THE UNIVERSITY
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RESTRICTED EPIGENETIC INHERITANCE OF H3K9 METHYLATION

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PREFACE

This thesis was composed by myself and the research presented is my own unless otherwise stated

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ABSTRACT

In most eukaryotes methylation of histone H3 on lysine 9 (H3K9me) is the key post-translational modification required for the assembly of constitutive heterochromatin at centromeres and other chromosomal regions. H3K9me is bound by the chromodomain proteins HP1/Swi6 and the Suv39/Clr4 H3K9 methyltransferase itself suggesting that, once established, H3K9me might act as an epigenetic mark that can transmit the chromatin state independently of the initiator signal. However, it has not been demonstrated that H3K9me does indeed act as an epigenetic mark. Fission yeast represents an excellent system to address this question since *S. pombe* lacks DNA methylation and H3K9me is catalysed by the unique, non-essential H3K9 methyltransferase Clr4. To determine whether H3K9me carries epigenetic properties it is important to uncouple H3K9me from genomic domains that have the intrinsic ability to recruit the heterochromatin machinery. One way to solve this problem is to isolate H3K9me from its original context and investigate whether at an ectopic site H3K9me can self-propagate through cell division. To accomplish this, we tethered regulatable TetR-Clr4 fusion protein at euchromatic loci in fission yeast. This resulted in the assembly of an extensive domain of H3K9me-dependent heterochromatin that is rapidly disassembled following TetR-Clr4 release. Strikingly, the inactivation of Epe1, a putative histone demethylase, is sufficient to maintain the silent H3K9me-dependent heterochromatin at the tethering sites through mitotic and meiotic cell divisions in absence of TetR-Clr4. These results indicate that H3K9me acts as an epigenetic mark to maintain heterochromatin domains; however, a regulatory mechanism dependent on Epe1 exists to actively remove H3K9me and thus prevent heterochromatin from being transmitted when assembled at inappropriate regions of the genome.

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ABBREVIATIONS

5-FOA	5-fluoro-orotic-acid
ade	adenine
adh	alcohol dehydrogenase
AHT	anhydrotetracycline
BAH	bromo-adjacent homology
bp	base pair
cdd	chromodomain deleted
cDNA	complementary deoxyribonucleic acid
CENPA	centromere protein A
ChIP	chromatin Immunoprecipitation
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide thriphosphate
dsRNA	double stranded ribonucleic acid
EDTA	ethylene di-amine tetra acetic acid
FACT	facilitates chromatin transcription
GFP	green fluorescent protein
HA	haemagglutinin
HDAC	histone deacetylase
Imr	inner most repeats
Kb	kilobase
KDa	kilodalton
LB	Luria Bertani medium
ME	malt extract
mRNA	messanger RNA
N-terminal	amino-terminal
Nmt	no message in thiamine
nt	nucleotide
ORF	open reading frame
otr	outer repeats
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PMG	pombe media glutamate

RDRC	RNA-directed RNA polymerase complex
RITS	RNA-mediated initiation of transcriptional silencing
RNA	ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
rpm	rotation per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
sir	silent information regulator
TBE	tris-borate EDTA
TetO	tetracycline operator
TetR	tetracycline repressor protein
tRNA	transfer RNA
ts	temperature sensitive
TSS	transcription start site
Tween	polyoxyethylenesorbitan monolaurate
ura	uracile
wt	wild type
YES	yeast extract supplemeted

CHAPTER 1: INTRODUCTION

1.1 Chromatin

1.1.1 Definition and function

In eukaryotes, the genome is compacted into a nucleoprotein complex called chromatin. The fundamental unit of the chromatin is the nucleosome composed of 147 base pairs of DNA wrapped around an octamer of histones. Each octamer is composed of a tetramer of H3/H4 flanked by two separate H2A/H2B dimers. In many organisms, histone H1 binds and protects the linker DNA between nucleosomes (Kornberg and Lorch, 1999). Histones are mostly globular except for their N-terminal tails that are unstructured. Histone tails are enriched in basic residues and subject to many different post-translational modifications (PTMs). Chromatin has several functions; it represents the first level of packaging of the genome in order for it to fit in the small nuclear compartment, it protects the DNA from degradation, it organizes the genome into highly structured chromosomes and it regulates gene expression. Chromatin is a highly dynamic structure and displays remarkable plasticity in allowing cellular processes such as DNA replication, DNA repair, transcription and mitotic condensation to occur and it allows for the acquisition of specific functions during cell differentiation in metazoa (Bell et al., 2011; Groth et al., 2007) (Leeb and Wutz, 2012; Vagnarelli, 2012).

1.1.2 Chromatin organization

Chromatin is divided into domains of differing DNA, protein and RNA composition with distinct functional properties. Initial observations using microscopic approaches defined two main chromatin types: euchromatin that displays low density staining and heterochromatin that remains condensed outside mitosis and generally localizes to the nuclear periphery (Bickmore and van Steensel, 2013; Grewal and Jia, 2007; Passarge, 1979). Later on, euchromatin was defined as open chromatin, relatively gene-rich and generally transcriptionally active in contrast to heterochromatin that represents a condensed, mostly transcriptionally repressed, gene poor environment (Grewal and Jia, 2007). More recently many new chromatin sub-types have been

described characterized by their specific composition in terms of DNA methylation, histone variants, histone PTMs and their nuclear localisation (Bickmore and van Steensel, 2013).

1.1.3 Regulation of chromatin structure and function

Many chromatin factors control nucleosome assembly, position or modification and participate in the assembly of the chromatin landscape (Lalonde et al., 2014; Park and Luger, 2008). By regulating chromatin composition and dynamics, these actors finely tune the properties of chromatin over different regions of the genome. This regulation makes the genome more accessible for transcription or replication, more condensed for mitotic entry, or increases compaction over repetitive elements to prevent DNA recombination.

Histone chaperones

As soon as they are synthetized, histones associate with histone chaperones that transport them into the nucleus, facilitate nucleosome assembly, disassembly and histone recycling during replication and transcription. Histone chaperones also ensure that a constant pool of soluble histones is available in cells that can be utilised under stress conditions. Specific chaperones are responsible for the incorporation of histone variants in chromatin (Avvakumov et al., 2011; Burgess and Zhang, 2013)

Histone remodelers

ATP-dependent histone remodelers are able to evict, slide or destabilize nucleosomes in chromatin and are therefore involved in many biological processes. Five classes of histone remodelers have been defined based on their biological activity (SWI/SNF, INO80, Swr1, ISWI and NuRD/Mi-2/CHD)(Clapier and Cairns, 2009; Wang et al., 2007{Clapier, 2009 #566). SWI/SNF remodelers, for example, help to open up the chromatin to make the DNA accessible to the transcription machinery, while ISWI remodelers position nucleosomes with a regular spacing in chromatin and are important for the reorganization of chromatin following replication.

Histone variants

Chromatin properties can also be modified by the incorporation of histone variants that share sequence similarities and structural properties with core histones but display specific features. Histone variants replace core histones at specific regions of the genome and thus confer new functions to the chromatin. The incorporation of the histone H3 variant CENP-A at centromeres is for instance at the basis of kinetochore assembly (Henikoff and Smith, 2015). H2AZ is present at the first nucleosomes of many genes in most eukaryotes and is thought to aid transcriptional initiation by destabilizing this nucleosome, although H2AZ is also associated with transcription pausing in budding yeast and plants (Henikoff and Smith, 2015; Talbert and Henikoff, 2010).

Histone post translational modifications (PTMs)

Histone PTMs have been proposed to affect chromatin structure and functions. To date histones have been shown to undergo lysine acetylation, arginine, lysine and glutamine methylation, phosphorylation, proline isomerization, ubiquitylation (Ub), ADP ribosylation, arginine citrullination, SUMOylation and carbonylation, most of which occurs on residues at the N-terminal tail of histone (Kouzarides, 2007; Tessarz et al., 2014). Many enzymes mediate the deposition and the removal of these histone PTMs. Specific patterns of individual histone modifications and chromatin associated factors are associated with particular biological processes and are highly conserved through evolution, suggesting that histone PTMs play important roles in cellular functions (Kouzarides, 2007). With this observation came the idea that a histone code might operate to regulate the chromatin state and chromatin changes (Barth and Imhof, 2010; Jenuwein and Allis, 2001; Turner, 2002). A particular set of histone modifications would impose a particular chromatin state and mediate specific biological processes such as transcription initiation, DNA repair or replication. What remains unclear is whether histone PTMs alone can dictate a chromatin state (without DNA dependent instructions) or whether they just result from changes dictated by DNA (such as transcription).

Histone PTMs can directly affect chromatin structure by altering the interaction between nucleosomes and DNA or the interaction histones within nucleosomes (Bannister and Kouzarides, 2011; Cuthbert et al., 2004; Kornberg and Lorch, 1999).

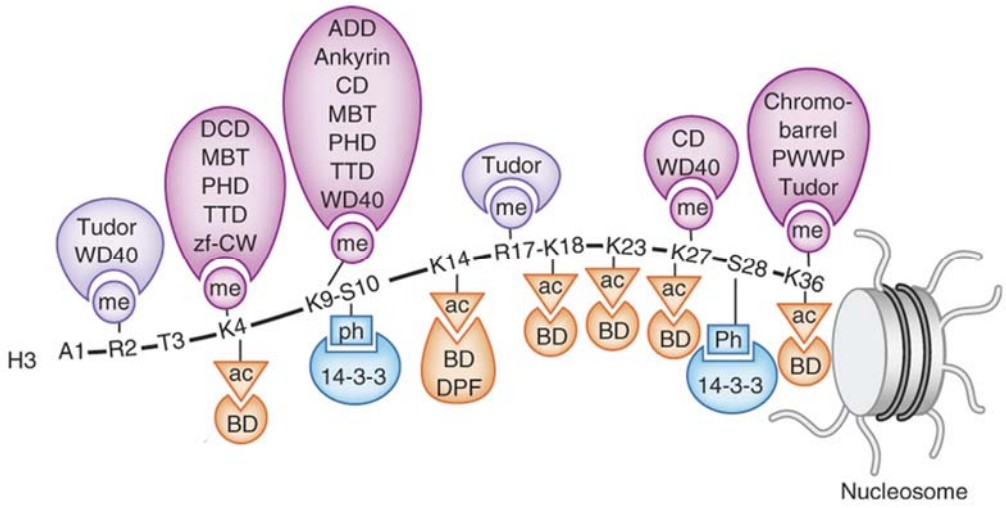


Figure 1.1: Overview of binding specificity of various histone binding domains for the N-terminal tail of histone H3

Histone readers bound to methylated (me) lysine, methylated (me) arginine, acetylated (ac) lysine and phosphorylated (ph) serine residues are indicated. Figure adapted from (Musselman, Lalonde et al. 2012).

Histone PTMs also create a specific binding platform for numerous effector proteins that associate with modified nucleosomes or free histones. A huge range of proteins carry domains that specifically bind various post-translational modified residues of histones (Figure 1.1). Histone methylation will be discussed more extensively as it is most relevant to this thesis.

-Histone methylation

Histones can be mono-, di- and tri- methylated on lysine residues and mono- and di-methylated on arginine residues (Figure 1.2). Histone methyltransferases use S adenosyl-L-methionine as a methyl donor to catalyse the reaction. With the exception of H3K79 methylation that is catalysed by the Dot1 protein, methylation of all other lysine residues in histones is carried by proteins containing a conserved SET catalytic domain. The SET domain proteins are a very large family with more than 100 members identified in humans (Qian and Zhou, 2006) while there are only nine arginine methyltransferases. Lysine methyltransferases (KMTs) are highly specific with respect to their substrates. Indeed, one KMT generally methylates a single residue and several KMTs might also be required to catalyse momo-, di- and trimethylation of a residue (Qian and Zhou, 2006). Methylated lysines in histones can be bound by several protein motifs such as chromodomain, tudor, MBT or PHD domains (Adams-Cioaba and Min, 2009; Musselman et al., 2012). Lysine methylation is associated with both transcriptional activation and transcriptional repression. H3K4, H3K36 and H3K79 methylation are usually found on actively transcribed regions while H3K9, H3K27 and H4K20 methylation are normally associated with silent chromatin. For many years histone methylation was thought to be a stable irreversible modification. This idea arose from studies of histone turnover rates in mammalian cells performed in the 1970s that suggested that the turnover rate of methylated K and R in histones was similar to the turnover rate of histones in general (Byvoet et al., 1972; Thomas et al., 1972). Although other studies showed that a small proportion of histone methyl groups do turnover, it was not until 2004 that the first histone demethylase was identified (Shi et al., 2004). So far, two classes of lysine demethylases have been described: the SWIRM and the JmjC domain containing demethylases (Klose and Zhang, 2007). SWIRM demethylases are flavin adenine dinucleotide (FAD)-dependent amine oxidases that demethylate mono and di-methylated lysines. The JmjC domain proteins promote demethylation via an

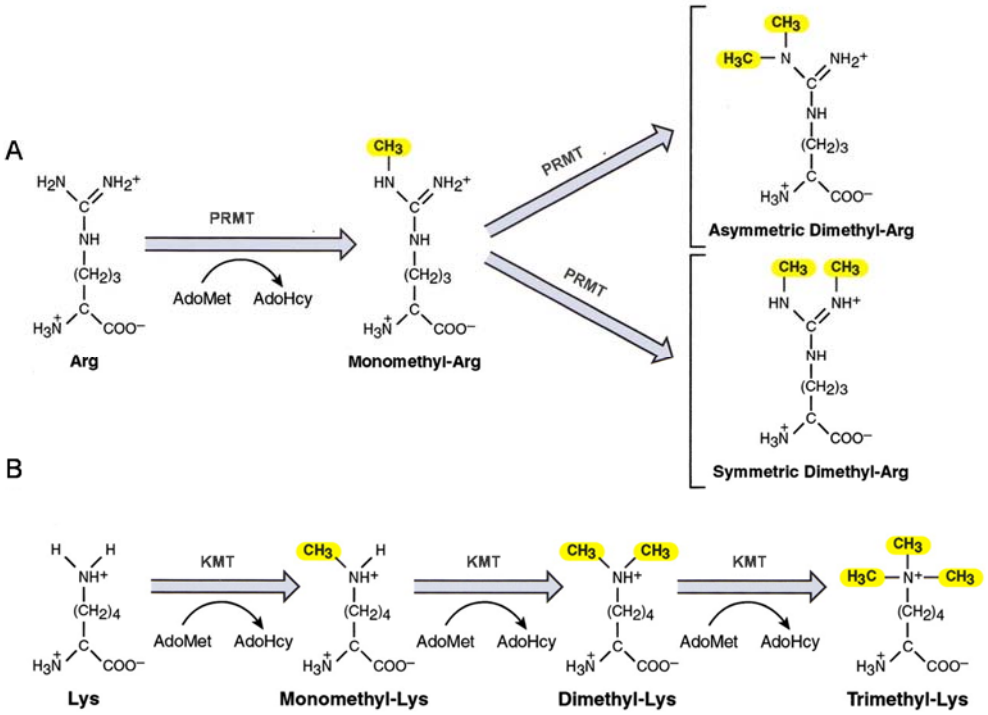


Figure 1.2: Arginine and lysine methylation

(*A*) Methylation of arginine by protein arginine methyl-transferases (PRMTs) into mono- and di-methyl-arginine. Di-methyl-arginine can be symmetric or asymmetric depending on the PRMT that catalyses the reaction.
(*B*) Methylation of lysine by lysine methyl transferases (KMTs) into mono-, di- and tri-methyl-lysine. Figure adapted from (Zhang and Reinberg 2001).

oxidative reaction that relies on Fe(II) and α-ketoglutarate as cofactors. Unlike the Amine-Oxidase demethylases, JmjC containing proteins can demethylate mono-, di- and tri- methylated lysine. In the case of arginine methylation, the human peptidylarginine deiminase PAD4 converts non-methylated and mono-methylated arginine into citrulline (Cuthbert et al., 2004; Wang et al., 2004).

Other mechanisms can also induce the loss of certain methylation marks from histones in chromatin. Histone tails can be cleaved by endopeptidases in a process called histone clipping that has been described in various organisms from yeast to humans. This results in the removal of the histone N-terminal tail and therefore the loss of all associated PTMs (Allis et al., 1980; Santos-Rosa et al., 2009; Vossaert et al., 2014).

1.1.4 Chromatin landscape associated with active transcription

In eukaryotes, transcriptionally active regions of the genome display relatively low nucleosome density and therefore a relatively open chromatin state. Specific conserved histone modifications are concentrated over specific regions of transcription units and their neighbouring regulatory regions (Figure 1.3). However, the role of all these histone PTMs with respect to the transcription process is not always known (Smolle and Workman, 2013). In most species the histone variant H2AZ marks promoter regions. It is found in the +1 nucleosome of transcribed genes. H2AZ is essential in humans but its function in gene expression is poorly understood (Smolle and Workman, 2013) although it is thought to destabilize the +1 nucleosome to aid transcription initiation (Henikoff and Smith, 2015).

Regions of the genome that are transcriptionally active are generally enriched in acetylated marks. Promoter regions of active genes often display high levels of acetylated residues: H3K9Ac, H3K14Ac and H4K16Ac (Owen-Hughes and Gkikopoulos, 2012; Smolle and Workman, 2013). Acetylation of histones disrupts the interaction between positively charged histones and negatively charged DNA. Acetylated histones are therefore less stable on the DNA and consequently acetylated chromatin adopts a more open configuration that is permissive to transcription. Acetylated lysine residues on nucleosomes also provide specific binding sites for other proteins that can affect the chromatin state (Verdone et al., 2005).

H3K4 methylation is usually found on active genes. Promoter regions are generally enriched in H3K4me3 while the bodies of transcription units (ORFs) are enriched in H3K4me2. H3K4 is methylated by the conserved H3K4 methyltransferase Set1 that is part of the Set1C/COMPASS complex. H3K4 methylation requires prior H2B ubiquitylation (Racine et al., 2012; Smolle and Workman, 2013). Methylated H3K4 facilitates the transcription process by creating a binding platform for proteins involved in chromatin remodeling and further histone modifications. For instance, BPTF, a component of the NURF chromatin-remodeling complex, binds H3K4me3 through its PHD domain and directs the activation of the H0XC8 gene in *Xenopus* embryos (Wysocka et al., 2006). Other methylated histone residues are also associated with active transcription. H3K36me2 and H3K36me3 are found over the entire transcription unit of active genes. In *S. cerevisiae,* H3K36me2/me3 recruits the RPD3S histone deacetylase (HDAC) complex to chromatin to remove acetyl groups from histones H3 and H4 and promote the reassembly of nucleosomes following the passage of RNAPII (Carrozza et al., 2005; Smolle and Workman, 2013). H3K79 methylation by the methyltransferase Dot1 family of proteins is also found over active genes in budding yeast, flies, mice and humans, however the mechanism that links H3K79me to active transcription is poorly understood

1.2 Heterochromatin

1.2.1 Characteristics and functions

Heterochromatin domains represent condensed regions of the genome that are generally repressed transcriptionally. Heterochromatin can be divided in two subtypes, constitutive heterochromatin and facultative heterochromatin (Trojer and Reinberg, 2007).

Constitutive heterochromatin is often assembled on repetitive sequences and in most eukaryotes is concentrated at centromeres and telomeres. These permanent blocks of heterochromatin play a role in the maintenance of the genome integrity (Grewal and Jia, 2007; Peng and Karpen, 2008; White and Allshire, 2008). They prevent recombination of repeat regions, silence dispersed repetitive elements derived from various transposable elements and play a role in nuclear organisation (Peng and Karpen, 2008). At centromeres, heterochromatin is important for accurate chromosome segregation (Bernard et al., 2001; Kellum and Alberts, 1995; Shimura

et al., 2011). In fission yeast Swi6, the counterpart of heterochromatin protein 1 (HP1), is required for the recruitment of cohesin to centromeres and heterochromatin promotes the establishment of the H3 histone variant CENP-A at centromere regions (Bernard et al., 2001; Folco et al., 2008; Kagansky et al., 2009).

Facultative heterochromatin is associated with a subset of genes that are only packaged into a heterochromatin-like structure in response to differentiation signals or environmental cues (Trojer and Reinberg, 2007),(Yamanaka et al., 2013), Zofall et al, 2012). X inactivation in mammals is a good example of facultative heterochromatin formation (discussed in section 1.2.2.B.1). Facultative heterochromatin is often characterized by an enrichment of H3K27me3, H3K9me3, H4K20me3, H2Aub1, hypoacetylated histones and macroH2A; however a common signature has not been found for all facultative heterochromatin loci (Trojer and Reinberg, 2007).

H3K9 methylation

In most organisms, histones tails are hypoacetylated at heterochromatin regions but histone H3 methylated on the lysine 9 (H3K9me) is highly enriched. H3K9me recruits chromodomain proteins such as HP1 that interact with and recruit other heterochromatin factors to trigger gene silencing. The machinery responsible for H3K9me-dependent heterochromatin formation is conserved from *S. pombe* to humans. H3K9me is catalysed by a HMT of the SET domain family. The *S. pombe* genome encodes a single H3K9 methyltransferase Clr4 that is responsible for mono-, di- and tri-methylation of H3K9 (Yamada et al., 2005). In higher eukaryotes, several H3K9 methyltransferase collaborate to methylate H3K9me. For example, in humans, G9a/GLP mostly direct mono- and di-methylation on H3K9, SetDB1 trimethylates H3K9, and Suv39h1/h2 methyltransferases mediate H3K9 di- and tri-methylation (Shinkai and Tachibana, 2011). The degree of conservation within the Clr4/Suv39 family is high since human SUV39H1 gene can partially rescue the silencing defect of a Drosophila *su(var)3-9* null mutant (Schotta et al., 2002).

H3K9me1/me2/me3 are concentrated over different regions in the genome (Barski et al., 2007; Rosenfeld et al., 2009). In mammals, H3K9me1 is mostly found at promoter and 5'UTR of active genes and, unlike H3K9me2/me3, is not enriched over gene poor regions (Barski et al., 2007). H3K9me2 and H3K9me3 are highly enriched over

heterochromatic regions including gene poor regions, centromeres and subtelomeric regions. However, in mammalian cells the distribution of H3K9me2 and H3K9me3 do not strongly overlap. Large domains of H3K9me2 chromatin referred to as LOCKs (Large Organised Chromatin K9-modifiifcation) are conserved in mice and humans and are observed in a cell type specific manner, indicating that they might play a role during cell differentiation (Wen et al., 2009). Long stretches of H3K9me3 are found in the genome, especially at pericentromere regions, that do not overlap with LOCKs. Moreover, H3K9me3 has also been found enriched over coding regions, suggesting that H3K9me3 might play a role in active transcription (Vakoc et al., 2005).

In most species, H3K9 methylation is recognised by HP1-like proteins that bind H3K9me directly via their chromodomains. HP1 proteins are composed of a chromodomain (CD) that binds methylated H3K9, a hinge domain and a chromoshadow domain (CSD). The CSD creates a binding surface that recruits additional proteins many of which contain a PxVxL motif (Richart et al., 2012; Smothers and Henikoff, 2000). The CSD itself contains a PxVxL motif, allowing HP1 proteins to dimerise. The ability of HP1 proteins to dimerise is thought to be important for heterochromatin spreading (Canzio et al., 2011

; Cowieson et al., 2000). Mammalian and *S. pombe* HP1/Swi6 can also bind RNA and DNA via their hinge domains and this has been proposed to regulate their localization (Keller et al.; Meehan et al., 2003; Muchardt et al., 2002). HP1/Swi6 acts as a platform that recruits a variety of proteins of different activities and functions. Both HP1 and Swi6 can directly associate with the H3K9 KMTs Suv39 and Clr4 in metazoa (mammals, *Drosophila)* and *S. pombe* respectively and this interaction plays a role in the spreading and maintenance of H3K9 methylation (Haldar et al., 2011; Yamamoto and Sonoda, 2003). There are several different HP1-related proteins in *S. pombe*, mammals and *Drosophila* which exhibit different localization patterns and distinct functions. In mammals, HP1α and HP1β associate with repressed promoters within euchromatin regions while HP1γ is enriched along with H3K9me3 on actively transcribed genes (Black and Whetstine, 2011).

Other heterochromatin associated histone modifications

In higher eukaryotes other histone PTMs and DNA modifications contribute along with H3K9 methylation to heterochromatin formation. Heterochromatin regions in many species are enriched in H3K27me3, H2Aub1 and 5-methyl cytosine (DNA

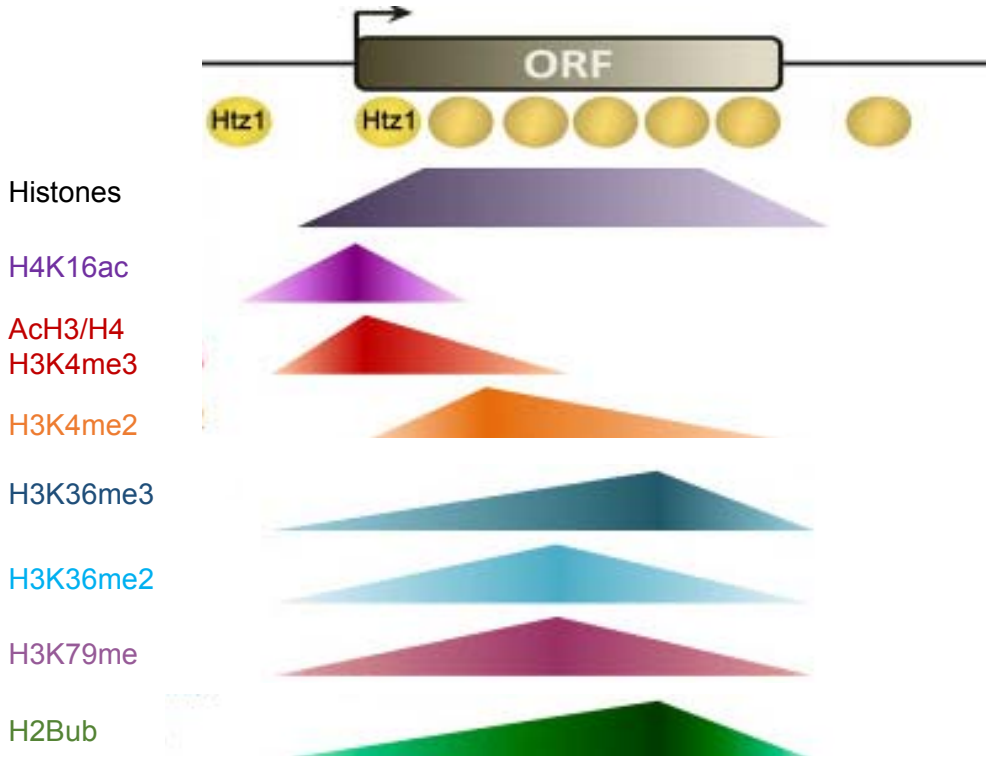


Figure 1.3: Distribution of nucleosomes and histone modifications across transcription units. These data have been collected from *S. cerevisiae* studies. Figure adapted from (Smolle and Workman 2013).

methylation). Notably these three modification are absent from *S. pombe*. In mammals, two Polycomb repressive complexes PRC1 and PRC2 are responsible for H2Aub1 on lysine 119 and di/trimethylation of H3K27, respectively (Margueron and Reinberg, 2011). H3K27me3 collaborates with H3K9me for heterochromatin assembly and these two PTMs overlap at many regions in the genome. The H3K9 methyltransferases GLP and G9a interact directly with PRC2 and participate in its recruitment to the chromatin (Mozzetta et al., 2014). Furthermore, HP1 binding to H3K9me3 seems to be enhanced by H3K27me3 (Boros et al., 2014). In plants and mammals, 5-MeC methylation of DNA also plays an important role in the establishment and the maintenance of H3K9me (Almouzni and Probst; Cheng, 2014; Jackson et al., 2002; Johnson et al., 2007; Soppe et al., 2002; Tariq et al., 2003).
In human cells, HP1 proteins provide a docking site for another KMT, Suv4-20h1/2, that tri-methylates H4K20 and promotes chromatin compaction (Schotta et al., 2004). H3K9me3 also facilitates tri-methylation of H3K64 which has been proposed to stabilise heterochromatin domains (Lange et al., 2013). In general the main features of heterochromatin are well conserved amongst species although heterochromatin domains in higher eukaryotes have additional complexity in which heterochromatin formation relies on more complex crosstalk between DNA methylation and various histone modifications. Some species also exhibit specific heterochromatin components, for example, *Arabidopsis thaliana* completely lacks HP1 related proteins and *S. cerevisiae* is devoid of both H3K9 methylation and HP-1 related proteins and relies on histone deacetylases for the formation of Sir2/3/4 dependent heterochromatin.

Heterochromatin-like structures in Saccharomyces cerevisiae

Unlike heterochromatin domains in *S. pombe* or higher eukaryotes, heterochromatin regions in *S. cerevisiae* do not rely on H3K9 methylation but depend entirely on histone deacetylases that are recruited to chromatin. Chromatin silencing in *S. cerevisiae* depends on the SIR complex, a trimer composed of SIR2, SIR3 and SIR4. Similar to heterochromatin in higher eukaryotes, this SIR-dependent heterochromatin creates a repressive environment, enriched in hypoacetylated histones. This SIR-dependent heterochromatin is mainly assembled over the silent mating type loci (HML and HMR) and subtelomeric regions, which are replicated late in S phase and located at the nuclear periphery or in the vicinity of the nucleolus (Grunstein and Gasser,

2013; Kueng et al., 2013). RNAPII transcription within the tandemly repeated rDNA is also repressed via a different complex called RENT (regulator of nucleolar silencing and telophase) in which Sir2 prevents recombination between rDNA repeats (Gottlieb and Esposito, 1989). At the silent mating type loci and subtelomeres, specific DNA sequences called silencers are responsible for the recruitment of the SIR proteins. Silencers are generally binding sites for specific factors such as Abf1, Rap1 and Orc1 that recruit Sir proteins to the chromatin. (Gilson et al., 1993; Kueng et al., 2013; Mishra and Shore, 1999; Triolo and Sternglanz, 1996).

The spreading of this Sir-dependent heterochromatin occurs in a sequential manner. Initially, Sir4 associated with Sir2 is recruited to the nucleation site. This dimer deacetylates H4K16 via Sir2 H4K16ac HDAC activity. This allows the recruitment of Sir3 that preferentially binds unacetylated nucleosomal histone H4 (Hecht et al., 1995). Sir3 then recruits additional Sir2-Sir4 dimer through its interaction with Sir4, thereby promoting the spreading of this silent chromatin over a large domain (King et al., 2006).

Nuclear localization

Chromosome localisation and the interaction between chromosomal domains is not random in the nucleus and the maintenance of this specific nuclear organisation is important for the maintenance of the genome integrity. Early studies using electron microscopy showed that condensed region of chromatin preferentially associate with the nuclear periphery and the nucleolus (Bickmore and van Steensel, 2013; Kind and van Steensel, 2010). However whether silent regions are specifically targeted to these nuclear compartments or whether positioning of a chromatin domain to the nuclear periphery or the nucleolus promotes silencing is still unclear. The localisation of a chromatin domain to the perinucleolus or the lamina does not necessarily lead to transcriptional silencing. In human cells, the artificial tethering of chromosomes to the nuclear periphery has been shown to promote silencing of some genes around the tethering sites, however many genes remain unaffected by the relocalisation to the nuclear periphery indicating that the nuclear periphery is actually permissive to transcription (Bickmore and van Steensel, 2013; Finlan et al., 2008). On the other hand, a recent report indicates that transcription activation or decondensation of chromatin by recruitment of an acidic peptide results in genes being released from the nuclear envelope (Therizols et al., 2014).

The mechanisms involved in the association of heterochromatin with the nucleolus and at the nuclear periphery are complex and seem to involve both DNA sequence and chromatin. In *C. elegans*, insertion of a heterochromatic array into a chromosome is sufficient to promote peripheral localisation of this domain (Meister et al., 2010)
H3K9me itself might be important for clustering heterochromatin regions, at the nuclear periphery since in *C. elegans* the two H3K9KMTs promote peripheral localisation of transgene repeats (Towbin et al., 2012). Similarly, the loss of the H3K9 KMT Clr4, in *S. pombe* has been reported to result in the dissociation of the mating type locus from the nuclear periphery (Alfredsson-Timmins et al., 2007). In human cells HP1alpha has been shown to directly interact with the inner nuclear membrane protein LBR both *in vitro* and *in vivo* and this might be important for targeting heterochromatin to the nuclear periphery (Ye et al., 1997).

1.2.2 Heterochromatin establishment

Heterochromatin domains assemble sequentially in three steps: establishment, spreading and maintenance (Rusche et al., 2003). The establishment of heterochromatin relies on inducer signals that recruit chromatin modifiers at a nucleation site and promote heterochromatin assembly. Heterochromatin can then spread in *cis* independently of the underlying sequence. Additional factors can be involved in the stable maintenance of heterochromatin and factors that are essential for heterochromatin establishment may be dispensable for its maintenance. In most organisms, the mechanisms that initiate heterochromatin establishment are poorly understood. Nevertheless, in all organisms heterochromatin is found enriched at repeat regions and transposon related elements suggesting a role for these specific sequences in recruiting heterochromatin factors. In some instances an increase in repeat copy number triggers heterochromatin formation. For example, an increase in copy number of macrosatellite repeats in the genome correlates with an increase of silencing of surrounding genes (Brahmachary et al., 2014). Similarly, in *Drosophila*, increasing the copy number of a transgene inserted in the genome promotes heterochromatin-induced gene silencing (also known as position effect variegation/PEV (Dorer and Henikoff, 1994).
It is well recognised that RNA interference (RNAi) is absolutely essential for the establishment of heterochromatin on centromeric repeats in fission yeast (Buscaino

et al., 2013; Partridge et al., 2007; Sadaie et al., 2004) (Reyes-Turcu et al.; Volpe et al., 2002). Increasingly more evidence also points towards a conserved role for RNAi in the assembly of heterochromatin in higher organisms (Jackson et al.).

1.2.2.A RNAi related pathways promote heterochromatin establishment

Small RNAs play an important role in regulating transcription throughout genomes of many organisms. Three types of small RNAs have been found to promote gene silencing: Piwi-interacting RNAs (piRNAs), short interfering RNAs (siRNAs) and micro RNAs (miRNAs) (Castel and Martienssen). These small effector RNAs are between 20 and 30 nucleotides in length and act either to repress transcription, or post-transcriptionally to destroy RNAs, from specific loci in a sequence dependent manner. The three classes of small RNAs associate with effector proteins from the Argonaute family that are necessary for mediating their function. Argonaute proteins contain a PIWI domain that in many cases exhibits RNase-H-like slicer activity. All three types of small RNAs promote post-transcriptional gene silencing (PTGS); in the case of siRNAs and piRNAs they induce transcript degradation while miRNAs inhibit translation of target mRNAs in the cytoplasm. Interestingly, small RNAs can also promote gene silencing at the transcriptional level by recruiting factors that induce heterochromatin assembly. Transcriptional gene silencing (TGS) has been well described in plants and fungi where siRNAs can target respectively the DNA methyltransferases or H3K9 methyltransferase activity to genomic loci. Recently, RNAi-dependent processes relying on piRNA have also been found to be involved in metazoan gene silencing phenomena, mainly in the germline (Castel and Martienssen, 2013; Gu and Elgin, 2013).

siRNA-dependent heterochromatin formation

-siRNA production (Figure4)

siRNAs originate from double stranded RNAs (dsRNAs) that are cleaved by the Dicer family of RNAse III like enzymes into 20 to 25 nucleotides siRNA duplexes with a hydroxyl group and two nucleotide overhangs at the 3'-end and a monophosphate group at the 5'-end. It is thought that Dicer-independent pathways might also lead to the production of siRNAs in several organisms (Halic and Moazed, 2010; Lee et al.,

2010). These small siRNAs duplexes are loaded into the effector Argonaute protein-containing RNA-Induced Silencing Complex (RISC). Prompted by slicing, one strand of the siRNA duplex dissociates while the remaining single strand of RNA serves to guide this activated RISC to complementary transcripts allowing its slicer to cleave and trigger their degradation (Castel and Martienssen, 2013).

-siRNA dependent DNA methylation in plants.

In *Arabidopsis thaliana*, heterochromatin is formed on repetitive DNA, various transposable elements and their derivatives by an RNA dependent DNA methylation process. Heterochromatin repeats are transcribed by a plant-specific RNA polymerase RNAPIV that physically associates with RNA-dependent RNA Polymerase 2 (RDRP2) to produce dsRNA. dsRNA are processed into siRNAs that recognize nascent homologous transcripts transcribed by RNAPV and target these loci for RNA-directed DNA methylation (Castel and Martienssen, 2013; Wassenegger et al., 1994). The RDM1 protein interacts with both the DNA methyltransferase DRM2 and AGO4, and thus bridges RNAi with DNA methylation and heterochromatin formation (Gao et al., 2010). The role of small RNAs in H3K9me assembly in *A. thaliana* is unclear; however there is a crosstalk between DNA methylation and H3K9 methylation. It is therefore probable that siRNA-directed DNA methylation subsequently promotes H3K9 methylation by recruiting the H3K9 methyltransferase KYP to chromatin (Johnson et al., 2007). In *A. thaliana*, siRNAs are particularly abundant in the germline. It has been observed that cells associated with germ cells go through a phase of DNA demethylation which results in expression of transposable elements (TEs). The reactivation of TEs results in the production of siRNAs that are thought to be transmitted to the gametes and thus ensuring the transmission of TEs in a silent state to the progeny (Slotkin et al., 2009).

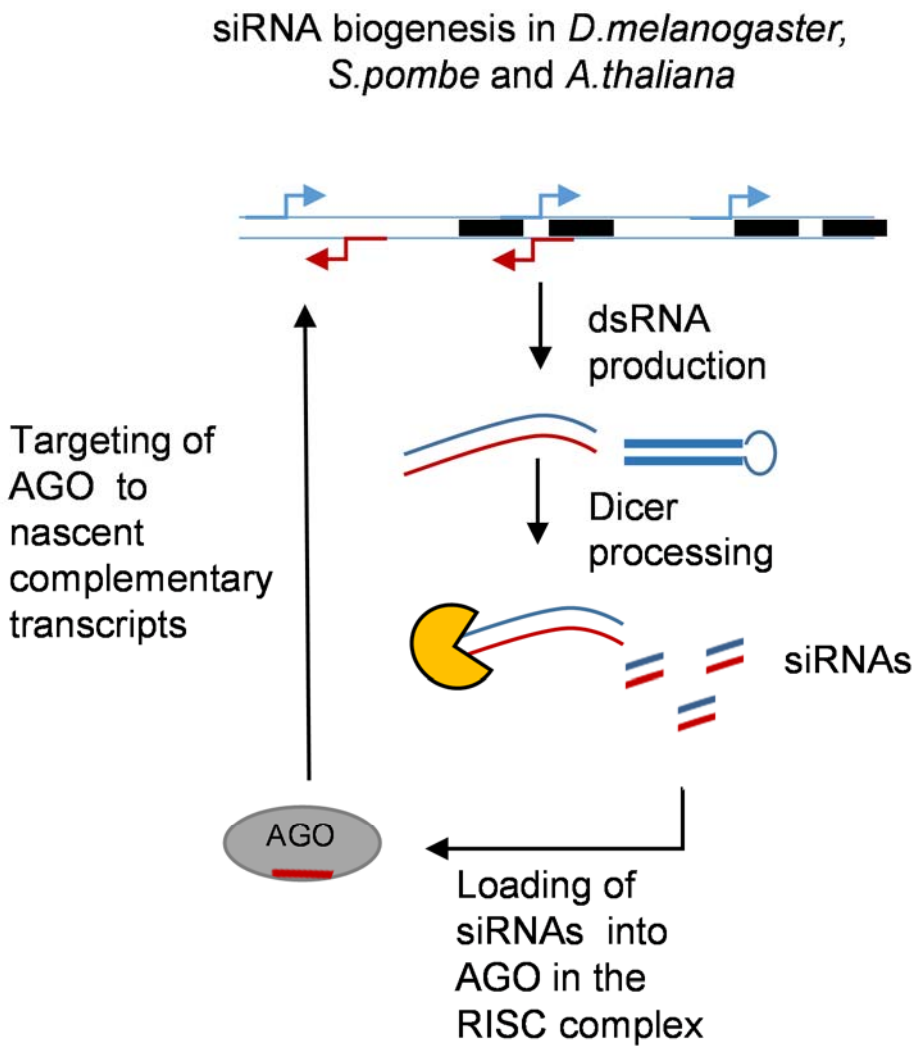


Figure 1.4: siRNA biogenesis.

dsRNA arising from convergent transcription, or repeats are processed by Dicer into siRNA duplexes that are loaded into AGO that is part of the RISC complex. One strand of the siRNA duplex is discarded and AGO bound to the siRNA targets RISC to nascent transcripts. Figure adapted from (Castel and Martienssen, 2013).

-siRNA dependent heterochromatin formation in metazoa

Although siRNAs can be detected in mammals, their cellular role remains unclear. In worms, siRNAs seem to trigger heterochromatin formation. Feeding worms with dsRNAs homologous to an endogenous gene results in H3K9me assembly over that target gene (Burkhart et al., 2011). In flies, H3K9me assembly also appears to be linked to siRNAs production since the deletion of ago2 or dcr2 leads to decreased levels of H3K9me at centromeres (Fagegaltier et al., 2009). However further investigation is required to conclusively demonstrate a causative role for siRNAs in the establishment of H3K9 methylation-dependent heterochromatin in metazoa.

- siRNA dependent H3K9me formation in S. pombe (described in details in 1.2.4)

piRNA mediated transcriptional gene silencing

piRNAs are the largest class of small RNAs. They are found in abundance in the germline in many metazoa and were thought to be specifically expressed in the gonad. However recent analyses also detected piRNAs in somatic cells from *Drosophila* and mammals (Castel and Martienssen; Gunawardane et al., 2007).

In *Drosophila* and mammals, piRNA pathways play a role in the assembly of heterochromatin on transposable elements, especially in the germline (Elgin and Reuter, 2013; Wang and Elgin, 2011). In *Drosophila*, deletion of Piwi from the germline results in loss of silencing of some TE elements and this is associated with loss of H3K9me and HP1 from these elements (Wang and Elgin). Whether this loss of TE silencing is caused by the loss of piRNAs themselves remains unclear. Piwi has been shown to interact directly with HP1alpha in a two hybrid screen suggesting that piRNAs and the associated machinery are likely to be involved in establishing heterochromatin (Brower-Toland et al., 2007).

1.2.2.B RNAi independent establishment of heterochromatin

Although small RNA Argonaute-related targeting mechanisms appear to be important for heterochromatin establishment in fission yeast and in higher eukaryotes, other mechanisms exist to promote heterochromatin nucleation. These mechanisms

include specific DNA binding factors, long non-coding RNAs and other RNA processing factors.

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) have been shown to induce gene silencing by recruiting chromatin modifiers to the chromatin either *in cis* or *in trans* depending on the RNA and the locus being studied.

In most mammals, the presence of two X chromosomes in females (XX) results in the inactivation of one of the two X chromosomes in order to prevent females from producing twice as much X chromosome gene transcripts as males (XY) in a process generally known as dosage compensation (Chaligne and Heard, 2014). X-inactivation is inherited through mitotic divisions and re-set during development. Following fertilisation, either the maternal or the paternal X chromosome is randomly inactivated in the embryo. Heterochromatin formation on the X chromosome is dependent on a specific long non-coding transcript called *Xist* (X-inactive-specific-transcript) and it is part of the X-inactivation center (Xic). *Xist* is transcribed from only one of the two X chromosomes and coats that X chromosome *in cis*. *Xist* is tightly regulated to ensure that it is only expressed and coats one X chromosome allowing the second X chromosome to remains active. Upon *Xist* coating, euchromatin-associated marks such as H3K4me2, H3K4me3 and H3K9ac are lost from that X chromosome. The chromosome becomes hypoacetylated and transcription by RNAPII is lost. H3K27me3, H3K9me2, H4K20me1 and H2AK119ub1 and DNA methylation at CGIs accumulate during the inactivation process. This combination of repressive marks results in the inactivation of that X chromosome. *Xist* lncRNA is thought to recruit the PRC1 and PRC2 complexes that are responsible for installing the H2AK119ub1 and H3K27me3 PTMs, respectively (Sarma et al., 2014). However, it remains unknown how Xist recruits these chromatin modifiers.

Other lncRNAs have also been shown to act *in trans* and target several genomic loci for heterochromatin assembly. In humans, the lncRNA HOTAIR interacts with GA-rich DNA motifs and recruits the PRC2 H3K27 methyltransferase that nucleates broad domains of H3K27me3 heterochromatin over HOTAIR target sites (Chu et al., 2011).

Determinant of Selective Removal (DSR) containing transcripts

In fission yeast, several meiotic genes assemble heterochromatin during vegetative growth in an RNAi-independent manner ((Harigaya et al., 2006; Tashiro et al., 2013; Zofall et al., 2012). During vegetative growth, transcripts of meiotic genes containing a specific motif known as a Determinant of Selective Removal (DSR) are recognized and bound by the Mmi1 protein. Mmi1 associates with Red1 which recruits Pla1, a polyA polymerase. Together these proteins facilitate the degradation of DSR-containing transcripts by the exosome (Sugiyama and Sugioka-Sugiyama, 2011; Yamanaka et al., 2010). It has been proposed that heterochromatin components are recruited to loci that produce DSR containing transcripts via Red1 (Zofall et al., 2012). Consistent with this, the insertion of a DSR in the 3'UTR of *ura4*$^{+}$ or *ade6*$^{+}$ genes is sufficient to assemble heterochromatin in a transcription-dependent manner (Tashiro et al., 2013; Zofall et al., 2012). However not all DSR-containing genes are enriched in H3K9 methylation and the levels of H3K9me detected at DSR containing genes such as *mei4*$^{+}$ or *ssm4*$^{+}$ are low compared to constitutive heterochromatin loci such as centromere repeats (Tashiro et al., 2013) (Ard et al., 2014). Moreover, silencing of the meiotic genes is maintained in the absence of Clr4, suggesting that although Clr4 is recruited via an interaction with Red1, H3K9me is not essential for silencing of these genes (Egan et al., 2014).

RNA processing machinery

The establishment of heterochromatin at fission yeast centromeric repeats requires functional RNAi (Buscaino et al., 2013; Sadaie et al., 2004). However, cells lacking the Mlo3 protein, which is involved in RNA transport and RNA quality control, bypass the need for RNAi in establishing heterochromatin at centromeres (Reyes-Turcu et al.). Cells lacking Mlo3 accumulate bidirectional centromeric transcripts and restore recruitment of the Clr4 containing complex CLRC to the centromeric repeats in the absence of RNAi. This indicates that the absence of a functional RNA surveillance machinery involving Mlo3 promotes RNAi-independent heterochromatin formation. Therefore, another pathway can operate in parallel to RNAi to promote heterochromatin formation at centromeres. It was proposed that this other pathway might involve non coding transcripts and the Rrp6 3'-5' exonuclease, a component of the exosome that is essential for heterochromatin formation in cells lacking Mlo3 and RNAi (Reyes-Turcu et al.).

DNA binding factors

Heterochromatin can be nucleated via DNA binding factors that directly recruit the heterochromatin components to chromatin. For example, in *Drososphila* the Polycomb repressive complex responsible for H3K27me3 methylation is targeted to specific DNA sequences called PcG response elements (PREs) (Simon and Kingston, 2009). PREs are often found in the vicinity of target genes that will be repressed if the Polycomb repressive complexes are recruited or remain active if the Trithorax complex associates. At the mating type locus in *S. pombe*, the transcription factors Atf1 and Pcr1 recruit heterochromatin components in parallel to RNAi; the telomere repeat binding protein Taz1 binds to the DNA and performs the same function at telomeres (Jia et al., 2004; Kanoh et al., 2005). In *S. cerevisiae*, the Sir proteins are also recruited by DNA binding factors such as Abf1, Orc1, and Rap1 that bind silencing elements at the silent mating type loci and initiate heterochromatin formation.

