin association of RITS by binding methylated H3K9 with high affinity[5], thereby creating a positive-feedback loop between siRNA biogenesis, RITS localization and H3K9 methylation. Hence, siRNA-programmed RITS acts as a specificity determinant for the recruitment of other RNAi complexes and chromatin-modifying enzymes to centromeres. However, an outstanding question is whether synthetic siRNAs can also function in this context, and thereby be used to trigger *de novo* formation of heterochromatin, particularly outside of centromeric repeats, to stably silence gene expression at will[1].

Small RNAs have crucial roles in endogenous chromatin-mediated processes also in plants, *Caenorhabditis elegans*, *Drosophila melanogaster* and ciliates. Their role in chromatin silencing can also be extended to mammalian cells, although the mechanisms and physiological pathways are less clear[1,6]. Yet, efforts to initiate chromatin modifications *in trans* by using siRNAs have been inherently difficult to achieve in all organisms. In plants, this is because the ability of siRNAs to induce DNA methylation at gene promoters is context-dependent and sensitive to pre-existing chromatin modifications[7]. And although siRNAs have been shown to promote DNA methylation *in trans* on homologous reporter transgenes in tobacco and *Arabidopsis*[8], it is unclear whether this is a general phenomenon for endogenous promoters. In mammalian cells, the introduction of siRNAs or hairpin RNAs has been reported to promote the modification of DNA and histones[9–11]. However, most small RNAs seem to mediate post-transcriptional gene silencing exclusively, and siRNA-mediated silencing of transcription does not necessarily require chromatin modification[12,13]. Consequently, the potential of synthetic siRNAs to trigger long-lasting gene repression in mammalian cells is debated. Similarly, although studies in *S. pombe* have shown that RNA-hairpin-derived siRNAs can promote H3K9 methylation *in trans* at a small number of loci[14,15], it is inefficient, locus-dependent, and the silent state observed is weak and highly unstable[14]. Rather, endogenous protein-coding genes seem to be refractory to siRNA-directed repression *in trans* in wild-type cells (Extended Data Figs 1 and 2). Therefore, it has been proposed that the ability of siRNAs to direct *de novo* formation of heterochromatin *in trans* is under strict control by mechanisms that have thus far remained elusive.

To identify putative suppressors of siRNA-mediated heterochromatin formation, we designed a small-RNA-mediated silencing (sms) forward genetic screen. We constructed a reporter strain (sms0), which expresses an RNA hairpin (ade6-hp) that is complementary to 250 nucleotides of *ade6*+ (Fig. 1a and Extended Data Fig. 1). We chose *ade6*+ as a reporter because *ade6* mutant cells form red colonies on limiting adenine indicator plates, whereas *ade6*+ cells appear white. Although the ade6-hp construct generated siRNAs complementary to *ade6*+ messenger RNAs, no red colonies were visible, demonstrating that *ade6*+ siRNAs cannot silence the *ade6*+ gene *in trans* in sms0 cells (Extended Data Figs 1b and 2). To screen for mutants that would enable *ade6*+ siRNAs to act *in trans*, we mutagenized sms0 cells with ethylmethanesulfonate (EMS). This revealed five *sms* mutants that are highly susceptible to *de novo* formation of heterochromatin and stable gene silencing by siRNAs that are acting *in trans* (Extended Data Fig. 3).

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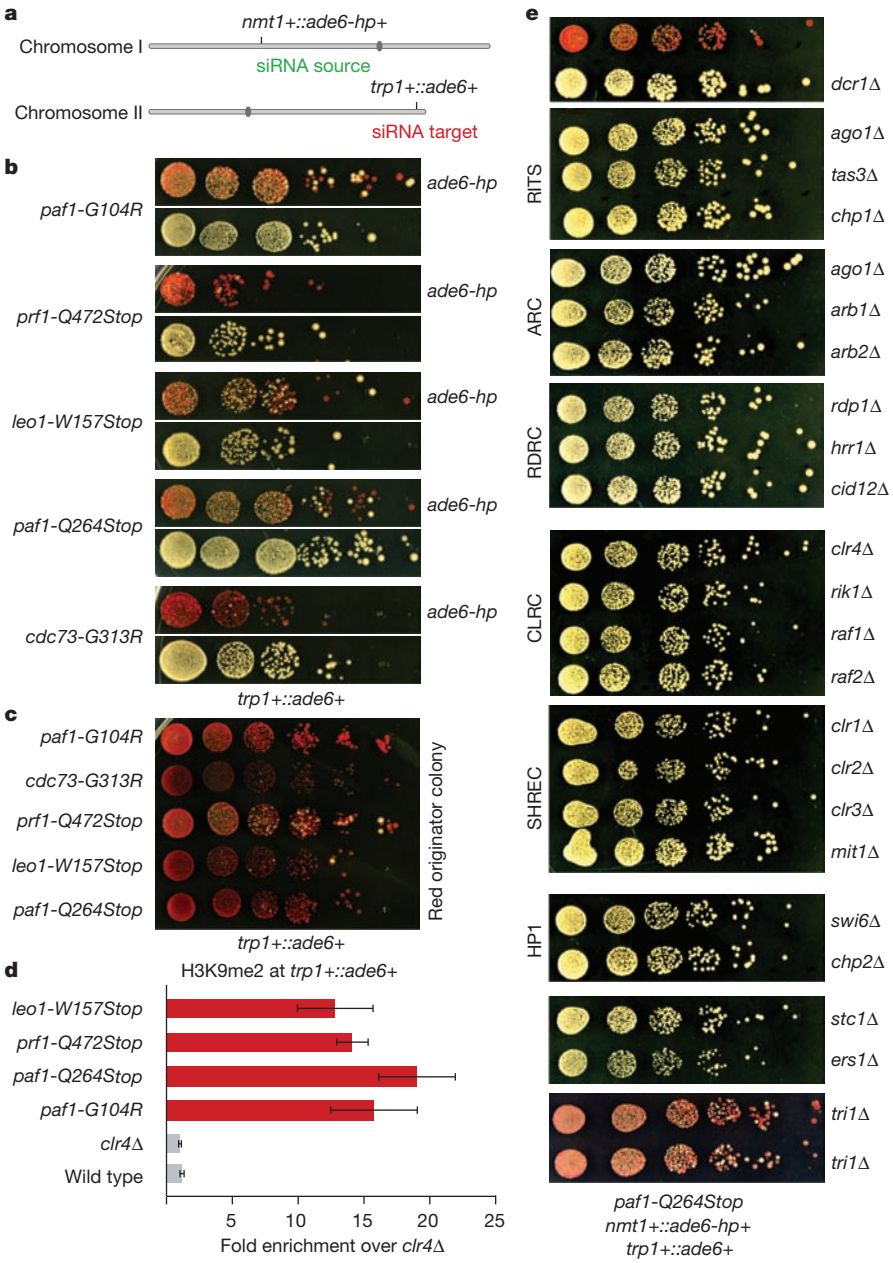

Figure 1 | siRNA-directed *de novo* formation of heterochromatin. **a**, The ade6-hp RNA producing locus and siRNA target *in trans* in the sms0 strain. **b**, Silencing assay performed with freshly generated Paf1C mutants. **c**, Silencing assay performed with red colonies from **b**. **d**, *ade6+* siRNAs direct the methylation of H3K9 *in trans* at the *trp1+::ade6+* locus in Paf1C mutant cells. Error bars, s.e.m.; $n = 3$ technical replicates. **e**, Gene silencing at the *trp1+::ade6+* locus depends on the same factors as constitutive heterochromatin at centromeric repeats. ARC, Argonaute siRNA chaperone complex.

To map the mutations in *sms* mutants, we re-sequenced the genomes of sms0 and backcrossed *sms* mutants using whole-genome next-generation sequencing. We mapped missense or nonsense mutations in the genes SPBC651.09c, SPAC664.03, SPBC13E7.08c and SPBC17G9.02c (Extended Data Fig. 3), whose homologues in budding yeast encode for protein subunits of the Paf1 complex. We therefore named SPAC664.03, SPBC13E7.08c and SPBC17G9.02c after the *S. cerevisiae* homologues *paf1+*, *leo1+* and *cdc73+*, respectively. SPBC651.09c has already been named as *prf1+* (ref. 16). To validate these as the causative mutations, we reconstituted the candidate point mutations in Paf1, Leo1, Cdc73 and Prf1 in sms0 cells. All five point mutations recapitulated the *sms* mutant phenotype in cells expressing ade6-hp siRNAs (Fig. 1b, c). As expected from the red colour assays, *ade6+* mRNA levels were reduced in all mutant strains. siRNA-mediated *ade6+* silencing was also observed in cells that express a carboxy-terminally 3×Flag-tagged version of the fifth Paf1C subunit Tpr1, which acts as a hypomorphic allele (Extended Data Fig. 4). Therefore, we have identified mutant alleles for the homologues of all five subunits of Paf1C that enable siRNAs to induce gene silencing *in trans*.

We next analysed whether other genes could also be silenced *in trans* in the Paf1C mutants. We first selected the endogenous *ura4+* gene, as this has been shown to be refractory to silencing by siRNAs acting *in trans*[14,15,17]. The *paf1-Q264Stop* mutation was introduced in a strain expressing *ura4+* siRNAs from a *ura4+* hairpin integrated at the *nmt1+* locus[15]. *ura4+* repression was monitored by growing cells on media containing 5-fluoroorotic acid (5-FOA), which is toxic to *ura4+* expressing cells. As expected, *paf1+* cells did not grow on 5-FOA-containing media, indicating that the *ura4+* gene is expressed. However, *paf1-Q264Stop* cells formed colonies on 5-FOA containing media, demonstrating siRNA-directed silencing of the endogenous *ura4+* locus (Extended Data Fig. 5a). Similarly, siRNAs generated at the heterochromatic *ura4+::5BoxB* locus[18] were able to silence a *leu1Δ::ura4+* reporter *in trans* in *paf1-Q264Stop* but not *paf1+* cells (Extended Data Fig. 5b), demonstrating that siRNAs generated from sources other than RNA stem–loop structures also direct *trans*-silencing in *paf1+* mutant cells. Finally, we also observed silencing of the endogenous *ade6+* gene when ade6-hp siRNAs were expressed from the *nmt1+* locus in *paf1-Q264Stop* cells (Extended Data Fig. 5c). In summary, Paf1C mutations

enabled siRNA-directed silencing *in trans* at all euchromatic loci that we tested.

The foregoing results indicated that *de novo* formation of heterochromatin was mediated by *trans*-acting siRNAs. Indeed, Paf1C mutants showed high H3K9 methylation at all *ade6+* siRNA target loci (Fig. 1d and Extended Data Fig. 6a–c), demonstrating that Paf1C prevents *trans*- as well as *cis*-acting siRNAs from directing methylation of H3K9. Further corroborating the formation of bona fide heterochromatin at the *ade6+* target locus, *ade6+* repression was dependent on components of SHREC (Snf2/histone deacetylase (HDAC) repressor complex) and CLRC, as well as the heterochromatin protein 1 (HP1) proteins Swi6 and Chp2, which are known to facilitate constitutive heterochromatin formation at centromeres (Fig. 1e). Finally, the formation of heterochromatin reduced transcriptional activity of the *ade6+* gene as evidenced by reduced H3K36 tri-methylation and RNA polymerase (Pol) II occupancy (Extended Data Fig. 6d, e). From these results we conclude that siRNAs can initiate the formation of heterochromatin and gene silencing, but that this is under strict negative control by Paf1C. This explains previous unsuccessful attempts to induce stable heterochromatin formation *in trans* using synthetic siRNAs.

Consistent with the formation of an epigenetically distinct chromatin domain at the siRNA target loci, cells in a population of freshly generated Paf1C mutants were either fully red or fully white. The latter gradually became red with increasing numbers of mitotic divisions, and once established, the silent state was remarkably stable (Fig. 1b, c). The fact that not all cells in a population of naive Paf1C mutant cells turned red immediately allowed us to determine the frequency of initiation of heterochromatin formation quantitatively. This analysis revealed that silencing in mitotic cells was efficiently established in *leo1-W157Stop* mutant cells, whereas *cdc73-G313R* cells were the least efficient (Fig. 2a). Descendants of a red colony switched to the white phenotype only sporadically in all Paf1C mutants, demonstrating that maintenance of heterochromatin is very robust in these cells (Fig. 2b). Interestingly, siRNA-directed *de novo* formation of heterochromatin was most efficient in meiosis. In 70% of all crosses between a naive *paf1-Q264Stop* mutant (white) and a *paf1+* cell, at least one of two *paf1-Q264Stop* spores had initiated *ade6+* repression (red) (Fig. 2c and Extended Data Fig. 7). We also observed highly efficient propagation of the silent state through meiosis, but only in descendants of spores that inherited the Paf1C mutation (Fig. 2d). Thus, siRNAs are sufficient to initiate the formation of very stable heterochromatin when Paf1C function is impaired.

