Regulation of heterochromatic RNA decay via heterochromatin protein 1 (HP1)

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SUMMARY

The central dogma of molecular biology describes the directional flow of biological information from DNA via RNA to protein. Information stored in DNA is copied to an mRNA molecule during the process of transcription. The mRNA is used as a template for translation, in which polypeptides are synthesized. The regulation of this process, which is conserved through all trees of life, has been a central field of study over the last decades.

The discovery that RNA not only serves a simple role as a mere copy, but is much more versatile has created a lot of excitement. For example, RNA molecules themselves can act as enzymes. In the ribosome, rRNAs comprise the catalytic core for peptide bond formation. snRNAs form the core of the splicing machinery. tRNAs are the adaptors and thereby the actual readers of the genetic code. Last but not least, in the RNAi pathway, small RNAs serve as guides to target silencing complexes to complementary RNAs. Altogether, these findings placed RNA at the center of eukaryotic genome regulation.

On the other hand, DNA in eukaryotic cells does not exist as a mere fibre, but is wrapped around the core histone octamer to form a nucleosome. Nucleosomes are the basic building blocks to form higher order chromatin structures. Besides its architectural role in chromosome segregation, genome stability and recombination, chromatin has also been linked to gene expression. In contrast to the rather gene-rich euchromatin, heterochromatin is a highly condensed and repressive structure, serves as a safe storage place for transposable elements and makes up a large fraction of the genome of higher eukaryotes. Repression or activation in different chromatin contexts involves covalent modifications on the histone proteins. The nature and combination of these modifications create different docking sites for various effector proteins that have either activating or repressing function.

Surprisingly, recent studies have suggested that a substantial fraction of the genome, although heterochromatic, is transcribed at least to a certain extent and many of those transcripts do not encode proteins. Moreover, fascinating mechanisms have been

discovered, in which the silencing of heterochromatic sequences involves RNA-dependent mechanisms. Altogether this suggests that the regulation of the genomic output in eukaryotes not only occurs at the level of transcription but to a substantial extent via co- or posttranscriptional gene silencing mechanisms (CTGS or PTGS, respectively). The cellular RNA decay machineries therefore have to be equipped with tools to specifically distinguish and degrade certain RNAs.

Generally, RNA decay mechanisms recognize aberrant features that are contained in the RNA molecule itself, for example the presence and length of a poly(A) tail at the 3'end. The RNAi pathway is triggered by the presence of short ssRNA molecules that are complementary to a target RNA and thereby lead to degradation. In some cases degradation induces feedback mechanisms back to chromatin resulting in histone modification and/or transcriptional modulation.

My work has identified a novel mechanism to regulate RNA decay, which is dependent on the chromatin context from which the RNA has been transcribed. This mechanism is independent of the actual RNA sequence/molecule but involves binding to the heterochromatin protein $HP1^{Swi6}$. I found that $HP1^{Swi6}$ binding to a heterochromatic transcript fulfils a checkpoint function, which mediates repression on at least two levels. First, $HP1^{Swi6}$ prevents translation of heterochromatic RNA by inhibiting association with ribosomes. This ensures repression even in the absence of RNA decay. Second $HP1^{Swi6}$ mediates elimination by capturing RNA at the site of transcription and escorting it to the degradation machinery. On a molecular level, this is achieved by RNA binding to the $HP1^{Swi6}$ hinge region. This renders the chromodomain structurally incompatible with stable H3K9me association leading to heterochromatin eviction and degradation of the RNA.

My data points towards a model in which binding of $HP1^{Swi6}$ to a heterochromatic RNA creates a heterochromatin-specific ribonucleoprotein (hsRNP) that is prone to degradation. Importantly, $HP1^{Swi6}$ can induce degradation of any RNA of heterochromatic origin, which could be a crucial feature to repress the expression of deleterious sequences and transposons. Last but not least, my work is the first example that demonstrates that RNAs can act as "repellents" for chromatin proteins.

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ABBREVIATIONS

(Hi-)Seq	High throughput (deep) sequencing
A. thaliana	*Arabidopsis thaliana*
Ago1	Argonaute 1
Air1	Protein Air1, Arginine methyltransferase-interacting RING finger protein 1
Arb1/2	Argonaute-binding protein 1/2
ARC	Argonaute siRNA chaperone
Asf1	Histone chaperone cia1, Anti-silencing function protein 1
ATF	Cyclic AMP-dependent transcription factor
Atf1	Transcription factor atf1
Aub	Aubergine
C. elegans	*Caenorhabditis elegans*
Cbx	Chromobox protein homolog
Ccq1	Coiled-coil quantitatively-enriched protein 1
CD	Chromo Domain
CENP-A	Histone H3-like centromeric protein
ChIP	Chromatin Immunoprecipitation
Chp1/2	Chromo domain-containing protein 1
CID	Histone H3-like centromeric protein cid, Centromere identifier protein
Cid12	Poly(A) RNA polymerase cid12, Caffeine-induced death protein 12
Cid14	Poly(A) RNA polymerase cid14, Caffeine-induced death protein 14
Clr1/2/3/4/6	Cryptic loci regulator protein 1/2/3/4/6
CLRC	Clr4-Rik1-Cul4 complex
CREB	Cyclic AMP-responsive element-binding protein

CSD	Chromoshadow Domain
CTCF	Transcriptional repressor CTCF
CTGS	Co-Transcriptional Gene Silencing
Cul4	Cullin-4
D. melanogaster	*Drosophila melanogaster*
Dam-ID	DNA adenine methyltransferase identification
Dcr1	Dicer 1
Ddb1/2	DNA damage-binding protein 1
DEAD-motif	Asp-Glu-Ala-Asp motif
DIM-2/5	Cytosine-specific methyltransferase DIM-2/5
Dis3	Exosome complex exonuclease dis3, Chromosome disjunction protein 3
disiRNA	Dicer-independent small interfering RNA
DMM-1	DNA methylation modulator - 1
dn	double null
DNA	Deoxyribonucleic Acid
Dnmt	DNA Methyltransferase
dsRNA	double-stranded RNA
E(z)	Enhancer of Zeste
endo-siRNA	endogenous small interfering RNA
Epe1	Putative JmjC domain-containing histone demethylation protein 1
ERV	Endogenous Retrovirus
ESET	ERG-associated protein with a SET domain, SETDB1
FISH	Fluorescent In Situ Hybridization
FLC	Flowering Locus
FRAP	Fluorescence Recovery After Photobleaching
G9a	Protein G9a, Histone-lysine N-methyltransferase EHMT2
GLP	G9a-like protein 1, Histone-lysine N-methyltransferase EHMT1
H3K27me	Histone H3 Lysine 27 methylation

H3K9me	Histone H3 Lysine 9 methylation
HDAC	Histone deacetylase
HIRA	histone cell cycle regulation defective homolog A
HKMT	Histone Lysine Methyltransferase
HOX	Homeobox protein
HP1	Heterochromatin Protein 1
HPL-1/2	Heterochromatin protein 1 homolog
Hrr1	Helicase required for RNAi-mediated heterochromatin assembly 1
hsRNP	heterochromatin-specific ribonucleoprotein
ICR	Imprinting Control Region
JmjC domain	Jumonji domain
LHP1	Chromo domain-containing protein LHP1, LIKE HETEROCHROMATIN PROTEIN 1
LIM domain	Lin11, Isl-1 & Mec-3 domain
lincRNA	long intergenic non-coding RNA
LINE	Long Interspersed Nucleotide Element
LTR	Long Terminal Repeat
mESC	mouse Embryonic Stem Cell
MIP	Methylation Induced Premeiotically
miRNA	micro RNA
Mit1	Chromatin remodeling factor mit1, Mi2-like interacting with clr3 protein 1
mRNA	Messenger RNA
MSUD	Meiotic Silencing Of Unpaired DNA
Mtr4	ATP-dependent RNA helicase mtr4
NAD	Nicotinamide adenine dinucleotide
ncRNA	non-coding RNA
NMR	Nuclear magnetic resonance
nt	nucleotide
NT	N-terminus

ORF	Open Reading Frame
Pc	Polycomb
PcG	Polycomb group
Pcr1	Transcription factor pcr1
Pdd1/3p	Programmed DNA degradation protein 1/3
PEV	Position Effect Variegation
piRNA	piwi-interacting RNA
Piwi	P-element induced wimpy testis
PolII	RNA Polymerase 2
PRC1/2	Polycomb repressive complex 1/2
PRE	PcG response elements
PROMPT	Promoter Upstream Transcripts
PTGS	Post-transcriptional gene silencing
PTM	Posttranslational Modification
RdDM	RNA-dependent DNA methylation
Rdp1	RNA-dependent RNA polymerase
RDRC	RNA-dependent RNA polymerase complex
RING	Really Interesting New Gene
RIP	Repeat-induced Point Mutation
RITS	RNA-induced transcriptional silencing
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
RRM	RNA recognition motif
rRNA	Ribosomal RNA
Rrp6	Exosome complex exonuclease rrp6, Ribosomal RNA-processing protein 6
Rz	Ribozyme
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
scnRNA	scan RNA

SET-domain	Suvar 3-9/Enhancer of zeste/Trithorax - domain
SETDB1	Histone-lysine N-methyltransferase SETDB1
SHREC	Snf2/Hdac-containing Repressor Complex
SINE	Short Interspersed Nucleotide Element
SIR	Silent Information Regulator
siRNA	small interfering RNA
snRNA	small nuclear RNA
sRNA	small RNA
ssRNA	single-stranded RNA
Stc1	LIM-like protein linking chromatin modification to RNAi, siRNA to chromatin protein 1
Su(var)	Supressor of variegation
Swi6	Chromatin-associated protein swi6, Switching gene swi6
Tas3	RNA-induced transcriptional silencing complex protein tas3, targeting complex subunit 3
Taz1	Telomere length regulator taz1
TE	Transposable Element
TFIIIC	Transcription factor tau subunit sfc
Tlh1/2	ATP-dependent DNA helicase tlh1/2, Sub-telomeric helicase RecQ homolog 1/2
TRAMP	Trf4/Air2/Mtr4 Polyadenylation
tRNA	Transfer RNA
Twi1p	Tetrahymena Piwi-related protein
YY1	Transcriptional repressor protein YY1, Yin and yang 1

INTRODUCTION

1. Chromatin: General properties and function

1.1. Heterochromatin and Euchromatin

The concept of two types of chromatin, heterochromatin and euchromatin, was proposed based on cytological observations made in the early 20th century. At this time it was noted for the first time that some parts of the genome (euchromatin) become invisible during interphase (Heitz, 1928). Heterochromatin, in contrast, remains condensed during the whole cell cycle and is functionally distinct from euchromatin. It replicates rather late during S-phase, is gene poor, often locates to the nuclear periphery and is transcriptionally less active (Grewal and Jia, 2007).

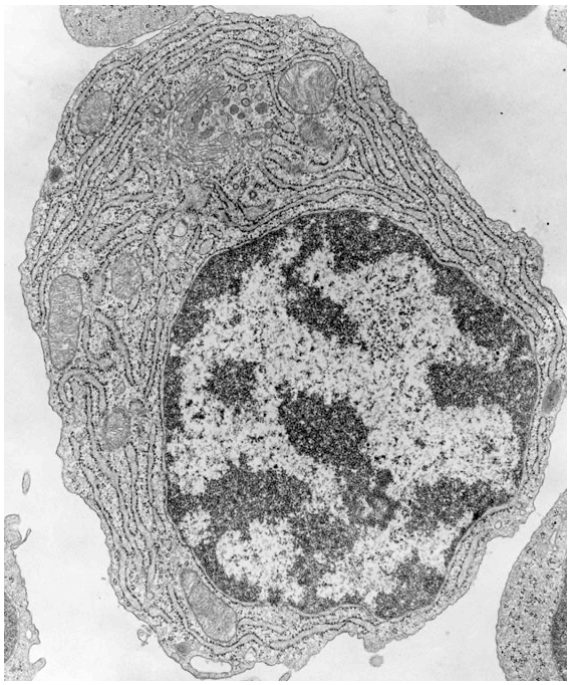

Figure 1 – Electron Micrograph of a plasma cell from bone marrow

Heterochromatin can be cytologically defined as a condensed structure within the nucleus of eukaryotic cells. In electron micrographs it is electron dense and therefore dark staining. This bone marrow cell shows the typical association of heterochromatin with the nuclear periphery.
EM picture: UCSF, Office of educational technology, cell structure lab

Heterochromatin can be further subdivided into *constitutive heterochromatin*, which is found at highly repetitive DNA elements surrounding centromeres and telomeres.

Thereby, besides its function in repressing the activity of transposable elements, heterochromatin contributes to the integrity and the maintenance of the overall chromosomal structure and mechanics (Buhler and Moazed, 2007; Richards and Elgin, 2002).

Facultative heterochromatin forms in a euchromatic environment and functions in the heritable and stable maintenance of gene expression patterns. One of the best-studied cases is the inactivation of the female X chromosome (XCI) in mammals during early development. Interestingly, silencing often involves the action of ncRNAs such as Xist in the case of XCI (Beisel and Paro, 2011; Chow and Heard, 2009).

1.2. Position Effect Variegation

Position Effect Variegation (PEV) is a conserved phenomenon that has first been described in *Drosophila melanogaster*. Hermann Muller identified a mutant fly, in which the eye color displayed a variegated expression resembling a red-white mosaic (Muller, 1930). The red eye color of wild-type flies is encoded by the *white* gene, which is normally expressed in every ommatidium. Due to an inversion on the X chromosome, the white gene in the mutant flies resides in close proximity to heterochromatin, resulting in the stable and heritable silencing of the *white* gene in some of the cells.

A conserved feature of classical PEV is the stochastic occurrence of the silencing, but once established, it is maintained stably throughout many cell divisions. The silenced domain may then spread in *cis* into adjacent regions that are several kilobases away (Huisinga et al., 2006). Levels of variegating gene expression were also found to depend in a dosage-dependent manner on the silencer levels (Eissenberg et al., 1992).

PEV silenced domains have an altered chromatin architecture. Variegating transgene inserts were shown to have a reduced accessibility to restriction digest and micrococcal nuclease treatment and were packaged in a more regular nucleosome array (Cryderman et al., 1998; Sun et al., 2001; Wallrath and Elgin, 1995). From these studies and cytological observations (Schotta et al., 2003; Zhimulev et al., 1988) it has been concluded that this compact structure limits the accessibility for transcription

factors and the transcription machinery itself. In this classical model, PEV and gene silencing would be caused by the compaction of heterochromatin into an inert and transcriptionally inactive structure. However, recent genome wide studies have challenged this view, because transcription was found to be more widespread throughout the entire genome than previously anticipated (Birney et al., 2007; Cheng et al., 2005; Kapranov et al., 2007).

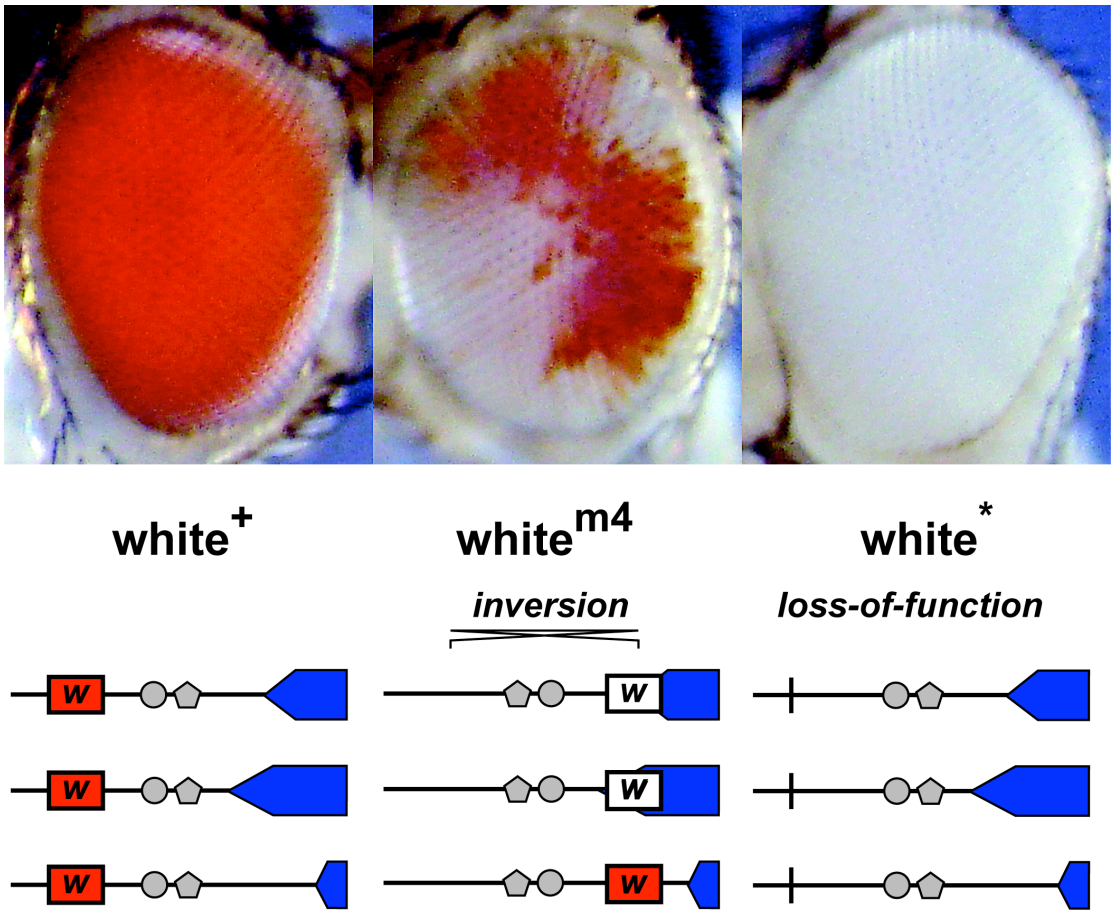

Figure 2 - Position Effect Variegation (PEV) in *D. melanogaster*

In wild-type cells, the *white* gene (red box), which encodes the red eye color in flies, is expressed from a euchromatic region. In *white*^{m4} mutant flies, an inversion positions the white gene in close proximity to heterochromatin (blue arrows) resulting in gene silencing. Stochastic spreading of heterochromatin into the *white* gene causes the variegated occurrence of the silencing (ON or OFF cells). Once established, however, the cells epigenetically transmit this state to their daughter cells. In *white* loss-of-function mutants, the absence of the gene product causes a complete white-eye phenotype. The grey circles/polygons illustrate genomic loci between the *white* locus and heterochromatin. Picture courtesy of Jonathan Schneiderman.

PEV phenomena have not only been described in flies (Eissenberg, 1989; Reuter and Wolff, 1981), but later on were also found in yeasts (Allshire et al., 1994; Allshire et al., 1995; Sandell and Zakian, 1992), plants (Matzke and Matzke, 1998) and mammals (Rakyan et al., 2002).

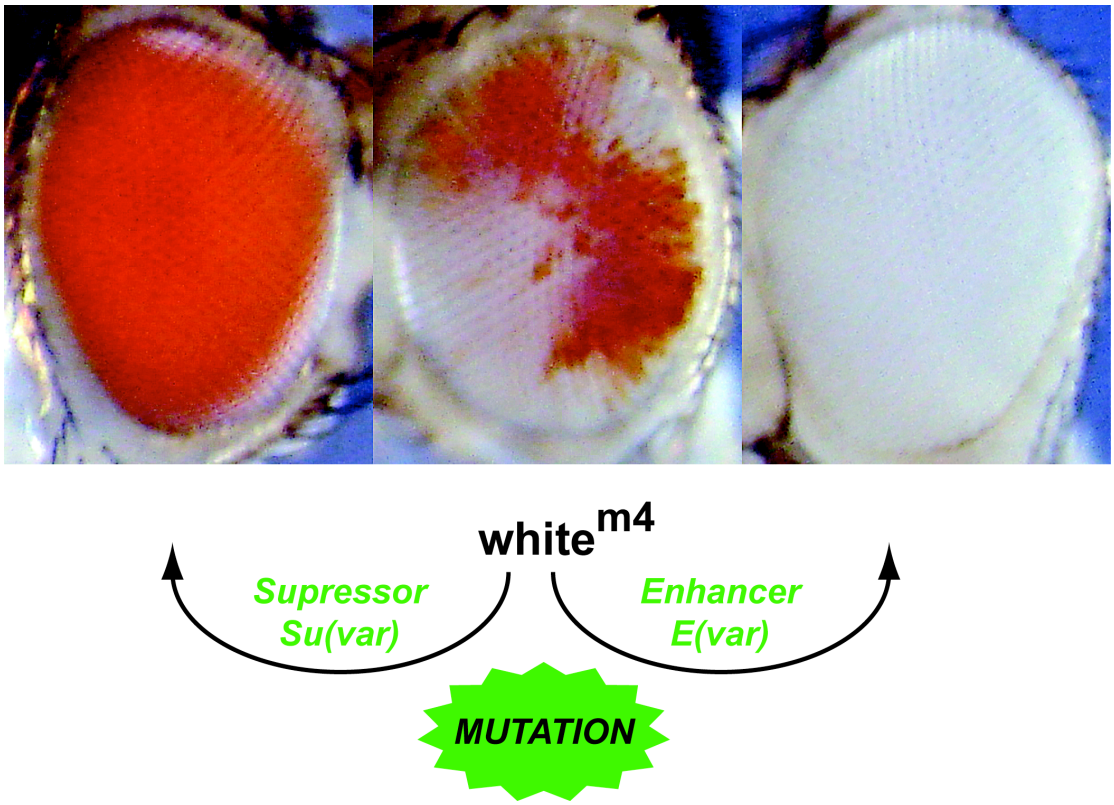

Figure 3 - PEV as a tool to genetically identify heterochromatin factors

Position Effect Variegation was used as a powerful model system to identify factors that are required for heterochromatin formation and maintenance. Supressor mutations (Su(var)) are genes that are required for heterochromatin formation. E(var) mutations encode genes that antagonize heterochromatin formation and spreading.

Most importantly, PEV has been instrumental to genetically identify the molecular components of heterochromatin silencing. Since the first publication of a PEV screen in the early 1980ies in *Drosophila* (Reuter and Wolff, 1981), this strategy has been applied to other organisms and has allowed understanding of heterochromatin biology on a molecular level.

1.3. Molecular components of chromatin structure

Histone proteins and their posttranslational modifications (PTM) have crucial roles in the regulation of eukaryotic genomes. Each chromatin unit, the nucleosome, contains 147 bp of DNA wrapped around an octamer composed of four core histone proteins H2A, H2B, H3 and H4 (Luger et al., 1997). To date, 130 PTMs on various sites in human histones have been identified (Tan et al., 2011). Histone modifications contribute to a plethora of different aspects of chromatin biology and genome regulation. Generally, one can distinguish two different mechanisms for the function of the histone modifications. First, histone modifications can directly influence the chromatin compaction by altering the inter-nucleosomal contacts or changing the net charge of the histones themselves. This has been demonstrated for acetylation, which neutralizes the basic charge of lysines and thereby affects chromatin compaction (Shogren-Knaak et al., 2006). Second, the modified histones serve as recruitment platforms for nonhistone proteins (Kouzarides, 2007). The same modification, depending on which histone residue it is placed, may have a different role. For example, whereas methylation at H3K4, H3K36 and H3K79 are positively correlated with active gene expression, it is linked to repression at H3K9, H3K27 or H4K20 (Lachner and Jenuwein, 2002). In the following, I will only focus on the molecular mechanisms involving heterochromatin repression.

1.4. Repressive histone modifications are a conserved molecular hallmark of heterochromatin

Heterochromatin assembly requires H3K9 methylation, which creates a binding site for HP1 proteins (Bannister et al., 2001; Ekwall et al., 1995; Lachner et al., 2001). H3K9 methylation and binding of HP1 is a conserved molecular hallmark of heterochromatin from fission yeast to humans. The H3K9 methylation is catalyzed by the conserved SET-domain containing Suv3-9 family of proteins (Nakayama et al., 2001; Rea et al., 2000).

In addition to providing the substrate for HP1 binding, it has been shown for some of the Suv3-9 family members that they promote spreading by binding to H3K9me themselves (Collins et al., 2008; Zhang et al., 2008). Furthermore, some of the Suv3-9 family members have been shown to directly interact with HP1 (Loyola et al., 2009; Nozawa et al., 2010). This system could, once initiated, provide a self-assembly mechanism to spread linearly along a chromosome fiber. Via dimerisation of the conserved Chromoshadow-domain, HP1 interacts with a plethora of PxVxL-containing proteins functioning in almost every aspect of nuclear biology (Brasher et al., 2000; Cowieson et al., 2000; Nozawa et al., 2010).

Less well understood are the molecular mechanisms to stop inappropriate enrichment of heterochromatic marks into neighboring euchromatic regions. There is evidence that subnuclear organization and higher order chromatin structures can limit spreading (Gaszner and Felsenfeld, 2006; Ishii et al., 2002). Furthermore, sequence specific DNA binding factors such as CTCF or YYI seem to contribute to boundary formation in higher eukaryotes. Other reports implicate tRNA, RNA polymerase III and/or TFIIIC and ncRNA in barrier formation (Lunyak et al., 2007; Noma et al., 2006; Scott et al., 2006). In fission yeast, $HP1^{Swi6}$ itself is involved in boundary formation and has been shown to recruit the antisilencing factor Epe1, whose specific degradation within heterochromatin is controlled by the Cul4-Ddb1/2 complex (Braun et al., 2011). A similar mechanism might be operating in the fungus *Neurospora crassa* (Honda et al., 2010).

2. Heterochromatin biology in unicellular organisms

Much of our knowledge about the molecular details of heterochromatin formation and maintenance is based on studies performed in yeasts. Whereas the findings made in *S. cerevisiae* were instrumental for a conceptual understanding of silencing, the molecular components are different from the ones that are present in higher eukaryotes. Therefore, more recently people have changed their focus to fission yeast, which uses an H3K9me-HP1 system, similar to the one found in higher eukaryotes, for repression.

In the following few paragraphs, I will first summarize our understanding of the heterochromatin-silencing pathways in unicellular eukaryotes. Based on this, I will introduce chromatin silencing systems in other eukaryotes with a particular focus on RNA and the involvement of HP1 and other chromodomain-containing proteins.

2.1. The *Saccharomyces cerevisiae* SIR repression mechanism

Position effect variegation is observed at the silent mating-type loci and near telomeres (telomere position effect, TPE) in budding yeast, however, it is absent from the 125bp centromeric region, which is not heterochromatic. Heterochromatin in budding yeast is different from fission yeast in that it lacks an RNAi component (see below) and does not contain H3K9me. However, it involves the action of the conserved Sir2 NAD-dependent histone deacetylase (Aparicio et al., 1991). The absence of any active marks seems to be sufficient to favour binding of the Sir2-3-4 complex (SIR: Silent Information Regulator). Cis-acting DNA sequences (nucleation sites) as well as binding proteins such as Rap1 and yKu are necessary to nucleate assembly and spreading of silent chromatin. Indeed, it seems that in budding yeast the silencing arises through compaction as well as the sterical hindrance of positive regulators of transcription. Furthermore, recruitment to the nuclear envelope seems to have a regulatory effect (Buhler and Gasser, 2009).

2.2. Heterochromatin silencing in *S.pombe*

Heterochromatin in fission yeast can be found at the centromeres, the telomeres and silent mating-type loci (Allshire et al., 1994; Allshire et al., 1995; Lorentz et al., 1992; Lorentz et al., 1994; Thon et al., 1994).

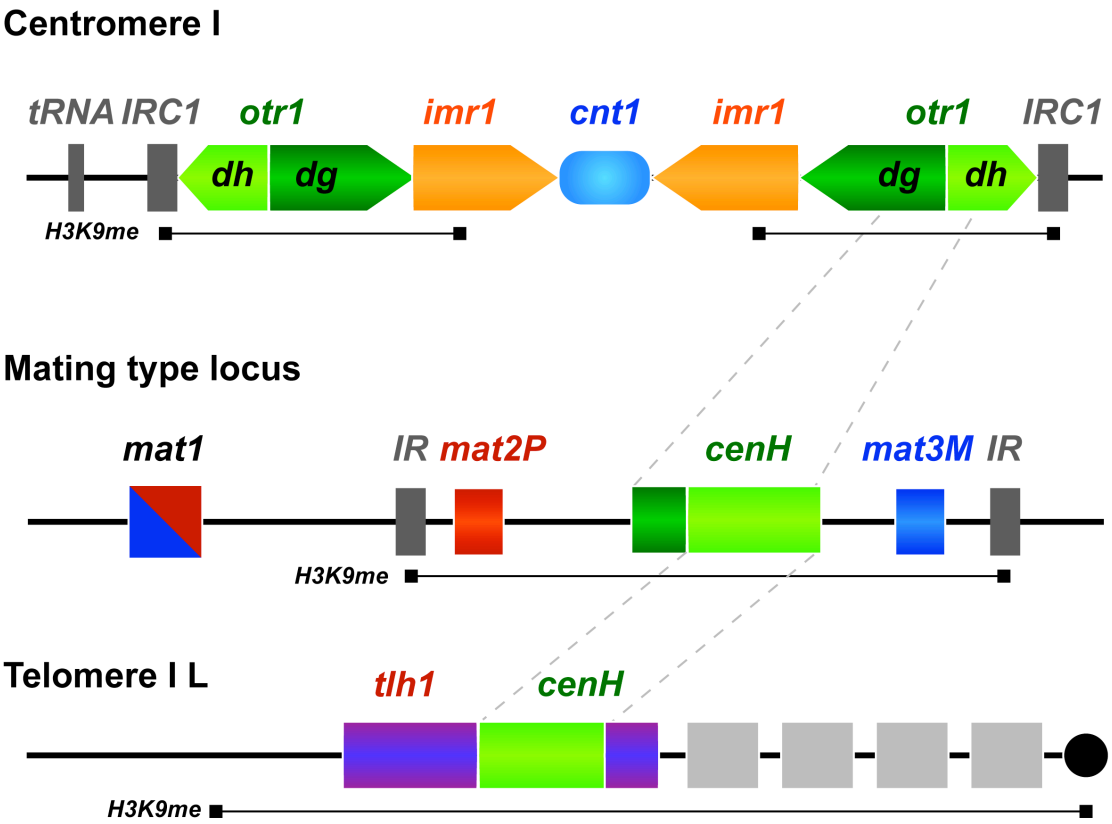

Figure 4 - Domain Architecture of centromeric, mating-type and telomeric heterochromatin in fission yeast

The Centromere is composed of a central core region (cnt1) which is the site of kinetochore assembly and contains the histone variant Cenp-A. This core is flanked by innermost repeats (imr1) and outermost repeats (otr1). The otr regions are composed of dg and dh repeat elements. The inverted repeat (IRC1) elements and tRNA genes mark transitions between heterochromatin and surrounding chromatin and serve as boundary elements. The mating-type locus contains the mat1, mat2 and mat3 genes, whereby the transcriptional status of the mat1 gene (P or M) determines the mating type of the cell. The cenH element is thought to be an RNAi-dependent nucleation center that acts in a parallel pathway with Atf1/Pcr1 mediated assembly of heterochromatin. A cenH like element can also be found within the subtelomeric tlh1/2+ ORFs. At the telomere, RNAi nucleates heterochromatin from this region in a parallel pathway with Taz1. Figure adapted from (Grewal and Jia, 2007).

The assembly of heterochromatin in fission yeast involves the stepwise action of several chromatin-modifying complexes. First, histone deacetylase-containing complexes act globally as well as locally on acetylated histones (Grewal et al., 1998; Shankaranarayana et al., 2003; Yamada et al., 2005). This is followed by histone methylation by Clr4 and binding of HP1 proteins, that will lead to spreading of the heterochromatic domains (Cam et al., 2005; Nakayama et al., 2000; Nakayama et al., 2001). The mechanisms for targeting, establishment and maintenance of heterochromatin at the different heterochromatic loci, however, show some distinct features.

2.2.1. RNAi-dependent heterochromatin formation at the centromere

At the centromeric domains silencing critically depends on RNAi. Deletion of any of the single RNAi genes encoding Dicer (Dcr1), Argonaute (Ago1) or RNA-directed RNA polymerase (Rdp1) leads to a loss of H3K9me and HP1 binding (Volpe et al., 2002). This is phenotypcially reflected by impaired centromere function (Provost et al., 2002). Both forward and reverse strand transcripts have been detected in RNAi mutants. Furthermore, siRNAs matching to centromeric repeats have been identified in wild-type cells (Buhler et al., 2008; Cam et al., 2005; Reinhart and Bartel, 2002).

Biochemical studies have led to the identification of a number of protein complexes involved in the formation of centromeric heterochromatin. It is thought that these complexes act together via physical interaction among themselves, with chromatin and nascent RNA and thereby establish and maintain heterochromatin in *cis*.

Ago1 is found together with Chp1, Tas3 and a single stranded sRNA in a complex termed RITS (RNA-induced initiation of transcriptional silencing complex) that localizes to centromeres (Verdel et al., 2004). Tethering of RITS to a nascent RNA is sufficient to establish heterochromatin (Buhler et al., 2006). Furthermore, the RITS complex couples recognition of nascent RNA to H3K9me-binding via the Chp1 chromodomain (Schalch et al., 2009).

Upon recognition of a nascent transcript, RITS recruits an RNA-dependent RNA polymerase (Rdp1). Rdp1 resides in the RDRC complex, which contains Rdp1, the poly(A) polymerase family member Cid12 and a putative RNA helicase Hrr1. RITS

and RDRC physically interact and both localize to the centromere and associate with centromeric transcripts (Motamedi et al., 2004). Synthesis of dsRNA triggers cleavage by Dcr1, which is physically associated with RDRC and centromeres (Colmenares et al., 2007; Woolcock et al., 2010).

The siRNA duplex is then loaded onto Ago1 with the help of a putative siRNA chaperone complex ARC, which contains a double stranded RNA along with two uncharacterized proteins Arb1 and Arb2 (Buker et al., 2007).

Being at the core of heterochromatin formation at centromeres, RITS couples RNAi to histone modification. Nascent transcript bound RITS recruits the H3K9-methyltransferase Clr4-containg complex CLRC via the LIM domain protein Stc1 (Bayne et al., 2010). Accordingly, tethering of Clr4 to a euchromatic locus leads to the formation of heterochromatin independently of RNAi (Kagansky et al., 2009).

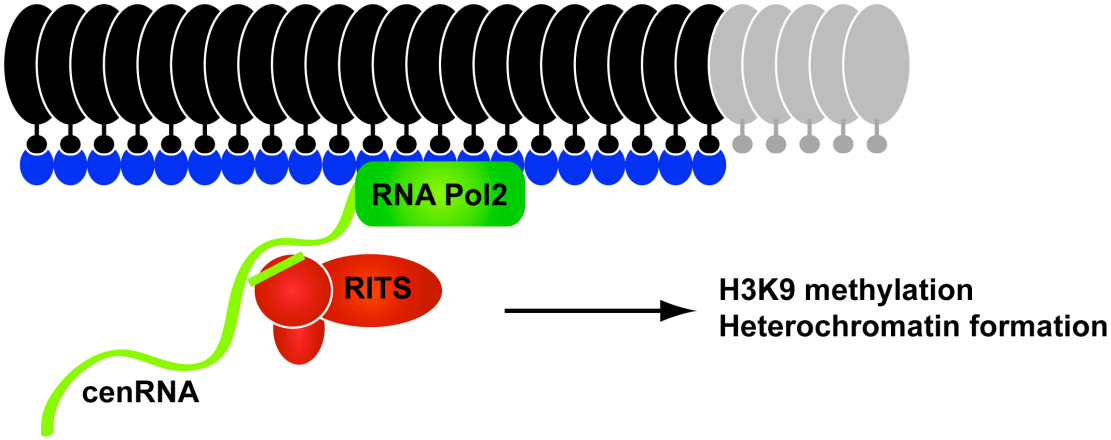

Figure 5 – Tethering of RITS to nascent transcript mediates heterochromatin formation

The RITS complex consisting of Ago1, Chp1 and Tas3 associates via siRNA-cenRNA base-pairing to centromeres. This results in the recruitment of several different complexes in *cis*, which are in turn responsible for H3K9 methylation, silencing, siRNA generation and creation of a positive feedback loop, which epigenetically maintains heterochromatin. Figure adapted from Bühler, 2007.