1.2.3 Heterochromatin spreading

Once nucleated, heterochromatin domains can spread in *cis,* independently of the underlying DNA sequence. This property of heterochromatin is at the origin of the position effect variegation (PEV), a type of silencing originally described in *Drosophila*; thus genes adjacent to transposable/repetitive elements can be repressed by heterochromatin, however, the degree of silencing in different cells varies due to stochastic heterochromatin spreading (Elgin and Reuter, 2013; Lewis, 1950). Similarly, the insertion of reporter genes into heterochromatin domains in fission yeast results in their silencing (Allshire et al., 1994) (Allshire et al., 1995; Nimmo et al., 1994; Thon et al., 1994). Mechanistically, heterochromatin domains can spread in *cis* because of a self-reinforcement mechanism involving H3K9me mediated by the Suv39/Clr4 H3K9 KMTs, HP1/Swi6, and HDACs. Pre-existing H3K9me can be bound by HP1/Swi6 and Suv39/Clr4 via their chromodomains allowing the methylation of H3K9 in neighbouring nucleosome and the spreading and reinforcement of H3K9me along the chromatin fibre (Zhang et al., 2008). Suv39/Clr4 is also recruited/stabilised via its direct interaction with HP1/Swi6 (Haldar et al., 2011; Schotta et al., 2002; Yamamoto and Sonoda, 2003). A similar reinforcement mechanism exists to promote the spreading of H3K27me along chromatin (Bonasio et al., 2010; Margueron et al.,

2009). Since heterochromatin can spread in *cis*, regulatory mechanisms must exist to prevent heterochromatin from spreading uncontrollably over and silencing essential genes.

-Heterochromatin/euchromatin borders

Several mechanisms have been described that prevent heterochromatin from spreading into euchromatic regions. The transition between heterochromatin and euchromatin regions is often not defined by clear boundaries but rather represents a broad transition domain between euchromatin and heterochromatin that can result in PEV within a population (Kimura and Horikoshi, 2004). The extent of heterochromatin spreading at these regions relies on the availability of heterochromatin and euchromatin components. In *S. cerevisiae* for example, the transition between heterochromatin and euchromatin at sub-telomeric regions occurs by competition between Sas2 HAT acetylation and Sir2 HDAC deacetylation of H4K16 residues. Both the loss of Sas2 and the overexpression of Sir3 result in increased spreading of heterochromatin from telomeres into chromosomes (Renauld et al., 1993; Suka et al., 2002). Similar broad transition zones exist at *S. pombe* telomeres where heterochromatin spreading seems also countered by a HAT, Mst2, which acetylates H3K14 (Wang et al., 2015)**.**

At some positions in the genome, specialised DNA elements called barriers, insulators or boundary elements are found at the junction between heterochromatin and euchromatin that prevent heterochromatin from affecting neighbouring euchromatic regions (Gaszner and Felsenfeld, 2006; Heger and Wiehe, 2014). Insulator elements create a nucleosome landscape at these boundaries over which heterochromatin is unable to spread and create a sharp transition between euchromatin and heterochromatin. Boundary elements often display an open nuclease sensitive chromatin configuration and are bound by various enzymatic activities that promote a euchromatin state (ie. HATs, H3K4 HMT or nucleosome remodelers) creating an environment that is resistant to heterochromatin spreading (Beisel and Paro, 2011; Gaszner and Felsenfeld, 2006). For example, the cHS4 insulator in chicken cells acts as a barrier at the β-globin locus by recruiting HATs and H3K4 HMT through the binding of two transcription factors USF1 and USF2 (West et al., 2004). In addition, tRNA genes are found to have barrier activity in yeast and human cells (Donze and Kamakaka, 2001; Noma et al., 2006; Raab et al., 2011; Scott

et al., 2007; Van Bortle and Corces, 2012; Wang et al., 2011). It has been proposed that the nucleosome depleted chromatin over tRNA genes is responsible for their function as boundary elements. tRNA gene barrier activity in yeast is dependent on the RNAPIII transcription factor TFIIIC (Noma et al., 2006; Valenzuela et al., 2009).
Histone demethylases have also been found to play a role at regions of transition between heterochromatin and euchromatin by promoting H3K9 demethylation. The fission yeast Lsd1/Lsd2 complex is enriched at heterochromatin boundaries and probably prevents heterochromatin spreading by demethylating H3K9me (Lan et al., 2007). Interestingly, *Drosophila* Lsd1 is also found at transition points between euchromatin and heterochromatin but unlike its fission yeast counterpart, *Drosophila* Lsd1 demethylates H3K4 and appears to protect heterochromatin domains by preventing H3K4me from spreading into heterochromatin regions (Chosed and Dent, 2007; Rudolph et al., 2007). In fission yeast, the putative H3K9 demethylase Epe1 also prevents heterochromatin from spreading over boundary regions at centromeres and the mating type locus (Ayoub et al., 2003; Trewick et al., 2007; Zofall and Grewal, 2006; Zofall et al., 2012).

1.2.4 Heterochromatin assembly in *Schizosaccharomyces pombe*

Several features make *S. pombe* an excellent organism in which to study H3K9-dependent heterochromatin. Heterochromatin is not essential for cell survival in *S. pombe*. In addition, heterochromatin assembly relies on a single H3K9 methyltransferase, Clr4, which is responsible for all mono-, di- and tri-methylation of H3K9. Furthermore, the absence of both DNA methylation and H3K27me, which have a close relationship with H3K9 methylation in other organisms (i.e. *C. elegans, Drosophila* or mammals), facilitates the study of factors that are directly involved in the assembly and maintenance of H3K9me-dependent heterochromatin.

RNAi-dependent establishment of heterochromatin

The initial recruitment of Clr4 to a nucleation site relies on diverse mechanisms depending on the heterochromatin site. The RNAi machinery has been shown to play a crucial role in establishing heterochromatin on centromere repeats, at telomeres and the silent mating type loci as well as at facultative heterochromatin islands (Figure 1.5) (Buscaino et al., 2013; Partridge et al., 2007; Reyes-Turcu et al.; Sadaie et al.,

2004). Particular types of repeat element, called *dg* and *dh* are present at centromeres, and *dg/dh* related repeats are also present between the silent *mat2* and *mat3* genes at the mating type locus and within subtelomeric repeats. All these repeats are the major source of siRNAs in fission yeast (Grewal and Klar, 1997). The convergent transcription of these repeats by RNAPII during S phase forms dsRNAs that are cleaved by the ribonuclease Dcr1 into [double stranded) siRNAs duplexes (Castel and Martienssen; Lejeune et al., 2011).

The siRNAs duplexes are loaded into Ago1 within the RNA-induced transcriptional silencing (RITS) complex with the aid of the chaperones Arb1 and Arb2 (Verdel et al., 2004) (Buker et al., 2007; Holoch and Moazed, 2015).

RITS complex

The RITS complex is composed of Ago1, Tas3 and the chromodomain protein Chp1. Ago1 has ribonuclease activity and cleaves the passenger strand of the siRNA duplex while the guide strand remains associated with RITS and guides the complex to complementary nascent transcripts that will be cleaved (Verdel et al., 2004). RITS recruits the Clr4 containing CLRC complex via the bridging protein Stc1 and the RITS component Chp1 stabilises RITS on the chromatin by interacting directly with H3K9me via its chromodomain (Bayne et al, 2010; Zhang et al, 2008)(He et al., 2013; Verdel et al., 2004). Together with Dcr1 and CLRC, RITS also promotes the recruitment of the RNA-dependent RNA polymerase (RDRC) complex to the chromatin (Motamedi et al., 2004; Sugiyama et al., 2005). Therefore, the RNAi machinery directs both PTGS and TGS. PTGS is indicated by the degradation of new repeat transcripts during transcription, and TGS by the recruitment of Clr4/CLRC that promotes heterochromatin formation and prevents RNAPII access to the chromatin through the majority of the cell cycle.

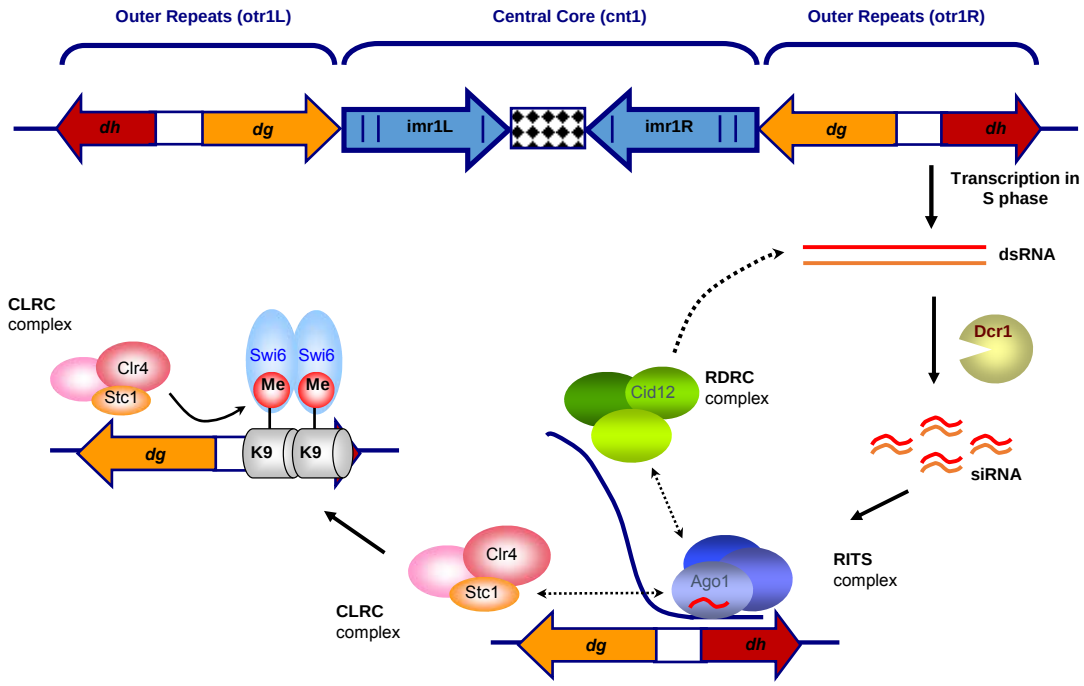


Figure 1.5: RNAi-directed heterochromatin assembly in fission yeast: example of centromeres.

RNAPII transcribes centromeric repeats (*dg* and *dh*) which generate dsRNAs that are processed by Dcr1 into siRNAs duplexes. siRNAs are loaded into Ago1 that is part of the RITS complex and target RITS to the nascent centromeric repeats by base complementarity. RITS interacts with RDRC which increases the production of siRNAs by Rdp1 RNA-dependent RNA polymerase activity. RITS also recruits the CLRC complex via an interaction with Stc1 which results in the methylation of H3K9 that is recognised by chromodomain proteins such as Swi6 that recruit heterochromatin factors involved in gene silencing. Figure adapted from (Lejeune et al., 2011).

RNA dependent RNA polymerase complex (RDRC)

The RNA dependent RNA polymerase complex (RDRC) is composed of the RNA dependent polymerase 1 (Rdp1), a putative RNA helicase (Hrr1) and a polyA polymerase (Cid12). RDRC generates further dsRNAs from the targeted repeat RNAs creating a positive feedback loop. Dcr1 binds with RDRC and RITS which couples RDRC-dependent dsRNA production with siRNA production (Colmenares et al., 2007). RDRC is also essential for silencing within centromere repeats (Motamedi et al., 2004) (Figure 6).

CLR4-like Clr4 complex (CLRC)

CLRC is composed of the unique H3K9 methyltransferase Clr4, Raf1, Raf2, Cul4 and Rik1 (Bayne et al., 2010; Hong et al., 2005; Horn et al., 2005; Jia et al., 2005. CLRC associates with both RITS and RDRC which help to its recruitment to centromeric repeats {Gerace, #351; Li et al., 2005; Rea et al., 2000; Thon et al., 2005). Interestingly, Clr4 is also involved in siRNA production, suggesting that the RNAi machinery and CLRC are part of a reinforcement loop. However, siRNA production and H3K9 methylation can be uncoupled. Mutations in Raf1 result in its dissociation from CLRC and abolishe H3K9 methylation without affecting siRNA production (Buscaino et al., 2012). Furthermore, tethering of Rik1 adjacent to a *ura4*$^{+}$ reporter induces RNAi-mediated silencing of *ura4*$^{+}$ independently of H3K9 methylation and other CLRC components (Gerace et al.).

Although the Clr4 SET domain alone exhibits H3K9 methyltransferase activity *in vitro*, its association with the other CLRC components is required to allow it to methylate H3K9 *in vivo* (Rea et al., 2000) (Zhang et al., 2008). The CLRC complex shows strong similarity with the conserved Cullin-RING ligase (CRL) family of E3 ubiquitin ligases. The CLRC complex was shown to exhibit E3 ubiquitin ligase activity *in vitro* (Horn et al., 2005), however we have been unable to detect this activity (Horn et al., 2005){Buscaino, 2012 #349}. The CLRC component, Cul4, is a Cullin protein and participates in a second ubiquitin ligase complex known as Cul4-Ddb1^{DCAF}. The cullins of these CRLs serve as scaffold proteins which are regulated by neddylation. The conserved adaptor protein for Cul4 is normally Ddb1. Rik1 resembles Ddb1 and

Raf1 shares similarity with known CRL substrate specifiers (DCAFs) (Buscaino et al., 2012). It is therefore plausible that CLRC acts as an E3 ubiquitin ligase and might regulate Clr4 H3K9me methyltransferase activity or the *in vivo* recruitment of Clr4 to chromatin (Buscaino et al., 2012; Kuscu et al., 2014). It has also been found that Clr4 methylates Mlo3 which itself is important for siRNA production and might regulate heterochromatin assembly (see above) (Zhang et al.).

The methylation of H3 on lysine 9 by Clr4 creates binding sites for chromodomain proteins. Both Clr4 enzymatic activity and H3K9me binding via Clr4 chromodomain are essential for spreading of heterochromatin. The HP1-related chromodomain proteins Swi6, Chp2 and the Chp1 chromodomain protein component of RITS also contribute to this process (Zhang et al., 2008).

***Chromodomain proteins and heterochromatin* spreading**

Although the initial recruitment of Clr4 to chromatin relies on diverse nucleation signals specific to the heterochromatic site, once established, the spreading of heterochromatin occurs in a similar fashion at all heterochromatin loci. H3K9me provides a binding site for the chromodomain proteins Swi6, Chp2, Chp1 and Clr4 HMT itself. These four chromodomain proteins display specific localization and functions and are all important for the maintenance of heterochromatin integrity (Sadaie et al., 2008; Thon and Verhein-Hansen, 2000).

Direct binding of Clr4 to H3K9me2/3 via its chromodomain promotes spreading of heterochromatin away from nucleation sites independently of the underlying DNA sequence (Fischer et al., 2009; Haldar et al., 2011; Nakayama et al., 2001; Sadaie et al., 2004; Sadaie et al., 2008; Zhang et al., 2008). The other chromodomain proteins also promote heterochromatin formation and spreading by directly recruiting Clr4/CLRC to chromatin. Swi6 was shown to interact directly with Clr4 both *in vitro* and *in vivo* (Haldar et al., 2011). Chp1 binding to H3K9me stabilizes the interaction of the RITS complex with chromatin and also promotes CLRC recruitment (Verdel et al., 2004).

Dimeric Swi6 (See *1.2.1.A)* has been proposed to directly affect chromatin structure by bridging nucleosomes together by binding H3K9me in two nucleosomes, this might result in increased condensation and reduced access to this chromatin (Canzio et al., 2011

). Swi6 also affects chromatin structure and function indirectly by associating with various partners that influence processes such as chromosome segregation, nuclear organization and replication. For example, Swi6 is responsible for cohesin recruitment to centromeres and telomeres via its interaction with Mis4 (Bernard et al., 2001; Fischer et al., 2009; Zeng et al., 2010).
Both Swi6 and Chp2 also contribute to the recruitment of the HDAC complexes which are essential to establish the hypoacetylated state required for TGS and the maintenance of heterochromatin integrity (Fischer et al., 2009).

Histone deacetylases: key players in heterochromatin establishment, spreading and maintenance

Silencing of transcription in heterochromatin regions is associated with hypoacetylated histones. Three complexes with HDAC activities have been shown to contribute to silencing at *S. pombe* heterochromatic regions: SHREC (Snf2/ Hdac-containing Repressor Complex) , the Clr6 complex and Sir2 (Buscaino et al., 2013; Ekwall, 2005; Freeman-Cook et al., 2005; Grewal et al., 1998; Sugiyama et al., 2007). In addition, the histone acetyltransferases Mst1 and Mst2 that acetylate H4K16 and H3K14, respectively, counteract heterochromatin formation since loss of either HAT strengthens heterochromatin silencing and promotes heterochromatin spreading (Reddy et al.; Wang et al., 2013).
SHREC is composed of Clr1, Clr2, Chp2, the histone H3K14 HDAC Clr3 and the chromatin remodeler Mit1 (Bjerling et al., 2002; Motamedi et al., 2008; Sugiyama et al., 2007). SHREC was proposed to limit RNAPII access to heterochromatin repeats (Buscaino et al., 2013). The loss of either Mit1 or Clr3 activity results in decreased nucleosome occupancy and partial loss of heterochromatin-dependent silencing (Creamer et al., 2014; Sugiyama et al., 2007). SHREC is recruited to heterochromatin by both Swi6 and Chp2 (Sadaie et al., 2008).
The essential Clr6 HDAC contributes to different complexes Clr6-CI and –CII (Nicolas et al., 2007). In Clr6-CI Clr6 associates with Pst2, Alp13 and Prw1 and is preferentially targeted to intragenic regions where it prevents antisense transcription of protein coding genes. It probably also prevents accumulation of transcripts from *dg*/*dh* repeats elements via its H3K9Ac deacetylase activity (Nakayama et al., 2003; Nicolas et al., 2007). Cells expressing mutant Clr6 display silencing defects at centromere repeats and at the mating type locus (Grewal et al., 1998).

The Sir2 HDAC, unlike SHREC and Clr6-CI, is recruited to chromatin independently of Swi6. Several studies suggest that Sir2 acts upstream of Swi6 and Clr4 to initiate heterochromatin formation (Alper et al., 2013)(Freeman-Cook et al., 2005). Sir2 belongs to the Sirtuin family of HDACs that utilizes NAD^+ as a cofactor. Sir2 has been shown to deacetylate H4K16ac, H3K4ac, H3K9ac and H3K14ac *in vitro* (Alper et al., 2013
; Shankaranarayana et al., 2003). *In vivo*, loss of Sir2 activity results in increased levels of H3K9ac within heterochromatic regions and this prevents H3K9 methylation and recruitment of chromodomain proteins Clr4, Swi6 and presumably Chp1 and Chp2 (Freeman-Cook et al., 2005; Shankaranarayana et al., 2003). Consequently cells lacking Sir2 display a moderate defects in heterochromatin integrity within centromere repeats (Shankaranarayana et al., 2003). However, further analyses using episomal plasmid-based minichromosomes carrying centromere repeat elements revealed that Sir2 plays an active role in the nucleation, spreading and maintenance of heterochromatin at centromeres in association with RNAi (Buscaino et al., 2013{Wang, 2013 #339). Genome-wide analyses of H3K9me2 profiles has also shown that Sir2 is essential for the integrity of subtelomeric heterochromatin and the facultative heterochromatin islands associated with nitrogen-starvation responsive genes (Zofall et al., 2012).

HDACs are key players for heterochromatin establishment, spreading and maintenance at all constitutive heterochromatin domains as well as conditional heterochromatin where they act in a redundant manner. Cells defective for just one of these HDACs do not display dramatic defects in the integrity of centromeric heterochromatin. However, the combined deletion of the genes encoding Sir2 and Clr3 results in the loss of heterochromatin from centromere repeats elements (Alper et al., 2013
; Buscaino et al., 2013). Clr3 and Clr6 HDACs have also been shown to have overlapping functions in maintaining silencing of tf2 retrotransposons and in maintaining heterochromatin at the mating type locus (Hansen et al., 2005; Kim et al., 2004).

Heterochromatin at centromeres

Centromeres are regions on each chromosome where the kinetochore assembles. In most organisms the location of kinetochores are specified by the assembly of centromere specific nucleosomes in which normal histone H3 is replaced by the histone H3 variant CENP-A. CENP-A is essential for kinetochore assembly in all organisms where it has been studied.

The *S. pombe* genome is carried on three chromosomes. The three centromeres occupy 35 kb (cen1), 65 kb (cen2) and 110 kb (cen3) (Figure 7). Each centromere is contains a central domain where CENP-A^{Cnp1} chromatin and the kinetochore assemble. The central domain is composed of a central core flanked by two innermost repeats (*imr*) that are unique to each centromere. The central domain is surrounded by outer repeats (*otr*) composed of *dg* and *dh* repeats. The number and arrangement of these otr-dg/dh repeats differs at the three centromeres (Figure 7) but all copies of each type of repeat are almost identical (Pidoux and Allshire, 2004). The *otr* repeats attract heterochromatin and they are delimited on both sides by tRNA gene clusters or IR (define) repeats that act as boundaries to prevent heterochromatin from spreading (Noma et al., 2006; Scott et al., 2006). RNAi is the main trigger for the initial recruitment of Clr4 and heterochromatin on these centromere repeats and is absolutely required for heterochromatin nucleation. However, RNAi is dispensable for the maintenance of heterochromatin on these centromere repeats (Buscaino et al., 2013; Partridge et al., 2007; Sadaie et al., 2004) (Reyes-Turcu et al.). Although the *otr* repeats are fully coated with heterochromatin, there are distinct siRNA hotspots over specific regions of these repeats (Buhler et al., 2008; Cam et al., 2005; Djupedal et al., 2009; Halic and Moazed, 2010; Zaratiegui et al., 2011). These siRNA hotspots are thought to represent the nucleation sites for heterochromatin formation that then spreads outwards along the entire centromeric repeat in a Sir2-dependent manner (Buscaino et al., 2013).

Heterochromatin is also assembled around centromeres in many other organisms suggesting that heterochromatin may contribute to centromere function. *S. pombe* centromeric heterochromatin is essential for the recruitment of cohesin over the *otr* repeats and accurate chromosome segregation (Bernard et al., 2001). This centromeric heterochromatin has also been shown to promote CENP-A establishment on the central domain to form functional centromeres (Folco et al., 2008; Kagansky et al., 2009).

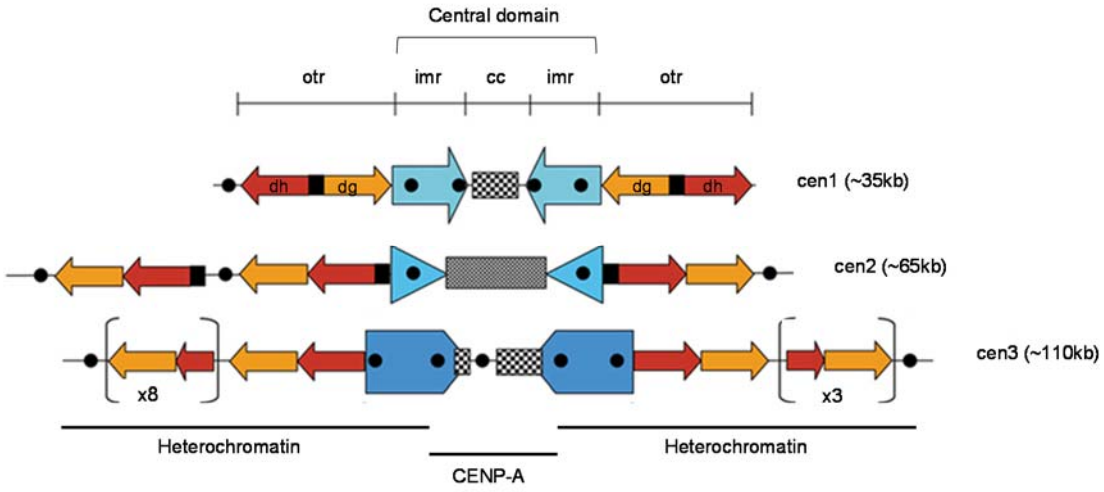


Figure 1.6: Fission yeast centromeres.

The three centromeres in *S. pombe* have a similar structure. A central core (*cc*) region is surrounded by two innermost repeats (*imr*) that are unique for each centromeres and outer repeats (*otr*) that are composed of repeats called *dg* and *dh* repeats found in a different arrangement at the three centromeres. The *otr* repeats and part of the *imr* assemble heterochromatin while the central core and the rest of the *imr* assemble CENP-A chromatin and correspond to the region where kinetochrores assemble. tRNA genes (black circle) are found at most boundary regions between heterochromatin domains and CENP-A chromatin or euchromatin outside centromeres. Figure adapted from (Partridge, 2008).

Heterochromatin at the mating type locus

The mating type locus constitutes a region of approximately 30 kb on chromosome 2. It is composed of three mating type genes. The *mat1*$^{+}$ gene is transcriptionally active and the products encoded by *mat1*$^{+}$ (*P* or *M)* dictates the mating type of a cell {Egel, 1984 #576}. Two silent donor loci, *mat2-P* and *mat3-M* provide the P or M information to the *mat1* locus in a directed recombination-mediated switching process. Both donor loci are embedded in a 20 kb domain of heterochromatin that silences expression and regulates recombination (Hall et al., 2002; Jia et al., 2004). The mating type of a haploid cell can be switched by the directed recombination of the *mat1* cassette with either *mat2-P* or *mat3-M* resulting in a change in the information encoded by *mat1*. The heterochromatic mat2-mat3 region (silent mating-type region) is flanked by two inverted repeats IR-R and IR-L elements that act as boundaries and prevent heterochromatin from spreading outwards over neighbouring genes (Noma et al., 2001). It has been shown that the expression of extra Swi6 (three copies of the *swi6+* gene) in cells lacking the IR-R boundary results in heterochromatin spreading an additional 8 kb along the chromosome from mat2-mat3. (Noma et al., 2006). A 4.3 kb sequence called *cenH* with 96% homology to the centromeric *otr* repeat lies between the mat2 and mat3 loci and promotes RNAi-dependent heterochromatin formation in this region (Grewal and Klar, 1997; Hall et al., 2002).

In contrast to centromere repeat heterochromatin, RNAi is not essential for the efficient establishment or maintenance of heterochromatin over the mat2-mat3 region (Hall et al., 2002; Jia et al., 2004; Kim et al., 2004). This lack of dependency on RNAi is because another pathway acts in parallel to recruit Clr4 at the silent mating type region. Two transcription factors Atf1 and Pcr1, previously shown to be involved in the regulation of gene expression in response to stress and sexual differentiation bind to a specific DNA sequence residing between *cenH* and *mat3* (Takeda et al., 1995; Watanabe and Yamamoto, 1996). Deletion of the gene coding Atf1 or Pcr1 alone does not significantly affect heterochromatin maintenance, however in the absence of both RNAi and Atf1 or Pcr1, heterochromatin is lost or strongly reduced. Furthermore in the absence of functional RNAi and Atf1 or Pcr1 heterochromatin can not be established at the mating type locus, suggesting that they act in parallel pathways (Jia et al., 2004; Kim et al., 2004). Atf1 and Pcr1 associate with Clr4 and Swi6 *in vitro* and

recruit the Clr3 HDAC to the *mat2-mat3* region to maintain heterochromatin (Jia et al., 2004; Yamada et al., 2005).

Heterochromatin at telomeres

Telomeres are repeat-rich nucleoprotein structures that protect chromosome ends from degradation and fusion with other chromosomes (Nimmo et al., 1998; Schoeftner and Blasco, 2009). Telomeres in most eukaryotes are known to assemble heterochromatin (Grewal and Jia, 2007). Heterochromatin at *S. pombe* telomeres extends 45-75 kb away from chromosome ends depending on the chromosome arm. This telomeric heterochromatin prevents recombination between telomeric repeats and is therefore important for the maintenance of telomere length (Bisht et al., 2008; Nimmo et al., 1998). Telomeric heterochromatin is assembled by two redundant pathways. First, the protein Taz1 binds directly to telomeric repeats and recruits other shelterin components such as Rap1, which mediates Clr4-dependent heterochromatin formation (Kanoh and Ishikawa, 2001). Second, the RNAi machinery acts on the subtelomeric regions of chromosome 1 and 2 which contain DNA repeat elements (tlh) that share homology with the *mat2-mat3 cenH* element and centromeric *dg*/*dh* repeats. These *tlh* elements are transcribed and processed by RNAi to promote Clr4 recruitment to telomeres. Only in cells lacking both Taz1 and RNAi is significant loss of H3K9me and Swi6 from telomeres observed, indicating that they operate redundantly to maintain heterochromatin at telomeres (Kanoh et al., 2005; Schoeftner and Blasco, 2009).

1.3 Epigenetic maintenance of heterochromatin domains

Once heterochromatin domains are established by nucleation and spreading, the chromatin state must be stably maintained through cell division. In some cases the maintenance of heterochromatin relies on the constant recruitment of the heterochromatin components by nucleation factors that replenish H3K9me levels. For example, at every fission yeast cell cycle, centromere repeats are transiently transcribed in S phase by RNA polymerase II to produce polyadenylated siRNA precursor transcripts which leads to the recruitment of Ago/RITS and Clr4/CLRC to homologous chromatin (Chen et al., 2008). Thus a peak of siRNA levels is observed soon after transcription of *dg*/*dh* repeats and simultaneously an increase in RNAi

components and CLRC components on chromatin is observed (Chen et al., 2008). H3K9me2 levels increase on centromere repeats in late S phase reaching their maximum in G2 (Chen et al., 2008; Kloc et al., 2008). It has also been proposed that heterochromatin maintenance by RNAi on centromere repeats may be coupled with, and required for, RNAPII release from the chromatin during replication in a process that prevents RNAPII and DNA polymerase collisions but results in heterochromatin formation (Zaratiegui et al.).

Interestingly, some factors that are required to establish heterochromatin are not necessarily essential or even important for its maintenance. RNAi in fission yeast is essential for establishing heterochromatin on centromere repeats. However, although transcriptional silencing is defective, reasonable levels of H3K9 methylation and heterochromatin components are maintained in the absence of RNAi. This partial maintenance of heterochromatin in cells lacking RNAi suggests that H3K9me might be epigenetically propagated (Buscaino et al., 2013; Motamedi et al., 2004; Partridge et al., 2007; Sadaie et al., 2004; Verdel et al., 2004; Volpe et al., 2002) (Reyes-Turcu et al.). In *Drosophila*, the Piwi Argonaute-related protein is important for the establishment of silencing of certain genes close to TE elements during early stages of development. However, silencing of these genes is unaffected by the loss of Piwi in later developmental stages. This finding indicates that, like RNAi in fission yeast, Piwi is important for establishing transcriptionally silent heterochromatin domains but is dispensable for their maintenance (Gu and Elgin, 2013).
During parent of origin specific imprinting, heterochromatin is differentially established at a locus on the paternal or the maternal chromosome during gametogenesis. Such heterochromatin domains are stably maintained in the adult offspring even though nothing within the DNA sequence differentiates the maternal allele from the paternal allele. Likewise, the random inactivation of one X chromosome in female mammals also shows that two identical DNA sequences can differentially assemble heterochromatin in the same cell, and that this heterochromatinised state is subsequently stably propagated on that same X chromosome through many mitotic cell divisions. Such observations show that following establishment, heterochromatin domains can self-propagate independently of the nucleation signal and therefore independently of the underlying DNA sequence on an epigenetic manner.

The epigenetic propagation of heterochromatin requires the stable maintenance of specific marks that can propagate the memory of the heterochromatic state through cell divisions. These epigenetic marks must be maintained through processes that dramatically alter chromatin such as transcription, replication, mitosis or DNA repair in order to propagate the heterochromatin state from one cell to its daughter cells. In high eukaryotes, DNA methylation was proposed to allow the epigenetic inheritance of heterochromatin domains. In this thesis I investigated whether H3K9me can act as an epigenetic mark and propagate a heterochromatin domain.

1.3.1 Definition of epigenetics

The term “epigenetic” was introduced by Waddington in the 1940s. Waddington used the phrase “epigenetic landscape” to define how the genome regulates developmental processes(Waddington, 2012). Later, Nanney defined an “epigenetic system” as a mechanism able to control what traits or genes are expressed in a specific cell (Nanney, 1958). Once DNA methylation was found to have heritable properties, the idea was conceived that specific traits, which play a role in cellular functions, might be transmitted from one cell to its daughter cells via differential DNA methylation as well as DNA sequence (Holliday, 1987; Holliday and Pugh, 1975). Now ‘epigenetic’ is broadly used to described distinctly different processes. Epigenetic is often very generally used to describe molecules or processes associated with chromatin which play a role in the regulation of the genome, for example, histone PTMs, chromatin remodelling, non-coding RNA associated events. In this study, a more narrow definition of epigenetics is adopted in which epigenetics is defined as the stable inheritance of a phenotype through mitotic and/or meiotic division, that occurs independently of changes in the DNA sequence (Bonasio, 2015; Bonasio et al., 2010). The epigenetic maintenance of phenotypes can be achieved via *trans*-acting epigenetic signals or *cis*-acting epigenetic marks. For example, epigenetic information carried by prions acts in *trans*. Prion proteins are transmitted from the cellular compartment (cytoplasm, nucleoplasm) of one cell to daughter cells. Prions are proteins that can adopt several structural conformations with at least one form being able to transmit its state by converting other forms of the prion protein to its own folded conformation (Halfmann and Lindquist, 2010). The phenotype associated with the “dominant” prion conformation is epigenetically inherited through cell divisions.

Many apparent epigenetic signals are directly associated with the specific locations on chromosomes where they act *in cis* to transmit the epigenetic state to daughter cells. 5-methyl cytosine methylation in DNA is a good example of a *cis* acting epigenetic signal since it can be accurately copied during replication in every cell cycle. DNA methylation marks heterochromatin regions in higher many eukaryotes such as humans or plants and is thought to be responsible for the epigenetic inheritance of heterochromatin domains such as imprinted loci (Kelsey and Feil, 2013). Most DNA methylation in mammalian cells occurs as 5-Methyl Cytosine (5-MeC) within the context of the symmetrical CG dinucleotide. Since DNA is replicated in a semi-conservative manner, 5-MeC within CpG on both strands of DNA generates two hemi-methylated daughter strands after replication. However, because maintenance DNA methyltransferase 1 (Dnmt1) travels with the replication fork, both hemi-methylated CpG dinucleotides are rapidly converted to the fully methylated state. In this way, the DNA methylation pattern on parental chromatin is copied and maintained on chromosomes in both daughter cells. (Goll and Bestor, 2005; Wigler et al., 1981).

Another well studied epigenetic mark associated with the chromosome is the H3 histone variant CENP-A that by itself dictates centromere formation independently of the underlying DNA sequence and allow epigenetic transmission of centromeres. It was for example shown in *Drosophila* that the tethering of CENP-A^{CID}-LacI to an extrachromosomal plasmid containing LacO arrays is sufficient to promote assembly of kinetochrores that can be stably maintained through several cell divisions after elimination of CENP-A^{CID}-LacI. CENP-A^{CID}-LacI is sufficient to recruit endogenous CENP-A on the extrachromosomal plasmid that then propagate the centromere on an epigenetic manner (Mendiburo et al., 2011).

1.3.2 Is H3K9me an epigenetic mark?

Although many post-translational modifications on histones are commonly referred to as ‘epigenetic modifications’ or ‘epigenetic marks’, it remains to be determined if any histone PTM can carry information through cell divisions into daughter cells without any involvement of associated cis-acting DNA sequences. Histone modifications such as phosphorylation and acetylation are very dynamic and display high turnover which makes them unlikely candidates for PTMs that allow the inheritance of a specific chromatin state (Barth and Imhof, 2010). In contrast mass spectrometry analyses of

the dynamics of heavy methyl groups incorporated into chromatin *in vivo* show that methyl groups on histones are very stable (Barth and Imhof, 2010). Histone methylation such as H3K9me2/me3 and H3K27me2/me3 that are associated with heterochromatin regions are particularly stable in the cells and therefore more likely to carry the memory of these specific chromatin states through cell division. (Zee et al, 2010).

Epigenetic marks need a propagation mechanism that allows their maintenance through cell division. In order for a specific histone modification to self-maintain, the propagation mechanism must accurately copy that histone PTM to the chromatin of both daughter chromatids during the replication process and also be able to restore any histone PTM lost due to enzymatic removal or histone turnover. Interestingly, histone PTMs such as H3K9me, H4K20me and H3K27me have an inbuilt system where the histone modifier (writer) is coupled to the methylated lysine binder (reader) directly or through a protein-protein interaction. This allows these PTMs to spread along chromatin fibres and reinforces their presence on chromatin (Bonasio et al., 2010; Margueron et al., 2009) (Kalakonda et al., 2008). For example, in a mechanism conserved from fission yeast to humans H3K9me is directly bound by the chromodomain present in the SUV39H1/Clr4 H3K9 methyltransferases and this feedback system is further strengthened by the interaction of these methyltransferases with the HP1/Swi6 proteins which also bind H3K9me through their chromodomains (Haldar et al., 2011; Yamamoto and Sonoda, 2003). It remains to be determined if these self-reinforcement loops are important or sufficient to maintain H3K9me through cell division and also other processes that disrupt chromatin such as transcription or DNA repair..

Maintenance of histone PTMs upon transcription

During transcription chromatin structure is altered to make the DNA template accessible to transcription factors and RNAPII. At least one H2A/H2B dimer needs to be evicted from the nucleosome to allow access to the transcription machinery and higher levels of transcription cause dissociation of H3/H4 tetramers (Smolle and Workman, 2013). However, to counter the potential loss of resident histones, a recycling process operates to maintain the chromatin state following transcription. Histone chaperones play an important role in the transcription-coupled disassembly,

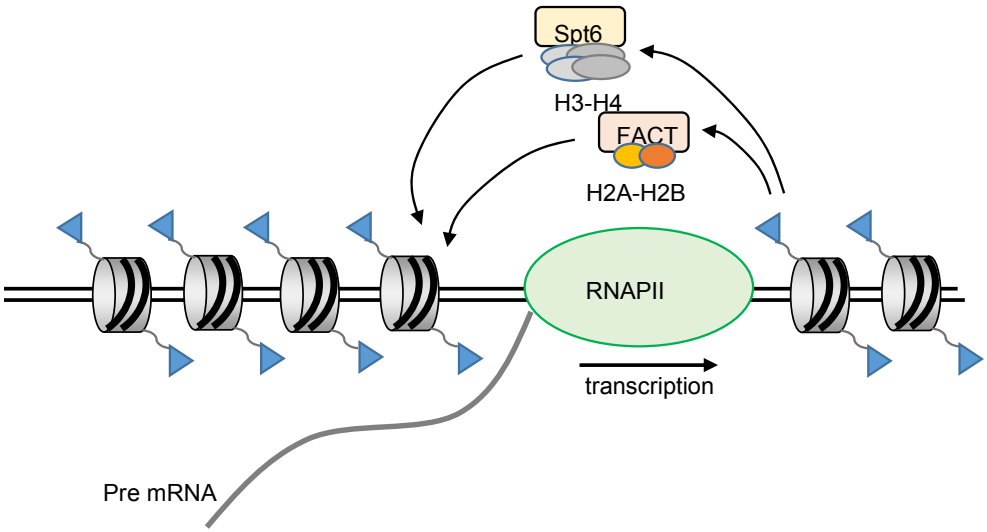


Figure 1.7: Nucleosome recycling during transcription.

Histones evicted from the chromatin by the passage of the RNAPII are recycled by the histones chaperones Spt6 and FACT that contribute to the reassembly of nucleosomes behind RNAPII. Histones PTMs are represented by the blue flag.

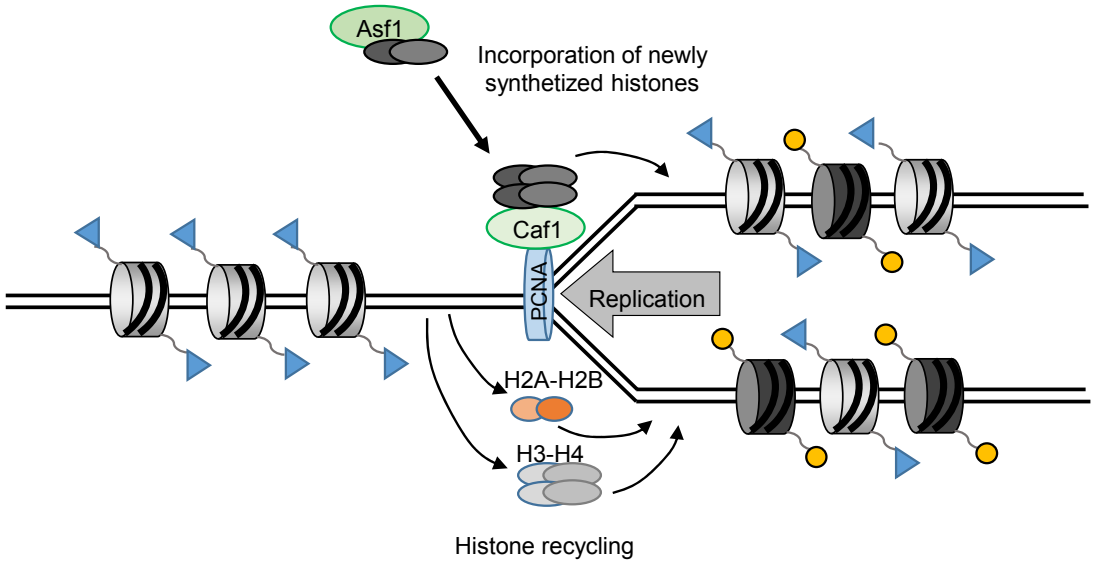


Figure 1.8: Assembly of nucleosomes behind the replication fork.

During replication, old histones are recycled via an unknown mechanism and reassemble nucleosomes (light grey) onto the two daughter strands of DNA that are thought to carry old PTMs (blue flag) and therefore carry the memory of the chromatin state to the daughter cells. Newly synthetized histones are incorporated into nucleosomes via the replication specific histone chaperone CAF1 that interacts with the replication associated protein PCNA (Proliferating cell nuclear antigen) and Asf1. New histones H3 and H4 are acetylated (yellow circle) when incorporated in the new nucleosomes (dark grey) on the daughter strands of DNA.

recycling and reassembly of nucleosomes. Spt6 associates preferentially with (H3-H4)$_2$ tetramers while FACT associates with both H2A-H2B dimers and (H3-H4)$_2$ tetramers and together promote RNAPII-coupled histone recycling (Rossetto et al., 2014) (Figure 1.7). This recycling mechanism allows histone PTMs to be maintained and reinstalled through transcription. Nevertheless, histone loss occurs as a result of transcription-dependent histone turnover in highly transcribed regions (Svensson et al., 2015) (Rufiange et al., 2007; Verzijlbergen et al., 2010). Histone chaperones such as Hira and Daxx for (H3-H4) or Nap1 for H2A-H2B dimers facilitate human chromatin reassembly by promoting the incorporation of newly synthetized histones (Avvakumov et al., 2011; Goldberg et al., 2010).

Maintenance of histone PTMs upon replication

The epigenetic propagation of a chromatin state requires it to be accurately copied following S phase to both daughter chromatin strands. During replication chromatin is disassembled to make DNA accessible to the replication machinery. Three events must occur with respect to replicating chromatin: disruption of chromatin, transfer of parental nucleosomes onto duplicated DNA and deposition of nascent histones to assemble new nucleosomes (Figure 1.8).

Parental histone recycling

Old histones from parental nucleosomes are recycled behind the replication fork where they are randomly distributed and reassembled as nucleosomes on both developing sister-chromatids. The mechanism of histone recycling at the replication fork is not fully understood (Avvakumov et al, 2011)(Burgess and Zhang, 2013). It is thought that during recycling, histone PTMs associated with parental histones are conserved, thus allowing the memory of the pre-existing chromatin state to be copied to both chromatids. Consistent with this hypothesis parental histone H3 has been shown to still carry tri-methylated K9 and K27 following replication, while these same modifications accumulate more slowly on newly deposited histones (Alabert et al., 2014). An alternative hypothesis is that the histone modifiers, rather than the histone PTMs themselves, might enable epigenetic memory and maintain the chromatin state through replication by travelling with the replication fork (Petruk et al., 2012). Indeed, during S phase in *Drosophila* embryos H3K4me and H3K27me are not detected, however, the H3K4 Trithorax and H3K27 Enhancer-of-Zeste KMTs remain associated

with chromatin and might be responsible for the restoration of H3K27me and H3K4me following replication.

Maturation of chromatin

New histones incorporated into the chromatin carry specific histone PTMs that facilitate their association with histone chaperones and therefore their assembly onto newly duplicated DNA. The histone chaperones CAF1 and Asf1 participate in H3-H4 deposition while FACT facilitates H2A-H2B deposition (Avvakumov et al., 2011) (Figure 9). In most eukaryotes, new histone H4 is acetylated on both K5 and K12 and new histone H3 is acetylated on several residues including lysine 56 (Loyola et al, 2006; Sobel et al, 1995)(Zhu and Reinberg, 2011)(Corpet & Almouzni, 2009; Masumoto et al, 2005). After replication has been completed the original chromatin structure needs to be restored. The newly incorporated histones are modified in a way that matches the PTMs that were present on the parental histones in a process called maturation of the chromatin. It now appears that the restoration of different histone PTMs to chromatin can actually be either coupled with or uncoupled from replication (Pesavento et al, 2008; Zee et al, 2010).

Restoration of full H3K9me levels following replication

Many chromatin modifiers have been shown to associate directly with the replication fork, particularly with PCNA or the PCNA-binding histone chaperone CAF1, and are thought to participate in the maturation of the chromatin following replication (Alabert and Groth, 2012; Groth et al., 2007). The mammalian SETDB1 KMT forms a complex with the replication fork associated histone chaperone CAF1 where it catalyzes H3K9 monomethylation on new soluble histones (Loyola et al., 2009). H3K9me1 histone is the favored substrate of the Suv39h1 KMT which catalyzes di- and tri-methylation of H3K9, thus providing a mechanism for the restoration of heterochromatin on newly assembled nucleosomes. HP1α also associates with CAF1 and thus might help restore heterochromatin after S phase (Dohke et al., 2008). What remains unclear is whether or not H3K9me on parental nucleosomes plays a role in the recruitment of these heterochromatin components to the replication fork and the restoration of heterochromatin.

1.3.3 Maintenance of H3K9 methylation and DNA methylation

The maintenance of histone H3K9me in higher eukaryotes such as mammals or plants may result from crosstalk between H3K9 me and 5-MeC methylation in DNA, and possibly other epigenetic marks. It has been proposed that DNA methylation could drive H3K9me at heterochromatin loci. H3K9me and DNA methylation are highly correlated across the genome and are both associated with silencing expression in plants and mammals (Law and Jacobsen, 2010; Rose and Klose, 2014). In fact, the mammalian SETDB1 and G9a H3K9 KMTs ,and HP1 are thought to be recruited to chromatin during replication via DNA methylation binding proteins (Corpet and Almouzni, 2009). During replication the MBD1 (methyl-CpG binding protein 1), which binds 5-MeCpG, associates with the H3K9 methyltransferase SetDB1 in a CAF1 dependent manner and is proposed to promote H3K9 methylation of H3 associated with CAF1 in H3-H4 tetramers (Reese et al., 2003; Sarraf and Stancheva, 2004). MBD1 also associates with SUV39h1 and HP1. (Dohke et al., 2008; Fujita et al., 2003; Huang et al., 2010). The multidomain protein UHRF1 in mammals could also create a bridge between DNA and H3K9 methylation since it can bind both H3K9me and hemi-methylated DNA and UHRF1 also interacts with DNMT1 and PCNA during replication (Rose and Klose, 2014). Together these observations suggest that the maintenance of H3K9me during cell division might be driven by DNA methylation. Consistent with DNA methylation underpinning the maintenance of H3K9me, it has been shown that a heterochromatin domain created at the *Oct4* locus by the artificial tethering of HP1α in mouse ES cells is partially dependent on DNA methylation (Hathaway et al., 2012). However, mouse ESCs lacking the three 5-MeC DNA methyltransferases have a similar distribution of H3K9me as wild-type cells, suggesting that H3K9me can be maintained at many heterochromatic regions independently of DNA methylation (Tsumura et al., 2006). Whether the maintenance of H3K9me at these loci is mediated by purely epigenetic processes has not been determined.