Notably, assembly of heterochromatin at the *ade6+* target gene was accompanied by the production of novel *ade6+* siRNAs that are not encoded in the ade6-hp and that accumulated to high levels (Fig. 2e). Thus, primary ade6-hp siRNAs trigger the production of highly abundant secondary *ade6+* siRNAs in Paf1C mutants. To test whether continuous production of siRNAs is necessary for sustaining the repressed state, we deleted genes encoding for RNAi factors and found that *ade6+* silencing was completely abolished in all canonical RNAi mutants. Deletion of *tri1+* resulted in moderate derepression of *ade6+* silencing, suggesting a minor contribution of this exonuclease to siRNA-mediated heterochromatin silencing (Fig. 1e). To test whether secondary siRNAs produced at the *ade6+* target locus are sufficient to maintain heterochromatin, we crossed a *trp1+::ade6+ paf1-Q264Stop ade6-hp+* strain (red) with a *trp1+::ade6+ paf1-Q264Stop* (white) strain. These crosses regularly produced spores that gave rise to red cells even in the absence of the *nmt1+::ade6-hp+* allele. The red phenotype was still visible after replica plating, demonstrating that heterochromatin can be maintained in the absence of the primary siRNAs for hundreds of mitotic cell divisions (Fig. 2f). These results demonstrate that siRNAs can induce an epigenetic change in gene expression in meiotic and mitotic cells, and that secondary siRNA production is sufficient to propagate the repressed state for many mitotic cell divisions independently of the primary siRNAs that triggered the epigenetic switch.

The highly conserved Paf1C is well known for promoting RNA Pol II transcription elongation and RNA 3′-end processing (Fig. 3a). Paf1C also governs transcription-coupled histone modifications and has connections to DNA damage repair, cell cycle progression, and other processes[19]. Given this broad function, we assessed the effect of our Paf1C mutations on genome expression. This analysis revealed that *paf1-G104R*, *paf1-Q264Stop*, *prf1-Q472Stop* and *leo1-W157Stop* impair repression of heterochromatin formation, without affecting RNA expression globally (Supplementary Information and Extended Data Fig. 8). This is consistent with our observation that *ade6+* expression is unaffected in Paf1C mutants in the absence of siRNAs (Fig. 1b). We did, however, detect a reduction in H3K36 tri-methylation and an increase in RNA Pol II occupancy on the *ade6+* gene in *paf1-Q264Stop* cells (Fig. 3b, c). This is consistent with the role of Paf1C in promoting transcription, and suggests that decelerated transcription kinetics in Paf1C mutants enables siRNA-directed epigenetic gene silencing. To dissect which of Paf1C's activities are most critical to prevent RNAi-mediated heterochromatin assembly, we interfered with transcription elongation, termination

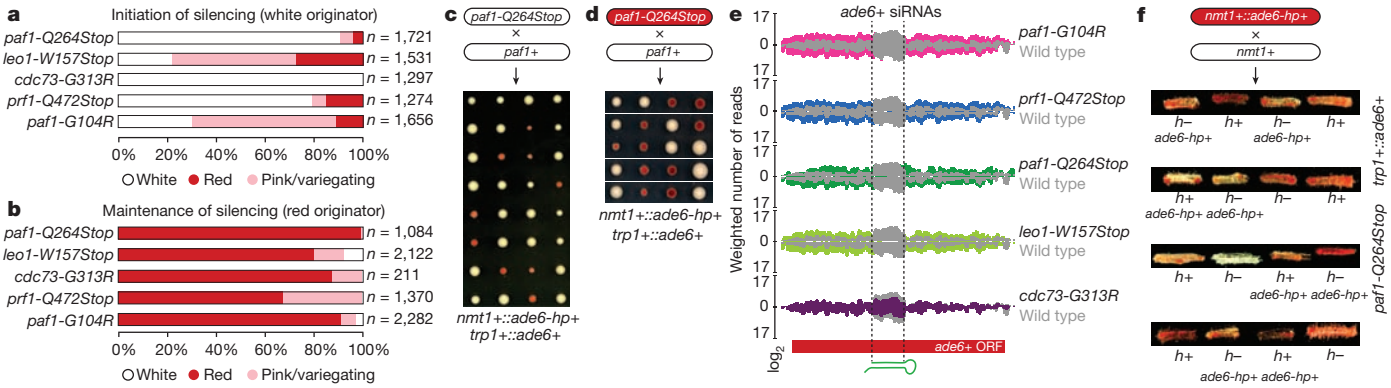

Figure 2 | siRNA-mediated epigenetic gene silencing. **a**, Percentage of naive Paf1C mutant cells that establish heterochromatin within 20–30 mitotic divisions. *n*, number of scored colonies. **b**, Stability of ectopic heterochromatin in mitotic cells. *n*, number of scored colonies. **c**, Initiation of heterochromatin formation during meiosis. Naive *paf1-Q264Stop* cells (white) were crossed with *paf1+* cells. Spore dissection of eight crosses is shown. **d**, Red *paf1-Q264Stop* cells (heterochromatic *ade6+*) were crossed with *paf1+* cells to assess stability of ectopic heterochromatin through meiosis. White descendants are *paf1+*. **e**, siRNA reads mapping to the *ade6+* locus in wild-type (grey) and Paf1C mutant (coloured) strains. Read counts were normalized to library size and are shown in $\log_2$ scale. Dashed lines mark the *ade6+* fragment targeted by the hairpin. **f**, Red *paf1-Q264Stop* cells (heterochromatic *ade6+*) carrying the *ade6+*-targeting hairpin (*ade6-hp+*) were crossed with *paf1-Q264Stop* cells without the hairpin to test hairpin requirement after initiation of silencing. Four spores derived from the cross were struck on yeast extract (YE) plates to assess the silencing phenotype. *h+* and *h−* denote mating types and *ade6-hp+* marks cells carrying the hairpin.

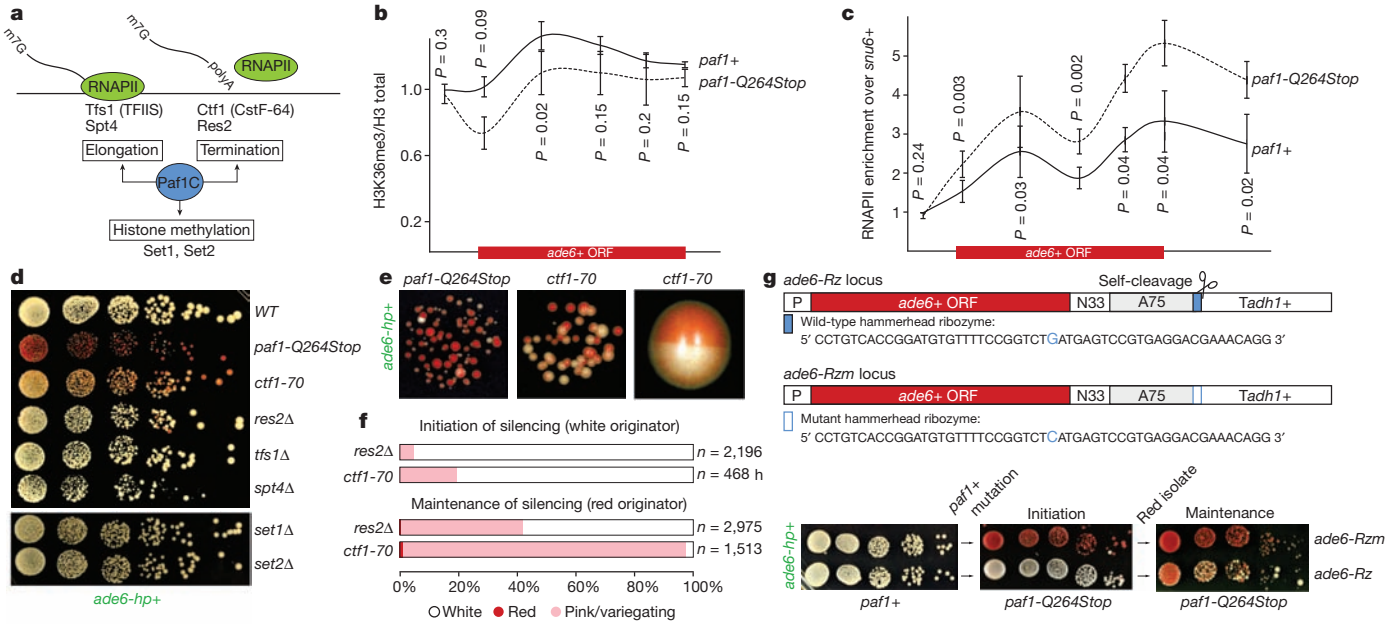

Figure 3 | Mechanism of repression. **a**, Paf1C governs RNA Pol II (RNAPII) transcription elongation, RNA 3′-end processing, and transcription-coupled histone modifications. **b**, **c**, Chromatin immunoprecipitation experiments to assess *ade6+* transcriptional activity in Paf1C mutant cells. H3K36me3 levels were normalized to total H3 levels. *snu6+* is transcribed by RNAPIII and serves as background control. ORF, open-reading frame. Error bars, s.e.m.; $n = 3$ independent biological replicates; *P* values were calculated using one-tailed Student's *t*-test. **d**, **e**, Silencing assays showing that *ade6+* siRNAs can initiate repression of *ade6+* in transcription termination mutants. Note the bi-stable state of repression in *ctf1-70* cells. Cells were grown on YE plates. **f**, Percentage of naive transcription termination defective cells (white originator) that establish heterochromatin within 20–30 mitotic divisions (initiation) and stability of ectopic heterochromatin in descendants thereof (maintenance). *n*, number of scored colonies. **g**, A 52-nucleotide wild-type or mutant hammerhead ribozyme sequence preceded by a templated polyA(75)-tail was integrated 33 nucleotides downstream of the *ade6+* stop codon (*ade6-Rz* or *ade6-Rzm*, respectively). **h**, Silencing assay showing that *ade6+* siRNAs stably repress *ade6-Rzm* but not *ade6-Rz* in *paf1* mutant cells. Note that *ade6-Rz* produces fully functional mRNA in *paf1+* cells.

or co-transcriptional histone modification directly by mutating genes encoding elongation factors (Tfs1 and Spt4), termination factors (Ctf1 and Res2), or histone methyltransferases (Set1 and Set2)[20,21] (Fig. 3a). We observed siRNA-mediated initiation of *ade6+* silencing in *ctf1-70* and *res2Δ* cells, but not in *tfs1Δ*, *spt4Δ*, *set1Δ* and *set2Δ* cells (Fig. 3d–f), demonstrating that impaired transcription termination but not elongation is sufficient to allow siRNA-directed repression. Notably, although impaired transcription termination in *ctf1-70* and *res2Δ* cells was sufficient to initiate silencing, the silent state was less stable than in *paf1-Q264Stop* mutant cells (Fig. 3e, f). This explains why our screen did not reveal mutations in transcription termination factors.

In *ctf1-70* cells, although RNA Pol II fails to terminate, the nascent RNA is still properly processed and released from the site of transcription[21]. This probably accounts for the less stable silencing in *ctf1-70* cells and suggests that the more severe phenotype of Paf1C mutants is due to the combined effects of impaired termination and nascent transcript release. Therefore, we tested whether artificially releasing the nascent transcript from the site of transcription partially alleviates siRNA-mediated heterochromatin formation in Paf1C mutant cells. To this end, we inserted a 52-nucleotide hammerhead ribozyme (Rz), preceded by a templated polyA (A_{75}) tail, downstream of the *ade6+* open reading frame (*ade6-Rz*) to induce self-cleavage of nascent *ade6+* transcripts (Fig. 3g). Indeed, initiation of silencing at the *ade6-Rz* locus was inefficient and the repressed state was poorly propagated in *paf1-Q264Stop* mutant cells. By contrast, silencing was very effective in cells that contain a single base change in the catalytic site of the ribozyme (*ade6-Rzm*) that abolishes self-cleavage (Fig. 3h). Thus, retaining the nascent transcript on chromatin is critical to stabilize the repressed state.

These results are consistent with a kinetic model for Paf1C function and demonstrate that proper transcription termination is crucial to prevent *de novo* formation of heterochromatin by siRNAs (Extended Data Fig. 9). This is further supported by the recent observation that termination sequences in the 3′ untranslated region of the *ura4+* gene inhibit the ability of siRNAs to promote heterochromatin formation[17] and is reminiscent of enhanced silencing phenotype (*esp*) mutations in *Arabidopsis thaliana*, which are in genes that encode for members of the cleavage polyadenylation specificity factor and cleavage stimulation factor complexes[22]. Importantly, our results show that impairment of both transcription termination and nascent transcript release is imperative to confer stability to the repressed state, although compromised transcription termination is sufficient to initiate the formation of bi-stable heterochromatin by *trans*-acting siRNAs.

Besides Dcr1-dependent siRNAs, Ago1 associates with Dcr1-independent small RNAs referred to as primal RNAs (priRNAs). priRNAs seem to be degradation products of abundant transcripts and could potentially trigger siRNA amplification and uncontrolled heterochromatic gene silencing[23]. Therefore, we speculated that the physiological function of Paf1C is to protect the genome from spurious priRNA-mediated heterochromatin formation. To investigate this we analysed whether Paf1C mutants would disclose genomic regions that could be potentially assembled into facultative heterochromatin by endogenous small RNAs. On the basis of our results, loci at which facultative heterochromatin forms in an RNAi-dependent manner are expected to show reduced RNA expression with a concomitant increase in siRNA production. As expected, the *nmt1+::ade6-hp+*, *trp1+::ade6+* and *ade6-704* loci fulfilled this criteria (Extended Data Fig. 10a). Moreover, we observed repression and siRNA production for genes flanking these loci, indicating spreading of heterochromatin into neighbouring genes, which occurred up to 6 kilobases (kb) up or downstream of the ade6-hp siRNA target sites. Indeed, we observed H3K9 methylation in this region in *leo1Δ* cells specifically (Extended Data Fig. 10b, c). In addition to these regions, we observed siRNA-directed silencing signatures at different, non *ade6+*-linked genomic loci, indicating that Paf1C may indeed function to protect the genome from illegitimate repression of protein coding genes by endogenous priRNAs. However, we did not recover the same sites repeatedly in the different Paf1C mutants

(Supplementary Table 1). This indicates that initiation of silencing at these sites occurred stochastically and that there are no specific sites primed for the formation of facultative heterochromatin in mitotic cells that are grown under standard laboratory conditions. Therefore, we conclude that Paf1C protects protein-coding genes from unwanted long-term silencing that might occur by chance, thereby restraining phenotypic variation and conferring epigenetic robustness to the organism.