2.2.2. Heterochromatin formation at the silent mating-type locus

At the mating-type locus, the H3K14 deacetylase Clr3 is targeted by the ATF/CREB family to a *REIII* heptamer sequence (Yamada et al., 2005). Clr3 is found in a complex with Mit1, Clr1, Clr2 as well as the chromodomain protein Chp2. The action of this complex limits RNA polymerase II access to heterochromatin and thereby mediates transcriptional gene silencing downstream of H3K9me (Motamedi et al., 2008;

Sugiyama et al., 2007). In addition, the global histone deacetylase Clr6 (Nicolas et al., 2007) interacts with the Asf1/HIRA histone chaperone complex to mediate chromatin remodeling in a pathway that requires $HP1^{Swi6}$ for spreading (Yamane et al., 2011). Apart from the pathway that requires Atf1/Pcr1 as a nucleation element, establishment but not maintenance of heterochromatin silencing at the mating-type locus also requires RNAi (Hall et al., 2002; Jia et al., 2004). The fact that RNAi is only required for establishment, but not for maintenance of heterochromatin formation defines this as a classical epigenetic event, in which a state (in this case heterochromatin) can be transmitted to subsequent generations even in the absence of the trigger (RNAi).

It is thought that targeting of the RITS complex occurs at a region that shares remarkable homology to the centromere (*cenH*). Indeed this region was found to be transcribed and de-novo localization of RITS to this region depends on Dicer (Dcr1). This data suggests that siRNAs generated by Dcr1 are targeting RITS *de novo* to the *cenH* region. It is possible that these sRNAs are produced from the centromere(s) and then act as guides for the recruitment of RNAi to the mat locus.

Once established, H3K9me and $HP1^{Swi6}$ are sufficient for heterochromatin maintenance at the mating-type locus (Noma et al., 2004). Mating-type silencing also depends on RNA turnover pathways (Buhler et al., 2007; Wang et al., 2008).

2.2.3. Telomeric Heterochromatin

In fission yeast, telomeric DNA consists of telomeric repeats that are about 300 bp long, which are flanked by rRNA genes on chromosome III and subtelomeric sequences that contain ORFs on chromosomes I and II. The telomere-specific recruiter Taz1 establishes H3K9me and $HP1^{Swi6}$ heterochromatin (Kanoh et al., 2005). Additionally, Ccq1 helps to recruit Chp2 and the SHREC complex for transcriptional silencing (Sugiyama et al., 2007).

The telomere-linked helicases *tlh1+* and *tlh2+* notably share extensive sequence homology with the *cenH* region found at centromeres suggesting that RNAi could be required. In support of this hypothesis, RITS associates with telomeres (Noma et al., 2004) and some cumulative effects can be seen in RNAi-Taz1 double mutant cells

(Kanoh et al., 2005). Nonetheless, it is important to note that in comparison to the centromere and the mating-type locus, the silencing mechanisms operating at the telomere are the least studied. More experiments are required to refine the role of RNAi and other targeting pathways at this locus (Buhler and Gasser, 2009; Hansen et al., 2006; Kanoh et al., 2005).

2.3. Heterochromatin silencing induces DNA elimination in *Tetrahymena thermophila*

T. thermophila contains an extreme example of genome regulation, in which RNAi-directed heterochromatin formation results in the complete elimination of a potentially harmful sequence from the genome (Mochizuki, 2011). Apart from the work in *S. pombe,* this is to date one of most convincing examples of nuclear RNAi and a direct involvement in heterochromatin formation.

Each cell of the ciliated protozoan *Tetrahymena* contains a germline micronucleus and a somatic macronucleus. The macronucleus lacks 15% of the DNA sequences found in the zygotic nucleus or the micronucleus due to DNA elimination occurring in developing macronuclei during late stages of conjugation. Many of the eliminated sequences are transposon-like repeats and other repetitive sequences.

This process requires the Ago protein Twi1p (Mochizuki et al., 2002). Normally Twi1p localizes to the cytoplasm, however during early stages of conjugation, it is directed to the parental macronucleus until the micronuclei undergoes meiosis followed by fertilization (Noto et al., 2010). Once the zygotic nucleus gives rise to the next generation micro- and macronuclei, Twi1p relocalizes to the newly formed macronucleus. This is accompanied by 28nt scnRNAs that are processed from bidirectional transcripts of the whole micronucleus genome. An RNA that is homologous to any sequence in the old macronucleus is degraded, possibly via base-pairing interaction of the Twi1p-scnRNA complex with nascent transcripts. However, the ones not matching a DNA sequence remain stable and are transferred to the new macronucleus. There, they participate in heterochromatinization via H3K9me and H3K27me and subsequent elimination of the DNA sequences.

Importantly this process requires two chromodomain proteins Pdd1p and Pdd3p that are both required for H3K9me establishment and recognition. Tethering of Pdd1p is sufficient to promote DNA excision. Additionally, Pdd1p also recognizes H3K27me3, which is catalyzed by a E(z) homolog. It is thought that a Twi1p-scnRNA complex recruits E(z) and subsequent recognition of H3K27me via the chromodomain protein Pdd1p. This in turn would mediate H3K9me and DNA elimination (Liu et al., 2004; Liu et al., 2007; Taverna et al., 2002).

It remains to be demonstrated whether a similar complex like RITS, composed of Pdd1p and Twi1p also exists in *Tetrahymena*. This would provide a missing biochemical link between RNAi and the chromatin modifying complexes.

2.4. A genome defense system in *Neurospora crassa* inactives repetitive sequences by mutation and subsequent heterochromatinization

The filamentous fungus *Neurospora crassa* has several mechanisms to suppress transposon invasion. In the vegetative stage, RNAi triggers post-transcriptional gene silencing (PTGS), a phenomenon that has been initially termed quelling. Similarly, RNAi has been implicated in MSUD (Meiotic silencing of unpaired DNA), the DNA damage response, a classical miRNA pathway and a novel silencing mechanism that uses Dicer-independent siRNAs (disiRNA) (Dang et al., 2011; Lee et al., 2009; Wei et al., 2012). However, it remains to be demonstrated whether any of these mechanisms is directly involved in chromatin modification.

During the sexual cycle, however, transposable elements and repetitive sequences are silenced by DNA methylation after their inactivation via a *Neurospora* specific defense system termed RIP (repeat-induced point mutation). RIP is a mechanism in which repeated sequences are specifically targeted for sequence alteration from G:C to A:T. These “edited” regions then become targeted by DNA methylation, which occurs unlike CpG in vertebrate cells, at cytosine residues in any sequence context (Rountree and Selker, 2010; Selker, 2002).

DNA methylation by DIM-2 occurs downstream of H3K9 methylation by DIM-5 and involves recruitment by HP1 (Freitag et al., 2004; Selker et al., 2003). In fact, DNA methylation, H3K9me3 and HP1 almost completely overlap and the H3K9 methylation machinery is sufficient to establish DNA methylation (Lewis et al., 2009). More recent studies demonstrated that HP1 is also involved in silencing independently of DNA methylation at the centromere (Honda et al., 2012). Interestingly, HP1 was also shown to interact with a JmjC domain protein DMM-1, which prevents spreading of heterochromatin into nearby genes (Honda et al., 2010). This is reminiscent of the situation in *S.pombe*, where $HP1^{Swi6}$ recruits the anti-silencer Epe1, which is involved in boundary formation.

It is still unclear how the RIPed sequences are recognized. It is interesting, that recognition of AT-rich DNA, which is characteristic of RIPed sequences, involves the DIM-5 complex that contains Cul4 and Ddb1, which is similar to the fission yeast CLRC complex (Jia et al., 2005; Zhao et al., 2009). Another open question is how these regions are actually silenced. Intriguingly, DNA methylation and H3K9me do not seem to inhibit transcription initiation (Rountree and Selker, 1997), suggesting that co- or posttranscriptional RNA turnover mechanisms might be operating (Barra et al., 2005).

2.5. An *Ascobulus Immersus* defense system epigenetically silences repetitive sequences

MIP (methylation-induced premeiotically) is a process in which duplicated copies of a gene become DNA methylated during the sexual phase. In contrast to RIP in *Neurospora crassa*, however, these sequences are not subjected to mutation (Barry et al., 1993). The silencing persists epigenetically even when only a single copy is inherited (Rhounim et al., 1992). The DNA methylation is catalyzed by Masc1 and possibly other redundant enzymes (Malagnac et al., 1997). An involvement of H3K9me or HP1 proteins has not been described. Very interestingly, truncated transcripts are produced from these MIPed regions, which have been attributed to RNA polymerase stalling (Barra et al., 2005). Nevertheless, it is interesting to speculate that post- or co-transcriptional degradation mechanisms could be acting as well.

3. Heterochromatin biology in multicellular eukaryotes

3.1. Silencing in *Drosophila melanogaster*

3.1.1. Occurrence and function of heterochromatin

About one third of the *D. melanogaster* genome is heterochromatic. It is mainly found at pericentric regions and the telomeres and consists largely of transposable elements (TEs). Molecularly, it is characterized by the presence of H3K9me, HP1 (encoded by the *Su(var)2-5* gene), but the absence of DNA methylation. The central core region of the centromere, like in fission yeast and higher eukaryotes, is enriched for the histone variant CID/CENP-A (Sullivan and Karpen, 2004).

Whilst the key function of heterochromatin in Drosophila is the silencing of transposable elements (TEs) and viruses, it is interesting that TEs are also important to maintain telomere function. In contrast to most other eukaryotes that use telomerase-generated short repeats, flies use arrays of retrotransposons for telomere homeostasis. Here, the silencing function of HP1a seems to be mediated by the chromodomain, however, the localization to telomeres is mediated by direct DNA binding (Mason et al., 2008).

Heterochromatin formation results in the loss of gene expression in position effect variegation (PEV), a paradigm that allowed genetic identification of many of the molecular components of heterochromatin in *D. melanogaster* (Eissenberg et al., 1990). In these pioneering studies, a dosage response was observed for many factors: additional copies of the variegator genes resulted in increased silencing, whereas reduction resulted in loss of PEV. Furthermore, insertion of repetitive reporter sequences into the genome is sufficient to cause PEV (Dorer and Henikoff, 1994).

HP1a directly interacts with Su(var)3-9 and this dual interaction with both the modified histone and the modifying activity has been suggested to form the core of the heterochromatin self-assembly and spreading machinery (Grewal and Elgin,

2007). Consistent with this model, tethering of HP1a to euchromatic sites is sufficient to induce gene silencing and bypasses the requirement of Su(var)3-9 (Li et al., 2003).

Intererstingly, in *Drosophila* HP1a is not limited to heterochromatin, but also associates with euchromatin and seems to be involved in positive regulation of gene expression. Furthermore, there are 4 other HP1s in *Drosophila*, which have euchromatic preference and/or are germline specific. It is however still poorly understood, how this dual role as a repressor and activator works on a mechanistic and molecular level (Piacentini et al., 2009; Vogel et al., 2009).

3.1.2. RNA and heterochromatin formation in Drosophila.

Small RNAs are major players in regulating gene expression and have been suggested to contribute to heterochromatin formation in *Drosophila*. In contrast to the well-established role of HP1s and H3K9me, there are still many open questions regarding the contribution of the RNAi machinery to heterochromatin formation.

In the *Drosophila* germline, the piRNA pathway is an essential RNAi mechanism for retrotransposon silencing and developmental gene regulation (Simonelig, 2011). piRNA pathway mutations cause defects in the germline and embryonic axis specification. Molecularly, it is based on the production of 24-30nt RNAs that associate with the Piwi-clade of Argonaute proteins. piRNAs are produced in a Dcr-independent manner from presumably single-stranded precursors which reside in distinct genomic clusters (Aravin et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007; Malone et al., 2009). Most of these clusters reside in heterochromatin, however, only a subset produces piRNAs. The piRNAs then act in trans to slice target transcripts. This occurs via three specialized Argonautes, the nuclear Piwi and the perinuclear Aubergine (Aub) and Ago3, which cooperate in a ping-pong amplification cycle to produce more piRNA. At the same time, this mechanism eliminates potentially harmful transposon transcripts. It is still unclear, how the initial precursor transcript is recognized and how the primary piRNA, that is required to start the ping-pong cycle, is produced. One possible explanation is that primary piRNAs could be inherited maternally (Brennecke et al., 2008).

Interestingly, an HP1 protein, Rhino, is required for piRNA production (Klattenhoff et al., 2009). Consistently, piRNA cluster transcription requires SETDB1-catalyzed H3K9me (Rangan et al., 2011). Furthermore, the putative nucleases Squash and Zucchini have been suggested to cleave the precursor transcripts, to produce such a primary piRNA. It is therefore tempting to speculate, that recognition of piRNA precursors via a chromatin protein (Rhino?) would trigger cleavage by Squash and Zucchini. These degradation products could then trigger the ping-pong amplification cycle via Piwi, Ago3 and Aub (Khurana and Theurkauf, 2010).
Because HP1a interacts with both Piwi and Su(var)3-9 in the soma, it has been hypothesized that germline piRNAs not only arise from heterochromatic regions but might also feedback to and guide chromatin modifications (Khurana and Theurkauf, 2010). There is currently data supporting as well as contradicting this hypothesis (Moshkovich and Lei, 2010; Wang and Elgin, 2011). More studies are required to finally address this issue.

A functionally distinct RNAi pathway operates in genome defense in somatic cells. This endo-siRNA pathway involves Dcr2-dependent sRNAs corresponding to transposon-derived sequences and a subset of mRNA stem loop structures, which direct Ago2 mediated target cleavage (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008). In contrast to *S. pombe* and plants, amplification by an RNA-dependent RNA polymerase seems to be absent in this case. Whilst the effector complexes are triggered by the presence of siRNAs, it is unclear whether recognition of the precursor transcripts by Dcr-2 involves a chromatin component.
Interestingly, endo-siRNAs have been linked to heterochromatin formation (Fagegaltier et al., 2009). Mutations in *piwi* and *spindle-E*, encoding a DEAD-motif RNA helicase, were previously shown to cause a loss of PEV in the soma and result in a dramatic redistribution of HP1a and reductions in H3K9me. Additionally, sRNAs corresponding to silenced PEV reporters have been identified (Brower-Toland et al., 2007; Haynes et al., 2006; Pal-Bhadra et al., 2004). On the other hand there is also conflicting data suggesting that RNAi is not involved in heterochromatin formation in

Drosophila (Moshkovich and Lei, 2010). It could be that RNAi influences heterochromatin only under certain circumstances and these effects could be locus specific.

In summary, the key function of heterochromatin is to repress transposon activity both in the *Drosophila* soma and germline. The repression mechanisms involve transcriptional inactivation and RNA degradation via RNAi. RNA degradation by RNAi can be triggered by transcription from a heterochromatic context. To what extent the various RNAi mechanisms may feedback to chromatin is still being debated and an intense field of study at the moment (Malone and Hannon, 2009).

3.1.3. Facultative heterochromatin formation via the polycomb system

In higher eukaryotes like *Drosophila*, another chromatin-based repression system is present, which is required for the stable and heritable maintenance of gene-expression patterns in different cell lineages. This is achieved by the Polycomb group (PcG) proteins, which regulate genes involved in developmental decisions, for example Hox genes. These proteins were first identified in *Drosophila* where mutations show characteristic defects in body patterning (Beisel and Paro, 2011).

Consistently, PcG targets are highly enriched for transcription factors and regulators of developmental pathways (Schwartz et al., 2006). The hallmark histone modifications associated with Pc repression are H3K27me3 and H2A-K119ub. In *Drosophila*, targeting of the Pc proteins occurs by defined *cis*-regulatory DNA elements (PcG response elements, PREs). These elements are characterized by a complex pattern of motifs that are in turn recognized by various sequence-specific DNA-binding proteins such as Pleiohomeotic (PHO), GAGA factor (GAF) or Zeste (Simon and Kingston, 2009).

Two protein complexes, PRC1 and PRC2, form the molecular core of the Polycomb repression system. PRC1 contains PC (Polycomb), SCE (Sex combs extra), PH (Polyhomeotic) and PSC (Posterior Sex combs). PC itself is the “reader” protein, that binds to H3K9me3 or H3K27me3. The complex then catalyzes H2A-K119 ubiquitylation via the SCE RING finger activity, which possibly mediates gene-

silencing via chromatin compaction and inhibition of RNA polymerase 2 elongation (Francis et al., 2004; Stock et al., 2007; Wang et al., 2004).

PRC2 contains E(Z) (Enhancer of Zeste), ESC (Extra sex combs), SU(Z)12 (Suppressor of Zeste), NURF55 (Nucleosome remodeling factor 55) and PCL (Polycomb-like). It contains both "readers" (ESC) and "writers" (EZH1) for the H3K27me3 mark, providing an inherent mechanism for the epigenetic propagation of the mark during cell division (Margueron et al., 2009; Schmitges et al., 2011). Although it is clear that the H3K27me mark is repressive, it is not known how this repression is achieved on a molecular level.

3.2. Heterochromatic loci are targeted by multiple silencing pathways in plants

Plants use a combination of H3K9me, DNA methylation and sRNA pathways for chromatin silencing. They are required to regulate gene expression and to protect the genome from parasitic DNA elements. In most of these cases, the silenced elements contain transposons and repetitive sequences such as inverted repeats (Chan et al., 2005; Lippman et al., 2004).

In the RNAi-dependent pathway (RdDM), 24nt siRNAs that originate from these regions promote heterochromatin formation (Chan et al., 2004). These 24nt sRNAs seem to be mobile and are able to transmit epigenetic modifications systemically within the plant (Molnar et al., 2010). The amplification mechanism for siRNA production from these DNA methylated regions occurs via a specialized transcription complex (Pol IV), an RNA-dependent RNA polymerase (RDR2) and a Dicer-complex (Dcl3, Hen1, Drb). Chromatin modifications are then triggered via Ago4. The interaction of Ago4 with a downstream polymerase PolV suggests that a nascent transcript model, like the one proposed for fission yeast, might apply. In support of this hypothesis, genetic experiments have shown that recruitment of the DNA methyltransferase Drm2 requires Ago4, and biochemically, this interaction could be mediated by Rdm1 (Gao et al., 2010). The initial primary RNAs that trigger Ago4

mediated silencing have been proposed to result from overlapping transcription by PolII (Matzke et al., 2009).

Whilst the crosstalk between RNAi and DNA methylation are well studied, the role of H3K9me in gene silencing is less understood. Guiding of the DNA methyltransferase CMT3 depends on the Kryptonite H3K9 methyltransferase (Chan, 2008; Jackson et al., 2002). Intriguingly, the chromodomain of CMT3 recognizes H3 tails that are simultaneously methylated at H3K9 (mediated by KYP) and H3K27 (Lindroth et al., 2004). While DNA methylation seems to transcriptionally silence transposons, a posttranscriptional layer operating via H3K9me seems to exist, too (Mirouze et al., 2009).

Studying H3K9me has been complicated by the fact that there are at least 29 active SET-domain proteins in Arabidopsis, of which 14 belong to the Su(var)3-9 group (Baumbusch et al., 2001). Indeed, the contribution of all these enzymes seem to be locus specific (Yu, 2009). Similarly, H3K9me3 is found at euchromatic regions and the exact nature of the heterochromatic histone "code" has not yet been clearly defined.

The *Arabidopsis* HP1 homolog LHP1 is not a component of constitutive heterochromatin, but rather recognizes H3K27me3 in vivo. It represses genes located within euchromatin, which is characteristic of polycomb (Pc) mediated repression (Libault et al., 2005; Turck et al., 2007; Zhang et al., 2007). The Pc system is best studied in the cold-induced repression at the FLOWERING LOCUS (FLC). The FLC is modified by both H3K9me2 and H3K27me2 (Bastow et al., 2004). The nuclear proteins FCA and FPA co-transcriptionally recognize aberrant RNA produced from the FLC locus, which in turn triggers epigenetic silencing. This pathway only acts in *cis* and lacks an siRNA amplification cycle that is characteristic of the classical RdDM (Baurle et al., 2007).

LHP1 is required for maintaining the repressed state of the FLC (Mylne et al., 2006; Sung et al., 2006). Consistently, mutations in LHP1 affect flowering time and plant architecture (Gaudin et al., 2001). On the contrary, they do not affect silencing of genes positioned in constitutive heterochromatin (Nakahigashi et al., 2005).

Interestingly, the ncRNA COLDAIR is required to recruit PRC2 to the FLC locus and maintain its repression via H3K27me in the cold (Heo and Sung, 2010). DCL4 seems to control the expression of the FCA gene via co-transcriptional cleavage of nascent read-through transcripts, which promotes transcription termination and FCA expression (Liu et al., 2012).

Generally, it seems that multiple silencing systems, acting in *cis* and *trans*, and integrating RNA components in different ways, cooperate to mediate chromatin modification and silencing. This variety might have evolutionary reasons, as plants lack adaptive immune systems like the ones that can be found in vertebrates.

3.3. Regulation of heterochromatin plasticity in *Caenorhabditis elegans*

Heterochromatic H3K9me, but no DNA methylation marks, are also found in the nematode *C. elegans*. Their chromosomes are holocentric and therefore do not contain pericentric repeats. However, heterochromatic regions are found at the chromosome ends and during meiosis (Wenzel et al., 2011).

H3K9me2 and H3K9me3 are differentially localized and generated by distinct enzymes: MET-2 (SETDB homolog) for H3K9me2 or MES-2 (E(z) homolog) for H3K9me3 (Bessler et al., 2010; Liu et al., 2010).

There are two HP1 proteins in *C. elegans*: HPL-1 and HPL-2, which are 48% identical and have partially redundant functions (Couteau et al., 2002; Schott et al., 2006). Another class of H3K9me-readers include MBT domains and have also been shown to specifically bind to H3K9me2/3 (Koester-Eiserfunke and Fischle, 2011).

Heterochromatin seems to function during early meiosis, when H3K9me2 marks accumulate on unpaired chromosomes (e.g. the male X chromosome). Interestingly, this process depends on some RNAi factors, such as the RNA-directed RNA polymerase EGO-1 and the Piwi/Ago protein CSR-1. The RNAi proteins seem to be required for selective accumulation of the heterochromatic marks on the unpaired chromosomes (She et al., 2009).

In line with these findings, it has been shown that endo-siRNAs can direct H3K9me (Burkhart et al., 2011; Burton et al., 2011). Repetitive transgenes are transcriptionally silenced when introduced into worms. This causes a *trans*-effect, which also silences the cognate endogenous genes. The repression depends on both RNAi and chromatin factors and results in decreased RNA Pol II occupancy on the silenced genes (Grishok et al., 2005; Robert et al., 2005; Sijen and Plasterk, 2003).

Heterochromatin is also required for transposon silencing in the *C. elegans* germline (Sijen and Plasterk, 2003). piRNAs trigger silencing, which is maintained by the HP1 homolog HPL-2, two methyltransferases and a nuclear Argonaute protein. This represents a multigenerational epigenetic inheritance mechanism, which is triggered by sRNAs (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012).

3.4. Heterochromatin in mammals

Constitutive heterochromatin in mammals is characterized by the presence of H3K9me, H4K20me, H3K27me1 and DNA methylation. H3K9me1, me2 and me3 occur at distinct loci throughout the genome and their plasticity is highly regulated through the concerted action of different enzymes (Rice et al., 2003). A fully comprehensive picture of the modifications and their regulation is not yet available. The reason is not only the complexity of mammalian development, but also the fact that genome-wide technologies, which are instrumental for the studies of such large genomes, have only become available recently and still are undergoing rapid development.

3.4.1. Occurrence of heterochromatic marks

In mouse embryonic stem cells (mESCs), which is the best-studied model system, H3K9me3 is highly correlated with the repressive mark H4K20me3. There is a strong enrichment at telomeres, pericentric satellite and long terminal repeats (LTRs). Interestingly, the enrichment at LTRs mainly reflects sequences that are known to produce dsRNA. H3K9me3 is also found on imprinting control regions (ICRs), whilst in this case on the other allele the active H3K4me3 mark is found. In some cases, the

H3K9me3 mark is able to spread from these repetitive regions to repress proximal sites (Martens et al., 2005; Meissner et al., 2008; Mikkelsen et al., 2007).

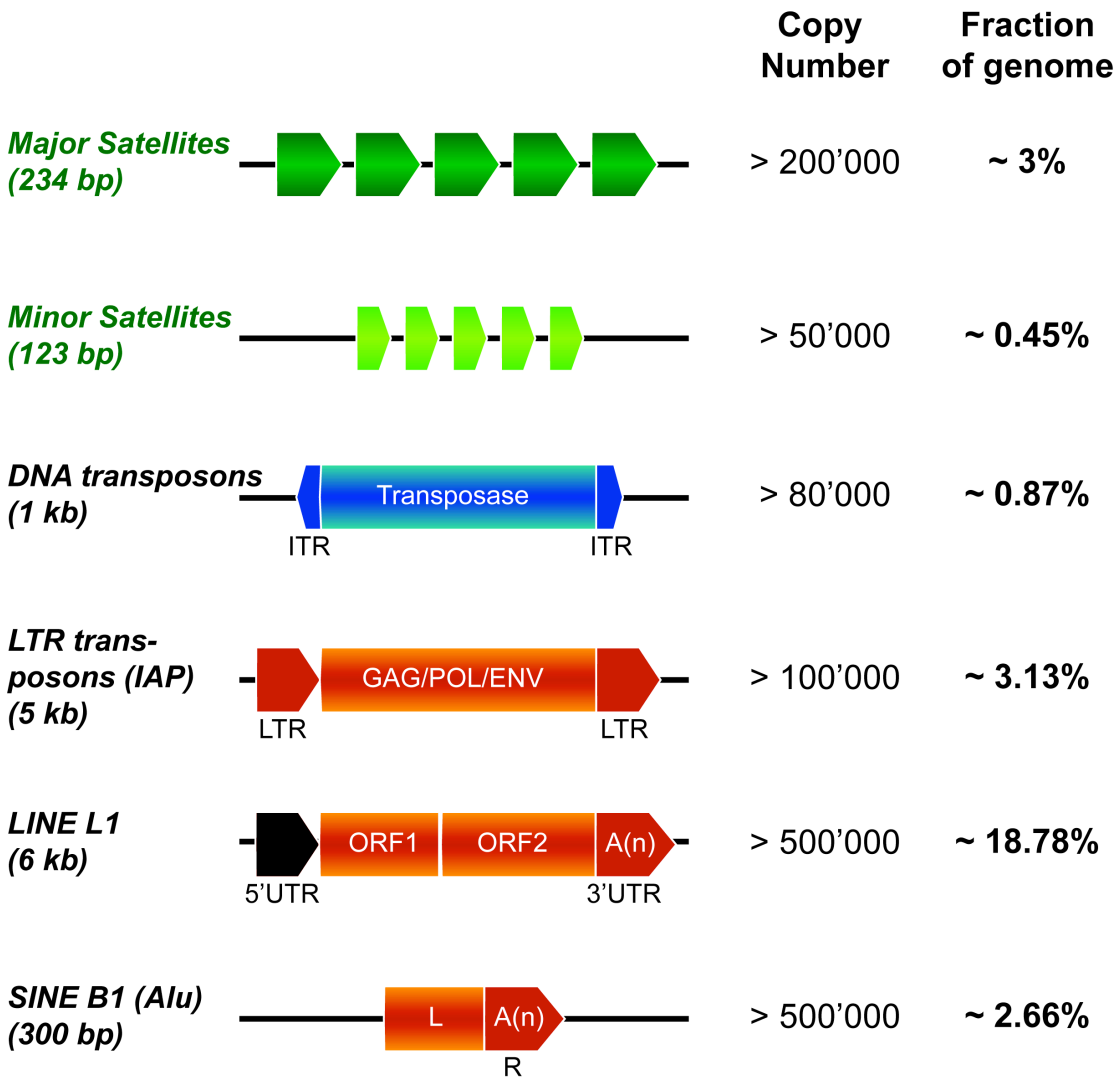

Figure 6 - Repetitive elements in the mouse genome

Major and minor satellite repeats are found in sequences surrounding the centromere. DNA transposons do not require an RNA intermediate for transposition but use a "cut and paste" mechanism. The LTR, LINE and SINE retrotransposition mechanism involves reverse transcription and insertion of the copy at a new site in the genome. SINEs require LINEs for their propagation, as they do not encode for proteins. H3K9me3 is found in satellite, DNA transposons and LTR repetitive elements. The LTRs enriched in H3K9me3 comprise elements from endogenous retroviruses (class I and II ERVs) and lose this mark during differentiation. Abbreviations: ITR, inverted terminal repeat; LTR, long terminal repeat; Gag, group-specific antigen (capsid proteins); Pol, polymerase; Env, envelope; LINE, long interspersed nucleotide element; SINE, short interspersed nucleotide element; L, Left monomer; R, Right monomer; Figure adapted from (Martens et al., 2005).

H3K9me2 can be found at quite high basal levels in mouse ES cells and during differentiation subtle local changes occur (Filion and van Steensel, 2009; Lienert et al., 2011). H3K9me1 has been studied in human T-cells, where it was surprisingly found to be associated with transcribed regions (Barski et al., 2007).

The H3K9me marks are in a dynamic regulation with other marks, a process that is still incompletely understood. For example, the HDAC Sirt1 and the HKMT Suv39h (see below) are functionally and physically linked (Vaquero et al., 2007). Another form of repressed chromatin, H4K20me, is linked to H3K9me, whereby the methylation state of each residue defines a distinct repressed region within the mammalian genome (Nishioka et al., 2002; Sims et al., 2006).

A conserved feature is the enrichment of the histone variant CENP-A in the central core of the centromere (Sullivan and Karpen, 2004). Furthermore, constitutive heterochromatin (H3K9me2) is not associated with the nuclear periphery in mammals (Guelen et al., 2008).

3.4.2. Deposition of heterochromatic marks ("Writers")

The deposition of the different H3K9me marks is very complex in mammals, as they contain several different SET-domain containing Histone-Lysine-Methyltransferases (HKMTs).

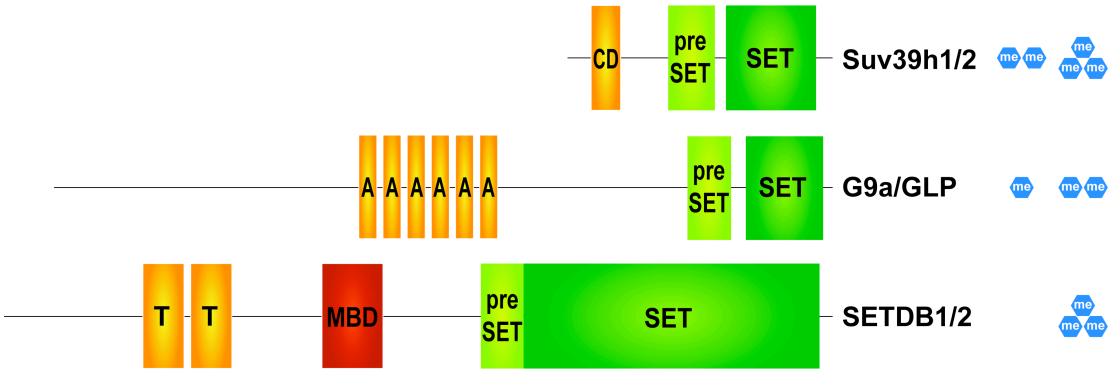

Figure 7 - Domain architecture of mammalian HKMTs

The three major mammalian Histone-Lysine-Methyltransferases (HKMTs) families all contain a pre-SET and SET-domain (green) that are catalytically active. The remaining parts differ considerably in size and domains. Suv39h1/2 contain a N-terminal chromodomain (CD, yellow) that binds H3K9me. The Ankyrin repeats (A) in the G9a/GLP family exert the same function and are H3K9me binding modules. SETDB1 contains two Tudor domains (T), which in other proteins are known to bind methylated lysines or dimethylated arginines. Additonally, it contains a DNA methyl-binding domain (MBD), which is required to couple H3K9me to DNA methylation. Suv39h1/2 catalyzes H3K9me2 and me3, G9a/GLP me1 and me2, SETDB1 me3 (shown as blue hexagons).

(a) Suv39h family

Suv39h1 and Suv39h2 are responsible for H3K9me2 and me3 in pericentromeric heterochromatin. Like their counterparts in other eukaryotes, they contain an N-terminal Chromdomain (CD). Enzymatic activity is inhibited by H3K9 acetylation (Ac) and H3S10 phosphorylation (P), however stimulated by H3K14Ac (Peters et al., 2003; Rea et al., 2000).

The single Suv39h1 and Suv39h2 knockout mice are viable suggesting that these enzymes act redundantly during embryogenesis. In adults, Suv39h2 is specifically expressed in adult testes and is important for organizing meiotic heterochromatin in the male germline (O'Carroll et al., 2000). Double mutant mice contain aberrant H3K9me at pericentric heterochromatin resulting in impaired viability, chromsome missegregation, the development of lymphomas due to genomic instability and infertility (Peters et al., 2001).

On a molecular level, Suv39h1/2 proteins create the substrate for and interact with HP1 proteins (Aagaard et al., 1999). HP1α and HP1β in turn interact with the de novo DNA methyltransferase Dnmt3b, which suggests that DNA methylation occurs downstream of H3K9me through recruitment via HP1. Consistently, pericentric localization of Dnmt3b and DNA methylation at major, but not minor satellite repeats is impaired in Suv39 dn ES cells. This is accompanied by the accumulation of major satellite transcripts (Lachner et al., 2001; Lehnertz et al., 2003; Martens et al., 2005).

Besides the function at pericentric heterochromatin, H3K9me and DNA methylation are also involved in telomere length homeostasis (Garcia-Cao et al., 2004; Gonzalo et al., 2006). Suv39h1 has also a role outside constitutive heterochromatin, as it was shown to act together with HP1 and Rb in a transcriptional co-repressor complex to regulate the cyclin E promoter. This process links heterochromatinization to cell cycle regulation (Nielsen et al., 2001b).

(b) G9a/GLP family

The major H3K9me1 and H3K9me2 HKMTs are G9a and GLP, which in contrast to Suv39h1/2 lack a chromodomain (CD) but instead contain N-terminal Ankyrin (Ank)

repats. The Ank repeats, however, seem to exert the same function as the CD, as they are methyllysine-binding modules (Collins et al., 2008). The loss of G9a or GLP affects mainly euchromatic regions and causes severe growth retardation and early lethality in mice. Consistently, these two proteins were found to form a heterodimeric complex that is required for their function in vivo (Tachibana et al., 2002; Tachibana et al., 2005). A self-enforcing spreading mechanism has been proposed based on the finding that G9a directly interacts with HP1 and Dnmt1, and HP1 interaction with Dnmt1 stimulates its enzymatic activity (Esteve et al., 2006; Smallwood et al., 2007). G9a is involved in silencing retrotransposons through CpG methylation. Interestingly, this is independent of the catalytic activity, H3K9me3 and HP1 recruitment (Dong et al., 2008). Recent studies have suggested that G9a is particularly required for silencing newly acquired proviruses but not for the maintenance of the silent state (Leung et al., 2011).

G9a acts on a number of non-histone targets, for example the transcription factor MyoD (Ling et al., 2012; Rathert et al., 2008). It would be interesting to address how much of the phenotypes caused by HKMTs mutation can be attributed to histone methylation versus non-histone substrates.

(c) SETDB1/2 family

Similar to G9a/GLP, the HKMT ESET/SETDB1 localizes predominantly to regions outside of constitutive heterochromatin and also lacks a N-terminal CD. Instead, SETDB1 contains two tudor domains that recognize methylated arginines. The KAP1 co-repressor recruits SETDB1 to promoters and coordinates histone methylation and HP1 deposition to mediate gene silencing (Schultz et al., 2002). Like the HP1-Suv39h1/2 pathway, DNA methylation is also coordinated with H3K9me by SETDB1. The methyl CpG binding protein MBD1 directly interacts with SETDB1 during replication (Sarraf and Stancheva, 2004). Interestingly, a SETDB1 interaction partner, mAM (ATF/CREB family member), stimulates the enzymatic activity to convert H3K9 di- to trimethyl (Wang et al., 2003).

In mESCs, SETDB1 seems to have a crucial function in controlling H3K9me3 of LTR-containing retroviruses (ERVs) and seems to function independently of DNA

methylation in these cells (Matsui et al., 2010). Aberrant activation of ERVs caused transcription of promoter-proximal viral elements into genes resulting in the production of chimeric transcripts (Karimi et al., 2011). Interestingly, HP1 proteins are dispensable for retroviral silencing (also see below), indicating that H3K9me3 serves a different role in maintaining these elements in a silent state (Maksakova et al., 2011). In contrast to SETDB1 and its euchromatic function, SETDB2 has been less studied but it seems to be involved in centromeric function (Falandry et al., 2010).