In plants, H3K9me and CHG DNA methylation are involved in a self-reinforcing loop that maintains heterochromatin regions. The H3K9 methyltransferase KRYPTONITE interacts directly with methylated CHG (where H= A,T or C) while the DNA methyltransferase CMT3 that methylates CHG binds H3K9me2 via both its BAH and chromo domains. Moreover, the loss of KRYPTONITE leads to large a decrease of DNA methylation (Jackson et al., 2002). Thus H3K9 methylation might participate with other epigenetic marks in the self-propagation of heterochromatin domains.

1.3.4 Transgenerational epigenetic inheritance

Epigenetic inheritance of traits through mitotic divisions is involved in many processes such as centromere maintenance, maintenance of imprinting, and the maintenance of the inactivated X chromosome in mammals. However, transgenerational epigenetic inheritance through the gametes and into progeny is clearly restricted. In many organisms an “epigenetic reprogramming” step occurs in the germline and/or at an early stage in the embryo resulting in the loss of chromatin associated marks such as DNA methylation and certain histone PTMs (Heard and Martienssen, 2014). These reprogramming events are important in order to reset chromatin by removing chromatin marks (DNA methylation & histone PTMs) acquired during development or resulting from changes in the environment and thus, may prevent the transmission of deleterious epialleles to the offspring (Heard and Martienssen, 2014). In mammals reprogramming occurs in two phases, in the germline and in the zygote. During these stages DNA methylation is removed by both active and passive demethylation (Seisenberger et al., 2013). The loss of DNA methylation in mouse germ cells, is also associated with the loss of H3K9me and H3K27me and consequently, loss of imprints and silencing of the inactive X chromosome. Nevertheless, imprinted loci are protected during zygotic DNA demethylation and a number of loci, mostly corresponding to repetitive DNA sequences, also escape demethylation in the germline (Messerschmidt, 2012). Those loci that escape reprograming may provide examples of transgenerational epigenetic inheritance.

DNA demethylation in the plant germline is restricted to asymmetric cytosines and does not target symetrical 5-MeCpG. The preservation of 5-MeCpG might explain why, unlike in mammals, transgenerational epigenetic inheritance is common in plants (Heard and Martienssen, 2014). In maize, the so called “cycling transposons” switch/cycle between active and silent states and the transcriptional state (silent or active) of particular transposon elements can be transmitted through generations epigenetically (Heard and Martienssen, 2014; Lisch, 2012). This class of transposon is silenced by DNA methylation and can sometimes influence the expression of nearby genes. For example, expression of a gene involved in seed and leaf color that is influenced by a cycling transposon causes changes in seed and leaf coloring that are epigenetically transmitted to progeny (Lisch, 2012).

Despite the presence of stringent reprogramming in mammals some examples of transgenerational epigenetic inheritance have been described. The inheritance of the

phenotype associated with the *agouti* allele A^{vy} has been extensively studies in mice. The Agouti protein causes yellowness of the coat and too much Agouti protein in mice leads to obesity. The expression of the *agouti* gene is dependent on the transcriptional state of a neighbouring retrotransposon IAP element. DNA methylation on the promoter of this IAP element correlates with silencing of the *agouti* gene resulting in a yellow-brown mottled or brown pseudoagouti coat colour. The transcription state of *agouti* in fathers does not influence the expression state of *agouti* in offspring, indicating that the epigenetic marks associated with *agouti* are lost in the male germline. However, mothers with a yellow coat (i.e. agouti expressed) give rise to a higher percentage of yellow offspring than expected, suggesting that the yellow phenotype is epigenetically transmitted through oogenesis from one generation to the next (Daxinger and Whitelaw, 2012).

Transgenerational epigenetic inheritance mediated by histones variants and histone PTMs?

Germline reprogramming in mammals results in loss, or a dramatic decrease, in the levels of many methylated lysine residues on histones, thus H3K9me and other histone PTMs are unlikely to extensively promote epigenetic inheritance to the offspring (Heard and Martienssen, 2014). Moreover, during spermatogenesis, the majority of histones are replaced by protamines making histones unreliable candidates for transmitting parental chromatin states to the offspring. However, a fraction of nucleosomes do remain associated with chromatin in the sperm despite the histone-protamine exchange and these might allow some degree of transgenerational inheritance at particular loci (Brykczynska et al., 2010). The histone H3 variant CENP-A was isolated from Bull sperm indicating that CENP-A escapes the histone/protamine exchange in the germ line potentially allowing centromeres to be epigenetically inherited in the progeny (Palmer et al., 1991). Nucleosomes carrying H3K9me3 also seem to be maintained in human spermatozoa and are even detected in early stage embryos (van de Werken et al., 2014). Thus, H3K9me3 might be involved in the maintenance of heterochromatin domains on particular chromosomal regions from one generation to the next. However, the pattern of PTM preservation is not conserved in mouse embryos since H3K9me3 is lost from paternal chromosomes and its reestablishment is dependent on Polycomb proteins (van de Werken et al., 2014).

Transgenerational inheritance of traits resulting from environmental changes

Phenotypes can clearly be epigenetically transmitted to the following generations, albeit at a low frequency. It has also been proposed that epigenetic changes in chromatin that were induced by the environment, through signaling pathways (temperature changes, shifts in nutrition), might also be transmitted and affect offspring. One example of this is the tongue sole fish which undergoes sex reversion in response to changes in temperature that are subsequently passed on to progeny (Bonasio, 2015). Young female (ZW) fish develop into fertile pseudo-males that, despite their female karyotype (ZW), resemble and behave like males (ZZ). Interestingly, the ZW offspring that received a Z chromosome from these pseudo-males also develop the pseudo-male phenotype even though they were not exposed to the same initiating temperature change. This heritability of the pseudo-male phenotype indicates that the changes induced on the Z chromosome are transmitted to offspring. However, it is clearly possible that the temperature change in the F0 might affect the Z chromosome in the germline of ZW mothers and therefore be responsible for the transmission of the phenotype to the F1 ZW offspring (Bonasio, 2015; Chen et al., 2014).

Another study has recently shown that F0 mice exposed to odour-induced fear conditioning give rise to F1 and F2 offspring that are more sensitive to that specific odour (Dias and Ressler, 2014). The increased sensitivity to the odour stimulus was found to be associated with a slight decrease in DNA methylation over the gene encoding the odourant receptor that responds to the stimulus. However, it was not shown whether this drop in DNA methylation was transmitted beyond the F1, thus it is unproven if the phenotype observed in response to this odour stimulation is transgenerational inherited.

Various studies so far suggest that the transmission of epigenetic traits through gametogenesis is not a frequent event, especially in mammals, and seems to be counteracted via robust reprogramming during gametogenesis and early embryogeneis. The few examples of transgenerational inheritance that have been described are often associated with certain mobile genetic elements (transposons etc) that appear to escape reprogramming events because their reactivation, even for a short period of time, would potentially be deleterious for the genome.

1.4 Aim of this study

The aim of this study is to determine whether H3K9 methylation can act as an epigenetic mark responsible for the maintenance of heterochromatin domains. In many eukaryotes, heterochromatin formation relies on a complex crosstalk between H3K9me, H3K27me and DNA methylation. It is therefore extremely challenging to study the role of H3K9me in heterochromatin maintenance in this context. *S. pombe* represents an ideal model organism to study the epigenetic properties of H3K9me because:

1. H3K9me heterochromatin is not essential which makes it amenable for studies using genetic approaches.
2. H3K9me is catalysed by a single SUV39 homolog Clr4 methyltransferase.
3. It lacks both DNA methylation and H3K27me that are interlinked with H3K9me-dependent heterochromatin establishment and maintenance in other eukaryotes.

In this study, a regulatable TetR-Clr4* protein was tethered to various euchromatic loci in the genome. The presence of TetR-Clr4* at ectopic sites is sufficient to promote heterochromatin formation characterised by H3K9me enrichment and gene silencing. The maintenance of this heterochromatin domain was then assessed upon TetR-Clr4* release from the tethering site to determine whether H3K9me is sufficient to epigenetically maintain a heterochromatin domain through mitotic and/or meiotic division.

Aim1: Characterise heterochromatin domains assembled upon tethering of TetR-Clr4* to a euchromatic locus.

Aim 2: Determine whether H3K9me is sufficient to propagate the ectopic heterochromatin domain upon release of TetR-Clr4*.

Aim 3: Determine whether H3K9me loss from the tethering site is dependent on specific phases of the cell cycle.

Aim 4: Determine the effect of transcription and histone turnover on the maintenance of H3K9 methylation.

Aim 5: Investigate the role of known and putative histone demethylases in H3K9me loss following TetR-Clr4* release from the tethering site.

CHAPTER 2: Materials and Methods

2.1 Fission Yeast Protocols

2.1.1 Mating and random spore analysis

Two strains from opposite mating types (h^+ and h^-) were mixed together in equal amounts on ME plates and left for 2 days at 25°C /32°C. For crosses of h^{90} strains with either h^+ or h^- cells, cells were mixed approximately at a 1:10 ratio with an excess of h^- or h^+ over h^{90}. On ME plates, cells are nitrogen starved which causes the cells to mate, undergo meiosis and subsequent sporulation. The formation of asci containing four spores was assessed by light microscopy. To isolate the spores from vegetative cells, cells were resuspended in 300 µl of 1:100 diluted glusulase (NEN) and incubated overnight at 36°C (25°C for 2 days for temperature sensitive). Vegetative cells and ascus walls are digested by the glusulase while spores remain intact. 4 ml of water was then added and 2 µl and 20 µl of the diluted spores were plated on selective media and grown at the appropriate temperature.

2.1.2 S. pombe transformation by electroporation

50 ml cultures were grown to log phase ($5x10^6$ to $1x10^7$ cells/ml) and harvested at 3500 rpm for 2 minutes. The pellet was washed three times in 10 ml of 1.2 M ice-cold sorbitol and resuspended in 500 µl of 1.2 M ice-cold sorbitol. 200 µl cells were added to 100 ng plasmid DNA or 10 µg linear DNA fragments in an ice-cold electroporation cuvette. Cells were pulsed using a Bio-Rad Gene Pulser II with settings of 2.25kV, 200Ω and 25µF. Cells were rapidly mixed with 500 µl of 1.2 M ice-cold sorbitol. Cells were then either directly plated on selective media or grown overnight in 10 ml of non-selective liquid medium before plating on selection.

2.1.3 Silencing assay

The expression levels of the *ade6*$^+$ marker gene can be assessed by conducting serial dilution assay or direct plating on medium containing a low amount of adenine. When *ade6*$^+$ is expressed, cells will form white colonies on medium containing limiting

adenine. If *ade6*$^+$ is repressed, cells accumulate an intermediate metabolite (amino-imidazole ribonucleotide) that gives a red colouring to the cells.

Serial dilution assay

Identical amounts of cells were resuspended in 200 µl of water at 5 x10^6 cells/ml followed by 1:5 serial dilutions made in sterile microtitre plates in dH_2O. 5 µl of each dilution was plated on the appropriate media: PMG full, PMG media containing limiting adenine (10 µg/ml) or on PMG medium containing limiting adenine supplemented with 10 µM anhydrotetracycline (AHT) and thiamine to asses of the silencing of the *4xtetO-ade6*$^+$ cassette. Cells were then incubated at the appropriate temperature (25°C or 32°C).

Random plating

An alternative to the serial dilution assay to assess the silencing of the *ade6*$^+$ marker gene was to directly plate cells onto PMG media containing limiting adenine (10 µg/ml). Thus, a large number of single colonies are obtained and the percentage of *ade6*$^+$ expressed and *ade6*$^+$ silenced colonies can be assessed easily.

2.1.4 Sectoring assay (performed by Alison Pidoux)

A sectoring assay was used to estimate rate of loss of silencing of the *4xtetO-ade6*$^+$ cassette upon TetR-Clr4* removal in an *epe1*Δ background. Red *epe1*Δ *tetR-clr4** *4xtetO-ade6*$^+$ colonies were picked from plates containing anhydrotetracycline (AHT; SIGMA), and re-plated onto +AHT plates. Resultant colonies were categorised as: half-sectored (half or more of the colony white, as diagrammed in Figure7.5.B) i.e. upon first division of the plated cell, silencing was lost in one daughter cell and maintained in the other; or red (any colony with red in both halves) in which silencing was maintained in both daughter cells (but may have been lost subsequently). Pure white colonies were excluded as they had already lost silencing at the time of plating. The number of 'half-sectored' colonies is expressed as a percentage of all red containing colonies to give an estimate of the rate of loss of silencing per division.

2.1.5 Cell cycle analyses

Analysis of H3K9me2 levels through the cell cycle upon TetR-Clr4 release.*

Pre-cultures of *cdc25-22* cells were grown overnight at 25°C. Cells were shifted at 36°C for 2h30min to obtain a synchronised population of cells arrested in late G2. AHT (10 µM) and thiamine were added to the medium to induce TetR-Clr4* removal

and synchronised cells were released into the cell cycle. Samples for H3K9me2 and FLAG ChIP analyses were collected before (0h), and every 20 min for 3h following AHT addition to assess H3K9me2 levels through the cell cycle. The septation index was assessed using light microscopy.

Analysis of H3K9me2 levels in G2-arrested cells upon TetR-Clr4 release.*

Pre-cultures of *cdc25-22* cells were grown overnight at 25°C. Cells were shifted at 36°C for 2h30min to obtain a synchronised population of cells arrested in G2/M. Synchronised cells were released into the cell cycle at 25°C for approximately 2h and, following their passage through S phase (that is concurrent with septation), cells were arrested a second time in G2/M by shifting the cells to 36°C in pre-warmed medium. AHT (10 μM) and thiamine were immediately added to the medium to induce TetR-Clr4* dissociation from *tetO*. Samples for H3K9me2 and FLAG ChIP analyses were collected before (0h), and 1h, 2h, 3h, 4h following AHT addition to assess H3K9me2 loss in arrested cells upon TetR-Clr4* release.

2.1.6 Recombination-induced tag exchange (RITE) to assess histone H3 turnover

Recombination-induced tag exchange (RITE) construct

The H3.2 Recombination-induced tag exchange (RITE) strain was constructed by PCR amplification of the HA/T7 RITE cassette followed by integration at the endogenous locus by homologous recombination. The RITE cassette is composed of a first epitope tag (HA) associated with a hygromycin resistance gene surrounded by 2 LoxP sites. A T7 epitope tag is situated downstream of the 2nd loxP site and will be expressed with the H3.2 gene upon Cre-EBD-induced recombination of the loxP sites. The Cre-EBD open reading frame was PCR amplified from pTW040 and recloned in pRAD15 vector under an attenuated *adh* promoter. The plasmid was integrated at the *ars1* locus using the *Mlu*I site. The RITE constructs were gift from Fred Van Leeuwen and cloned in *S. pombe* by Manu Shukla (Allshire lab).

Histone turnover experimental design

Pre-cultures were grown overnight at 25°C in medium supplemented with hygromycin to select against cells that have prematurely undergone recombination. Cells were shifted to 36°C for 2h30 min to obtain a synchronised population of cells arrested in G2/M. Synchronised cells were released into the cell cycle at 25°C for approximately

2h and, following their passage through S phase, arrested a second time in G2/M by shifting the cells to 36°C in pre-warmed medium devoid of hygromycin (Duchefa Biochemie). Simultaneously, ß-estradiol (1 µM; Sigma) was added to the medium to induce recombination-induced tag exchange. Samples for western blotting and total H3 and H3-T7 ChIP analyses were collected before (0h) and at indicated times following Cre-EBD activation. Cells were also plated on non-selective medium before and after Cre-EBD activation and replica-plated onto plates supplemented with hygromycin to assess of the loss of the hygromycin resistance gene that is recombined out following swapping of the RITE cassette.

2.1.8 Media and Growth

Growth conditions

S. pombe was grown at indicated temperatures between 25ºC and 36ºC; 32 ºC being the permissive temperature for wild type strains. Wild type haploid strains will grow with the following generation times when in log phase:

medium	**temperature °C**	**generation time**
Rich medium (YE)	25	3h
	32	2h10
	36	2h
Minimal medium (PMG)	25	4h
	32	2h39
	36	2h20

Media

All solutions were made up to a final volume in dH_2O and autoclaved unless otherwise stated.

PMG liquid in 900ml:

Pthallic acid	3 g
di-sodium orthophosphate	2.2 g
glutamic acid	3.75 g
D-glucose anhydrous	20 g
vitamins 1000x	1 ml
minerals 10,000x	0.1 ml
salts 50x	20 ml

PMG agar in 900ml:

Pthallic acid	3 g	
di-sodium orthophosphate	2.2 g	
glutamic acid	3.75 g	
D-glucose anhydrous		20 g
vitamins 1000x	1 ml	
minerals 10,000x	0.1 ml	
salts 50x	20 ml	
agar (OXOID)		20 g

YES liquid in 900ml:

Yeast extract (DIFCO)	5 g	
D-glucose anhydrous		30 g
Adenine (Sigma)	0.2 g	
Arginine (Sigma)	0.2 g	
Lysine (Sigma)	0.2 g	
Histidine (Sigma)	0.2 g	
Uracil (Sigma)	0.2 g	
Leucine (Sigma)	0.2 g	

YES agar in 900ml:

Yeast extract (DIFCO)	5 g	
D-glucose anhydrous		30 g
Adenine (Sigma)	0.2 g	
Arginine (Sigma)	0.2 g	
Lysine (Sigma)	0.2 g	
Histidine (Sigma)	0.2 g	
Uracil (Sigma)	0.2 g	
Leucine (Sigma)	0.2 g	
agar (OXOID)		20 g

ME plates in 900ml:

Malt extract (OXOID)	27 g
Adenine (Sigma)	0.2 g
Arginine (Sigma)	0.2 g
Histidine (Sigma)	0.2 g
Uracil (Sigma)	0.2 g
Leucine (Sigma)	0.2 g

Vitamins 1000x (100ml):

Inositol	1 g
Biotin	1 mg
Pantothenic acid	0.5 g
Nicotinic acid	1 g

⇨ Filter sterilised

Salts 50x:

Potassium chloride	50 g
di-sodium sulphate	2 g
Magnesium chloride	53.5 g
Calcium chloride	1 g

Minerals 10,000x (100ml):

$FeCl_{2}.6H_2O$	2 g
Molybdic acid	1.6 g
Boric acid	5 g
$MnSO_4$	4 g
ZnSO	4 g
$CuSO_4.5H_2O$	0.4 g
Citric acid	10 g

⇨ Filter sterilised

Supplement stocks:

Adenine 50x (Sigma)	5 g/L
Arginine 100x (Sigma)	10 g/L
Lysine 100x (Sigma)	10 g/L
Histidine 100x (Sigma)	10 g/L
Uracil 20x (Sigma)	2 g/L
Leucine 100x (Sigma)	10 g/L

Additional supplements:

Fluoroorotic acid (FOA) (Melford Laboratories): final concentration of 1 g/500ml

Thiamine (Sigma): 15μM thiamine (5 μg/ml; from filter sterilized 2000x stock at 10 mg/ml)

Nourseothricin (cloNAT) (Werner BioAgents): final concentration of 2000x

Geneticin (G418) (Gibco) : final concentration of 0.1 mg/ml.

Hygromycin B(Hyg) (Duchefa Biochemie) : 0.123 mg/ml final

Anhydrotetracycline hydrochloride vetranal (Sigma)(AHT): 10 μM final. 20mM stock in DMSO was kept at -20. Before addition in the medium 250 μl of this conc stock was added into 5 ml DMSO and supplemented to 500 ml medium

ß-estradiol (Sigma): 1 μM final. 1000x stock made in ethanol

2.2 DNA protocols

2.2.1 *S. pombe* colony PCR

A small amount of yeast cells was incubated in 10µl of SPZ (1.2M sorbitol, 100mM sodium phosphate, 2.5mg/ml zymolyase-100T (MP Biomedical)) at 37°C for approximately 20min. 50µl of H20 was added to the cells and 5µl of this crude extract was used as a PCR template.

2.2.2 Genomic DNA preparation from *S. pombe*

10 ml of culture was harvested at 3500 rpm for 2 minutes. The pellet was resuspended in 250 µl of DNA buffer (0.1 M Tris pH8; 0.1 M NaCl; 1 mM EDTA; 1% SDS) and 250 µl of phenol. Cells were then mechanically lysed using acid-washed glass beads (Sigma). The DNA was isolated by phenol/chloroform extraction and ethanol precipitated. 1/10 volume of Sodium Acetate (3 M, pH 5.2) was added followed by 2.5 to 3X volume of 100% ethanol. The sample was incubated on ice for 15min (or more) and harvested at 4°C for 30minutes at 14000g. The DNA pellet was rinsed with 70% ethanol followed by 15 centrifugation. The pellet was resuspended in 50 µl TE and stored at -20°C.

2.2.3 PCR reaction

Taq polymerase reaction

DNA amplification using Taq polymerase (Roche) was used for yeast and bacteria colony PCR that does not require precise amplification.

10xPCR buffer
250 µM dNTPs
1 µM primer
0.5U taq (Roche)
Template DNA (about 10 ng)
dH_2O

PCR program:
94°C for 4 minutes
94°C for 30 sec ┐
55°C for 30 sec │ X29 cycles
72°C for 1min ┘
72°C for 5min

Pfx amplification:

Accurate DNA amplification using Pfx (Life Technologies) was used to amplify DNA fragments for integration in the genome or for sequencing reactions.

2X Pfx buffer
1 mM $MgSO_4$
0.3 mM dNTPs
0.5 µM each primer
to 20 µl dH2O
~100 ng DNA
0.1 µl Pfx

PCR program :
94°C for 5 minutes
94°C for 30 sec
55°C for 30 sec
68°C 1min/kb
X35 cycles
68°C for 10min

2.2.4 Agarose gel electrophoresis (AGE)

AGE was used to analyse the size of DNA fragments. Agarose was dissolved in 1X TBE. This technique is used to determine the size of a DNA fragment. Agarose is dissolved in TBE (55g boric acid, 9.3g EDTA, 108g Trizma base) to make an appropriate percentage gel usually between 0.8 and 1.5% agarose. Ethidium bromide at 0.03ug/ml (Sigma) was added to the agarose gel. Orange G loading buffer (30% Glycerol; 0.25% orange G) was added to the DNA samples before loading the gel. After migration, The DNA was visualized by UV using a transilluminator.

2.2.5 Sequencing

ABI Prism BigDye Terminator Cycle sequencing kit was used for the sequencing reaction.

Sequencing reaction:
Big dye Terminator mix: 2ul
BigDye buffer: 2ul
0.32 µM primer,
template DNA as recommend by manufacturers (1-1000 ng for PCR products)
dH_2O up to 10ul.

Sequencing program:

95°C for 5 minutes
95°C for 30 seconds
ramp 1°C per second to 55°C
55°C for 15 seconds
ramp 1°C per second to 64°C
64°C for 4 minutes
] x25 cycles

Analyses of the sequence was then done by a sequencing service.

2.2.6 Construction of sib1 and vps1302 tethering sites

Insertion of the *tetO* sequence into *vps1302* and *sib1* was done in two steps. First, the *ura4*$^{+}$ gene was amplified by Pfx using long oligos with 80bp homology with *sib1* or *vps1302*. This DNA fragment was gel purified using a gel purification kit from Qiagen. 10 μl of this product was transformed into *S. pombe* by electroporation. The linear DNA fragment integrates efficiently by homologous recombination. Positive integrants were selected on PMG-uracil plates and verified by colony PCR. In a second step, the *tetO* sequence(s) was amplified by PCR from gDNA containing the *4xteto-ade6*$^{+}$ cassette using Pfx polymerase. 100 bp long oligos with 20 bp homology with the sequence adjacent to the *tetO* array and 80 bp homology with *vps1302* or *sib1* were used. The DNA fragment was then gel purified and transformed into *vps1302:ura4* and *sib1:ura4* strains as before. Positive integrants were selected on PMG+FOA and colony PCR before sequencing of the insertion locus.

Replacement of sib1 and vps1302 promoter with ura4+ or adh1, adh11, adh81 promoters

As for *tetO* insertion within *sib1* and *vps1302* ORFs, the insertion of *adh* promoters to replace the endogenous promoter of these two genes was done in two consecutive steps. The promoter was first replaced by the *ura4* gene that was then replaced by *adh1, adh11* or *adh81* promoters.

2.3 RNA protocols

2.3.1 RNA extraction

10 ml of culture in log phase was harvested and washed twice in dH_20; then RNA extraction was performed using an RNeasy Miniprep kit (Qiagen). 15 μg of RNA was treated using Turbo DNAseI (Life Technologies) and incubated at 37°C for 1h. A second step of DNAse treatment was performed by adding a further 2 µl of Turbo DNaseI for 1h. RNA clean-up using the RNeasy miniprep kit was then performed and RNA was quantified using the Nanodrop.

2.3.2 Reverse transcription

1 µg of RNA/sample was incubated with 1 µl of random hexamer (100 ng/ul), 1 µl of dNTPs mix (10 mM) and 3 µl of dH_2O for 10min at 65°C for a denaturing step. Samples were then incubated on ice. For cDNA synthesis, 4 µl of Superscript buffer (5X), 1 µl of DTT 0.1M, 1 µl of Superscript enzyme III and 1 µl of dH_2O were added to the reaction. The samples were then incubated for 5 min at 25°C, 1h at 50°C and finally 15min at 70°C. A –RT control was made in which the superscript enzyme III was not added to the reaction. cDNA diluted 1:2 was used as a template for qPCR reactions.

2.4 Protein protocols

2.4.1 Protein extraction

A 10 ml of culture was grown to log phase and harvested at 3500 rpm for 2 minutes. The pellet was resuspended in 1 ml dH_2O and transferred to 2 ml round-bottomed screw-cap tubes. Cells were harvested and the supernatant was discarded. The cell pellet was resuspended in 2X protein sample buffer (50 mM Tris-HCl pH6.8, 2% SDS, 2 mM EDTA, 0.03% bromophenol blue 10% glycerol) supplemented with 5 μM PMSF and 20 µl/ml of β-mercaptoethanol at a concentration of $5x10^7$ cells per 100 ul. 1X volume of acid washed glass beads (Sigma) was added to the sample and alternately vortexed vigorously and boiled over the next 5-10 min. The supernatant was clarified and loaded on a protein gel.

2.4.2 Western Blot

10 to 20 ul of protein prep were loaded on NuPAGE Bis-Tris gels (Life technologies). The protein gels were run at 200V in 1X NuPage MOPS or MES buffer from Life Technologies depending on the size of the protein of interest. Proteins were transferred to a nitrocellulose membrane in 1X transfer buffer (Life Technologies) supplemented with 10% methanol in a semi-dry transfer. The transfer was performed in an XCell Blot Module (Invitrogen) 1h at 32 Volts for 1 gel. The membrane was then

stained with red Ponceau (Sigma) to confirm transfer and blocked for 30min to 1h at room temperature in PBS+0.1% Tween-20 +5% Milk. The membrane was incubated overnight at 4°C with the primary antibody in blocking solution (PBS +0.1 Tween-20+5% milk). Three washes of 10 minutes in PBS + 0.1% Tween were carried out followed by addition of the HRP-coupled secondary antibody for 1 hour at RT. Three washes of 10 min in PBS+ 0.1% Tween were performed. The protein of interest were detected by luminescence using an Enhanced Chemi-Luminescence kit (Amersham).

Antibodies used for western blot analyses
T7 antibody : abcam 9138. Goat polyclonal.1/1000 in PBS 0,1%tween 5%milk
HA antibody: anti HA 12CA5 mouse monoclonal Ab 1:1000 in PBS 0,1%tween 5%milk.

2.4.3 Chromatin immuno-precipitation (ChIP)

50 ml of cells grown to log phase were fixed for 15min in 1% paraformaldehyde (Sigma) at RT unless stated otherwise. The cells were washed twice in ice-cold PBS and the cell pellet was frozen. Cells were resuspended in 350 µl of cold lysis buffer supplemented with 1 mM PMSF and protease inhibitors (1:100) (Sigma). 500 ul of glass beads were added to the samples in order to mechanically break the cells by bead beating (Biospec Products). The cell lysate was recovered then sonicated (20 min, 30 s on/off cycle) using a BioRuptor sonicator (Diagenode) in order to fragment the chromatin in pieces of approximately 300 to 900 bp. Two spins of 10 minutes were performed at 13500 rpm at 4°C to clarify the lysate. The supernatant was transferred to fresh tubes before pre-clearing of the samples with 25 µl of pre-washed protein G dynabeads (Roche) or 25 µl of protein G agarose (Roche) depending on the antibody used for 30 min-1h at 4°C. After pre-clearing, the samples were transferred to new tubes. 10 µl of lysate was retained as an INPUT control. 300 µl is used for the immunoprecipitaion. 25 µl of pre-washed protein G agarose or Dynabeads were added to the lysate along with the antibody of interest at the appropriate concentration. The immunoprecipitation was carried out overnight at 4°C. The beads were washed briefly in lysis buffer, 10 minutes in lysis buffer containing 0.5 M NaCl, 10 minutes in wash buffer and briefly in TE pH8. 100 µl of 10% Chelex resin (Bio-Rad) was added in each INPUT and IP samples and boiled for 12 min to extract the DNA. The samples were then incubated for 30min at 55°C with 2.5 µl of Proteinase

K (10 mg/ml) before inactivation of the proteinase K 10min at 100°C. The samples were then recovered in fresh tubes and analysed by qPCR.

Lysis buffer: 140 mM NaCl, 50 mM HEPES-KOH pH7.5, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate
Lysis buffer 0.5M Nacl: 500 mM NaCl, 50 mM HEPES-KOH pH7.5, 1 mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate
Wash buffer: 10 mM Tris-HCl pH8, 0.25 M lithium chloride, 0.5% NP-40, 1 mM EDTA, 0.5% sodium deoxycholate
TE: 10 mM Tris-HCl pH8, 1 mM EDTA

2.4.4 Antibodies used for ChIP

For H3K9me2 ChIPs, One microliter monoclonal H3K9me2 antibody (m5.1.1; a kind gift from Takeshi Urano) was used with Protein G agarose beads (Roche). For all other ChIPs, Protein G Dynabeads (Life Technologies) were used along with 1.5 µl anti-Flag M2 antibody (Sigma, F1804), 2 µl anti-H3K9me3 (39161 active motif), 2 µl anti-H3 antibody (ab1791, Abcam), 3 µl 4H8 anti-Rpb1 CTD antibody (2629S, Cell Signalling Technology), 10 µl anti-Swi6 rabbit serum, 3 µl or 1.5 µl anti-T7 antibody (9138, abcam), 4µl anti Chp1 rabbit polyclonal (18191, Abcam), 3µl anti-H3K4me3 (39159 active motif), 3µl anti-H3K36me3 (61101, active motif).

2.4.5 Quantitative PCR (qPCR)

The qPCRs were performed in 96 or 384 well plates (Roche) with 10 ul of PCR reaction/well.
Each reaction consists of 5 ul of Light Cycler 480 SybrGreen Master Mix (Roche), 0.05 of each primer (100 µM), 1.9 µl of dH_2O and 3 µl of DNA template. For ChIP samples, the IP sample was diluted 1:20 and the INPUT sample 1:200 for the PCR. For cDNA samples, the cDNA was diluted 1:2.

PCR program:
95°C for 2 minutes
95°C for 20 seconds
55°C for 20 seconds
72°C for 20 seconds
] x55 cycles

The data were analysed with the Light Cycler 480 Software 1.5 (Roche). All ChIP enrichments were calculated as % DNA immunoprecipitated at the locus of interest relative to the corresponding input samples, and normalized to % DNA immunoprecipitated at the *act1*$^{+}$ locus. Histograms represent data averaged over three biological replicates. Error bars represent standard deviations from three biological replicates.

2.6 Bacterial Protocols

2.6.1 Transformation

1 to 5 ng of plasmid of interest was transformed in 30 μl of DH5α (Invitrogen). The DNA and cells were mixed and incubated on ice for 20 min, heatshocked for 45 sec in a waterbath at 42°C and left on ice for 1 min before addition of 700 µl of LB. Cells were then grown for 45 min at 37°C and plated onto LB media with the appropriate selection.

2.6.2 Plasmid miniprep

A single colony of a bacteria containing the plasmid of interest was grown in 3ml of LB + selection at 37°C overnight. The cells were harvested for 5 minutes at 3500rpm and plasmids were extracted using a QIAGEN miniprep kit and eluted in dH2O.

2.6.3 .Bacterial Media

LB medium (1litre):

Sodium chloride	10 g
Bacto tryptone	10 g
Bacto yeast extract	5 g

LB agar (1 litre):

Sodium chloride	10 g
acto tryptone	10 g
Bacto yeast extract	5 g
Bacto agar	15 g

Supplement

Ampicillin:	100 μg/ml
Carbenicillin:	50 μg/ml

2.7 Strains used in this thesis

Table: strains		
FY	**Relevant genotype**	
A2730	ura4::[4xTetO+B2+B3:B38:B37-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A7874	ago1::KANMX ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A8770	dcr1::NatMX6 ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A9823	ura4::[4xTetO-ade6] cdc25-22(ts)ars1 :prad15-cre-EBD-LEU2 .2-lox--gR-Lox-T7 ade6-210 leu1-32	
A9181	sib1:[4xTet0]+ ura4-D18 ade6-704 leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd cc2:his3 his3D1	
A9280	promoter(sib1)::rad1 sib1:[4xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ura4-D18 his3D1 ade6-704 cc2:his3	
A9282	promoter(sib1)::rad11 sib1:[4xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ura4-D18 his3D1 ade6-704 cc2:his3	
A9285	promoter(sib1)::rad81 sib1:[4xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ura4-D18 his3D1 ade6-704 cc2:his3	
A9822	promoter(sib1)::ura4 sib1:[4xTet0] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ura4-D18 his3D1 ade6210/704 cc2:his3	
A9113	vps1302:[1xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ade6-210/704 ura4-D18 his3D1 cc2:his3	
A9117	promoter(vps1302)::rad1 vps1302:[1xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ade6-210/704 ura4-D18 his3D1 cc2:his3	
A9119	promoter(vps1302)::rad11 vps1302:[1xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ade6-210/704 ura4-D18 his3D1 cc2:his3	
A9120	promoter(vps1302)::rad81 vps1302:[1xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ade6-210/704 ura4-D18 his3D1 cc2:his3	
A9546	promoter(vps1302)::ura4 vps1302:[1xTetO] leu1+:nmt81xTetR"off"-2FLAG-clr4-cdd ade6-210/704 ura4-D18 his3D1 cc2:his3	
A8414	ura4::[4xTetO-ade6] ade6-DNN leu1+:nmt81xTetR"OFF"-2xFlag-clr4-cdd cdc25-22(ts)	
B0294	epe1::KANMX ura4::[4xTetO-ade6+] ade6-DNN leu1-32	F1
B0302	epe1::KANMX ura4::[4xTetO-ade6+] ade6-DNN leu1-32	F2
B0321	ura4::[4xTetO-ade6+] ade6DNN leu1-32	F2xwt
B0322	ura4::[4xTetO-ade6+] ade6DN/N epe1::ura4	
A8976	epe1::KANMX ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
B0109	epe1 K314A ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
B0110	epe1 97A ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
B0574	dcr1::NatMX6, Tis2::KANMX, ura4::[4xTetO-ade6+], ade6-DNN, leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
B0159	epe1::KanMX ago1::KANMX ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A8436	ura4::[4xTetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd jmj1::kan	
A8439	ura4::[4xTetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd msc1::kan	
A9551	lid2 JmjD::leu2 ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A9552	ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd spLsd2m(KK861862AA)-13Myc::KanR	
A9571	spLsd1m(KK613614AA)-3:NatR ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A9574	Jmj4::NATR ura4::[4xTetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	
A8437	ura4::[4xTetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd jmj2::kan	
8678	ura4::[4xtetO-ade6+] his3- ade6DN/N	
B0198	tis2:kanMX ura4::[4xtetO-ade6+] ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd clr3::HYGR	
B0156	cdt2::ura+ ade6-DN/N ura4::[4xTetO-ade6+] leu1+nmt81xTetR"off"-2xFlag-clr4-cdd	
B0107	ura4::[4xTetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd sir2::NAT Tis2::KANMX4	
A8441	ura4::[4xtetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd sir2::NAT	
A2795	ade6-DN/N ura4::[4xTetO-ade6+] clr4::hgrMX6 leu1+:nmt81xTetR"off"- 2xFLAG-clr4-cdd	
B0148	swi6:NATR ura4:[4xTetO-ade6+] ade6-DNN leu1+nmt81xTetR"off"-2xFlag-clr4-cdd epe1::KanMX	
B0178	swi6:NATR, ura4::[4xTetO-ade6+], ade6-DNN leu1+:nmt81xTetR"off"-2xFLAGclr4-cdd	
B0560	epe1::KANMX, ura4::[4xTetO-ade6+], ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd, GBD-clr4-Delta CD- hgrMX6	
B0563	ura4::[4xTetO-ade6+], ade6-DNN leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd, GBD-clr4-cdd-hgrMX6	
B0566	epe1::KANMX, clr4::hgrMX6, ura4::[4xTetO-ade6+], ade6-DNN, leu1+:nmt81xTetR"off"-2xFLAG-clr4-cdd	

2.8 Primers used in this study

qPCR/RT primers

qChIP +3kb from tetO
ura4_L1_fwd TCAGCATATCTTTCCACACTTG
ura4_L1_rev TGAGAGGGAGTTTGAACCG

qChIP +4kb from tetO
ura4_L2_fwd CAGTTAGTTTCAGGTTTCCC
ura4_L2_rev GCAGAGTAATGGTGATTGG

qChIP +5kb from tetO
ura4_L3_fwd ATCTCCTTCCCTCCTTTAACG
ura4_L3_rev ATGCTGACGCTCTGTGTAC

qChIP +7kb from tetO
ura4_L5_fwd TTATCAAGCGTTGTTCATATAATTG
ura4_L5_rev AGTGTCGCACCATCTAAGG

qChIP +12kb from tetO
ura4_L10_fwd ACCAGTAGTGAAACGACGAAATC
ura4_L10_rev GCCCGTATGCTCCCTTGC

qChIP/qRT ade6
ade6 for CATGGAAATTGCAGTGATGG
ade6 rev CGAGCAGGGGCATATACTAAA

qChIP/qRT -0,5kb from tetO
qura4 5'for TCGCAGACATTGGAAATACC
qura4 5'rev ATGGCAATTTGTGATATGAGC

qChIP -1kb from tetO
ura4_R1_rev ACTCAGCATTCTTTCTCTAAATAGG
ura4_R1_fwd ACAAACAAATACACTAGGTAAATCG

qChIP -5kb from tetO
ura4_R5_fwd TATACAACTGCTGCTAATCCTAG
ura4_R5_rev CGCCTCTAATTTCCCTTTCG

qChIP -10kb from tetO
ura4_R10_rev TCGTTCAACTCAACCGTAGG
ura4_R10_fwd ATGCCCATTAAATCTCTTCACTG

qChIP/qRT act1
Q_ACT_F GGTTTCGCTGGAGATGATG
Q_ACT_R ATACCACGCTTGCTTTGAG

qChIP dg
Q_DG_F AATTGTGGTGGTGTGGTAATA
Q_DG_R GGGTTCATCGTTTCCATTCAG

qChIP/qRT mug135
qmug 135 fwd GGACATTAGATAGTCTGGATG
qmug 135 rev CACTCTGCTTTATCCTCTTC

qChIP/qRT new 25
qnew 25 fwd GTTTGCTGCAATTATTGCTG

qnew25 rev GAGGACTTTGAGAAATGGAG

qChIP/qRT pmp20
qpmp20 fwd GCTGTCGGATCTACTTTGCC
qpmp20 rev CAAGGAGGAGTGAAAGCGCC

qChIP/qRT tam14
qtam14 fwd CGGTTCAAACTCCCTCTCAG
qtam14 rev GAGGATGTTTGGCAACATAAC

qChIP/qRT vps1302 +0,1kb from tetO
PA198 GTAACAGCTGGAGTACTGAC
PA199 GACCACTTGCTTTCGATC

qChIP/qRT vps1302 -1kb from tetO
PA067 CAGACTGGCTACAATTTAAG
PA068 GGTACTATCAAATGAAACGC

qChIP/qRT sib1 -0,1kb from tetO
PA077 CTTAATAGCAGAGACATTTG
PA219 CGAGCAAGACTCTTAAAGG

qChIP/qRT sib1 -0,1kb from tetO
PA224 CATTTGTAAGTCCTTTGCTC
PA064 CACCATGTCTTATATGATGC

qChIP IRC
IRC-L1 tgctgaatgtaaccaacatca
IRC-R1 gcctcaattgcctattagtgct

qChIP/ sib1 1kb from tetO
PA220 GAGCATAATCATGTTGAATC
PA221 GCAGAAAGTACGGTATTGCC

qChIP/ sib1 -0.5kb from tetO
PA222 GTAGGACGGACGATCAAATC
A223 CCAACTGCTGCTTTCGAAG

qChIP vps1302 +2kb from tetO
PA225 GTAATATGCCCTGCCTTAG
PA226 CACAGATTGTTCTGAAGGAG

qChIP vps1302 +5kb from tetO
PA227 CAGGTTTAACAACCATTCCC
PA063 CTAACATTGAACTTTCCAG

qChIP vps1302 +1kb from tetO
PA228 CTAGAGTTAAAGGAATCTG
16C6 qPCR for2 1kb CCAATCAGTAAAATACTTGGC

Construct of vps1302 tethering site

spbc16C6.02
revCTTGTGAAGTTGATTTGGAAGCATCAGCAACAAATACCAAAGATGTATTTCTAATGTC
AAACCGAACAGATAAACTGATGGCAAATTACTTTTTATTCCC
primer for ura4 insertion at 4534 bp from atg of vps1302. this primers shows homology with 3'UTR of ura4

spbc16C6.02 for
GAAAGTCCTGATGATGTACAGTCTCTTCAAGCTGAGAGCTCAGTCAGTACTCCAGCTGT
TACAGCAGCATCTGAAAAATCaagcttagctacaaatcccac
primer for ura4 insertion at 4534 bp from atg of vps1302. this primers shows homology with 5'UTR of ura4

16C6 teto rev bis
CTTGTGAAGTTGATTTGGAAGCATC
AGCAACAAATACCAAAGATGTATTTCTAATGTCAAACCGAACAGATAAACTGATGGGCT
GGATTTCGTTTACCTC
amplification of tet0 for insertion in vps1302. this primer shows homology with 5' UTR of ade6 to amplify tet0 from the ura4:4xteto-ade6+ cassette.

16C6 for tetO bis
GAAAGTCCTGATGATGTACAGTCTCTTCAAGCTGAGAGCTCAGTCAGTACTCCAGCTGT
TACAGCAGCATCTGAAAAATCCAAGGCCTACTAGTGCATG
amplification of teto for insertion in vps1302.

16C6 ura4
5’revCTAAGAGTCGATTTAAAAAATCTTTTTCGTTAGTTTCGTATTTAATACAAAAGACTTA
CTTGCTAGTAACCCTTCTAACATGCAAATTACTTTTTATTCCC
primer for ura4 insertion at the promoter region of vps1302. this primers shows homology with 3'UTR of ura4

16C6 5’ ura for
CATATTCTAATGGATTTCTTGAAATTTTTACAAACGTAAACGAGATTTTTCTTTTCTTGAAA
ATGTCTGTTACTTAGCTaagcttagctacaaatcccac
primer for ura4 insertion at the promoter region of vps1302. this primers shows homology with 5'UTR of ura4

16C6 rad
for2CATATTCTAATGGATTTCTTGAAATTTTTACAAACGTAAACGAGATTTTTCTTTTCTTG
AAAATGTCTGTTACTTAGCTCCCTACAACAACTAAGAAAATG
amplification of adh promoters for insertion in 5' of 16C6

16C6 rad
rev2CTAAGAGTCGATTTAAAAAATCTTTTTCGTTAGTTTCGTATTTAATACAAAAGACTTA
CTTGCTAGTAACCCTTCTAACATCTCGACATATGGGCAATTC
amplification of adh promoters for insertion in 5' of 16C6

Construct of sib1 tethering site

Sib1 for ura3
CCTGAGCAAAGGACTTACAAATGCAACCTTTCTATTTCTCTTTCCCAAATTGATGACTTA
TGTAACGGGTTTTCATTTAaagcttagctacaaatcccac
primer for ura4 insertion at 4636 from sib1 ATG. this primers shows homology with 5'UTR of ura4

spac23G3.02c ura4 rev
GTTACCAAAGCAAATGTCTCTGCTATTAAGCAAAAAAGATAATGTTTTAGCCCAACAACA
TTGCAAAAACGTTGATGTAGGCAAATTACTTTTTATTCCC
primer for ura4 insertion at 4636 from Sib1 ATG. this primers shows homology with 3'UTR of ura4

Sib1 for tetO bis

CCTGAGCAAAGGACTTACAAATGCAACCTTTCTATTTCTCTTTCCCAAATTGATGACTTA
TGTAACGGGTTTTCATTTACAAGGCCTACTAGTGCATG
amplification of teto for insertion in sib1.

Sib1 tetO rev bisGTTACCAAAGCAAATGTCTCTGCTATTAAGCAAAAAAGATA
ATGTTTTAGCCCAACAACATTGCAAAAACGTTGATGTAGGGCTGGATTTCGTTTACCTCa
mplification of tet0 for insertion in sib1. this primer shows homology with 5' UTR of ade6 to amplify tet0 from the ura4:4xteto-ade6+ cassette.

Sib1 ura4 5’ rev
GGGGCATCACTTGACGGCCGCATAGGACCTAATTGAGAAATTTGAGTTGAGAAAGCAT
CTTCTTCATTTGAATTCTTCATGCAAATTACTTTTTATTCCC
primer for ura4 insertion at the promoter region of sib1. this primers shows homology with 5'UTR of ura4

Sib1 ura4 5’ for
CATCGATTAAATGAGACATCaagcttagctacaaatcccac
primer for ura4 insertion at the promoter region of sib1. this primers shows homology with 3'UTR of ura4

Sib1 long homol for
GAACTATGCGGAAGCAAATCTGGAGTAAAGCGAAAAGGAAGTGGTAAGTGTGTTACAA
CACATTTTTTTTAATAGAATGGCATCGATTAAATGAGACATC
increase sib1 homology for ura4 and adh promoter insertion in 5' of sib1

23G3 rad rev2
GGGGCATCACTTGACGGCCGCATAGGACCTAATTGAGAAATTTGAGTTGAGAAAGCAT
CTTCTTCATTTGAATTCTTCATCTCGACATATGGGCAATTC
amplification of adh promoters for insertion in 5' of sib1

Sib1 rad for
CATCGATTAAATGAGACATCCCCTACAACAACTAAGAAAATG
amplification of adh promoters for insertion in 5' of sib1

CHAPTER 3: Characterisation of an ectopic heterochromatin domain assembled by tethering Clr4 methyltransferase activity to a euchromatic locus

INTRODUCTION

The aim of this study is to determine whether once established, a heterochromatin domain is able to self-maintain through cell divisions epigenetically, i.e. independently of a specific DNA sequence. It is extremely challenging to study the epigenetic function of H3K9 methylation (H3K9me) in its natural context such as centromeres, telomeres or the mating type locus, where initiator signals such as the RNAi machinery or DNA binding factors recruit the H3K9 methyltransferase to chromatin during every cell cycle (Chen et al., 2008; Kloc et al., 2008). To determine whether H3K9me can act as a true epigenetic mark it is essential to uncouple H3K9me from genomic domains that have the intrinsic ability to form heterochromatin via DNA sequence dependent mechanisms. One way to solve this problem is to isolate H3K9me from its normal endogenous context and investigate whether H3K9me can persist at an ectopic site through one or more cell divisions.

Several studies have shown that the chromatin landscape can be altered by tethering chromatin modifiers to ectopic places in the genome (Hathaway et al., 2012; Kagansky et al., 2009; Li et al., 2003; Lustig et al., 1996). Tethering of the chromodomain protein HP1α to an active *Oct4* gene in mouse cells is sufficient to assemble heterochromatin and promote silencing of *Oct4* (Hathaway et al., 2012). In *S. pombe,* expression of H3K9 methyltransferase (HMTase) Clr4 fused to the DNA binding domain from the *S. cerevisiae* Gal4 protein (GBD) allows Clr4 activity to be tethered at Gal4 binding sites (*gbs*) inserted at the *ura4* locus. This tethered Clr4-GBD protein also results in the assembly of a heterochromatin domain that spreads over 18-20kb (Kagansky et al., 2009). This synthetic heterochromatin domain is characterized by an enrichment of H3K9me2, the recruitment of the chromodomain protein Swi6 (homologue of HP1), and the silencing of underlying genes that occurs independently of the RNAi (Kagansky et al., 2009). In this configuration, the ectopic heterochromatin formed relies on tethered Clr4 alone since the underlying sequence cannot initiate heterochromatin formation.