In summary, we discovered that synthetic siRNAs are highly effective in directing locus-independent assembly of heterochromatin that can be stably maintained through mitosis and meiosis only when Paf1C activity is impaired. A remarkable observation of our study is that the newly established heterochromatin was inherited for hundreds of cell divisions across generations in Paf1C mutant cells, even in the absence of the primary siRNAs that triggered the assembly of heterochromatin. This phenomenon complies with the classical definition of epigenetics[24] (that is, that it is heritable even in the absence of the initiating signal) and highlights fundamental roles of Paf1C and the RNAi machinery in building up epigenetic memory. This mechanism is also reminiscent of RNA-mediated epigenetic phenomena in higher eukaryotes such as paramutation[25] and RNA-induced epigenetic silencing (RNAe)[26]. RNAe is a phenomenon in which small RNAs of the *C. elegans* Piwi pathway can initiate transgene silencing that is extremely stable across generations even in the absence of the initiating Piwi protein. Yet, not all Piwi pathway RNAs trigger RNAe (ref. 27). Similarly, generation of siRNAs is necessary but not sufficient for paramutation in maize[28]. Thus, Paf1C may also have a regulatory role in paramutation and/or RNAe. Notably, Paf1C is known to help maintain expression of transcription factors required for pluripotency in human and mouse embryonic stem cells and prevent expression of genes involved in lineage specification[29,30], which may also involve small RNAs and chromatin regulation.

The ability to induce long-lasting and sequence specific gene silencing by transient delivery of synthetic siRNAs without changing the underlying DNA sequence will not only enable fundamental research on mechanisms that confer epigenetic memory, but may also open up new avenues in biotechnology and broaden the spectrum of the potential applications of RNAi-based therapeutics. Epigenetic control over gene expression is of particular interest in plant biotechnology, as this would circumvent the generation of genetically modified organisms.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.S., K.M.K., V.F. and J.B. generated strains and performed experiments; Y.S. performed the sms screen; the genome-wide small RNA and gene expression data were analysed by K.M.K.; M.B.S. designed and performed the computational analysis of the mutant genome resequencing data; M.B. designed experiments and prepared the manuscript. All authors discussed the results and commented on the manuscript.

Author Information Genome-wide data sets are deposited at GEO under the accession number GSE59171. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.B. (marc.buehler@fmi.ch).

METHODS

Strains and plasmids. Fission yeast strains were grown at 30 °C in YES medium. All strains were constructed following a PCR-based protocol[31] or by standard mating and sporulation. Plasmids and strains generated in this study are shown in Supplementary Tables 2 and 3.

EMS mutagenesis, hit selection and backcrossing. Exponentially growing *sms0* (SPB464) cells were washed and resuspended in 50 mM K-phosphate buffer (pH 7.0) and treated with EMS (final concentration 2.5%) for 150 min. An equal volume of freshly prepared 10% sodium thiosulfate was then added. Cells were washed with water and subsequently resuspended in YES. EMS treatment resulted in ~50% cell viability. To screen for mutants in which *ade6+* expression was silenced, cells were spread on YE plates. About 350,000 colonies were examined and pink colonies were selected for further evaluation. Positive hits were backcrossed four times with the parental strains SPB464 or SPB1788, depending on mating type.

Silencing assays. To assess *ura4+* expression, serial tenfold dilutions of the respective strains were plated on PMGc (non-selective, NS) or on PMGc plates containing 2 mg ml^{-1} 5-FOA. To assess *ade6+* expression, serial tenfold dilutions of the respective strains were plated on YES and YE plates.

Assessment of initiation versus maintenance of ectopic heterochromatin formation. Mutant strains were seeded on YE plates and single-cell-derived red or white colonies were selected. Colonies were resuspended in water and 100–500 cells were seeded on YE plates, which were then incubated at 30 °C for 3 days. Images of the plates were acquired after one night at 4 °C and colonies were counted automatically using Matlab (The MathWorks) and ImageJ Software (National Institutes of Health).

RNA isolation and cDNA synthesis. RNA isolation and cDNA synthesis was performed as described previously[32].

Quantitative real-time PCR. Real-time PCR on cDNA samples and ChIP DNA was performed as described[33] using a Bio-Rad CFX96 Real-Time System using SsoAdvanced SYBR Green supermix (Bio-Rad). Primer sequences are given in Supplementary Table 4.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) experiments were performed as previously described[33] with minor modifications. In brief, *S. pombe* cells were fixed with 1% formaldehyde for 15 min and then lysed in buffer containing 50 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. Chromatin was sheared with a Bioruptor (Diagenode). The following antibodies were used in this study: histone H3K9me2-specific mouse monoclonal antibody from Wako (MABI0307), histone H3-specific rabbit polyclonal antibody from Abcam (ab1791), histone H3K36me3-specific rabbit polyclonal antibody from Abcam (ab9050), and RNA polymerase II mouse monoclonal antibody from Covance (8WG16).

Small RNA sequencing. Total RNA was isolated from exponentially growing cells using the hot phenol method[34]. The RNA was fractionated using RNeasy Midi columns (Qiagen) following the RNA cleanup protocol provided by the manufacturer. The flow-through fraction was precipitated ('small RNA' fraction). Aliquots (25 μg) of the small RNA fraction were separated by 17.5% PAGE and the 18–28-nucleotide population purified. Libraries were prepared using the Illumina TruSeqTM small RNA preparation protocol (RS-930-1012). The 145–160-nucleotide population was isolated and the library sequenced on an Illumina HiSeq2000. Small RNA reads were aligned as described previously[32] with two mismatches allowed.

Whole-genome sequencing. Cells from an overnight culture were collected, washed once with water and flash frozen in liquid nitrogen. Cells were spheroplasted in spheroplast buffer (1.2 M sorbitol, 100 mM $KHPO_4$, pH 7.5, 0.5 mg ml^{-1} zymolyase (Zymo Research), 1 mg ml^{-1} lysing enzyme from *Trichoderma harzianum* (Sigma)). Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). Barcoded genomic DNA libraries for Illumina next-generation sequencing were prepared from 50 ng genomic DNA using the Nextera DNA Sample Preparation Kit (Illumina). Libraries were pooled equimolarly and sequenced on one lane of a HiSeq2000 machine (Illumina). Basecalling was done with RTA 1.13.48 (Illumina) software and for the demultiplexing CASAVA_v1.8.0 (Illumina) was used. For each strain, between 8.7 and 25.5 million (mean of 14.2 million) 50-nucleotide reads were generated and aligned to the *Schizosaccharomyces pombe* 972h- genome assembly (obtained on 17 September 2008 from http://www.broad.mit.edu/annotation/genome/schizosaccharomyces_group/MultiDownloads.html) using 'bwa' (ref. 35, version 0.7.4) with default parameters, but only retaining single-hit alignments ('bwa samse -n 1' and selecting alignments with 'X0:i:1'), resulting in a genome coverage between 26 and 85-fold (mean of 44-fold). The alignments were converted to BAM format, sorted and indexed using 'samtools' (ref. 36, version 0.1.19). Potential PCR duplicates were removed using 'MarkDuplicates' from 'Picards' (http://picard.sourceforge.net/, version 1.92). Sequence variants were identified using GATK (ref. 37, version 2.5.2) indel realignment and base quality score recalibration using a set of high confidence variants identified in an initial step as known variants, followed by single nucleotide polymorphism (SNP) and INDEL discovery and genotyping for each individual strain using standard hard filtering parameters, resulting in a total of 270–274 sequence variations (mean of 280) in each strain compared to the reference genome (406 unique variations in total over all strains). Finally, variations were filtered to retain only high quality single nucleotide variations (QUAL $\geq$ 50) of EMS type (G|C to A|T) with an alellic balance $\geq$ 0.9 (homozygous) that were not also identified in the parental strain (sms0), reducing the number of variations per strain to a number between 2 and 8 (mean of 4.6).

Expression profiling. RNA was isolated from cells collected at an attenuance (*D*) of 600 nm of 0.5 ($D_{600\,nm} = 0.5$) using the hot phenol method[34]. The isolated RNA was processed according to the GeneChip Whole Transcript Double-Stranded Target Assay Manual from Affymetrix using the GeneChip *S. pombe* Tiling 1.0FR. All tiling arrays were processed in R[38] using bioconductor[39] and the packages tilingArray[40] and preprocessCore. The arrays were RMA background-corrected, quantile-normalized, and $\log_2$-transformed on the oligonucleotide level using the following command: expr <−log$_2$(normalize.quantiles(rma.background.correct(exprs(readCel2eSet (filenames,rotated = TRUE))))). Oligonucleotide coordinates were intersected with the genome annotation and used to calculate average expression levels for individual genomic features (excluding those with <10 oligonucleotides) as well as broader annotation categories. In the latter case, multimapping oligonucleotides were counted only once per category (avoiding multiple counts from the same oligonucleotide).

Gene nomenclature. The proteins PAF1p, CDC73p, RTF1p, LEO1p and CTR9p form a stable complex in *S. cerevisiae* (Paf1C). The systematic IDs of the genes encoding the *S. pombe* homologues of these proteins are SPAC664.03, SPBC17G9.02c, SPBC651.09c, SPBC13E7.08c and SPAC27D7.14c, respectively. The *CTR9* homologue SPAC27D7.14c is currently annotated as Tpr1. The RTF1 homologue SPBC651.09c is currently annotated as PAF-related factor 1 (*prf1+*), because *rtf1+* is already used for an unrelated gene (SPAC22F8.07c). Therefore, we refer to SPAC664.03, SPBC17G9.02c, SPBC651.09c, SPBC13E7.08c and SPAC27D7.14c as *paf1+*, *cdc73+*, *prf1+*, *leo1+* and *tpr1+*, respectively, in this paper.

Statistics. A one-tailed Student's *t*-test was used, with $P < 0.05$ as the significance level. No statistical methods were used to predetermine sample size.