3.4.3. Occurrence and function of mammalian HP1s ("readers")

Apart from the HKMTs, HP1 proteins are the second key component of heterochromatin. There are three HP1 homolouges in humans and mice, which share almost the same sequence between the two species and are highly conserved among each other. In vivo, HP1α is exclusively localized to heterochromatin, whereas HP1β and predominantly HP1γ are also found in euchromatin (Maison and Almouzni, 2004; Minc et al., 2000). While these conclusions were mainly based on microscopical observations, recently published ChIP-Seq data confirmed that 89% of the human HP1γ (Cbx3) associates with genic regions, which do not contain H3K9me2/3 marks (Smallwood et al., 2012).

In vivo targeting of either HP1α or HP1β is sufficient to recruit SETDB1, mediate H3K9me3, chromatin condensation and stable gene repression (Ayyanathan et al., 2003; Verschure et al., 2005). Heterochromatin formation results in repression and PEV (Hiragami-Hamada et al., 2009). By transiently tethering HP1α to an ectopic locus, the dynamics of heterochromatin formation and spreading could be resolved (Hathaway et al., 2012). This study demonstrated that a newly formed heterochromatic allele was epigenetically inherited even when the tethered HP1α was removed. Surprisingly, HP1 is highly dynamic, which suggests that the silencing effects do not occur through formation of a static network (Cheutin et al., 2003). Accordingly, HP1α dissociates from centromeres upon heat shock, however this does not lead to a loss of H3K9me or decompaction of chromatin (Velichko et al., 2010). These findings reflect our poor understanding of the actual molecular mechanism of HP1 silencing and its other functions in (euchromatic) genome regulation that are

seemingly contradictory. For example, human HP1γ associates with RNA Polymerase II to recruit splicing factors to euchromatic genes (Smallwood et al., 2012).

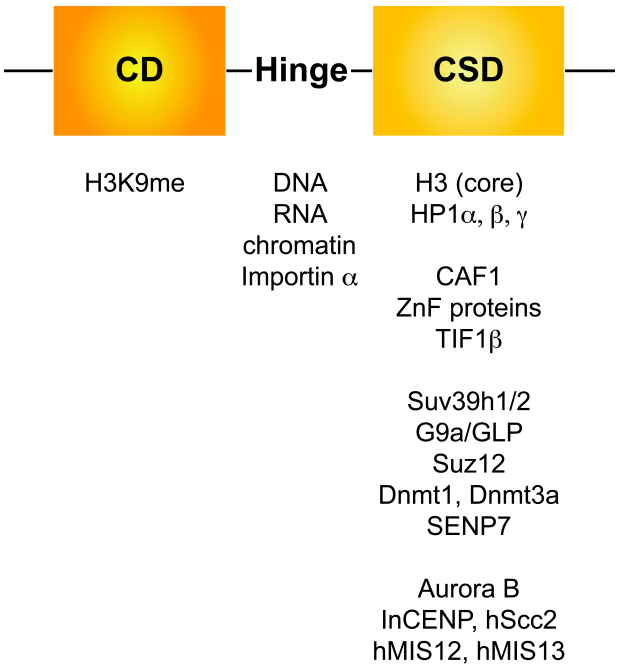

Figure 8 - HP1 interaction partners

HP1 proteins contain a N-terminal Chromodomain (CD) and a C-terminal Chromoshadow-Domain (CSD), which is separated by the intrinsically unstructured Hinge region. The CD of HP1 was shown to be an H3K9me binding module. The hinge region binds nucleic acids, chromatin as well as Importins. The CSD domain binds a plethora of factors mostly through recognition of a PxVxL peptide motif, which requires HP1 homo- and heterodimerization. This includes chromatin remodelers and transcriptional repressors (CAF1, ZnF proteins, TIF1b), Histone-modifiers and enzymes (HKMTs, the PRC2 member Suz12, SENP7) as well as kinetochore and cohesin complexes (Aurora B, InCENP, hScc2, hMIS12 and 13).

HP1 proteins interact with a plethora of factors including H3K9-methylated histones (Bannister et al., 2001; Kaustov et al., 2010; Lachner et al., 2001), the core histone H3 (Richart et al., 2012), chromatin remodelers, cell cycle regulators, DNA damage response proteins, DNA methyltransferases (Nozawa et al., 2010), RNA and DNA (Maison et al., 2011; Muchardt et al., 2002; Sugimoto et al., 1996). Many of the protein interaction partners contain a PxVxL pentapeptide motif, which is recognized by the chromoshadow domain (CSD) dimer (Cowieson et al., 2000; Smothers and Henikoff, 2000). The CSD forms both homo- and heterodimers *in vivo* and *in vitro* (Nielsen et al., 2001a).

Additionally, HP1 proteins are posttranslationally modified and this contributes to their plastic regulation. For example, phosphorylated HP1γ serves as a marker for transcription elongation and has impaired silencing activity (Lomberk et al., 2006). Phosphorylated HP1β is released from H3K9me heterochromatin and initiates a signaling cascade promoting the DNA damage response (Ayoub et al., 2008). HP1α is

sumoylated *in vivo* and this modification is required for its targeting to heterochromatin. *In vitro*, sumoylated HP1α interacts with RNA, however in vivo experiments identifying HP1α RNA targets and linking RNA binding mechanistically to heterochromatin formation are still missing (Maison et al., 2011; Muchardt et al., 2002).

The variety of binding partners, posttranslational modifications and the ability to hetero- and homodimerize could explain the multifaceted role of the HP1 proteins, which possibly expand their function from being classical silencers. Cbx5 (HP1α) null mice are viable and fertile exhibiting no obvious abnormality. Cbx1 (HP1β) null mutation results in perinatal lethality in mice (Aucott et al., 2008). These animals have defects in forming neuromuscular junctions and display aberrant cortex formation. Cbx3 (HP1γ) deficient mice hardly ever reach adulthood and are characterized by severe hypogonadism and loss of germ cells. There is indication that this could be caused by ectopic expression of the normally silenced L1 retrotransposon (Abe et al., 2011; Brown et al., 2010). On the other hand, it has been demonstrated that HP1s alone or in combination do not contribute to the silencing of proviral ERVs, as their combined depletion did not lead to the derepression of these elements (Maksakova et al., 2011). This could reflect functional redundancy with other H3K9me readers or point towards a silencing model that involves H3K9me but not HP1s.

3.4.4. The mammalian Polycomb group proteins

The Polycomb repression system is also found in mammalian cells, in which the Pc group proteins are usually found at promoter regions of their target genes (Beisel and Paro, 2011). Like in *Drosophila*, they are essential for regulation of developmental genes during differentiation (Boyer et al., 2006; Lee et al., 2006; Mohn et al., 2008). Accordingly, mutation of Pc proteins leads to early lethality in mice (Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004).

The mammlian PcG proteins reside in two different complexes, PRC1 and PRC2. PRC1 contains mammalian Cbx2, 4, 6, 7 and 8, which contain an N-terminal chromodomain (CD). Whilst the *Drosophila* Pc CD binds H3K27me3, the mammalian counterparts recognize either H3K9me3 or H3K27me3, both or none of

these marks. Intriguingly, the CD of Cbx7, which binds both H3K9me3 and H3K27me3 also binds RNA (Bernstein et al., 2006).

The PRC2 complex contains the catalytic activity that mediates H3K27-methylation (E(z)) and recognizes the same via the seven-bladed beta-propeller domain of Eed. This system could work like the Su(var)3-9 – HP1 mechanism in a self-reinforcing loop, that promotes propagation and maintenance of heterochromatin during DNA replication (Margueron et al., 2009). RNA binding has been also described for several PRC2 complex members, although the affinities are relatively low (Simon and Kingston, 2009; Zhao et al., 2010).

In contrast to *Drosophila*, where PcG proteins are recruited through specific *cis*-acting DNA sequences (polycomb response elements, PRE), the mechanisms in mammals are more complex. It involves the action of DNA binding proteins, DNA elements such as CpG islands and ncRNAs. It has been suggested that RNA binding is involved in polycomb recruitment, conceptually analogous to the sRNA-mediated recruitment of RITS to nascent heterochromatic RNA in *S.pombe*. For example, the ncRNA HOTAIR is required for repression of the HOXD cluster. Intriguingly, this RNA is transcribed from another HOX cluster to repress HOXD in *trans* (Rinn et al., 2007). The Xist ncRNA is required for X-chromosome inactivation in *cis*, where it acts together with a number of other ncRNAs for dosage compensation in the somatic cells of female mammals (Chow and Heard, 2009). This process is facilitated by the activity of certain retrotransposons that are transcribed from heterochromatin and processed into sRNAs (Chow et al., 2010).

4. RNA turnover and chromatin-dependent gene silencing

4.1. Introduction

As described above, transcription and the involvement of RNA in heterochromatic gene silencing has been described in a number of systems. Consistently, recent genome wide studies have discovered that transcription occurs more widespread throughout the entire genome than previously anticipated (Cheng et al., 2005; Kapranov et al., 2007; Ponjavic et al., 2007; Willingham et al., 2006; Yamada et al., 2003). Paradoxically, heterochromatin, although compact and silent, can be transcribed at least to a certain extent and a number of functionally important RNAs have been identified that arise from heterochromatic regions (Buhler et al., 2007; Chow et al., 2010; Motamedi et al., 2004; Nagano et al., 2008; Volpe et al., 2002; Zhao et al., 2008). Similarly, silencing of heterochromatin in fission yeast requires active transcription (Djupedal et al., 2005; Kato et al., 2005). This suggests that heterochromatin is kept silent on at least two levels: First, via transcriptional gene silencing, which limits the accessibility of RNA Polymerase 2 to heterochromatic genes. Second, via RNA degradation, which operates downstream of transcription. It is important to note, that this mode of RNA degradation depends on the locus from which the RNA is transcribed.

4.2. Transcriptional gene silencing

In fission yeast, transcriptional gene silencing (TGS) is mediated by the SHREC complex, which regulates nucleosome positioning and limits RNA Polymerase 2 access to heterochromatin. It consists of histone deacetylases and chromatin remodelers (Sugiyama et al., 2007). The SHREC complex member Clr3 is also part of a second complex (SHREC2), which contains the HP1 homologue Chp2 and thereby links H3K9me to H3K14 deacetylation leading to transcriptional repression. Consistently, increased RNA Polymerase 2 occupancy is detected in SHREC and

SHREC2 mutants (Motamedi et al., 2008; Yamada et al., 2005). In other organisms, TGS can be found, too. This includes budding yeast (Johnson et al., 2009), *Drosophila* (Francis et al., 2004; Sigova et al., 2006; Stock et al., 2007), plants (Stams et al., 1998) and human cells (Lu and Gilbert, 2007). It should be noted, however, that most of the conclusions drawn in higher eukaryotes are based on indirect results, for example steady-state RNA levels. In the future, nuclear run-ons, in-vitro transcription assays and RNA Polymerase 2 ChIP experiments will have to confirm these conclusions.

4.3. Chromatin-dependent RNA turnover

While transcriptional gene silencing is an important repression mechanism, the production of RNA and rapid degradation from heterochromatic regions has been reported, too. For example, heterochromatin formation inhibits transcription only from the forward but not the reverse strand, indicating rapid turnover (Volpe et al., 2002). RNA Polymerase II is enriched in G2 cells within centromeric heterochromatin when compared to an untranscribed gene. Similarly, Rpb2 mutation affects silencing and siRNA generation (Djupedal et al., 2005; Kato et al., 2005).

RNA Polymerase 2 levels and transcription rates are unchanged, if ectopic heterochromatin and silencing is produced via RITS tethering (Buhler et al., 2006). Additionally, RNA polymerase II occupancy at reporter genes does not substantially increase in heterochromatin mutants, although silencing is lost and siRNAs can be detected (Buhler et al., 2007). Importantly, silencing of nascent transcripts via RNAi is *cis*-restricted (Buhler et al., 2006). Under normal circumstances, sRNAs are not able to act in *trans* (Iida et al., 2008). Last but not least, deletion of the non-canonical poly(A) polymerase Cid14 leads to a loss of silencing, while the integrity of heterochromatin and RNA Polymerase 2 levels are unchanged. This shows that active turnover is acting on heterochromatic transcripts in a process occurring downstream of H3K9me (Buhler et al., 2007; Buhler et al., 2006).

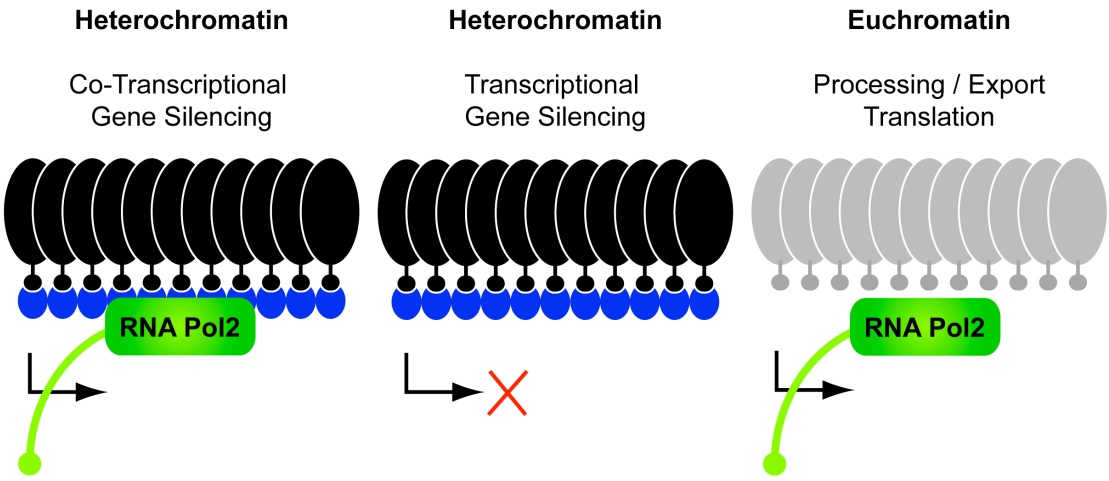

Figure 9 - Co-transcriptional gene silencing (CTGS)

Co-transcriptional gene silencing (CTGS) refers to a silencing mechanism, in which RNA degradation is triggered by the chromatin context from which the transcript is produced. This mechanism operates along with transcriptional gene silencing (TGS) to silence heterochromatic genes. Euchromatic genes are transcribed, processed, exported from the nucleus and translated.

4.4. Function of transcription and RNA turnover in silencing

The above reports clearly demonstrate that heterochromatin can be transcribed and active turnover contributes to the silencing. Indeed, there are a number of examples demonstrating that this is not only reflecting transcriptional noise and its elimination, but is also functionally relevant. In the following, I list some of the possible mechanisms together with one representative example for each case.

- The act of transcription itself is required for a certain process (e.g. chromatin remodeling) and the RNA is a simple byproduct that has to be eliminated (Hirota et al., 2008).
- The RNA itself is needed (e.g. as a recruitment platform, scaffold, decoy) but its abundance is under control by RNA decay (Zhao et al., 2008).
- The degradation product is needed (e.g. small RNAs) (Buhler et al., 2006; Volpe et al., 2002)
- The RNA arises from a deleterious element (e.g. transposons) and is therefore kept silenced by RNA degradation (Brennecke et al., 2007).
- Spurious transcription and degradation of certain RNAs is used to keep endogenous genes in a “poised” state, which allows the rapid activation of those genes under certain environmental conditions (Woolcock et al., 2012).

4.5. Regulating RNA decay in a chromatin-dependent manner

To distinguish between "normal" and "aberrant" is one of the central themes in RNA degradation. Generally, quality control steps act multiple times before the RNA reaches its final functional destination. This involves recognition of an aberrant feature in the RNA and/or non-functional RNP (Doma and Parker, 2007).

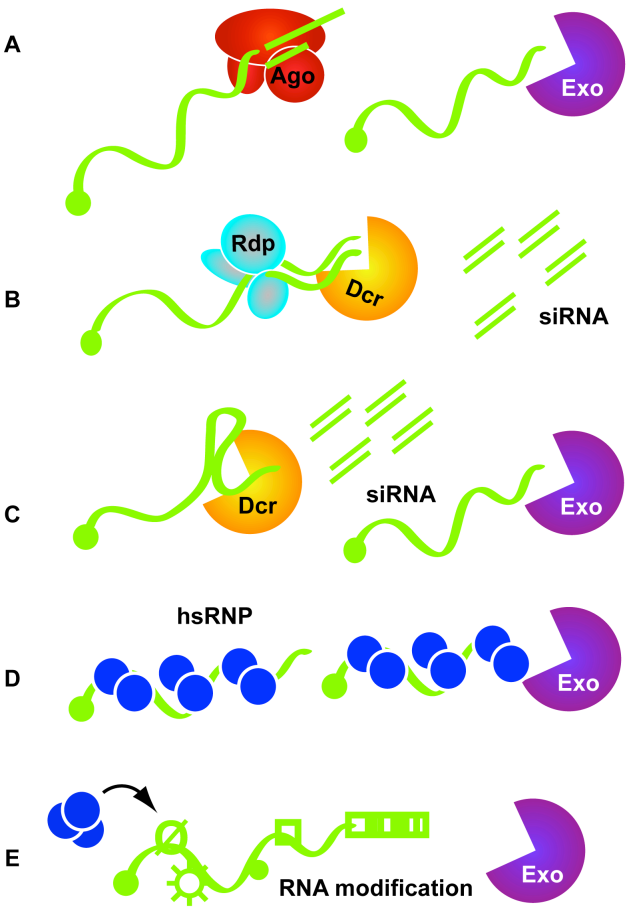

Figure 10 - Possible mechanisms inducing chromatin-dependent RNA decay

Hypothetical mechanisms, by which chromatin-dependent RNA degradation is made specific: A) Ago contains slicer activity. Co-transcriptional slicing leads to the release of a nascent transcript containing a free 3'end, which is inducing exosome mediated decay. B) Rdp synthesizes double-stranded RNAs that are degraded by Dcr. The siRNAs are not necessarily stable and/or functional *per se*. C) Dcr directly cleaves nascent RNA through physical association with chromatin. This requires hairpin formation. D) A heterochromatin-specific RNP is co-transcriptionally formed. This RNP "tags" the RNA for degradation. E) The RNA is edited in a chromatin-specific manner (e.g. methylation). Chromatin-specific editing of the RNA triggers degradation. Mechanisms A) to C) specifically occur in the vicinity of chromatin through physical interactions of these proteins with the genome. Degradation in D) and E) could occur elsewhere in the cell.

4.5.1. RNAi triggers RNA degradation by physical association with its target genes

Chromatin-dependent RNA degradation is *cis*-restricted and depends on the locus from which the RNA is transcribed (Buhler, 2009; Buhler et al., 2007). Accordingly, RNA degradation in some instances takes place in direct association with a locus, a process referred to as co-transcriptional gene silencing (CTGS). In *S. pombe*, synthesis

of precursor transcripts that generate sRNAs and synthesis of nascent RNA for siRNA-mediated recruitment of RITS are required for silencing (Buhler et al., 2006). Consistently, the nuclease Dcr1 was found to associate with centromeres (Woolcock et al., 2010). Interestingly, Dcr1 also associates with euchromatin to repress stress response genes demonstrating a physiological role for CTGS (Woolcock et al., 2012).

4.5.2. Specificity in RNAi-independent turnover mechanisms

The *S. pombe* RNAi pathway is only required for heterochromatin silencing at the centromere, but its inactivation does not affect heterochromatin maintenance at the silent mating type locus and the telomeres. In *S. cerevisiae*, which has lost the RNAi machinery, the exosome is involved in the nuclear turnover of cryptic transcripts that are produced from supposedly transcriptionally inactive regions (LaCava et al., 2005; Vasiljeva et al., 2008; Wyers et al., 2005).

Mutations in components of the nuclear exosome and the TRAMP complex member Cid14 lead to a loss of heterochromatin silencing in fission yeast. Interestingly, the integrity of heterochromatin (H3K9me, Pol II occupancy) is unchanged in these mutants (Buhler et al., 2007). This suggests that RNA decay by the exosome and/or Cid14 can be specifically triggered, if a transcript is of heterochromatic origin.

The TRAMP complex is an important co-factor for the exosome and mediates specific recognition and degradation of aberrant transcripts in *S. cerevisiae* (Vanacova et al., 2005). Recognition is in some instances achieved by a short poly(A) tail that allows the cell to distinguish the substrates from regular mRNAs, which contain long poly(A) tails. Nonetheless, poly(A) activity of TRAMP is not always needed for degradation (Houseley et al., 2007; Rougemaille et al., 2007) and silencing of heterochromatin in *S. pombe* does not require the TRAMP complex member Air1 (Buhler et al., 2007). Therefore, it was unclear, how the TRAMP complex and the exosome specifically contribute to heterochromatin silencing in *S.pombe* and whether this contribution is direct.

4.6. Major questions related to chromatin-dependent RNA turnover

1. How is transcription in different heterochromatic contexts regulated?
2. What are the proteins and chromatin modifications that recognize and trigger chromatin-dependent RNA turnover?
3. Which decay machineries are used in which genomic and cellular context?
4. Is the transcription *per se* or the actual transcript needed for function?
5. If the transcript is functional, does this depend on the precursor transcript or the degradation product? What is the mechanistic basis of the function?

5. Aim of this thesis

The findings made by Bühler et al. (2007) suggest that RNAi-independent turnover mechanisms contribute to heterochromatin silencing in *S. pombe*. Genetic and biochemical experiments demonstrated that Cid14 is required for transcript degradation downstream of heterochromatin integrity. The molecular mechanism, however, how Cid14 would specifically recognize and degrade these transcripts remained unclear. I set out to identify the molecular checkpoint that determines specificity taking advantage of various biochemical approaches as well as the awesome power of yeast genetics.

RESULTS

1. Manuscript 1 / see Appendix

Proteomic and functional analysis of the noncanonical poly(A) polymerase Cid14
Keller, C., Woolcock, K., Hess, D., Buhler, M.
RNA. 2010 Jun;16(6):1124-9. Epub 2010 Apr 19.

Previous work demonstrated that efficient silencing of transgene insertions depends on the non-canoncial poly(A) polymerase Cid14. Analogous to the situation in *S. cerevisiae*, Cid14 was found to reside in a TRAMP-like complex consisting of Cid14, Air1 and Mtr4. However, Air1 is dispensable for efficient silencing of heterochromatin. Furthermore, no protein was identified that would biochemically link Cid14 to a chromatin silencing function and attempts to crosslink Cid14 to DNA failed (Buhler et al., 2007; Wang et al., 2008).

I therefore revisited affinity purifications to better characterize the Cid14-interaction network in order to find new factors that would link Cid14 to heterochromatic gene silencing. Purifications under varying conditions revealed that Cid14 forms a very stable complex with Air1, which is absolutely required for association with the putative exosome co-factor Mtr4. In the absence of the *air1+* gene, we did not find another Air protein co-purifying with Cid14, and similarly, no other Cid protein was identified in Air purifications in the absence of *cid14+*. This demonstrates that the very stable Cid14-Air1 interaction forms the core of a single fission yeast TRAMP complex. I also found that Cid14 interacts with a higher molecular weight complex that represents a 60S ribosomal subunit assembly interaction network. This finding provided a biochemical link to the known function of Cid14 in pre-rRNA processing (Win et al., 2006). Despite extensive analysis, I was not able to identify factors that would link Cid14 physically to heterochromatin.

The finding that the Cid14-Air1 interaction is absolutely required for TRAMP integrity was surprising, as silencing of transgene insertions is independent of Air1, but dependent on Mtr4. My biochemical experiments, however, ruled out the possibility that another Air protein could act redundantly in this complex or that Cid14 could interact with Mtr4 in the absence of Air1. I therefore wanted to test the functional contribution of Air1 and Cid14 to gene silencing on a genome wide scale. For this, I assessed expression in *wild-type*, *cid14Δ* and *air1Δ* cells using *S. pombe* tiling arrays, which cover the entire genome.

The analysis made by Katrina Woolcock showed that only about half of the genes that are repressed by Cid14 also depend on repression by Air1. On the other hand, a large number of genes are repressed by Air1 independently of Cid14. The same observations were made for genes that are downregulated upon deletion of either *cid14+* or *air1+*. This demonstrates that although Cid14 and Air1 are linked tightly on a biochemical level, they can function independently of each other.

Last but not least, I wanted to check how many genes that are repressed by Cid14 are of heterochromatic origin. This analysis revealed that in some instances these genes were H3K9 methylated, but there was no absolute correlation. Indeed, there are a number of genes that are H3K9 methylated but not repressed by Cid14. This is, however, consistent with the idea that Cid14 functions in a parallel pathway with RNAi-mediated turnover to silence heterochromatic genes (Buhler et al., 2007; Buhler et al., 2008). The heterochromatic genes, which were repressed by Cid14, were mainly telomeric and subtelomeric transcripts as well as a number of meiotic genes residing in heterochromatic islands (Zofall et al., 2012).

2. Manuscript 2 / see Appendix

HP1^{Swi6} Mediates the Recognition and Destruction of Heterochromatic RNA Transcripts

Keller, C., Adaixo, R., Stunnenberg, R., Woolcock, K. J., Hiller, S., Buhler, M.

Mol Cell. 2012 Jul 27;47(2):215-27. Epub 2012 Jun 7.

Highlighted in:

Silent decision: HP1 protein escorts heterochromatic RNAs to their destiny.

Ren, J., Martienssen RA. EMBO J. 2012 Jun 15

Chromatin: RNA eviction by HP1

Schuldt, A. Nat Rev Mol Cell Biol. 2012 Jul 11;13(8):478-9

Should I stay or should I go? Chromodomain proteins seal the fate of heterochromatic transcripts in fission yeast.

Creamer KM, Partridge JF. Mol Cell. 2012 Jul 27;47(2):153-5.

The results from my proteomic studies indicated that Cid14 is unlikely to recognize transcripts directly on chromatin, but rather acts downstream of a factor that is escorting the heterochromatic RNA to the degradation machinery. I decided to find this "checkpoint" using a classical PEV approach taking advantage of Yeast genetics. In these experiments, I surprisingly found that loss of Cid14 only caused a derepression of heterochromatic genes on the RNA but not the protein level. Knowing that these transcripts are properly processed and have coding potential *per se*, I postulated the existence of a factor that recognizes and binds nascent heterochromatic RNA to mediate their degradation. In the absence of Cid14, heterochromatin integrity is unaffected, which sustains a functional checkpoint that prevents translation of heterochromatic RNA most likely via nuclear retention.

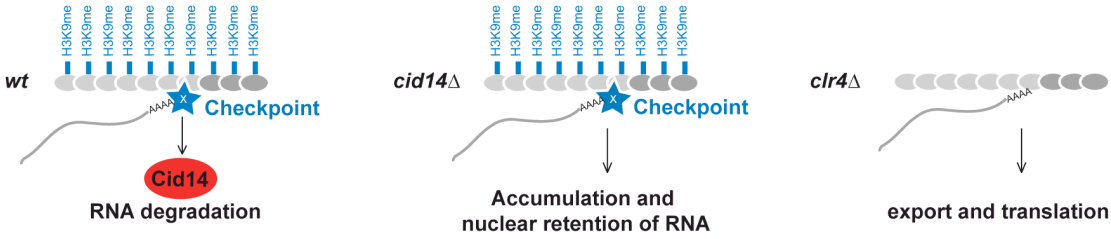

Figure 11 - Checkpoint model for heterochromatin-mediated RNA decay

A checkpoint factor (blue star) that recognizes both RNA and H3K9me escorts nascent transcripts to the degradation machinery (red). In the absence of Cid14-mediated degradation, H3K9me and a functional checkpoint are intact, leading to loss of RNA degradation but not translational inhibition. Translational inhibition occurs through packaging into a heterochromatin specific RNP (hsRNP), which most likely causes nuclear retention. In *clr4Δ* cells, H3K9me marks and the checkpoint are lost, which leads to export and translation.

My subsequent experiments showed that this checkpoint factor is the fission yeast HP1 protein Swi6. I showed that Swi6 directly binds RNA and that this interaction occurs via the hinge region. I was able to create an RNA binding mutant that does not affect the molecular properties of the rest of the molecule (e.g. overall fold, H3K9me interaction). *In vivo*, this mutant rescues H3K9me defects that are found in *swi6Δ* cells and also localizes properly to heterochromatic domains. This demonstrates that RNA binding is neither required to maintain H3K9me nor for the recruitment of Swi6 to H3K9me-chromatin. However, consistent with the postulated function as a checkpoint, I found that heterochromatic RNA and protein levels are derepressed in an RNA binding mutant. This suggests that Swi6 is involved in silencing on at least two levels. First, it assembles the heterochromatic transcripts into a ribonucleoparticle (hsRNP) that is not competent for translation and thereby inhibits expression on the protein level. Second, Swi6 mediates RNA decay and thereby inhibits expression on the RNA level.

Using NMR spectroscopy, we could explain these findings on a molecular basis. In solution RNA titration experiments revealed that structural changes upon RNA binding were not limited to the hinge region but surprisingly also affected the chromodomain. This pointed towards the intriguing possibility that RNA binding could affect H3K9me binding. We confirmed this idea with SPR experiments and found that RNA competes with Swi6 binding to H3K9me. Based on this data, we postulate a model in which RNA binding renders the chromodomain structurally incompatible with stable H3K9me association leading to heterochromatin eviction and degradation of the RNA.

My data has several important implications. Binding to $HP1^{Swi6}$ can induce degradation of any RNA of heterochromatic origin, which could be a crucial feature to repress the expression of deleterious sequences and transposons. It shows how repression is achieved on a mechanistic level: via RNA degradation and creation of a translationally incompetent hsRNP. Furthermore, it provides a possible explanation for why HP1 proteins are so highly dynamic and why RNA degradation factors like Cid14 have not been recovered previously in PEV screens. Last but not least, my work is the first example, which demonstrates that RNAs can act as “repellents” for chromatin proteins.

DISCUSSION

1. Composition and functional relation of the TRAMP complex to heterochromatin silencing

1.1. The role of the fission yeast TRAMP complex

My work demonstrated that Cid14 is part of a stable complex together with Air1, which forms the core of a single TRAMP complex in fission yeast (Keller et al., 2010). It was shown before, however, that Air1 is dispensable for heterochromatic gene silencing. This discrepancy is somewhat surprising, as silencing also depends on Mtr4 (Buhler et al., 2007) and the interaction of Cid14 with Mtr4 absolutely requires Air1. Furthermore, stimulation of the exosome activity in *S. cerevisiae* requires an intact TRAMP complex (LaCava et al., 2005).

Taken together, this suggests that the silencing functions of Mtr4 and Cid14 are independent and achieved outside the TRAMP complex. It is possible that Cid14 mediates degradation of heterochromatic RNA independently of the exosome. Consistently, *rrp6Δ cid14Δ* double mutant cells are lethal suggesting that they are genetically operating in different pathways. In line of these findings, it is interesting, that poly(A) polymerase activity is dispensable for silencing (Claudia Keller, unpublished) and that degradation in *S. cerevisiae* does not always require activity. Additionally, it was found that Mtr4p has functions outside of TRAMP (LaCava et al., 2005). It could be that Cid14, rather than acting as an enzyme, is a recruiter for different RNA degrading activities. If these interactions occur very transient, this could be an explanation, why we did not pick them up in our proteomic analysis. Finding the exo- and/or endonucleases that are responsible for degradation would be crucial to understand Cid14-mediated gene regulation on a mechanistic level. Furthermore, I suggest to perform gene expression analysis in Cid14DADA catalytic

inactive mutants, to be able to functionally separate the poly(A) polymerase from the recruitment function.

I also found that Air1 and Cid14, although tightly linked, share only a small number of common substrates. Air1 could serve as an adaptor for certain RNAs, whereas for other targets the recruitment might be different (e.g. Swi6-dependent, see below). The properties and requirements of such RNAs, however, have not been defined, yet. My genome-wide analysis showed that Cid14 is involved in the degradation of a subset of heterochromatic substrates including telomeric and meiotic transcripts. However a number of heterochromatic genes are not affected in *cid14Δ* cells. This is consistent with the finding that various RNA turnover mechanism act together to ensure proper silencing (Buhler et al., 2007). My subsequent work has addressed the molecular details, how RNA turnover is triggered by the presence of H3K9 methylation (see below).

1.2. A conserved role for the TRAMP complex?

Whereas the TRAMP complex has been extensively studied in yeast, the existence and function of a human TRAMP complex are not yet clear. A stable complex does not seem to exist, however, hTRF4-2 (Trf4p) and ZCCHC7 (Air2p) can be found in hRRP6 and hMTR4 precipitates (Lubas et al., 2011). This complex localizes to nucleoli, suggesting that a conserved function could be the involvement in rRNA maturation (LaCava et al., 2005; Win et al., 2006). hMtr4 is also found in the trimeric NEXT complex consisting of hMTR4, the Zn-knuckle protein ZCCHC8, and the putative RNA binding protein RBM7. This complex is required for the degradation of promoter upstream transcripts (PROMPTs) (Lubas et al., 2011; Preker et al., 2008). It is interesting that in contrast to the yeast exosome the human Rrp6 more efficiently degrades structured substrates on its own (Januszyk et al., 2011). Therefore, there might be no absolute need for a co-factor, which stimulates enzymatic activity. However, co-factors could be used to help recruiting the exosome to different kinds of substrates. Clearly, there are more studies required to define the substrates of the exosome and all the putative co-factors using state-of-the-art techniques covering the entire genome (Deep Sequencing).

2. HP1^{Swi6} defines an hsRNP that triggers heterochromatin-dependent RNA decay

A number of recent genome wide studies have suggested that transcription occurs more widespread throughout the entire genome than previously anticipated (Barski et al., 2007; Birney et al., 2007; Cheng et al., 2005; Yamada et al., 2003). This includes heterochromatic regions that are characterized by H3K9me and HP1 binding (Buhler et al., 2007; Maison et al., 2011; Volpe et al., 2002). HP1s are highly dynamic (Cheutin et al., 2004; Cheutin et al., 2003) and they can be found in euchromatic regions, too (Libault et al., 2005; Minc et al., 2000; Piacentini et al., 2009). This suggests that silencing via H3K9me bound HP1s is unlikely to be caused through the formation of a static network that renders these regions refractory for transcription. Instead, RNAs produced from these regions are not expressed because they are highly unstable and/or can't be translated by the ribosome. Consistently, the existence "cryptic" transcription and rapid degradation has been observed in a number of organisms (Buhler et al., 2007; Kapranov et al., 2007; Lemieux et al., 2011; Preker et al., 2008; Wilhelm et al., 2008; Woolcock et al., 2010; Woolcock et al., 2012; Wyers et al., 2005). The mechanisms for specificity of degradation are very diverse. For example, in the case of RNAi-mediated CTGS it occurs through direct association of the Dcr1 nuclease with the target genes at nuclear pores. Interestingly, this is independent of heterochromatin formation (Woolcock et al., 2012). For Cid14-mediated degradation, the transcription from a heterochromatic context seems to be the trigger, however, how this would be achieved on a molecular level was unknown (Buhler et al., 2007).

2.1. HP1 inhibits expression on the protein and RNA level

My work has shown, that in fission yeast, HP1^{Swi6} controls heterochromatin silencing by triggering RNA degradation and creation of a translationally incompetent hsRNP. Importantly, the transcripts seem to be properly processed and recognition occurs independently of the actual sequence and/or presence of an ORF (Keller et al., 2012). Inhibiting expression both on the RNA and protein level might be of particular

importance, because in the absence of RNA degradation, silencing is still maintained at the protein level via sequestration. In this sense, inhibition of translation, which is most likely caused by nuclear retention, serves as a backup mechanism to ensure proper silencing.

Conceptually similar examples have alreaday been reported. In *Arabidopsis*, MET1 mutants display an increased expression of the transposon Evadé, however, this transposon is not able to transpose. This suggests that the inhibition of expression occurs at both the RNA and protein level. Interestingly, it was shown that the repression mechanism operating postranscriptionally in MET1 mutants requires the KYP H3K9 methyltransferase (Mirouze et al., 2009). Although the contribution of the HP1 homologue LHP1 has not been addressed in this study, it could be the presence of H3K9me that prevents translation via binding to an HP1 homolog resulting in RNA sequestration in the nucleus.