In this study, a TetR/tetO system was used to tether Clr4 H3K9 HMTase at the *ura4* locus. An array containing four *tetO* sites (*4xtetO*), 30 bp upstream of an *ade6*$^{+}$ reporter gene was homologously recombined into the *ura4* locus on chromosome III (*ura4:4xtetO-ade6*$^{+}$). The construct expressing the TetR^{off}-2xFLAG-Clr4-cdd fusion protein (abbreviated TetR-Clr4*), was integrated at the *leu1* locus and regulated by the thiamine repressible *nmt81* promoter (Figure 3.1). The tetracycline repressor (TetR^{off}) binds its target *tetO* sequence with high affinity in the absence of tetracycline. Addition of tetracycline changes the conformation of TetR^{off} resulting in its dissociation from *tetO*. Consequently, this tethering system allows the inducible removal of TetR-Clr4* from the *ura4:4xtetO-ade6*$^{+}$ tethering site upon tetracycline addition and subsequent analysis to determine if heterochromatin is maintained at this ectopic locus following TetR-Clr4* release. Clr4 is known to be recruited to the chromatin through its interaction with H3K9me2/me3 via its chromodomain (Zhang et al., 2008). It has previously been shown that the removal of the chromodomain from Clr4 allowed more efficient silencing with tethered Clr4 (Clr4-GBD) at an ectopic locus. Deletion of the chromodomain appears to prevent extensive recruitment of Clr4-GBD to other H3K9me-rich locations in the genome (Kagansky et al., 2009). Taking this into account, the TetR-Clr4* (TetR-2xflag-Clr4-cdd*)* fusion protein used in this study was constructed so as not to contain the Clr4 chromodomain (Figure 3.1). In addition to TetR-Clr4*, all strains utilised also express wild-type Clr4 from its endogenous gene. Thus this wild-type Clr4 can potentially bind H3K9me at the ectopic site via its chromodomain and allow the methylation of new histone H3 in neighbouring nucleosomes following replication.

3.1 TetR-Clr4* induces gene repression over a broad domain surrounding the tethering site

Heterochromatin regions such as centromeres, telomeres and the mating type locus are transcriptionally repressed through most of the cell cycle. The insertion of a reporter gene within these heterochromatin domains leads to silencing of the reporter gene (Allshire et al., 1994; Nimmo et al., 1994; Thon et al., 1994).

Expression of the *ura4:4xtetO-ade6*$^{+}$ reporter gene was assessed in cells where TetR-Clr4* was bound to the tethering site. Cells were grown for many cell divisions (>20) to allow sufficient time for TetR-Clr4* to establish a heterochromatin domain over the

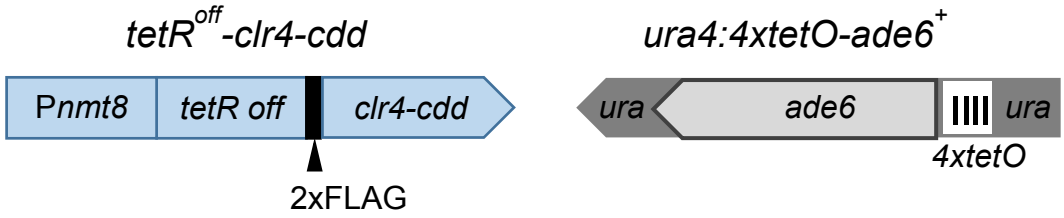


Figure 3.1: Diagram of constructs used to tether Clr4 H3K9 methyltransferase activity upstream of the *ade6*$^{+}$ reporter gene. tetR^{off} -2xflag-clr4-cdd (tetR-clr4*) integrated at *leu1*$^{+}$ (left panel) and the *4xtetO-ade6*$^{+}$ cassette integrated at the *ura4* locus (right panel)

ura4:4xtetO-ade6^{+} locus. On indicator plates containing limiting adenine (1/10th ade), *ade6*$^{+}$ cells form white colonies whereas *ade6*$^{-}$ cells accumulate a metabolic intermediate that results in red colonies. Cells containing the *ura4:4xtetO-ade6*$^{+}$ cassette were first tested in this colony colour assay. Cells containing TetR-Clr4* bound to the tethering site formed red colonies, indicating that the *4xtetO-ade6*$^{+}$ reporter genes was repressed (Figure 3.2.B). Silencing of the *ura4:4xtetO-ade6*$^{+}$ reporter was confirmed by measuring transcript levels by qRTPCR. RNA levels of the *ade6*$^{+}$ gene were found to be reduced by more than 75% (primer pairs +1.0 kb from the tethering site) when TetR-Clr4* is bound to the tethering site. Transcript levels from genes at the vicinity of the *ura4:4xtetO-ade6*$^{+}$ (up to 5 kb from the tethering site for *mug135*$^{+}$) were also reduced 3 to 5 fold depending on the gene (Figure 3.2.C). Chromatin immunoprecipitation (ChIP) was performed to assess RNA polymerase II (RNAPII) levels at *ade6*$^{+}$ and at the disrupted *ura4* gene. Consistent with transcriptional silencing, RNAPII association with the *ade6*$^{+}$ gene was decreased when TetR-Clr4* was bound (Figure 3.2.D). Normally, the H3K4me3 post-translational modification is enriched at active promoters and H3K36me3 accumulates over gene bodies as a result of transcriptional elongation. ChIP analysis for H3K4me3 and H3K36me3 showed that when TetR-Clr4* was bound to *4xtetO-ade6*$^{+}$ the levels of both modifications was reduced 2-3 fold in cells containing TetR-Clr4* (Figure 3.3). These analyses demonstrate that the tethering of TetR-Clr4* to the *4xtetO-ade6*$^{+}$ locus leads to a significant reduction of transcriptional activity at the *ade6*$^{+}$ reporter gene and at genes at the vicinity.

3.2 TetR-Clr4* promotes H3K9 methylation over a 10 kb region surrounding the tethering site.

Heterochromatin can spread along the chromatin fibre using H3K9 methylation to recruit the chromodomain proteins Swi6, Chp1, Chp2 and Clr4 itself, and together with HDACs, promote H3K9 methylation of adjacent nucleosomes. To assess the extent to which the heterochromatin domain extends around the *4xtetO-ade6*$^{+}$ tethering site, anti-H3K9me2 ChIP was performed followed by qPCR analyses using primer pairs designed to detect specific sequences at increasing distances from the *4xtetO*/TetR-Clr4* tethering site. H3K9me2 enrichment was detected at 1, 3, 4, 5 and 12 kb to the left, and at 0.5, 1, 5, and 10 kb to the right, of the tethering site (Figure 3.4). In presence of TetR-Clr4* bound to *4xtetO-ade6*$^{+}$, an enrichment of H3K9me2,

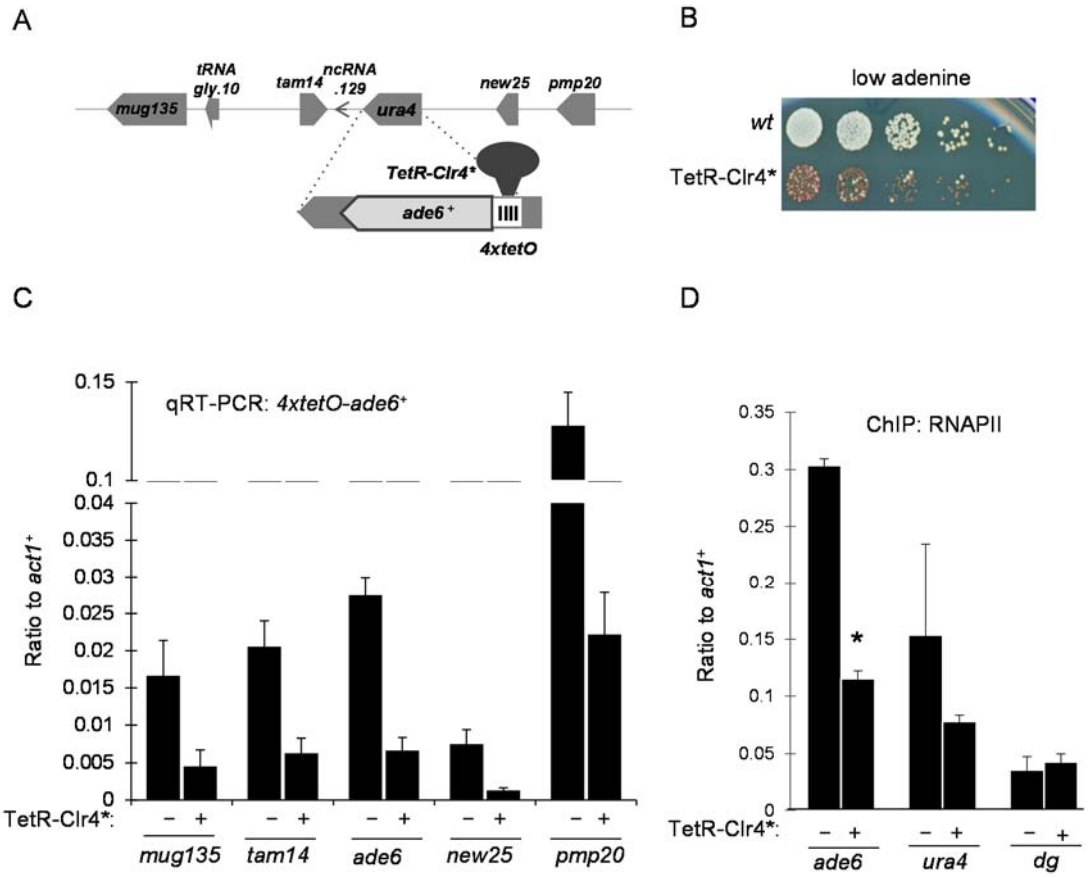


Figure 3.2: Tethering Clr4 H3K9 methyltransferase activity at the *ura4* locus causes silencing of the nearby genes.

(A) Positions of *4xtetO*, tethered TetR-Clr4* beside *ade6*$^+$ at *ura4*, Dumbbells indicate primer pairs.
(B) Colony colour assay to assess silencing of *4xtetO-ade6*$^+$. In the presence of TetR-Clr4*, *ade6*$^+$ is silenced causing colonies to be red on medium containing low adenine. In the absence of TetR-Clr4*, *ade6*$^+$ is expressed and the colonies are white.
(C) qRT-PCR analysis of transcript levels of genes at the vicinity of the tethering site; relative to *act1*$^+$, compared to levels in cells lacking tethered Clr4 (-). Data are means ± SD (*n* = 3 biological replicates).
(D) ChIP analysis of RNAPII levels associated with *ura4:4teto-ade6*$^+$ in presence or absence of TetR-Clr4*. RNAPII levels were assessed in *ade6*$^+$ reporter gene, in 5' of *ura4* and at centromeric repeats (*dg)* that are known to have low RNAPII occupancy. Data are means ± SD (*n* = 3 biological replicates) (t-test *P<0.05).*

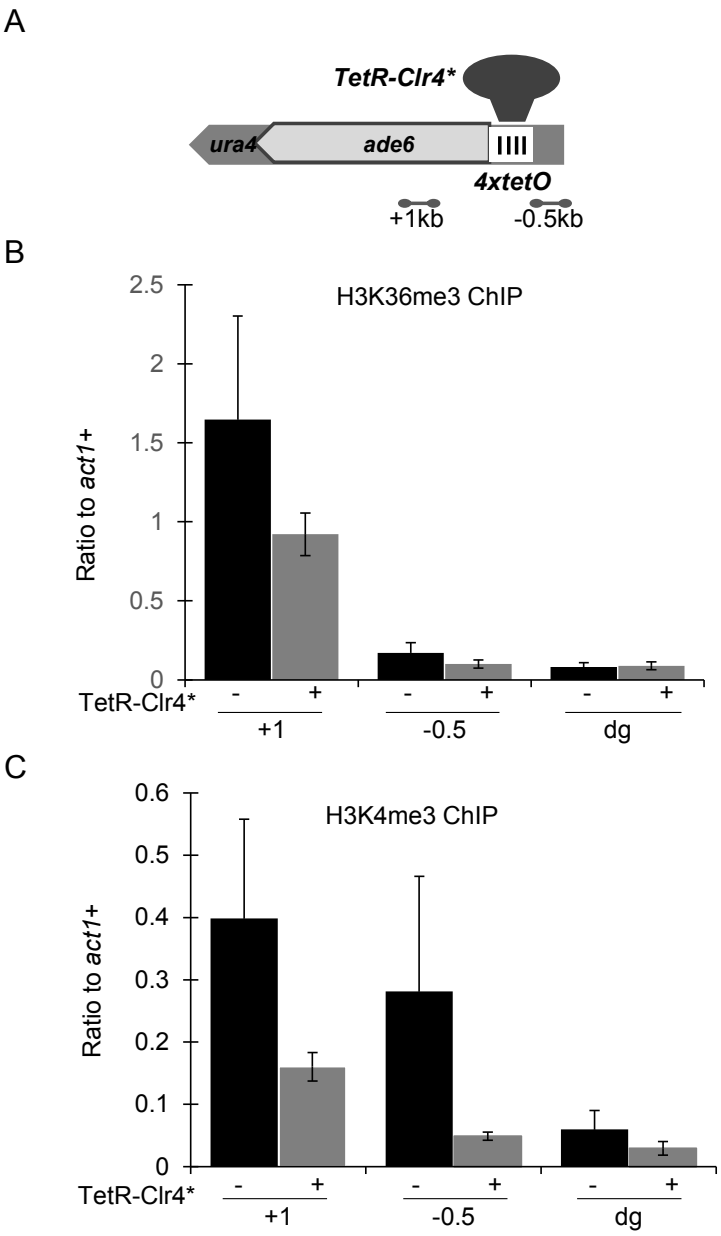
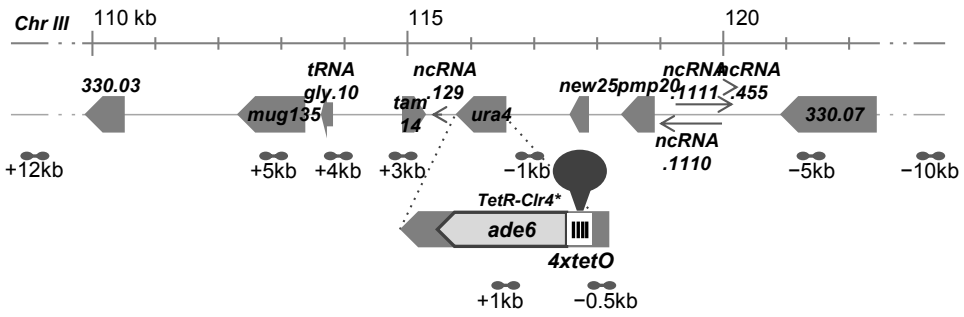


Figure 3.3: Tethering of Clr4 H3K9 methyltransferase activity results in a decrease of histone post-translational modifications associated with active transcription.

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*.
(B) ChIP analysis of H3K36me3 level at 0,5kb, 1kb from 4x*tetO* and at centromeric repeats *(dg)* in cells lacking TetR-Clr4* (-) and in cells containing TetR-Clr4* bound to the tethering site (+); relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)
(C) ChIP analysis of H3K4me3 level at 0,5kb, 1kb from 4x*tetO* and at centromeric repeats *(dg)* in cells lacking TetR-Clr4* (-) and in cells containing TetR-Clr4* bound to the tethering site (+), relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)
Low levels of H3K36me3 and H3K4me3 are found at centromeric heterochromatin repeats (*dg*) that are transcriptionally inactive through the majority of the cell cycle.

A



B

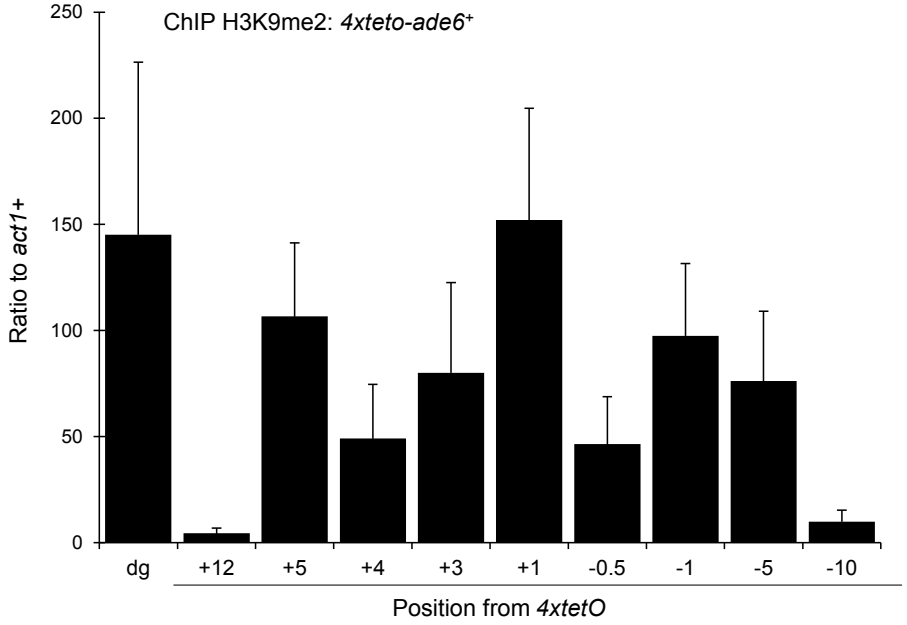


Figure 3.4: Tethering of Clr4 at the *ura4* locus promotes assembly of H3K9me2 several kilobases away from the tethering site.

(A) Positions of *4xtetO*, tethered TetR-Clr4* beside *ade6*$^+$ at *ura4*, and surrounding *S. pombe* chromosome III genes. Dumbbells indicate primer pairs.
(B) ChIP analysis of H3K9me2 *at ura4:4teto-ade6*$^+$ in the presence of bound TetR-Clr4*; data are means ± SD (*n* = 3 biological replicates); relative to *act1*$^+$. H3K9me2 levels at the tethering site are compared to those at centromeric repeats (*dg*).

47- to 150-fold greater than the highly expressed control *act1*$^{+}$ gene was detected at all sites assessed up to 5 kb away from the tethering site. Enrichment of H3K9me2 was only 4-fold and 10-fold over the *act1*$^{+}$ control 12 kb and 10 kb to the left and right of *4xtetO-ade6*$^{+}$, respectively. This indicates that the tethering of TetR-Clr4* at the *4xtetO-ade6*$^{+}$ locus leads to the formation of a domain of H3K9 methylated chromatin that spreads over 5-10 kb on both sides of the *4xtetO* tethering site. The levels of H3K9me2 detected close to the tethering site are similar to those of the endogenous centromeric repeats *dg* where RNAi-directed heterochromatin is normally concentrated (Figure 3.4). These analyses show that the tethering of TetR-Clr4* at the *ura4* locus creates a domain of ectopic H3K9me-dependent heterochromatin similar in size to the one previously described with the GBD-Clr4 tethering system (Kagansky et al., 2009).

3.3 RNAi is not required to form heterochromatin when TetR-Clr4* is tethered at the *4xtetO-ade6*$^{+}$ locus

At centromeric repeats, RNAi is essential for heterochromatin establishment but is also important for heterochromatin maintenance (Buscaino et al., 2013; Motamedi et al., 2004; Partridge et al., 2007; Reyes-Turcu et al., 2011
; Sadaie et al., 2004; Verdel et al., 2004; Volpe et al., 2002). During every cell cycle, convergent centromeric repeats are transcribed in S phase by RNAPII and form dsRNAs that are processed by the protein Dcr1 into siRNAs (Chen et al., 2008; Kloc et al., 2008). siRNAs target the RNAi effector complex containing Ago1 (RITS) to centromeric repeats and promote recruitment of Clr4 containing complex CLRC and replenishment of H3K9 methylation (Motamedi et al., 2004; Verdel et al., 2004; Volpe et al., 2002). The absence of a functional RNAi pathway results in loss of siRNAs, a significant decrease of H3K9 methylation and heterochromatin from centromere repeats and a large increase in their transcription. The importance of RNAi for maintaining H3K9 methylation and silencing was therefore investigated when TetR-Clr4* was bound at the *4xtetO-ade6*$^{+}$ locus (Figure 3.5). ChIP analysis revealed that significant amounts of the chromodomain protein Chp1, a component of the RITS complex, were detected at the ectopically silenced locus (Figure 3.5.B). Since Chp1 binds H3K9me via its chromodomain (Petrie et al., 2005), it is possible that it recruits other components of the RITS RNAi effector complex to the tethering site that might trigger the production of homologous siRNAs that then mediate the maintenance of

heterochromatin maintenance. To test this possibility, cells with the TetR-Clr4*-dependent heterochromatin domain formed over the *ura4:4xtetO-ade6*$^{+}$ locus were crossed with cells lacking the RNAi pathway due to deletion of the genes encoding either Dicer (*dcr1Δ*) or Argonaute (*ago1Δ*). The maintenance of heterochromatin over *4xtetO-ade6*$^{+}$ was then assessed in the *dcr1Δ* or *ago1Δ* relative to wild-type cells when TetR-Clr4* was tethered. H3K9me2 levels were assessed by ChIP in *wt* cells or cells lacking RNAi (*dcr1Δ* or *ago1Δ*) at 1 kb and 7 kb away from the tethering site (Figure 3.5.C). Levels of H3K9me2 were found to be not significantly different between *wt* and either of the RNAi mutants (Figure 3.5.C). Furthermore, both *dcr1*∆ and *ago1*∆ form red/*4xtetO-ade6*$^{+}$ colonies on plates containing limiting adenine, indicating that RNAi is not required for silencing of *ura4:4xtetO-ade6*$^{+}$ expression when TetR-Clr4* is bound (Figure 3.5.D). These results show that RNAi is not required for TetR-Clr4* mediated heterochromatin formation and silencing of *4xtetO-ade6*$^{+}$ which is consistent with previous observations using the GBD-Clr4* tethering at the *ura4* locus (Kagansky et al., 2009).

3.4 The expression of TetR-Clr4* affects heterochromatin integrity at centromeric repeats

Centromeric repeats retain significant levels of H3K9me2 in the absence of RNAi (Buscaino et al., 2013; Volpe et al., 2002). Strikingly, in the absence of RNAi (*dcr1Δ* or *ago1Δ*), in cells expressing TetR-Clr4* and carrying the *ura4:4xtetO-ade6*$^{+}$ reporter, H3K9me2 is completely lost from centromere repeats (*dg*; Figure 3.5.C). This finding indicates that the formation of this domain of ectopic heterochromatin by TetR-Clr4* affects the maintenance of residual H3K9me at centromeres. Consistent with this observation, levels of H3K9me2, and the chromodomain proteins Swi6 and Chp1 are decreased by approximately half over these centromere repeats when TetR-Clr4* is tethered at *ura4:4xtetO-ade6*$^{+}$ relative to wild-type cells that lack this ectopic heterochromatin (Figure 3.6.A-C).

It is possible that the TetR-Clr4* fusion protein, lacking the Clr4 chromodomain, competes with the full length endogenous Clr4 at centromeric repeats and affects heterochromatin maintenance and spreading that relies on Clr4 carrying a functional chromodomain (Zhang et al., 2008). To test this hypothesis, the presence of TetR-Clr4* at centromeric repeats was assessed by ChIP. ChIP analysis showed TetR-Clr4* to be significantly enriched close to the *tetO* array (0.5 kb) but that enrichment

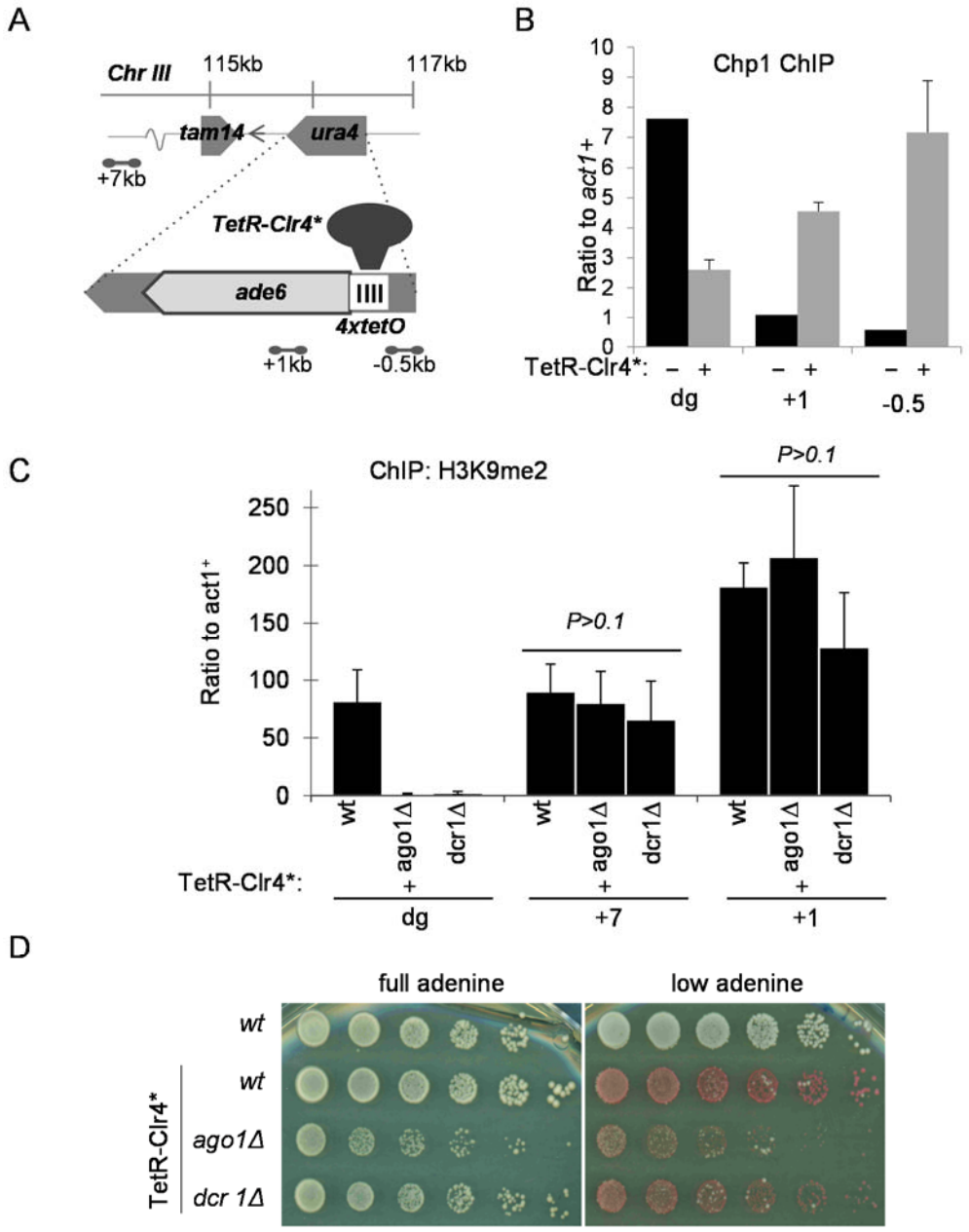


Figure 3.5: RNAi is not required for heterochromatin assembly at *ura4.*

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*
(B) ChIP analysis of Chp1 levels at *4xTetO-ade6*$^{+}$ and at centromeric repeats (*dg*) in presence or absence of TetR-Clr4* bound to *4xtetO*; relative to *act1*$^{+}$.
(C) ChIP analysis of H3K9me2 levels at the tethering site and at centromeric repeats (*dg*) in presence (wt) or absence of a functional RNAi machinery (*dcr1Δ, ago1 Δ)*; relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates).
(D) Assay for silencing of *4xtetO-ade6*$^{+}$. In presence of TetR-Clr4* in the cells, *ade6*$^{+}$ is silenced in *wt* as in *dcr1Δ* and *ago1 Δ* which causes red coloring of the cells on medium containing low amount of adenine (1/10th). In absence of TetR-Clr4*, *ade6*$^{+}$ is expressed and the cells are white.

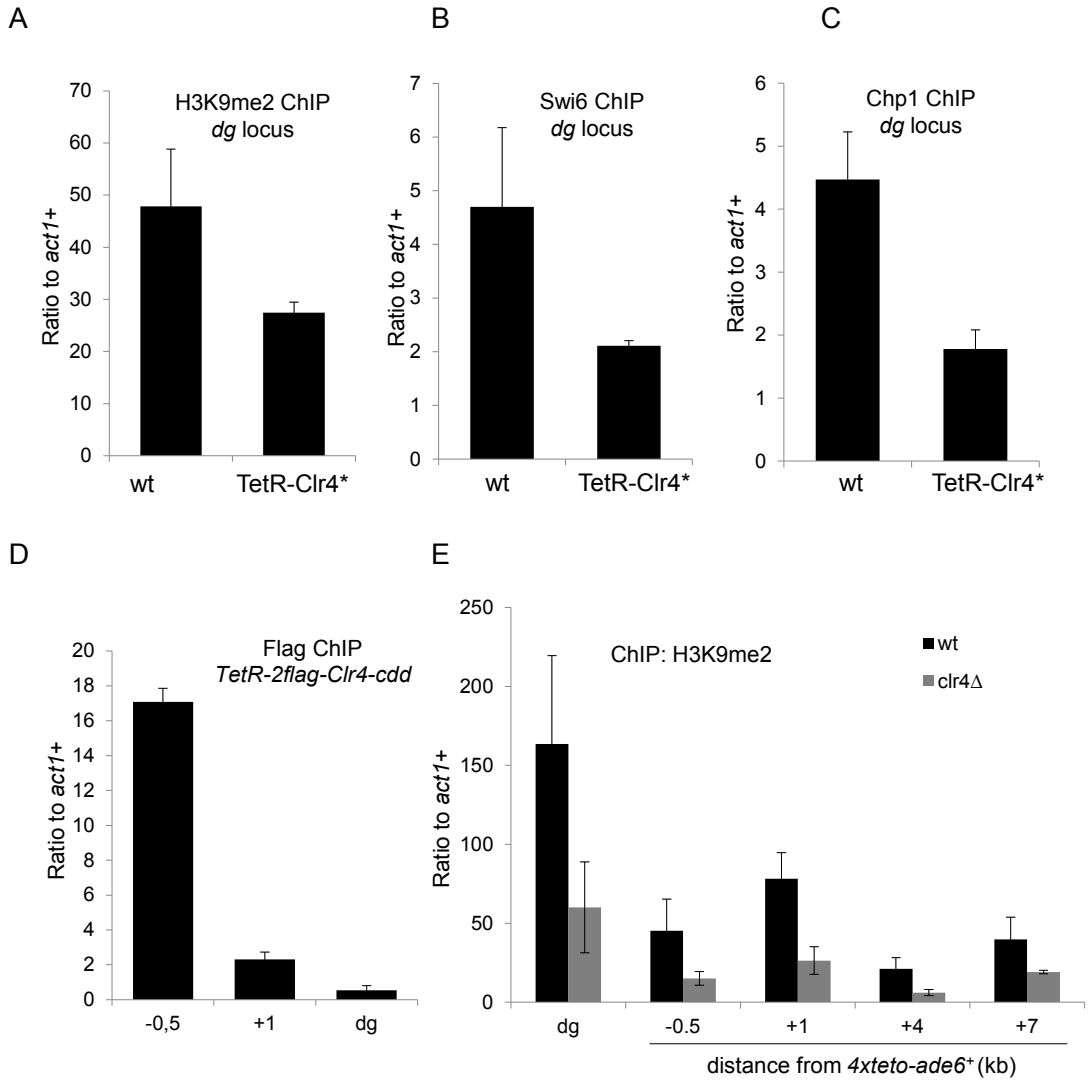


Figure 3.6: Expression of TetR-Clr4* affects moderately heterochromatin integrity at centromeres

(A-C) ChIP analysis of H3K9me2 (A), Swi6 (B) and Chp1 (C) levels at heterochromatic centromeric repeats (*dg*) in presence (TetR-Clr4*) or absence (wt) of TetR-Clr4* in the cells.
(D) ChIP analysis to assess FLAG-tagged TetR-Clr4* presence at centromeric repeats (*dg*) and around the tethering site, relative to *act1*$^{+}$. TetR-Clr4* is not detectable at centromeric repeats and is detected at the tethering site.
(E) ChIP analysis of H3K9me2 levels at heterochromatic centromere repeats (*dg*) and at the tethering site in presence (wt) or absence of endogenous *clr4*$^{+}$ (*clr4*Δ), relative to *act1*$^{+}$ when TetR-Clr4* is bound to *4xtetO*. H3K9me2 is present at heterochromatic centromeric repeats and up to 7kbs away from the tethering site in absence of endogenous *clr4*$^{+}$.
Data are means ± SD (*n* = 3 biological replicates)

was much lower just 1 kb from the tethering site, however, TetR-Clr4* was not detected over centromeric repeats. This finding suggests that the stable recruitment of TetR-Clr4* to chromatin is mainly restricted to the *tetO* array (Figure 3.6.D) However, unlike normal *clr4Δ* cells (not shown) some H3K9me2 was clearly detected over endogenous centromere repeats (*dg*) in cells expressing TetR-Clr4* but lacking endogenous *clr4*$^{+}$ (Figure 3.6.E). This indicates that although TetR-Clr4* is not detectable at centromeres by ChIP and despite its lack of chromodomain, TetR-Clr4* at least transiently visits centromeric repeats where it can direct significant levels of H3K9 methylation. Although TetR-Clr4* lacks the Clr4 chromodomain it is possible that it can still be recruited to endogenous centromere repeats via an interaction with heterochromatin components such as the chromodomain protein Swi6 that interacts directly with Clr4 or Stc1 that is essential for the RNAi-dependent recruitment of the Clr4 containing complex CLRC to centromeric repeats (Bayne et al., 2010; Haldar et al., 2011). Since Clr4 chromodomain is important for heterochromatin spreading, it is possible that TetR-Clr4* lacking Clr4 chromodomain might also act as dominant negative by preventing endogenous Clr4 from associating with chromatin and promote H3K9 methylation-dependent heterochromatin spreading along the chromatin fibre (Zhang et al., 2008).

DISCUSSION

The above analyses show that the tethering of Clr4 H3K9 methyltransferase activity to an ectopic locus using the TetR-Clr4*/*4xtetO* system is sufficient to establish a domain of heterochromatin domain over a substantial chromosomal region. This finding is consistent with results reported in other studies using similar approaches to tether Clr4 (Kagansky et al., 2009; Ragunathan et al., 2015). The resulting ectopic heterochromatin domain shares several characteristics with those described for endogenous heterochromatin:

- it is enriched in methylated H3K9 chromatin
- chromodomain proteins such as Chp1 are recruited
- genes embedded within this heterochromatin are transcriptionally silenced.

The ectopic heterochromatin domain created by tethered TetR-Clr4* spreads several kilobase pairs outwards from the tethering site, however, in the absence of

endogenous *clr4*$^{+}$ in the cells the levels of H3K9me2 around *4xtetO* are reduced suggesting that endogenous Clr4 with its chromodomain intact might also be recruited to the tethering site and allow H3K9 methylation and associated silencing to spread away from the tethering site.

These and previous analyses indicate that two elements influence the levels of H3K9 methylation mediated by an ectopic silencer tethering site: (i) the distance from the nucleation site and (ii) the nature of the underlying DNA sequence (Kagansky et al., 2009; Wheeler et al., 2009). The enrichment profile indicates that H3K9me2 is distributed in an almost symmetrical pattern around the tethering site and tends to decrease with distance from the nucleation site. The heterochromatin domains formed on the outer centromere repeats and over the mat2-mat3 region of the mating type locus are delimited by boundary elements that prevent heterochromatin expanding over neighbouring euchromatic regions (Noma et al., 2001; Scott et al., 2007). Because such boundary elements are absent in the vicinity of the *4xtetO* tethering site, it is likely that the transition from heterochromatin to euchromatin is variable and results from competition between pro-silencing and pro-expression factors so that a broad domain may be formed where both repressive and active factors and associated histone PTMs coexist (Kimura and Horikoshi, 2004). This could be tested by performing ChIP to detect transcription associated histone acetylation and methylation across the domain. Moreover, the insertion of known boundary elements on either or both sides of the tethering site would test if such elements can limit the degree of spreading and create a discrete edges to the ectopic heterochromatin domain.

H3K9me2 enrichment around the tethering site also depends on the underlying DNA sequence. Some DNA sequences are more prone to heterochromatin assembly than others (Kagansky et al., 2009; Wheeler et al., 2009). For instance the level of H3K9me2 enrichment on the remaining promoter region of *ura4*, that is 0.5 kb away from *4xtetO*, is low compared to its enrichment on the *mug135*$^{+}$ gene, 5 kb from *4xtetO* (Figure 3.4.B). In a previous study, when a 1.6 kb fragment of centromeric outer repeat DNA (L5 fragment) was inserted at different genomic locations it was found that the extent of the heterochromatin domain formed depended on the specific insertion site (Wheeler et al., 2009). Insertion of a transcriptionally inert DNA adjacent to L5 allowed heterochromatin to spread further away from the nucleation site. In

contrast, the presence of highly transcribed genes appeared to prevent spreading and thus, such transcription units may act as barriers to heterochromatin. Thus the size of the heterochromatin domain formed at ectopic locations as a result of spreading from the initiating site is dependent on the nature of the cis-acting sequences and chromatin in its path.

It should also be noted that essential genes are present in the vicinity of the *ura4* gene. The *mug135*$^{+}$ and *alg11*$^{+}$ genes residing 2 and 7.5 kb, respectively, from the *ura4* insertion site are both essential for cell viability. The formation of a domain of heterochromatin around the *ura4* locus might therefore be detrimental for cell survival and the long-term maintenance of heterochromatin over the entire domain might be selected against. In agreement with this possibility, the growth of *4xtetO-ade6*$^{+}$ cells was found to be faster in the absence of Tetr-Clr4* bound to the *4xtetO* sites (data not shown).

The tethering of TetR-Clr4* to *4xtetO-ade6*$^{+}$ creates a broad heterochromatin domain at the euchromatic locus that is enriched in H3K9me2 and leads to the silencing of the genes in the vicinity. It was investigated in the following chapter whether this ectopic heterochromatin is able to self-propagate upon removal of TetR-Clr4* from the tethering site.

CHAPTER 4: Ectopic histone H3K9 methylated heterochromatin formed at a euchromatic locus is not heritable

INTRODUCTION

In fission yeast, an active RNAi pathway is essential for the establishment and maintenance of full heterochromatin over the centromeric outer repeats (*dg/dh*). Every cell cycle, a burst of repeat transcription and siRNA production by the RNAi machinery is required to reinforce H3K9 methylation and heterochromatin on these centromere repeat elements following replication (Chen et al., 2008; Kloc et al., 2008). However, the loss of RNAi from wild-type cells where heterochromatin is fully assembled on centromere repeats does not result in the complete loss of H3K9me-dependent heterochromatin, indicating that some characteristics of heterochromatin can be maintained independently of RNAi (Buscaino et al., 2013; Partridge et al., 2007; Sadaie et al., 2004). These and other analyses lead to the proposal that following establishment, H3K9me-dependent heterochromatin domains in fission yeast might be transmitted epigenetically through cell division (self-propagation) with H3K9 methylation acting as the epigenetic mark which, following replication, allows replenishment of the heterochromatin domain through the direct binding of the chromodomain of Clr4 to H3K9me (Bannister et al., 2001; Zhang et al., 2008 {Bannister, 2001 #160). Nevertheless, it cannot be ruled out that other nucleation signals acting independently from RNAi and Atf1/Pcr1 at the mating type locus, are involved in maintaining H3K9 methylation at centromeres and the mating type loci.

Since the domain of synthetic H3K9me-dependent heterochromatin over the *ura4:4xtetO-ade6*$^{+}$ locus is assembled independently of the underlying DNA sequence and relies entirely on TetR-Clr4* tethering, it provides an excellent system with which to test if H3K9 methylation associated silent chromatin can be copied from parental nucleosomes during replication so that it is maintained in daughter cells. In this chapter, I investigate whether the addition of tetracycline allows release of TetR-Clr4* from the *4xtetO* tethering sites and maintenance of heterochromatin at the ectopic locus.

4.1 H3K9 methylation is rapidly lost upon removal of TetR-Clr4* from the tethering site

To investigate whether the large heterochromatin domain encompassing the *ura4:4xtetO-ade6*$^{+}$ tethering site can be epigenetically transmitted in progeny cells following release of TetR-Clr4* from its binding sites, time course experiments were performed. The tetracycline derivative anhydrotetracycline (AHT) has been shown to be a more effective inducer of *tetO* based promoters in fission yeast (Erler et al., 2006). The addition of AHT is expected to induce a conformational change in TetR-Clr4* that results in its release from the *tetO* sequence. To ensure that TetR-Clr4* was efficiently lost from the tethering site, transcription of the *nmt81-tetR-clr4** construct that produces the TetR-Clr4* fusion protein was also repressed by the addition of thiamine that negatively regulates the expression from the *nmt* promoter.

A time course experiment with time points taken over the period of an hour was performed to assess the initial presence of TetR-Clr4* (TetR-2xflag-Clr4-cdd) at *ura4:4xtetO-ade6*$^{+}$ and its release following AHT addition. Anti-FLAG antibodies were used to immunoprecipitate TetR-Clr4* in ChIP experiments. Subsequent qPCR showed that TetR-Clr4* was not detectable at the tethering site 5 minutes after AHT and thiamine addition. Thus, this regimen results in the rapid and complete release of TetR-Clr4* from *ura4:4xtetO-ade6*$^{+}$ (Figure 4.1.A: performed in collaboration with Sandra Catania). To check if growth in the presence of AHT and thiamine affects endogenous heterochromatin integrity, H3K9me2 levels were assessed at centromere outer repeat elements (*dg*) (Figure 4.1.B). No change in the levels of H3K9me2 associated with centromere repeats was detected when cells were grown in the presence or absence of AHT and thiamine. Thus this treatment does not interfere with endogenous H3K9me-dependent heterochromatin integrity (Figure 4.1.B).

Next, ChIP was performed to determine the dynamics of H3K9 methylation over the *ura4:4xtetO-ade6*$^{+}$ synthetic heterochromatin domain following release of TetR-Clr4*. Again, anti-FLAG CHIP confirmed that TetR-Clr4* was stably released from the tethering site following AHT/thiamine addition (Figure 4.2.C). Upon TetR-Clr4* release, qPCR showed that at all nine locations tested, H3K9me2 levels rapidly declined. At T0 the highest levels of H3K9 methylation were detected within 1 kb of the *4xtetO* tethering site but this signal was reduced by more than 90% within six hours after AHT/Thiamine addition (Figure 4.2.A-B). For fission yeast in these conditions of log phase growth, six hours represent between 2 and 3 cell divisions for the entire population. A higher resolution time course was also performed to assess H3K9me2 levels at 20 minute intervals following TetR-Clr4* release by AHT/thiamine addition (Figure 4.2.D). In this time course, although H3K9me2 loss was not immediate, a slight reduction in H3K9me levels was evident 60 minutes after AHT treatment, declining to 25% of T0 levels by 200 minutes.

A third time course experiment was performed to simultaneously assess H3K9me2, TetR-Clr4* and H3 levels on *4xtetO-ade6*$^{+}$ after addition of AHT plus thiamine (Figure 4.3). The resulting ChIP analyses show that although H3K9me2 levels -0.5 and +1 kb from the TetR-Clr4* tethering site rapidly decline, H3 levels remain relatively steady over this period. This time course experiment demonstrates that the loss of H3K9me2 from the *ura4:4xtetO-ade6*$^{+}$ locus within 10 hours does not result from loss of histone H3 at the locations tested. However, 24 and 48h hours after AHT addition a slight drop in histone H3 levels was seen +1 kb from the TetR-Clr4* tethering site (Figure 4.3). Heterochromatin domains are known to have higher nucleosome occupancy and less histone nucleosome turnover than expressed genes (Aygun et al., 2013; Garcia et al.). Thus the loss of H3 detected is perhaps caused by the disassembly of heterochromatin and the resulting re-expression from promoters within what was a synthetic heterochromatin domains.

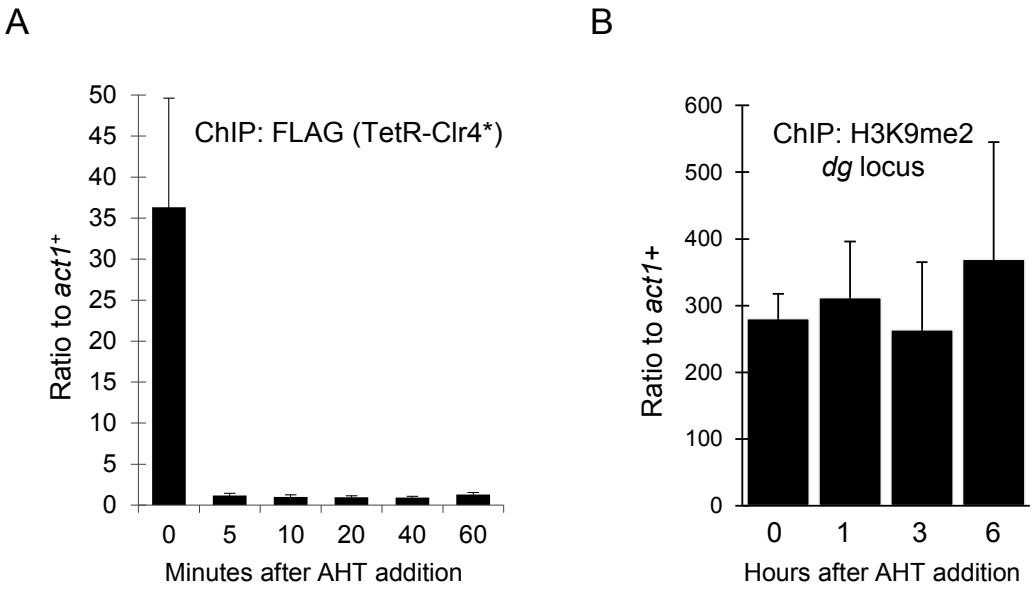


Figure 4.1: Addition of AHT and thiamine to the medium results in the rapid loss of TetR-Clr4* from the tethering site and does not affect heterochromatin at centromeres.