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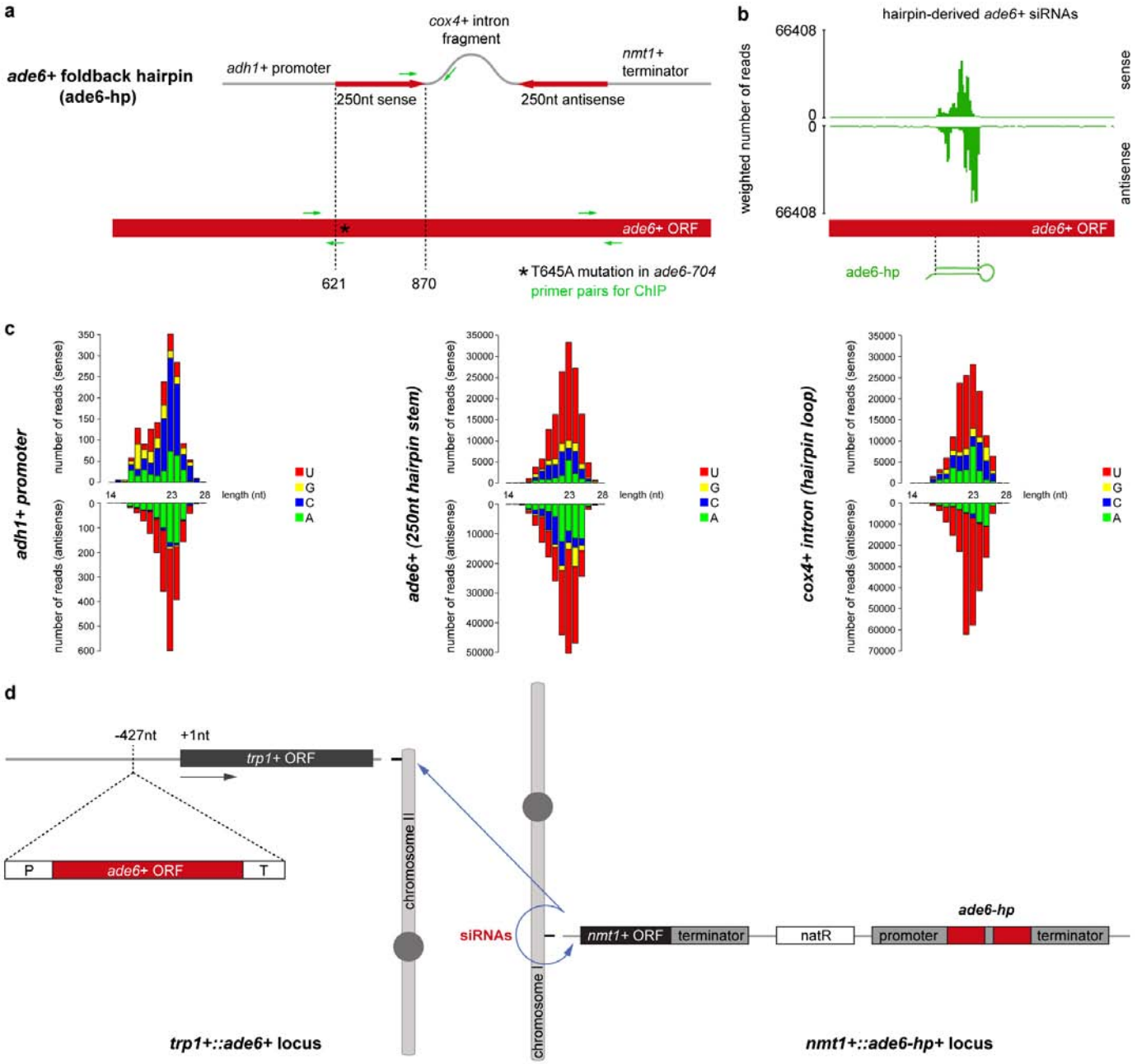
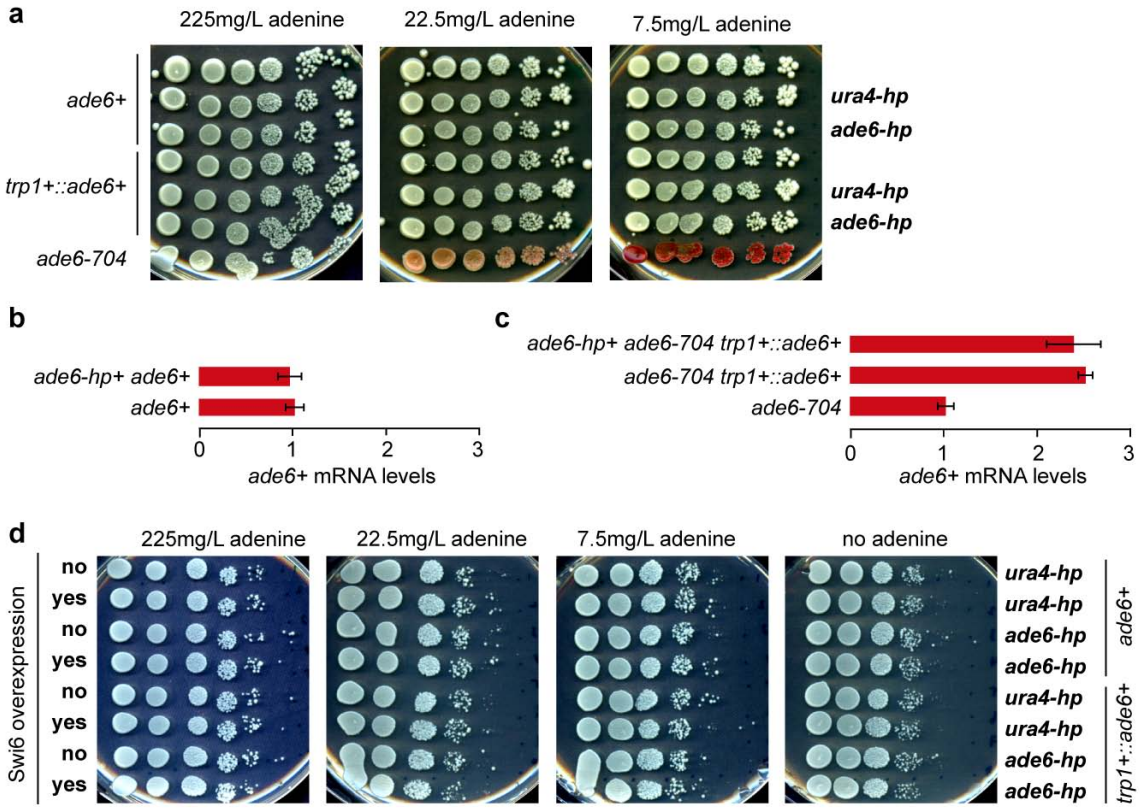
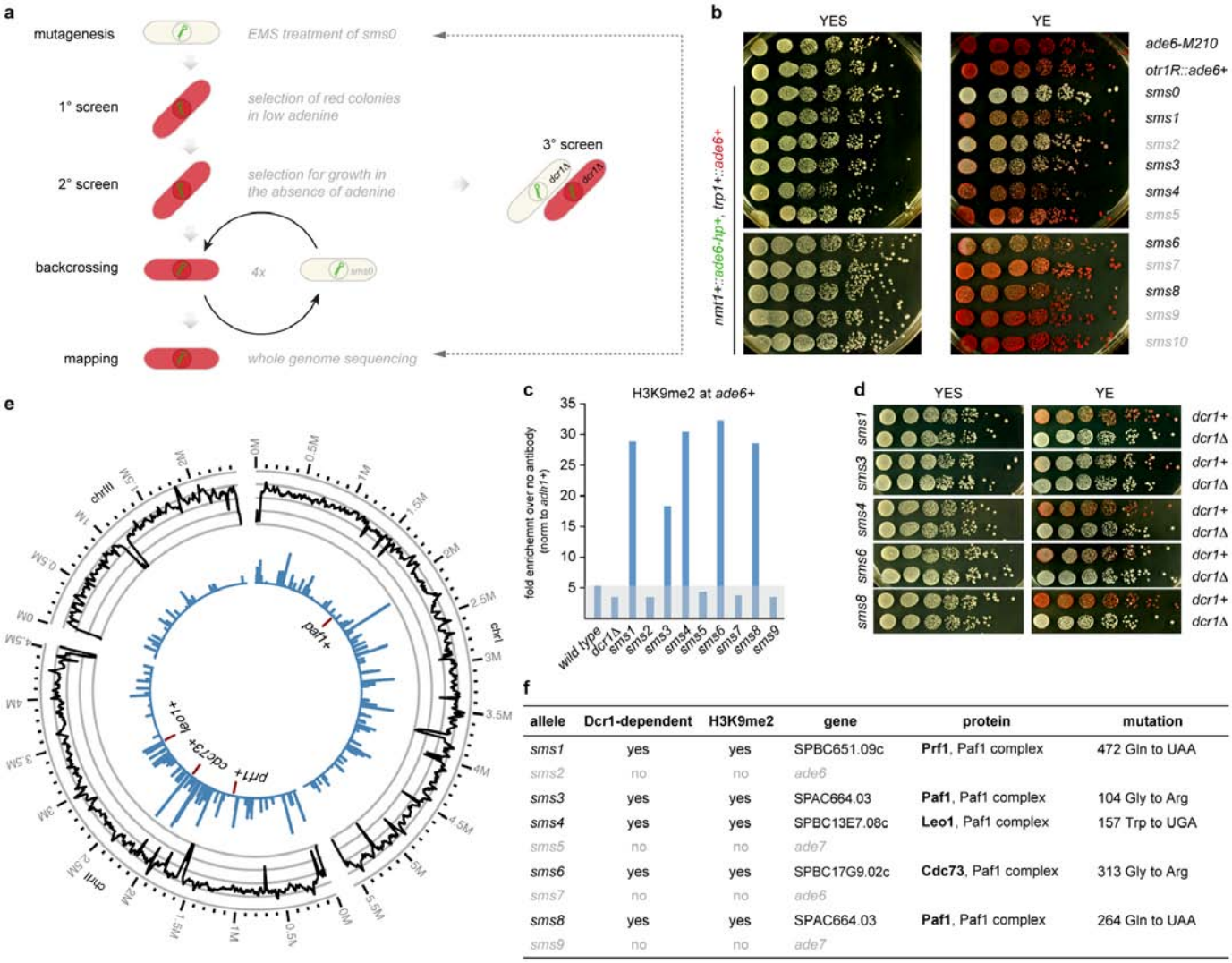


Extended Data Figure 1 | Design of the *ade6+* RNA hairpin (ade6-hp) construct that expresses abundant sense and antisense (primary) siRNAs. **a**, The RNA stem–loop construct consists of a 250-nucleotide-long *ade6+* fragment, followed by a *cox4+* intronic sequence and the reverse complement of the *ade6+* fragment. The promoter sequence of the *adh1+* gene drives expression of the RNA hairpin. Transcription of the construct is terminated by the termination signals of the *nmt1+* gene. The construct was provided by T. Iida. **b**, **c**, Small RNA sequencing revealed that the RNA stem is converted into sense and antisense siRNAs covering the 250-nucleotide stretch from the *ade6+* open reading frame (nucleotides 621–870). Furthermore, sense and antisense siRNAs mapping to the *cox4+* intronic and *adh1+* promoter sequences are also generated when this construct is expressed in wild-type cells. ORF, open reading frame. Asterisk denotes the point mutation (Thr645Ala) in the *ade6-704* loss of function allele. Green arrows indicate forward and reverse primers that were used for PCR in ChIP experiments. **d**, Schematic diagram depicting origin and target(s) of synthetic ade6-hp siRNAs. The ade6-hp expression cassette (**a**) was inserted into the *nmt1+* locus on chromosome I by homologous recombination. The ade6-hp-containing plasmid was linearized with *PmlI*, which cuts in the middle of the *nmt1+* terminator sequence, and transformed into *ade6-704* cells. Thereby, the ade6-hp construct was inserted downstream of the *nmt1+* gene. The nourseothricin (Nat)-resistance cassette linked to the ade6-hp construct allowed selection of positive transformants. It also allows assessment of spreading of repressive heterochromatin that is nucleated by the ade6-hp siRNAs *in cis* (see Extended Data Fig. 7b). A wild-type copy of the *ade6+* gene was inserted upstream of the *trp1+* gene on chromosome II by homologous recombination. Because the endogenous *ade6-704* allele is non-functional, positive transformants could be selected by growth in the absence of adenine. In Paf1C mutant cells, ade6-hp-derived siRNAs either act *in cis* to assemble heterochromatin at the *nmt1+* locus (chromosome I), or *in trans* to direct the formation of heterochromatin at the *trp1+::ade6+* (chromosome II) and *ade6-704* (chromosome III) loci.

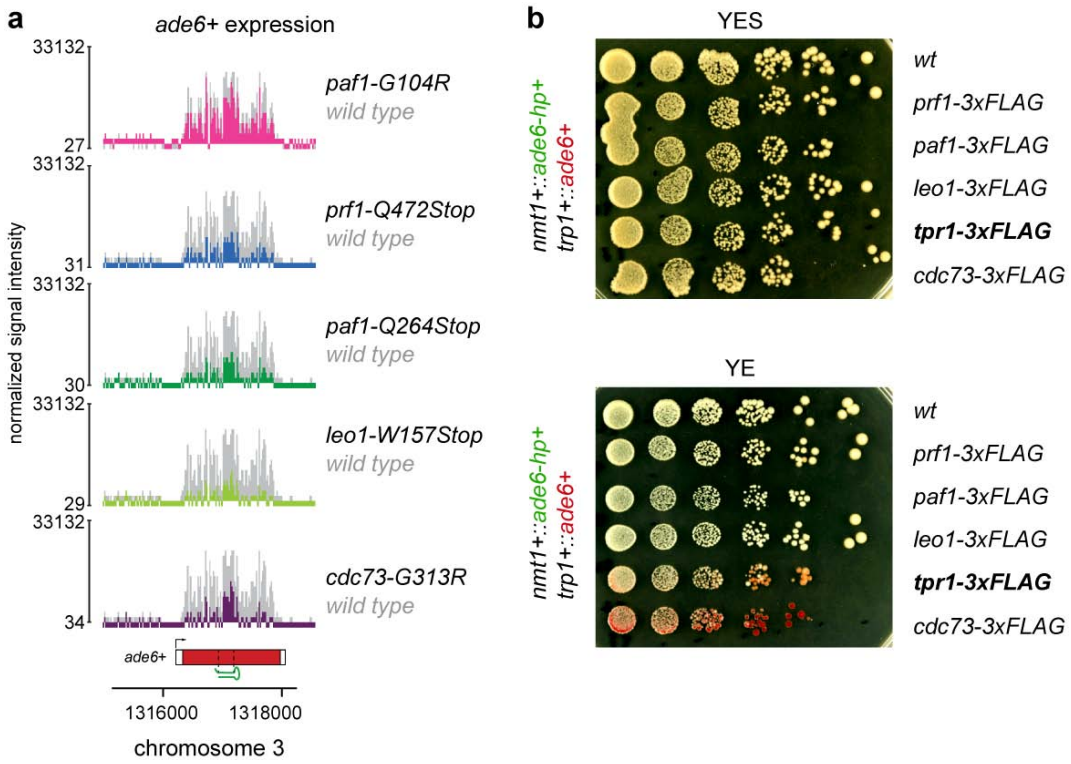


Extended Data Figure 2 | Silencing assays demonstrating the inability of synthetic siRNAs to act *in trans* in Paf1C wild-type cells. **a**, *ade6+* silencing assays were performed with cells expressing synthetic ade6-hp siRNAs, ura4-hp siRNAs or no siRNAs. The ability of ade6-hp siRNAs to silence either the endogenous *ade6+* gene or the *trp1+::ade6+* reporter gene was assessed at different adenine concentrations. *ade6-704* cells were used as positive control. **b**, **c**, *ade6+* mRNA levels were determined by quantitative RT–PCR and normalized to *act1+* mRNA. One representative biological replicate is shown. Error bars, s.d. **d**, *ade6+* silencing assays demonstrating that neither the endogenous *ade6+* gene nor the *trp1+::ade6+* reporter gene becomes repressed by *trans-acting* ade6-hp siRNAs, even upon overexpression of the heterochromatin protein Swi6.