Similarly, the expression of HLA-3C is controlled on two levels in human NK cells: RNA degradation and RNA sequestration. In the presence of enzymatic active MEX-3C, this protein controls degradation of the HLA-3C mRNA via binding to the transcript and recruitment of cytoplasmic exonucleases. In the absence of enzymatic activity, binding to the mRNA is sufficient for RNA sequestration resulting in decreased HLA-A protein levels at the cell surface. Via this mechanism, MEX-3C like $HP1^{Swi6}$ integrates both RNA degradation and translational competence via sequestration (Cano et al., 2012).

2.2. HP1 connects heterochromatin transcription to degradation by Cid14 via formation of a hsRNP

RNA degradation is achieved by $HP1^{Swi6}$-mediated recruitment of RNA decay factors like Cid14. Because $HP1^{Swi6}$ and Cid14 are found at all heterochromatic regions and Swi6 binds RNA without sequence preference it is possible that any RNA of heterochromatic origin is subjected to RNA degradation (Cam et al., 2005; Woolcock et al., 2010). Therefore, if a cell is able to “store” any sequence, for example a

deleterious element such as a transposon, in heterochromatin, this is sufficient for repression.

Cid14 is recruited to the mating-type as well as the telomeric sequences in an $HP1^{Swi6}$-dependent manner. Consistently, $HP1^{Swi6}$ mediates degradation by Cid14 at these two loci (Keller et al., 2012). How $HP1^{Swi6}$ recruits Cid14 at these loci is an unresolved question. Although Cid14 contains two PxVxL motifs (Cowieson et al., 2000; Smothers and Henikoff, 2000), recombinant Swi6 and Cid14 do not interact *in vitro* (Claudia Keller, unpublished) suggesting that the recruitment network requires other factors and/or posttranslational modifications. Dam-ID experiments in nls-swi6-KR25A strains are currently being performed, to address whether RNA binding is required to establish an interaction network for Cid14 recruitment. Because H3K9me is intact in this mutant at the mating-type and telomeric loci, it would also allow us to refine the role of the H3K9me mark versus $HP1^{Swi6}$ itself in the recruitment of Cid14.

Additionally, I suggest to revisit $HP1^{Swi6}$ affinity purifications under different conditions. It is possible that this hsRNP complex has not been identified so far, because in these standard purifications the soluble (non-chromatin associated) fraction was enriched (Fischer et al., 2009; Motamedi et al., 2008). Quantitative proteomic approaches including the nls-swi6-KR25A allele as a negative control would be instrumental to define the hsRNP. Additionally, we could identify PTMs in an unbiased way and shed light on their contribution to the formation of the hsRNP.

2.3. Cid14 association with centromeres and euchromatin

Interestingly, Cid14 association with the centromere is not lost in *swi6Δ* cells and similarly, accumulation of centromeric transcripts is not observed in *cid14Δ* cells. It is very likely that at the centromere redundant turnover and recruitment mechanisms are acting. To answer the question, whether Cid14 plays a redundant role in RNA turnover at the centromere, we would require separate-of-function alleles for other RNA turnover pathways (such as RNAi). These alleles should only affect decay but not H3K9methylation, which maintains the $HP1^{Swi6}$ checkpoint.

The fact that H3K9me is not lost at the centromere, however absent at the telomeres and mat loci in *swi6Δ* cells could imply that H3K9me is indeed required for Cid14

recruitment. One could directly test this hypothesis by performing Cid14 Dam-ID experiments in a *clr4Δ* (the fission yeast Su(var)3-9 homologue) background, which lacks H3K9me at all heterochromatic loci. On the other hand, Cid14 also associates with euchromatin, excluding the possibility that H3K9me is the only trigger for chromatin association.

Finally, it is interesting that recruitment of the RNAi machinery to centromeres does not require H3K9me (Woolcock et al., 2010). Cid14 associates with mating-type and telomeric loci and triggers transcript degradation depending on the presence of $HP1^{Swi6}$ and/or H3K9me. Therefore, two different RNA turnover mechanisms that act together seem to have different recruitment and trigger mechanisms.

2.4. Cid14 mediates heterochromatic RNA turnover

Our Dam-ID experiments demonstrate that Cid14 associates with telomeric and mating-type heterochromatin in an $HP1^{Swi6}$-dependent manner. Together with our genome wide expression data, this shows that the contribution of Cid14 to silencing is direct. Nonetheless, it is still enigmatic how Cid14 triggers RNA turnover.

In vitro, an intact TRAMP complex is required to stimulate degradation by the exosome (LaCava et al., 2005). Nonetheless, silencing is intact in *air1Δ* cells, in which the interaction of Cid14 with Mtr4 is lost (Keller et al., 2010). We do not find any exosome components in our pulldowns from *wild-type* or *air1Δ* cells and do not recover TRAMP in exosome purifications (Claudia Keller, unpublished).

Poly(A) activity of Trf4 is required for degradation via the exosome (Vanacova et al., 2005). Recombinant Cid14 shows robust poly(A) activity and does not destabilize RNAs on its own (Buhler et al., 2007; Buhler et al., 2008). Activity, however, is dispensable for silencing (Claudia Keller, unpublished) and we do not detect aberrant polyadenylation of heterochromatic transcripts in *cid14* mutants (Keller et al., 2012). So does Cid14 really act via polyadenylation and recruitment of the exosome to degrade heterochromatic targets?

I suggest two possibilities: Cid14 acts via the exosome, but uses different redundant pathways and mechanisms, which back up each other in the absence of another component. For example, Dis3 could replace Rrp6 and vice versa. This redundancy

occurring *in vivo* could explain seemingly contradictory data from genetic and biochemical experiments (Buhler et al., 2007; Kinoshita et al., 1991; Wang et al., 2008; Woolcock et al., 2012).

Alternatively, Cid14 could recruit a so far unidentified, novel nuclease. Recruitment could occur via direct transient interaction. A kinetic competition model could apply (Doma and Parker, 2007), in which inhibition of nuclear export by RNA binding to Cid14 eventually triggers degradation. Consistent with this model, changing the kinetics of hsRNP formation by introducing a ribozyme at the 3'end of PEV reporters escape the $HP1^{Swi6}$-Cid14 checkpoint leading to protein expression in *cid14Δ* cells (Claudia Keller, unpublished).

Our extensive attempts to identify such nucleases biochemically have failed so far, probably due to low abundance or very transient interaction. Genetic screening would be possible, if one could perform a PEV screen that directly assesses heterochromatic RNA levels. Mutation of this downstream factor should show a similar phenotype like a *cid14* mutant, if the genetic connection is linear.

2.5. HP1 inhibits translation of heterochromatic RNA

Translational repression is observed in *cid14* mutant cells, where RNA degradation is absent, however H3K9me and the checkpoint function are still intact. The mechanistic basis of this is not clear, mainly because we were unable to directly localize heterochromatic RNAs by microsopic or biochemical methods. The most likely hypothesis, however, is that $HP1^{Swi6}$ traps heterochromatic RNA in the nucleus. This would explain, why heterochromatic RNAs in *cid14Δ* cells do not accumulate to the same extent as in *clr4Δ* or *swi6Δ* cells. Inefficient RNA processing and export leads to RNA degradation by the exosome (Jensen et al., 2003). It is therefore likely, that inhibition of nuclear export by $HP1^{Swi6}$ can at some point induce RNA degradation by other mechanisms. At the moment, we try to confirm this hypothesis using ultrasensitive RNA imaging approaches.

2.5.1. Design of a PEV screen that detects changes in RNA levels

An important impact of my findings is, that they explain why RNA degradation factors were not previously identified in PEV screens: In the absence of RNA degradation, expression is inhibited at the protein level. PEV screens, however, assess changes in heterochromatic gene expression at the protein level (Baur et al., 2001; Bayne et al., 2008; Reuter and Wolff, 1981; Smith et al., 1999). It would be therefore very interesting to design a PEV screen that is able to identify changes at the RNA level. Either this requires a genetic trick, in which the translational repression by the $HP1^{Swi6}$ checkpoint is bypassed. Alternatively, one needs any signal that linearly correlates with the amount of RNA specifically produced from heterochromatin. RNA fluorescent in situ hybridization (RNA FISH) can't be used on a genome wide scale due to the extensive sample preparations and optimizations that are required in fission yeast. Heterochromatic RNAs that are tagged with an aptamer (e.g. MS2) which bind to GFP-tagged fusion proteins (e.g. MS2-CP-GFP) are not suitable either, because the co-expressed fusion protein is always expressed at the same level.

(a) Strategy 1: Combound-based fluorescence screen

Therefore, I suggest to perform on a compound-based screen. The heterochromatic RNA is tagged with the Spinach aptamer, which binds to the DFHBI compound (Paige et al., 2011). A conformational change upon binding of the compound to the aptamer leads to fluorescence, giving rise to a signal that correlates to the amount of RNA. This would allow to quantitatively assess heterochromatic RNA levels. One could then perform chemical mutagenesis or alternatively use available deletion libraries (Kim et al., 2010b) to screen for factors that increase heterochromatic RNA levels. Crucial to the use of this technique is that the compound is not fluorescent, if it is not bound to the aptamer. This is the case for the Spinach-DFHBI method.

In trial experiments, I was not able to detect any fluorescent signal using this system (Claudia Keller, unpublished). However, it could be that the yeast cell wall is not permeable for DFHBI or the compound is immediately pumped out. Additionally, it is not clear, yet, whether the Spinach system is suitable for lowly expressed transcripts and gives rise to a signal that is high enough to be detected above background in

fission yeast. Given the advantage of the system, I however think that it is worthwhile to perform more experiments to optimize this system.

(b) Strategy 2: Genetically bypassing HP1^{Swi6} by Rz-termination

An alternative to the proposed Spinach-approach would be to use a Ribozyme (Rz)-tagged heterochromatic reporter system containing synthetic poly(A) tails (Dower et al., 2004). The beauty of this system is that a single point mutant of the Rz is available, which makes this an ideal control strain. As already mentioned, preliminary data suggests that rapid termination escapes translational repression but not degradation by HP1^{Swi6} (Claudia Keller, unpublished; also see Figure 12).

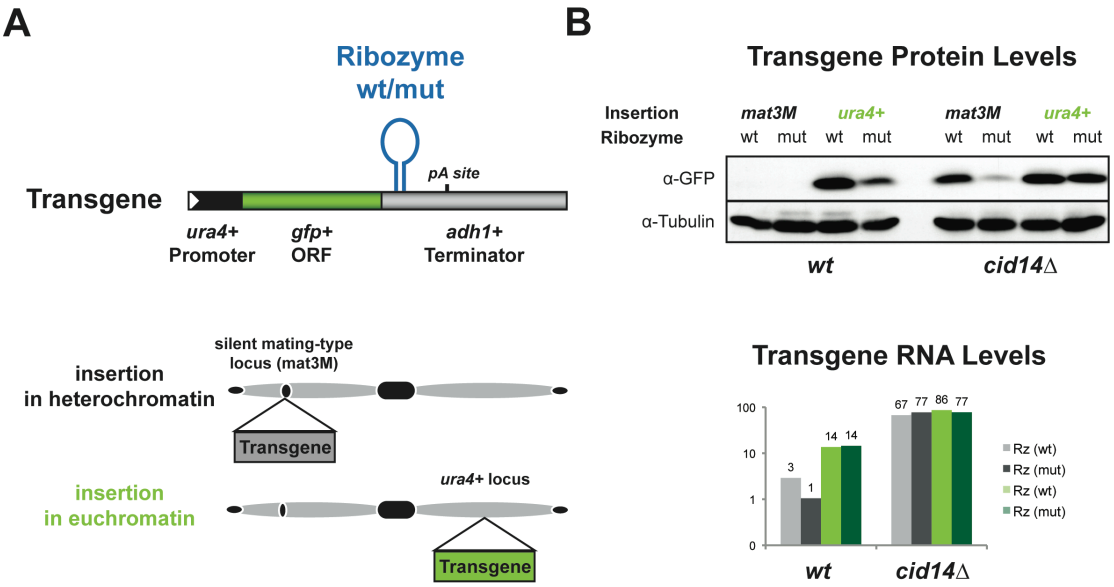

Figure 12 - Ribozyme-mediated termination escapes the HP1^{Swi6} checkpoint

A) The gfp+ reporter ORF is followed by a Ribozyme, which is placed in the Tadh1 terminator upstream of the first canonical poly(A) site. The whole reporter is driven by a *ura4+* promoter. This cassette is integrated at the heterochromatic mating-type locus (*mat3M*) or the euchromatic *ura4+* locus. B) In *wild-type* (wt) cells, ribozyme containing reporter constructs are regularly silenced, when introduced at the heterochromatic *mat3M* locus. Cid14-mediated degradation contributes to the silencing. Consequently, deletion of *cid14+* results in transcript accumulation in both the wt and mut Rz reporter strains. However, normally terminated transcripts (Rz mut) are not translated, because of the HP1^{Swi6} checkpoint. Rapid termination by a Rz escapes this checkpoint leading to protein production in *cid14Δ* cells. At the euchromatic *ura4+* locus, Rz termination has no effect on the amount of protein produced in a *cid14+* mutant background.

In *wild-type* cells, GFP-Rz reporters are silenced by Cid14-mediated RNA degradation. Consistent with our published data, *mat3M::gfp-Rz+* and *mat3M::gfp-Rz(mut)* RNA levels are derepressed in *cid14Δ* cells. However, there is a large difference on the protein level between the Rz (wt) and the Rz (mut) reporter. Under

canonical termination conditions (Rz mut) the HP1^{Swi6} checkpoint prevents translation of heterochromatic RNA, however, this is bypassed if the transcript is terminated with a Rz (wt). Importantly, the difference in protein levels between Rz (wt) and Rz (mut) is only seen, if the reporter is inserted in a heterochromatic context, demonstrating that *cid14Δ* cells have no general translation or processing defects. I therefore predict, that more factors like *cid14+* could be identified by genetic screens carried out in these Rz reporter strains.

Ideally, a genome-wide screen would be performed using a sensitive click beetle luciferase assay, in which reporter levels can be quantitatively assessed directly in the culture medium in a multiwell format. (Shimada and Buhler, 2012)

3. Chromodomain proteins integrate RNA and H3K9me binding

3.1. Molecular properties of HP1^{Swi6} RNA binding

Our work has demonstrated that HP1^{Swi6} directly binds RNA and that this binding occurs via the intrinsically unstructured hinge region. The CD and CSD as isolated domains do not bind RNA, however, the NT and CD change molecular architecture upon RNA binding. This indicated, that RNA binding influences binding to H3K9me (see below).

Furthermore, I was able to create a mutant that fails to bind RNA but retains the folds of the CD and CSD. In this mutant, all 25 positively charged residues (Arg/Lys) of the hinge region are mutated to Ala (KR25A). From our NMR studies, however, we know that only 13 resonances from the hinge region show changes upon binding to a 20mer RNA. Because we lack a complete resonance assignment of the hinge region so far, we do not know whether the hinge-RNA interaction is exclusively occurring at Lys/Arg or involves other amino acids, too.

There are well-described examples of RNA binding to unstructured regions in the literature (Weiss and Narayana, 1998). For the ones that have been characterized by structural biology methods, nearly half of the hydrogen bonds to RNA are formed by

Arg, Lys and the main chain NH groups. Importantly, for RNA-protein complexes, the ribose (2'OH) contributes largely to these interactions (Bahadur et al., 2008). This could explain, why $HP1^{Swi6}$ does not bind DNA. Given the importance of this group, it would be interesting to test whether HP1s are able to bind 2'O-methylated RNAs such as piRNAs (Tian et al., 2011). On the other hand, the Arg/Lys-rich properties of the hinge region might explain, why RNA binding is sequence and length independent, as these amino acids favor hydrogen bonding and van-der-Waals contacts to the phosphate backbone of the RNA (Ellis et al., 2007). Similarly, it would be worthwile testing whether longer RNAs induce more changes in the hinge region (maybe up to 25, if all Lys/Arg are involved).

To get more insights into the molecular nature of $HP1^{Swi6}$ RNA recognition, I suggest assigning the $HP1^{Swi6}$ RNA-protein interaction surface, with the ultimate goal to solve the solution structure by NMR spectroscopy.

Based on such data, we could test, whether a certain spacing of positively charged residues and/or a motif is required for RNA binding. This would help to find such motifs in HP1s and other proteins, for example in RNA binding proteins, which have been identified by recent proteomic studies (Baltz et al., 2012).

3.2. RNA and H3K9me binding are competitive processes

Our NMR data revealed that structural changes upon RNA binding to $HP1^{Swi6}$ are not limited to the hinge region, however, also affect the CD and NT. This pointed towards the intriguing possibility that RNA and H3K9me binding are connected. The *in vivo* data of the RNA binding mutant suggested that these events are competitive. We confirmed this idea using Surface Plasmon Resonance (SPR). Therefore, $HP1^{Swi6}$ dissociates from heterochromatin when complexed with RNA. So far we do not have a complete resonance assignment of the NT-CD-hinge construct, which is a prerequisite for getting a NMR solution structure. This would be a crucial experiment to understand the competition mechanism on a molecular level.

In vivo, we propose that these competitive events form the molecular basis of heterochromatic RNA transcript degradation as discussed above. Importantly, H3K9me and $HP1^{Swi6}$ recruitment to this mark is not altered globally in nls-swi6-

KR25A mutants, suggesting that RNA binding is not required for the structural maintenance of heterochromatin. Importantly, the properties and function of HP1^{Swi6} RNA binding seem to be different from the mammalian HP1s (Maison et al., 2002; Maison et al., 2011; Muchardt et al., 2002).

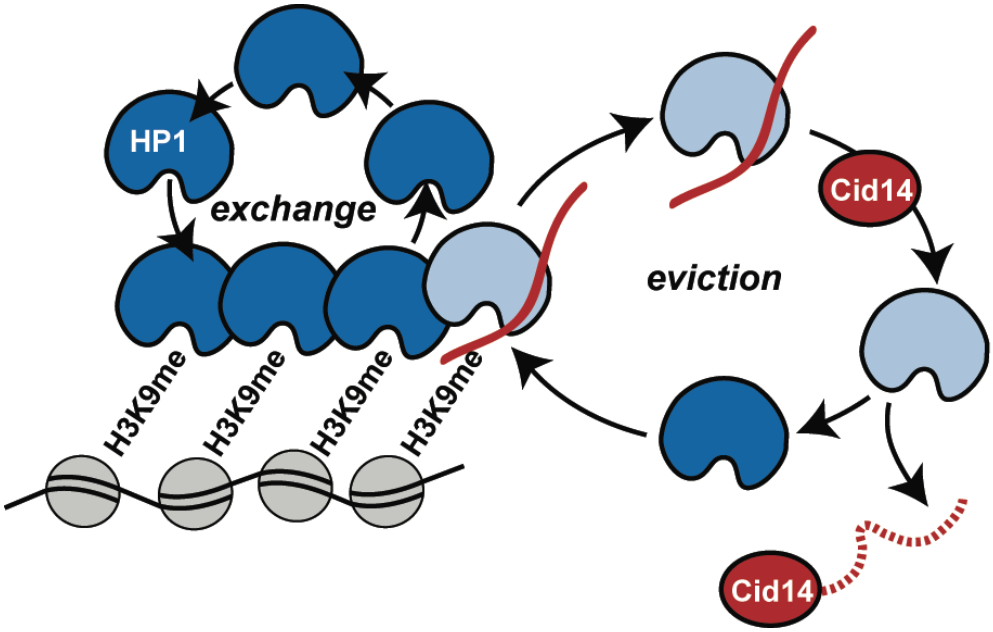

Figure 13 - Model for HP1 mediated degradation of heterochromatic RNA by competitive H3K9me and RNA binding

HP1^{Swi6} proteins associate with H3K9-methylated nucleosomes (gray) only transiently and readily exchange from heterochromatin (dark blue). This continuous exchange of HP1^{Swi6} prevents saturation of heterochromatin with RNA. In case transcription within heterochromatin occurs, HP1^{Swi6} binds the newly synthesized RNA (red) and dissociates from H3K9 methylated nucleosomes as a result of competition between RNA and the histone tail for HP1^{Swi6} binding (light blue). Subsequently, the RNA is passed on to Cid14 (red), which in turn initiates RNA degradation. The RNA produced from highly transcribed regions at the heterochromatin boundaries could serve as repellents and inhibit heterochromatin spread into euchromatin.

The finding that RNA counteracts H3K9me binding is particularly interesting, if one considers that heterochromatin boundaries are usually characterized by the presence of very highly transcribed genes (Cam et al., 2005; Takahashi et al., 1991). This includes tRNA genes, whose transcriptional activity is required for boundary formation (Noma et al., 2006; Scott et al., 2006). RNA has been also linked to boundary formation in mammalian cells (Lunyak et al., 2007). I therefore propose, that RNA-mediated eviction of HP1^{Swi6} is the molecular mechanism underlying heterochromatin boundary formation. This hypothesis predicts that in an HP1^{Swi6} RNA binding mutant H3K9me marks spread across the heterochromatin boundary

into neighboring euchromatic regions. Furthermore, it is possible that eviction of $HP1^{Swi6}$ induces degradation of the actual precursor/boundary-RNA via a Cid14-dependent mechanism. To address these questions, we are currently performing genome-wide ChIP-Seq and sRNA-Seq experiments.

3.3. Regulation and specificity of $HP1^{Swi6}$ RNA binding

3.3.1. Regulation via a dynamic organization of hsRNPs in nuclear foci

Fission yeast $HP1^{Swi6}$ localizes to nuclear foci corresponding to the main heterochromatic loci (Ekwall et al., 1995) . It is highly dynamic and exchanges with rapid kinetics on the ensemble level, but always remains closely associated with H3K9me chromatin (Cheutin et al., 2004). RNA and H3K9me binding are competitive processes, which causes preferential recruitment of RNA-free $HP1^{Swi6}$. This could explain why induction of RNA degradation is specific to heterochromatic regions, as local concentration of $HP1^{Swi6}$ ensures preferential capturing of heterochromatic RNA on the ensemble level. The local concentration outside these regions is not high enough, so $HP1^{Swi6}$ cannot effectively compete with other proteins comprising a "regular" mRNP.

One could test this idea by largely overexpressing $HP1^{Swi6}$. This would increase the concentration of $HP1^{Swi6}$ outside the foci in the nucleoplasm. In this case, $HP1^{Swi6}$ RNA binding would occur on non-heterochromatic targets and maybe also induce their degradation. Such a gain-of-function allele should induce global gene expression changes, which correlates to the targets that are newly bound by $HP1^{Swi6}$. Indeed, overexpressed *swi6+* has been reported to enhance gene silencing within heterochromatin (Nakayama et al., 2000) and causes both up- and downregulation of a large number of genes (Wiren et al., 2005). Interestingly, these gene expression changes are much more pronounced than the effects in *swi6Δ* cells, indicating a gain-of-function. I speculate, that this is caused by ectopic RNA binding. To confirm this hypothesis, I suggest to perform gene expression analysis using tiling arrays and comparing *swi6* with *nls-swi6-KR25A* overexpressing cells.

On the other hand, other factors, which increase heterochromatin specificity, could be acting *in vivo*. For example, heterochromatin-specific proteins could be required to link HP1^{Swi6} to Cid14. Our Dam-ID experiments indeed point towards this possibility. Factors, which prevent RNA binding off-chromatin, could contribute to an additional layer of regulation, too. To get more insights into these mechanisms, it is again very crucial to identify the components of the hsRNP via proteomic methods (see above).

Last but not least, it is important to note, that also RNA binding is highly dynamic. This indicates, that RNA does not remain tethered firmly to HP1^{Swi6} *in vitro*. *In vivo*, this could be an important feature to very quickly hand over the transcript to the RNA degradation machinery. If RNA binding affects H3K9me binding, one would expect to see changes in the dynamic association of HP1^{Swi6} with heterochromatin. However, preliminary FRAP data from our lab suggest that *wild-type* have *nls-swi6-KR25A* cells display identical kinetic behaviour on the ensemble level (Rieka Stunnenberg, & Claudia Keller, unpublished). This probably reflects the fact that in bulk heterochromatin of *wild-type* cells H3K9me-CD interactions largely outnumber RNA-hinge interactions. Demonstration of changed kinetics might only be possible in a situation, where this balance is shifted towards higher RNA levels, without affecting the number of H3K9me histone tails. Preliminary experiments, in which this balance is shifted by deleting *cid14+*, indeed confirm this hypothesis (Claudia Keller & Rieka Stunnenberg, unpublished).

3.3.2. Inhibition of RNA binding in the cytoplasm

Once HP1^{Swi6} is synthesized in the cytoplasm, it would be formally possible that it binds a transcript in the cytoplasm. However, this is not likely to happen *in vivo*. Firstly, because RNAs appear hardly ever "naked" in the cytoplasm. They are covered with a plethora of factors that are required for many processes, for example translation (Hieronymus and Silver, 2004). Second, the hinge region also acts as a nuclear localization signal (NLS) (Keller et al., 2012; Wang et al., 2000). The nuclear localization of HP1^{Swi6} is disrupted in the swi6-KR25A mutant, showing that the residues that are required for RNA binding and nuclear localization at least partially overlap. Importin α directly binds to canonical NLS sequences with an affinity that is

considerably stronger than the HP1^{Swi6} RNA interactions (Goldfarb et al., 2004). Therefore, it is likely that Importin α binding to the hinge region not only translocates the protein to the nucleus but also inhibits binding of RNA in the cytoplasm. Interestingly, interaction of the hinge region with Importins was recently reported for human HP1α (Nozawa et al., 2010). Preliminary experiments with HP1^{Swi6} indicate that the same could hold true for fission yeast (Claudia Keller, unpublished).

3.3.3. Regulation in a cell-cycle dependent manner?

During mitosis, HP1^{Swi6} is lost from centromeres via H3S10 phosphorylation. This is thought to heterochromatic transcription and accumulation of RNA in G1/S phase. The rapid processing into sRNA promotes recruitment of RITS and the restoration of H3K9me and HP1^{Swi6} binding (Chen et al., 2008; Kloc et al., 2008; Li et al., 2011).
Is it possible, that during mitosis, HP1^{Swi6} binds to other RNA targets, because its local concentration is shifted? Or is RNA binding off-chromatin during these cell cycle stages inhibited for example via posttranslational modifications? Our current experiments did not address this issue at all, as unsynchronized fission yeast cells are mainly residing in the G2 phase and the M/G1 phase is very short. It would be interesting to analyze the properties of the nls-swi6-KR25A mutant in other cell cycle stages. As mentioned above, a starting point would be a proteomic analysis of HP1^{Swi6} complexes and PTMs in different cell cycle stages.

3.4. Conservation of HP1 RNA binding

3.4.1. Role of HP1 RNA binding in mammals

The hinge region, in contrast to the chromo- (CD) and chromoshadow-domain (CSD), is poorly conserved and much shorter in higher eukaryotes. It is therefore somewhat surprising, that HP1α also binds RNA via the hinge region (Muchardt et al., 2002). Assuming that the mere presence of Lys/Arg in an unstructured hinge region is enough, it would be expected that all the mammalian HP1s bind RNA. Consistently, we find that human and mouse HP1α, HP1β and HP1γ directly bind to RNA with similar affinity and binding mode (Veronika Ostapcuk & Claudia Keller, unpublished) although earlier experiments have suggested different binding

properties (Muchardt et al., 2002). The affinities of the mammalian HP1s to RNA are about 10-fold weaker than HP1^{Swi6}, which might reflect a smaller total number of amino acids including Lys/Arg in the hinge region (13 versus 25 in HP1^{Swi6}). It is likely, that this is the reason, why mutation of 3 Lys is already enough to abolish RNA binding (Muchardt et al., 2002), whereas in HP1^{Swi6}, mutation of 3 or 7 positively charged residues has no effect *in vitro* and *in vivo* (Claudia Keller, unpublished).

Figure 14 - Alignment of Swi6 with the human HP1 homologs

Whereas the CD and the CSD (blue border) are highly conserved from fission yeast to humans, the hinge region (green) is considerably smaller and shows less sequence conservation. The residues important for HP1α RNA binding (Muchardt et al., 2002) of are shown in red.

Several reports address the function of HP1 RNA binding in human cells. *In vitro* HP1α is a sequence-unspecific RNA binder (Muchardt et al., 2002). These authors were able to create a point mutant (3K>A), which fails to localize to the characteristic heterochromatic foci. Similarly, RNase treatment of human cells leads to dispersal of HP1α foci, which can be restored upon addition of nuclear RNA (Maison et al., 2002). In vitro, sumoylated HP1a preferentially binds to RNA. NEM treated nuclear extracts were shown to interact specifically with in-vitro transcribed RNA. In these pulldowns,

Sumo-HP1α is only detected with Major Forward, but not Reverse and Minor Satellite transcripts, indicating sequence specificity. From immunostaining experiments using different mutants it was concluded that sumoylation of the hinge region is required for *de novo* recruitment of HP1α (Maison et al., 2011). Consistent with our in vitro data, it was shown in another report that all the human HP1s interact with TERRA RNA, linking these proteins to a function telomere homeostasis (Deng et al., 2009). Another link of HP1s to RNA biology is the fact that the phenotypes that are caused by HP1γ disruption are similar to Miwi2-/- (Carmell et al., 2007) and Dnmt3L-/- mice (Bourc'his et al., 2001). Based on all these findings, the current model is that RNA binding is involved in HP1 recruitment to heterochromatin. Importantly, these properties of human HP1s seem to be fundamentally different from the fission yeast $HP1^{Swi6}$, in which RNA binding to the hinge region is inducing eviction (also see discussion below on other CD proteins in *S. pombe*).

For future studies, it will be instrumental to revisit the biochemical properties of HP1 RNA binding. This includes structural work by NMR spectroscopy as well as proteomic studies to find interaction partners of HP1s bound to RNA. This will be important to obtain point mutants, which are needed to address the functional role *in vivo*. Whole genome approaches (H3K9me and HP1 ChIP-Seq) instead of immunofluorescence are required to more directly assess the importance of HP1 RNA binding in establishing and maintaining heterochromatic domains. I also suggest to identify direct RNA targets *in vivo* in an unbiased approach using CLIP-Seq combined with expression analysis in RNA binding mutants. All these experiments should be performed in cells, in which HP1s are endogenously functioning in a certain biological process, for example in the male germline, where HP1γ might be involved in transposon silencing (Brown et al., 2010).

3.4.2. Connection of HP1 proteins to RNA in other eukaryotes

The HP1 protein Rhino, has been linked to transposon silencing via the piRNA pathway in the *Drosophila* germline (Klattenhoff et al., 2009). Rhino is a rapidly evolving HP1 member, which is expressed in ovaries. It has been suggested, that the function of this rapid evolution is to keep the activity of transposons in check

(Vermaak et al., 2005). It is interesting, that the hinge region of Rhino is even longer than the one of HP1^{Swi6} and furthermore, the overall amino acid composition is much more basic. It is therefore possible, that Rhino is an RNA binding protein that functions like HP1^{Swi6}. Rhino could recognize transposon transcripts and escort them to the RNA degradation machinery to ensure efficient elimination of transposon RNA.

	Rhino		Swi6	
	#	%	#	%
Ala (A)	14	3.30%	12	3.70%
Arg (R)	**19**	**4.50%**	**14**	**4.30%**
Asn (N)	19	4.50%	17	5.20%
Asp (D)	**26**	**6.20%**	**25**	**7.60%**
Cys (C)	5	1.20%	3	0.90%
Gln (Q)	15	3.60%	8	2.40%
Glu (E)	**25**	**6.00%**	**47**	**14.30%**
Gly (G)	24	5.70%	17	5.20%
His (H)	9	2.20%	9	2.70%
Ile (I)	23	5.50%	13	4.00%
Leu (L)	27	6.50%	13	4.00%
Lys (K)	**43**	**10.30%**	**40**	**12.20%**
Met (M)	12	2.90%	4	1.20%
Phe (F)	13	3.10%	4	1.20%
Pro (P)	28	6.70%	21	6.40%
Ser (S)	59	14.10%	40	12.20%
Thr (T)	24	5.70%	15	4.60%
Trp (W)	4	1.00%	5	1.50%
Tyr (Y)	6	1.40%	9	2.70%
Val (V)	23	5.50%	12	3.70%
Pyl (O)	0	0.00%	0	0.00%
Sec (U)	0	0.00%	0	0.00%
Total	418		328	

Figure 15 - Amino acid composition of Rhino versus Swi6

The number and % of the amino acids in Rhino and Swi6 are compared. Positively charged amino acids are marked in blue, negatively charged ones in red. Rhino is considerably longer and also contains less negatively charged amino acids.

In *Arabidopsis* LHP1 is required for maintaining the repressed state of the FLC locus, which controls vernalization and flowering (Mylne et al., 2006; Sung et al., 2006). Biochemically, LHP1 has not been studied extensively. It is known to bind both H3K9me and H3K27me *in vitro*. However, whether LHP1 binds RNA has not been addressed in those reports (Turck et al., 2007). LHP1 is slightly larger than HP1^{Swi6} and the presence of positively charged residues in the hinge region are conserved (see Figure 16). It would be very interesting to test, whether LHP1 RNA binding is involved in regulating FLC via direct association with this gene.

Figure 16 - Alignment of Swi6 with LHP1

The sequences of *S. pombe* Swi6 and *A. thaliana* LHP1 show remarkable conservation, which is not only limited to the CD and CSD (grey letters). This also includes a number of Lys and Arg, which could potentially be involved in RNA binding.

3.5. RNA binding in other chromatin proteins

3.5.1. Chromdomain proteins as RNA interaction modules

(a) Fission yeast CD proteins

HP1^{Swi6} is not the only fission yeast CD protein that binds RNA. Chp1 binds RNA via an RRM and the CD. This is very interesting, because the Swi6 CD does not bind RNA but overall, these domains are structurally very similar. Indeed, RNA binding is contributed by a unique positively charged surface in the C-terminal α-helix of the Chp1 CD (Ishida et al., 2012). Furthermore, the Clr4 CD is also able to bind RNA, however, only when bound to H3K9me. In contrast to Swi6, where binding of RNA induces changes that negatively affect H3K9me binding, the opposite is true for the CDs of Clr4 and Chp1. RITS tethering via RNA therefore occurs on two levels. sRNA-nascent transcript interaction via Ago1 and Chp1 CD interaction, which effectively tethers it to H3K9me chromatin. For the Clr4 CD the same function could apply. Therefore, CD proteins integrate RNA binding to functionally control very different processes: Tethering in the case of Chp1 and Clr4 versus eviction and RNA degradation in the case of Swi6 (Creamer and Partridge, 2012; Keller et al., 2012).

Again, it is worthwhile emphasizing again, that RNA crucially affects three hallmark proteins of heterochromatin, a region that has been suggested to be refractory to transcription for a long time. Last but not least, the fourth fission yeast CD protein Chp2 does not seem to bind RNA (Motamedi et al., 2008) and consistently, the Swi6 hinge region is unable to function in Chp2 (Sadaie et al., 2008).

(b) CD proteins in other organisms

CD proteins binding to RNA have been described in other organisms, too. The CD of the *Drosophila* histone acetyltransferase MOF is an RNA binding module (Akhtar et al., 2000). However, it should be noted that this domain adopts a β-barrel fold, which is different from canonical CDs (Nielsen et al., 2005). Therefore this module is also called chromo-barrel domain, which is structurally more similar to Tudor domains. Interestingly, this report demonstrated that the MOF CD is necessary but not sufficient for RNA binding. Similarly, the CD of MSL3, another protein involved in dosage compensation and required for H4K16ac, binds nucleic acids, too. Upon binding to DNA, the CD of MSL3 switches its properties and recognizes the repressive mark H4K20 (Kim et al., 2010a).

The mouse PcG protein Cbx7 binds RNA via the CD. Interestingly, Cbx7 contains affinity for both H3K9me and H3K27me and is enriched in facultative heterochromatin. The interaction of Cbx7 with RNA was suggested to be required for heterochromatin recruitment (Bernstein et al., 2006). Consistently, repression of the INK4b/ARF/INK4a locus by Cbx7 requires both H3K27me recognition and binding to the ncRNA ANRIL (Yap et al., 2010).