(**A**) ChIP time course to assess FLAG-tagged TetR-Clr4* release from *ura4:4xtetO-ade6*$^{+}$, following AHT and thiamine addition; relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates).
(**B**) ChIP time course to assess H3K9me2 levels at endogenous centromeric repeats (*dg)* following AHT and thiamine addition; relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates). H3K9me2 levels at *dg* did not change during the time course following AHT addition (t-test *P*≥*0.38*)

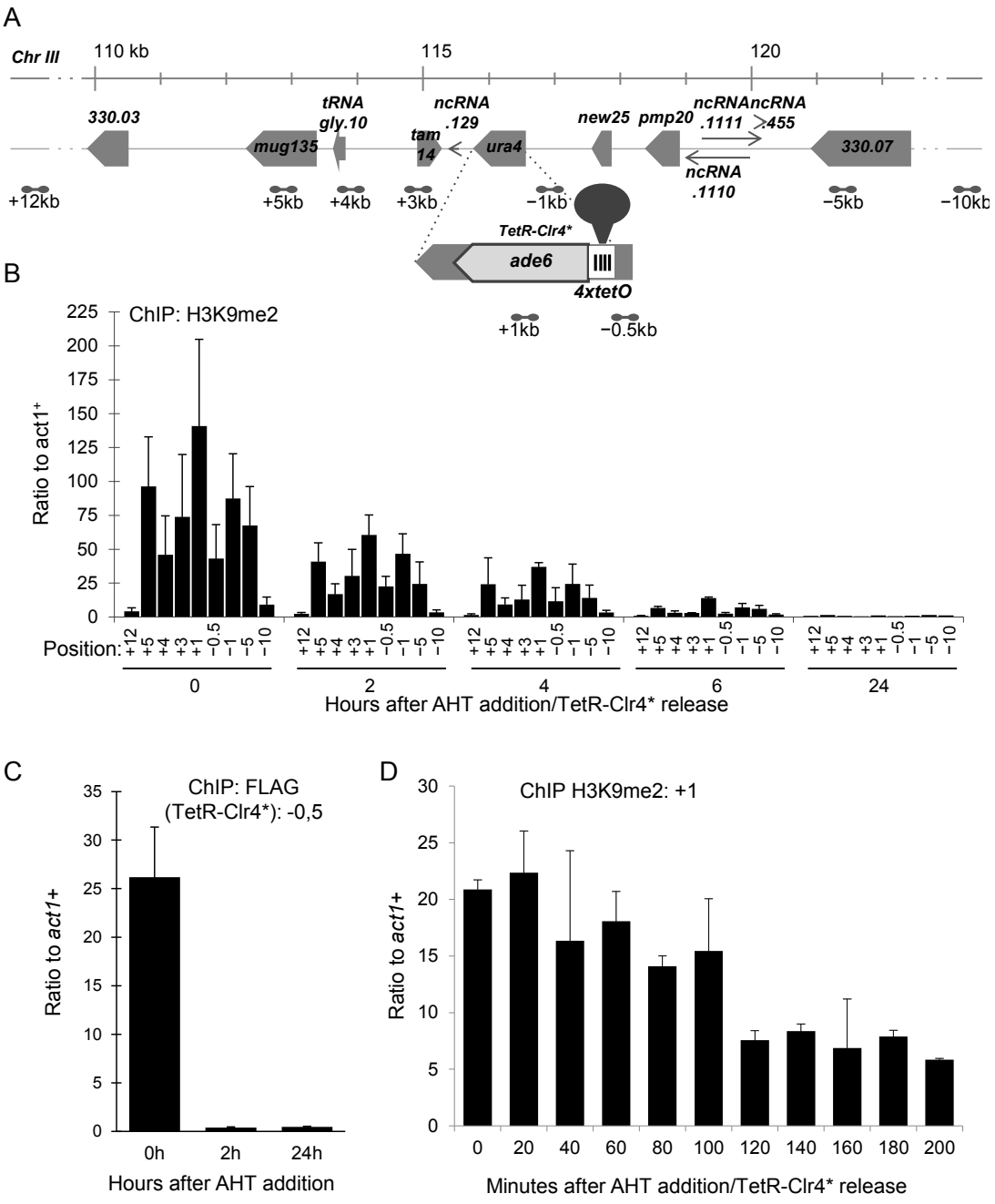


Figure 4.2: H3K9me2 is rapidly lost from 4xtetO-ade6+ upon TetR-Clr4* release

(A) Positions of 4xtetO, tethered TetR-Clr4* beside ade6+ at ura4, and surrounding S. pombe chromosome III genes. Dumbbells indicate primer pairs.
(B and C) ChIP time course of H3K9me2 (B) and FLAG-TetR-Clr4* (C) levels around the tethering site following AHT addition using indicated primers; relative to *act1*$^{+}$. Data are mean ±SD (*n=3* biological replicates).
(D) ChIP time course of H3K9me2 levels on *4xtetO-ade6*$^{+}$ following AHT addition; relative to *act1*$^{+}$. H3K9me2 levels were assessed every 20min following AHT treatment. Data are means ± SD (*n* = 3 biological replicates)

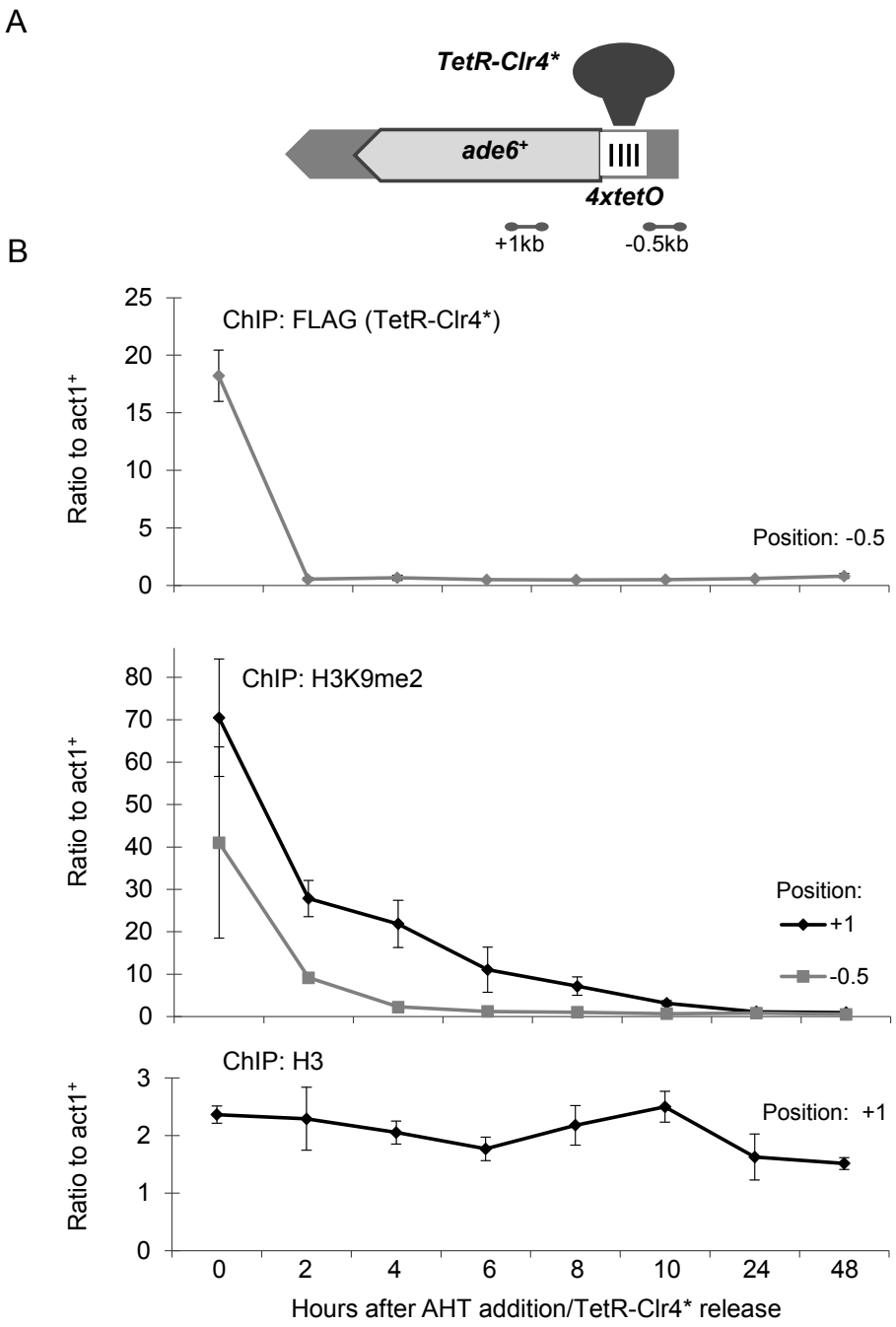


Figure 4.3: H3K9 methylation is rapidly lost upon release of tethered TetR-Clr4* methyltransferase from *4xtetO-ade6*$^+$

(A) 4xtetO-ade6$^+$ test locus with the position of primer pairs relative to the tethering site.
(B) ChIP time course to assess FLAG-tagged TetR-Clr4* (top panel), H3K9me2 (middle panel) and total H3 levels (bottom panel) on *4xtetO-ade6*$^+$ following AHT addition/TetR-Clr4* release, using indicated primer pairs (n=3); relative to *act1*$^+$. Independent experiment from Figure 4.2.A. Data are means ± SD (*n* = 3 biological replicates).

4.2 H3K9me2, H3K9me3 and their reader, Swi6, are rapidly lost form the synthetic heterochromatin domain upon release of TetR-Clr4*

In fission yeast, both H3K9me2 and H3K9me3 have been detected in heterochromatin at the mating-type locus and centromere (Al-Sady et al., 2013; Yamada et al., 2005). The di- and tri- methylated states of lysine 9 in histone H3, however, have different properties and may recruit specific chromodomain proteins with different efficiency. Indeed the Clr4 chromodomain protein displays a higher affinity for H3K9me3 than H3K9me2 while Swi6 has a similar affinity for both H3K9me2 and H3K9me3 (Al-Sady et al., 2013). To determine whether the loss of H3K9me2 correlates with a loss of H3K9me3 and whether these two modifications observe the same dynamics following TetR-Clr4* release, H3K9me2 and H3K9me3 ChIP were performed 0, 2.5 and 5 hours following AHT/thiamine addition and both H3K9me2 and H3K9me3 levels monitored -0.5, +1.0, +4.0 and +5.0 kb from the *4xtetO* tethering site (Figure 4.4). At T0, both H3K9me2 and H3K9me3 showed a similar pattern of enrichment around the tethering site except that close to the tethering site (-0.5 kb) where H3K9me3 appeared to be proportionally higher than H3K9me2 prior to the release of TetR-Clr4* from *4xtetO-ade6*$^{+}$. One explanation for this is that constitutively bound Clr4 activity at the *4xtetO* sites drives lysine 9 on nearby nucleosomes into the tri-methylated state and that this is less likely with increasing distance from the tethering site. At T2.5 and T5 hours, after AHT addition both H3K9me2 and H3K9me3 rapidly disappeared from all positions monitored around the tethering site with similar dynamics (Figure 4.4.B). After 5 hours more than 80% of the original signals for H3K9me2 and H3K9me3 has been lost. The chromodomain of Swi6 binds both H3K9me2 and H3K9me3 Consistent with loss of methylated H3K9, anti-Swi6 ChIP revealed that Swi6 was also lost from the *ura4:4xtetO-ade6*$^{+}$ locus after release of TetR-Clr4* (Figure 4.5; Swi6 ChIP performed by Alison Pidoux).

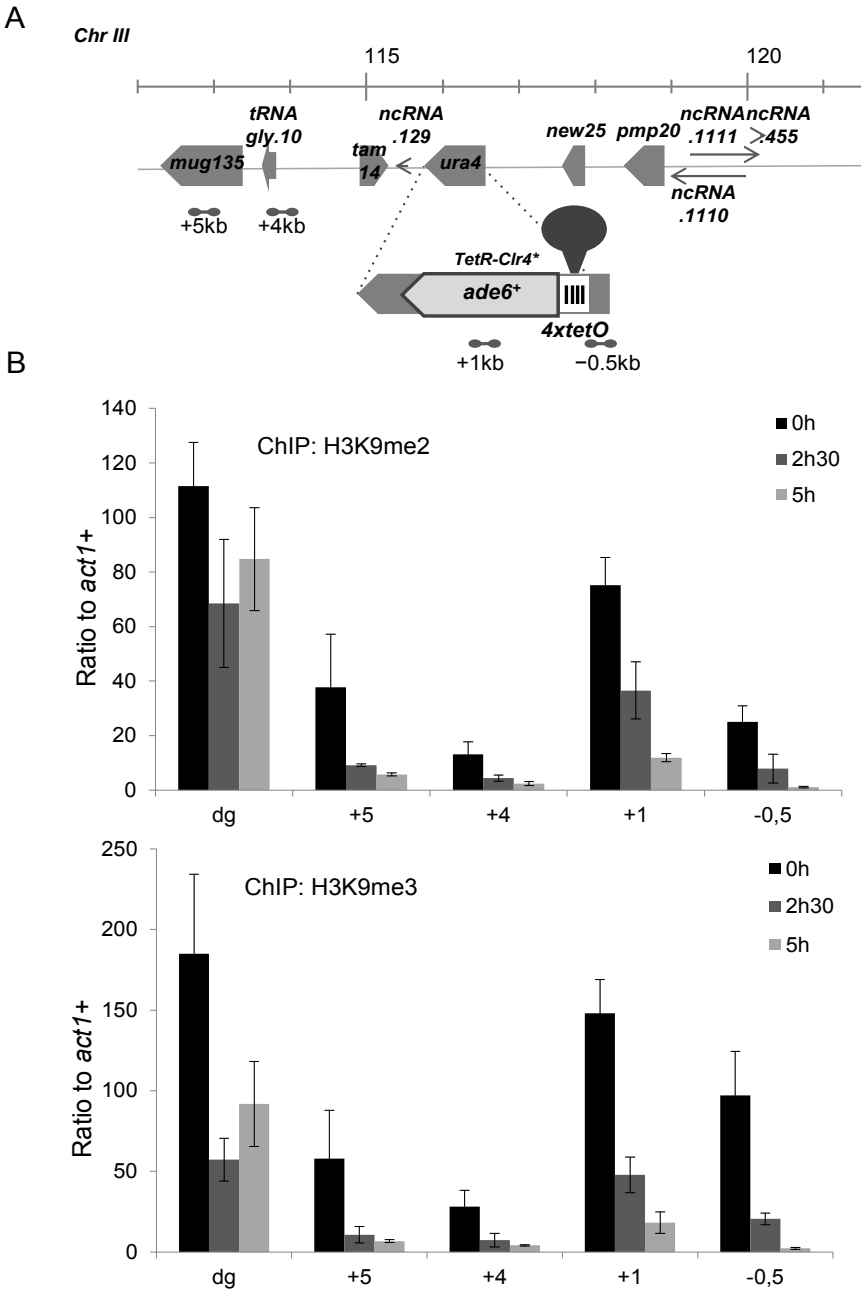
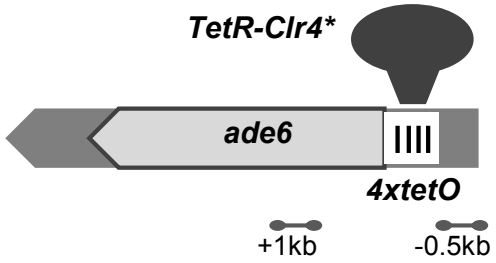


Figure 4.4: Loss of H3K9me2 and H3K9me3 from *4xtetO-ade6*$^{+}$ following *TetR-Clr4*release.*

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*.

(B) ChIP analysis of H3K9me2 (top panel) and H3K9me3 (bottom panel) levels at the tethering site before (0h), or 2h30 and 5h following thiamine and AHT treatment. H3K9me levels were assessed at -0.5kb, 1kb, 4kb and 5kb from the tethering site and compared to H3K9m2/me3 levels at centromeric repeats (*dg*); relative to *act1*$^{+}$. H3K9me2 and H3K9me3 display a similar pattern of enrichment around the tethering site and are both very rapidly lost from the *ura4* locus upon TetR-Clr4* release. Data are means ± SD (*n* = 3 biological replicates)

A



B

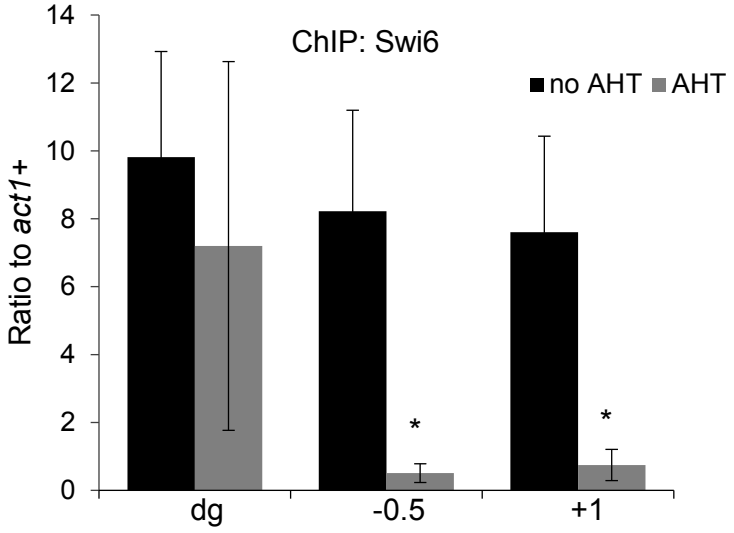


Figure 4.5: Loss of Swi6 chromodomain protein from 4xtetO-ade6+ following TetR-Clr4* release.
(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*.
(B) ChIP analysis of Swi6 enrichment at the tethering site in strains containing TetR-Clr4* (no AHT) or not (AHT) at the tethering site; relative to *act1*$^{+}$. Swi6 levels at the tethering site are comparable to Swi6 levels at centromeric repeats (*dg*) in the presence of TetR-Clr4* bound to *4xtetO-ade6*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)

4.3 Rapid loss of silencing at *4xtetO-ade6*$^{+}$ upon TetR-Clr4* release.

To analyse in more detail the effect that release of TetR-Clr4* has on the expression of the *4xtetO-ade6*$^{+}$ reporter gene, *4xtetO-ade6*$^{+}$ silencing was assessed following AHT and thiamine addition. Cells were initially grown on plates lacking both thiamine and AHT (TetR-Clr4* "ON") to allow full expression of TetR-Clr4* and thus ensure robust synthetic heterochromatin assembly over the region. Subsequently these cells were plated on limited adenine (1/10th Ade) indicator plates with or without thiamine and AHT (10 µM). After growth for 2 to 3 days on plates containing AHT and thiamine, silencing of the *4xtetO-ade6*$^{+}$ reporter gene was completely lost as indicated by the formation of white colonies. In contrast the *4xtetO-ade6*$^{+}$ reporter gene was clearly repressed in plates lacking AHT and thiamine since the same cells form red colonies (Figure 4.6.B). This result demonstrates that silencing of *4xtetO-ade6*$^{+}$ can not be maintained when TetR-Clr4* is released. qRT-PCR experiments were also performed to assess the expression of the *4xtetO-ade6*$^{+}$ reporter gene in a time course experiment over a 24 hour period following TetR-Clr4* release. *ade6*$^{+}$ transcription was found to begin to increase by only 1.7 fold and 2.4 fold after 4 and 6 hours respectively, and reach a maximum of 4-fold only 24 hours after AHT addition (Figure 4.6.C). These data indicate that the loss of silencing from the *4xtetO-ade6*$^{+}$ gene occurs only gradually following the more rapid decline of H3K9me2 from the gene.

To determine how other histone post-translational modifications were affected by TetR-Clr4* release, ChIP was performed to assess the enrichment of two histone modifications normally associated with active transcription on the *4xtetO-ade6*$^{+}$ reporter gene in the same time course experiment that was used to monitor *4xtetO-ade6*$^{+}$ expression levels. ChIP was performed to monitor in parallel H3K9me2 (heterochromatin), H3K4me3 (transcription initiation from promoters) and H3K36me3 (transcription elongation) (Figure 4.7). Following TetR-Clr4* release, as expected H3K9me2 levels at – 0.5 and +0.1 kb from *4xtetO* declined by >65% in 2 and >85% within 6 hours (Figure 4.7.B). Surprisingly, H3K4me3 levels on *4xtetO-ade6*$^{+}$ remained low even at T8 hours but then increased 2-fold between 8 hours and 24 hours of TetR-Clr4* release (Figure 4.7.C). H3K36me3 levels on *4xtetO-ade6*$^{+}$ were found to increase by 2-fold and reach the enrichment observed in cells lacking TetR-Clr4* within 6 hours of TetR-Clr4* release (Figure 4.7.D).

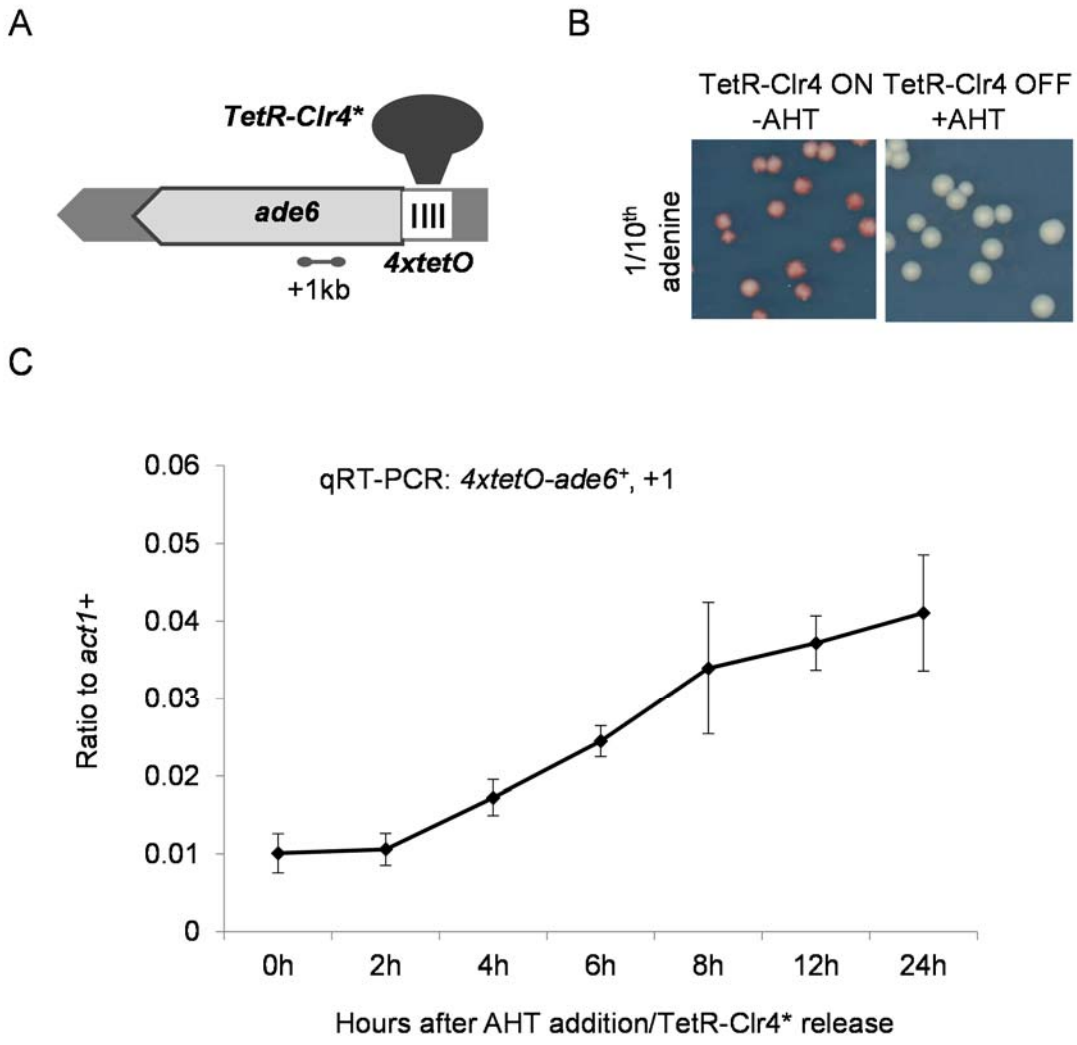


Figure 4.6: Transcription is rapidly restored following TetR-Clr4* removal from the ectopic site

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pair relative to tethered TetR-Clr4*
(B) Silencing assay at *4xtetO-ade6*$^{+}$ in presence of TetR-Clr4* bound to 4x*tetO* (TetR-Clr4* "ON") or 2-3 days upon AHT addition (TetR-Clr4* "OFF").
(C) qRT-PCR analysis of *4xtetO-ade6*$^{+}$ transcript levels before (0h) and 2h, 4h, 6h, 8h, 12h and 24h after TetR-Clr4*release, relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)

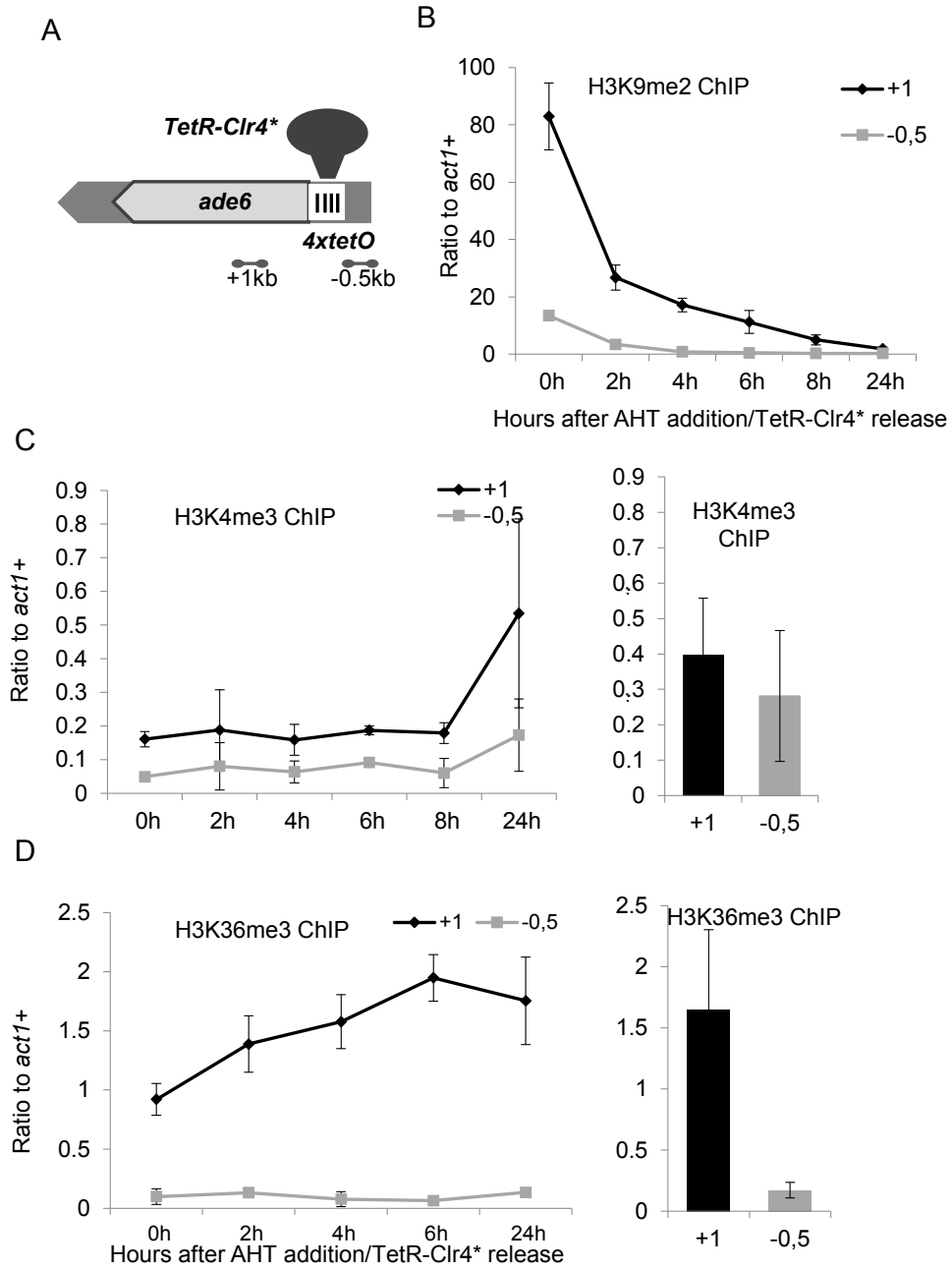


Figure 4.7: Histone modifications associated with active transcription are rapidly restored following TetR-Clr4*removal from the ectopic site

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*

(B) ChIP analysis of H3K9me2 enrichment at 0,5kb and 1kb from *tetO*, 0h, 2h, 4h, 6h, 8h, and 24h following TetR-Clr4* release from the tethering site, relative to *act1*$^{+}$.

(C) ChIP analysis of H3K36me3 level at 0,5kb and 1kb from *tetO*, 0h, 2h, 4h, 6h, and 24h following TetR-Clr4* release relative to *act1*$^{+}$. H3K36me3 level at the tethering site in cells lacking TetR-Clr4* is indicated on the right panel (*clr4*$^{+}$)

(D) ChIP analysis of H3K4me3 level at 0,5kb and 1kb from *tetO*, 0h, 2h, 4h, 6h, 8h and 24h following TetR-Clr4* release relative to *act1*$^{+}$. H3K4me3 level at the tethering site in cells lacking TetR-Clr4* is indicated on the right panel (*clr4*$^{+}$).

Data are means ± SD (*n* = 3 biological replicates)

DISCUSSION

The analyses presented show that the tethered Clr4 histone H3 lysine 9 methyltransferase activity can induce a domain of heterochromatin that is comparable in size and amplitude to that formed over the silent mating-type locus. However, despite this, when tethered Clr4 activity is released from the tethering site, H3K9 methylation, Swi6 and reporter gene silencing are rapidly lost, allowing the acquisition of active histone PTMs and expression of the reporter gene. It is important to note that the cells employed express wild-type Clr4 H3K9 methyltransferase as well as all the core RNAi and heterochromatin components, thus the chromodomains of Clr4, Swi6, Chp1 and Chp2 should be able to recognize H3K9 methylation on parental nucleosomes and copy it to neighbouring nascent nucleosomes. However, the results presented demonstrate that H3K9 methylation and the resultant ectopic heterochromatin domain can not be self-propagated in the absence of the initiating TetR-Clr4* fusion proteinat this specific site.

Constant binding of TetR-Clr4* at *4xtetO-ade6*$^{+}$ is required to maintain H3K9me. Once it is released H3K9me is rapidly lost. This loss of H3K9me is followed by the restoration of a euchromatic state as indicated by the increase of *ade6*$^{+}$ transcripts and H3K36me3 levels during the time course and finally the later appearance of H3K4me3 on *4xtetO-ade6*$^{+}$. It was surprising that H3K4me3 levels remained low until the later stages of the time course when it is restored to the levels observed in cells lacking TetR-Clr4*. The increase in both *ade6*$^{+}$ RNA levels and H3K36me3 levels prior to accumulation of H3K4me3 suggests that H3K4me3 is not required to induce transcription and that this modification occurs after transcription has been more fully activated.

Although other analyses show that H3K9me2 enrichment around the tethering site varies according to the underlying sequence context (see Chapter 3), the dynamics of H3K9me loss from all sites tested around the *4xtetO-ade6*$^{+}$ is similar suggesting that the process responsible for H3K9me2 loss occurs independently from underlying genes and associated cis-acting sequences.

The results obtained in this chapter indicate that H3K9me heterochromatin might lack a self-propagation system that would allow H3K9me to be maintained through cell division and therefore transmit the memory of the chromatin state epigenetically to the daughter cells. In this scenario, the maintenance of H3K9me heterochromatin

would rely entirely on signals dictated by the DNA sequence such as RNAi that promote heterochromatin assembly at every cell cycle. Another possibility is that the epigenetic transmission of H3K9 methylation is specifically counteracted at euchromatic loci such as the *4xtetO-ade6*$^{+}$ test locus.

Although the ectopic heterochromatin domain formed around the *4xtetO-ade6*$^{+}$ tethering site resembles endogenous heterochromatin in many respects, it remains possible that this locus contains particular features that somehow prevent the persistence of H3K9me. In cycling cells, the level of RNA polymerase II and the enrichment of H3K4me3 and H3K36me3 that are associated with active transcription are higher at the ectopic heterochromatin domain than at centromeric repeats although reduced compared to cells lacking TetR-Clr4* (Figure 3.2.D and Figure 3.3). Although transcription is clearly repressed by the assembly of TetR-Clr4*-induced heterochromatin, the transcriptional activity of genes within this synthetic heterochromatin domain might be higher or of a different quality than that which occurs on the outer repeats at centromeres. Since proteins involved in maintaining repression at centromere repeats such as the histone deacetylases Sir2 and Clr3 are important for heterochromatin maintenance, it is possible that the transcriptional activity at the ectopic site might counteract the maintenance of heterochromatin at the ectopic site by promoting transcription-induced histone turnover (Buscaino et al., 2013; Sugiyama et al., 2007).

It is well known in other systems that heterochromatin loci are generally localized to the nuclear envelope or to nuclei and that this specific organisation of the genome is biologically relevant and might be important for maintenance of silencing at heterochromatic regions (Bickmore and van Steensel, 2013). However whether silent regions are specifically targeted to theses nuclear compartments or whether positioning of a chromatin domain to the nuclear periphery or the nucleolus promotes silencing is still unclear. In human cells, the artificial tethering of chromosomes to the nuclear periphery has been shown to promote silencing of some genes around the tethering sites, however many genes remain unaffected by the relocalisation to the nuclear periphery indicating that the nuclear periphery is actually permissive to transcription (Finlan et al., 2008). In *S. pombe*, the presence of constitutive heterochromatin domains at the nuclear envelope might contribute to the maintenance of heterochromatin domains possibly by creating a micro-environment at the nuclear periphery enriched in factors that promote heterochromatin

maintenance such as HDACs (Mizuguchi et al., 2014; Steglich et al., 2012). Therefore, it would be of interest to test whether the *ura4:4xtetO-ade6*$^{+}$ locus has a particular nuclear localisation and whether this is altered when TetR-Clr4* is tethered. Moreover, the contribution of nuclear localisation to heterochromatin stability could be tested by artificially tethering the *ura4:4xtetO-ade6*$^{+}$ locus to the nuclear periphery and determining if this counteracts H3K9me loss and promotes the epigenetic maintenance of the heterochromatin domain.

The results presented so far show that although H3K9me can recruit Clr4 directly and indirectly via Swi6 to chromatin, its mere presence is not sufficient for a domain of H3K9me-dependent heterochromatin to be maintained over an ectopic locus. When assembled ectopically at the single euchromatic tethering site tested, H3K9me was shown to be rapidly lost from the entire chromatin domain. There are three specific processes that are expected to cause the loss of H3K9 methylation from any locus:

1. Histone turnover that is often a result of transcription during which H3 can be evicted and cause loss of H3K9me.

2. Passive dilution as a result of the lack of a transmission mechanism that 'copies' H3K9 methylation from neighbouring parental nucleosomes and places it on newly deposited nucleosomes during or following replication of the heterochromatin domain.

3. Finally the rapid loss of H3K9me from the ectopic site could be caused by an active mechanism that removes H3K9me from dispersed ectopic heterochromatin locations in the nucleus and thus prevents sporadic heterochromatin formation with potentially deleterious outcomes.

These three possible mechanisms of H3K9me loss from ectopic heterochromatin domains are investigated in the following chapters.

CHAPTER 5: Heterochromatin is lost in a cell cycle-independent manner at the tethering site

INTRODUCTION

Histone modifications are dynamic during the cell cycle (Probst et al., 2009). Two phases of the cell cycle might be critical for the stability of H3K9 methylation and the preservation of heterochromatin regions. The events associated with S phase and mitosis might explain the loss of H3K9me and heterochromatin disassembly that is observed when TetR-Clr4* is released from *4xtetO-ade6*$^{+}$.

i) S phase:

When DNA is replicated during S phase nucleosomes need to be disassembled to give access to the replication machinery at the replication fork. Parental histones are recycled and re-deposited onto the two daughter strands of DNA (Avvakumov et al., 2011). Thus, post-translational modifications associated with the parental histones are predicted to carry the memory of the chromatin state behind the replication fork (Benson et al., 2006; Margueron and Reinberg, 2010; Probst et al., 2009). New histones are incorporated in an acetylated state and this acetylation needs to be removed and PTMs matching the modification pattern of neighbouring parental histones would need to be installed to preserve that same chromatin state in a particular region in both daughter cells (Probst et al., 2009). Detailed analyses have shown that in fission yeast, RNAi machinery is critical for the restoration of full H3K9me levels and heterochromatin integrity on centromere repeats following replication. It is at this stage that RNAPII transcription of the repeats increases, allowing engagement by homologous siRNAs loaded in Ago1/RITS. RITS recruits the Clr4-containing CLRC complex to the centromeric repeats which leads to the replenishment of H3K9 methylation (Chen et al., 2008; Kloc et al., 2008). Thus, RNAi reinforces H3K9me and accurately restores the heterochromatin domains following S phase. However, it is unlikely for RNAi to operate at the *4xtetO-ade6*$^{+}$ synthetic heterochromatin domain since it is not recruited by the underlying DNA sequence and it was shown in Chapter 1 that RNAi is not important for heterochromatin maintenance when TetR-Clr4* is bound to *tetO*. Thus, upon TetR-Clr4* release from the tethering site, the maintenance of H3K9me would rely on a self-propagating epigenetic mechanism to be maintained in S phase. If histone PTMs are not transferred with the parental histones to daughter strands of DNA, the heterochromatin domain will be

disassembled upon passage of the replication fork. On the other hand, if parental histones carrying H3K9me are recycled onto the chromatin but heterochromatin does not carry a reinforcing mechanism to modify the newly incorporated histones, H3K9me levels will be diluted by half with each round of each replication in the following cell cycles.

ii) Mitosis:

In many systems, chromosomes dramatically condense in preparation for mitosis which is associated with changes in the chromatin landscape (Perez-Cadahia et al., 2009; Vagnarelli, 2012). In fission yeast, histone H3 serine 10 becomes phosphorylated in preparation for mitosis which interferes with binding of chromodomain proteins to a peptide methylated at H3K9 at heterochromatin regions (Chen et al., 2008; Kloc et al., 2008). Time course analyses show that H3S10 phosphorylation increases on the outer repeats of centromere during mitosis, which associates with a release of Swi6 from its H3K9me docking site. However, live cell imaging shows that some Swi6-GFP reside at centromeres throughout mitosis (Pidoux et al., 2000). Decreased Swi6 at centromeres in mitosis also suggests that centromeric heterochromatin is partly disassembled which might contribute to the loss of H3K9me from *4xtetO-ade6*$^{+}$ observed following TetR-Clr4* release from the tethering site (Chen et al., 2008; Kloc et al., 2008; Li et al., 2013).

In the preceding chapters H3K9me was shown to be rapidly lost from the *ura4:4xtetO-ade6*$^{+}$ locus upon TetR-Clr4* release from the tethering site in cycling cells (Chapter 4). Here I investigate if passage through S phase or mitosis contributes to the loss of H3K9me and heterochromatin surrounding the tethering site.

RESULTS

5.1 H3K9me2 rapidly declines in non-cycling cells blocked in G2

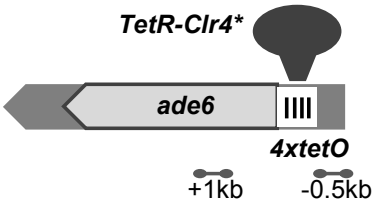
To determine if the loss of H3K9me from around the *4xtetO-ade6*$^{+}$ tethering site occurs during a specific phase of the cell cycle, the *cdc25-22* ts (temperature sensitive) mutant was used to block the cell cycle in late G2 prior to entry into mitosis. Cdc25 is a phosphatase that dephosphorylates the Cdc2 kinase (Millar et al., 1991). Cdc2 is inactive when phosphorylated which prevents cells entering mitosis (Millar et al., 1991). Cells carrying the *cdc25-22* mutation grow normally at 25^{o}C but at 36^{o}C the phosphatase is inactive so cells accumulate in G2. A prolonged arrest (>4 hours)

at G2 phase is detrimental for the cells, therefore a double block regimen was used where a first G2 block was performed by shifting the *cdc25-22* cells for 2h 30 from 25^{o}C to 36°C in order to synchronise most cells in G2. This initial block was then released by returning the cells to 25^{o}C so that cells re-enter the cell cycle and arrive at the next G2 simultaneously. The G2 synchronised cells were returned to 36^{o}C and maintained in G2 for four hours. At the start of the second G2 arrest, AHT was added to prevent TetR-Clr4* from binding to the tethering site. A proportion of cells were harvested at hourly intervals from T0 to T4 for ChIP to assess H3K9me levels over the *4xtetO-ade6*$^{+}$ reporter in these G2 arrested cells. (Figure 5.1.B). Cell density was also monitored during this entire double *cdc25-22* block synchronisation regimen and cells doubled as expected between the two G2 arrests and then remained constant throughout the second G2 arrest following AHT addition. These cell counts indicate that cultures were efficiently released from the first G2 block, went through replication and remained arrested during the second G2 block (Figure 5.1.C). Anti-Flag ChIP revealed that TetR-Clr4* was present at the tethering site at T0 and was efficiently released from *4xtetO-ade6*$^{+}$ within an hour of AHT addition (Figure 5.1.D). H3K9me2 ChIP revealed that following TetR-Clr4* release, H3K9me2 levels dropped by more than 75% within 4 hours (Figure 5.1.D). From this result it can be concluded that the loss of H3K9me from *4xtetO-ade6*$^{+}$ in cycling cells is not due to, or at least not solely due to, the passage of cells through replication or mitosis.

5.2 H3K9me2 steadily declines through the cell cycle

The above experiment shows that release of the initiator TetR-Clr4* results in H3K9 methylation loss from the ectopic locus in G2 arrested cells. To determine if this loss of H3K9 methylation is intensified at specific phases of the cell cycle, cell cultures which were synchronised in late G2 by a three hour single *cdc25-22* block at 36^{o}C, were then released at 25^{o}C into the cell cycle along with the addition of AHT. A proportion of cells were harvested at 20 or 40 minute intervals over 3 hours. To monitor the level of synchrony the % of septation (cytokinesis) was measured at each time point. Septation correlates with the peak of replication in fission yeast. Septated cells were

A



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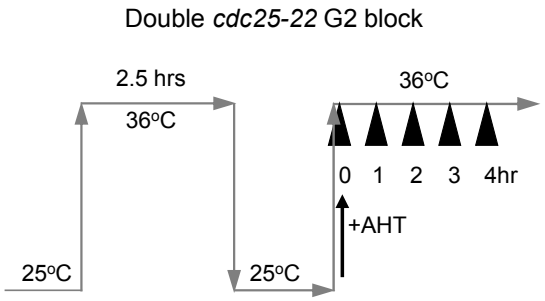

C

	Cell concentration [ ].10^6 cell/ml					
	1st G2 block	2nd G2 block				
culture		0h	1h	2h	3h	4h
replicate 1	1.73	3.38	3.5	3.425	3.3	3.35
replicate 2	3.9	7.6	7.6	7.75	7.05	6.8
replicate 3	2.36	4.125	4.725	4.15	4.575	4.4

D

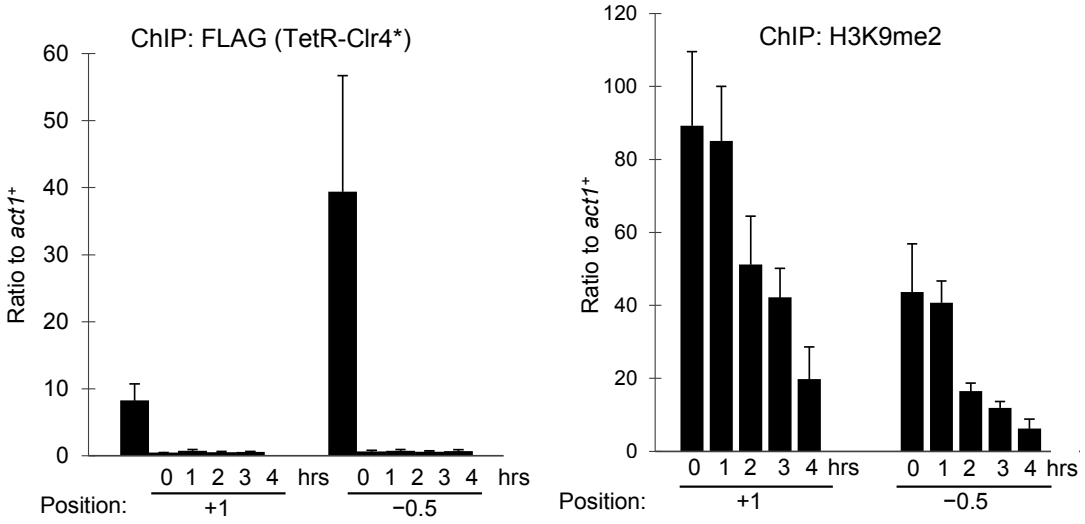

Figure 5.1: H3K9 methylation rapidly declines in non-cycling cells

(A) *4xtetO-ade6*⁺ test locus with the position of primer pairs relative to tethered TetR-Clr4*

(B) Experimental set up: double G2 block. *cdc25-22 ts* cells were synchronised in a first cell cycle block in G2 (shift to 36 °C) . Synchronised cells were released into the cell cycle (shift to 25 °C) and arrested once more in late G2 (36°C) when TetR-Clr4* release was induced by AHT addition (+AHT).

(C) The cell concentration through the G2 arrests was assessed to ensure that cells were efficiently synchronised and remain arrested in late G2 through the second cell cycle block.

(D) ChIP analysis of H3K9me2 (right panel) and TetR-Clr4* levels (left panel) in double-blocked *cdc25-22* G2 cells. H3K9me2 and TetR-Clr4 enrichment were assessed through the 2nd G2 arrest 0h,1h, 2h, 3h and 4h following TetR-Clr4* release. Data are mean ±SD (*n=3* biological replicate) relative to *act1*⁺.

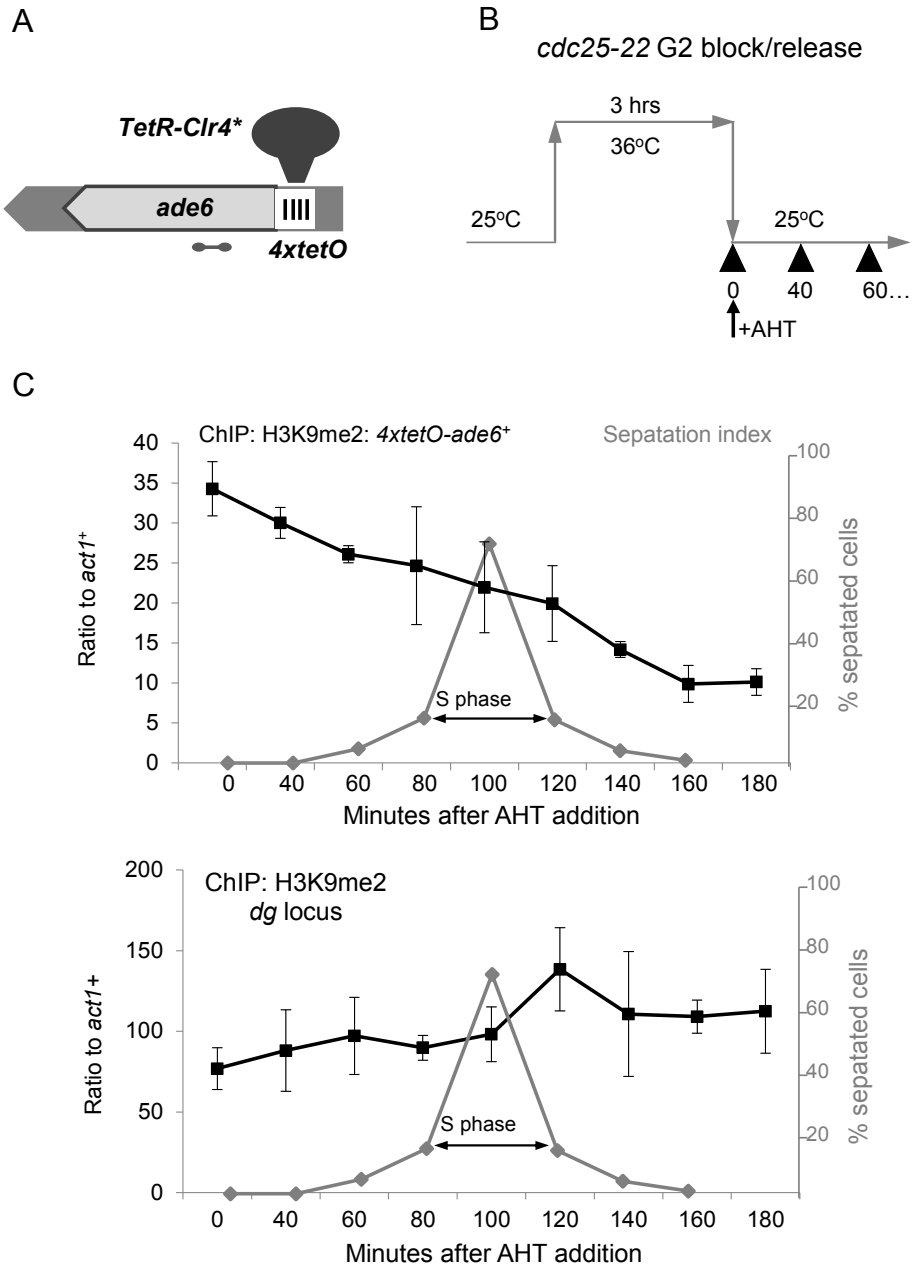

Figure 5.2: H3K9 methylation rapidly and steadily declines through the cell cycle

(A) 4xtetO-ade6^{+} test locus with the position of primer pair relative to tethered TetR-Clr4*

(B) Experiment set up: *cdc25-22* cells were synchronised in late G2 before addition of AHT and thiamine in the medium and simultaneous release of synchronised cells into the cell cycle. Samples were collected 40 min following AHT treatment and then every 20 min for H3K9me2 ChIP analysis.