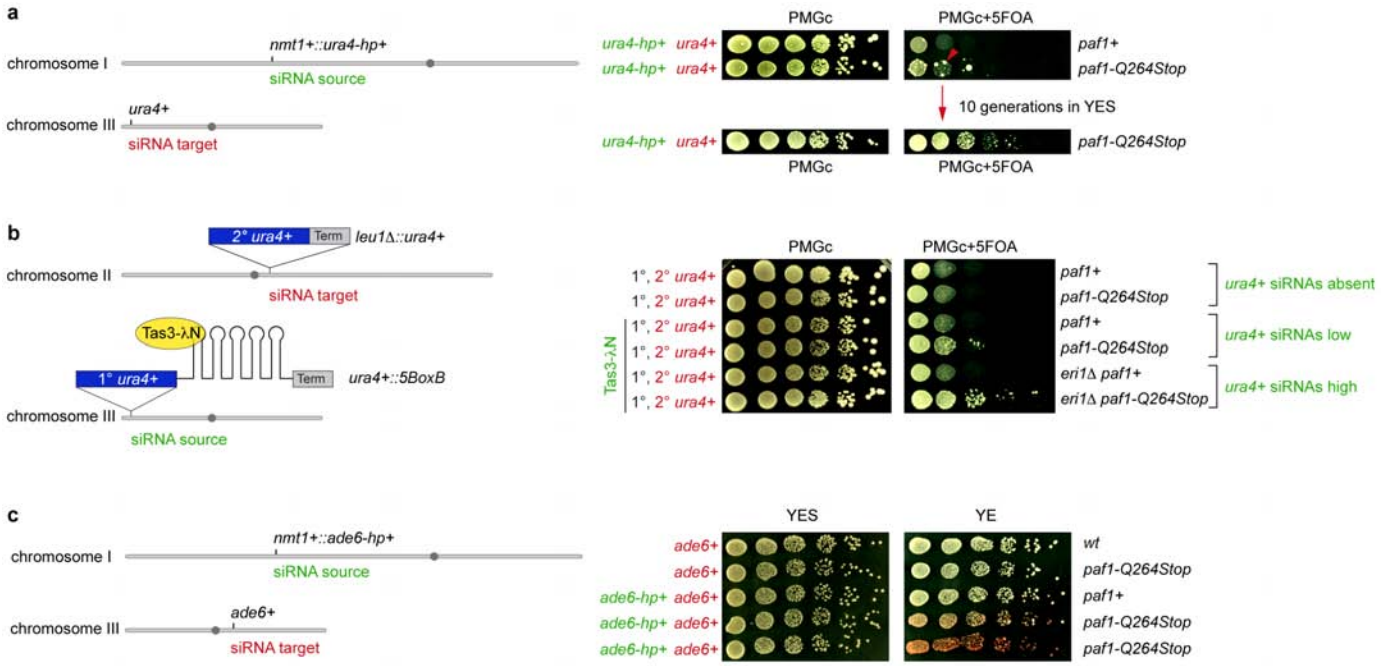


allele	Dcr1-dependent	H3K9me2	gene	protein	mutation
sms1	yes	yes	SPBC651.09c	**Prf1**, Paf1 complex	472 Gln to UAA
sms2	no	no	*ade6*		
sms3	yes	yes	SPAC664.03	**Paf1**, Paf1 complex	104 Gly to Arg
sms4	yes	yes	SPBC13E7.08c	**Leo1**, Paf1 complex	157 Trp to UGA
sms5	no	no	*ade7*		
sms6	yes	yes	SPBC17G9.02c	**Cdc73**, Paf1 complex	313 Gly to Arg
sms7	no	no	*ade6*		
sms8	yes	yes	SPAC664.03	**Paf1**, Paf1 complex	264 Gln to UAA
sms9	no	no	*ade7*		

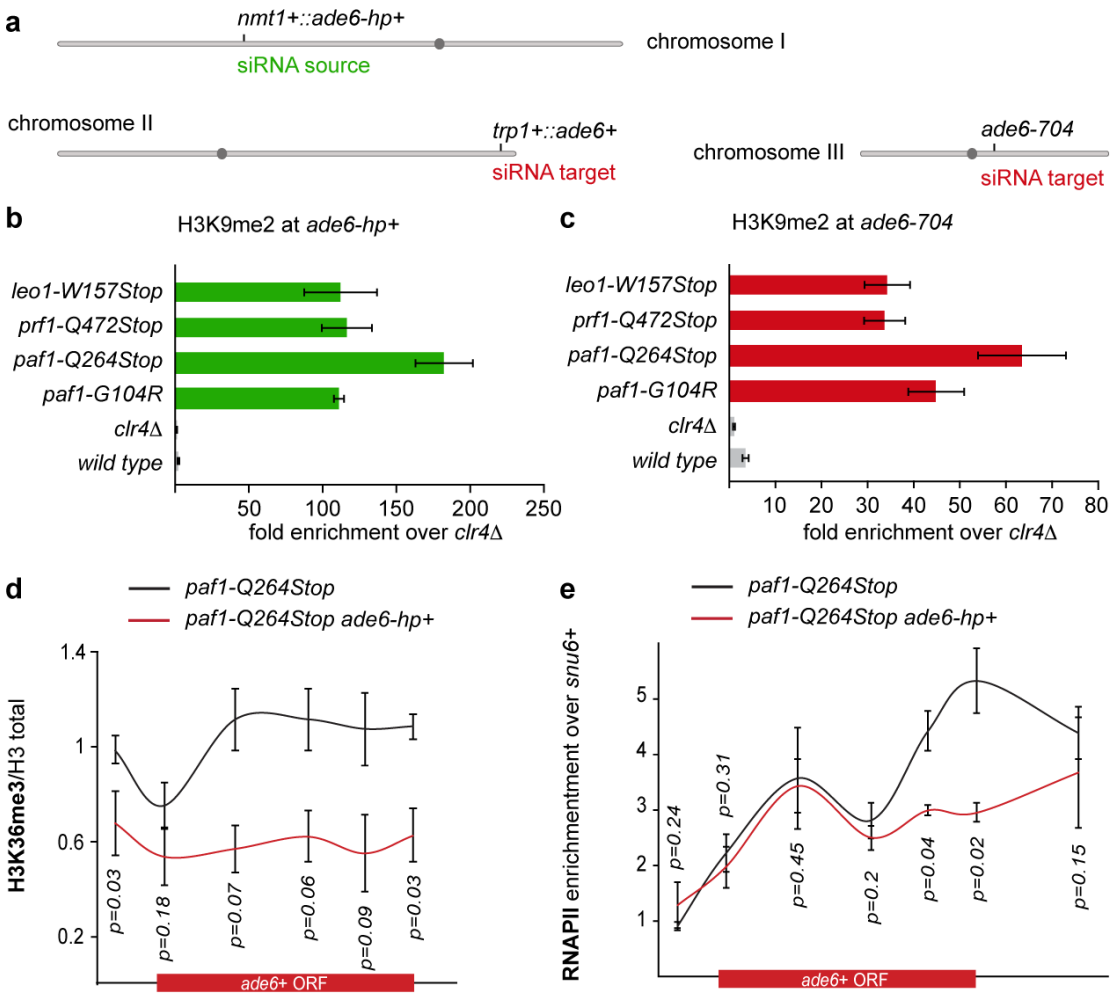
Extended Data Figure 3 | Sms forward genetic screen identifies five true positive hits that enable siRNAs to methylate H3K9 at the *ade6+* gene *in trans*. **a**, Workflow of the EMS mutagenesis screen. We mutagenized sms0 cells, which express abundant siRNAs complementary to the *ade6+* gene (indicated by green hairpin), with EMS (primary screen). Subsequently, we tested the positive red colonies for growth in the absence of adenine to select against loss-of-function mutations in the adenine biosynthesis pathway (secondary screen). In hits that remained positive after the secondary screen, *dcr1+* was deleted to identify truly siRNA-dependent hits (tertiary screen). For mapping of causative mutations by whole-genome next-generation sequencing, positive hits were backcrossed four times. **b**, *sms1-10* mutants show the red *ade6+* silencing phenotype on YE plates, which segregated through four successive backcrosses for all 10 mutants. The *ade6-M210* loss-of-function allele and *ade6+* inserted within centromeric heterochromatin (*otr1R::ade6+*) serve as positive controls. **c**, ChIP experiment demonstrating methylation of H3K9 at the *ade6+* target loci in *sms1*, *3*, *4*, *6* and *8*. One representative biological replicate is shown. **d**, *ade6+* silencing in *sms1*, *3*, *4*, *6* and *8* is Dcr1-dependent. **e**, Resequencing of EMS-mutagenized *S. pombe* strains. From outside to inside, the tracks show the genomic location, the average coverage per window of 10 kb (black line, scale from 0 to 30), the number of sequence variations identified before filtering in all strains per window of 10 kb (blue bars, scale from 0 to 90) and the five mutations that passed the filtering and overlapped with Paf1C genes (red lines, the two mutations in Paf1 are too close to be resolved individually). **f**, Table lists mutations mapped by whole-genome sequencing. In Dcr1-dependent mutants, we mapped mutations in the genes SPBC651.09c, SPAC664.03, SPBC13E7.08c and SPBC17G9.02c whose homologues in budding yeast encode for protein subunits of the Paf1 complex.



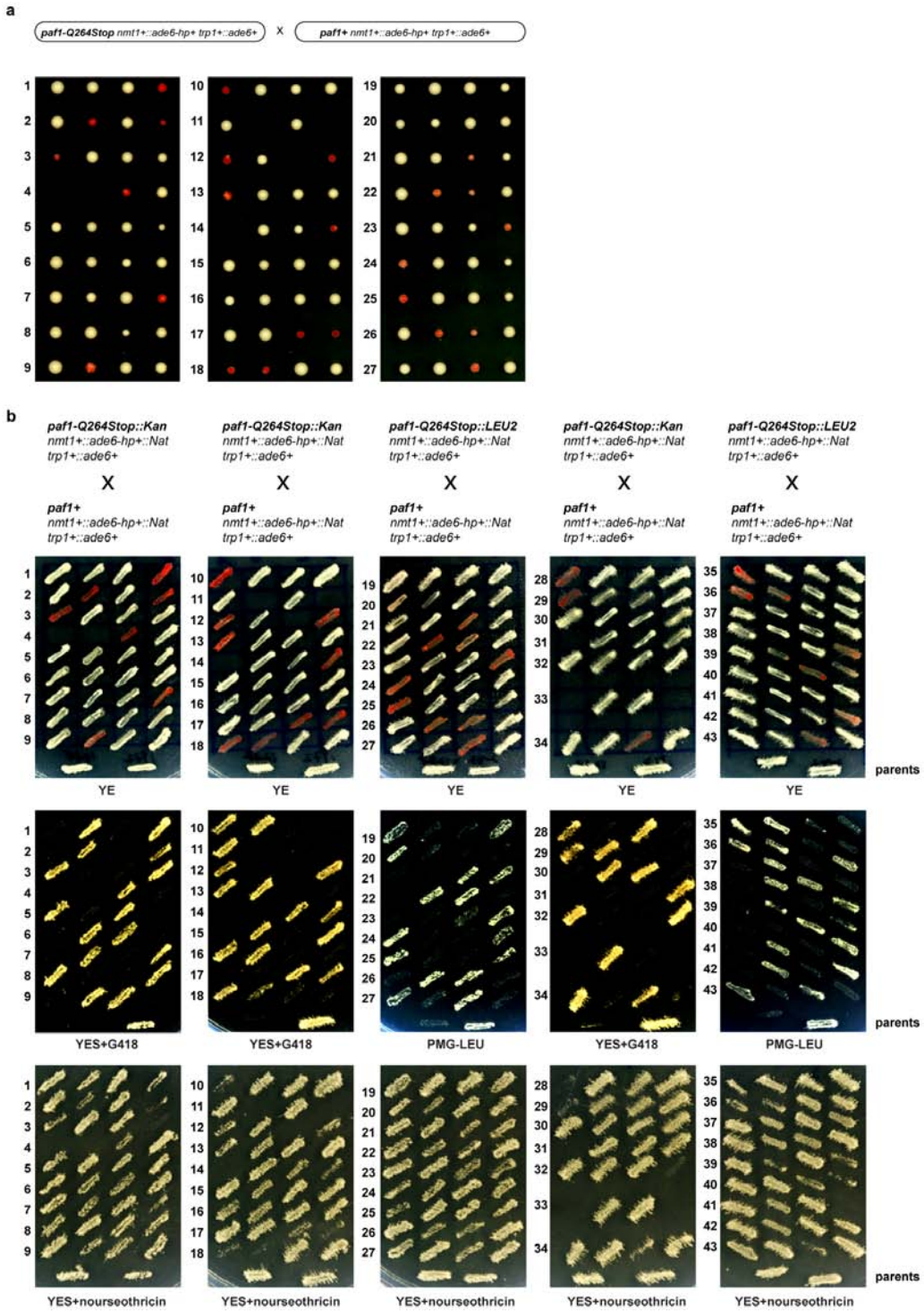
Extended Data Figure 4 | Mutant alleles for the homologues of all five subunits of Paf1C enable siRNAs to induce gene silencing *in trans*. **a**, *ade6+* siRNAs reduce *ade6+* mRNA levels in all Paf1C mutant strains identified in this study. Whole-genome tiling arrays were used to assess gene expression in the mutant cells indicated. *y* axis is in linear scale. **b**, C-terminally tagged Tpr1 and Cdc73 are hypomorphic. Full deletions of the *tpr1+* and *cdc73+* genes cause retarded growth phenotypes (Extended Data Fig. 8c). By contrast, *tpr1-3xFLAG* and *cdc73-3xFLAG* grow normally, and display ade6-hp siRNA-mediated repression of the *ade6+* gene.