It is interesting that CDs are also found in some transposable elements, for example the Gypsy LTR family as well as the Tf1 LTR-retrotransposon in *S.pombe*. The exact function has not been defined yet, however, it was speculated that they might direct their integration into heterochromatin (Hizi and Levin, 2005; Novikova, 2009). It would be interesting to test, whether these proteins bind RNA.

Taken together, this reflects a whole repertoire of CD proteins, which can be modulated in different ways by RNA binding.

3.5.2. Other chromatin proteins and the role of ncRNAs

The discovery that a large proportion of the genome is transcribed into RNAs that do not encode for proteins has created a lot of excitement recently. Whereas it was debated for a long time whether these transcripts just reflect noise, recent studies suggested that these molecules are functional. The authors discovered a large number of lincRNAs based on a chromatin signature (K4-K36) that is characteristic of transcribed genes. Indeed, these lincRNAs seem to be regulated in a cell-type specific manner and show evolutionary conservation (Guttman et al., 2009; Guttman et al., 2011).

Currently, there are many reports trying to address the role of ncRNAs in regulating chromatin proteins (Guttman and Rinn, 2012). In the following, I want to name some of those examples, knowing, however, that a comprehensive picture of the molecular mechanisms underlying ncRNA function is not yet available. Particularly, it is very difficult to distinguish, whether the ncRNA itself, a ncRNA degradation product or the act of transcription of the ncRNA as such are needed for a certain function.

Cis-regulating ncRNAs are suggested to remain tethered to the locus from where they are transcribed and influence regulatory proteins by virtue of association directly on chromatin. For example, the Xist RNA is required for dosage compensation and was implicated in the recruitment of Polycomb to the inactive X chromosome (Chu et al., 2011; Zhao et al., 2008). Similarly, the SET-domain protein Ash1 is targeted to the Ultrabithorax (Ubx) by association with TRE transcripts produced from the Ubx locus (Sanchez-Elsner et al., 2006). *Trans*-acting ncRNA can exert their function on genomic regions that are far away from where they are transcribed or even on another chromosome. The ncRNA HOTAIR directly binds to PRC2 and is required for its targeting in human cells (Rinn et al., 2007). However, the function of this ncRNA does not seem to be conserved in mice, suggesting that it is rapidly evolving or redundant mechanism mask its function (Schorderet and Duboule, 2011). Similarly, some ncRNAs have been suggested to be involved in relocating the polycomb protein Pc2 within the nucleus to dynamically control gene expression (Yang et al., 2011). Others have been shown to act as enhancers where they might be guides for DNA looping (Orom et al., 2010). Intriguingly, ncRNA transcription from one allele could be an

elegant mechanism to maintain allele specific repression during genomic imprinting. This has been demonstrated for the Air ncRNA that recruits the HKMT G9a selectively to the allele that has to be repressed (Nagano et al., 2008).

Whereby in these examples, the ncRNA seems to act in targeting chromatin-modifying complexes to certain loci, alternative functions have also been described. ncRNA can serve as co-factors for certain enzymes by acting as ligands or allosteric regulators. Such a function was demonstrated for the ncRNA CCND1, which inhibits the CBP/p300 Histone-Acetyltransferase function and therefore leads to transcription repression (Wang et al., 2008b). Similarly, ncRNA act as scaffolds, for example the TERC RNA in telomerase. Last but not least, in the "decoy" function, ncRNAs prevent protein complexes from binding their regulatory targets. A ncRNA transcribed upstream of the DHFR promoter was shown to interact with TFIIB and dissociate the pre-initiation complex (PIC) efficiently from chromatin leading to gene repression (Martianov et al., 2007).

Finally, there are reports, where the act of transcription but not the ncRNA is required. Here the RNA itself, can be regarded as a byproduct. For example, chromatin remodeling of the fission yeast *fbp1+* gene is induced upon transcription of several ncRNAs (Hirota et al., 2008). In *S. cerevisiae*, intergenic transcription controls nucleosome positioning and gene expression by transcriptional interference (Hainer et al., 2010). The same is true for the PHO5 gene, in which ncRNA transcription enhances chromatin pasticity to mediate activation of this gene (Uhler et al., 2007).

Last but not least, in some instances the ncRNA only serves as a precursor for the production of sRNA species. For a discussion of their role in genome regulation, I refer to recently published reviews (Malone and Hannon, 2009; Wilusz et al., 2009).

In conclusion, many functions of RNAs and their role in regulating gene expression are being discovered at the moment. The current repertoire of proteins that are regulated by ncRNAs is dominated by chromatin factors. It will be interesting to see, whether ncRNA function expands beyond chromatin biology in the near future. Indeed, it was predicted, that lncRNA might at some point rival sRNAs and proteins being a novel class of biological molecules (Wang and Chang, 2011). Like proteins, the abundance of these molecules has to be tightly regulated and I therefore envision, that studying ncRNA synthesis and turnover is an attractive field for future studies.

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APPENDIX

1. Manuscript 1: Proteomic and functional analysis of the noncanonical poly(A) polymerase Cid14

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Proteomic and functional analysis of the noncanonical poly(A) polymerase Cid14

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ABSTRACT

The fission yeast Cid14 protein belongs to a family of noncanonical poly(A) polymerases which have been implicated in a broad range of biological functions. Here we describe an extensive Cid14 protein–protein interaction network and its biochemical dissection. Cid14 most stably interacts with the zinc-knuckle protein Air1 to form the Cid14–Air1 complex (CAC). Providing a link to ribosomal RNA processing, Cid14 sediments with 60S ribosomal subunits and copurifies with 60S assembly factors. In contrast, no physical link to chromatin has been identified, although gene expression profiling revealed that efficient silencing of a few heterochromatic genes depends on Cid14 and/or Air1.

Keywords: TRAMP; CAC; heterochromatin silencing; ribosome biogenesis; poly(A) polymerase; Cid14

INTRODUCTION

Proper gene expression requires polyadenylation of most eukaryotic mRNA 3′ ends by the canonical poly(A) polymerase (PAP), which has been shown to be important for RNA export, translation, and RNA stabilization. Besides the canonical PAP, eukaryotic cells possess noncanonical PAPs, which have been implicated in a broad range of biological processes and are conserved from yeast to humans. The fission yeast *Schizosaccharomyces pombe* encodes six noncanonical PAPs: Cid1, Cid11, Cid12, Cid13, Cid14, and Cid16 (Stevenson and Norbury 2006). Although initially classified as noncanonical PAPs, some of these enzymes have been demonstrated to add U residues (Kwak and Wickens 2007; Rissland et al. 2007). Cid14 is a nuclear enzyme which preferentially adds purines to RNA substrates in vitro, functions in ribosomal RNA (rRNA) processing and heterochromatic gene silencing, and is required for faithful chromosome segregation, proper siRNA generation by the RNA interference (RNAi) pathway, and maintenance of genomic integrity of the ribosomal DNA (rDNA) locus (Win et al. 2006; Bühler et al. 2007, 2008; Wang et al. 2008; Bühler 2009).

Cid14 is a functional ortholog of the two noncanonical PAPs, Trf4p/5p, found in the distantly related budding yeast *Saccharomyces cerevisiae* (Win et al. 2006). Both Trf4p and Trf5p are found together with predicted zinc-knuckle proteins Air1p/2p and the helicase Mtr4p in complexes termed TRAMP4 (Trf4p–Air1p/2p–Mtr4p; LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005) and TRAMP5 (Trf5p–Air1p–Mtr4p; Houseley and Tollervey 2006). The TRAMP complexes are considered to be cofactors of the yeast nuclear exosome that functions to process or degrade RNAs (Mitchell et al. 1997; Mitchell and Tollervey 2000).

Here we report the existence of a single TRAMP-like complex in *S. pombe*, consisting of Mtr4, Cid14, and Air1. Whereas Air1 and Cid14 form a stable complex, the association with Mtr4 is weak and occurs only in the presence of both Cid14 and Air1. Moreover, Cid14 sediments with 60S ribosomal subunits and copurifies with 60S assembly factors, providing a link to its role in ribosomal RNA processing. Previously we have shown that efficient silencing of transgene insertions at heterochromatic loci depends on Cid14 (Bühler et al. 2007). Here we demonstrate that silencing of a few endogenous heterochromatic genes depends on Cid14. In contrast to the factors implicated in ribosome biogenesis, no components have been identified that would link Cid14 to chromatin. Therefore, we propose that Cid14 functions off chromatin to control gene expression.

RESULTS AND DISCUSSION

Cid14 stably associates with the zinc-knuckle protein Air1

Previously, we have shown that Cid14 copurifies with a large number of proteins, including ribosomal proteins

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(RPs) and two proteins that are homologs of the budding yeast Mtr4p and Air1p/2p (Bühler et al. 2007). To better characterize this protein–protein interaction network, we revisited affinity chromatography under various conditions. We started our analysis by tandem affinity purifications of fully functional C-terminally TAP-tagged Cid14 (Cid14-TAP; Bühler et al. 2007) at different salt concentrations followed by analysis of the purification by SDS polyacrylamide gel electrophoresis and mass spectrometry. LC-MS/MS analysis of tryptic digests of protein mixtures of Cid14-TAP and control purifications revealed that Cid14 copurifies specifically with Air1 (*SPBP35G2.08c*), Mtr4 (*SPAC6F12.16c*), and a large number of RPs at 150 mM NaCl (Fig. 1A,D). Association with Mtr4 and RPs was gradually lost with increasing NaCl concentrations or upon treatment with RNase I (Fig. 1B,D). The only detectable interaction preserved at 500 mM NaCl was with Air1 (Fig. 1A,D; Supplemental Table S1). Thus, Cid14 and Air1 form a stable complex, independently of Mtr4. We refer to this complex as Cid14–Air1 Complex (CAC). Similarly, Trf4p has been shown to stably associate with either Air1p or Air2p in *S. cerevisiae*. Interestingly, this interaction is necessary for PAP activity (Vanacova et al. 2005; Wyers et al. 2005). In contrast, Cid14 shows PAP activity independently of Air1 (Bühler et al. 2007).

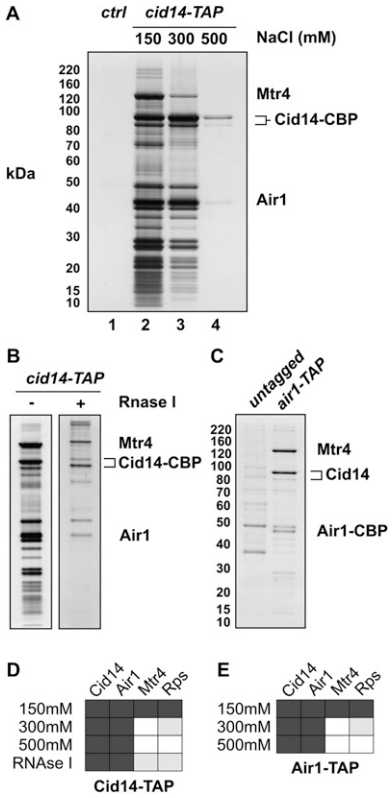


FIGURE 1. Cid14 interacts with Air1, Mtr4, and ribosomal proteins. (*A*) Silver-stained gel showing Cid14-TAP purifications under increasing salt conditions. The positions of Mtr4 (126 kDa), Cid14-CBP (83 kDa), a Cid14 degradation product, Air1 (35 kDa), and a molecular weight marker (*left*) are indicated. CBP, calmodulin binding peptide. (*B*) Silver-stained gel showing an RNase-treated Cid14-TAP purification. Five-hundred units of RNase I were added after the TEV-cleavage reaction for 1 h at RT. (*C*) Silver-stained gel showing an Air1-TAP purification (150 mM NaCl). (*D*,*E*) Table summarizing the LC-MS/MS results of the TAP purifications under various conditions (see also Supplemental Table S1). TAP elutions were TCA-precipitated and processed for LC-MS/MS analysis. RPs, ribosomal proteins. Black, gray, and white boxes indicate peptides that are, respectively, of high abundance, medium abundance, or absent in LC-MS/MS.

Mtr4, Cid14, and Air1 form a TRAMP-like complex in *S. pombe*

At low salt concentrations, Cid14 reproducibly copurified with Mtr4 and Air1, suggesting that a TRAMP-like complex also exists in *S. pombe*. To verify this, we constructed a strain expressing a C-terminally TAP-tagged Air1 protein (Air1-TAP). Affinity purification followed by mass spectrometry identified Cid14 and Mtr4 as Air1-interacting proteins, as well as RPs (Fig. 1C). Mtr4 and RP association was also sensitive to high salt concentrations (Fig. 1E and data not shown). Similarly, 500 mM NaCl washes have been demonstrated to dissociate Mtr4p from Trf4p, Trf5p, and Air2p in *S. cerevisiae* (LaCava et al. 2005). RNAse treatment of the Cid14-TAP complex bound to IgG beads prior to release by TEV cleavage did not abolish the recovery of Air1 and Mtr4 (Fig. 1B), whereas binding of RPs, in particular 40S ribosomal proteins, was reduced (Fig. 1B,D; Supplemental Table S1). This makes it unlikely that Mtr4, Cid14, and Air1 interact via substrate RNAs. Based on these results, we conclude that a TRAMP-like complex does exist in *S. pombe*.

In *S. cerevisiae*, Trf4p can either interact with Air1p or Air2p, suggesting the existence of two TRAMP complexes containing either Air1p or Air2p associated with Trf4p and Mtr4p (Wyers et al. 2005). Although *S. pombe* encodes for more than one Air1p/2p homolog, we consistently identified Air1 by LC-MS/MS from Cid14-TAP purifications (Supplemental Table S1). To rule out that a related zinc-knuckle protein could substitute in the absence of Air1, we purified Cid14-TAP expressed in *air1Δ* cells. These purifications did not reveal any other Air1 homologs associating with Cid14 (Fig. 2C,E; Supplemental Table S1). Thus, Air1 is the sole zinc-knuckle protein interacting with Cid14. Furthermore, we purified Air1-TAP from *cid14Δ* cells and found no other Cid14 homologs copurifying with Air1 (Fig. 2D). In conclusion, the association of CAC with Mtr4 represents the only TRAMP-like complex in *S. pombe*. Importantly, Cid14-TAP purifications from *air1Δ* cells revealed that Mtr4 no longer interacts with Cid14 in the absence of Air1 (Fig. 2C,E). This may suggest that Air1 mediates the interaction with Mtr4. However, Mtr4 was also lost when we purified Air1-TAP from *cid14Δ* cells (Fig. 2D,E). Therefore, an intact CAC complex is required for TRAMP formation in fission yeast.

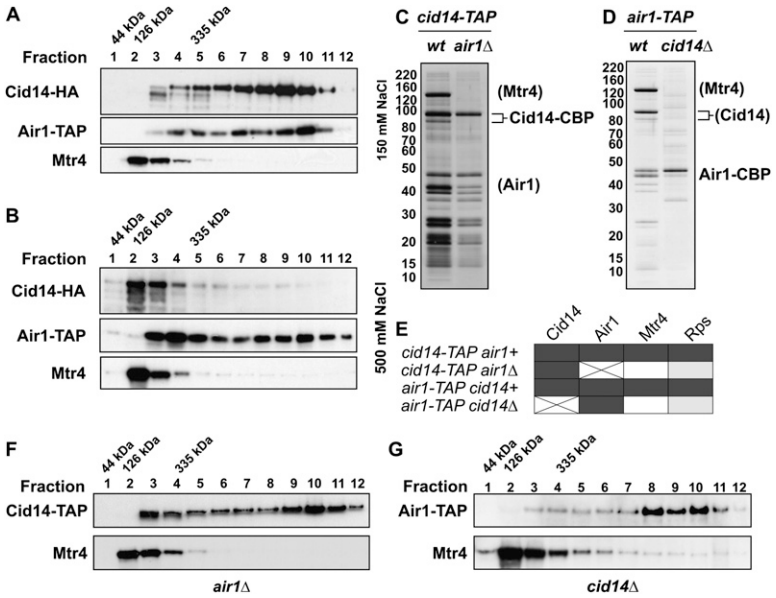


FIGURE 2. Cid14 resides in high and low molecular weight complexes. (*A*) Sucrose gradient fractionation under low salt conditions (150 mM NaCl). Individual fractions from sucrose density gradients were analyzed by Western blotting with antibodies recognizing Cid14-HA, Air1-TAP, or Mtr4. (*B*) Sucrose gradient fractionation under high salt conditions (500 mM NaCl). The analysis was performed as in *A*. (*C*) Silver-stained gel showing Cid14-TAP purifications performed with wild-type and *air1Δ* cells. (*D*) Silver-stained gel showing Air1-TAP purifications performed with wild-type and *cid14Δ* cells. (*C*,*D*) Salt concentration was 150 mM. (*E*) Table summarizing LC-MS/MS results of TAP purifications shown in *C* and *D*. Black, gray, and white boxes indicate peptides that are of high abundance, medium abundance, and absent in LC-MS/MS, respectively. (*F*,*G*) *S. pombe* lysates from *cid14-TAP air1Δ* and *air1-TAP cid14Δ* cells were separated by sucrose density gradient centrifugation. (*A*,*B*,*F*,*G*) *S. pombe* total cell lysates were loaded onto an 18%–54% sucrose gradient and protein complexes were separated by ultracentrifugation at 39,000 rpm for 18 h.

Cid14 associates with 60S ribosomal subunits and assembly factors

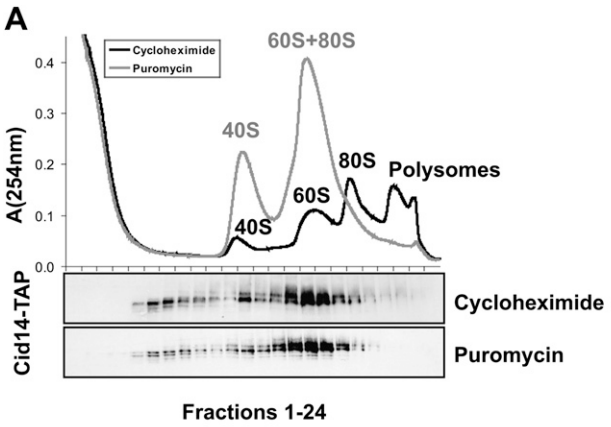
The results described above show that Cid14 resides in at least two biochemically distinct protein complexes, CAC and TRAMP. Importantly, Cid14 has previously been shown by gel filtration experiments to be part of a complex much larger than CAC and TRAMP (Win et al. 2006). Consistently, sucrose gradient fractionation indicated that Cid14 is part of both low and high molecular weight protein assemblies (Fig. 2A). We observed the same for Air1, but not Mtr4. Mtr4 sedimented mainly in fraction 2, which represents its own molecular weight of ~126 kDa (Fig. 2A,B). Thus, Mtr4 seems unlikely to be a stable component of any larger protein assemblies. Furthermore, only a small fraction of the Mtr4 population seems to be associated with CAC to form *sp*TRAMP, similar to what has also been described for Mtr4p in *S. cerevisiae* (LaCava et al. 2005).

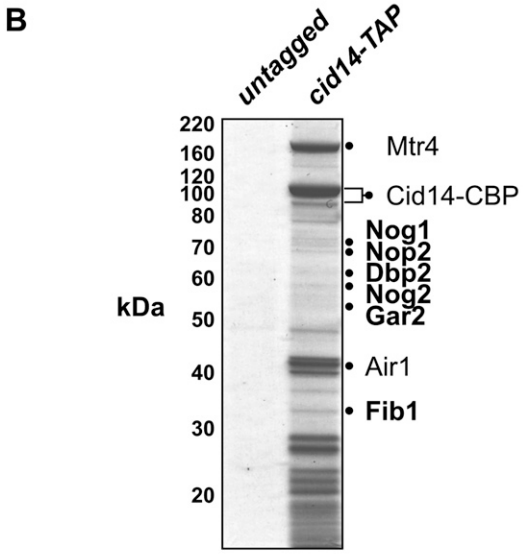
The high number of copurifying RPs and the sedimentation of Cid14 in high molecular weight fractions is indicative of an association with ribosomes. Interestingly, Cid14 has been reported to be involved in 25S rRNA processing (Win et al. 2006), suggesting that Cid14 might interact with ribosomal proteins during assembly of the large ribosomal subunit. Therefore, we performed ribosome fractionation on sucrose gradients ranging from 10% to 50% by centrifugation for 15 h followed by Western blotting to detect Cid14-TAP. Consistent with its known role in 25S rRNA processing, Cid14 was mainly detected in fractions representing the 60S large ribosomal subunit (Fig. 3A). Importantly, five proteins known to be involved in 60S biogenesis could be identified by LC-MS/MS after reducing the complexity of our Cid14-TAP purification by SDS-PAGE separation and performing the tryptic digest on individual gel bands (Fig. 3B). Thus, we conclude that the higher molecular weight Cid14 complex represents a 60S ribosomal subunit assembly protein–protein interaction network.

Silencing of a few endogenous heterochromatic genes depends on Cid14

Previously we have shown that efficient silencing of transgene insertions at heterochromatic loci depends on Cid14 (Buhler et al. 2007). However, it remained to be tested to what extent Cid14 also functions in heterochromatic silencing of endogenous genes and whether this depends on an intact TRAMP complex. Therefore, we hybridized total RNA isolated from wild-type, *cid14Δ*, and *air1Δ* cells to affymetrix tiling arrays. Taking the average of two biological replicates and using a cutoff of 1.5-fold, 149 and 323 genes were shown to be up-regulated in *cid14Δ* and *air1Δ* cells, respectively, while 73 and 86 were down-regulated (Fig. 4A,B). Interestingly, the genes differentially expressed in *cid14Δ* and *air1Δ* cells overlapped only partially, suggesting that Air1 and Cid14 can also function outside the CAC or TRAMP complexes (Fig. 4A,D). Consistent with this, we noticed that both Cid14 and Air1 associated with high molecular weight protein assemblies independently of each other (Fig. 2F,G).

Comparing the expression in *cid14Δ* to previously published H3K9me2 and $HP1^{Swi6}$ ChIP-on-chip data (Cam et al. 2005) revealed that only a small set of heterochromatic genes was up-regulated in *cid14Δ* (Fig. 4E; Supplemental Table S2). The majority of these are subtelomeric genes, as previously described (Wang et al. 2008). Importantly, not all of these heterochromatic genes were up-regulated in *air1Δ* cells, suggesting that an intact CAC and/or TRAMP complex is not always necessary to





Protein	GeneDB	kDa	%	#	function
Nop2	SPBP8B7.20c	68.9	7	3	60S biogenesis
Nog1	SPBC651.01c	72.8	10	5	60S biogenesis
Gar2	SPAC140.02	53	12	4	40S
Dbp2	SPBP8B7.16c	61.5	4	2	60S
"Nog2"	SPAC6F6.03c	59.9	4	2	60S
Fib1	SPBC2D10.10c	32	12	3	40S/60S

FIGURE 3. Cid14 associates with 60S ribosomal subunits and 60S ribosome assembly factors. (*A*) Sedimentation behavior of Cid14-TAP in 10%–50% ribosome sucrose gradients. UV profile (OD at 254 nm) with ribosomal subunits, mono- and polysomes is indicated. Samples were treated with cycloheximide to stabilize or puromycin to disrupt polysomes. Twenty-four fractions were collected and analyzed by Western blotting against Cid14-TAP. (*B*) Cid14-TAP and control purifications from 20 g of cells were separated by SDS-PAGE followed by Coomassie-staining. Bands were cut out and LC-MS/MS analysis was performed on in-gel processed samples. Positions of the bands and the corresponding proteins indentified by LC-MS/MS are indicated. %, percent sequence coverage; # number of unique peptides.

silence heterochromatin (Supplemental Table S2). Although future work on Air1 and its RNA binding properties will be required to rule out alternative functions, we speculate that Air1 functions as an RNA adaptor to support the association of Cid14 with its substrate. Depending on yet to be determined characteristics of a Cid14 substrate, Air1 might be more or less important for this.

Conclusions

Our findings that Cid14 associates with 60S ribosomal subunits and with proteins known to be involved in 60S biogenesis strongly suggest that Cid14 is directly involved in the assembly of pre-ribosomes. This is further supported

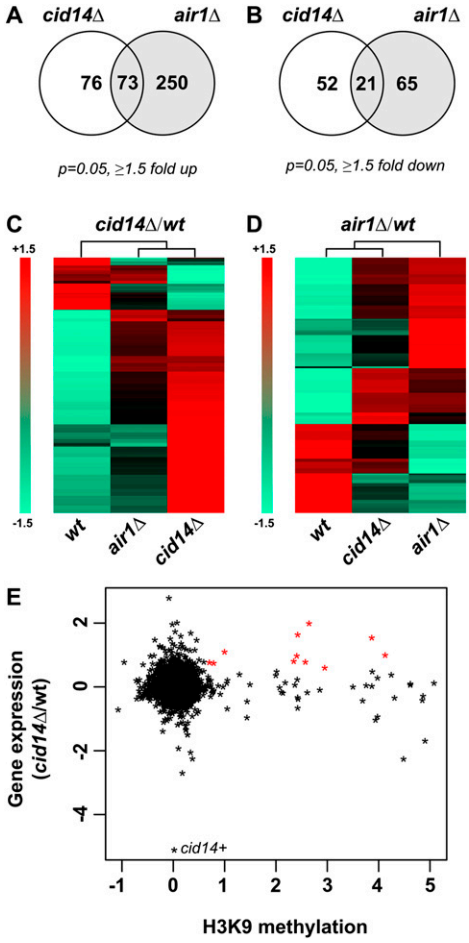


FIGURE 4. Differential gene expression in *cid14Δ* and *air1Δ* cells. (*A*,*B*) Venn diagrams showing the number of genes up- or down-regulated at least 1.5-fold in *cid14Δ* or *air1Δ* cells compared to wild type ($P = 0.05$) on *S. pombe* tiling arrays. Two biological replicates were analyzed. (*C*) Heatmap displaying the genes which were up- or down-regulated at least 1.5-fold ($P = 0.05$) in *cid14Δ* cells compared to wild type on *S. pombe* tiling arrays. (*D*) Heatmap displaying the genes which were up- or down-regulated at least 1.5-fold ($P = 0.05$) in *air1Δ* cells compared to wild type on *S. pombe* tiling arrays. (*C*,*D*) Artificially scaled expression values are shown for the strains indicated (−1.5 is set for the gene with the lowest expression and +1.5 is set for the gene with the highest expression). (*E*) Comparison of genes up-regulated in *cid14Δ* cells to previously published H3K9me2 ChIP-on-chip data (Cam et al. 2005). Asterisks in red indicate those genes that have a value >0.6 (log scale) in both the expression and ChIP experiments (listed in Supplemental Table S2).

by the nucleolar localization of Cid14 and its role in rRNA metabolism (Win et al. 2006; C Keller and M Bühler, unpubl.). Therefore, we propose that Cid14 is physically linked to ribosome biogenesis. Cid14 also functions in eliminating a variety of RNAs, amongst them transcripts originating from subtelomeric heterochromatin (this study; Bühler et al. 2007; Wang et al. 2008). In contrast to RPs and ribosome assembly factors, we were not able to identify proteins which could link Cid14 to heterochromatin physically. Therefore, we favor a model in which Cid14 recognizes and eliminates heterochromatic RNAs off chromatin. Finally, our biochemical and functional data suggest that Cid14 may at least partially function outside of an intact TRAMP complex. Future work will be required to elucidate substrate characteristics and requirements for Cid14 and/or the TRAMP complex.

MATERIALS AND METHODS

Strains and plasmids

Fission yeast strains used in this study are described in Supplemental Table S3 and were grown at 30°C in YES (Yeast Extract with Supplements). All strains were constructed following a standard PCR-based protocol (Bahler et al. 1998).

Tandem affinity purification

A 2-L culture of TAP-tagged *S. pombe* cells (OD at 600 nm ≈ 2) was pelleted, washed once in ice-cold PBS, resuspended in 1/4 pellet volume of lysis buffer (6 mM Na_2HPO_4, 4 mM $NaH_2PO_4•H_2O$, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 50 mM NaF, 4 μg/mL leupeptin, 0.1 mM Na_3VO_4, 1 mM PMSF, 1× Protease Inhibitors Complete EDTA free [Roche]), and pelleted into N_2(l). Ten grams of cells were then disrupted by cryo-grinding with a Retsch MM 400 (3 × 3 min at 30 Hz). Sixteen milliliters of lysis buffer was added to the powder and stirred for ca. 15 min in the cold room. The salt concentration was now adjusted, if required. The lysate was spun for 25 min at 12,000 rpm (4°C). The supernatant was then incubated with 200 μL of IgG-Sepharose for 2 h at 4°C on a rocker. The beads were transferred to a column and washed three times with 10 mL of washing buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40) and once with TEV-cleavage buffer (10 mM Tris-HCl [pH 8.0], 150 mM KOAc, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). The TEV-cleavage reaction was performed using 50 U of acTEV (Invitrogen) in 1 mL of TEV-cleavage buffer for 1 h at 25°C. Where indicated, the RNase I (Ambion) treatment was subsequently performed for 1 h at RT using 500 U. The eluate was then transferred to a new column and the old column was washed out with 0.5 mL of TEV-c buffer. Three milliliters of Calmodulin-binding buffer (CAM-B: 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM $Mg[OAc]_2$, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol), 4.5 μL of 1 M $CaCl_2$, and 150 μL of Calmodulin-Sepharose were added and incubated for 1 h at 4°C on a rocker. The beads were washed twice with 1.5 mL CAM-B (0.1% NP-40) and once with 1.5 mL CAM-B (0.02% NP-40). The purified proteins were eluted from the column using 1 mL of CAM-E (=CAM-B, but replacing the $CaCl_2$ with 10 mM EGTA). The eluate was split into two aliquots and each of them was TCA-precipitated. One pellet was resuspended in 1× LDS sample buffer and run on a 4%–12% NuPAGE gel (Invitrogen) using MOPS buffer followed by silver- or Coomassie-staining (Colloidal Blue Staining kit, Invitrogen). The other pellet was used for mass spectrometric analysis.

Sucrose density gradient centrifugation

The lysate was prepared as for the TAP purifications (0.5 g of cryo ground powder). After the high-speed spin, 300 μL of the lysate was loaded onto a 18%–54% sucrose gradient (buffered with 20 mM Tris-HCl [pH 7.5], 150 mM KCl, 1 mM DTT, 1 mM PMSF). Complexes were separated by ultracentrifugation for 18 h at 39,000 rpm (4°C) with an SW40 rotor (Beckman). The gradient was unloaded from the bottom with 70% sucrose. Twelve fractions of 1 mL were taken using a fraction collector while reading the absorbance at 254 nm with a UV reader. Twenty-eight microliters of the fractions was separated on a 4%–12% NuPAGE gel, blotted to nitrocellulose (1.5 h at 200 mA), and the proteins of interest were detected using the ECL system. Antibodies were used at 1:10,000 (α-PAP, Sigma), 1:20 (α-HA, FMI monoclonal antibody), 1:3000 (α-Mtr4, custom polyclonal, Eurogentec).

For the ribosome fractionation, 100 μg/mL cycloheximide or 1 mM puromycin was added to 250 mL of an exponentially growing *S. pombe* culture. The culture was incubated for another 10 min at 30°C and then pelleted. The cells were washed once and then resuspended in 0.5 mL of lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1× Protease Inhibitors Complete EDTA-free [Roche], 100 μg/mL Cycloheximide or 1 mM puromycin). For the puromycin treated sample, the $MgCl_2$ concentration in all buffers was reduced to 1 mM. One milliliter of glass beads was added and the cells were disrupted using a bead-beater (4 × 30 sec). The lysate was spun for 15 min at 16,000 rpm, 4°C, and then 300 μL of the supernatant was loaded onto a 10%–50% gradient, which was prepared as described above. The ultracentrifugation was carried out for 15 h at 27,000 rpm in a SW40 rotor (Beckman). In this case, 24 fractions of 0.5 mL were collected as described above.

Mass spectrometry

SDS-PAGE separated proteins and TCA-precipitated and acetone-washed protein pellets were reduced with TCEP, alkylated with iodoacetamide, and digested with trypsin. The generated peptides were analyzed by NanoLC-MSMS on a 4000Q Trap as described (Supplemental Table S1; Hess et al. 2008). The proteins were identified with Mascot searching Uniprot 15.6 (Perkins et al. 1999).

S. pombe tiling arrays and data analysis

RNA was isolated from cells collected at OD_{600} = 0.5 using the hot phenol method (Leeds et al. 1991). The isolated RNA was processed according to the GeneChip Whole Transcript (WT) Double-Stranded Target Assay Manual from Affymetrix using the GeneChip *S. pombe* Tiling 1.0FR. For analysis of the tiling arrays, an R-based script was used, which is available upon request. We used the genome and annotations from the *S. pombe* Genome

Project (http://www.sanger.ac.uk/Projects/S_pombe/). The oligos from the Affymetrix .BPMAP file were remapped using bowtie and the .GFF file was used to map them to the genes. The resulting .CDF file is available upon request. The expression data from *cid14Δ* was compared to ChIP-on-chip data for H3K9me2 (Cam et al. 2005) by plotting, for each annotated element, enrichment/input for the ChIP data against *cid14Δ*/wt for the expression data. The average of two biological replicates was taken for each data set.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org. Tiling array data are reposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE20905.

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HP1^{Swi6} Mediates the Recognition and Destruction of Heterochromatic RNA Transcripts

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SUMMARY

HP1 proteins are major components of heterochromatin, which is generally perceived to be an inert and transcriptionally inactive chromatin structure. Yet, HP1 binding to chromatin is highly dynamic and robust silencing of heterochromatic genes can involve RNA processing. Here, we demonstrate by a combination of in vivo and in vitro experiments that the fission yeast HP1^{Swi6} protein guarantees tight repression of heterochromatic genes through RNA sequestration and degradation. Stimulated by positively charged residues in the hinge region, RNA competes with methylated histone H3K9 for binding to the chromodomain of HP1^{Swi6}. Hence, HP1^{Swi6} binding to RNA is incompatible with stable heterochromatin association. We propose a model in which an ensemble of HP1^{Swi6} proteins functions as a heterochromatin-specific checkpoint, capturing and priming heterochromatic RNAs for the RNA degradation machinery. Sustaining a functional checkpoint requires continuous exchange of HP1^{Swi6} within heterochromatin, which explains the dynamic localization of HP1 proteins on heterochromatin.

INTRODUCTION

Heterochromatin is a distinct chromatin structure that is late replicating, gene poor, and rich in transposons or other parasitic genomic elements. Heterochromatic structures are required for proper centromere function, repression of recombination, sister chromatid cohesion, and the maintenance of telomere stability, and they also play an essential role in heritable gene silencing in a variety of organisms from yeast to humans (Grewal and Jia, 2007). One hallmark of heterochromatin is its association with members of the highly conserved heterochromatin protein 1 (HP1) family of proteins (James and Elgin, 1986). HP1 proteins consist of an N-terminal chromodomain (CD) and a structurally related C-terminal chromo shadow domain (CSD), separated by a hinge region. The CSD can mediate homodimerization of HP1 and binding to other proteins through a degenerate pentapeptide motif, PxVxL (Cowieson et al., 2000; Smothers and Henikoff, 2000). The CD binds the N-terminal tail of histone H3 when it is di- or trimethylated with high specificity but low affinity (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001; Lachner et al., 2001; Nielsen et al., 2002) and the hinge region has been implicated in nucleic acid binding (Muchardt et al., 2002). The fission yeast *Schizosaccharomyces pombe* contains two HP1 homologs, HP1^{Chp2} and HP1^{Swi6}, which both bind to methylated lysine 9 of histone H3 (H3K9) and are involved in heterochromatin silencing (Grewal and Jia, 2007). In contrast to other eukaryotes, *S. pombe* contains only a single member of the SUV39 histone methyltransferase family of proteins, Clr4, which is responsible for the methylation of H3K9 (Nakayama et al., 2001).