(C) ChIP analysis of H3K9me2 enrichment at 1kb from *tetO* (top panel), and at heterochromatic centromere repeats *dg* (bottom panel) following TetR-Clr4* release from the tethering site, relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)

found to peak at approximately 75% at T100 minutes. H3K9me2 levels on the *4xtetO-ade6*$^{+}$ reporter were assessed by ChIP (Figure 5.2) and this revealed that H3K9me2 levels steadily declined by 70% over this single synchronous cell cycle. Strikingly, the rate of H3K9me2 loss did not appear to accelerate during S phase which is concomitant with septation (T80, T100 and T120). If the new nucleosomes carrying newly synthetized histone H3 were not methylated during this period, then the pre-existing H3K9 methylation would be diluted by half and thus a drop of about 50% in H3K9me2 levels should have been seen between T80 and T120 when the majority of cells pass through S phase. The fact that no dramatic drop is seen in H3K9me2 during this period suggests that H3K9 methylation is faithfully replenished following replication of the *4xtetO-ade6*$^{+}$ reporter. Importantly, over this same time course experiment H3K9me2 levels over the centromeric outer repeats (*dg*) remain relatively stable. Thus, on centromeric repeats there is not much change in associated H3K9me levels through the cell cycle although a slight increase may occur in early G2 (T120) which corresponds to RNAi-dependent H3K9me replenishment. The increase in H3K9me2 observed on centromeric repeats in G2 in this experiment is not as great as that previously reported by others (Chen et al., 2008; Kloc et al., 2008).

DISCUSSION

The analyses presented above show that the loss of H3K9me from *4xtetO-ade6*$^{+}$ following TetR-Clr4* release is not dependent on passage through the cell cycle. Indeed, H3K9me2 was steadily lost throughout a synchronous cell cycle and rate of H3K9me2 loss did not increase during S phase or mitosis.

H3K9me2 levels on *4xtetO-ade6*$^{+}$ do not decrease by half during replication, as would be anticipated in the absence of a mechanism to restore H3K9me on newly incorporated histones. Therefore, it can be concluded that the new histone H3 incorporated into nucleosomes over the *4xtetO-ade6*$^{+}$ locus upon S phase can be methylated in order to match the modifications of parental histones (Kim and Huberman, 2001). H3K9me2 can therefore be maintained and propagated through S phase. Presumably, the mechanism of maintenance involves first, the recycling of parental lysine 9 methylated histone H3 and the reassembly of nucleosomes using old histones, and second, the recognition of this parental H3K9 methylation by the chromodomain readers which direct Clr4 to install the same H3K9 PTM on neighbouring newly assembled nucleosomes.

These analyses imply that even in the absence of the TetR-Clr4* heterochromatin initiator at the *4xtetO-ade6*$^{+}$ reporter, the endogenous Clr4 H3K9 methyltransferase must be recruited, most probably by direct binding via its chromodomain to parental H3K9me nucleosomes and indirectly via Swi6, allowing it to boost H3K9 methylation levels following S phase. H3K9me must therefore be able to direct self-propagation through replication although this process is not sufficient to prevent H3K9me loss from the ectopic domain of heterochromatin.

CHAPTER 6: Investigating the role of histone turnover in the loss of H3K9 methylation from ectopic Clr4 tethering sites

INTRODUCTION

In the preceding Chapter it was found that the loss of H3K9me2 from the *4xtetO-ade6*$^{+}$ tethering site does not result from the passive dilution of H3K9 methylated H3 as a result of DNA replication or any other cell cycle associated events. It is known that transcription of chromatin requires the disassembly and reassembly of nucleosomes in front and behind RNAPII, respectively (Smolle and Workman, 2013). Histones are recycled so that nucleosomes are reassembled using the same histones that were present in front of RNAPII. However, these RNAPII coupled remodelling events can result in the loss of pre-existing histones from the template which then need to be replaced with new histones (Svensson et al., 2015; Verzijlbergen et al., 2010). Therefore, it is possible that following the release of TetR-Clr4* from the *4xtetO-ade6*$^{+}$ tethering site, the loss of H3K9me2 is caused by transcription-coupled histone turnover. In this chapter the possibility that such transcription-coupled histone replacement processes are involved in the removal of H3K9 methylation following TetR-Clr4 release is investigated.

Early studies of the dynamics of the different histone subunits in nucleosomes of chromatin showed that although the H2B-H2A dimer undergoes very frequent exchange events during transcription, the nucleosome core composed of H3-H4 tetramer is very stable (Kimura and Cook, 2001). This is consistent with the expression profile of histone H3 that is mostly expressed just prior to S phase, suggesting that new histones H3 are mostly incorporated during replication and not extensively outside S phase as a result of histone turnover. However more recent studies revealed that the individual histone subunits within chromatin are more dynamic than originally thought. In *Drosophila* and Humans, a specific histone H3 variant H3.3 was shown to be assembled into the chromatin in place of canonical histone H3 as a result of histone exchange outside S phase (Chow et al., 2005; Mito et al., 2005). Unlike H3.1, H3.3 is expressed throughout the cell cycle and its assembly relies on the histone chaperone HIRA. In fission yeast, only one subtype of H3 exists and it resembles H3.3, thus it is likely that all *S. pombe* histone H3 is subject

to both replication-coupled assembly and replication-independent transcription-coupled turnover (Ahmad and Henikoff, 2002).

From yeast to mammals, the turnover of histones is not identical across the genome. Regulatory regions such as promoters and enhancers display high histone turnover compared to the rest of the genome (Huang and Zhu, 2014) (Svensson et al., 2015). At gene bodies, histone turnover appears to correlate with transcriptional activity with higher histone turnover taking place in highly transcribed genes (Jamai et al., 2007; Svensson et al., 2015)(Huang and Zhu. 2014). Regions of high histone turnover correlate with hyperacetylated domains. Histone acetylation weakens histone-DNA association by neutralizing the positive charge on lysine residues which causes acetylated histones to turnover more rapidly compared to unmodified or methylated histones (Barth and Imhof, 2010; Solis et al., 2004).

The nucleosomes in heterochromatin regions are generally hypoacetylated and display low histone turnover compared to the rest of the genome in fission yeast (Aygun et al., 2013; Choi et al., 2005; Garcia et al., 2010). Disruption of heterochromatin integrity in *S. pombe* by loss of Clr4 H3K9 HMTase, Swi6 or the Clr3 HDAC results in higher rates of histone H3 turnover on regions that are normally assembled in heterochromatin. Elevated histone turnover is perhaps not surprising since loss of heterochromatin integrity results in increased histone acetylation and transcription within these regions, however, low histone turnover itself may be important for the maintenance of heterochromatin integrity (Aygun et al., 2013).

Given the connection between transcription and histone turnover, it is possible that the maintenance of H3K9 methylation-dependent heterochromatin might be more favoured in regions of the genome that display low levels of histone H3 turnover. Thus tethering TetR-Clr4* to *tetO* sites inserted at a locus that displays low histone H3 turnover may slow down or prevent H3K9me loss upon TetR-Clr4* release compared to the *ura4:4xtetO-ade6*$^{+}$ test locus. In this chapter, the relationship between transcription, histone turnover and H3K9me-heteochormatin persistence is explored.

Replication independent histone turnover was first assessed using the Recombination Induced Tag Exchange (RITE) in G2 blocked cells. Using these data two broad regions of low transcription and low histone turnover were selected for insertion of *tetO* sites to which TetR-Clr4* was tethered. Subsequently the maintenance of heterochromatin upon TetR-Clr4* release was assessed at these tethering sites. Finally low to high levels of transcription were imposed on both regions to test if the

degree of transcription affects the establishment and/or maintenance of H3K9 methylation over these regions.

RESULTS

6.1 Recombination induced tag exchange (RITE) system as a tool to study histone turnover

The RITE system was developed as a method that allows the accurate assessment of protein turnover in budding yeast (Verzijlbergen et al., 2010). The RITE system involves the tagging of a gene of interest with two epitope tags separated by in-frame LoxP sites and a selectable marker. The first epitope tag can be irreversibly swapped to the second tag upon the activation of the inducible CRE recombinase so that all pre-existing old protein carries the first tag while, following the genetic switch, newly synthesized protein carries the second epitope tag. The RITE system consists of two components:

i) the RITE tagging cassette, which is inserted by homologous recombination as an in-frame fusion to the 3' end of the ORF of interest. The first C-terminal tag (HA-epitope) is surrounded by two in-frame LoxP sites with a hygromycin resistance marker gene downstream of the stop codon at the region encoding the HA-tag. The second LoxP site is followed by a second epitope tag (T7-epitope) and a second stop codon (Figure 6.1.A).

ii) a constitutively expressed hormone regulatable Cre recombinase which mediates the epitope switch. The Cre recombinase is fused to an Estrogen Binding Domain (EBD) that sequesters Cre in the cytosol with a complex composed of Hsp90 (Logie and Stewart, 1995). Upon addition of β-estradiol, Cre-EBD is released from Hsp90 and enters the nucleus where it induces the genetic switch of the epitope tags (HA->T7) within the RITE cassette by recombination between the two in-frame LoxP.

The RITE system has proven to be an excellent tool for measuring histone H3 turnover across the genome in *S. cerevisiae* (Verzijlbergen et al., 2010). We therefore adapted the RITE system for use in *S. pombe*.

There are three dispersed genes that code for the histone H3 in *S. pombe*: *hht1, hht2* and *hht3*. While *hht1* and *hht3* are mostly transcribed just prior to S phase, *hht2* is expressed more constantly through the cell cycle (Takayama and Takahashi, 2007).

The *hht2* (H3.2) gene was tagged with the RITE cassette at its 3' end in order to assess histone H3 turnover at regions of interest for us (this was performed by Manu Shukla in the Allshire lab). A construct that expresses the Cre-EBD recombinase was inserted at the *ars1* locus under the control of *adh15* attenuated version of the constitutive *adh1 gene* promoter. To assess the efficiency of recombination and hence H3->T7 tag swap, cells were exposed to β-estradiol for 2 hours to activate LoxP-LoxP recombination in the nucleus and subsequently plated on medium lacking β-estradiol at different times. The number of cells that performed the tag exchange was estimated by measuring the percentage of cells that had lost the hygromycin resistance marker as a result of Cre-mediated LoxP-LoxP recombination. In cycling cells 81% of cells lost hygromycin resistance after two hours and this increased to 99% after 6 hours (Figure 6.1.B). At the protein level, two hours after addition of β-estradiol, new histone H3.2 tagged with the T7 epitope (H3-T7) was detectable by western analysis in extracts from these cells. A decrease in the levels of old histone H3.2 tagged with HA epitope (H3-HA) was detectable about 4 hours after Cre activation (Figure 6.1.C).

These data show that the RITE system allows turnover of histone H3 to be monitored in *S. pombe* without overexpression of an additional tagged H3 gene as used previously (Aygun et al., 2013). As only one of the three H3 genes is tagged, only approximately one third of all H3 is tagged and this tagged H3 acts as a trace for histone exchange without compromising endogenous H3 function. The HA->T7 tag exchange is rapid and efficient upon Cre-EBD release into the nucleus so that the majority of the LoxP sites recombine and new H3-T7 tagged histone H3 were easily detectable within 2 hours following β-estradiol addition. The use of the RITE system should therefore allow replication-independent incorporation of new histone H3 to be assessed in regions of interest within a short time frame following Cre-EBD induced recombination.

6.2 Selecting loci with low histone turnover

The region around the original *ura4:4xtetO-ade6*$^{+}$ tethering site is quite complex. Upstream and downstream of the *4xtetO* sites there are many coding and non-coding genes, LTR elements and essential genes and these may have hindered the retention and heritability of heterochromatin at this locus following TetR-Clr4* release. To investigate the role of histone turnover in the loss of H3K9me from ectopic

heterochromatin loci, two new TetR-Clr4* tethering sites were selected at which the maintenance of H3K9me was assessed. The new TetR-Clr4* tethering sites were chosen to fulfil four criteria:

i) they must be as far as possible from known regulatory elements such as strong promoters that may hinder the maintenance of H3K9me dependent heterochromatin.

ii) to avoid selection against heterochromatin assembly there should be no essential genes at the vicinity of the tethering sites.

iii) they should display low levels of transcription as assessed by measurement of steady state RNA levels.

iv) there should be of low histone H3 turnover over these regions as assessed using the RITE system.

The tethering of TetR-Clr4* at loci that fulfil the above criteria would more rigorously test whether a domain of ectopic H3K9me-dependent heterochromatin established within a relatively "neutral" region with respect to expression and histone H3 turnover, and containing no essential genes, allows H3K9me to persist after TetR-Clr4* release.

There are actually very few, if any, large domains that could be considered of "neutral" sequence based on the above criteria in the *S. pombe* genome. For this reason we decided to tether TetR-Clr4* within two non-essential large genes that have long open reading frames and that are expressed at relatively low levels. The *vps1302*$^{+}$ and *sib1*$^{+}$ genes are 9.2 and 15.25 kb long, respectively. RNA seq data (generated by Eun Choi and Sandra Catania, Allshire lab), shows that the steady state level of RNA generated by these two genes is relatively low (Figure 6.2.A). Both genes are amongst the 25% of genes that display the lowest level of steady state RNAs. qRT-PCR data confirmed that both *vps1302*$^{+}$ and *sib1*$^{+}$ have low steady state RNA levels since their detected RNA levels were found to be approximately 100 fold lower than the highly transcribed *act1*$^{+}$ gene and three times lower than *ade6*$^{+}$ in the *ura4:4xtetO-ade6*$^{+}$ locus. ChIP was also performed with antibodies that recognise the unmodified CTD of RNAPII (total RNAPII) to asses RNAPII occupancy on these genes. Consistent with the RNA analysis, RNAPII occupancy was found to be 25 and 50% lower on the *vps1302*$^{+}$ and *sib1*$^{+}$ genes relative to the *ade6*$^{+}$ gene.

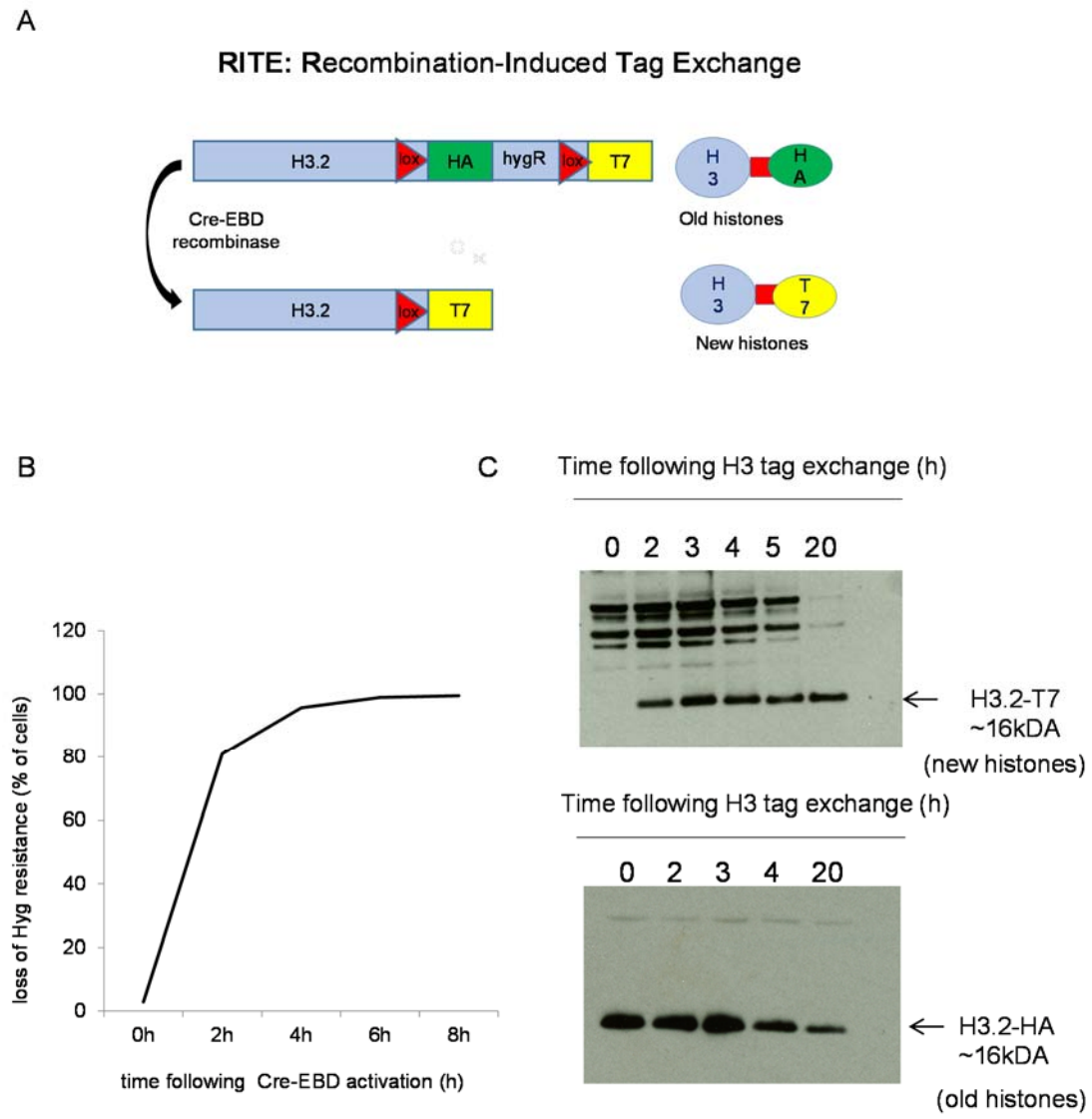

Figure 6.1: The RITE system as a tool to determine histone H3 turnover.

(A) Diagram of the RITE genetic switch cassette.
(B) Analysis of loss of the hygromycin resistance upon Cre-EBD activation. Cells were plated 0,2,4,6 and 8h following Cre activation on non-selective medium and replica plated on YES+hygromycin plates. The percentage of cells that lost the resistance to Hyg are plotted.
(C) Western Blot analysis of H3-T7 (new histones) and H3-HA (old histones) following Cre-EBD activation.

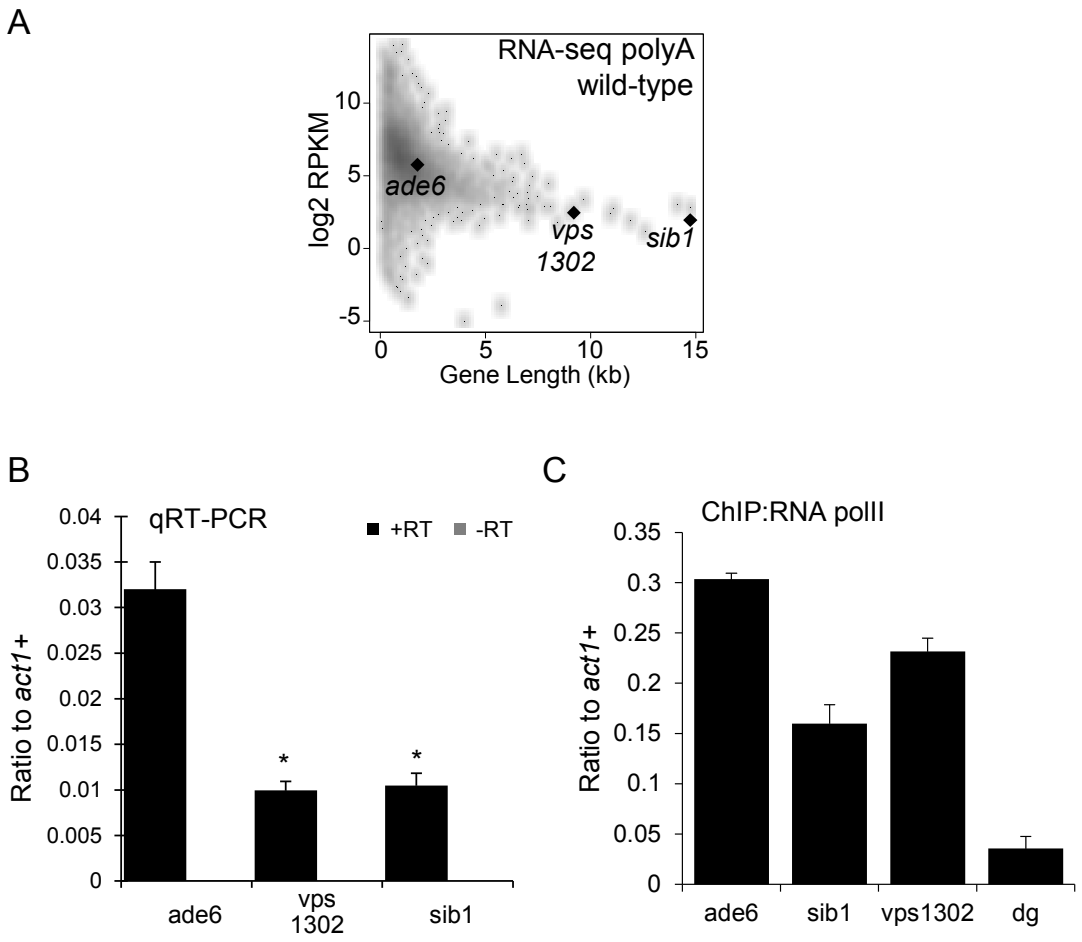

Figure 6.2: Selection of two new tethering sites for TetR-Clr4* tethering displaying low levels of transcription.

(A) Read distribution (log_2RPKM) from *S. pombe* polyA RNA-seq relative to gene length. *ade6*$^+$, *sib1*$^+$ and *vps1302*$^+$ indicated. (Experiment and analysis performed by Sandra Catania, Eun Choi and Pin Tong).
(B) qRT-PCR analysis of *ura4:4teto-ade6*$^+$*, sib1* $^+$ and *vps1302* $^+$ transcript levels relative to a control transcript *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates)
(C) ChIP analysis of RNAPII levels associated with *ura4:4teto-ade6*$^+$*, sib1* and *vps1302,* relative to *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates)

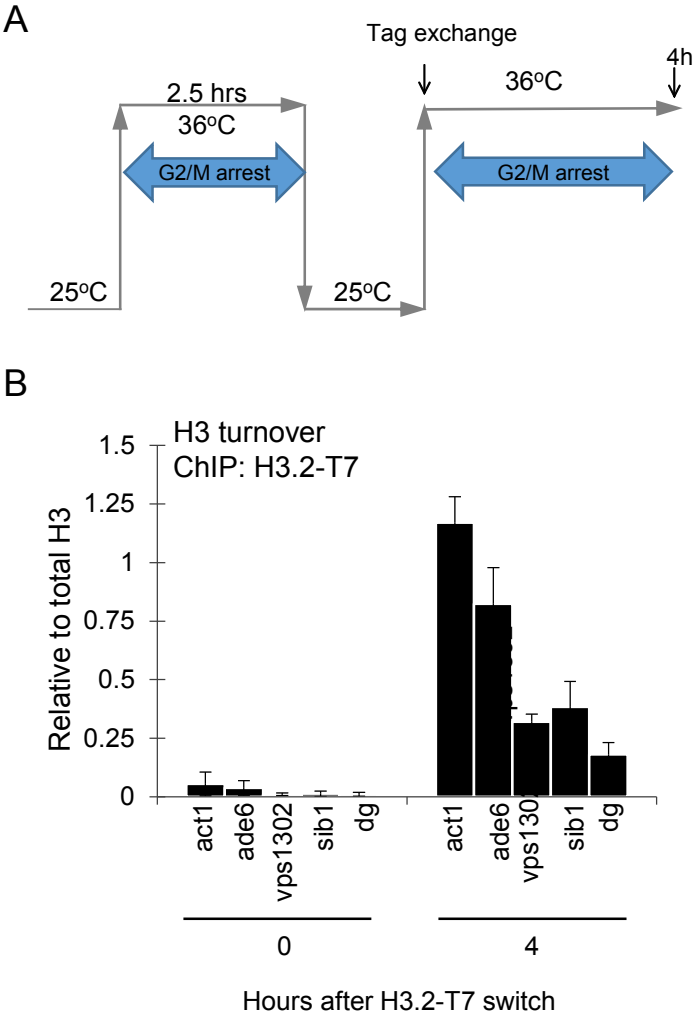

Figure 6.3: Determination of H3 replication-independent histone turnover in *sib1*⁺ and *vps1302*⁺

(A) Experimental set up: *cdc25-22* cells containing the RITE system and the *ura4:4xtetO-ade6*⁺ *cassette* were synchronised by performing a first G2/M block at 36°C for 2h30min. Synchronised cells were released into the cell cycle and arrested a second time in G2/M by shifting the cells at 36°C just after S phase. Simultaneously, ß-estradiol (1µM) was added to the medium to induce recombination-induced tag exchange. Samples for total H3 and H3-T7 ChIP analyses were collected before (0h) and 4h following Cre-EBD activation.
(B) ChIP analysis of T7 level relative to total H3 before (0h) and 4h ß-estradiol addition in cells arrested in late G2. Data are means ± SD (*n* = 3 biological replicates)

In order to assess H3 turnover on the *vps1302*$^{+}$ and *sib1*$^{+}$ genes, *cdc25-22* cells were subjected to two consecutive G2 arrests so that a high proportion of cells enter the second block synchronously in G2. The entry of the Cre-EBD recombinase into the nucleus was induced at the beginning of the second arrest by the addition of 1 μM β-estradiol to the culture. A proportion of cells were harvested before (T0) and four hours (T4) after Cre-EBD induction. ChIP was then performed to assess total H3 and newly incorporated H3.2-T7 levels associated with various genes before and after the HA->T7 tag swap (Figure 6.3). These analyses showed high enrichment of H3.2- T7 relative to total H3 over the *act1*$^{+}$ gene four hours after induction of the tag exchange. This was as expected for a highly transcribed gene such as *act1*$^{+}$. Heterochromatin regions have been shown to display low histone turnover (Aygun et al.). In agreement with this, heterochromatic centromere repeats (*dg*) were 5-fold less enriched of new H3-T7 relative to H3 compared to the *act1*$^{+}$ gene. In agreement with their low level of expression both the *vps1302*$^{+}$ and *sib1*$^{+}$ genes exhibit low turnover of histone H3; the enrichment of H3-T7 relative to total H3 in the middle of either gene was approximately 4 fold lower than that of *act1*$^{+}$ and 2 to 3 fold lower than that detected at the original *ura4:4xtetO-ade6*$^{+}$ test locus Since the body of both the *vps1302*$^{+}$ and *sib1*$^{+}$ genes satisfied our criteria of low expression, low histone H3 turnover and the middle of their open reading frames are distant from promoters or annotated regulatory elements, they provided good additional test sites for the tethering of TetR-Clr4*.

6.3 Investigating the role of histone turnover in loss of H3K9 methylation at ectopic sites following TetR-Clr4* removal from the tethering site

To allow tethering of the TetR-Clr4* fusion protein within the *vps1302*$^{+}$ and *sib1*$^{+}$ genes , *1xtetO* and *4xtetO* sites were inserted by homologous recombination into the middle of *vps1302*$^{+}$ and *sib1*$^{+}$ genes, respectively (Figure 6.4.A). Expression of TetR-Clr4* allowed the accumulation of high levels of H3K9 methylated chromatin at both of these tethering sites. Limited analyses was performed but at the *vps1302:1xtetO* locus, H3K9me2 was found to spread several kilobases away from the *1xtetO* tethering site in both directions, however, no significant H3K9me2 enrichment was detected -5 kb away, near the promoter of this gene. For the *sib1:4xtetO* locus, H3K9me2 was found to be highly enriched at +5 kb away towards the 3' end of the *sib1* gene (Figure 6.4.B). To determine if H3K9me2 can persist longer at these two genes with low expression and low histone H3 turnover, AHT was added to cultures

and the presence of H3K9 methylation was monitored by ChIP on cells harvested at time points T0, T3 and T6 hours. Similar to what was observed at the original *ura4:4xtetO-ade6*$^{+}$ test locus, the release of TetR-Clr4* resulted in the very rapid loss of H3K9me2 from the *vps1302:1xtetO* and *sib:4xtetO* tethering sites; more than 90% of H3K9me2 was lost within 6 hours from locations flanking both tethering sites (Figure 6.4.C).

To simultaneously compare the dynamics of H3K9me2 loss following TetR-Clr4* release at all three ectopic tethering loci, a strain containing *ura4:4xtetO-ade6*$^{+}$, *vps1302:1xtetO* and *sib:4xtetO* and expressing the TetR-Clr4* fusion protein was constructed. Anti-FLAG ChIP confirmed that TetR-Clr4* (TetR-2xFLAG-Clr4-cdd) was present at the three tethering sites, although substantially higher levels of TetR-Clr4* were detected at the *sib:4xtetO* than either *ura4:4xtetO-ade6*$^{+}$or *vps1302:1xtetO* (Figure 6.5). Further analysis revealed that three hours after AHT addition the TetR-Clr4* was efficiently released from all three tethering sites (Figure 6.5). ChIP was then performed to assess H3K9me2 associated with the *ura4:4xtetO-ade6*$^{+}$, *vps1302:1xtetO* and *sib:4xtetO* loci before and three hours following TetR-Clr4* release (Figure 6.5). In this pilot experiment, H3K9 methylation was monitored approximately 0.5 kb from the *tetO* sites at each locus and the initial level of H3K9 methylation detected at each tethering site differs. The highest levels of H3K9me2, 110 fold relative to the *act1*$^{+}$ gene, were detected 0.5 kb upstream of the *4xtetO* sites in the *sib1* gene. In these same cells lower H3K9me2 levels were detected near the tethering sites of *vps1302:1xtetO* (60 fold/*act1*$^{+}$) and *ura4:4xtetO-ade6*$^{+}$ (40 fold/*act1*$^{+}$). Three hours following TetR-Clr4* release, 42% of the starting level of H3K9me2 remained at the *vps1302:1xtetO* locus whereas H3K9me2 at the *ura4:4xtetO-ade6*$^{+}$ and *sib:4xtetO* was reduced to 25% and 30% respectively. This indicates that H3K9me2 loss might be occurring more rapidly at the manipulated *ura4* and *sib1* loci than that at *vps1302* locus. However, further time points following TetR-Clr4* release and analysis of H3K9 methylation levels at regular intervals surrounding each of the three tethering sites would be required to allow firm conclusion in respect to differences in the dynamics of H3K9me2 loss.

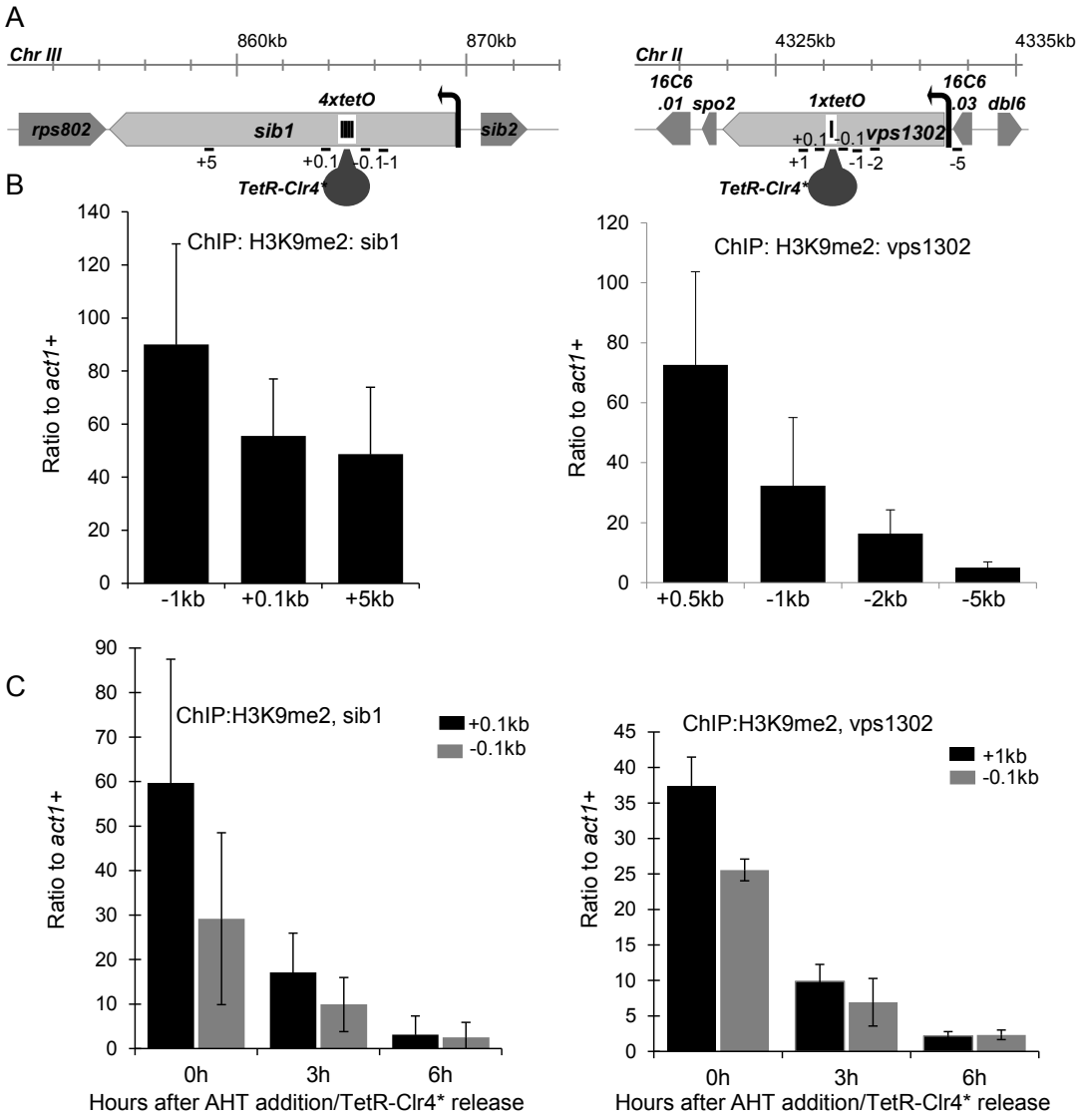

Figure 6.4: H3K9me2 is rapidly lost upon TetR-Clr4* removal from *sib1* and *vps1302*

(A) Diagram of constructs: *sib1:4xtetO* and *vps1302:1xtetO*
(B) ChIP analysis of H3K9me2 levels at *vps1302* and *sib1* loci, relative to *act1*$^{+}$, (n=3).
(C) ChIP analysis of H3K9me2 level following TetR-Clr4* release from *sib1* and *vps1302,* relative to *act1*$^{+}$, (n=3).

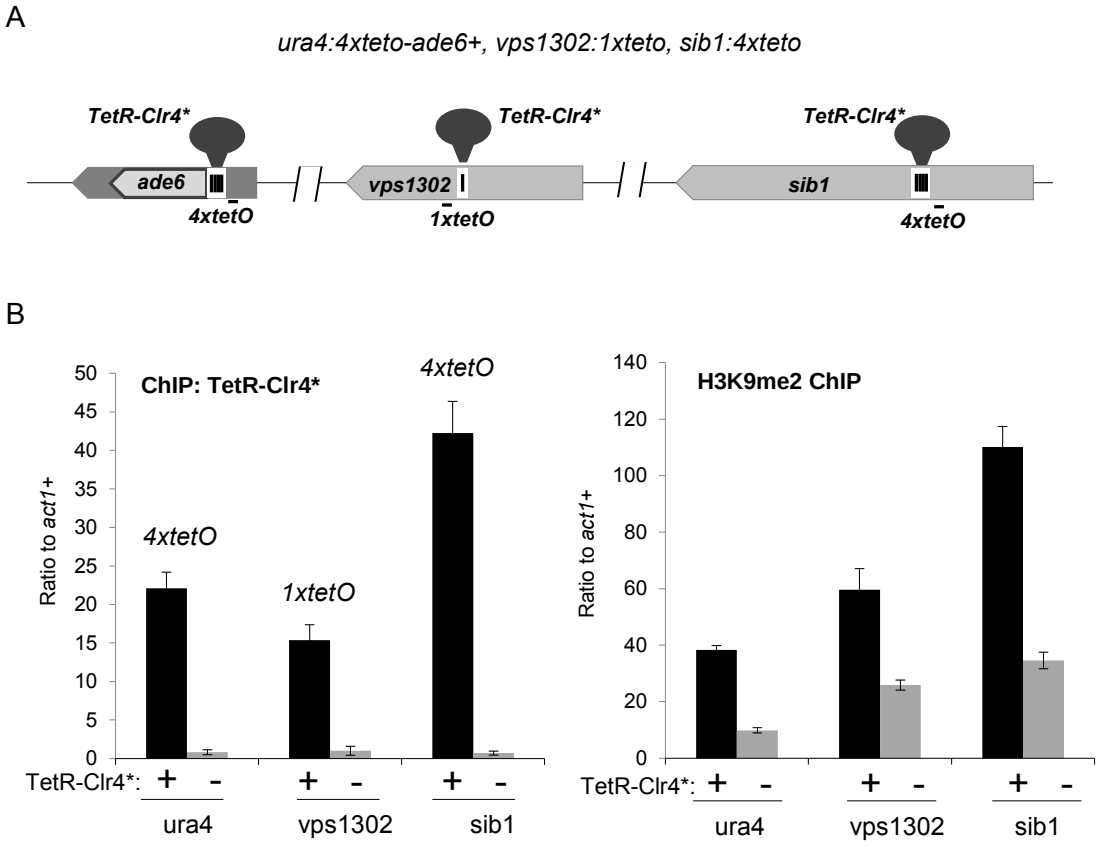

Figure 6.5: Comparison of H3K9me2 assembly and maintenance at *ura4, sib1* and *vps1302* in a strain containing the three tethering sites (*ura4:4xteto-ade6+, sib1:4xteto, vps1302:1xteto)*

(A) Positions of *4xtetO*, *1xtetO* and tethered TetR-Clr4* at *ura4, vps1302* and *sib1*, Dumbbells indicate primer pairs.
(B) ChIP analysis of TetR-Clr4* (left panel) and H3K9me2 levels (Right panel) in presence or three hours after TetR-Clr4* release from the tethering sites. Data are means ± SD (*n* = 3 biological replicates)

6.4 Investigating the role of transcription in H3K9 methylation establishment and maintenance at the TetR-Clr4* tethering sites

To determine if the loss of H3K9me2 from tethering sites is linked to the levels of transcription, the wild-type promoters of *sib1* and *vps1302* were replaced with different attenuated versions of the constitutive *adh1* promoter. The *adh81*, *adh11* and *adh1* version have been shown to have low, medium and strong transcription strength (Sakuno et al., 2009). Additional strains were constructed in which the endogenous promoter driving *vps1302*$^+$ and *sib1*$^+$ were deleted and replaced with the *ura4*$^+$ gene (no promoter) (Figure 6.6.A and Figure 6.7.A). Analyses of *vps1302* (Figure 6.6) and *sib1* (Figure 6.7) expression showed that the RNA level produced by the different versions of the two genes increased according to the promoter strength. *vps1302* RNA levels were 1% of *act1*$^+$ RNA levels in the no promoter strains and reached up to 40% of *act1*$^+$ RNA levels when *vps1302* was under the control of the strong wild-type *adh1* promoter. Similar results were obtained for the manipulated versions of the *sib1* gene. *sib1* RNA levels detected were 1% of *act1*$^+$ RNA levels in absence of a promoter. The strong *adh1* promoter produced much higher levels of *sib1* RNA, with an RNA level that reached 54% of *act1*$^+$ RNA levels.

To determine if the level of transcription across the *tetO* tethering sites within the *vps1302* and *sib1* affects the level of H3K9 methylation established and its ability to be retained following TetR-Clr4* release, CHIP was performed. Strains carrying *vps1302:1xtetO* or *sib1:4xtetO* with no, its own, low, medium or high *adh* promoters driving expression were used. TetR-Clr4* was tethered to the *tetO* sites in all strains and H3K9 methylation was monitored before, 3h and 6 hours after Tetr-Clr4* release by addition of AHT (Figure 6.8 and Figure 6.9).

ChIP analyses revealed that most H3K9me2 was lost from the tethering sites at *vps1302* and *sib1* within 6 hours of TetR-Clr4* release. This rapid loss of H3K9me2 occurred regardless of the strength of promoter driving transcription through the *vps1302* or *sib1* tethering sites or even the lack of a promoter on the associated gene. In conclusion, the tethering of TetR-Clr4* to a genomic region displaying low expression levels and low histone turnover is not sufficient to allow the maintenance of H3K9me at an ectopic locus.

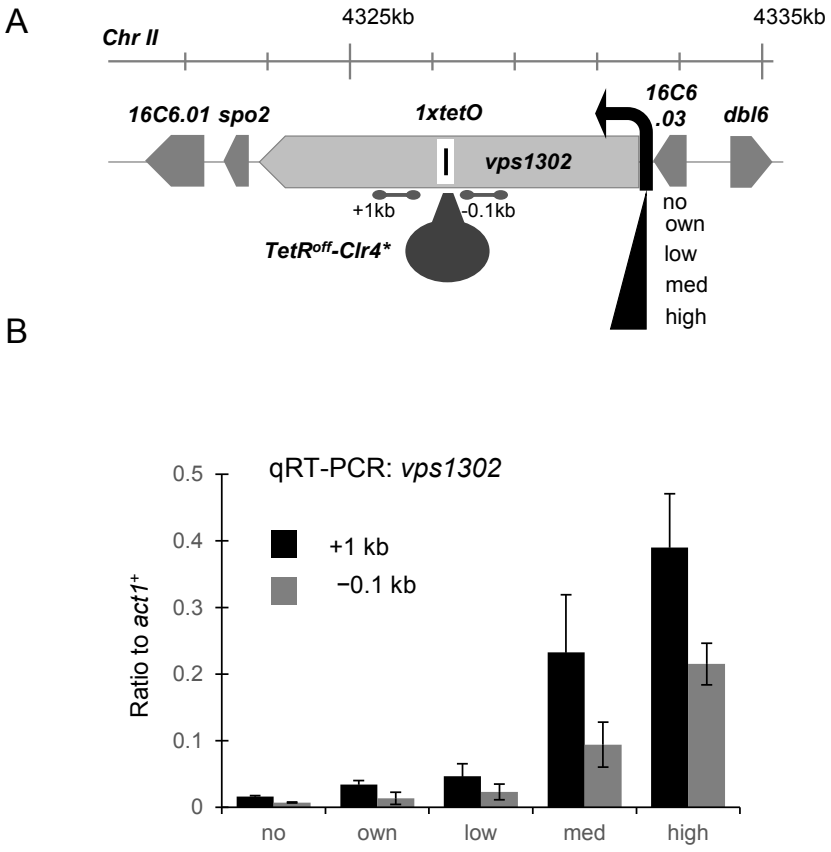

Figure 6.6: Replacement of *vps1302* promoter with different attenuated versions of the constitutive *adh1* promoter

(A) Position of *tetO* sites and tethered TetR-Clr4* within the *vps1302* locus on chromosome III. The *own* promoters of *vps1302* was deleted by replacement with *ura4*$^+$ (*no*) or swapped to the *low (adh81)*, medium (*med: adh11)*), or *high (adh1)* versions of the constitutive *adh1* promoter.
(B) RT-PCR analysis to monitor expression levels on *no*, *own, low-adh81*, med-*adh11* and *high-adh1* promoter versions of *vps1302*; relative to *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates)

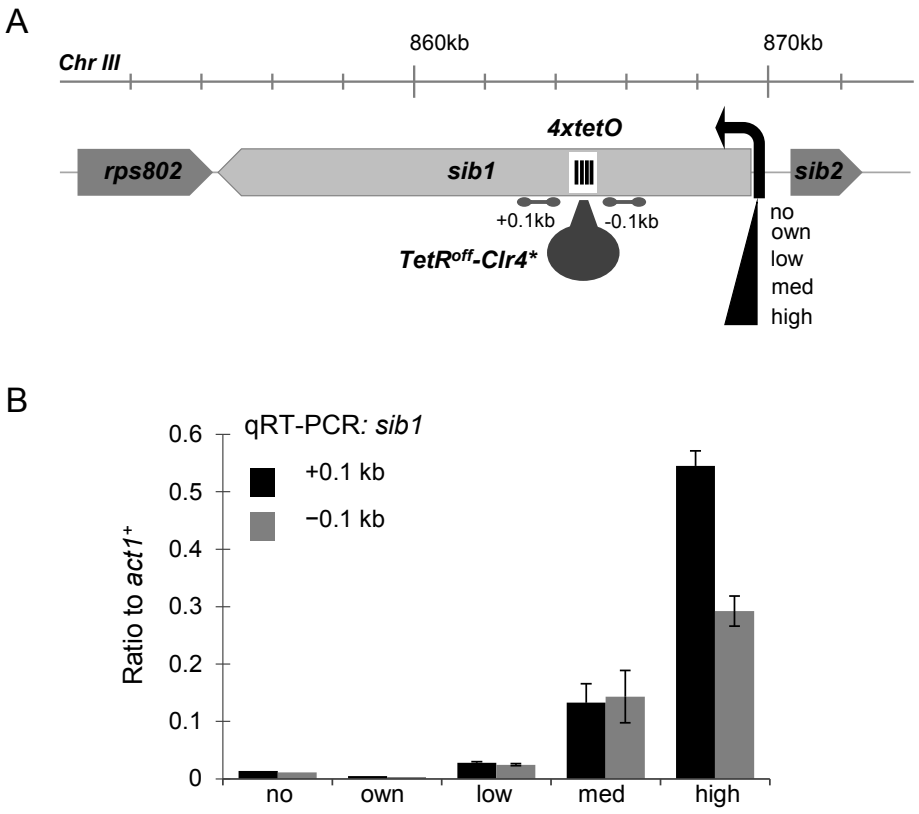

Figure 6.7: Replacement of *sib1* promoter with different attenuated versions of the constitutive *adh1* promoter

(A) Position of *tetO* sites and tethered TetR-Clr4* within the *sib1* locus on chromosome III. The *own* promoters of *sib1* was deleted by replacement with *ura4*$^{+}$ (*no*) or swapped to the *low (adh81)*, medium (*med: adh11)*), or *high (adh1)* versions of the constitutive *adh1* promoter.
(B) RT-PCR analysis to monitor expression levels on *no*, *own, low-adh81*, med-*adh11* and *high-adh1* promoter versions of *sib1*; relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)

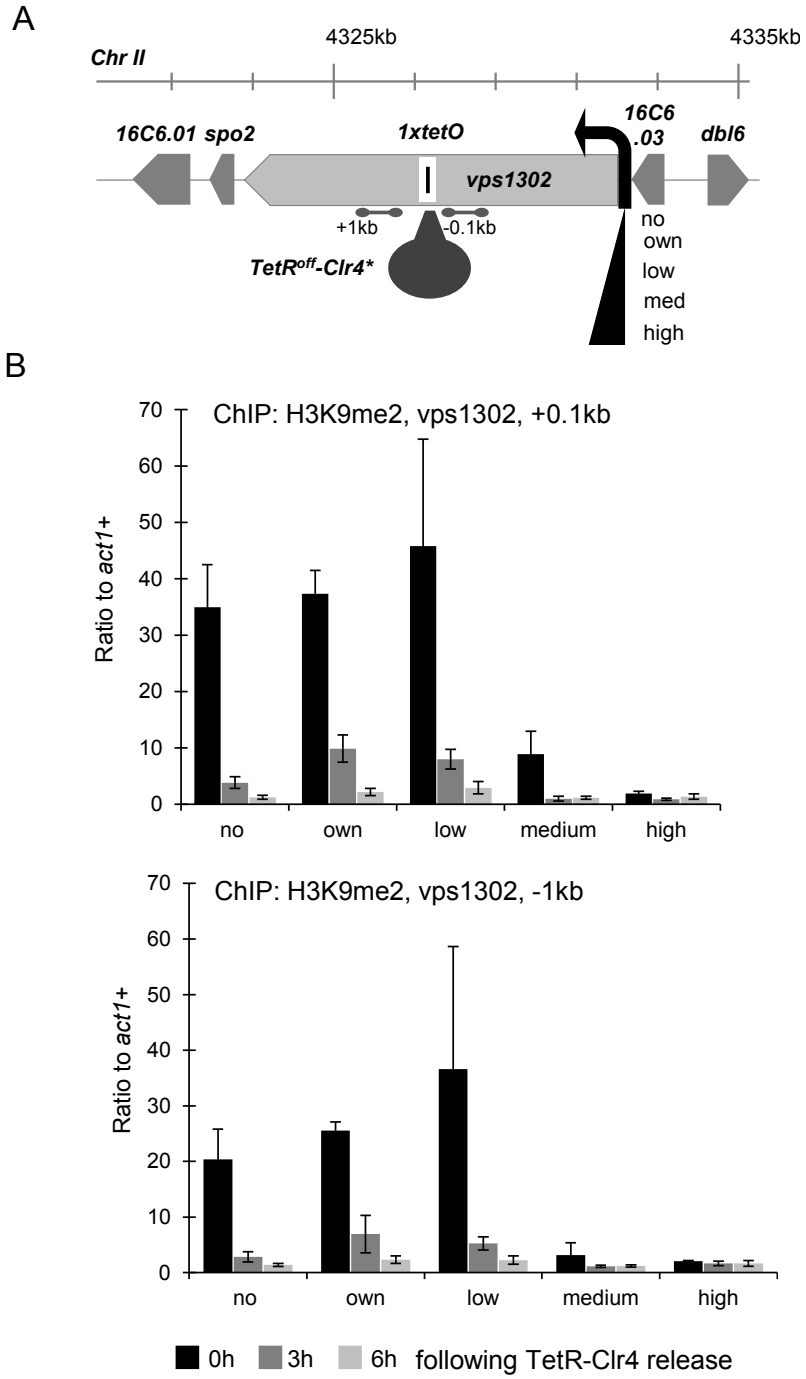

Figure 6.8: High expression prevents establishment of H3K9 methylation by tethered Clr4 methyltransferase in *vps1302*

(A) Position of *tetO* sites and tethered TetR-Clr4* within the *vps1302* locus on chromosome III. The *own* promoters of *vps1302* was deleted by replacement with *ura4*$^+$ (*no*) or swapped to the *low*, medium (*med*), or *high* versions of the constitutive *adh1* promoter.
(B) ChIP analysis of H3K9me2 levels on *no*, *own, low-adh81*, *med-adh11* and *high-adh1* promoter versions of *vps1302*, 0h, 3h and 6h following TetR-Clr4* release; relative to *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates).