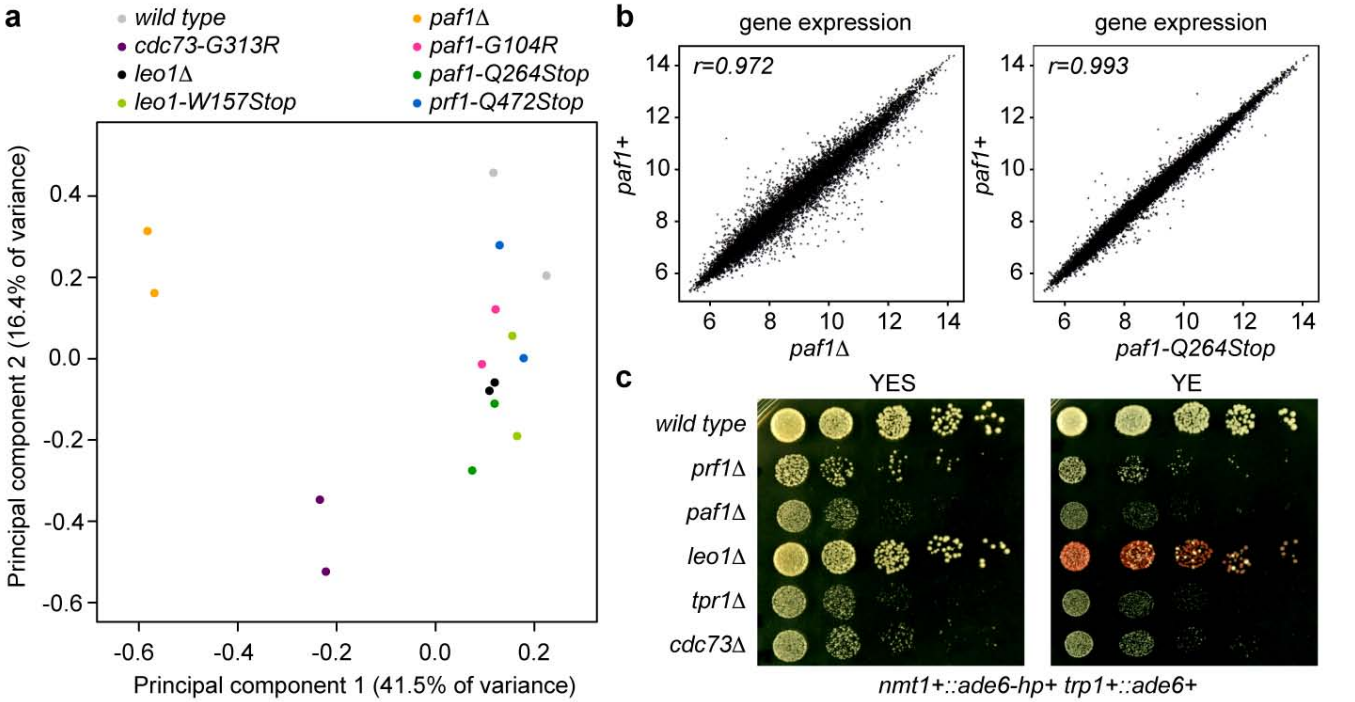
Extended Data Figure 5 | Expression of synthetic siRNAs in *paf1-Q264Stop* cells is sufficient to trigger stable repression of protein coding genes *in trans*. **a**, Left, the *paf1-Q264Stop* mutation was introduced into cells that express synthetic ura4-hp siRNAs[15]. Right, wild-type (*paf1+*) and *paf1-Q264Stop* were grown in the presence or absence of 5-FOA. Red arrow indicates *paf1-Q264Stop* colonies growing on FOA-containing medium. Note that these colonies could be propagated in non-selective medium without losing the repressed state. **b**, In *S. pombe*, artificial tethering of the RITS complex to mRNA expressed from the endogenous *ura4+* locus using the phage λN protein results in *de novo* generation of *ura4+* siRNAs. These siRNAs load onto RITS and are necessary to establish heterochromatin at the *ura4+* locus *in cis*. However, like ura4-hp siRNAs, they are incapable of triggering the repression of a second *ura4+* locus *in trans*[18]. To test whether *ura4+* siRNAs produced as a result of Tas3λN tethering to *ura4+::5BoxB* mRNA (chromosome III) can act *in trans* to silence a second *ura4+* allele (*leu1Δ::ura4+*, chromosome II), *paf1+* was mutated and *ura4+* repression was assessed by FOA silencing assays. Whereas 5-FOA was toxic to both *paf1+* and *paf1-Q264Stop* cells in the absence of *ura4+* siRNAs (Tas3 not fused to λN), FOA-resistant colonies appeared upon Tas3-λN tethering, demonstrating that siRNAs generated from the *ura4+::5BoxB* locus can initiate repression of the second *ura4+* copy expressed from the *leu1+* locus. Notably, siRNA-mediated *ura4+* repression *in trans* was more pronounced in the absence of the RNase Eri1. We have previously shown that the levels of *ura4+::5BoxB*-derived siRNA are higher in *eri1Δ* cells[41]. We note that *trans*-silencing of the second *ura4+* allele occasionally occurs in *paf1+* cells in the absence of Eri1 (ref. 18). However, in contrast to *paf1-Q264Stop* cells, the repressed state of *ura4+* is not stably propagated. Hairpin symbols downstream of the *ura4+* ORF denote BoxB sequences. They form stem–loop structures when transcribed and are bound by the λN protein. **c**, *ade6+* silencing assay demonstrating that also the endogenous *ade6+* gene is repressed if ade6-hp siRNAs are expressed from the *nmt1+* locus in *paf1-Q264Stop* cells. Silencing assay was performed with two freshly generated (naive) *paf1-Q264Stop* mutant strains. A few white colonies in which heterochromatin has not yet formed are discernable. Such white colonies were picked to determine heterochromatin initiation frequencies shown in Fig. 2.



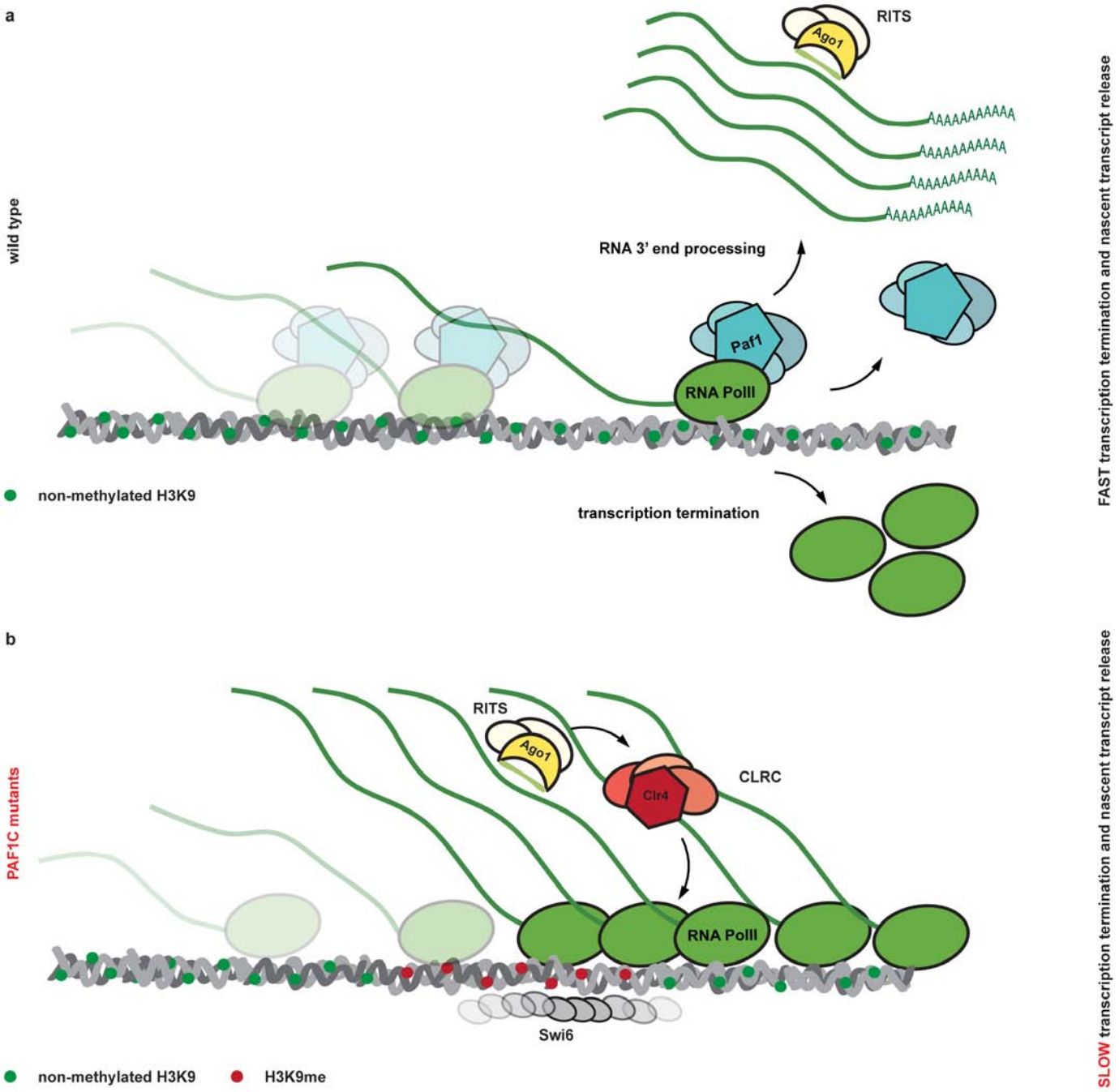
Extended Data Figure 6 | *ade6+* siRNAs trigger *de novo* methylation of H3K9 at homologous *ade6+* sequences *in cis* and *in trans*. **a**, ade6-hp RNA producing locus and siRNA target loci *in trans* in the sms0 strain. *ade6-704* is a loss-of-function allele of the endogenous *ade6+* gene and serves as a positive control in the silencing assays. **b**, **c**, *ade6+* siRNAs direct the methylation of H3K9 at *ade6* targets *in cis* (green) and *in trans* (red) in Paf1C mutant cells. H3K9me2 for *trp1+::ade6+* is shown in Fig. 1d. Quantitative PCR was performed with locus-specific primers. Error bars, s.e.m.; $n = 3$ technical replicates. **d**, **e**, ChIP experiments to assess *ade6+* transcriptional activity. H3K36me3 levels were normalized to total H3 levels. *snu6+* is transcribed by RNAPIII and serves as background control. Error bars, s.e.m.; $n = 3$ independent biological replicates; *P* values were calculated using the one-tailed Student's *t*-test.



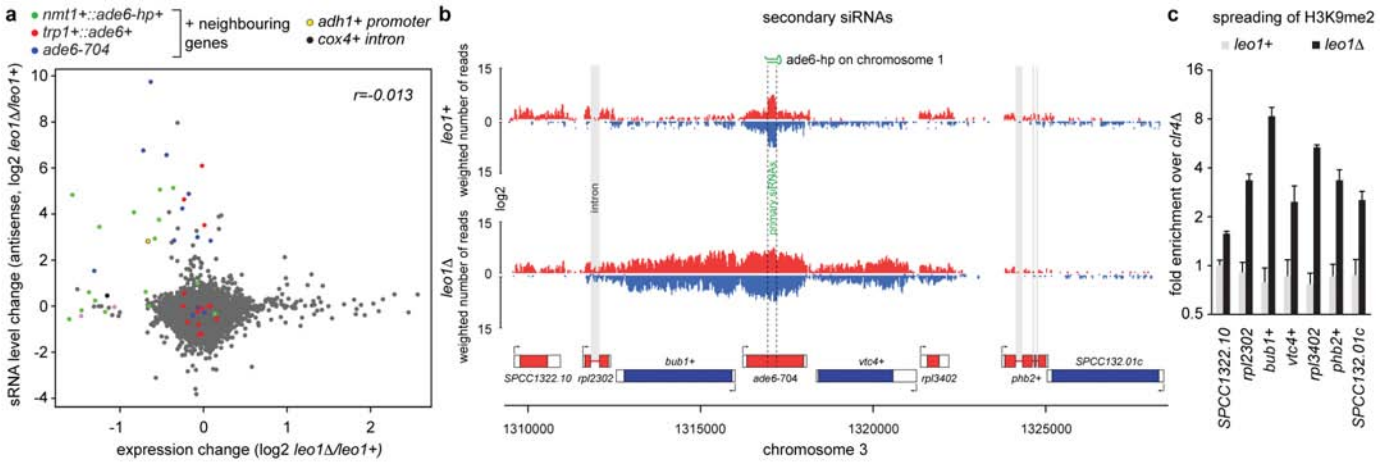
Extended Data Figure 7 | Pronounced siRNA-directed heterochromatin formation *in trans* during meiosis. **a**, White (naive) cells that had not yet established heterochromatin at the *trp1+::ade6+* locus were isolated from populations of freshly generated *paf1-Q264Stop* strains and crossed with *paf1+* cells. Both mating partners expressed ade6-hp siRNAs and contained the same *trp1+::ade6+* reporter. Spores were dissected on YE plates and incubated for 3–4 days at 30 °C. Note the non-Mendelian inheritance pattern of the parental white phenotype and the high incidence of heterochromatin formation (red phenotype) in *paf1-Q264Stop* cells after meiosis. **b**, Spores from 43 tetrads were dissected in total. Colonies formed by the individual spores (**a**) were then struck on YE plates and incubated for 3–4 days at 30 °C, followed by replica-plating onto YES-G418 and YES+nourseothricin (Nat) plates for genotyping. Thus, the cells visible on the YE plates have gone through roughly 50–80 mitotic divisions after mating and sporulation. This analysis shows that *de novo* formation of heterochromatin by *trans*-acting siRNAs during meiosis occurs more frequently than in mitosis. However, once established, heterochromatin is remarkably stable in mitotic cells (see also Fig. 2). Notably, growth of some *paf1-Q264Stop* descendants was reduced on YES+Nat plates, demonstrating spreading of heterochromatin into the neighbouring Nat-resistance cassette that marks the *nmt1+::ade6-hp+* locus (see Extended Data Fig. 1). Note that genes repressed by heterochromatin can be derepressed under strong negative selection. Thus, this observation indicates extraordinary repressive activity of the heterochromatin that forms *in cis* at the ade6-hp siRNA-producing locus. Finally, *paf1+* cells (no growth on YES-G418 or PMG-LEU) never turned red, demonstrating the high repressive activity of Paf1. This explains unsatisfactory results of previous attempts to induce the formation of stable heterochromatin *in trans* by expressing synthetic siRNAs.