Heterochromatin is generally perceived to be a structurally rigid and static chromatin compartment that is inaccessible to the transcription machinery, yet several findings challenge this view. For example, the H3K9 methyl-binding affinity of HP1 proteins can be rather low, and their association with heterochromatin is surprisingly dynamic (Cheutin et al., 2004, 2003; Festenstein et al., 2003; Schalch et al., 2009). Furthermore, recent work has revealed that both RNAi-dependent and -independent RNA turnover mechanisms are crucial for the quiescence of heterochromatic sequences in *S. pombe*, indicating that silencing of heterochromatin does not occur exclusively at the transcriptional level (Bühler et al., 2007). Repression of marker genes when inserted into heterochromatin depends on the noncanonical poly(A) polymerase Cid14, which is thought to target the heterochromatic RNA for degradation via the RNA exosome and/or the RNAi pathway. Similarly, silencing of subtelomeric genes marked by H3K9 methylation also depends on Cid14 (Keller et al., 2010; Wang et al., 2008). Importantly, heterochromatic gene silencing is impaired in Cid14 mutant strains, yet heterochromatin remains intact (Bühler et al., 2007). Thus, some level of transcription within heterochromatin is possible, and pathways to cope with the unwanted heterochromatic RNA do exist (Bühler, 2009). However, the mechanism of specific recognition of heterochromatic transcripts and thus their targeting for the Cid14-dependent degradation has remained elusive.

HP1^{Swi6}, one of the two *S. pombe* heterochromatin proteins, is best known for its critical role in proper centromere function. In *swi6* mutant cells, centromeres lag on the spindle during anaphase, and chromosomes are lost at a high rate (Ekwall et al., 1995). This is associated with a failure in the recruitment of cohesin to pericentromeric heterochromatin (Bernard et al.,

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2001; Nonaka et al., 2002). Thus, one function of HP1^{Swi6} is the attraction of a high concentration of cohesin to *S. pombe* centromeres, which guarantees proper chromosome segregation. HP1^{Swi6} has also been implicated in the recruitment of cohesin outside constitutive heterochromatin, thus regulating transcription termination between convergent gene pairs (Gullerova and Proudfoot, 2008). Besides cohesin subunits, HP1^{Swi6} also copurifies with a diverse set of other nuclear nonhistone proteins that are involved in a variety of nuclear functions such as chromatin remodelling and DNA replication (Fischer et al., 2009; Motamedi et al., 2008). Even though many of these interactions remain to be confirmed, HP1^{Swi6} may partner with many different factors and ensure genomic integrity. Apart from these functions, HP1^{Swi6} is also required for heterochromatic gene silencing, but on a mechanistic level this is poorly understood.

Here, we demonstrate that HP1^{Swi6} serves a general function linking transcription within heterochromatin to downstream RNA turnover. HP1^{Swi6} binds RNA via a molecular mechanism that involves the hinge region, the CD, and the N-terminal domain. Rather than tethering heterochromatic transcripts to chromatin, HP1^{Swi6} complexed with RNA dissociates from H3K9-methylated nucleosomes and escorts its associated RNAs to the RNA decay machinery. This detachment of HP1^{Swi6} from chromatin results from a competition mechanism that combines the interactions of RNA and methylated H3K9 to HP1^{Swi6} on the single-molecule level with dynamic exchange between the histone-bound and -unbound HP1^{Swi6} ensemble. Our results provide an explanation for the dynamic localization of HP1 proteins on heterochromatin and reveal insights into the role of RNA in the regulation of higher order chromatin structures.

RESULTS

Heterochromatic mRNA Transcripts Are Not Translated into Protein

Previous work revealed that the noncanonical polyA-polymerase Cid14 processes or eliminates a variety of RNA targets to control processes such as the maintenance of genomic integrity, meiotic differentiation, ribosomal RNA maturation, and heterochromatic gene silencing (Keller et al., 2010; Wang et al., 2008; Win et al., 2006). The effect of *cid14+* mutations on heterochromatin silencing has previously been studied using the *ura4+* reporter gene/5-FOA assay (Bühler et al., 2007). Because this assay does not allow a quantification of the resulting protein levels, and because it is also compromised by a general sensitivity of *cid14+* mutant cells to 5-FOA (Figure S1), we created reporter strains carrying a *gfp+* transgene inserted at the innermost centromeric repeat region (*imr1R::gfp+*) or at the *mat3M* locus (*mat3M::gfp+*) (Figure 1A). Consistent with previous results (Bühler et al., 2007), heterochromatic *gfp+* mRNA levels from centromeric locations increased significantly in *clr4Δ* and *dcr1Δ* cells, but only modestly in *cid14Δ* cells (Figure 1B), with no corresponding increase in GFP protein levels upon *cid14+* deletion (Figures 1C and S1A). Therefore, Cid14 plays a redundant role, if any at all, in the silencing of a reporter gene located in centromeric heterochromatin. In contrast, deleting the *cid14+* gene resulted in strongly elevated *gfp+* mRNA levels from the mating-type locus. Unexpectedly, however, this was not accompanied by a concomitant increase in GFP protein levels (Figures 1D and E).

To test whether mRNAs originating from heterochromatic genes engage in translation at all, we set out to profile their association with polyribosomes (Figure 1F). *S. pombe* cell lysates were separated on sucrose gradients and RNA was extracted from the individual fractions. The relative amount of a given mRNA in each fraction was then quantified by quantitative real-time RT-PCR (qRT-PCR). As expected, *act1+* mRNA was highly enriched, whereas the nuclear U6 snRNA was absent from the polysomal fractions (Figure 1F). When transcribed from its endogenous locus, mRNA encoded by the *ura4+* gene was also highly enriched in polysomes (data not shown). Similarly, *ura4+* mRNA originating from a *mat3M::ura4+* reporter was found in the polysomal fractions in the absence of the H3K9 methyltransferase Clr4. However, no considerable association with polysomes was observed for heterochromatic *ura4+* reporter mRNA in wild-type or *cid14Δ* cells (Figure 1F).

Thus, although heterochromatic mRNAs can be over 10-fold more abundant in *cid14Δ* cells than in wild-type cells, they are not translated into protein effectively.

HP1^{Swi6} Functions as an H3K9 Methylation-Specific Checkpoint to Assemble Translationally Incompetent Ribonucleoprotein Particles

Atypical processing of 5′ or 3′ ends of heterochromatic mRNAs could explain why heterochromatic mRNAs do not engage in translation. However, our analysis of mRNA termini revealed no major differences between heterochromatic and euchromatic transcripts (Figure S2 and data not shown), suggesting that heterochromatic mRNAs per se do not contain aberrant features that would signal their destruction or render them translationally inactive. Rather, transcripts emerging from heterochromatin are more likely to be channeled into the RNA decay pathway by the assembly of a heterochromatin-specific ribonucleoprotein particle (hsRNP). Therefore, we postulate the existence of an H3K9 methylation-specific checkpoint that would function on chromatin and assemble emerging transcripts into hsRNPs that are translationally incompetent and prone for degradation (Figure 2A).

Obvious candidates for proteins that could function as such a checkpoint are HP1 proteins, because they have been reported to have affinity for both H3K9-methylated histone H3 tails and RNA. Therefore, HP1 proteins might capture heterochromatic RNAs in an H3K9 methylation-specific manner. The *S. pombe* genome contains two HP1 homologs, HP1^{Chp2} and HP1^{Swi6}. Interestingly, even though HP1^{Swi6} is essential for the full repression of heterochromatin, its contribution to transcriptional gene silencing is minimal. Furthermore, heterochromatic RNAs have been observed to copurify with HP1^{Swi6} but not HP1^{Chp2} (Motamedi et al., 2008).

Therefore, we tested whether heterochromatic mRNAs would become translated in cells lacking HP1^{Swi6}. Consistent with the checkpoint model, GFP protein expression from the *mat3M::gfp+* allele was restored in *swi6Δ* and *swi6Δ cid14Δ* cells (Figure 2B). However, deletion of *swi6+* also resulted in a significant reduction in H3K9me2 at *mat3M::gfp+* (Figure 2C),

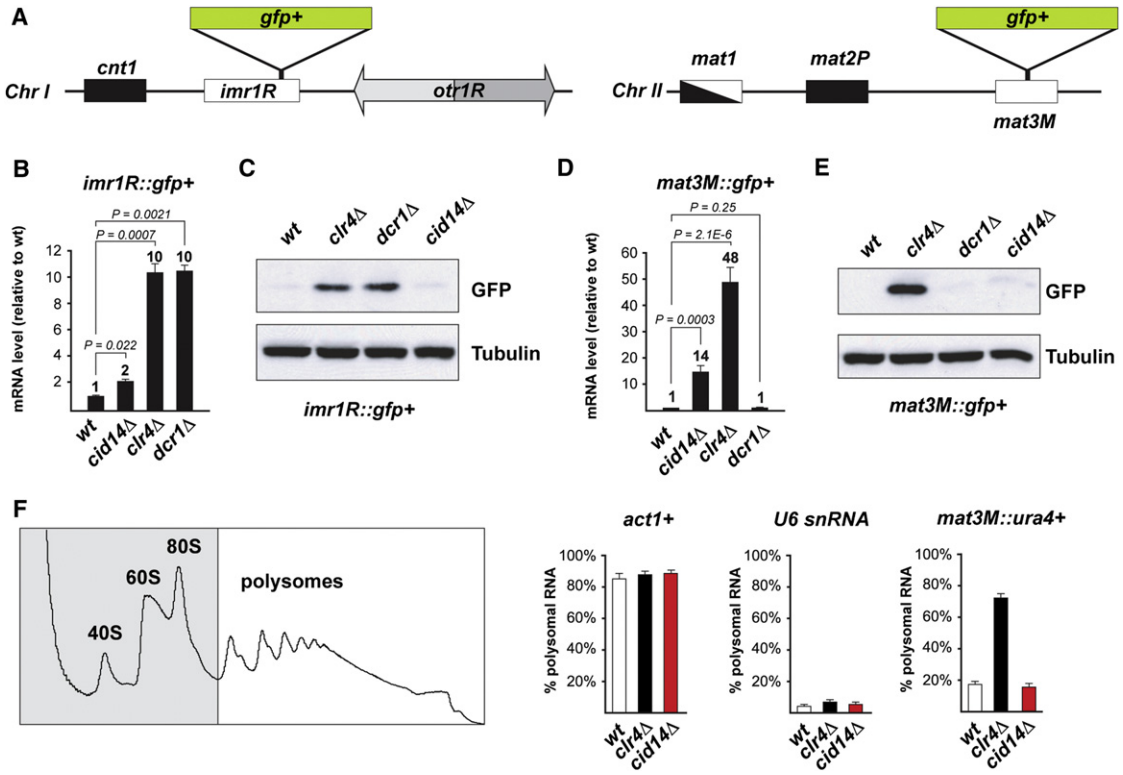

Figure 1. Heterochromatic mRNAs Are Not Translated into Protein

(A) Diagram representing DNA organization at the centromere of chromosome I and at the mating-type locus (chromosome II). cnt1, central core; imr1, innermost repeats; otr1, outermost repeat. *gfp+* reporter transgenes are driven by the *ura4+* promoter, whereas the ORF is followed by a natMX6 cassette (Tadh1 terminator).

(B) Quantitative real-time RT-PCR showing *gfp+* mRNA levels in *imr1R::gfp+* cells. Mean values normalized to *act1+* are shown (n = 3). Error bars represent SEM; p values were calculated using the Student's t test.

(C) Western blot showing GFP protein levels in *imr1R::gfp+* cells. Total protein from an equivalent number of cells was extracted by TCA. Tubulin served as a loading control.

(D) Quantitative real-time RT-PCR showing *gfp+* mRNA levels in *mat3M::gfp+* cells. Mean values normalized to *act1+* are shown (n = 14). Error bars represent SEM, p values were calculated using the Student's t test.

(E) Western blot showing GFP protein levels in *mat3M::gfp+* cells. Total protein from an equivalent number of cells was extracted by TCA. Tubulin served as a loading control.

(F) A representative polysome profile (OD 254 nm) with monosomal (fractions 1–5) and polysomal fractions (fractions 6–12 polysomal) is shown on the left. RNA levels were determined by quantitative real-time RT-PCR and the enrichment in the polysomal fraction was calculated as a percentage of the total. Error bars represent SEM. Act1+ RNA and U6 snRNA served as positive and negative controls, respectively.

not allowing us to definitely assign the checkpoint function to HP1^{Swi6}. In contrast, deletion of *swi6+* or *cid14+* or both did not significantly lower H3K9 methylation levels at the subtelomeric *tlh1/2+* genes, yet resulted in a strong upregulation of the respective mRNAs (Figures 2D and 2E). Importantly, association of *tlh1/2+* mRNA with polysomes was only observed in cells lacking *swi6+* but not *cid14+* (Figure 2F). These results place HP1^{Swi6} upstream of Cid14 and directly support a model in which HP1^{Swi6} acts on H3K9-methylated nucleosomes and promotes the assembly of translationally incompetent hsRNPs.

HP1^{Swi6} Binds RNA via the Hinge Region

The above results implicate HP1^{Swi6} in the checkpoint model as the H3K9 methylation "reader," yet it was not clear whether HP1^{Swi6} itself or any of its interacting proteins could capture heterochromatic RNAs. Whereas RNA-binding affinity has been demonstrated for mammalian HP1α (Muchardt et al., 2002), it was not known whether fission yeast HP1^{Swi6} can bind RNA directly. We purified recombinant HP1^{Swi6} and performed electrophoretic mobility shift assays (EMSA) using various RNA and DNA probes. In these assays, recombinant HP1^{Swi6} bound efficiently to the different RNAs but only weakly to DNA (Figure 3B). Furthermore, RNA binding could be competed with unlabeled RNA probes (Figure S3). HP1^{Swi6} consists of four domains: An N-terminal domain (NTD, residues 1–74), which is presumably flexibly disordered; a chromodomain (CD, residues 75–139), which binds K9-methylated histone tails (Bannister et al., 2001); a hinge region (H, residues 140–264); and a C-terminal chromo shadow domain (CSD, residues 265–328) (Cowieson et al., 2000). The hinge region of mammalian HP1α has been implicated in RNA binding (Muchardt et al., 2002). To test whether the hinge region also confers RNA binding

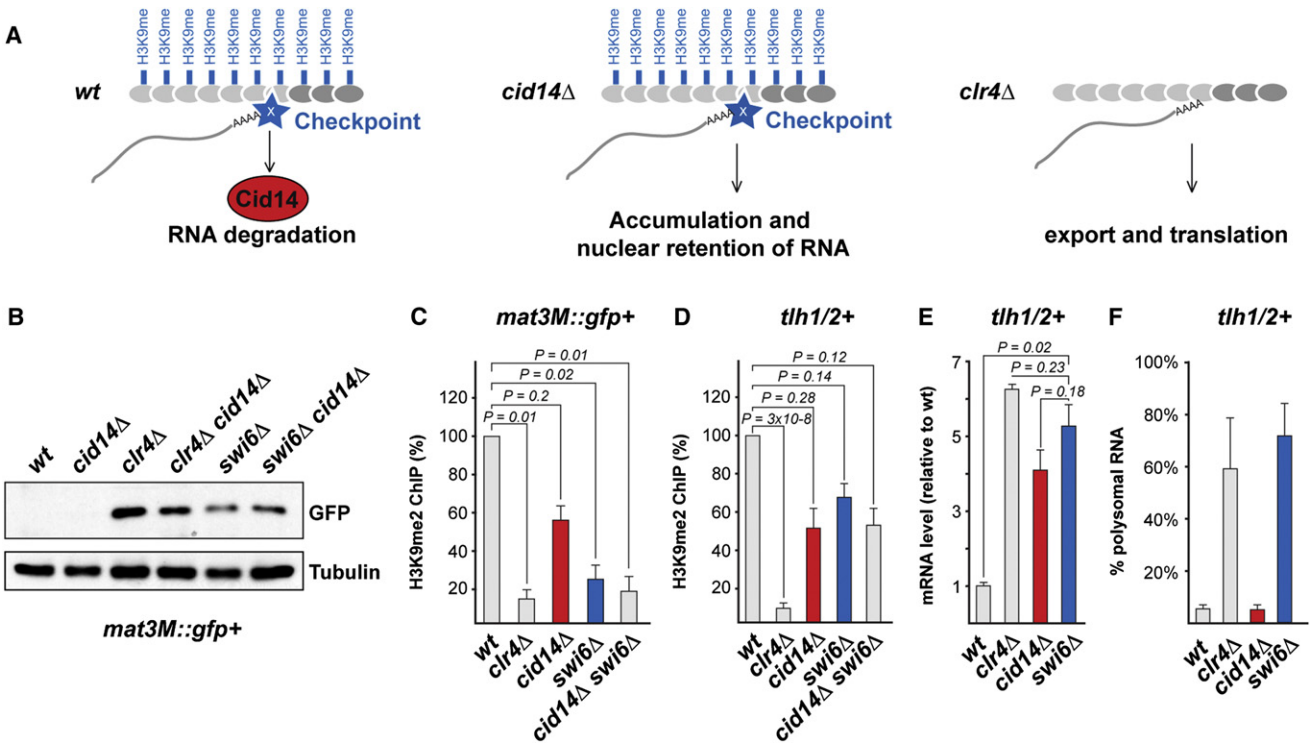

Figure 2. HP1^{Swi6} Prevents Translation of Heterochromatic RNAs

(A) Checkpoint model for the specific recognition of mRNA originating from heterochromatin. When H3K9 is unmethylated (*clr4Δ* or euchromatin), the checkpoint cannot assemble and mRNAs are exported and translated. In *WT* and *cid14Δ* cells, the checkpoint assembles on H3K9 methylated nucleosomes and captures heterochromatic mRNA transcripts. Eventually, these mRNAs are degraded in a Cid14-dependent manner. In the absence of Cid14 (*cid14Δ*), heterochromatic mRNAs accumulate but are not translated because they are retained by the checkpoint.

(B) Western blot showing GFP protein levels in *mat3M::gfp+* cells. Total protein from an equivalent number of cells was extracted by TCA. Tubulin served as a loading control.

(C and D) ChIP experiment showing that H3K9me2 levels at *mat3M::gfp+* are significantly reduced in *swi6Δ* and *cid14Δ swi6Δ* cells but not in *cid14Δ* cells. H3K9me2 levels at the telomeric *tlh1+* and *tlh2+* genes are not significantly reduced in *cid14Δ*, *swi6Δ*, and *cid14Δ swi6Δ* cells. Enrichment was determined by quantitative real-time PCR. Mean values normalized to *act1+* are shown (n = 4). Error bars represent SEM, p values were calculated using the Student's t test.

(E) *tlh1/2+* mRNA levels were determined by quantitative real-time RT-PCR. Mean values normalized to *act1+* are shown (n = 9). Error bars represent SEM, p values were calculated using the Student's t test.

(F) *tlh1/2+* mRNA associates with polysomes in *swi6Δ* but not in *cid14Δ* cells, although total mRNA levels are not significantly different in *swi6Δ* and *cid14Δ* cells (E). Enrichment of *tlh1/2+* mRNA in polysomal fractions of the indicated mutants was determined by polysome profiling as in Figure 1F. Error bars represent SEM.

properties to HP1^{Swi6}, we purified recombinant CD, hinge, and CSD. In contrast to the CD and the CSD, the isolated hinge region was sufficient for strong RNA binding (Figure 3B). By using NMR chemical shift titrations monitored on amide resonances in the flexible hinge region, we determined the binding constant of full-length HP1^{Swi6} to a 20-mer RNA as 38 ± 13 μM (Figure 3C). These results demonstrate that HP1^{Swi6} is able to bind RNA alone and that the hinge region is substantially involved in this binding interaction.

Design of an HP1^{Swi6} Mutant that Affects RNA but Not H3K9me Binding

Because heterochromatin at certain loci disintegrates upon removal of the *swi6+* gene (Figure 2C), we aimed to develop an HP1^{Swi6} mutant with compromised RNA- but normal H3K9me-binding affinity. Therefore, we mutated the positively charged residues of the hinge region, 20 lysines and 5 arginines, to alanines (Figure 4A). For the resulting mutant protein, HP1^{Swi6}-KR25A, RNA binding was indeed drastically reduced when compared to the wild-type protein (Figure 4B). For the subsequent use of the protein in vivo, we assessed the impact of these 25 mutations on protein architecture by solution NMR spectroscopy using recombinant HP1^{Swi6} and HP1^{Swi6}-KR25A protein. Based on the full-length proteins and subconstructs thereof, we established complete sequence-specific resonance assignments for the isolated CD (residues 75–139) (Figure S4A), as well as domain-specific resonance assignments for the NTD, the hinge region, and the CSD of wild-type HP1^{Swi6}. The chemical shift dispersion and intensities of the resonances in full-length HP1^{Swi6} indicated the CD and the CSD to be folded domains and the NTD and the hinge region to be flexibly unfolded polypeptide segments, as expected from predictions of the secondary structure. Analysis of the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ secondary chemical shifts of the isolated CD indicates three β-strands and one large α-helix at the C-terminal end of the domain (Figure S4E), which is well in agreement with the known

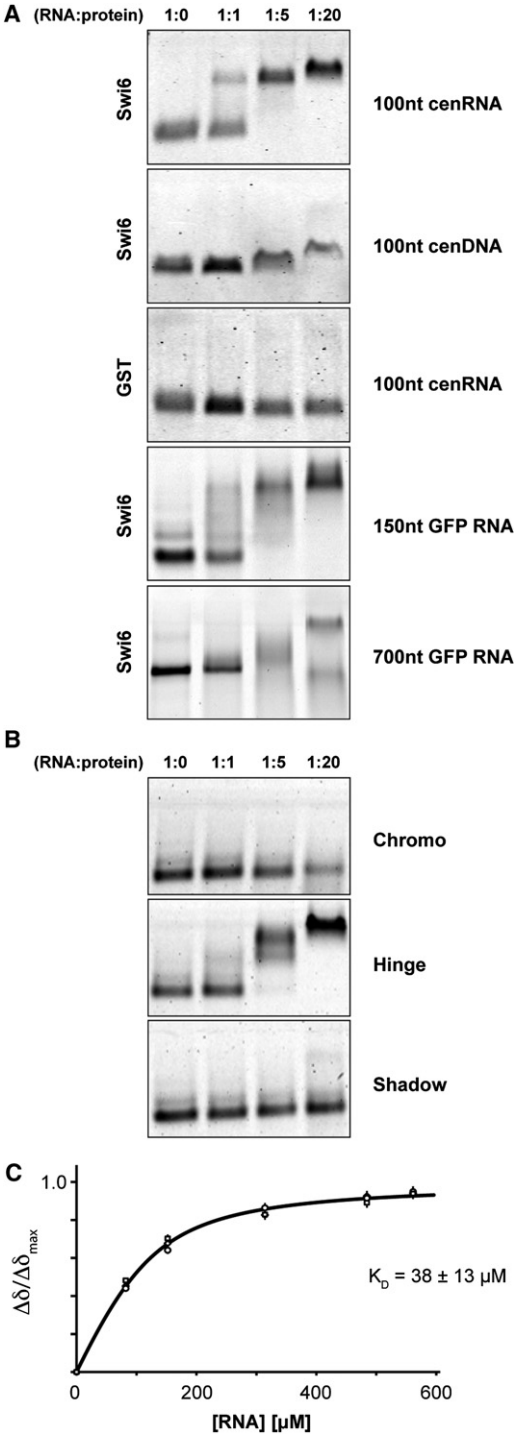

Figure 3. HP1^{Swi6} Is an RNA-Binding Protein

(A and B) Electrophoretic mobility shift assay (EMSA) using recombinant HP1^{Swi6}, HP1^{Swi6} subdomains or GST and different substrate nucleic acids (see Supplemental Information). RNA probes were labeled with fluorescein-UTP by in vitro transcription. DNA probes were produced by standard PCR. Protein-nucleic acid complexes were separated on 1.6%-TB agarose gels and the signal detected using a typhoon scanner.

(C) NMR chemical shift perturbation assay. The open circles are combined amide chemical shifts $\Delta\delta = \sqrt{0.04 \cdot \Delta\delta(^{15}\mathrm{N})^2 + \Delta\delta(^{1}\mathrm{H})^2}$ of three selected amide resonances plotted versus the RNA concentration. The line is the result of a nonlinear least-squares fit of a single binding curve to the data. The resulting dissociation constant K_D is indicated.

secondary structure elements in the homologous human chromobox homolog 3 (Kaustov et al., 2011). Importantly, 2D [^{15}N,^{1}H]-TROSY NMR spectra revealed the subspectra for the CD, the CSD, and the NTD, but not the hinge region of recombinant HP1^{Swi6}-KR25A, to be essentially identical to wild-type HP1^{Swi6} (Figures 4D and 4E). Thus, the 25 Lys and Arg to Ala mutations in the hinge region abolish RNA binding without affecting the global fold of the CD and CSD domains or having a structural effect on the unfolded NTD. Binding to methylated H3K9 is, therefore, expected to be maintained in the HP1^{Swi6}-KR25A mutant. This we could confirm by surface plasmon resonance (SPR) measurements (Figure 4C). The binding constants of wild-type and HP1^{Swi6}-KR25A to an immobilized peptide corresponding to residues 1–20 of a K9 trimethylated histone H3 tail (H3K9me3 peptide) (2.5 ± 0.5 μM and 7.8 ± 0.8 μM, respectively), were akin to and in correspondence with published values for the individual domains (Jacobs and Khorasanizadeh, 2002; Schalch et al., 2009).

Silencing but Not the Integrity of Heterochromatin Is Affected in the HP1^{Swi6} RNA-Binding Mutant

To study the functional relevance of RNA binding through the hinge region of HP1^{Swi6}, we replaced the endogenous *swi6+* open reading frame (ORF) with the HP1^{Swi6}-KR25A mutant ORF. Consistent with previous results that assigned a nuclear localization signal (NLS) function to the hinge region (Wang et al., 2000), we observed that the HP1^{Swi6}-KR25A protein localized mainly to the cytoplasm (Figure S5A and data not shown). Therefore, we added an N-terminal SV40 NLS to the wild-type and mutant HP1^{Swi6} alleles, which restored the characteristic heterochromatic foci in the nucleus and the specific association with RNA from heterochromatic regions (Figures 5A and S5B–S5F). Furthermore, in contrast to *swi6Δ* cells, neither NLS-HP1^{Swi6}- nor NLS-HP1^{Swi6}-KR25A-expressing cells were sensitive to thiabendazole (TBZ), showing that RNA binding to HP1^{Swi6} is not required for proper chromosome segregation (Figure 5B). Importantly, the H3K9 methylation defect observed at the *mat3M::gfp+* locus in *swi6Δ* cells (Figure 2C) was rescued by the *nls-swi6-KR25A* allele (Figure 5D). Similarly, H3K9 methylation within telomeric heterochromatin remained unaffected in *nls-swi6-KR25A* cells (Figures 5E and 5F).

These results demonstrate that neither H3K9 methylation nor recruitment of HP1^{Swi6} to heterochromatin depend on RNA binding through the hinge region of HP1^{Swi6}. However, silencing of heterochromatic genes was nonfunctional in *nls-swi6-KR25A* cells (Figures 5G–5J). Thus, RNA binding to HP1^{Swi6} is required for full repression of heterochromatic genes but dispensable for the integrity of heterochromatin. In summary, with *nls-swi6-KR25A* we created a separation-of-function allele of HP1^{Swi6} that fails to repress heterochromatic genes but still fulfills its architectural roles, with no impact on H3K9 methylation or chromosome segregation.

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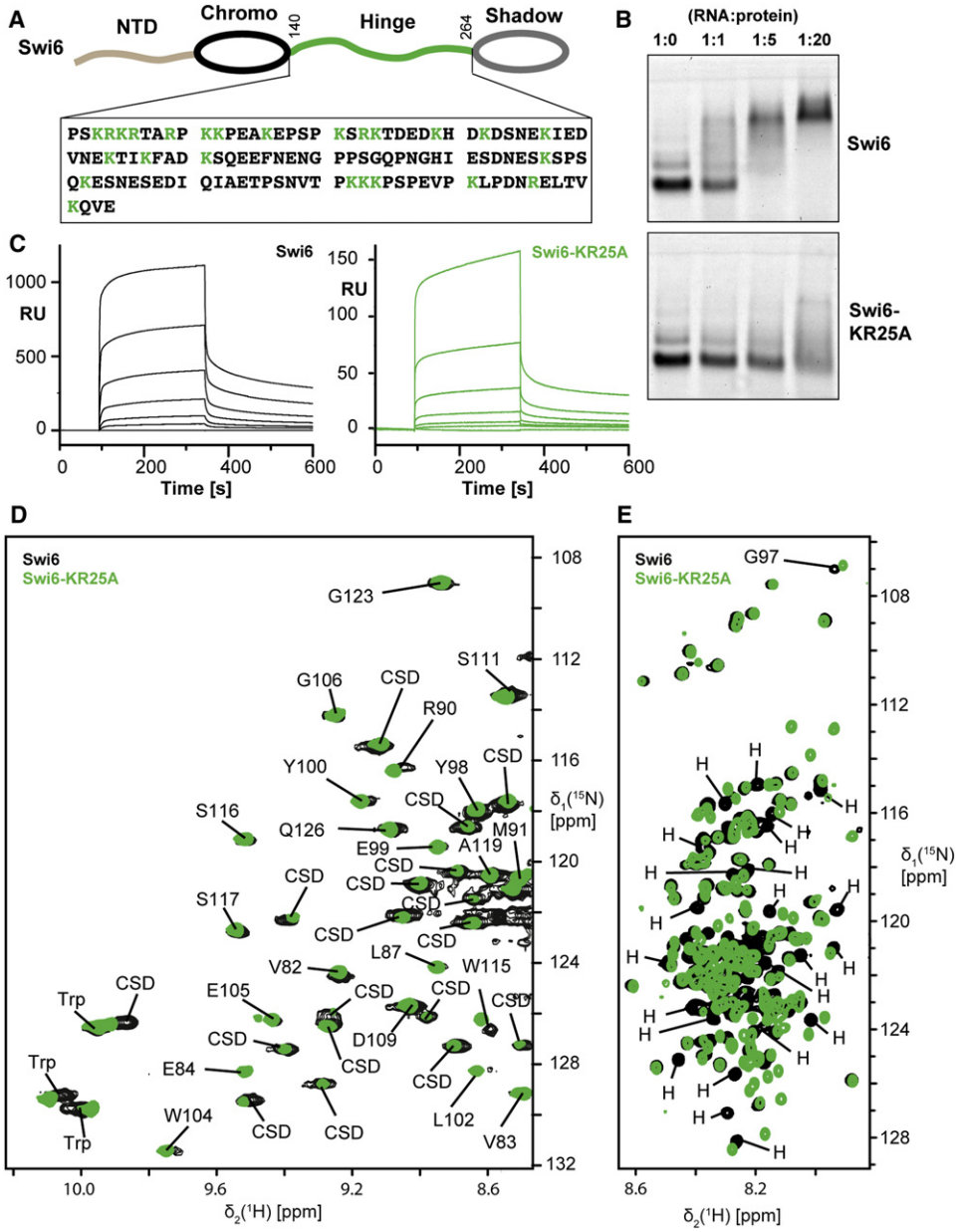

Figure 4. Characterization of HP1^{Swi6}-KR25A

(A) Domain architecture of HP1^{Swi6}. The two folded domains are indicated as ellipses, the two flexible domains as wavy lines. The amino acid sequence of the hinge region (residues 140–264) is given below. Lys and Arg residues that are mutated to Ala in the HP1^{Swi6}-KR25A protein are marked in green.

(B) EMSA showing that RNA binding of HP1^{Swi6}-KR25A is strongly impaired compared with the wild-type protein. A 100 nt centromeric RNA probe was used.

(C) SPR sensorgrams for binding of HP1^{Swi6} (black) and HP1^{Swi6}-KR25A (green) to an H3K9me3 surface. The protein concentrations are from bottom to top 0, 0.015, 0.047, 0.15, 0.43, 1.3, and 3.8 μM.

(D and E) Comparison of 2D [^{15}N,^{1}H]-TROSY correlation spectra of HP1^{Swi6} (black) and HP1^{Swi6}-KR25A (green). In (D), the downfield region of the spectrum is plotted at a low base level, showing mainly resonances from folded parts of the proteins. The sequence-specific resonance assignments for the CD and domain-specific assignments for the CSD (labeled "CSD") are indicated. In (E), the random-coil region of the same spectra are plotted at high base level, showing mainly resonances from the flexibly disordered NTD and hinge region. Domain-specific resonance assignments are shown for those resonances that are altered by the KR25A mutations. These are all located in the hinge region ("H"). The complete domain-specific resonance assignments are given in Figure S4.

HP1^{Swi6} Binding to K9 Methylated Histone H3 Is Highly Dynamic

Consistent with published results (Cheutin et al., 2004), fluorescence recovery after photobleaching (FRAP) experiments revealed that HP1^{Swi6} proteins are highly dynamic at the cellular ensemble level in vivo (Figure S5A). For proteins that are bound tightly to chromatin, recovery kinetics can be expected to be slow or not detectable, as observed for the telomere-binding

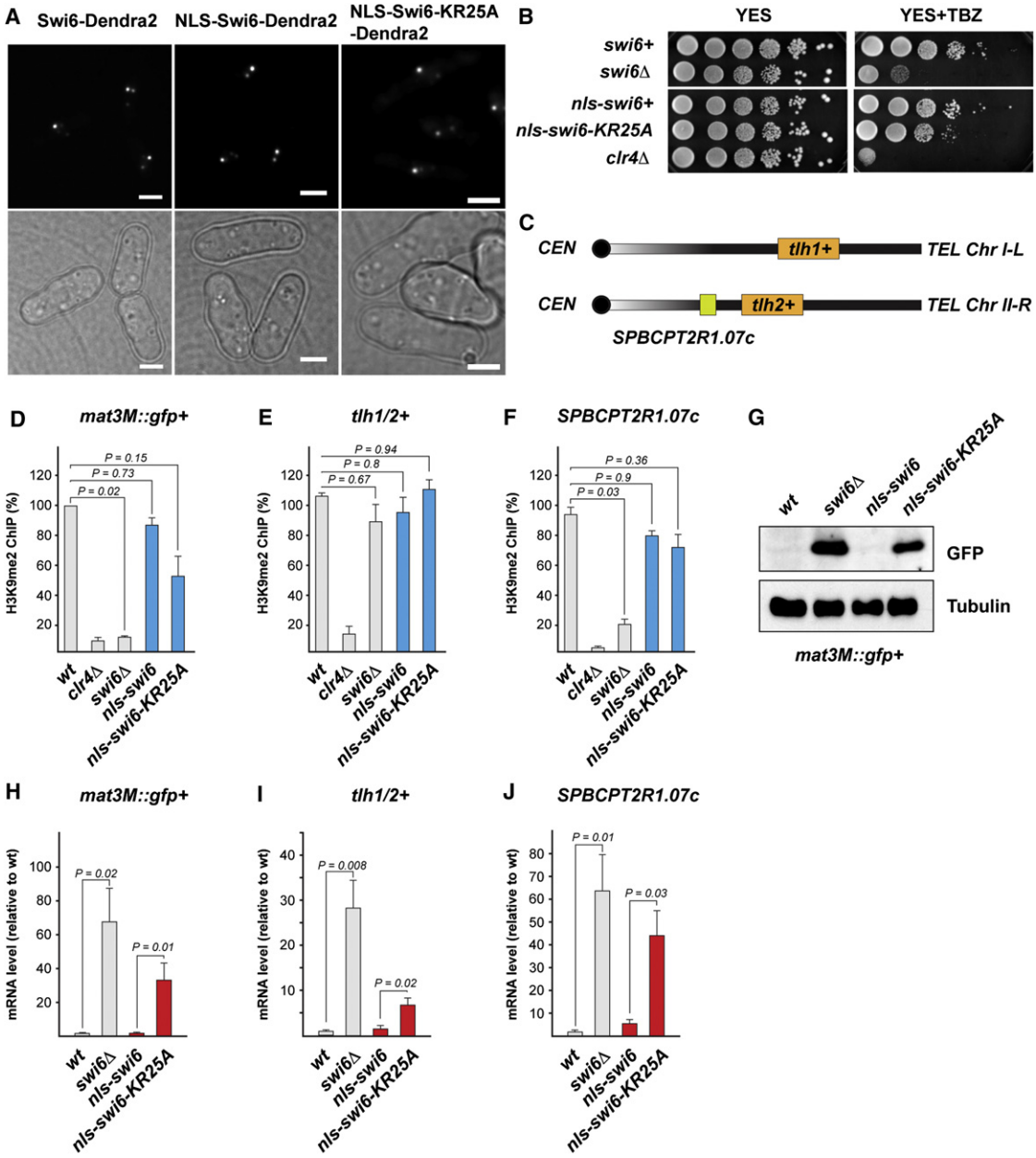

Figure 5. RNA Binding through the Hinge Region of HP1^{Swi6} Is Required for Silencing but Not Maintenance of Heterochromatin

(A) Microscopy of living *S. pombe* cells expressing C-terminally Dendra2-tagged HP1^{Swi6} variants driven from the endogenous promoter. Cells were grown in YES medium at 30°C. To restore nuclear localization of the HP1^{Swi6}-KR25A mutant (Figure S4), a SV40 NLS was added N-terminally. Scale bar = 2 μm.