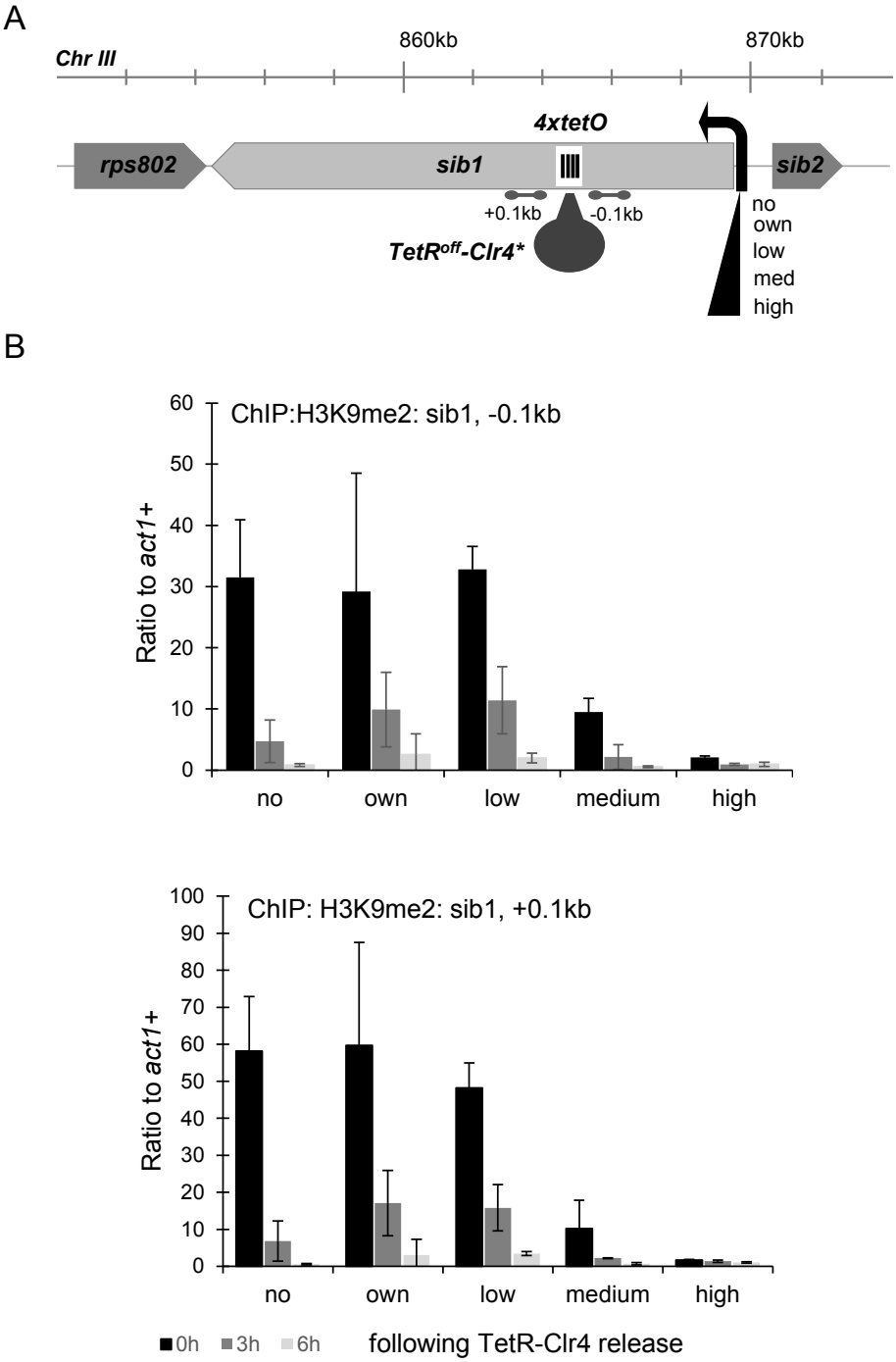

Figure 6.9: High expression prevents establishment of H3K9 methylation by tethered Clr4 methyltransferase in *sib1*

(A) Position of *tetO* sites and tethered TetR-Clr4* within the *sib1* locus on chromosome III. The *own* promoters of *sib1* was deleted by replacement with *ura4*$^+$ (*no*) or swapped to the *low*, medium (*med*), or *high* versions of the constitutive *adh1* promoter.
(B) ChIP analysis of H3K9me2 levels on *no*, *own, low-adh81*, *med-adh11* and *high-adh1* promoter versions of *sib1*, 0h, 3h and 6h following TetR-Clr4* release; relative to *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates).

Strikingly, it is noticeable that the initial H3K9me2 levels are dramatically lower when high levels of expression are driven over the tethering sites placed within the *vps1302* and *sib1* genes (Figure 6.8 and Figure 6.9). When under the control of the strongest *adh1* promoter, H3K9me2 at *sib1* and *vps1302* tethering sites was barely detectable; only an enrichment approximately 2 fold relative to the *act1*$^{+}$ gene was detected adjacent to the inserted *tetO* sites at both loci. This finding indicates that H3K9 methylation cannot be established in an environment of high transcription. Thus high levels of transcription appear to prevent the assembly of heterochromatin at a euchromatic site.

DISCUSSION

In this chapter, heterochromatin maintenance at ectopic locations was assessed at two new loci in order to determine whether the lack of H3K9me maintenance observed at *ura4:4xtetO-ade6*$^{+}$ can be explained by a general feature of ectopic heterochromatin or is specific to the *ura4* locus. The aim was to provide a potentially more suitable environment with features such as low transcription and low histone turnover and determine if such an environment stabilises H3K9me retention following release of tethered Clr4.

From the results in this chapter, it is clear that the assembly of the TetR-Clr4* heterochromatin domain is affected by the level of transcription at the tethering site since high transcription prevents efficient assembly of H3K9me2 domains. Moreover, in a strain containing the three tethering sites, H3K9me2 is more enriched at the low-expressed gene *sib1* than at the *ura4* locus even though both sites contain identical *4xtetO* sites. It is possible that high transcription competes with the heterochromatin machinery at the tethering site by recruiting transcription associated proteins such as RNAPII and HAT, that might compete with heterochromatin components for the association with the chromatin but also modify the chromatin by addition of active marks (H3K4me3, H3K9ac, H3K14ac) that counteract heterochromatin spreading (Buscaino et al., 2013). High transcription might also prevent heterochromatin assembly by increasing the histone turnover at those loci thus promoting the eviction of H3K9 methylated histones H3. Histone H3 turnover at the *vsp1302* and *sib1* genes under the control of *adh* promoters of different strength could be assessed using the RITE system to address this question.

As well as the transcriptional activity, the size of the tethering array might also influence heterochromatin assembly at the tethering site. *vps1302:1xtetO* has a single *tetO* binding site for TetR inserted in the ORF and assembles a smaller heterochromatin domain (both in amplitude and broadness) compared to *sib1* into which *4xtetO* were inserted. Although *vps1302:1xtetO* might be less permissive for heterochromatin assembly than *sib1:4xtetO*, it is possible that the multiple *tetO* elements promotes the assembly of a broader heterochromatin domain by stably recruiting more TetR-Clr4* to the chromatin. Consistent with this hypothesis, TetR-Clr4 tethering to a *10xtetO* array at the *ura4* locus results in the formation of a broader heterochromatin domain than the one obtained from a *4xtetO* array inserted at the same locus in this study (Ragunathan et al., 2015). It has however to be noted that in that study, Ragunathan et al use a full length TetR-Clr4 that is not deleted of Clr4 chromodomain which might favour heterochromatin spreading. ChIP sequencing experiments could be used to better assess and compare the size of the heterochromatin domains formed at the three tethering loci present in the same cells.

Although, high levels of transcription prevents heterochromatin assembly, tethering TetR-Clr4* within an environment of low transcription and low histone turnover away from essential genes and regulatory sequences was found to be insufficient to allow H3K9me maintenance at these euchromatic tethering loci. Heterochromatin is lost very rapidly from all tethering sites upon TetR-Clr4* release and this loss seems to be independent of the level of expression or the size of the heterochromatin domain formed at the tethering site. H3K9me is not maintained longer at the broader heterochromatin domain formed over the *sib1:4xtetO* locus compared to the smaller domain at the *vps1302:1xtetO* locus. However, time course experiments taking samples for H3K9me2 ChIP at shorter intervals following TetR-Clr4* release should be performed in a strain containing the three *tetO* sites in order to assess more accurately the dynamics of H3K9me loss form these distinct tethering loci.

From the data presented in Chapters 5 it was evident that H3K9me can direct a mechanism that replenishes its levels and prevent its dilution with unmodified new H3 assembled during replication. In this chapter, I have shown that H3K9me loss upon TetR-Clr4* release cannot be prevented or delayed when a domain of synthetic ectopic heterochromatin is assembled over regions of low histone turnover. It is therefore likely that the rapid loss of H3K9me-dependent ectopic heterochromatin at all ectopic locations and under all conditions tested is due to an active mechanism

that either removes methyl groups from resident H3K9 methylated nucleosomes or evicts the histones carrying this mark and thus prevents the transmission of H3K9 methylation and heterochromatin when it is assembled at inappropriate loci.

CHAPTER 7: Investigating the role of histone demethylases in H3K9 methylation removal after TetR-Clr4* release from an ectopic heterochromatin locus

INTRODUCTION

The results presented in Chapters 4, 5 and 6 show that an established domain of H3K9 methylated chromatin can not be retained at ectopic *tetO* containing loci following release of the TetR-Clr4* initiator. However, the loss of H3K9me is not caused by dilution through replication, suggesting that a maintenance mechanism can operate during S phase to install the H3K9me mark on new histone H3 and restore H3K9me (Chapter 5). In addition, since the tethering of TetR-Clr4* within genes that exhibit low levels of transcription and histone H3 turnover did not improve H3K9me maintenance, the rapid loss of H3K9me from these ectopic loci can not be caused by transcription-coupled histone turnover. It is possible that the failure to retain H3K9 methylation at the three tested loci reflects some general feature of euchromatin and that, rather than being passively diluted during replication or evicted by transcription-induced histone turnover, H3K9me might be actively removed by demethylation upon TetR-Clr4* release. This chapter investigates whether histone demethylases are responsible for removing H3K9 methylation from histones at TetR-Clr4* dependent ectopic heterochromatin loci.

Phenotypic, biochemical and bioinformatic analyses have identified nine genes encoding putative lysine demethylases in *S. pombe* containing conserved JmjC or FAD-dependent amine oxidase domains SWIRM. The Lsd1 and Lsd2 proteins are members of the SWIRM family while the Jmj1, 2, 3 ,4, Msc1, Lid2 and Epe1 contain a JmjC domain (Table 7.1). A complex containing both the Lsd1 and Lsd2 amino-oxidase containing proteins has previously been shown to display H3K9me demethylase activity *in vitro* and *in vivo* (Lan et al., 2007). Lsd1 and Lsd2 were also shown to be enriched at the promoters of active genes and at boundary regions that lie adjacent to centromeres and the silent mating type locus. Mutation in these proteins results in increased H3K9me3 over some promoter regions and the spreading of H3K9 methylation beyond its normal boundaries (Gordon et al., 2007; Lan et al., 2007).

JMJC lysine demethylases		
Jmj1	Poorly characterised	
Jmj2	H3K4me2/me3 demethylase	(Huarte, Lan et al. 2007)
Jmj3	Poorly characterised	
Jmj4	Poorly chracterised	
Msc1	No demethylase activity	(Klose, Kallin et al. 2006)
Lid2	H3K4me3 demethylase	(Li, Huarte et al. 2008)
Epe1	Putative H3K9 demethylase	(Trewick, et al. 2007)(Trewick, et al. 2005)
FAD-dependent amine oxidase		
Lsd1	H3K9me/ H3K4me demethylase	(Gordon, et al. 2007)(Lan, et al. 2007)
Lsd2	H3K9me/ H3K4me demethylase	(Gordon, et al. 2007)(Lan, et al. 2007)

Table 7.1 Putative histone demethylases and their catalytic activity in *S. pombe.*

Defective Lsd1 or Lsd2 also leads to an increase in total H3K4me levels, suggesting that they may also act as H3K4me demethylases (Gordon et al., 2007). Epe1 is a putative JmjC histone demethylase that is predicted to have H3K9me demethylase activity. Similar to Lsd1 and Lsd2, loss of Epe1 function also results in the formation of heterochromatin at ectopic locations and the spreading of heterochromatin beyond its normal boundaries at centromeres, telomeres and the mating type locus (Ayoub et al., 2003; Trewick et al., 2007; Zofall and Grewal, 2006; Zofall et al., 2012). However, despite numerous attempts, no enzymatic activity has been detected for Epe1 *in vitro* (Tsukada et al., 2006). A candidate approach was adopted to determine whether any of the nine putative histone demethylases is involved in removing H3K9 methylation from *ura4:4xtetO-ade6*$^{+}$ after TetR-Clr4* release from the tethering site by addition of AHT.

RESULTS

7.1 None of the known or putative lysine demethylase affects the integrity of synthetic heterochromatin

To determine if any of the putative demethylase affects heterochromatin integrity at the tethering site when TetR-Clr4* is bound to *4xtetO-ade6*$^{+}$ (-AHT), serial dilution growth assays to assess *ura4:4xtetO-ade6*$^{+}$ silencing and ChIP to measure H3K9me2 levels on *4xtetO-ade6*$^{+}$ were performed on wild-type cells and *epe1*Δ, *jmj1*Δ*, jmj2*Δ*, jmj4*Δ*, lid2-jmj*Δ*, lsd1-K613A,K614A, lsd2-K861A,K862A* and *msc1*Δ mutants (Figure 7.1: experiments performed in collaboration with Alison Pidoux, Allshire lab). *jmj3* mutants were not included in these analyses since cells carrying a *jmj3* gene deletion are inviable and no existing viable mutants have been described. In the remaining eight demethylase mutants tested, silencing of the *ura4:4xtetO-ade6*$^{+}$ reporter gene when TetR-Clr4* is bound (-AHT) is unaffected (Figure 7.1A). However, paler-coloured colonies were formed by *jmj4*Δ*, lid2-jmj*Δ *and lsd1-K613A-K614A* mutants compared to wild-type on –AHT indicator plates. This colony colour assay therefore suggests that the activities of Jmj4, Lid2 or Lsd1 may contribute to the integrity of TetR-Clr4*-mediated heterochromatin to some extent. In agreement with this, H3K9me was enriched at the tethering site in all mutants, however, lower H3K9me2 levels were detected in the *jmj4*Δ*, lid2-jmj*Δ *and lsd1-K613A-K614A* mutants compared to wild-type cells (Figure 7.1B).

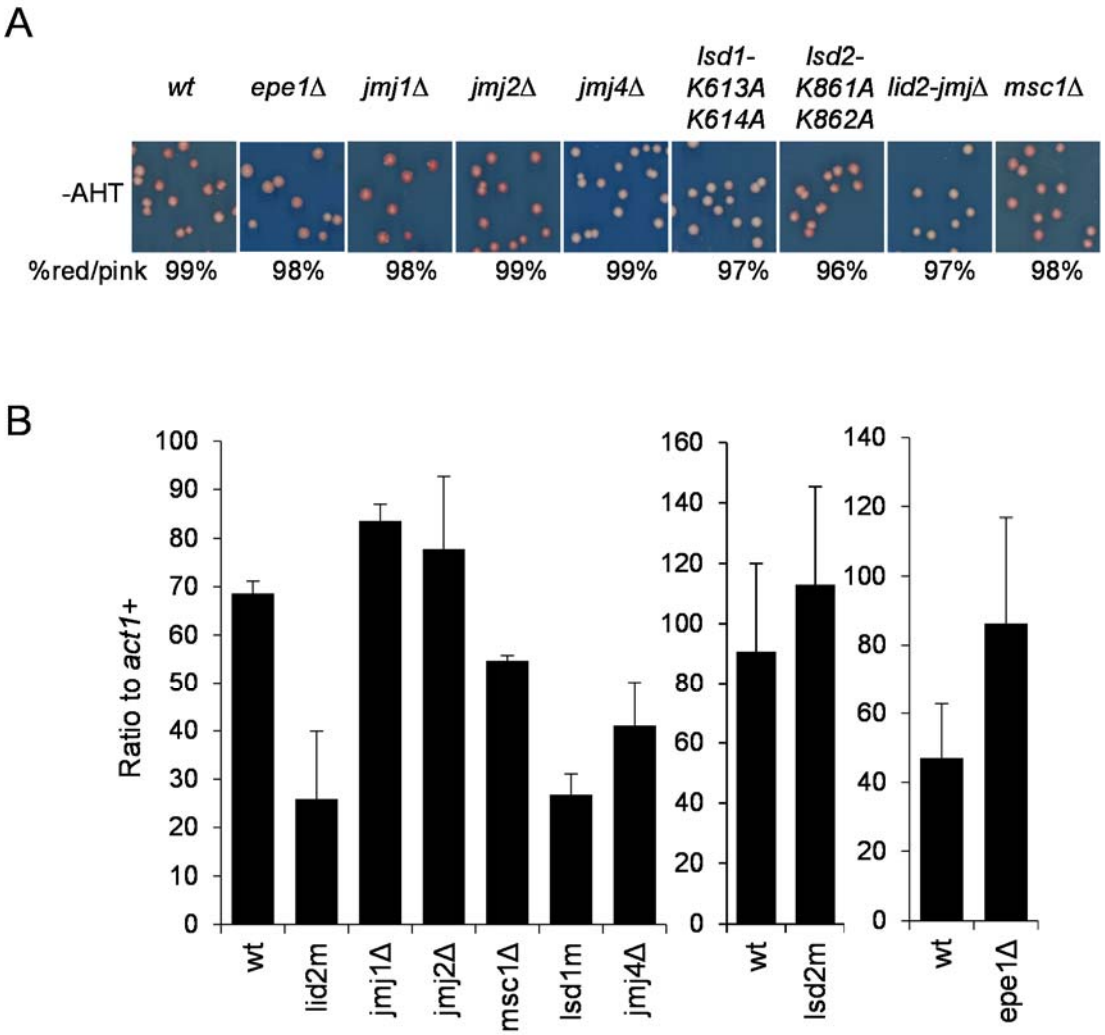

Figure 7.1: heterochromatin is assembled at *4xtetO-ade6*$^{+}$ in the 8 demethylases mutants tested.

(A) Colony colour assay showing that *4xtetO-ade6*$^{+}$ red/pink silent colonies persist in *epe1*Δ, *jmj1*Δ, *jmj2*Δ, *jmj4*Δ, *lid2-jmj*Δ, *lsd1-K613A,K614A lsd2-K861A,K862A* or *msc1*Δ mutants, in cells with *TetR-Clr4** bound to the tethering site (-AHT).
(B) ChIP analysis of H3K9me2 level at the tethering site in *wt* and demethylases mutants in cells containing *TetR-Clr4** bound to the tethering site (-AHT); relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates).

Loss of a specific H3K9 demethylase would perhaps be expected to enhance H3K9 methylation levels at the *4xtetO-ade6*$^+$ tethering site and cause increased silencing of the *4xtetO-ade6*$^+$ reporter. However, none of the tested demethylase mutants displayed a significant increase in *4xtetO- ade6*$^+$ silencing (i.e. darker red colonies on indicator plates), nor was a significant increase in the levels of H3K9me2 levels detected on *4xtetO-ade6*$^+$. This suggests that none of these known or putative lysine demethylases act to counteract heterochromatin assembly over *4xtetO-ade6*$^+$ when TetR-Clr4* is bound to the *tetO* sites.

7.2 Epe1 counteracts silencing around *4xtetO-ade6*$^+$ when TetR-Clr4* is bound to the tethering site

Epe1 is known to interact with heterochromatin in *S. pombe* through a direct interaction with Swi6. Moreover cells lacking Epe1 also rescue silencing defects caused by the loss of RNAi at centromeres, consistent with a role for Epe1 in anti-silencing (Trewick et al., 2005; Trewick et al., 2007; Zofall and Grewal, 2006). The integrity of the heterochromatin formed by TetR-Clr4* when tethered to the *4xtetO-ade6*$^+$ locus was therefore examined in more detail in cells lacking Epe1 (*epe1*Δ*)*. Analysis of H3K9me2 enrichment in wild-type (wt) and *epe1*Δ cells around *4xtetO-ade6*$^+$ revealed that H3K9me2 levels were not significantly affected by the loss of Epe1 when TetR-Clr4* was still bound (Figure 7.2.B). However, transcription is further repressed in *epe1*Δ cells for four of the five tested genes in the vicinity of the *4xtetO* tethering site. The expression of the highly expressed *pmp20*$^+$ gene was decreased by 5-fold in the absence of Epe1 (Figure 7.2.C). The fact that no corresponding increase in H3K9me2 levels was detected on these genes may indicate that Epe1 regulates the expression of these genes independently of any putative H3K9 demethylase activity. Epe1 has been shown to compete with Clr3/SHREC HDAC complex for the association with Swi6 and Chp2 to heterochromatin regions (Shimada et al., 2009). It is therefore possible that Epe1 might prevent silencing of the genes that flank the tethering site by hindering the recruitment of the Clr3/SHREC HDAC complex by Swi6 and Chp2. It is also possible that in the absence of a putative H3K9 demethylase such as Epe1, more H3K9me2 is converted to the trimethylated H3K9me3 state; however, as with H3K9me2, the levels of H3K9me3 detected at the TetR-Clr4* tethering site were not significantly different between wt and *epe1*Δ cells. Thus the di- and tri- methylated forms of H3K9 may not be a substrate for demethylation by Epe1 when TetR-Clr4* is bound to these *tetO* sites (Figure 7.3.B).

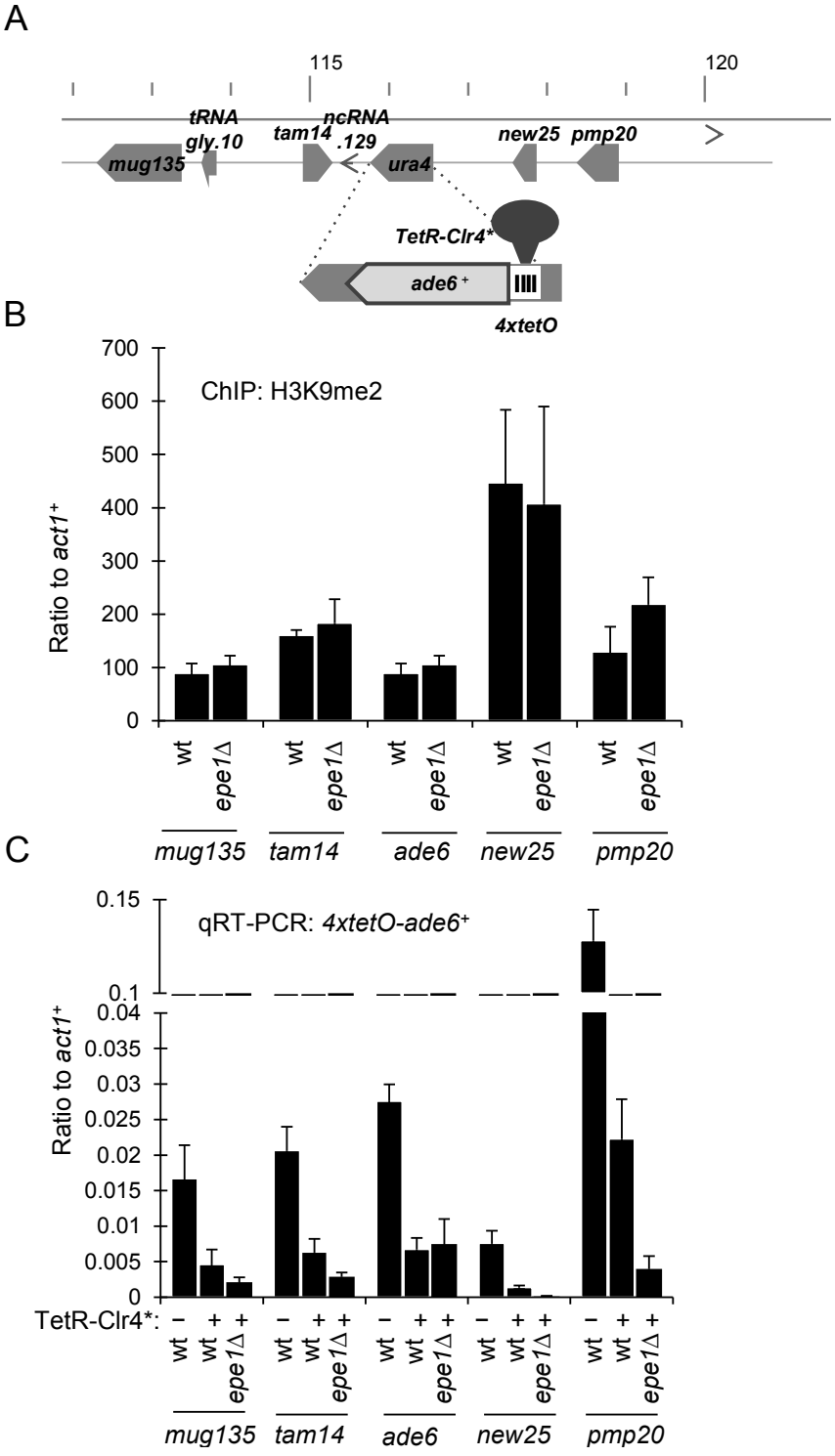

Figure 7.2: Epe1 counteracts gene silencing at *4xtetO-ade6*$^+$.
(A) *4xtetO-ade6*$^+$ test locus with the position of primer pairs relative to tethered TetR-Clr4*.
(B) ChIP analysis of H3K9me2 level on *mug135*$^+$, *tam14*$^+$, *4xtetO-ade6*$^+$, *new25*$^+$ and *pmp20*$^+$ in wild type and *epe1*Δ relative to *act1*$^+$; (n=3).
(C) RT-PCR analysis to monitor expression levels of *mug135*$^+$, *tam14*$^+$, *4xtetO-ade6*$^+$, *new25*$^+$ and *pmp20*$^+$ in wild type and *epe1*Δ relative to *act1*$^+$. Data are means ± SD (*n* = 3 biological replicates)

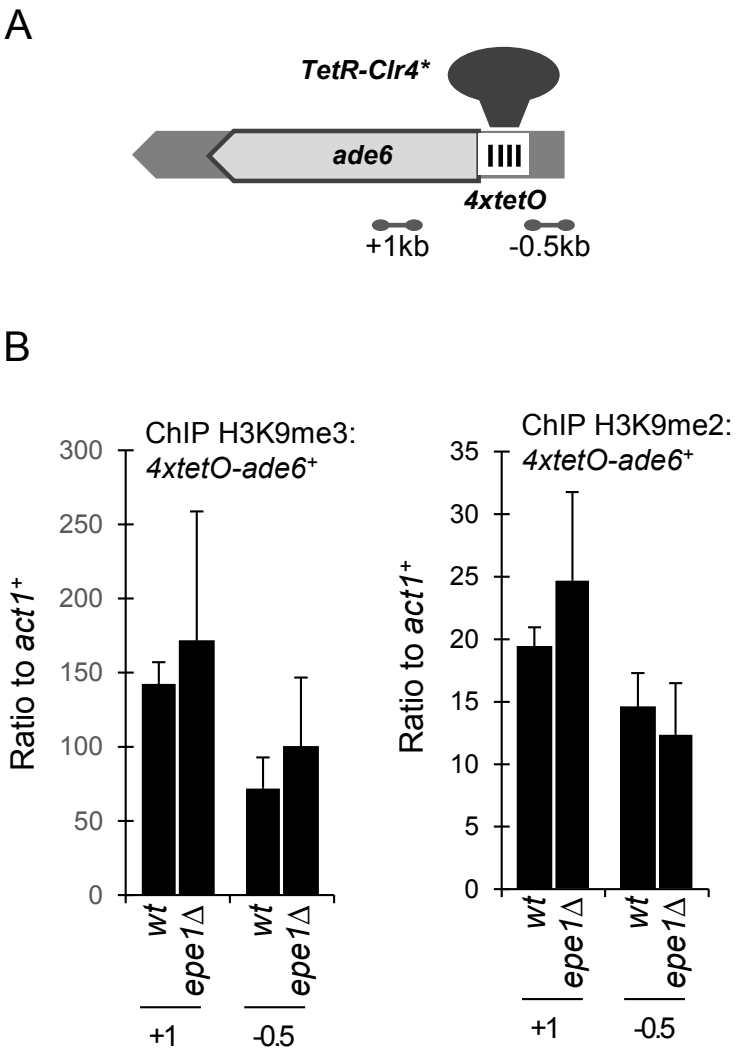

Figure 7.3: H3K9me2 and H3K9me3 levels remain unchanged at the tethering site in cells lacking Epe1.

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*
(B) ChIP analysis of H3K9m3 (left panel) and H3K9me2 (right panel) levels at *4xtetO-ade6*$^{+}$ in wild type and *epe1*Δ when TetR-Clr4* is bound to *4xtetO-ade6*$^{+}$, relative to *act1*$^{+}$. Data are means ± SD (*n* = 3 biological replicates).

Epe1 counteracts the silencing of several genes within the heterochromatin domain formed by TetR-Clr4* bound to *4xtetO-ade6*$^{+}$. On centromere repeat elements, continued transcriptional repression during most of the cell cycle is important for the maintenance of heterochromatin. It is therefore possible that the anti-silencing activity of Epe1 might somehow prevent the maintenance of H3K9me and thus heterochromatin silencing within the *4xtetO-ade6*$^{+}$ locus upon TetR-Clr4* release.

7.3 Lack of Epe1 in cells allows maintenance of *4xtetO-ade6*$^{+}$ silencing upon TetR-Clr4* release

The loss of *ura4:4xtetO-ade6*$^{+}$ silencing resulting from TetR-Clr4* release may be caused by Epe1 or the other known and putative demethylases (KDMs). To determine if any of these KDMs are involved, expression of the *4xtetO-ade6*$^{+}$ gene was assessed in wild-type, *epe1*Δ, *jmj1*Δ, *jmj2*Δ, *jmj4*Δ, *lid2-jmj*Δ, *lsd1-K613A,K614A, lsd2-K861A,K862A* and *msc1*Δ cells by colony formation on indicator plates containing AHT, which releases TetR-Clr4* from the *tetO* sites. Plating on +AHT plates resulted in the loss of *4xtetO-ade6*$^{+}$ silencing in wild-type and all KDM mutants except for *epe1*Δ that formed red/pink *ade6*-repressed colonies (Figure 7.4.A). 83% of *epe1*Δ cells formed red/pink colonies, demonstrating that silencing of *ura4:4xtetO-ade6*$^{+}$ is maintained in the absence of Epe1 even after TetR-Clr4* release from the tethering site (+AHT). Since approximately 20 divisions are required to form an average sized colony, this demonstrates that in the absence of Epe1, silencing of *ura4:4xtetO-ade6*$^{+}$ can be maintained over many cell divisions following TetR-Clr4* dissociation from *ura4:4xtetO-ade6*$^{+}$. This result suggests that Epe1 might prevent the epigenetic transmission of heterochromatin at ectopic loci.

To further investigate the maintenance of silencing, successive platings of red *4xtetO-ade6*$^{+}$ *epe1*Δ silenced colonies were performed on +AHT indicator plates (TetR-Clr4* released) (Figure 7.5.A). The colonies formed on the 4th replating had undergone approximately 80 divisions without TetR-Clr4* being bound and yet they still formed red silent colonies. This indicates that in the absence of Epe1, silent heterochromatin can be maintained at this ectopic locus for many generations. The colonies formed by *ura4:4xtetO-ade6*$^{+}$ *epe1*Δ cells on +AHT on indicator plates frequently displayed red/pink and white sectors, indicating that the silent state is semi-stable (Figure 7.5.B). Since all colonies originate from single cells the counting of sectored colonies allows the frequency of switching from silent to expressed state to be estimated.

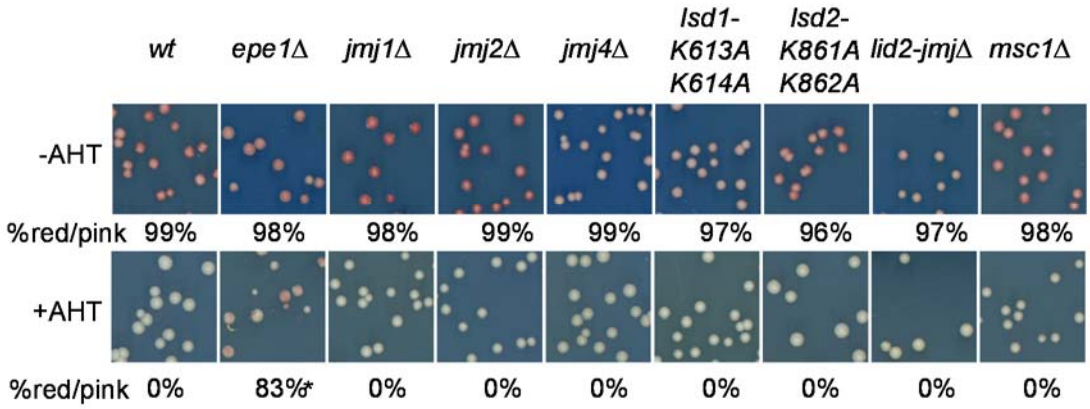

Figure 7.4: *4xtetO-ade6*$^{+}$ reporter gene remains silent in cells lacking Epe1 following TetR-Clr4* release.

Colony colour assay showing *4xtetO-ade6*$^{+}$ silencing in presence (-AHT) or absence (+AHT) of TetR-Clr4* bound to the tethering site. red/pink silent colonies persist in *epe1*Δ mutants, but not wt, *jmj1*Δ*, jmj2*Δ*, jmj4*Δ*, lid2-jmj*Δ*, lsd1-K613A,K614A lsd2-K861A,K862A* or *msc1*Δ mutants, in the absence of tethered *TetR-Clr4** (+AHT). In *epe1*Δ in absence of *TetR-Clr4** , 83%* cells form red/pink colonies. We observe 6% of red colonies, 77% of pink/sectored colonies and 17% of white colonies.

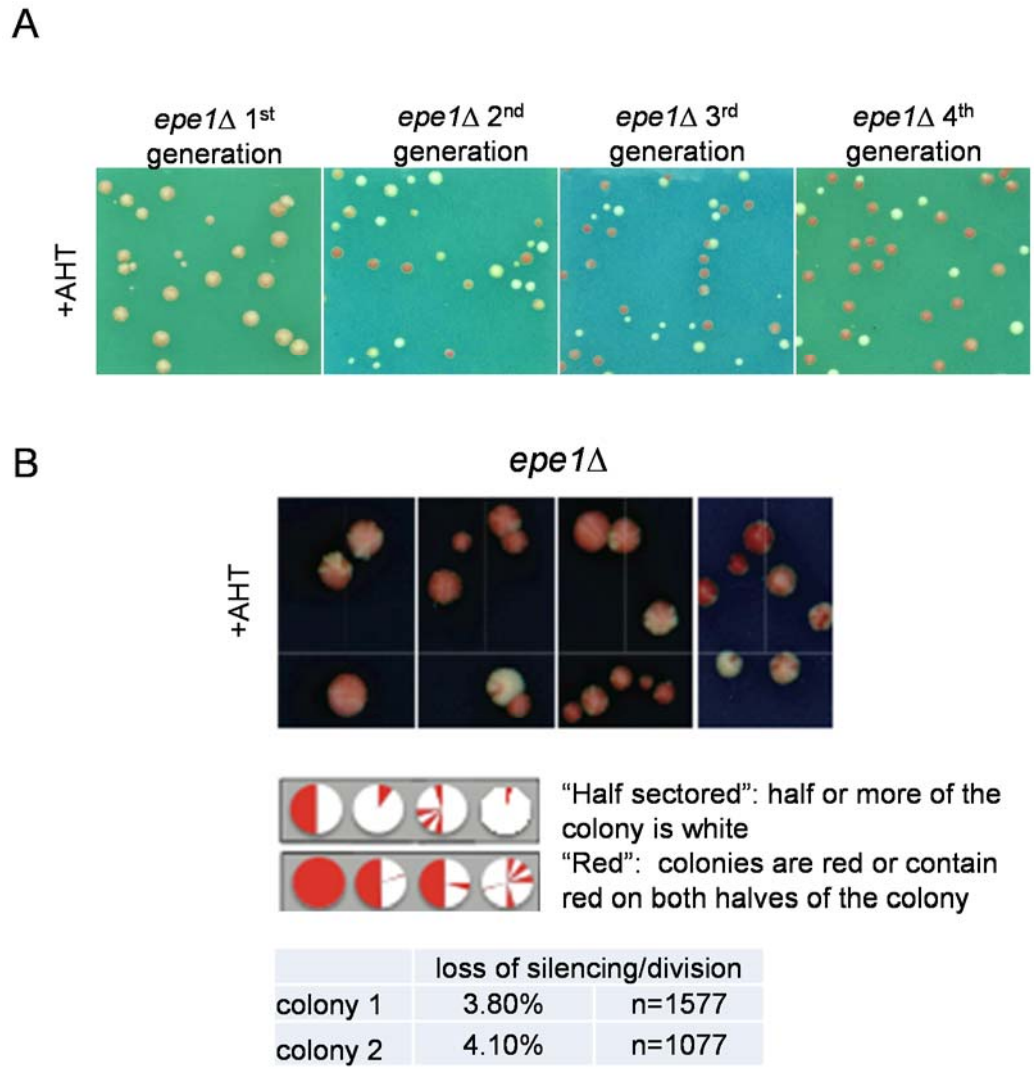

Figure 7.5: *4xtetO-ade6*⁺ remains silent through many cell divisions in the absence of Epe1 after TetR-Clr4* release.

(A) Silencing assay showing that *4xtetO-ade6*⁺ red/pink silent colonies persist in *epe1*Δ in absence of TetR-Clr4* (+ AHT) for many generations. Successive plating of *epe1*Δ red colonies from +AHT plates were plated on fresh +AHT plates. More than 20 divisions are required to form a colony, therefore on the 4th plating, cells were grown in absence of TetR-Clr4* for approximately 80 divisions.
(B) *epe1*Δ colonies form sectors on +AHT, indicating that the silent state is semi-stable.
A sectoring assay (bottom panel) was performed to estimate the rate of loss of silencing per division (experiment performed by Alison Pidoux).

A sectoring assay was therefore performed to estimate the rate of loss of silencing per division. Red *epe1*Δ *tetR-clr4* 4xtetO-ade6*$^+$ colonies were picked from +AHT plates, and re-plated onto fresh +AHT plates. Resultant colonies were categorised as: half-sectored (half or more of the colony was white) in which silencing of *4xtetO-ade6*$^+$ was lost during the first division in one daughter cell and maintained in the other; or red (any colony with red sectors in both halves of the colony) in which silencing was maintained in both initial daughter cells (but may have been lost subsequently in some daughter cells). Pure white colonies were excluded as they must have already lost silencing of *4xtetO-ade6*$^+$ at the time of plating. The number of 'half-sectored' colonies as a percentage of all colonies containing red colour gives an estimate of the rate of loss of *4xtetO-ade6*$^+$ silencing per division. Such analyses revealed that silencing of *4xtetO-ade6*$^+$ is lost at a rate of loss of ~4%/division (Figure 7.5.B analyses performed by Alison Pidoux; Allshire lab). Therefore it can be concluded that in the absence of Epe1 silencing of *4xtetO-ade6*$^+$ can be maintained epigenetically through many cell divisions.

7.4 Heterochromatin loss from *ura4:4xteto-ade6*$^+$ following TetR-Clr4* release requires an intact JmjC demethylase domain in Epe1

Based on the presence of a JmjC domain, Epe1 is a putative 2-OG-Fe(II)-dependent dioxygenase that binds Fe(II) and 2-oxyglutarate via a conserved arginine or lysine residue downstream of the iron-binding motif (Tsukada et al., 2006). The reaction catalyzed allows these JmjC containing proteins to act as lysine demethylases. The maintenance of *4xtetO-ade6*$^+$ silencing upon TetR-Clr4* release was therefore also assessed in cells expressing versions of the Epe1 protein that carry mutations in the putative catalytic domain. The *epe1-H297A* mutation is defective in the Fe(II) binding site whereas *epe1-K314A* is unable to bind the required co-factor 2-oxyglutarate. As with *epe1*Δ, both these enzyme inactivating mutations allowed maintenance of *4xtetO-ade6*$^+$ silencing upon TetR-Clr4* release (Figure 7.6.A). The percentage of red/pink *4xtetO-ade6*$^+$ silent colonies formed on indicator plates containing AHT was 65% for *epe1*Δ, 77% for *epe1-H297A* and *78%* for *epe1-K314A* cells. In contrast, 100% of colonies formed by *epe1*$^+$ wild type cells were white, indicating that *4xtetO-ade6*$^+$ silencing was lost (for detailed colony counts see Table.7.2; this work was performed in collaboration with Alison Pidoux; Allshire Lab).

To determine whether the maintenance of *4xtetO-ade6*$^+$ silencing in *epe1* mutants

after TetR-Clr4* removal was due to the maintenance of H3K9me at the ectopic locus, red-*4xtetO-ade6*$^+$ silent colonies formed by *epe1*Δ, *epe1-H297A* and *epe1-K314A* cells were picked from -AHT (TetR-Clr4* bound) and +AHT (TetR-Clr4* unbound) plates and grown in both –AHT and +AHT containing media. Cells were harvested and processed for ChIP analysis. Anti-FLAG ChIP to detect TetR-Clr4* at the tethering site showed that TetR-Clr4* was released from *4xtetO-ade6*$^+$ following growth in the presence of AHT treatment in all *epe1* mutants (Figure 7.6.C). H3K9me2 ChIP analyses revealed that *epe1*Δ, *epe1-H297A* and *epe1-K314A* cells retained at least 50% of their initial H3K9me2 levels at the *4xtetO-ade6*$^+$ locus whereas H3K9me2 was lost from *4xtetO-ade6*$^+$ in wild-type cells (Figure 7.6.D). These results show that Epe1 prevents heterochromatin maintenance over *4xtetO-ade6*$^+$ and strongly suggest that Epe1 acts as a demethylase that removes H3K9me when it is assembled at inappropriate places.

7.5 Heterochromatin maintenance at *4xtetO-ade6*$^+$ in *epe1*Δ cells requires intact Swi6 and Clr4 chromodomain

Clr4 is the only H3K9 methyltransferase in *S. pombe* (Nakayama et al., 2001). The fact that H3K9me2 persists on *4xtetO-ade6*$^+$ when TetR-Clr4* is released from the tethering site demonstrates that endogenous Clr4 must be recruited to *4xtetO-ade6*$^+$ where it ensures that the heterochromatin state is propagated through cell division in a sequence independent manner. It is known that Clr4 binds H3K9me directly via its chromodomain and indirectly via its interaction with Swi6, thus providing a read-write mechanism that allows heterochromatin to spread along chromatin from nucleation sites (Haldar et al., 2011; Zhang et al., 2008). To determine if the interaction of endogenous Clr4 with H3K9me via its chromodomain and Swi6 are involved in promoting heterochromatin maintenance at *4xtetO-ade6*$^+$ in the absence of Epe1, the persistence of H3K9me2 was assessed in *epe1*Δ cells that also lack Swi6 (*swi6*Δ), Clr4 (*clr4*Δ) or just the Clr4 chromodomain (*clr4-cdd*). *4xtetO-ade6*$^+$ silencing and H3K9me2 levels were assessed in these three mutants at the tethering site when TetR-Clr4* is bound to *4xtetO-ade6*$^+$ (-AHT) or released from *4xtetO-ade6*$^+$ (+AHT) in wild type or *epe1*Δ cells. When TetR-Clr4* is bound to *4xtetO-ade6*$^+$ (-AHT), both *clr4*Δ and *clr4-cdd* form red/pink *ade6*$^+$ repressed colonies and assemble H3K9me2 at the tethering site (Figure 7.7).