Extended Data Figure 8 | Effect of Paf1C mutations on global gene expression and silencing. **a**, The effect of the Paf1C mutations on genome expression was assessed by hybridizing total RNA to whole-genome tiling arrays. The parental wild-type strain, all Paf1C point mutations discovered in the screen, and full deletions of the *paf1*+ and *leo1*+ genes were included in the analysis. To compare the genome-wide expression profiles of the mutants with the wild-type strain, a principal component analysis (PCA) was performed on the data obtained for two biological replicates of each strain. Principal component (PC) 1 and 2 explained 41.5% and 16.4% of the variance between samples and were selected for visualization, revealing that *cdc73-G313R* and *paf1*Δ cells are most different from wild-type cells. All the other mutants clustered together in a group of samples that also includes wild type, demonstrating that RNA steady-state levels are only minimally affected in these mutants. Note that *leo1*Δ is more similar to wild type than *paf1*Δ, as well as that *paf1*Δ clusters separately from the Paf1C point mutants. **b**, Pairwise comparisons of gene expression between wild-type and *paf1* mutant strains. **c**, *leo1*Δ cells have no growth defect but are susceptible for *de novo* formation of heterochromatin by siRNAs acting *in trans*. These results suggest that Leo1 might be a bona fide repressor of small-RNA-mediated heterochromatin formation.



Extended Data Figure 9 | Kinetic model for Paf1C-mediated repression of siRNA-directed heterochromatin formation. **a**, Paf1C facilitates rapid transcription and release of the nascent transcript from the DNA template. Because the kinetics of transcription termination and RNA 3′ end processing is faster than RITS binding and CLRC recruitment, stable heterochromatin and long-lasting gene silencing cannot be established. **b**, In Paf1C mutant cells identified in this study, elongation of RNA polymerase II, termination of transcription, and the release of the nascent transcript from the site of transcription is decelerated. This results in an accumulation of RNA polymerases that are associated with nascent transcripts, opening up a window of opportunity for the siRNA-guided RITS complex to base-pair with nascent transcripts and recruit CLRC. Consequently, highly stable and repressive heterochromatin is assembled, which is accompanied by the generation of secondary siRNAs covering the entire locus (not depicted in this scheme). Notably, our results demonstrate that impaired transcription termination but not elongation is sufficient to allow silencing. However, to confer robustness to the repressed state, both transcription termination and release of the RNA transcript from the site of transcription must be impaired concomitantly.



Extended Data Figure 10 | Formation of ectopic heterochromatin. **a**, Differential gene expression compared to differential antisense siRNA expression in *leo1*Δ. Gene expression profiles were obtained with whole-genome tiling arrays and small RNA profiles by deep sequencing. Genes neighbouring the *nmt1+::ade6-hp+*, *trp1+::ade6+* and *ade6-704* loci are marked in colour (see also Supplementary Table 1). **b**, siRNA reads mapping to the *ade6-704* locus in *leo1+* and *leo1*Δ strains. Red, plus strand; blue, minus strand. Intronic *rpl2302* siRNAs in *leo1*Δ cells indicate co-transcriptional double-stranded RNA synthesis by RDRC before splicing. **c**, ChIP experiment showing H3K9me2 enrichments on genes surrounding the *ade6-704* locus in *leo1+* and *leo1*Δ cells. Enrichments were calculated relative to background levels obtained in *clr4*Δ cells and normalized to *adh1+*. Error bars, s.d.; mean of $n = 2$ independent biological replicates.

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MOLECULAR BIOLOGY

RNA interference hangs by a thread

The Paf1 protein complex in fission yeast has been found to protect protein-coding genes from inhibition by RNA-mediated silencing of transcription, by stimulating the release of nascent transcripts from DNA.

MIKEL ZARATIEGUI

RNA interference (RNAi) is a mechanism of gene regulation that uses small RNAs called short interfering RNAs (siRNAs) to silence the expression of specific targets that have complementary nucleotide sequences. This can occur through post-transcriptional silencing, which degrades the target transcript, or through modification of chromatin (the complex of proteins, RNA and DNA in which DNA is packaged in the cell), which prevents transcription from initiating. RNAi always causes transcript degradation, but its effect on chromatin is bewilderingly inconsistent; only some targets in some organisms exhibit RNAi-mediated chromatin modifications, despite the evolutionary conservation of this mechanism. In a paper published on *Nature*'s website today, Kowalik *et al.*[1] report that the target transcript must remain associated with the site of transcription for RNAi to act on chromatin, providing a possible explanation for this variability.

Researchers can exploit RNAi to inhibit any RNA sequence of choice, simply by introducing a source of siRNA, such as a double-stranded RNA molecule that can be processed to siRNA by the nuclease enzyme Dicer. During transcription, siRNA can hybridize with a complementary nascent transcript and induce the deposition of silencing chromatin marks, leading to the formation of an inheritable repressive type of chromatin called heterochromatin. The inheritance of silencing chromatin modifications down generations of cells is an example of epigenetic memory.

By forming complexes with Argonaute effector proteins, siRNA can locate and silence target sequences even if they are located at distant sites, a process called silencing *in trans*. This phenomenon is crucial for repressing the transcription of dispersed repetitive genomic elements, such as transposons[2], which must be silenced to prevent them from 'jumping' around the genome and introducing harmful mutations. However, when researchers try to take advantage of this phenomenon to induce heterochromatin in mammalian protein-coding genes, the process seldom works. Instead, it produces erratic results that depend in perplexing ways on the target gene and on pre-existing chromatin modifications.

Similarly disappointing results have been reported for fission yeast (*Schizosaccharomyces pombe*). The yeast's siRNA precursors cannot induce heterochromatin formation in target genes[3], although certain non-protein-coding sequences at the centre of the yeast's chromosomes are robustly silenced by RNAi. Reasoning that suppressive mechanisms might protect protein-coding genes from RNAi, Kowalik and colleagues performed a genetic screen in *S. pombe*, looking for mutations that allowed silencing *in trans*. Strikingly, all the mutations they obtained arose in proteins of the RNA polymerase-associated factor 1 complex (Paf1C).

The authors report that several different siRNA precursors can reliably induce heterochromatin formation when Paf1C is mutated. The silenced DNA regions exhibit all the hallmarks of endogenous heterochromatin, including decreased transcription, generation of target-derived (secondary) siRNA molecules, and addition of a methyl group to the amino-acid residue lysine 9 of the chromatin protein histone 3 (a modification known as H3K9me). Kowalik *et al.* found that, once established, siRNA-induced heterochromatin can be inherited across generations of cells, even in the absence of the original siRNA source, as long as Paf1C is still mutated. This suggests that the cycle of secondary siRNA generation and H3K9me deposition that arises after heterochromatin initially forms can induce its own inheritance (Fig. 1). Silencing marks are re-established after each chromatin replication, preventing their dilution and satisfying even the strictest definitions of epigenetic inheritance.

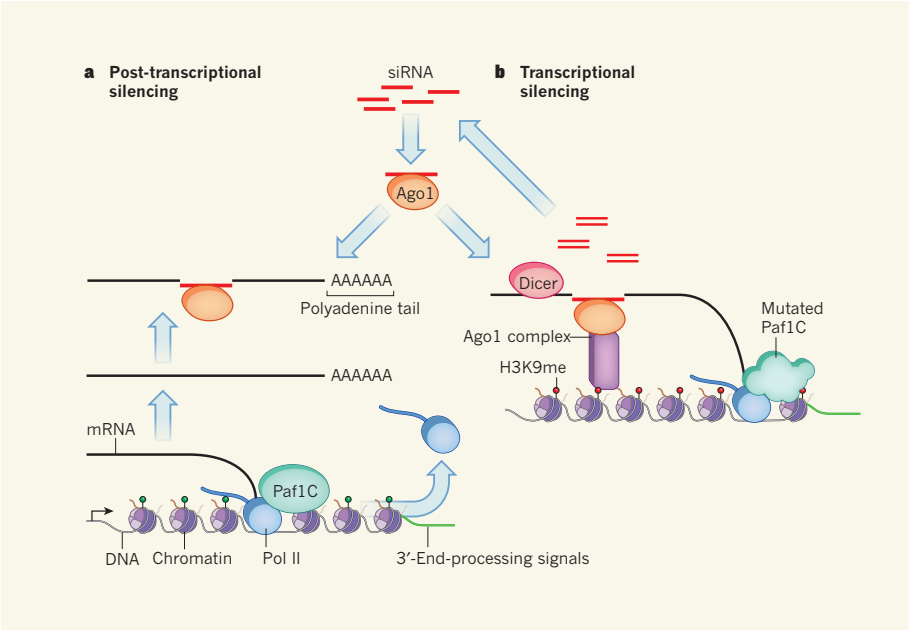

Figure 1 | Interfering with interference. Short interfering RNA (siRNA) forms complexes with the Argonaute 1 (Ago1) protein, enabling it to bind to and cleave complementary transcripts. **a**, In fission yeast, the Paf1 protein complex (Paf1C) promotes both transcript elongation by the enzyme RNA polymerase II (Pol II) and 3′-end processing, which consists of the release of Pol II, and cleavage and polyadenylation of messenger RNA, releasing the mRNA from the site of transcription. The released transcript is silenced post-transcriptionally by siRNA. **b**, Kowalik *et al.*[1] report that, if mutations compromise Paf1C activity, the uncleaved transcript is retained at the site of transcription. Ago1–siRNA forms complexes with other proteins, which together mediate the induction of chromatin-silencing modifications such as H3K9me, and the initiation of a self-reinforcing cycle of secondary siRNA production, in which mRNA is cleaved by siRNA, and the cleaved transcript is processed to siRNA by the nuclease enzyme Dicer.

How does Paf1C prevent RNAi from silencing chromatin? The authors tackled this question by genetically inhibiting proteins involved in each of the three main functions regulated by Paf1C: chromatin modification; elongation of messenger RNA during transcription; and processing of the 3′ end of mRNA as transcription terminates[4]. During 3′-end processing, transcripts are cleaved and a 'polyadenine tail' is added to stabilize the mRNA and permit normal translation. Interfering with chromatin modification or transcript elongation had no effect on heterochromatin formation. However, mutations in Ctf1 and Res2, two proteins required for cleavage and polyadenylation of mRNA, allowed *in trans* silencing to occur, albeit less efficiently than in the Paf1C mutants.

Conversely, Kowalik *et al.* demonstrate that bypassing 3′-end processing, by using a ribozyme molecule that cleaves off and releases the nascent transcript from DNA before termination, prevents *in trans* silencing even when Paf1C is mutated. Thus, it seems that the expeditious 3′-end processing of protein-coding genes protects them from RNAi by denying siRNA a docking platform — a nascent transcript still attached to the site of transcription. This process is probably necessary to prevent spurious heterochromatin formation by runaway RNAi activity, because the authors observe stochastic formation of heterochromatin in abnormal, protein-coding locations when Paf1C activity is compromised.

On a fundamental level, these results highlight the inextricable relationship between RNAi and mRNA processing. Both share the same targets, which are transcribed by the enzyme RNA polymerase II, and the two processes share much cross-regulation. It seems that the two mechanisms converge in 3′-end processing. Almost a decade ago, genetic screens searching for enhancers of post-transcriptional RNAi in plants yielded factors involved in 3′-end processing[5], a harbinger of the effect shown here for transcriptional silencing. Strikingly, it seems that Dicer can release some nascent transcripts[6,7], usurping the role of the normal cleavage and polyadenylation machinery in certain situations. Together with the known influence of chromatin state on 3′-end processing[8], a picture is emerging of a network of regulatory activities that enhance or prevent chromatin silencing in different targets.