(B) In contrast to *swi6*Δ cells, cells expressing the RNA-binding mutant NLS-HP1^{Swi6}-KR25A are not sensitive to thiabendazole (TBZ), indicating that chromosome segregation is normal. Cells were spotted on YES agar plates containing either 0 or 14 mg/l TBZ.

(C) Schematic diagram showing the location of three heterochromatic genes at the telomeres of chromosome I and II. *tlh1*+ and *tlh2*+ produce identical transcripts (Mandell et al., 2005). CEN, centromere; TEL, chromosome end.

(D–F) ChIP experiments demonstrating that H3K9me2 levels are not significantly reduced at *mat3M::gfp*+ (D), *tlh1/2*+ (E), and *SPBCPT2R1.07c* (F) in *nls-swi6*+ and *nls-swi6-KR25A* cells compared with wild-type cells. Mean values normalized to *act1*+ are shown (n = 4). Error bars represent SEM, p values were calculated using the Student's t test.

(G) Western blot showing GFP protein levels in *mat3M::gfp*+ cells. Total protein from an equivalent number of cells was extracted by TCA. Tubulin serves as a loading control.

(H–J) Quantitative real-time RT-PCR showing *mat3M::gfp*+ (I), *tlh1/2*+ (K), or SPBCPT2R1.07c (L) transcript levels in the respective mutants. Mean values normalized to *act1*+ are shown (n = 5). Error bars represent SEM, p values were generated using the Student's t test.

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protein Taz1 (Figure S5B). This is not the case for HP1^{Swi6}, for which fluorescence recovered rapidly after photobleaching with an exponential lifetime of 1.8 ± 0.1 s (Figure S5C). This dynamic exchange of the HP1^{Swi6} ensemble from chromatin in vivo is qualitatively consistent with the rapid exchange dynamics we observed in NMR peptide titration experiments in vitro. We found that the resonances of the CD involved in H3K9me3 peptide binding underwent line broadening due to intermediate chemical exchange. This indicates kinetic on/off rates for the exchange between bound and unbound forms of individual HP1^{Swi6} molecules in the range of about 0.01–1.0 ms^{-1}, corresponding to lifetimes of 1–100 ms. These in vivo and in vitro data thus demonstrate the highly dynamic behavior of HP1^{Swi6} and rule out the possibility that individual HP1^{Swi6} molecules remain tightly bound to heterochromatin for minutes or longer. Therefore, HP1^{Swi6} alone cannot tether heterochromatic RNAs to chromatin.

Localization of the HP1^{Swi6} Interaction Sites with RNA and H3K9me

To obtain insight into the interactions of HP1^{Swi6} with RNA and methylated H3K9 at the atomic level, we used NMR chemical shift perturbation to identify residues structurally involved in these interactions. To this end, we monitored amide moiety chemical shifts, which are sensitive to structural changes of the polypeptide backbone. For the interaction of full-length HP1^{Swi6} with the H3K9me3 peptide, we observed chemical shift changes for 21 out of the 65 residues in the CD, as well as for one tryptophan side chain indole moiety (Figures 6A and 6B). The location of these residues in the amino acid sequence in HP1^{Swi6} corresponds to the location of the known binding pocket for the peptide in homologous domains (Jacobs and Khorasanizadeh, 2002; Kaustov et al., 2011; Nielsen et al., 2002). No significant chemical shift changes occurred for the backbone amide resonances of the CSD, but smaller chemical shift perturbations were observed for 8 residues of the N-terminal domain and 1 residue of the hinge region (Figure 6B). On the other hand, interaction with 20-mer-GFP RNA induced chemical shift changes for resonances of three different domains: 13 residues from the hinge region, 19 from the CD, and 10 from the N-terminal domain (Figures 6C and 6D). Furthermore, all resonances of the CD underwent line broadening at intermediate RNA concentrations due to intermediate exchange indicating kinetic on/off rate constants for RNA binding below about 1 ms^{-1}.

These data show that binding of RNA as well as binding of H3K9me3 peptide to HP1^{Swi6} occurs by a molecular mechanism that includes structural changes in three domains of HP1^{Swi6}. The observation that these interaction sites partially overlap thereby points toward the intriguing possibility that histone tail and RNA binding are not independent. Rather, these could be competitive processes, meaning that HP1^{Swi6} dissociates from H3K9-methylated nucleosomes when complexed with RNA. Consistent with this idea, steady-state competition assays using SPR showed competitive behavior (Figure 6E). At substoichiometric RNA:HP1^{Swi6} ratios, the initial SPR response increased. This can be rationalized by the dimeric nature of HP1^{Swi6} caused by its CSD, which leads to complexes with 2 RNA and 2 peptide-binding sites. At concentrations above stoichiometry, however, the SPR response decreased with increasing RNA concentration, indicating competition for the peptide surface. Importantly, the 20-mer GFP-RNA did not bind to the immobilized peptide surface in a control experiment under the same buffer conditions (Figure S6D). Furthermore, binding of the HP1^{Swi6} -KR25A mutant to H3K9me was insensitive and noncompetitive to the addition of RNA (Figures 6E and S6D).

In summary, our results implicate a mechanism by which RNA and methylated H3K9 compete for HP1^{Swi6} binding at the ensemble as well as the single-molecule level. Binding of RNA to HP1^{Swi6} structurally involves the hinge, the CD, and the NTD and impedes binding of HP1^{Swi6} to methylated H3K9. Thus, rather than tethering RNA to heterochromatin firmly, HP1^{Swi6} dynamically complexes with RNA and dissociates from H3K9-methylated nucleosomes.

Cid14 Functions in the Vicinity of Heterochromatin

The above results have established HP1^{Swi6} as a crucial constituent of hsRNPs, tagging RNAs as a result of their heterochromatic origin and priming them for degradation. Importantly, the dynamic properties of HP1^{Swi6} imply that the degradation of heterochromatic RNA originating from telomeres and the mating-type locus occurs off chromatin, but it is unclear whether Cid14 would join the hsRNP before or after dissociation from H3K9 methylated nucleosomes. If it would occur before dissociation from heterochromatin, it should be possible to crosslink Cid14 to telomeres or the mating-type locus. However, ChIP experiments did not show enrichment of Cid14 at these loci (data not shown), suggesting that Cid14 joins the HP1^{Swi6}/RNA complex only after dissociation from heterochromatin.

To test whether this still occurs in close proximity to heterochromatin, we employed the DNA adenine methyltransferase identification method (DamID, Figure 7A), a sensitive chromatin profiling technique that is suited to capture indirect or transient protein–chromatin interactions. We generated strains that express HP1^{Swi6} and Cid14 fused to the Dam DNA methyltransferase (Figure 7A; Woolcock et al., 2011) and assessed GATC methylation throughout the *S. pombe* genome using tiling arrays. As expected, HP1^{Swi6} was highly enriched at the mating-type locus, the centromeres, and the telomeric regions when compared to a Dam-only control (Figure 7B). Similarly, GATC methylation within the different heterochromatic regions was also observed for Dam-Cid14, demonstrating that Cid14 resides in close proximity to heterochromatin. Importantly, GATC methylation by Dam-Cid14 at the mating-type locus and telomeres is fully dependent on HP1^{Swi6} and not as strong as for Dam-HP1^{Swi6} (Figure 7C). This indicates that Cid14 joins hsRNPs after assembly and dissociation from heterochromatin at the mating-type region and the telomeres.

In conclusion, these results demonstrate that Cid14 resides in the vicinity of heterochromatin and that heterochromatic RNA originating from telomeres or the mating-type locus is delivered to Cid14 in a close spatial and temporal correlation to the dissociation of HP1^{Swi6} from H3K9-methylated nucleosomes. We speculate that the actual degradation of heterochromatic RNA might also occur near heterochromatin. The functional relevance of the HP1^{Swi6}-independent association of Cid14 with centromeric heterochromatin remains unknown.

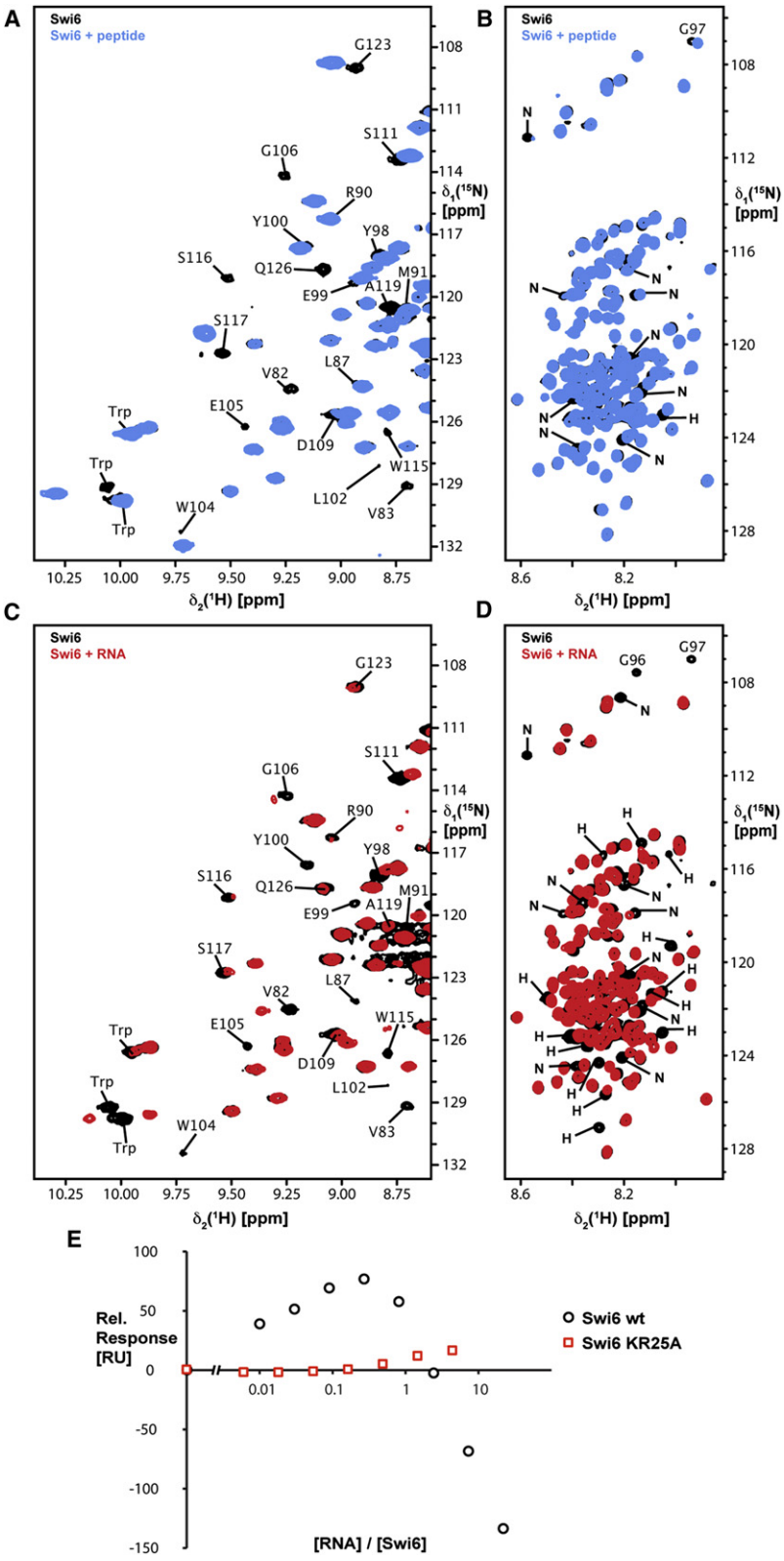

Figure 6. Localization and Competition of the HP1^{Swi6} Interactions

(A–D) Overlay of 2D [^{15}N,^{1}H]-TROSY correlation spectra of HP1^{Swi6}. The spectra are plotted in (A) and (C) at low base level, showing mainly resonance peaks from the two folded domains CD and CSD. The spectra are plotted in (B) and (D) at high base level, showing mainly resonances from the flexibly unfolded hinge and N-terminal domains. Residue type and number indicate sequence-specific resonance assignments for the CD. "H," "N," and "Trp" denote resonances from the hinge region, the NTD, and tryptophan side chains, respectively. (A and B) Black: HP1^{Swi6}; blue: 138 μM HP1^{Swi6} + 513 μM H3K9me3 peptide. (C and D) Black: HP1^{Swi6}; red: 95 μM HP1^{Swi6} + 560 μM RNA.

(E) SPR responses for competitive binding of H3K9me3 and RNA to HP1^{Swi6}. A constant concentration of 1 μM HP1^{Swi6} (black circles) or 5 μM HP1^{Swi6}-KR25A (red squares) with increasing concentrations of 20-mer GFP-RNA was injected to the H3K9me3 surface. The maximal SPR response after 200 s injection is plotted versus the RNA:protein concentration ratio. For each of the two proteins, the response in the absence of RNA was set to zero (raw data, see Figure S6D).

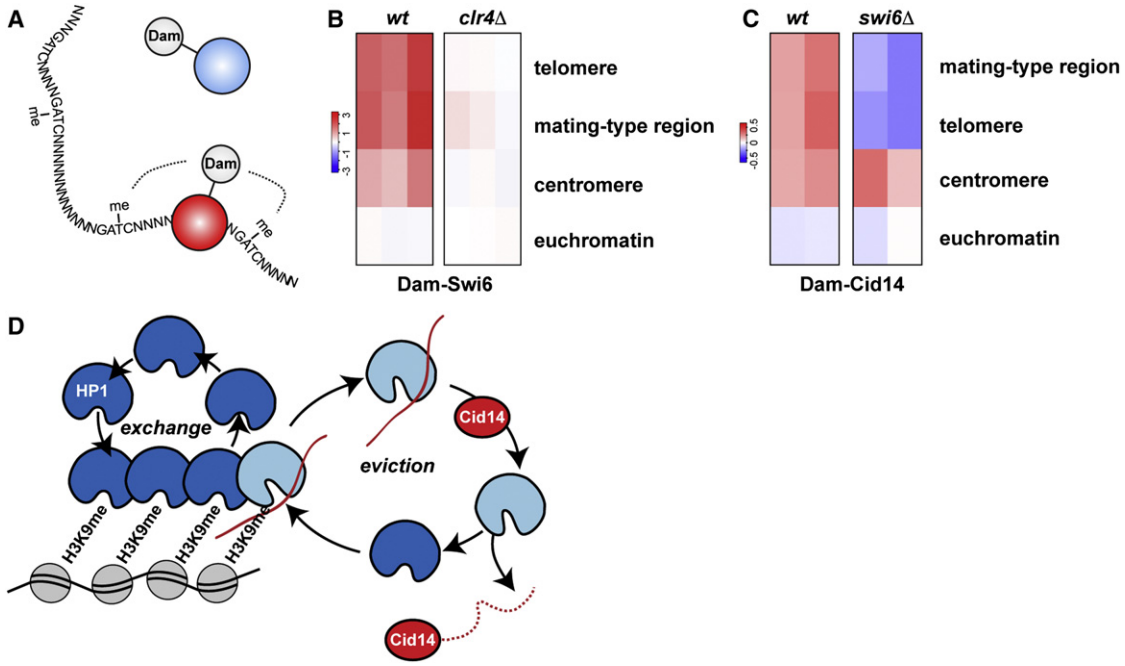

Figure 7. Cid14 Functions in the Vicinity of Heterochromatin

(A) In DamID, a Dam fusion protein is expressed at very low levels. On interaction of the fusion protein with chromatin (red), Dam methylates the adenine in the sequence context of GATC, which can be mapped by a methylation-specific PCR protocol.

(B and C) HP1^{Swi6} and Cid14 enrichments from DamID experiments (log_2) at chromosomal regions.

(D) Model for HP1^{Swi6}-mediated degradation of heterochromatic RNA. HP1^{Swi6} proteins associate with H3K9-methylated nucleosomes (gray) only transiently and readily exchange from heterochromatin (dark blue). This continuous exchange of HP1^{Swi6} prevents saturation of heterochromatin with RNA. In case transcription within heterochromatin occurs, HP1^{Swi6} binds the newly synthesized RNA (red) and dissociates from H3K9 methylated nucleosomes as a result of competition between RNA and the histone tail for HP1^{Swi6} binding (light blue). Subsequently, the RNA is passed on to Cid14 (red), which in turn initiates RNA degradation.

DISCUSSION

Association of HP1 Proteins with RNA

It was recognized earlier that proteins involved in chromatin regulation have the ability to bind RNA, although the functional relevance of this interaction has remained elusive. RNA binding was first demonstrated for the CDs of MOF and MSL-3, proteins involved in dosage compensation in *Drosophila* (Akhtar et al., 2000). For mammalian HP1α, the hinge region has been implicated in RNA binding (Muchardt et al., 2002). Here we demonstrate that HP1^{Swi6}, the fission yeast homolog of HP1α, can also bind RNA directly. Importantly, we have found that the interaction of HP1^{Swi6} with RNA mechanistically includes the hinge region, the CD, and the NTD, a property that could be easily overlooked when working with isolated domains. Therefore, it will be interesting to revisit the RNA-binding properties of other HP1 proteins, such as mammalian HP1α, β, or γ, by approaches similar to those in this study. It might be that different HP1 isoforms display important differences in their interaction with RNA, which could reveal novel insights into their functional diversification. It will also be very interesting to elucidate the structural basis of the RNA and peptide binding of HP1^{Swi6} at the atomic level, which should give additional insights into the biophysical nature of their competitive binding mechanism.

It has been speculated that the functional relevance of the RNA affinities of HP1α or the dosage compensation complex might be the targeting to chromatin by major satellite or roX noncoding RNAs, respectively (Akhtar et al., 2000; Maison et al., 2002, 2011). In such a model, RNA is proposed to be involved structurally in the assembly of a higher order chromatin structure by serving as a recruitment platform. This is unlikely to apply to *S. pombe* HP1^{Swi6}, as neither H3K9 methylation nor recruitment of HP1^{Swi6} to heterochromatin depends on RNA binding. In contrast, RNA bound to HP1^{Swi6} dissociates from chromatin as a result of exchange with the cellular HP1^{Swi6} ensemble and a decrease in affinity for methylated H3K9.

Stable Repression of Heterochromatin through RNA Sequestration and Degradation

The results of our work reinforce previous findings that heterochromatin is not always refractory to transcription, yet is tightly repressed. We demonstrate here that HP1^{Swi6} assures coupling between heterochromatin transcription and RNA turnover by serving as an H3K9 methylation-specific checkpoint. Based on the data presented, we propose a model for the action of the HP1^{Swi6} ensemble, which dynamically exchanges with the bulk in a maintenance cycle. Free RNA is captured in the eviction cycle and passed on to the degradation machinery. Constant flux of RNA-unbound HP1^{Swi6} from the bulk ensemble prevents

saturation of heterochromatin with RNA. Competition between RNA and methylated H3K9 for HP1^{Swi6} binding at the ensemble level guarantees that RNA-free HP1^{Swi6} is preferably recruited to heterochromatin, thereby sustaining a functional checkpoint on the H3K9-methylated nucleosome and ensuring constant turnover of heterochromatic RNAs (Figure 7C).

In our model, HP1^{Swi6} functions on chromatin to bind to and assemble emerging heterochromatic transcripts into special RNPs, which we refer to as hsRNPs. Thereby, HP1^{Swi6} guarantees specific and tight repression of heterochromatic genes on at least two levels. First, HP1^{Swi6} prevents protein synthesis by sequestration of mRNAs from ribosomes, most likely through nuclear retention. Thus, a heterochromatic mRNA remains repressed even in the absence of RNA degradation. This explains why classical PEV screens failed to recover RNA decay factors such as Cid14. Notably, Cid14 itself is involved in the processing of ribosomal RNA and also associates with 60S ribosomal proteins (Keller et al., 2010; Win et al., 2006), raising the possibility that loss of Cid14 might result in a general defect in translation. However, association of euchromatic mRNAs with polyribosomes, as well as protein expression levels, remain unaffected in *cid14Δ* cells (Figure 1 and data not shown), strongly arguing against such an indirect effect. Second, the HP1^{Swi6} ensemble ensures elimination of heterochromatic mRNAs by capturing the RNA at the site of transcription and escorting it to the degradation machinery. Rather than the classical features of an aberrant RNA, such as a truncated open reading frame or defective 5′ or 3′ ends, our data suggests that it is the physical association of a heterochromatic mRNA with HP1^{Swi6} that primes it for destruction. We note that artificial tethering of HP1^{Swi6} to a euchromatic mRNA does not result in RNA degradation (data not shown), suggesting that canonical mRNPs are immune to HP1^{Swi6}-mediated RNA turnover. Furthermore, since the kinetics of RNA binding to HP1^{Swi6} are fast, the hsRNPs may be stabilized by additional factors. However, at this point we can only speculate on such contributions by additional proteins or other molecules.

Concluding Remarks

In this study, we have discovered a function for one of the fission yeast HP1 proteins that provides the missing link between transcriptional origin and Cid14-dependent degradation of heterochromatic mRNAs. Our results highlight the role of RNA as a negative regulator of HP1^{Swi6} binding to chromatin and provide insights into the repression of heterochromatic domains at a posttranscriptional level. The high degree of conservation of HP1 proteins and heterochromatin-mediated gene silencing phenomena suggest that our findings may also apply to other eukaryotes.

Our work has revealed that HP1^{Swi6}, in addition to its role in proper centromere function, also guarantees tight repression of heterochromatic genes through RNA sequestration and degradation. Interestingly, the *Drosophila* HP1 protein Rhino has been linked recently to the piRNA pathway (Klattenhoff et al., 2009). In analogy to our checkpoint model, Rhino may bind the initial sense transcript at the heterochromatic transposon locus and subsequently escort it to the perinuclear "nuage" structure, where it can enter the ping-pong amplification cycle. Thus, rather than forming repressive chromatin, Rhino might specify the recognition and ensure efficient elimination of transposon RNA.

Finally, our results add another layer of complexity to the crosstalk between RNA and chromatin. In contrast to the emerging theme that RNA can serve as a scaffold to assemble, recruit, or guide chromatin-modifying complexes to their respective targets (Wang and Chang, 2011), we demonstrate that they may also function as "repellents." RNA-mediated eviction might be a possible mechanism that counteracts HP1 spreading along the chromatin fiber or the formation of ectopic heterochromatin. Importantly, neither coding potential nor stability is important for an RNA to function as a repellent, offering a possible molecular function for the many short-lived, low-abundant noncoding RNAs that are present in the eukaryotic cell.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Fission yeast strains and plasmids used in this study are described in Supplemental Information.

Western Blot and Polysome Profiling

Total proteins from exponentially growing cells were extracted using TCA and separated by SDS-PAGE. Antibodies for western blotting were used at the following concentrations: GFP (Roche; 1:3000), tubulin (Woods et al., 1989; 1:5000), Swi6 (Bioacademia; 1:10,000). Polysome profiling is described in Supplemental Information.

Chromatin Immunoprecipitation and Gene Expression Analysis

RNA isolation, cDNA synthesis, and quantitative RT-PCR was performed as described in Emmerth et al. (2010). Chromatin immunoprecipitation (ChIP) was performed as described in Bühler et al. (2006), using 2.5 μg of an antibody against dimethylated H3K9 (Kimura et al., 2008).

Electrophoretic Mobility Shift Assay (EMSA)

The desired amount of protein was diluted into 9 μl of 1 × electrophoretic mobility shift assay (EMSA) buffer (20 mM HEPES-KOH [pH 7.5], 100 mM KCl, 0.05% NP-40) and incubated for 10 min at RT. The substrate was added, incubated at 30°C for 30 min, and followed by gel electrophoresis (1.6% TB-agarose). Fluorescently labeled RNA was detected using a TyphoonTM 9400 Gel Scanner. RNA labeling is described in Supplemental Information.

Recombinant Protein Expression and Purification for NMR

Expression and purification was performed as described in Supplemental Information with the following modifications. Bacteria were grown in 6 l of M9 minimal medium containing ^{15}N-NH_4Cl as a nitrogen source. Induction was carried out using 0.5 mM IPTG. The lysate was incubated with 10 ml of glutathione-sepharose FF (GE). The protein was released from the glutathione-resin by TEV-cleavage o/n at 4°C using acTEV (Invitrogen). This was followed by Source15Q ion exchange chromatography (GE Healthcare). The purification was completed by size exclusion chromatography (Superdex 200; GE Healthcare) in 50 mM MES pH 6.5, 100 mM KCl, 5 mM DTT. The purified complex was concentrated to 100 μM by centrifugal filtration.

Solution NMR Spectroscopy and SPR

NMR experiments were performed on Bruker 800 MHz and 600 MHz spectrometers. The sequence-specific resonance assignments for the isolated HP1^{Swi6} CD (residues 75–139) were obtained from the two APSY-type experiments 4D APSY-HNCACB (15 projections) and 5D APSY-HNCOCACB (16 projections) (Gossert et al., 2011; Hiller et al., 2005, 2007) and subsequent automated backbone assignment by the algorithm MATCH (Volk et al., 2008). For SPR, samples were analyzed using a Biacore T-100 instrument (GE Healthcare). Further details are given in Supplemental Information.

DamID

DamID was carried out as previously published (Woolcock et al., 2011). Coordinates of heterochromatic regions are given in Supplemental Information.

ACCESSION NUMBERS

DamID data sets were deposited under accession number GSE36956 (NCBI Gene Expression Omnibus).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, Supplemental References, and five tables and can be found with this article online at doi:10.1016/j.molcel.2012.05.009.

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Supplemental Information

HP1^{Swi6} Mediates the Recognition and Destruction of Heterochromatic RNA Transcripts

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Supplemental Data

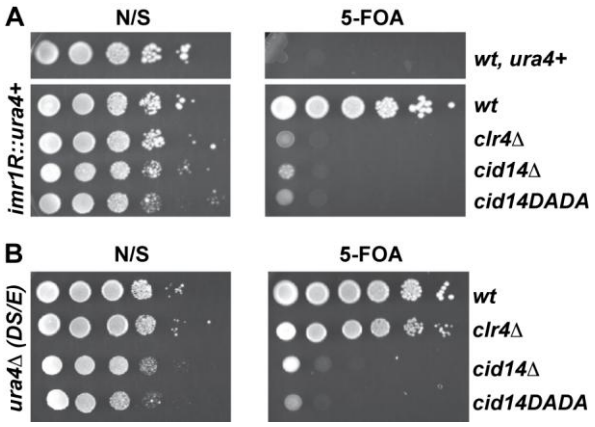

Figure S1, Related to Figure1. The Noncanonical PolyA-polymerase Cid14 Confers Resistance to 5-FOA

(A and B) Cells were spotted on PMG agar plates containing either 0 or 2 mg/L 5-FOA. The *ura4+* gene encodes orotidine 5'-phosphate decarboxylase, which converts 5-FOA to toxic 5-fluorouracil. Therefore, cells can only grow on 5-FOA containing medium if the *ura4+* gene is absent or silenced.

(A) Growth on 5-FOA containing medium indicates that the centromeric *ura4+* reporter (*imr1R::ura4+*) is efficiently silenced by heterochromatin. Deletion of the gene encoding the histone H3 methyltransferase Clr4 (*clr4*Δ) disrupts heterochromatin and silencing of *imr1R::ura4+* is lost. Similar to *clr4*Δ cells, cells lacking the *cid14+* gene or cells expressing a catalytically inactive Cid14 (*cid14DADA*) cannot grow on 5-FOA.

(B) Cells lacking a functional *ura4+* gene (*ura4DS/E*) grow on 5-FOA media in the absence of heterochromatin (*clr4*Δ), but not in the absence of Cid14 (*cid14*Δ) or if Cid14 has lost its polyadenylation activity (*cid14DADA).* Therefore, the inability of *imr1R::ura4+* cells to grow on 5-FOA when expressing *cid14*$^{+}$ mutants (A) is unlikely to result from defective heterochromatin silencing. Rather, 5-FOA is converted into 5-fluorouracil or another toxic substance by a *ura4+*-independent, endogenous pathway that becomes activated in the absence of Cid14. Alternatively, 5-FOA itself is toxic, but is usually degraded by an enzyme that is only expressed in the presence of functional Cid14.

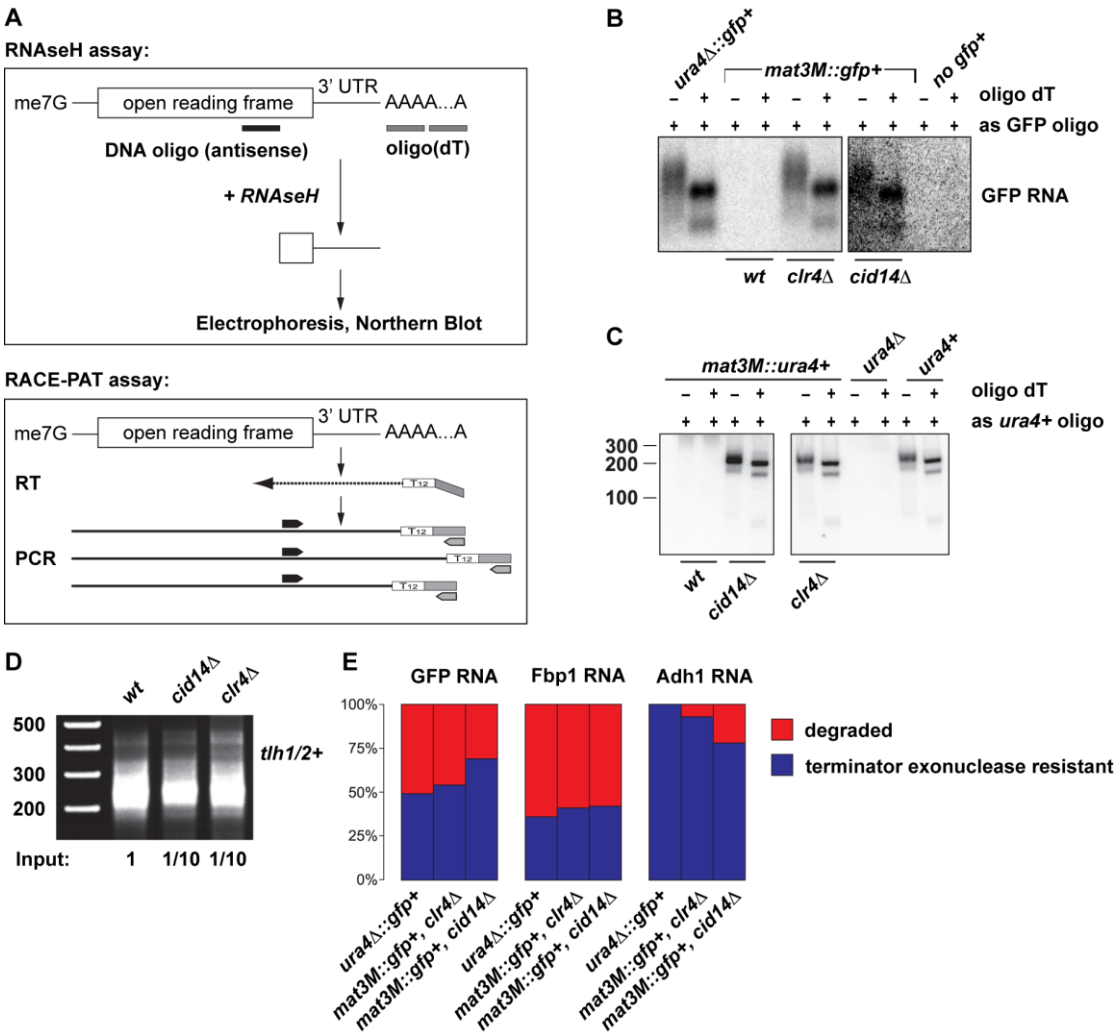

Figure S2, Related to Figure 2. Heterochromatic mRNAs Are Properly Processed

(A) Schematic diagram outlining RNAseH and RACE-PAT assays, which were used to assess the polyadenylation status of heterochromatic RNAs in different mutants.

(B) Evaluation of the polyadenylation status of heterochromatic *mat3M::gfp+* mRNAs by the RNaseH assay. Total RNA was separated on an agarose gel and transferred to a nylon membrane. GFP RNA was detected using specific ^{32}P-labelled DNA oligos. In the presence of oligodT (+oligodT), polyA tails are degraded by RNAseH. The appearance of two bands upon polyA tail removal is consistent with the presence of two major polyadenylation sites in the Tadh1 terminator present in this *gfp+* reporter. As expected, the distal site is used more frequently. The smear in the -oligodT lanes indicates the heterogenous polyA tail length of the GFP mRNA. Importantly, no major qualitative differences can be observed for euchromatic or heterochromatic GFP mRNAs in *wt, clr4Δ, or cid14Δ* cells.

(C) The polyadenylation status of heterochromatic *mat3M::ura4+* mRNAs was assessed as in B. Instead of agarose, polyacrylamide was used to separate RNAseH treated RNA.

(D) RACE-PAT assay to determine the polyadenylation state of *tlh1/2+* mRNAs. 1/10th of the RT reaction was used as input for the PCR in *cid14Δ* and *clr4Δ* cells.

(E) Total RNA was extracted from cells expressing either *gfp+* from a euchromatic (*ura4Δ::gfp+*) or heterochromatic (*mat3M::gfp+*) locus. The RNA was subsequently treated with terminator 5'-phosphate dependent exonuclease, which selectively degrades 5'-monophosphorylated RNA, while leaving 5'-me7G-capped RNA intact. The efficiency of the reaction was determined by comparing the amount of degraded 25S and 18S RNA (5'-monophosphorylated) versus 5S RNA (stable) on a Agilent Total RNA Nano Chip. The relative amount of a given RNA was quantified in untreated and exonuclease-treated samples by quantitative real-time RT-PCR. The terminator exonuclease resistant population reflects the relative amount of 5'-me7G-capped RNA.

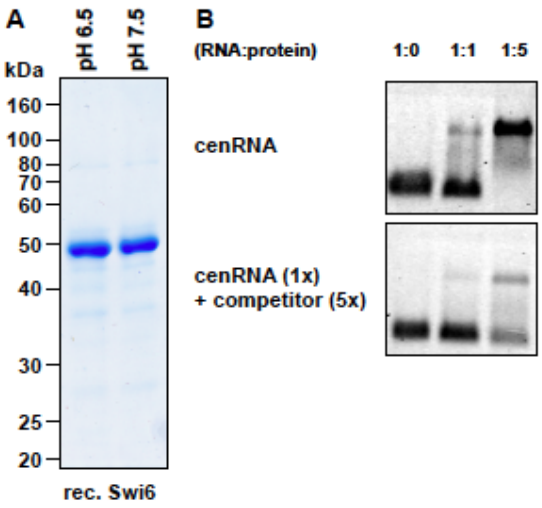

Figure S3, Related to Figure 3. HP1^{Swi6} Is an RNA-Binding Protein
(A) SDS-PAGE of the recombinant HP1^{Swi6} proteins that were used for NMR and SPR (pH 6.5), or EMSA (pH 7.5).
(B) EMSA demonstrating that binding of HP1^{Swi6} to a fluorescently labelled RNA probe can be competed by an unlabelled RNA probe.