But the most exciting implication is the potential to leverage this knowledge to induce targeted heterochromatin formation in different organisms, including humans. Mechanisms of RNAi-mediated chromatin modification might be universally conserved after all, with the variability observed in different targets and models stemming from differences in the efficiency of nascent transcript release at target sequences. In *S. pombe*, the transcripts naturally targeted for RNAi-mediated heterochromatin formation exhibit notoriously inefficient termination of transcription[9], which explains why their silencing is independent of Paf1C activity. Furthermore, a protein-coding reporter gene can be efficiently silenced *in trans* in this yeast if its 3′-end-processing signals are mutated[10]. Perhaps, then, we will be able to silence genes reliably and inheritably at will, by inhibiting transcription termination[11] and simultaneously using a specific RNAi trigger. In this way, RNAi would finally fulfil its promise and join other technologies, such as CRISPR/dCas9 systems, that enable editing of the epigenome. ■

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11 Appendix

11.1 Supplementary tables

Table 7 Gene repressed in the Paf1 complex mutants.

Gene ID		Chromosome	Location	Feature type	Expression fold change	small RNAs	
						yes/no	change in mutant
prf1-Q472Stop	SPNCRNA.1234	chr3	1837499-1840092	long ncRNA	-2.836	yes	down
	SPNCRNA.10	chr3	1837689-1838380	long ncRNA	-2.695	yes	up
	SPCC1223.03c	chr3	1840594-1843300	pre-mRNA	-2.224	no	
	SPAC1296.02	chr1	711413-711767	intron	-2.217	yes	NC
	SPNCRNA.504	chr3	1839578-1840266	long ncRNA	-1.930	yes	NC
	SPCC1322.13	chr3	1316243-1318070	pre-mRNA	-1.834	yes	up
	SPCC1223.01	chr3	1834599-1837464	pre-mRNA	-1.732	yes	up
	SPNCRNA.1235	chr3	1839787-1844115	long ncRNA	-1.724	yes	down
	SPCC1223.02	chr3	1838335-1839525	pre-mRNA	-1.640	yes	up
	SPCC1322.14c	chr3	1318375-1321275	pre-mRNA	-1.279	yes	up
	SPCC2H8.02	chr3	827911-828545	5' UTR	-0.897	no	
	SPCC285.17	chr3	1831913-1834169	pre-mRNA	-0.828	no	
	SPBC14F5.12c	chr2	4182411-4182642	5' UTR	-0.812	no	
	SPAC1296.02	chr1	711238-712354	pre-mRNA	-0.804	yes	NC
	SPBC1861.02	chr2	4133286-4133635	3' UTR	-0.777	no	
	SPAC2H10.04	chr1	5272461-5272835	pre-mRNA	-0.746	no	
	SPAC4C5.04	chr1	1198224-1198458	5' UTR	-0.727	no	
	SPNCRNA.511	chr3	2011061-2012433	long ncRNA	-0.724	no	
	SPCC13B11.01	chr3	1589427-1592889	pre-mRNA	-0.709	yes	up
	SPBC19C7.12c	chr2	2842901-2845922	3' UTR	-0.708	no	
paf1-Q264Stop	SPNCRNA.1234	chr3	1837499-1840092	long ncRNA	-2.540	yes	down
	SPNCRNA.10	chr3	1837689-1838380	long ncRNA	-2.456	no	
	SPCC1322.13	chr3	1316243-1318070	pre-mRNA	-2.366	yes	up
	SPAC1296.02	chr1	711413-711767	intron	-2.334	yes	up
	SPCC1223.03c	chr3	1840594-1843300	pre-mRNA	-1.972	yes	up
	SPCC1223.01	chr3	1834599-1837464	pre-mRNA	-1.536	yes	up
	SPNCRNA.1235	chr3	1839787-1844115	long ncRNA	-1.484	yes	NC
	SPNCRNA.504	chr3	1839578-1840266	long ncRNA	-1.379	yes	NC
	SPCC1322.14c	chr3	1318375-1321275	pre-mRNA	-1.265	yes	up
	SPAC664.03	chr1	1708728-1710347	pre-mRNA	-1.225	yes	up
	SPCC1223.02	chr3	1838335-1839525	pre-mRNA	-1.199	yes	up
	SPNCRNA.511	chr3	2011061-2012433	long ncRNA	-1.131	no	
	SPNCRNA.1254	chr3	2011026-2012559	long ncRNA	-1.055	no	
	SPAC1296.02	chr1	711238-712354	pre-mRNA	-1.019	yes	up
	SPBC1861.02	chr2	4133286-4133635	3' UTR	-0.992	no	
	SPNCRNA.763	chr1	1813439-1815920	long ncRNA	-0.975	no	
	SPAC2H10.04	chr1	5272461-5272835	pre-mRNA	-0.947	no	
	SPCC13B11.01	chr3	1589427-1591358	5' UTR	-0.892	yes	up
	SPNCRNA.17	chr2	1777375-1777788	long ncRNA	-0.891	no	
	SPBC1271.10c	chr2	347590-350376	pre-mRNA	-0.879	no	
paf1-G104R	SPNCRNA.1234	chr3	1837499-1840092	long ncRNA	-1.729	yes	NC
	SPNCRNA.10	chr3	1837689-1838380	long ncRNA	-1.671	yes	up
	SPCC1223.03c	chr3	1840594-1843300	pre-mRNA	-1.381	no	
	SPCC1223.02	chr3	1838335-1839525	pre-mRNA	-1.327	yes	up
	SPNCRNA.504	chr3	1839578-1840266	long ncRNA	-1.212	yes	NC
	SPAC1296.02	chr1	711413-711767	intron	-1.184	yes	NC
	SPNCRNA.1235	chr3	1839787-1844115	long ncRNA	-1.170	yes	NC
	SPBC13E7.07	chr2	3053333-3055355	3' UTR	-1.148	no	
	SPCC1322.13	chr3	1316243-1318070	pre-mRNA	-1.065	yes	up
	SPCC1223.01	chr3	1834599-1837464	pre-mRNA	-0.978	yes	up
	SPAC2H10.04	chr1	5272461-5272835	pre-mRNA	-0.897	no	
	SPBC83.10	chr2	1528476-1528831	3' UTR	-0.844	no	
	SPAC8C9.07	chr1	3654213-3654875	pre-mRNA	-0.682	no	

	SPCC1620.10	chr3	2163164-2164505	pre-mRNA	-0.678	no	
	SPBC1271.10c	chr2	347590-350376	pre-mRNA	-0.667	no	
	SPNCRNA.944	chr1	3876714-3877850	long ncRNA	-0.665	no	
	SPAC5H10.13c	chr1	167766-168809	3' UTR	-0.665	no	
	SPAC16E8.06c	chr1	3508514-3510077	pre-mRNA	-0.645	no	
	SPAC607.02c	chr1	2040405-2040627	5' UTR	-0.641	no	
	SPBC16E9.02c	chr2	1918006-1920215	pre-mRNA	-0.635	no	
leo1-W157Stop	SPNCRNA.1234	chr 3	1837499-1840092	long ncRNA	-2.745	yes	down
	SPNCRNA.10	chr 3	1837689-1838380	long ncRNA	-2.619	no	
	SPCC1322.13	chr 3	1316243-1318070	pre-mRNA	-2.513	yes	up
	SPAC1296.02	chr 1	711413-711767	intron	-2.466	yes	NC
	SPCC1223.03c	chr 3	1840594-1843300	pre-mRNA	-2.262	no	
	SPNCRNA.504	chr 3	1839578-1840266	long ncRNA	-2.021	yes	down
	SPAC186.05c	chr 1	5540032-5541502	pre-mRNA	-1.969	no	
	SPCC1223.02	chr 3	1838335-1839525	pre-mRNA	-1.838	yes	up
	SPNCRNA.1235	chr 3	1839787-1844115	long ncRNA	-1.690	yes	down
	SPCC1322.14c	chr 3	1318375-1321275	pre-mRNA	-1.546	yes	up
	SPCC1223.01	chr 3	1834599-1837464	pre-mRNA	-1.396	yes	up
	SPNCRNA.511	chr 3	2011061-2012433	long ncRNA	-1.178	no	
	SPNCRNA.1254	chr 3	2011026-2012559	long ncRNA	-1.115	no	
	SPAC2H10.04	chr 1	5272461-5272835	pre-mRNA	-1.107	no	
	SPAC1296.02	chr 1	711238-712354	pre-mRNA	-1.015	yes	NC
	SPAC18B11.03c	chr 1	311920-314040	pre-mRNA	-0.875	no	
	SPAC750.01	chr 1	5555716-5556768	pre-mRNA	-0.850	no	
	SPNCRNA.585	chr 3	2010261-2010800	long ncRNA	-0.826	no	
	SPNCRNA.1063	chr 1	5266525-5269973	long ncRNA	-0.755	no	
	SPNCRNA.17	chr 2	1777375-1777788	long ncRNA	-0.753	no	
leo1Δ	SPNCRNA.10	chr 3	1837689-1838380	long ncRNA	-1.597	yes	up
	SPNCRNA.1234	chr 3	1837499-1840092	long ncRNA	-1.588	yes	NC
	SPCC1223.03c	chr 3	1840594-1843300	pre-mRNA	-1.317	no	
	SPAC1296.02	chr 1	711413-711767	intron	-1.308	yes	NC
	SPCC2H8.02	chr 3	827911-828545	5' UTR	-1.276	no	
	SPNCRNA.1235	chr 3	1839787-1844115	long ncRNA	-1.046	yes	NC
	SPCC1322.13	chr 3	1316243-1318070	pre-mRNA	-1.038	yes	up
	SPAC186.05c	chr 1	5540032-5541502	pre-mRNA	-0.922	no	
	SPCC1223.01	chr 3	1834599-1837464	pre-mRNA	-0.912	yes	up
	SPNCRNA.511	chr 3	2011061-2012433	long ncRNA	-0.909	no	
	SPNCRNA.504	chr 3	1839578-1840266	long ncRNA	-0.895	yes	NC
	SPNCRNA.1254	chr 3	2011026-2012559	long ncRNA	-0.861	no	
	SPAC2H10.04	chr 1	5272461-5272835	pre-mRNA	-0.841	no	
	SPCC1223.02	chr 3	1838335-1839525	pre-mRNA	-0.804	yes	up
	SPAC186.05c	chr 1	5541139-5541502	5' UTR	-0.796	no	
	SPBC13E7.08c	chr 2	3052520-3055287	pre-mRNA	-0.765	no	
	SPBC13E7.07	chr 2	3052449-3055355	pre-mRNA	-0.697	no	
	SPBC83.10	chr 2	1528476-1528831	3' UTR	-0.657	no	
	SPAC9E9.01	chr 1	4435202-4435509	3' UTR	-0.643	no	
	SPNCRNA.17	chr 2	1777375-1777788	long ncRNA	-0.641	no	
cdc73-G313R	SPNCRNA.1234	chr3	1837499-1840092	long ncRNA	-2.353	yes	down
	SPCC1322.13	chr3	1316243-1318070	pre-mRNA	-2.231	yes	up
	SPNCRNA.10	chr3	1837689-1838380	long ncRNA	-2.221	no	
	SPAC186.05c	chr1	5540032-5541502	pre-mRNA	-1.771	no	
	SPCC1223.03c	chr3	1840594-1843300	pre-mRNA	-1.727	no	
	SPCC794.12c	chr3	275261-275513	3' UTR	-1.523	no	
	SPNCRNA.511	chr3	2011061-2012433	long ncRNA	-1.490	no	
	SPAC5H10.03	chr1	152573-152909	3' UTR	-1.473	no	
	SPNCRNA.763	chr1	1813439-1815920	long ncRNA	-1.463	no	
	SPNCRNA.411	chr2	2901243-2902541	long ncRNA	-1.451	no	
	SPBC317.01	chr2	3627758-3628658	3' UTR	-1.391	yes	down
	SPNCRNA.1254	chr3	2011026-2012559	long ncRNA	-1.390	no	
	SPNCRNA.1264	chr3	2117021-2118767	long ncRNA	-1.378	no	
	SPCC1223.01	chr3	1834599-1837464	pre-mRNA	-1.362	no	
	SPNCRNA.523	chr3	2381299-2381802	long ncRNA	-1.333	no	
	SPNCRNA.716	chr1	1146598-1148060	long ncRNA	-1.332	no	
	SPNCRNA.1096	chr3	37658-39319	long ncRNA	-1.311	no	
	SPNCRNA.1561	chr2	2901109-2902665	long ncRNA	-1.309	no	
	SPNCRNA.889	chr1	3138602-3139458	long ncRNA	-1.249	no	
	SPNCRNA.1192	chr3	1400016-1400808	long ncRNA	-1.234	no	

This table lists the 20 most repressed genomic features in each of the Paf1 complex mutant strains, based on the genome-wide expression profiles generated with tiling microarrays. Column '*Expression fold change*' shows average fold change in expression level between the mutant and the wild type strain (log2 scale). Column '*Small RNAs –yes/no*' indicates whether small RNAs originating from the respective loci were detected in our deep sequencing data set from either wild type or the mutant strain. Column '*Small RNAs – change in mutant'* describes whether small RNAs number increases or decreases in the respective mutant strain. 'NC' – no change.