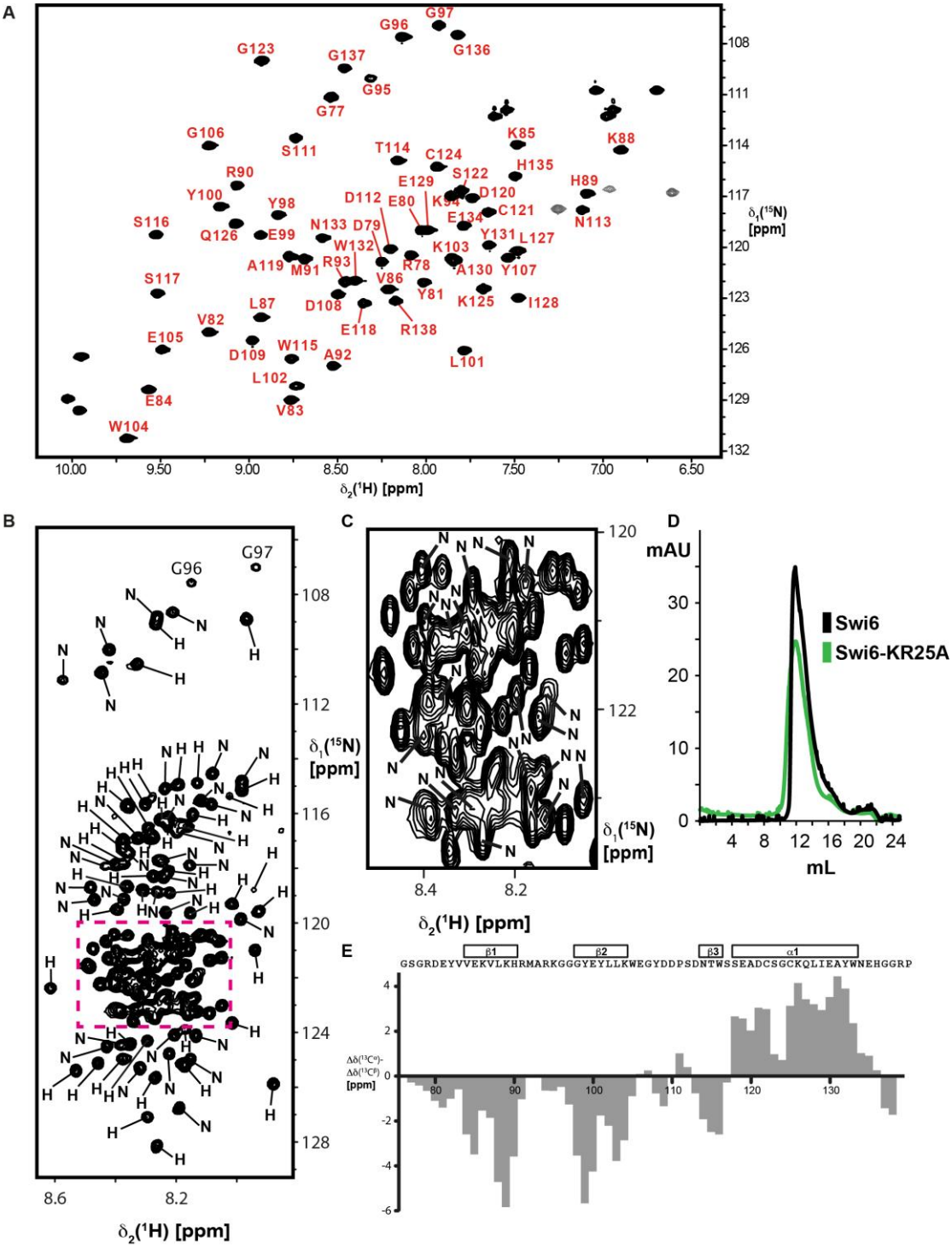

Figure S4, Related to Figure 4. Creation of a Mutant HP1^{Swi6} that Fails to Bind RNA but Keeps Its Other Molecular Properties

(A) 2D [^{15}N,^{1}H]-HSQC spectrum of the isolated CD (residues 75–139). Sequence-specific resonance assignments are indicated.

(B) Domain-specific assignments for the amide resonances arising from flexibly disordered segments of the polypeptide chain. On a 2D [^{15}N,^{1}H]-TROSY spectrum of full-length HP1^{Swi6}, the residues are identified which are part of the hinge region ("H") and the N-terminal domain ("N"). The part in red dashed lines is shown enlarged in (C).

(C) Enlargement of the central part of the spectrum (B). Resonances from the N-terminal domain are indicated "N". All other resonances belong to the hinge region.

(D) The chromatograms of $HP1^{Swi6}$ and $HP1^{Swi6}$-KR25A that were loaded onto a Superdex200 size exclusion column show that the KR25A mutation does not affect the dimeric state of protein.
(E) Secondary chemical shifts for the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts of the isolated CD (residues 75–139) relative to random coil values. Above the amino acid sequence of the domain, the secondary structure elements inferred from these shifts, three β-strands and one α-helix, are indicated.

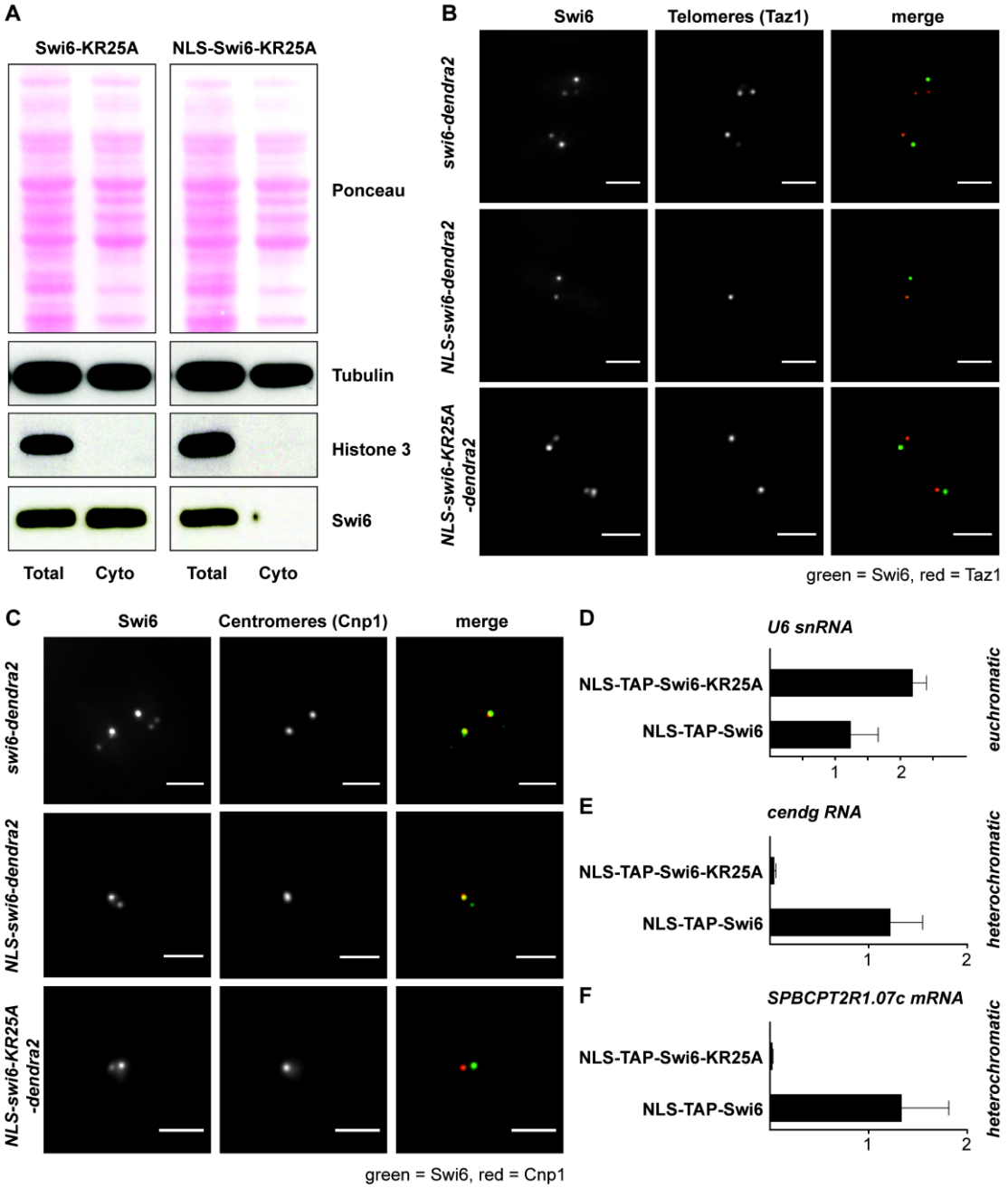

Figure S5, Related to Figure 5. RNA Binding through the Hinge Region of HP1^{Swi6} Is Required for Silencing but Not Maintenance of Heterochromatin

(A) Cells expressing HP1^{Swi6}-KR25A with or without an SV40 NLS were fractionated into total and cytoplasmic fractions. Proteins were separated by SDS-PAGE and detected by Western blot. Cytoplasmic Tubulin and nuclear Histone H3 serve as fractionation controls.

(B) Microscopy of living *S. pombe* cells co-expressing C-terminally Dendra2-tagged HP1^{Swi6} variants and C-terminally mCherry-tagged Taz1 driven from their endogenous promoters. Cells were grown in YES medium at 30°C. To restore nuclear localization of the HP1^{Swi6}-KR25A mutant (Figure S4), a SV40 NLS was added N-terminally.

(C) Microscopy of living *S. pombe* cells co-expressing C-terminally Dendra2-tagged HP1^{Swi6} variants and C-terminally mCherry-tagged Cnp1 driven from their endogenous promoters. Cells were grown in YES medium at 30°C. To restore nuclear localization of the HP1^{Swi6}-KR25A mutant (Figure S4), a SV40 NLS was added N-terminally.

(D-F) RIP experiment demonstrating that HP1^{Swi6} but not HP1^{Swi6}-KR25A interacts with heterochromatic RNA in vivo. TAP-tagged Swi6 was immunoprecipitated and the RNA was

isolated followed by cDNA synthesis. The amount of co-immunoprecipitated RNA was quantified by quantitative real-time RT-PCR and normalized to act1+ mRNA. The amount of RNA co-immunoprecipitated with $HP1^{Swi6}$-KR25A is shown relative to the amount of RNA that co-immunoprecipitates with $HP1^{Swi6}$. As a control for unspecific background RNA binding in this pulldown experiment, U6 snRNA levels were measured (D).

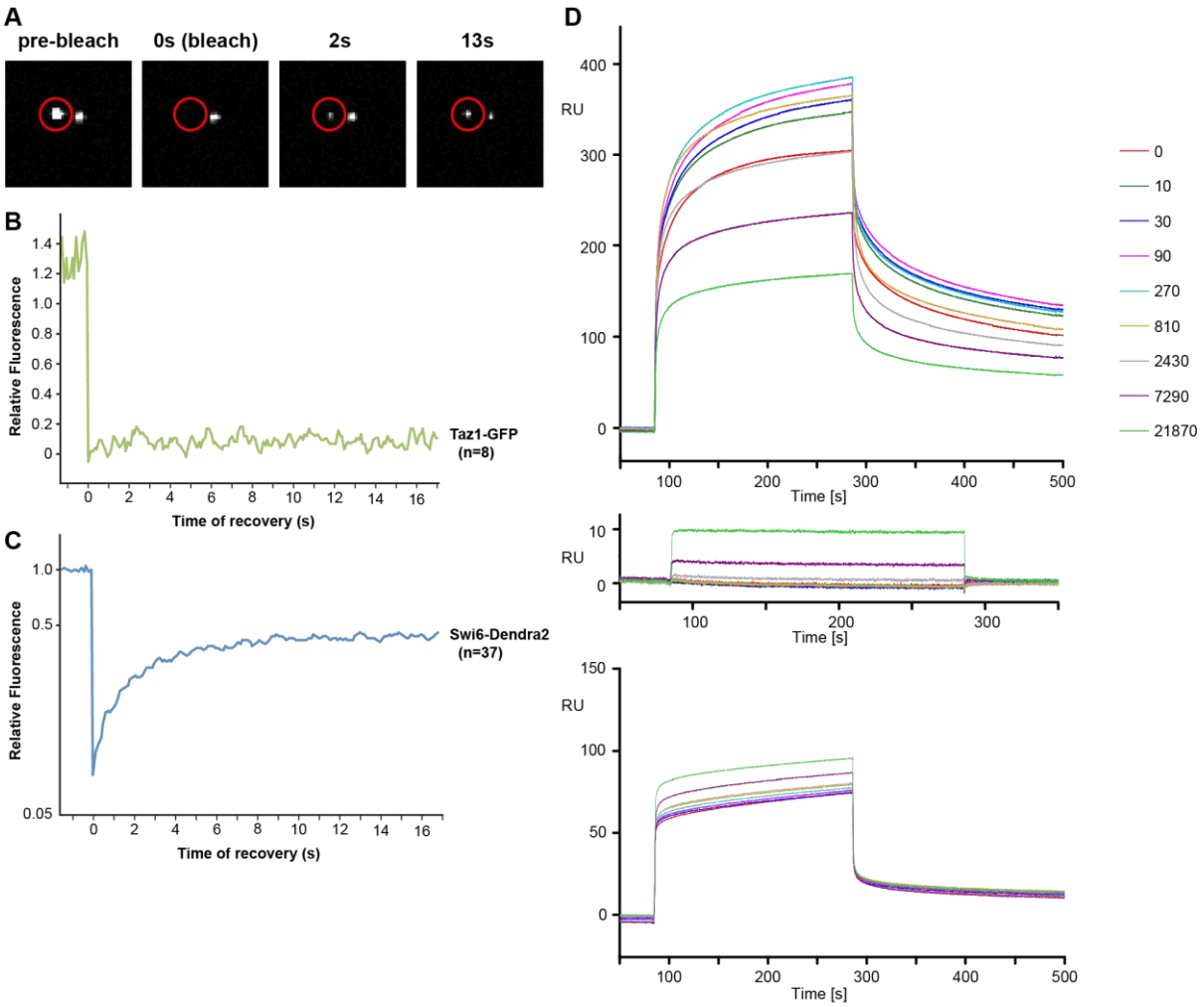

Figure S6, Related to Figure 6. Dynamic Exchange of HP1^{Swi6} from Chromatin on the Ensemble Level In Vivo and the Influence of RNA Binding on H3K9me Binding Properties

(A) Representative images of Fluorescence Recovery After Photobleaching (FRAP) performed with a cell expressing HP1^{Swi6}-Dendra2. Pictures were taken before, immediately after, 2 seconds after, and 13 seconds after photobleaching. Red circle indicates the heterochromatin focus subjected to photobleaching.

(B) FRAP analysis of cells expressing Taz1-GFP. Average relative fluorescence intensities of 8 bleached foci (cells) with a gliding time-average of 3 frames are shown.

(C) FRAP analysis of cells expressing HP1^{Swi6}-Dendra2. The fluorescence intensities were normalized to an unbleached focus in the same image. Average relative fluorescence intensities of 37 bleached foci (cells) with a gliding time-average of 3 frames are shown.

(D) SPR responses for competitive binding of H3K9me3 and RNA to HP1^{Swi6}. Top panel: A constant concentration of 1 µM HP1^{Swi6} with increasing concentrations of 20mer GFP-RNA was injected to an H3K9me3 surface for 200 s. The color code indicates the RNA concentrations in nM. Middle panel: same experiment without HP1^{Swi6}, showing that RNA does not bind to the peptide surface under the experimental conditions used. Bottom panel: same experiment as in the top panel but using 5 µM HP1^{Swi6} KR25A instead of 1 µM HP1^{Swi6} wild-type.

Supplemental Experimental Procedures

Strains and Plasmids

Fission yeast strains were grown at 30°C in YES. All strains were constructed following a PCR-based protocol (Bahler et al., 1998) or standard mating and sporulation. Point mutations were created using the QuickChange Lightning Site-directed mutagenesis kit (Stratagene).
The Swi6-KR25A hinge region fragment was created by gene synthesis (Integrated DNA Technologies, Inc.) and linked by a fusion PCR strategy to give rise to Swi6-KR25A. This was then cloned into a bacterial GST-expression vector. This plasmid was either transformed into bacteria for recombinant protein expression or used as a template for PCR-based gene targeting in *S. pombe*. All Swi6 mutant strains were created by transformation into a *swi6Δ::ura3+* (c.a.) strain (ORF deletion) followed by counterselection on 5-FOA. The Dendra2 protein sequence (Chudakov et al., 2007) was reverse translated *in silico* using yeast codons. The template for PCR-based gene targeting was created by gene synthesis (Integrated DNA Technologies, Inc.) followed by cloning into a pFA6a-link-plasmid series vector (Sheff and Thorn, 2004),
All strains were confirmed by sequencing. Plasmid sequences and detailed maps are available upon request.

Silencing Assays

Serial 10-fold dilutions of the strains indicated were plated on PMGc (nonselective, NS) or on PMGc plates containing 2 mg/mL 5-Fluoroorotic Acid. For ade6-reporter strains, the cells were spotted on YES, YE low ade (22.5 mg/L adenine). For TBZ assays, the cells were spotted on YES plates containing 14 µg/mL Thiabendazole (TBZ) (Sigma T5535).

RNA Probe Labelling for EMSA

DNA templates were generated by PCR on *S. pombe* genomic DNA using primers containing T7 polymerase promoter sequences. In-vitro transcription was performed using the T7 RNA polymerase MEGA script kit (Ambion). For the synthesis of labeled probes, a mix of 0.6 mM UTP and 0.4 mM Fluorescin-UTP (Roche, 2.5 mM) was used. The reaction was carried out for 1h at 37°C followed by a 15 min incubation with 1 µL Turbo DNaseI (37°C). The reaction was phenol-chloroform extracted and purified over G50 spin columns (Amersham) to remove unincorporated nucleotides.

Recombinant Protein Expression and Purification

Recombinant proteins were expressed as N-terminal GST-fusion proteins in Rosetta (BL21) bacteria. A 1L culture was grown in LB + antibiotics until OD_{600}=0.4. The cells were grown for another 2h at 20°C (OD_{600} around 0.6), followed by induction of expression of the GST-fusion proteins with 0.5 mM IPTG. The culture was grown o/n at 20°C. The cells were pelleted, washed and frozen in N_2(l). For protein purification, the cell pellet was resuspended in 5 pellet volumes of lysis buffer (25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Triton-X100 + Protease Inhibitors) and sonicated 6 x 30 sec at 50%. The lysate was spun at 16'000 rpm, 4°C for 30 minutes and cleared by filtration (0.45 µm). The extract was incubated with 1 mL of glutathione-agarose (Sigma) and rotated for 2h at 4°C. After 3 washes (25 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% Triton-X100) the protein was eluted using 50 mM reduced glutathione. The eluate was dialysed o/n into 50 mM HEPES (pH 7.5), 200 mM KCl, 10% Glycerol. This recombinant protein was used for EMSA.

Polysome Profiling

A detailed protocol for polysome profiling in *S. pombe* is available upon request. Briefly, 50 mL of cells were grown to an OD of 0.5-0.6. Cycloheximide was added to a final concentration of 100 µg/µL and the culture was incubated for another 10 min at 30°C. The cells were pelleted and flash frozen in N_2(l). Lysis was performed by bead-beating in 200 µL of lysis buffer and 500 µL glass beads followed by removing insoluble material by centrifugation. 140 OD_{260} were loaded onto a 15-60% sucrose gradient and separated by ultracentrifugation for 2h at 39'000 rpm (Beckman SW40 rotor). The gradient was unloaded from the bottom with 70% sucrose. Fractions were collected while monitoring the absorbance at 254 nm. RNA was isolated from the fractions using phenol-chloroform extraction followed by isopropanol precipitation. RNA recovery was determined by UV absorbance. cDNA was synthesized from 500 ng RNA using the Affinity Script Multiple Temperature cDNA synthesis kit (Stratagene)

and subsequently quantified by qRT-PCR. The data was analyzed as described in (Ding and Grosshans, 2009), calculating the RNA enrichment relative to the total amount of RNA in a given fraction.

mRNA Polyadenylation State Assays

The polyadenylation state of mRNAs was assayed by RACE-PAT or oligo(dT)/RNase H-Northern analysis as described in (Salles et al., 1999). To increase resolution in the RNAseH-Northern assay, an oligo (mb1314) that anneals 100 bp before the STOP codon of the GFP ORF was included in the RNaseH cleavage reaction. RNA was isolated from 50 mL of exponentially growing cells using the hot phenol method. 50 µg of total RNA was incubated with 2 uL mb1314 (100 µM) and with or without oligo dT (10 µL of 100 ng/µL) in a total volume of 68 µL. This was incubated at 65°C for 5 min and slowly cooled down to RT. 8 µL of 10 x buffer, 1 µL RNasIn Plus (Promega) and 1.5 µL RNaseH (New England Biolabs) were added followed by a 30 min incubation at 37°C. The RNA was phenol-chloform extracted followed by ethanol precipitation. The pellet was resuspended in 20 µL of 100% formamide, denatured and separated on a 2.4% MOPS-agarose gel. After capillary transfer in 20 x SSC to a positively charged nylon membrane and UV crosslinking, PNK-labelled oligos (mb1315/mb1316) were hybridized o/n at 35°C. The membrane was washed 3 x 15 min in 0.5 x SSC, 0.1% Triton-X100 at 35°C. Signal was detected using a Phosphorscreen.

5'-Dependent Terminator Exonuclease Assay

Total RNA was isolated using the hot phenol method. The RNA was subjected to DNase digestion using the Absolutely RNA Miniprep Kit (Stratagene). 1 µg RNA was treated with 1 µL of terminator 5'phosphate-dependent exonuclease (Epicentre) for 2h at 30°C. Control reactions were incubated for 2h at 30°C in the absence of the enzyme. The reaction was terminated by phenol-chloroform extraction followed by isopropanol precipitation.1/10th of the reaction was analyzed on a Agilent Bioanalyzer 2100 (Eukaryote Total RNA Nano Chip). 500 ng of RNA was used for cDNA synthesis and quantification by qRT-PCR.

Live Cell Imaging and FRAP Analysis

Imaging was performed on an Olympus IX81 microscope equipped with a Yokogawa CSU-X1 spinning disk, a UPlanFLN 40x/1.3 objective, a CascadeII camera (Photometrics, AZ), a 491nm laser line (Cobolt, Sweden), a Semrock Di01-T488/568 dichroic and a Semrock FF01-525/40-25 emission filter. All devices were piloted with the software Metamorph (Molecular Devices Inc, CA). For FRAP experiments, a UGA-40 module (Rapp-Optoelectronics, Hamburg) equipped with a 473nm laser line and a chroma Z405/473rpc-xt dichroic was installed on the setup. In Metamorph, image acquisition was done using the live replay menu with an exposure time of 100ms and binning 2 for the camera. The bleaching region was a diffraction-limited spot, bleach time was 20ms. The acquired images were analyzed using the open source Fiji software (Walter et al., 2010). The fluorescence intensities were normalized to an unbleached focus in the same image and pre-bleach intensities were averaged and set to 1. Growth conditions for live cell microscopy were described in (Emmerth et al., 2010). Images were acquired at room temperature.

Solution NMR Spectroscopy

The sequence-specific resonance assignments for the isolated HP1^{Swi6} CD (residues 75–140) were obtained using a 750 µM sample of [*U*-^{13}C,^{15}N]-labeled CD sample in 50 mM MES-KOH pH 6.5 buffer with 100 mM KCl, 5 mM DTT and 5%/95% D_2O/H_2O. The assignments were obtained from the two triple-resonance APSY-type experiments 4D APSY-HNCACB (15 projections) and 5D APSY-HNCOCACB (16 projections) (Gossert et al., 2010; Hiller et al., 2005; Hiller et al., 2007) and subsequent automated backbone assignment by the algorithm MATCH (Volk et al., 2008). These experiments were recorded at 25°C on a Bruker 600 MHz spectrometer equipped with a room-temperature triple-resonance probe in a total experiment time of 63 h. The assignments of the CD were transferred to full-length HP1^{Swi6} by a comparison of the [^{15}N,^{1}H]-correlation patterns, which were found to be highly similar (Figs. 4 & S4). The domain-specific resonance assignments of the NTD, the hinge region and the CSD were obtained by identifying the individual substracta from HP1^{Swi6} subconstructs: isolated CD, CD+hinge, CD+hinge+CSD, NTD+CD+hinge.

The NMR titration experiments were performed at 25°C on a Bruker 800 MHz spectrometer equipped with a cryogenic triple-resonance probe. 2D [^{15}N,^{1}H]-TROSY experiments

(Pervushin et al., 1997) of 50–120 μM samples of [*U*-^{15}N]-Swi6 in 50 mM MES-KOH pH 6.5 buffer with 100 mM KCl, 5 mM DTT and 5%/95% D_2O/H_2O were recorded. Typically, 1024 and 90 complex points were recorded in the direct and indirect dimension, respectively in total experiment times of 8–12 h for each spectrum. H3K9me3 peptide from a 1 mM stock solution or 20mer GFP-RNA from a 3 mM stock solution of the same buffer were added.

Surface Plasmon Resonance (SPR)
Samples were analyzed using a Biacore T-100 instrument (GE Healthcare). H3K9me3 peptide was covalently bound to a CM5 chip by amine coupling achieving a final density of 1985 RU. All measurements were recorded as subtracted sensorgrams relative to a flow channel with blank amine immobilisation. Sensorgrams were recorded at 25 °C and flow rate of 50μl min^{-1} using 25 mM NaP_i pH 7.0, 150 mM KCl, 5mM DTT, 0.1% P20, 62.5 μg ml^{-1} BSA and 5% Glycerol as running buffer. All samples were diluted in running buffer prior to injection. Each sample was injected for 200 sec and dissociation was recorded for 300 sec. A regeneration step was performed at the end of each cycle by injecting 5 mM NaOH for 30 sec followed by a stabilization period of 50 sec. For the determination of binding constants, increasing concentrations of HP1^{Swi6} or HP1^{Swi6}-KR25A were injected. For the competition assay, samples of 1 μM Swi6 with increasing amounts of RNA were injected.

Dam-ID
DamID was carried out as previously published (Woolcock et al., 2011). Average enrichment values were calculated for all the oligos overlapping the major heterochromatic regions: mating type locus (chromosome 2, 2114000-2137000), telomeres (chromosome 1, 1-20000 and 5571500-5579133; chromosome 2, 4516200-4539804), and centromeres (chromosome 1, 3753687-3789421, chromosome 2, 1602264-1644747, chromosome 3, 1070904-1137003).

RNA Immunoprecipitation (RIP)
RIP was performed essentially as described in (Gilbert and Svejstrup, 2006). IgG-dynabeads (expoxy-coupled) that have been pre-blocked using E.coli tRNA were used for the immunoprecipitation of the TAP-tagged proteins. An additional DNaseI-digestion step was included before the cDNA synthesis with random primers.

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Supplemental Tables

Table S1. Plasmids

pMB247	pFA6a-Cid14DADA-TAP-hphMX6	Template for PCR based gene targeting (Creation of Cid14DADA::TAP allele)
pMB680	pGEX	empty n-term GST fusion vector with TEV and Thrombin cleavage site
pMB714	pGEX-Swi6	Swi6 purification (N-term GST, TEV and Thrombin cleavage site)
pMB715	pGEX-Swi6-KR25A	Swi6-KR25A purification (N-term GST, TEV and Thrombin cleavage site)
pMB776	pGEX-Swi6-CD	Swi6-Chromodomain purification (N-term GST, TEV and Thrombin cleavage site)
pMB768	pFA6a-link-Dendra2-hphMX	Template for PCR based gene targeting (C-term Dendra2 tagging)

Table S2. Primers for Real-Time PCR

		Forward Primer	***Reverse Primer***
cendg	mb549/mb550	AAGGAATGTGCCTCGTCAAATT	TGCTTCACGGTATTTTTTGAAATC
cendh	mb551/mb552	GTATTTGGATTCCATCGGTACTATGG	ACTACATCGACACAGAAAAGAAAACAA
gfp+	mb820/mb821	CGAAAGATCCCAACGAAAAGAG	TCCCAGCAGCTGTTACAAACTC
ura4+	mb553/554	TACAAAATTGCTTCTTGGGCTCAT	AGACCACGTCCCAAAGGTAAAC
tlh1/2+	mb682/683	CGTGTGCAAGCCGTCAAA	GCTCGAGTTGTGCTGAAATGTC
SPBCPT2R1.07c	mb3006/mb3007	TGGTGTTGCTCCAAAGTGTAGTGGA	GACAGTTGCCTCCGGTAAATGGATTC
Control Genes			
U6 snRNA	mb1281/mb1282	GATCTTCGGATCACTTTGGTCAA	TGTCGCAGTGTCATCCTTGTG
act1+	mb555/mb556	TCCTCATGCTATCATGCGTCTT	CCACGCTCCATGAGAATCTTC
fbp1+	mb557/mb558	CTGGCCAGCTTATTCAACTTCAT	GATTTCGTCGAGATCTTTTTTCATG

Table S3. Primers for RNaseH Assays

		Target	***Purpose***
mb1315	TTACAAACTCAAGAAGGACCATGTGGTCTCTC	GFP	probe
mb1316	TTTGTATAGTTCATCCATGCCATGTGTAATCCCA	GFP	probe
mb1314	GATTGTGTGGACAGGTAATGG	GFP	cleavage

Table S4. RNA Probes

		Length	***Purpose***
20-GFP-RNA	AUGGGUAAAGGAGAAGAACU	20nt	NMR
150-GFP-RNA	ggAGUAAAGGAGAAGAACUUUUCACUGGAGUUGUCCCAAUUCUUGUUGAAUUAGA UGGUGAUGUUAAUGGGCACAAAUUUUCUGUCAGUGGAGAGGGUGAAGGUGAUGC AACAUACGGAAAACUUACCCUUAAAUUUAUUUGCACUACUG	150nt	EMSA
700-GFP-RNA	ggAGUAAAGGAGAAGAACUUUUCACUGGAGUUGUCCCAAUUCUUGUUGAAUUAGA UGGUGAUGUUAAUGGGCACAAAUUUUCUGUCAGUGGAGAGGGUGAAGGUGAUGC AACAUACGGAAAACUUACCCUUAAAUUUAUUUGCACUACUGGAAAACUACCUGUU CCAUGGCCAACACUUGUCACUACUUUCACUUAUGGUGUUCAAUGCUUUUCAAGAU ACCCAGAUCAUAUGAAACGGCAUGACUUUUUCAAGAGUGCCAUGCCCGAAGGUUA UGUACAGGAAAGAACUAUAUUUUUCAAAGAUGACGGGAACUACAAGACACGUGCU GAAGUCAAGUUUGAAGGUGAUACCCUUGUUAAUAGAAUCGAGUUAAAAGGUAUUG AUUUUAAAGAAGAUGGAAACAUUCUUGGACACAAAUUGGAAUACAACUAUAACUC ACACAAUGUAUACAUCAUGGCAGACAAACAAAAGAAUGGAAUCAAAGUUAACUUCA AAAUUAGACACAACAUUGAAGAUGGAAGCGUUCAACUAGCAGACCAUUAUCAACA	711nt	EMSA

	AAAUACUCCAAUUGGCGAUGGCCCUGUCCUUUUACCAGACAACCAUUACCUGUCC ACACAAUCUGCCCUUUCGAAAGAUCCCAACGAAAAGAGAGACCACAUGGUCCUUC UUGAGUUUGUAACAGCUGCUGGGAUUACACAUGGCAUGGAUGAACUAUACAAA		
100-cen-RNA	ggCGUGCGAUCGGGCCGCGACUGGCCAUUUUCAAGGAUAUAUCGAAUCAAAUUUA GGUAUUGCUCUUCUUCUGUAUUUCUAUAUUCGGAGGAAGUAAAU	99nt	EMSA

Table S5. Strain Table

		Figure	Genotype	Source	Comment
spb28	wt	1	h+ leu1-32 ura4D18 oriI ade6-M216 imr1R(Nco1)::gfp+::natMX	*	gfp+ driven by ura4+ promoter
spb38	clr4Δ	1	h+ leu1-32 ura4D18 oriI ade6-M216 imr1R(Nco1)::gfp+::natMX clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb36	dcr1Δ	1	h+ leu1-32 ura4D18 oriI ade6-M216 imr1R(Nco1)::gfp+::natMX dcr1Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb313	cid14Δ	1	h+ leu1-32 ura4D18 oriI ade6-M216 imr1R(Nco1)::gfp+::natMX cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb342	wt	1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb360	clr4Δ	1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb361	dcr1Δ	1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 dcr1Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb374	cid14Δ	1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb342	wt	2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb374	cid14Δ	2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb360	clr4Δ	2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb535	cid14Δ clr4Δ	2	h90 mat3M(EcoRV)::gfp+::natMX ura4- leu1-32 ade6- clr4D::hph cid14Δ::kanMX	*	gfp+ is driven by ura4+ promoter; ura4D18 or DS/E; ade6-M210 or 216
spb721	swi6Δ	2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6Δ::ura3+	*	gfp+ driven by ura4+ promoter
spb723	cid14Δ swi6Δ	2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6Δ::ura3+ cid14Δ::kan	*	gfp+ driven by ura4+ promoter
spb1071	swi6-Dendra2	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6-Dendra2::hphMX	*	gfp+ driven by ura4+ promoter
spb1240	nls-swi6-Dendra2	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6-Dendra2::hphMX	*	gfp+ driven by ura4+ promoter
spb1241	nls-swi6-KR25A-Dendra2	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6-KR25A-Dendra2::hphMX	*	gfp+ driven by ura4+ promoter
spb342	wt	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb360	clr4Δ	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb939	swi6Δ	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6Δ::ura3+ (ORF deletion only)	*	gfp+ driven by ura4+ promoter
spb1226	nls-swi6	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6	*	gfp+ driven by ura4+ promoter
spb1227	nls-swi6-KR25A	4	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6-KR25A	*	gfp+ driven by ura4+ promoter

spb435	dam	6	h+ leu1-32 ade6-M216 ura4Δ::nmt1(81x)-dam-myc-kan		
spb436	dam-cid14	6	h+ leu1-32 ade6-M216 ura4Δ::nmt1(81x)-dam-myc-cid14-kan		
spb1386	dam swi6Δ	6	h+ leu1-32 ade6-M216 ura4Δ::nmt1(81x)-dam-myc-kan swi6Δ::ura3+ (ORF deletion only)		
spb1387	dam-cid14 swi6Δ	6	h+ leu1-32 ade6-M216 ura4Δ::nmt1(81x)-dam-myc-cid14-kan swi6Δ::ura3+ (ORF deletion only)		

spb65	wt, ura4+	S1	972h-	1	
spb221	wt	S1	h- imr1R(Nco1)::gfp+::natMX	*	gfp+ driven by ura4+ promoter
spb295	clr4Δ	S1	h- imr1R(Nco1)::gfp+::natMX clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb294	cid14Δ	S1	h- imr1R(Nco1)::gfp+::natMX cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb373	cid14DADA	S1	h- imr1R(Nco1)::gfp+::natMX cid14DADA-TAP::hphMX	*	gfp+ driven by ura4+ promoter
spb342	wt	S1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb360	clr4Δ	S1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb374	cid14Δ	S1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb739	cid14DADA	S1	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 cid14DADA-TAP::hphMX	*	gfp+ driven by ura4+ promoter

spb29	wt, ura4+	S2	h+ otr1R(SphI)::ura4+ leu1-32 ade6-M210 ura4Δ::gfp::natMX	*	end. ura+ ORF replaced with gfp+
spb342	wt	S2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb360	clr4Δ	S2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 clr4Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb374	cid14Δ	S2	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 cid14Δ::kanMX	*	gfp+ driven by ura4+ promoter
spb76	no gfp	S2	h90 mat3M(EcoRV)::ura4+ ura4-DS/E leu1-32 ade6-M210	2	

spb342	wt	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210	*	gfp+ driven by ura4+ promoter
spb1055	swi6-KR25A	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6-KR25A	*	gfp+ driven by ura4+ promoter
spb1468	swi6-Dendra2 cnp1-mCherry	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 swi6-Dendra2::hphMX cnp1-mCherry::kanMX	*	gfp+ driven by ura4+ promoter
spb1450	nls-swi6-Dendra2 cnp1-mCherry	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6-Dendra2::hphMX cnp1-mCherry::kanMX	*	gfp+ driven by ura4+ promoter
spb1469	nls-swi6-KR25A-Dendra2 cnp1-mCherry	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 nls-swi6-KR25A-Dendra2::hphMX cnp1-mCherry::kanMX	*	gfp+ driven by ura4+ promoter
spb1439	NLS-TAP-Swi6	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 NLS-TAP-Swi6	*	gfp+ driven by ura4+ promoter
Spb1493	NLS-TAP-Swi6-KR25A	S5	h90 mat3M(EcoRV)::gfp+::natMX ura4-DS/E leu1-32 ade6-M210 NLS-TAP-Swi6-KR25A	*	gfp+ driven by ura4+ promoter

Source: *this study, [1]Charles Hoffmann, [2]Danesh Moazed