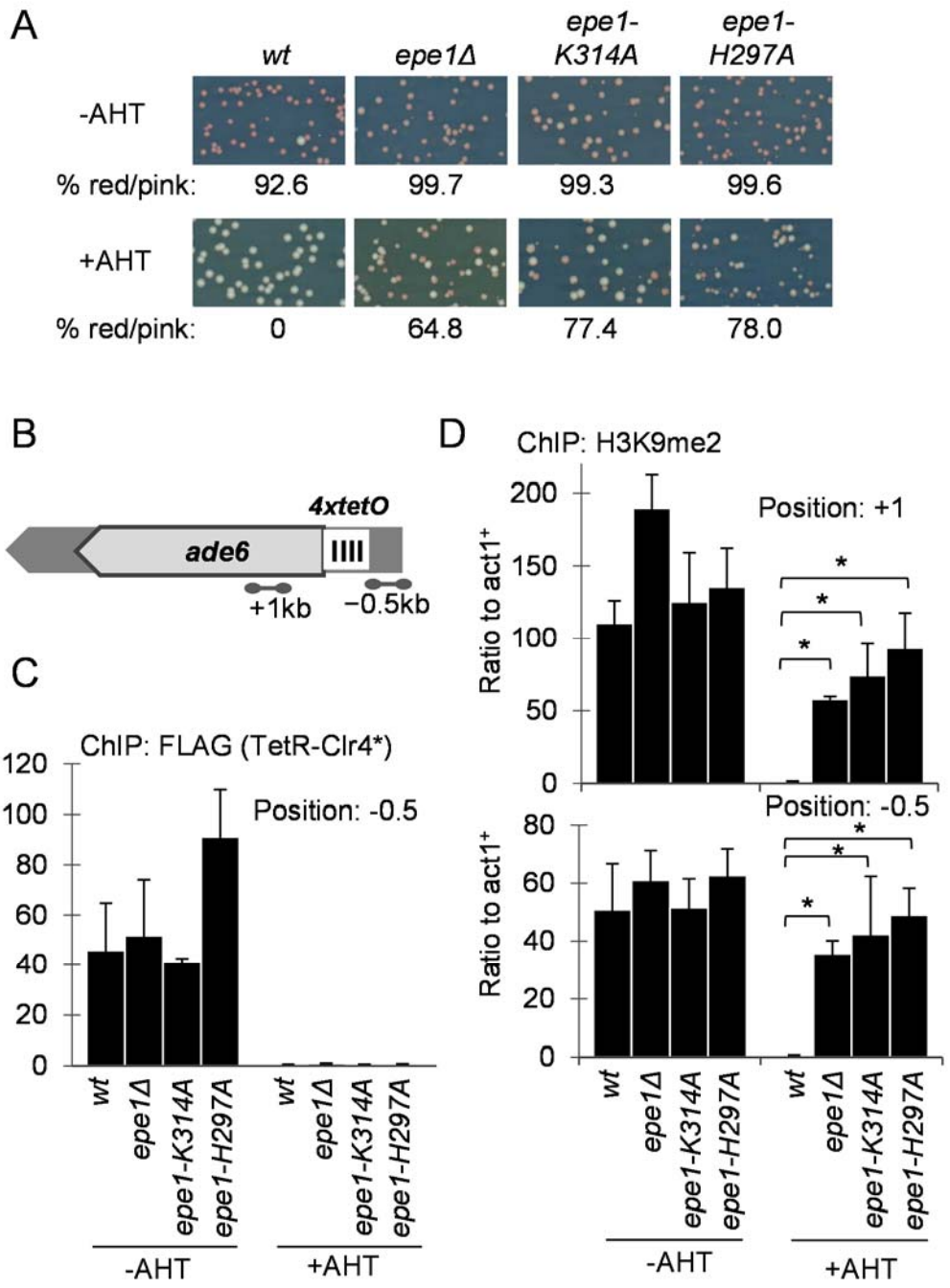

Figure 7.6: heterochromatin loss at *ura4:4xtetO-ade6*⁺ upon TetR-Clr4* release depends on an intact Epe1 JmjC demethylase domain.

(A) Colony colour assay showing that *4xtetO-ade6*⁺ red/pink silent colonies persist in *epe1*Δ, epe1-K314A and epe1-H297A mutants but not wt, in the absence of tethered *TetR-Clr4** (+AHT).

(B) Position of the primers used relative to *4xtetO-ade6*⁺

(C) ChIP analysis of TetR-Clr4* level at the tethering site in wild-type, *epe1*Δ, *epe1-K314A* and *epe1-H297A* cells carrying *4xtetO-ade6*⁺ in presence or absence of AHT, relative to act1⁺. Data are means ± SD (*n* = 3 biological replicates).

(D) ChIP analysis of H3K9me2 level in wild-type, *epe1*Δ, *epe1-K314A* and *epe1-H297A* cells carrying *4xtetO-ade6*⁺ with (-AHT) or without (+AHT) tethered TetR-Clr4*, relative to act1⁺. Data are mean ±SD (*n=3* biological replicates), *P<0.05* (t-test).

genotype	colony plated (n)	% white	% pale pink /sectored	% red/pink	% any pink	colony plated AHT (n)	% white	% pale pink /sectored	% red/pink	% any pink
						AHT	AHT	AHT	AHT	AHT
wt	1 (473)	1.1	0	98.9	98.9	1 (437)	100	0	0	0
wt	2 (184)	0.5	0	99.5	99.5	2 (187)	100	0	0	0
wt	3 (332)	0.6	0	99.4	99.4	3 (341)	100	0	0	0
wt	4 (106)	33.0	0	67.0	67.0	4 (102)	100	0	0	0
wt	5 (99)	9.1	0	90.9	90.9	5 (96)	100	0	0	0
wt	6 (472)	0	0	100	100	6 (410)	100	0	0	0
	average	**7.4**	**0**	**92.6**	**92.6**		**100**	**0**	**0**	**0**
epe1D	1 (45)	0	0	100	100	1 (46)	76.1	8.7	15.2	23.9
epe1D	2 (85)	0	1.2	98.8	100	2 (82)	46.3	34.1	19.5	53.7
epe1D	3 (317)	0	0.3	99.7	100	3 (270)	16.3	67.4	16.3	83.7
epe1D	4 (628)	0	0	100	100	4 (651)	5.2	79.6	15.2	94.8
epe1D	5 (104)	1.9	0	98.1	98.1	5 (173)	33.5	45.7	20.8	66.5
epe1D	6 (90)	0	0	100	100	6 (158)	33.5	51.9	14.6	66.5
	average	**0.3**	**0.2**	**99.4**	**99.7**		**35.2**	**47.9**	**16.9**	**64.8**
epe1-K314A	1 (342)	0.3	0	99.7	99.7	1 (311)	5.1	71.7	23.2	94.9
epe1-K314A	2 (49)	2.0	0	98.0	98.0	2 (49)	16.3	73.5	10.2	83.7
epe1-K314A	3 (72)	0	0	100	100	3 (52)	42.3	53.8	3.8	57.7
epe1-K314A	4 (151)	0	0	100	100	4 (151)	29.8	60.9	9.3	70.2
epe1-K314A	5 (67)	0	0	100	100	5 (61)	29.5	50.8	19.7	70.5
epe1-K314A	6 (174)	1.7	1.1	97.1	98.3	6 (263)	12.5	72.6	14.8	87.5
	average	**0.7**	**0.2**	**99.1**	**99.3**		**22.6**	**63.9**	**13.5**	**77.4**
epe1-H297A	1 (551)	0.4	0	99.6	99.6	1 (270)	9.6	64.1	26.3	90.4
epe1-H297A	2 (133)	0	0.8	99.2	100	2 (123)	30.1	60.2	9.8	69.9
epe1-H297A	3 (622)	0	0	99.8	100	3 (233)	12.9	76.4	10.7	87.1
epe1-H297A	4 (43)	0	0	100	100	4 (41)	43.9	36.6	19.5	56.1
epe1-H297A	5 (106)	1.9	0	98.1	98.1	5 (121)	14.9	67.8	17.4	85.1
epe1-H297A	6 (122)	0	0	100	100	6 (136)	20.6	59.6	19.9	79.4
	average	**0.4**	**0.2**	**99.5**	**99.6**		**22.0**	**60.8**	**17.3**	**78.0**

Table 7.2: Silencing of *4xtetO-ade6*$^+$ in wild-type and *epe1* mutants.

Silencing of *4xtetO-ade6*$^+$ is indicated by red/pink colony colour. For each strain, six single red colonies were picked from limiting adenine plates and resuspended in dH_2O before plating on minimal medium containing limiting adenine, or on the same medium containing AHT and thiamine to release/repress TetR-Clr4*. After 4-5 days of growth at 32^{o}C, colonies were classified according to colour. Colonies exhibiting any pink coloring were included pale pink/sectored category, those with robust colouring were classified as red/pink. Percentages of the different categories are reported, along with average percentages for the six colonies.

Thus, neither endogenous Clr4 nor the chromodomain of Clr4 are essential for directing heterochromatin formation at the tethering site. This heterochromatin must rely on TetR-Clr4* as the only source of H3K9 methyltransferase activity. Interestingly, *swi6*Δ cells form no red/ade6$^+$-repressed colonies when TetR-Clr4* is bound and H3K9me2 is not detected at the tethering site either in *epe1*$^+$ or *epe1*Δ backgrounds. This result indicates that Swi6 is essential for establishing H3K9me at the tethering site. It is likely that Swi6 promotes heterochromatin assembly at the *4xtetO-ade6*$^+$ locus by recruiting HDACs such as Clr3 that are known to be recruited via Swi6 (Sugiyama et al., 2007; Yamada et al., 2005) and have previously been shown to be essential for the formation of ectopic heterochromatin via tethered Clr4 at the *ura4* locus (Kagansky et al., 2009).

Following TetR-Clr4* release in the above strains (Figure 7.7; +AHT), only the *epe1*Δ single mutant maintains *4xtetO-ade6*$^+$ silencing and retains H3K9me2 at the tethering site. In the absence of Clr4 or the Clr4 chromodomain, *4xtetO-ade6*$^+$ silencing was lost and H3K9me2 at *4xtetO-ade6*$^+$ was not detected. These data confirm that the maintenance of H3K9me-dependent heterochromatin at this ectopic tethering site requires endogenous Clr4 with a functional chromodomain. At endogenous centromere repeat elements (*dg*), H3K9me2 was detected in the absence of endogenous Clr4 when TetR-Clr4* was expressed in cells, indicating that TetR-Clr4* can rescue the lack of heterochromatin exhibited by *clr4*Δ cells. However, as expected, H3K9me2 was lost from endogenous centromeres when TetR-Clr4* was released from *4xtetO-ade6*$^+$ and repressed by thiamine (+AHT) in *clr4*Δ cells since such cells are devoid of H3K9 methyltransferase activity (Figure 7.7). These results suggest that H3K9me maintenance at *4xtetO-ade6*$^+$ relies on a read-write mechanism in which H3K9me recruits endogenous Clr4 to the ectopic heterochromatin domain via its chromodomain and through its ability to associate with Swi6. Thus, both Swi6 and Clr4 act to maintain H3K9me at this ectopic locus. Endogenous Clr4 presumably methylates new histone H3 incorporated into the chromatin during replication, or histone H3 incorporated as a result of histone turnover, and also resident histone H3 that has been demethylated and thus can maintain high H3K9me level at the tethering site in the absence of Epe1.

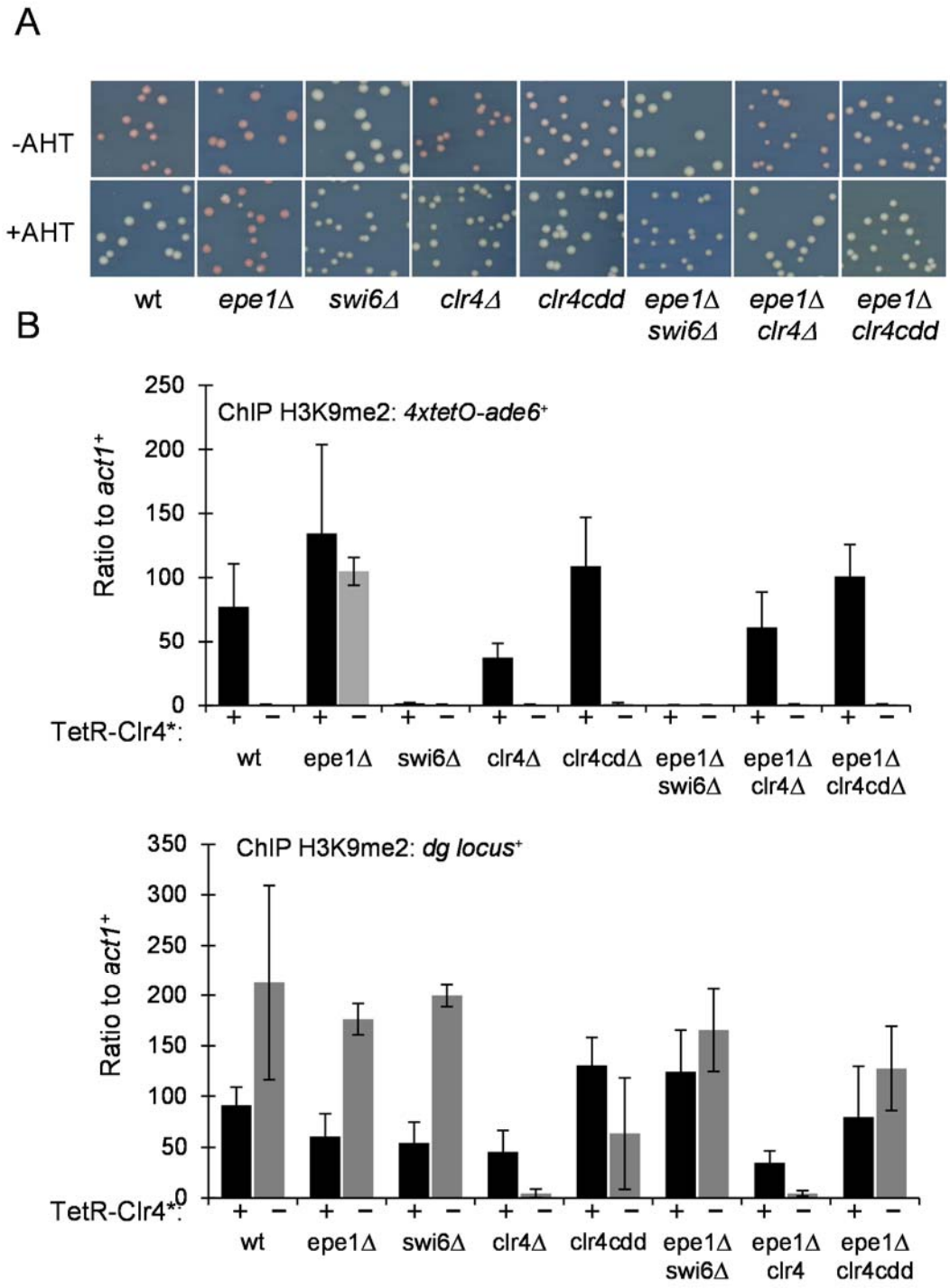

Figure 7.7: H3K9me maintenance on *4xtetO-ade6*$^{+}$ in *epe1*Δ depends on endogenous Clr4 chromodomain and Swi6

(A) Colony colour assay showing that *4xtetO-ade6*$^{+}$ red-pink silent colonies are lost in *epe1*Δ *clr4*Δ *and epe1*Δ *clr4cdd in* the absence of TetR-Clr4* (+AHT). The absence of Swi6 prevents *4xtetO-ade6*$^{+}$ silencing in cells with TetR-Clr4* at the tethering site (-AHT). *cdd*= chromodomain deleted.

(B) ChIP analysis of H3K9me2 level to monitor H3K9me2 enrichment on *4xtetO-ade6*$^{+}$ in wt *vs epe1*Δ, in cells lacking *clr4*, s*wi6* and *clr4* chromodomain (cd) in presence or absence of TetR-Clr4* in the cells; relative to *act1*$^{+}$. n=3, data are mean +/-SD.

7.6 Heterochromatin maintenance at *4xtetO-ade6*$^+$ does not depend on RNAi

At endogenous centromere repeats the maintenance of heterochromatin requires an active RNAi pathway (Chen et al., 2008; Kloc et al., 2008; Verdel et al., 2004; Volpe et al., 2002). Interestingly, loss of Epe1 was found to rescue silencing defects at heterochromatic centromere repeats in RNAi mutants (Trewick et al., 2007). This observation suggests that RNAi-dependent reinforcement of heterochromatin at centromeres might be important to counteract Epe1-dependent loss of heterochromatin stability.

Results presented in Chapter 3 showed that RNAi is not required for heterochromatin assembly at *4xtetO-ade6*$^+$ when TetR-Clr4* is bound to the tethering site. It is however possible that the RNAi machinery recruited via heterochromatin to the *4xtetO-ade6*$^+$ locus induces siRNA production from associated RNAs and it is these siRNAs that act to promote Clr4 recruitment and thus H3K9 maintenance in *epe1*Δ cells after TetR-Clr4* release. Here, the role of RNAi in the maintenance of heterochromatin after TetR-Clr4* release at *4xtetO-ade6*$^+$ is investigated. The *4xtetO-ade6*$^+$ was found to remain silent in many colonies after TetR-Clr4* release in *epe1Δ dcr1*Δ and *epe1*Δ *ago1*Δ double mutant cells. Moreover, moderate to high levels of H3K9me2 was retained at *4xtetO-ade6*$^+$ in these cells (Figure 7.8). This demonstrates that the RNAi machinery is not essential for H3K9me maintenance at the tethering site in the absence of Epe1.

Remarkably, in *dcr1*Δ and *ago1*Δ single mutants a few pale pink/ade6$^+$ repressed colonies were formed on +AHT (TetR-Clr4* released) indicator plates (Figure 7.8.A; not easily distinguishable on resulting images). Consistent with this observation, following release of TetR-Clr4* (+AHT) a 6 and 13 fold enrichment of H3K9me2 was still observed on *4xtetO-ade6*$^+$ relative to *act1*$^+$ in *dcr1*Δ and *ago1*Δ cells, respectively (Figure7.8.B). As expected, H3K9me2 was completely lost from *4xtetO-ade6*$^+$ in wild-type cells and retained in *epe1Δ* cells (Figure 7.8.B). This surprising result shows that in the absence of RNAi, H3K9me heterochromatin can be partially maintained at an ectopic site by epigenetic processes even when Epe1 is present in the cells. One explanation is that the defect in heterochromatin formation at centromeres that occurs in cells lacking RNAi allows the released heterochromatin proteins that form a larger pool of available components which can associate with *4xtetO-ade6*$^+$ and thus compete more effectively with the anti-silencing activity of Epe1 after TetR-Clr4* release.

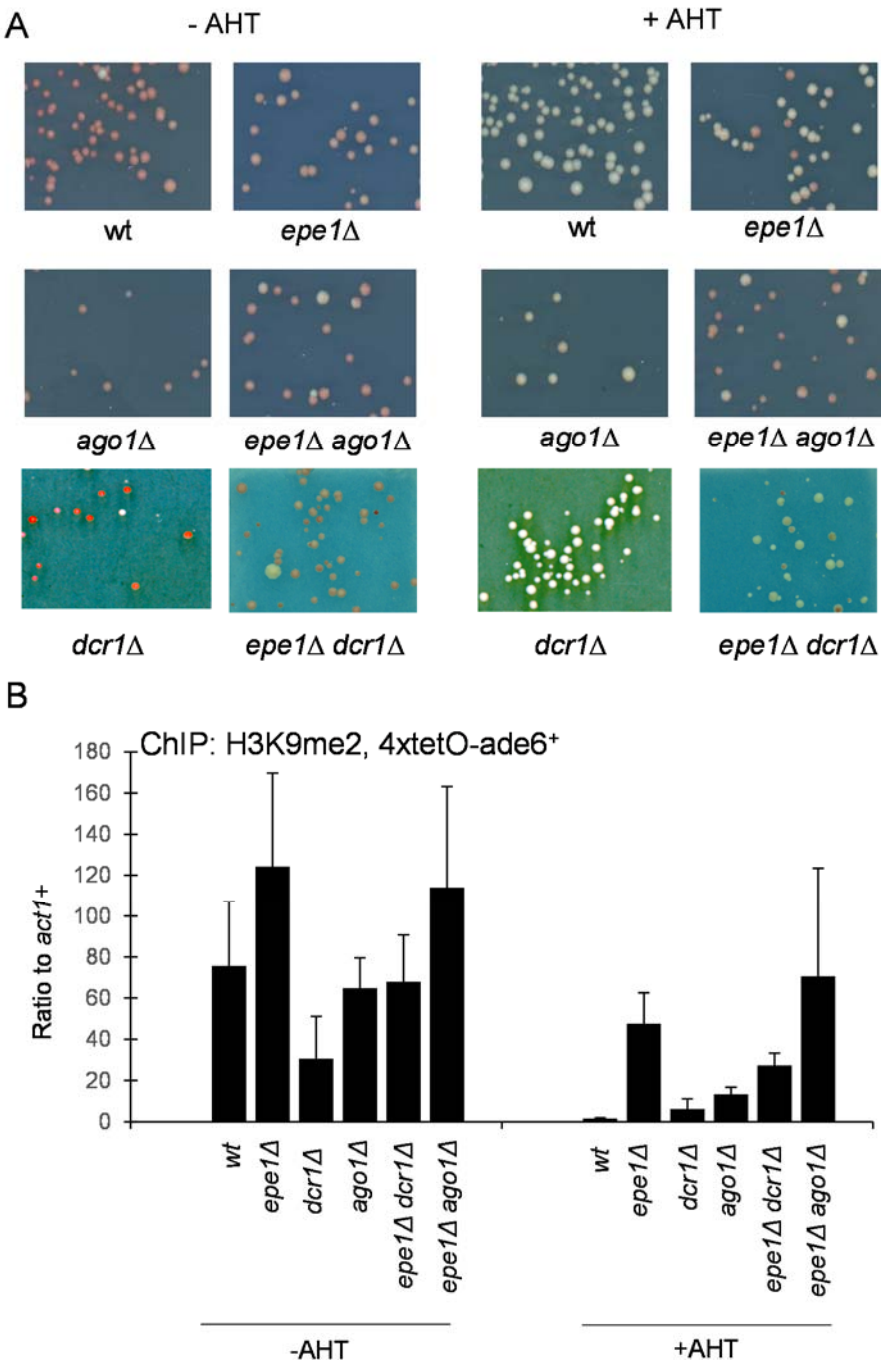

Figure 7.8. RNAi is dispensable for heterochromatin maintenance at *4xtetO-ade6+* in *epe1*Δ

(A) Colony colour assay showing that *4xtetO-ade6*$^{+}$ red/pink silent colonies persist in *epe1*Δ *ago1*Δ *and* in *epe1*Δ *dcr1*Δ in absence of TetR-Clr4* (+AHT).

(B) ChIP analysis of H3K9me2 levels showing that H3K9me2 is partially retained in *epe1*Δ *ago1*Δ *and* in *epe1*Δ *dcr1*Δ in absence of TetR-Clr4* (+AHT). Interestingly, *ago1*Δ and *dcr1*Δ retain low levels of H3K9me2 on +AHT. This may be due to the release of heterochromatin components from RNAi-dependent heterochromatin regions in absence of RNAi that might provide a larger pool of heterochromatin factors available to counteract Epe1 activity at *4xtetO-ade6*$^{+}$. Data are means ± SD (*n* = 3 biological replicates)

It is also possible that the absence of RNAi directly affects Epe1 protein levels, or its activity at the tethering site or more generally in the cells. These results indicate that in the absence of Epe1, heterochromatin is able to self-propagate independently of RNAi at the tethering site and strengthens the idea that H3K9 methylation alone can direct an epigenetic memory system that maintains heterochromatin domains.

7.7 Loss of Epe1 allows maintenance of heterochromatin at *4xtetO-ade6*$^+$ through meiotic divisions

The above analyses show that a domain of heterochromatin can be established ectopically by tethering Clr4 activity to a euchromatic locus. This silent heterochromatin domain persists without direct tethering of Clr4 through multiple cell divisions in cells lacking Epe1, but not in wild-type cells. But can this heterochromatin be copied and passed on through gametes and the process of mating into the next generation? To address this question, the maintenance of heterochromatin at *4xtetO-ade6*$^+$ through mating, meiotic divisions and sporulation was next investigated. Silencing and H3K9me2 were assessed at *4xtetO-ade6*$^+$ in the resulting progeny after one and two rounds of mating during which the construct expressing TetR-Clr4* was crossed out. *epe1*Δ *4xtetO-ade6*$^+$ *tetR-clr4** cells (F0) were crossed to *epe1*Δ cells lacking both *4xtetO-ade6*$^+$ and *tetR-clr4**. F1 *epe1*Δ *4xtetO-ade6*$^+$ progeny that lacked *tetR-clr4** were selected and crossed again to *epe1*Δ cells that lacked *4xtetO-ade6*$^+$ and *tetR-clr4**. The tethering site in the resulting F2 *epe1*Δ *4xtetO-ade6*$^+$ progeny had never been directly bound by TetR-Clr4* in the same nucleus. FLAG ChIP to detect TetR-Clr4* confirmed that it was bound to *4xtetO-ade6*$^+$ in F0 cells but was absent from the F1 and F2 *epe1*Δ progeny containing *4xtetO-ade6*$^+$ all grown in the absence of AHT which would allow TetR-Clr4* to bind *4xtetO-ade6*$^+$ if it was present (Figure 7.9).

Remarkably a high proportion of these F2 *epe1*Δ *4xtetO-ade6*$^+$ progeny that lacked TetR-Clr4* formed red-pink/*ade6*$^+$-repressed colonies indicating that the *4xtetO-ade6*$^+$ reporter remains silent in these cells (Figure 7.10.A). qRT-PCR analyses confirmed that RNA levels generated by *4xtetO-ade6*$^+$ were significantly lower in the F2 than in wild-type cells that were never exposed to TetR-Clr4* and thus did not establish heterochromatin at *4xtetO-ade6*$^+$ (Figure 7.10.C). Consistent with this result, high levels of H3K9me2 were retained over *4xtetO-ade6*$^+$ in F2 cells (Figure 7.11). As a control, *epe1*$^+$ *4xtetO-ade6*$^+$ progeny lacking *tetR-clr4** were obtained from a cross between these F2 *epe1*Δ *4xtetO-ade6*$^+$ cells and wild-type *epe1*$^+$ cells.

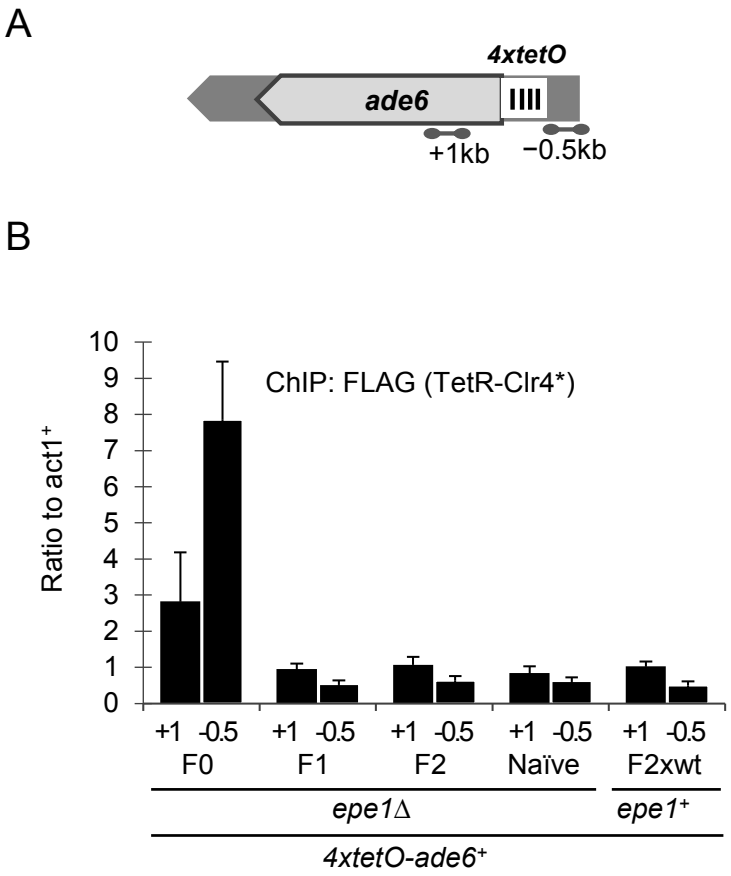

Figure 7.9: F1 and F2 progeny lack TetR-Clr4* in the cells

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs.
(B) ChIP analysis of TetR-Clr4 levels on *4xtetO-ade6*$^{+}$ in indicated cell types, in absence of AHT in the medium, relative to *act1*$^{+}$. Data are mean ±SD (*n=3* biological replicates).

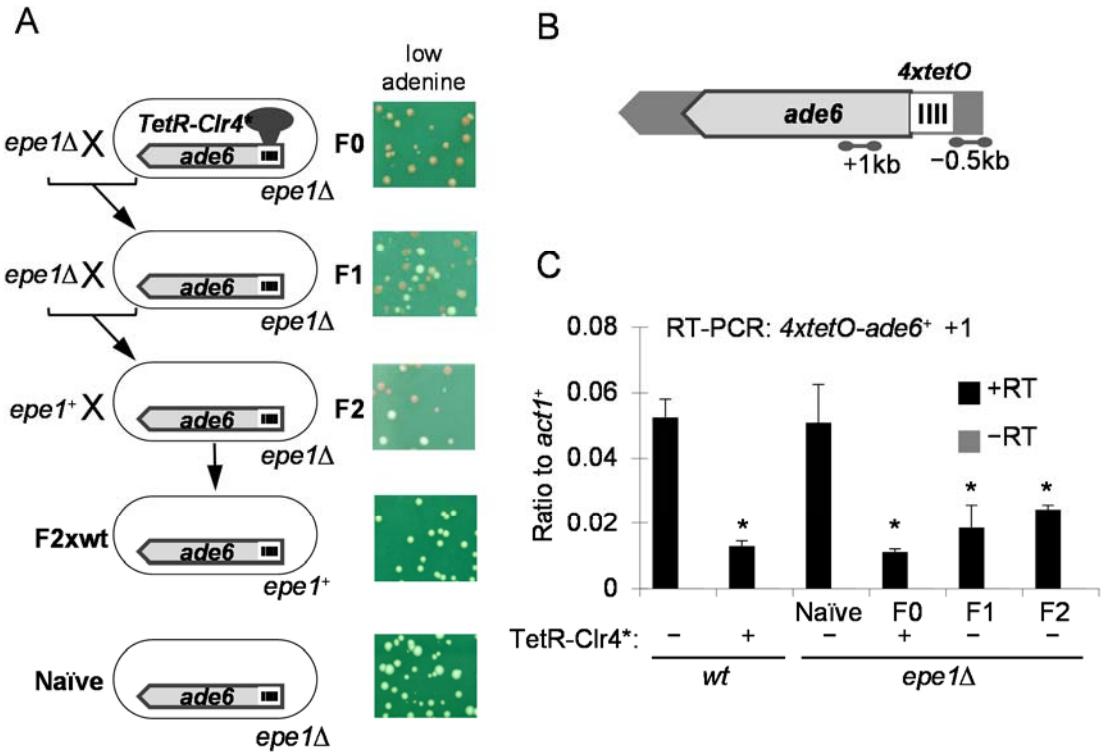

Figure 7.10: *epe1* mutants retain *4xtetO-ade6*$^+$ silencing in absence of TetR-Clr4* through meiosis

(A) F0 *epe1*Δ *4xtetO-ade6*$^+$ *tetR-clr4** cells were crossed to *epe1*Δ lacking TetR-Clr4* and *4xtetO-ade6*$^+$. F1 progeny *epe1*Δ *4xtetO-ade6*$^+$ lacking tetR-clr4* were crossed to *epe1*Δ cells, generating *epe1*Δ *4xtetO-ade6*$^+$ F2 progeny. *epe1*$^+$ F2xwt progeny were produced by crossing *epe1*$^+$ into *epe1*Δ *4xtetO-ade6*$^+$ F2 cells. Naïve *epe1*Δ *4xtetO-ade6*$^+$ cells never expressed TetR-Clr4*. Colour assays to assess *4xtetO-ade6*$^+$ *silencing* in the different cell types is presented on the right.
(B) *4xtetO-ade6*$^+$ test locus with the position of primer pairs.
(C) qRT-PCR to assess transcription of *4xtetO-ade6*$^+$ in indicated cell types, relative to *act1*$^+$. Data are mean ±SD (*n=3* biological replicates). *P<0.05* (t-test).

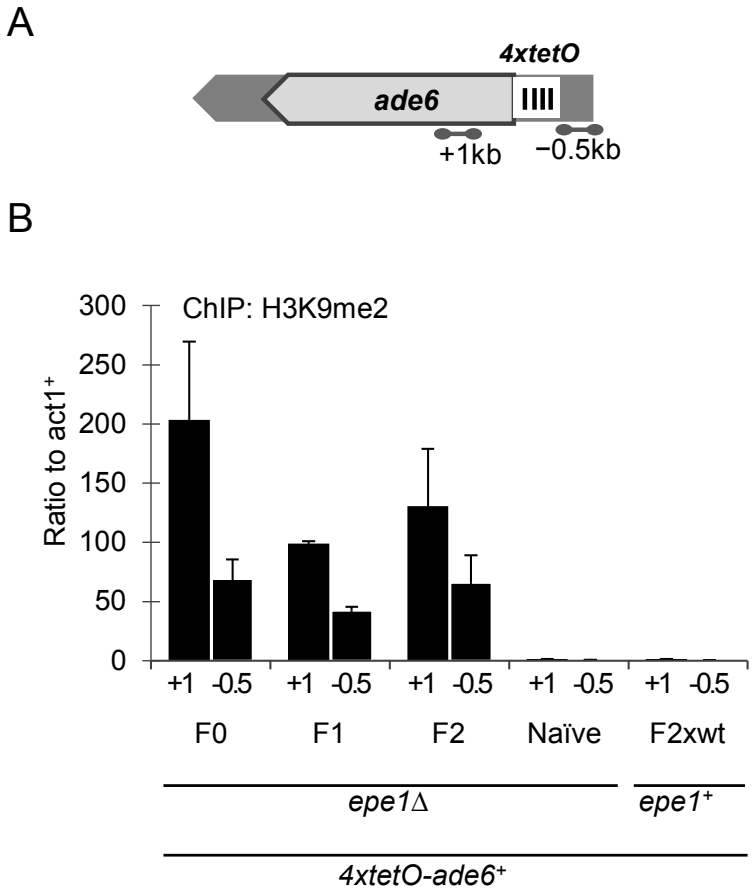

Figure 7.11 *epe1* mutants retain H3K9me2 at *4xtetO-ade6*$^+$ in absence of TetR-Clr4* through meiosis

(A) *4xtetO-ade6*$^+$ test locus with the position of primer pairs.
(B) ChIP analysis of H3K9me2 levels on *4xtetO-ade6*$^+$ in indicated cell types, relative to *act1*$^+$. Data are mean ±SD (*n=3* biological replicates).

Heterochromatin was lost from *4xtetO-ade6*$^+$ in these *epe1*$^+$ cells as indicated by the formation of fully white/*ade6*$^+$ expressed colonies, (Figure 7.10) and loss of H3K9me2 from *4xtetO-ade6*$^+$ (Figure 7.11). This indicates that the addition of functional Epe1 to *epe1*Δ cells carrying heterochromatin at *4xtetO-ade6*$^+$ results in the removal of H3K9me2, and the loss of silencing at the tethering site. Moreover, genetically identical naïve *epe1*Δ *4xtetO-ade6*$^+$ cells that were never exposed to the TetR-Clr4* initiator, formed only white/*ade6*$^+$-expressing colonies and as expected H3K9me2 was absent from *4xtetO-ade6*$^+$ (Figure 7.10 and Figure 11). This confirms that *epe1*Δ *4xtetO-ade6*$^+$ cells can not assemble heterochromatin at *4xtetO-ade6*$^+$ independently of the expression of TetR-Clr4* which directs H3K9 methylation to the *4xtetO-ade6*$^+$ site.

The above results demonstrate that in the absence of Epe1, H3K9me can persist through multiple mitotic and meiotic divisions, in the absence of the heterochromatin initiator TetR-Clr4* bound to *4xtetO-ade6*$^+$*.* Thus, heterochromatin formed by methylation on H3K9 has epigenetic properties that allow heterochromatin to be copied and maintained at an ectopic locus in the complete absence of the triggering nucleation signal. However, it is clear that a regulatory mechanism directed by Epe1 normally prevents the epigenetic inheritance of heterochromatin domains formed at inappropriate places in the genome.

7.8 Clr3 may promote heterochromatin maintenance by antagonising Epe1 recruitment to the chromatin

It is clear from the data presented that Epe1 prevents the epigenetic propagation of H3K9me; however, it remains unclear how Epe1 counteracts H3K9me2 maintenance at *4xtetO-ade6*$^+$. The fact that the catalytic domain of Epe1 is essential to prevent H3K9me inheritance suggests that Epe1 is likely to act as an H3K9me demethylase that removes H3K9me and heterochromatin from *4xtetO-ade6*$^+$. However, Epe1 might also counteract heterochromatin maintenance indirectly by preventing H3K9 methylation. Epe1 and the HDACs Clr3 and Sir2 have been shown to have opposing effects on heterochromatin regions and Epe1 was proposed to counteract both Clr3 and Sir2 HDACs activity (Shimada et al., 2009; Wang et al., 2013).

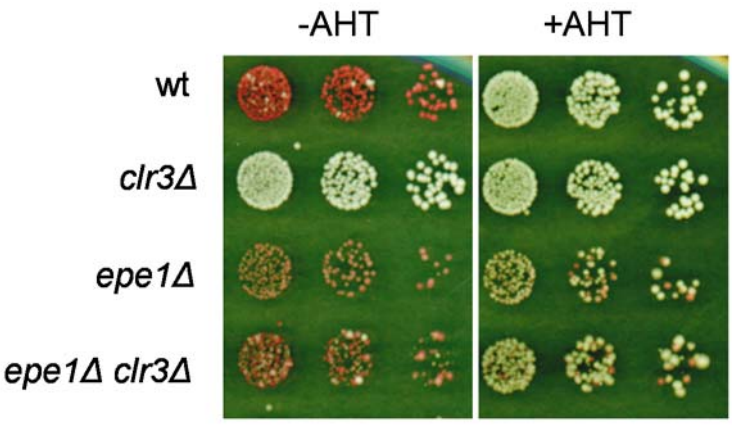

Figure 7.12: Clr3 promotes heterochromatin maintenance by counteracting Epe1 activity

Colony colour assay showing that *epe1*Δ rescues *4xtetO-ade6*$^{+}$ silencing in clr3Δ cells in cells with TetR-Clr4* bound to *4xtetO* (-AHT). *4xtetO-ade6*$^{+}$ silencing is also maintained in *epe1Δ clr3Δ* after TetR-Clr4* release (+AHT).

The histone deacetylases Sir2 and Clr3 play an important role in maintaining heterochromatin integrity at centromere repeats and the silent mating type locus (Buscaino et al., 2013; Yamada et al., 2005). Sir2 and Clr3 act in parallel with RNAi to maintain heterochromatin on centromere repeats. These two deacetylases were proposed to promote heterochromatin maintenance by suppressing transcription from centromeric repeats (Buscaino et al., 2013). Here, the interaction between Epe1 and the HDACs Clr3 and Sir2 was further investigated to determine whether Epe1 might prevent the maintenance of H3K9me at *4xtetO-ade6*$^+$ by counteracting Clr3 and Sir2 activity, or if Sir2 and Clr3 promote heterochromatin maintenance by counteracting Epe1 activity.

When TetR-Clr4* was bound to *4xtetO-ade6*$^+$, *clr3*Δ cells were observed to form only white/ade6$^+$ expressed colonies, suggesting that cells lacking Clr3 fail to assemble ectopic heterochromatin, which is consistent with previous studies (Kagansky et al., 2009) (Figure 7.12). Remarkably, in cells that lacked both Clr3 and Epe1, silencing of *4xtetO-ade6*$^+$ was restored as indicated by the formation of red/ade6$^+$ repressed colonies on –AHT plates where TetR-Clr4* is bound and was even maintained to some extent after TetR-Clr4* release (+AHT; Figure 7.12). This result suggests that Clr3 HDAC is not necessary for maintaining heterochromatin at the tethering site. Both Clr3 and Epe1 have been shown to associate with Swi6 and their association with Swi6 has been proposed to be mutually exclusive (Shimada et al., 2009). It therefore seems likely that Clr3 promotes heterochromatin maintenance at *4xtetO-ade6*$^+$ by antagonising Epe1 recruitment to the chromatin.

7.9 Epe1 and the bromodomain protein Bdf2 might prevent Sir2-dependent heterochromatin maintenance

Sir2 deacetylase activity has been shown to be essential for establishment, spreading and maintenance of heterochromatin (Alper et al., 2013
; Buscaino et al., 2013; Shankaranarayana et al., 2003). At centromeric boundaries, Epe1 together with the bromodomain protein Bdf2 counteract Sir2-dependent heterochromatin spreading into regions distal to the heterochromatic centromere repeats (Wang et al., 2013). Epe1 was shown to recruit Bdf2 to these boundary regions where it binds H4K16ac and protects H4K16ac from deacetylation by Sir2 and thereby prevents spreading of H3K9me and heterochromatin. To determine whether Epe1 might prevent heterochromatin maintenance at *4xtetO-ade6*$^+$ using a

similar mechanism, the role of Bdf2 and Sir2 in heterochromatin assembly and maintenance was assessed at *4xtetO-ade6*$^{+}$ before and after TetR-Clr4* release. Consistent with previous studies (Kagansky et al., 2009), Sir2 is essential for heterochromatin assembly at Clr4 tethering sites (Figure 7.13.B-C): *sir2*Δ cells formed white/ade6^{+} expressing colonies when TetR-Clr4* was bound and H3K9me2 was not enriched on *4xtetO-ade6*$^{+}$ (Figure 7.13.B-C). Deletion of *epe1*$^{+}$ was not sufficient to restore *4xtetO-ade6*$^{+}$ silencing in *sir2*Δ cells (Figure 7.13.B), suggesting that Sir2 is essential for the maintenance of heterochromatin over *4xtetO-ade6*$^{+}$ and does not act by antagonizing Epe1 function.

Interestingly, in cells lacking the bromodomain protein Bdf2, red/*4xtetO-ade6*$^{+}$ repressed colonies were formed after TetR-Clr4* release (+AHT) (Figure 7.13D). Thus, like Epe1, Bdf2 prevents heterochromatin maintenance at the tethering site. Bdf2 protects H4K16ac from Sir2-dependent deacetylation at boundaries (Wang et al., 2013). It is possible that Epe1 and Bdf2 antagonize heterochromatin spreading and maintenance at the *tetO* tethering site by preventing Sir2 dependent H4K16 deacetylation. To test this, H4K16ac levels were assessed at *4xtetO-ade6*$^{+}$ in wild-type, *epe1*Δ and *bdf2*Δ cells when TetR-Clr4* is bound to *4xtetO*. The presence of TetR-Clr4* bound to *4xtetO* reduces H4K16ac levels by two to three fold which is expected since the assembly of heterochromatin is associated with H4K16 hypoacetylation (Alper et al., 2013). In *epe1*Δ and *bdf2*Δ mutants, even less H4K16ac was detected at the tethering site when TetR-Clr4* is bound to *4xtetO-ade6*$^{+}$, suggesting that in cells lacking Epe1 or Bdf2, H4K16ac might be more available for deacetylation by Sir2. Epe1 and Bdf2 might promote heterochromatin disassembly at the ectopic heterochromatin locus upon TetR-Clr4* release by protecting H4K16ac from Sir2-dependent deacetylation and thus counteract Sir2-dependent heterochromatin spreading and maintenance.

In conclusion, Epe1 might prevent heterochromatin inheritance at *4xtetO-ade6*$^{+}$ by two different means:

i) Epe1 probably demethylates H3K9me resulting in the rapid removal of H3K9me from the tethering site upon TetR-Clr4* release.

ii) Epe1 might also recruit Bdf2 to *4xtetO-ade6*$^{+}$ which then protects H4K16ac from Sir2-dependent deacetylation and thus hinder Sir2-dependent heterochromatin spreading and maintenance.

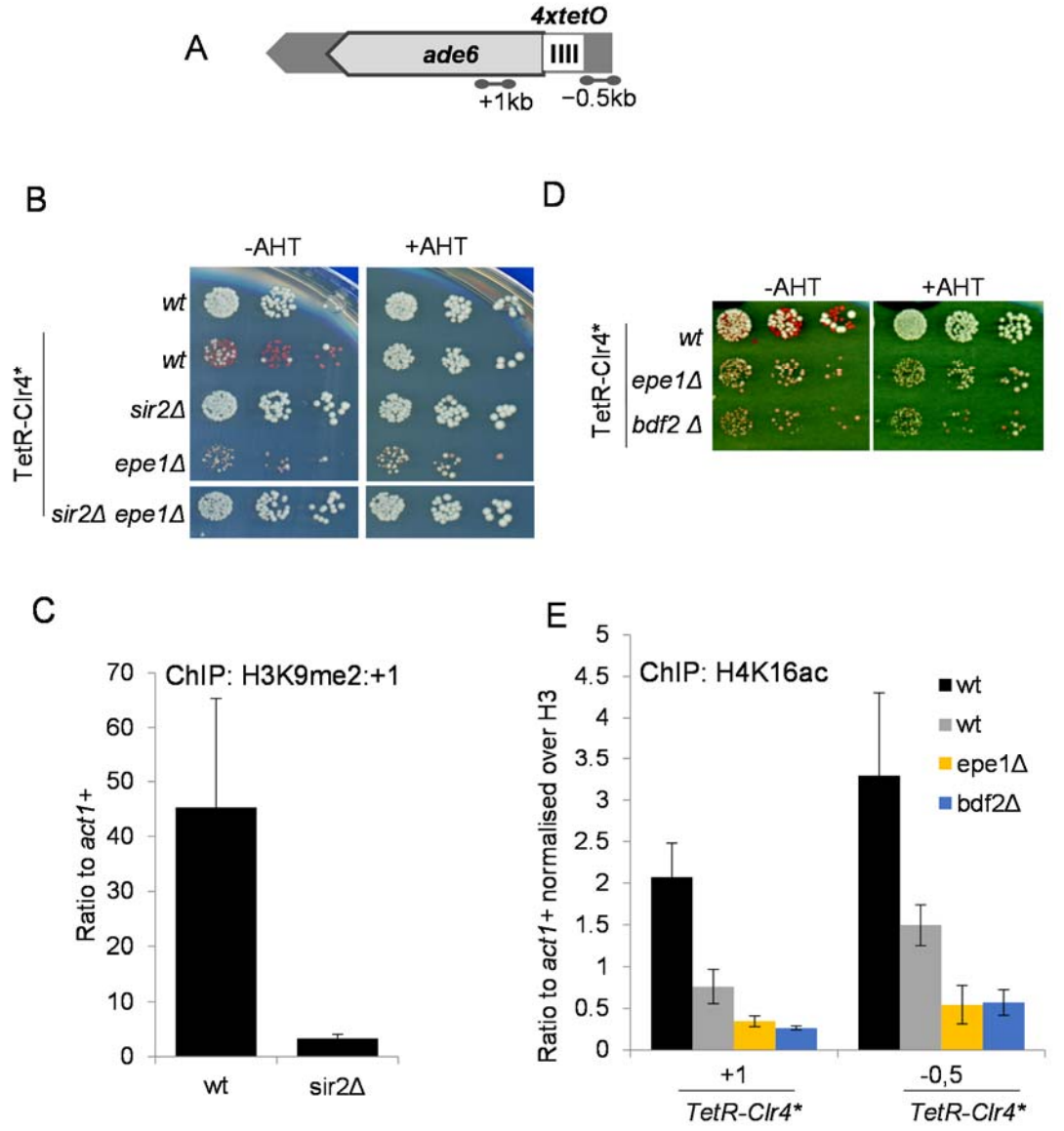

Figure 7.13: Epe1 and Bdf2 might counteract Sir2-dependent heterochromatin maintenance at *4xtetO-ade6*$^{+}$.

(A) *4xtetO-ade6*$^{+}$ test locus with the position of primer pairs relative to tethered TetR-Clr4*
(B) Colony colour assay showing that *epe1*Δ does not restore *4xtetO-ade6*$^{+}$ silencing in *sir2*Δ cells in the presence (-AHT) and the absence (+AHT of TetR-Clr4*.
(C) ChIP analysis of H3K9me2 levels at *4xtetO-ade6*$^{+}$ in wild type and *sir2*Δ cells when TetR-Clr4* is bound to the tethering site, relative to *act1*$^{+}$.
(D) Colony colour assay showing that *bdf2*Δ maintains *4xtetO-ade6*$^{+}$ silencing in the absence of TetR-Clr4* (+AHT).
(E) ChIP analysis of H4K16ac levels at *4xtetO-ade6*$^{+}$ in cells lacking Epe1 and Bdf2 when TetR-Clr4* is bound to *4xtetO-ade6*$^{+}$, relative to *act1*$^{+}$.
Data are means ± SD (*n* = 3 biological replicates)

7.10 Regulation of Epe1 by the Cul4-Ddb1(Cdt)² ubiquitin ligase is not restricted to endogenous heterochromatin regions

It is so far unclear whether Epe1 is regulated in order to target specifically heterochromatin assembled inappropriately in the genome or whether all heterochromatic loci are targeted equally by Epe1 activity. Epe1 levels have been shown to be regulated by the Cul4-Ddb1(Cdt)² ubiquitin ligase that polyubiquitinylates Epe1 directing it for proteasome degradation (Braun et al., 2011). In the absence of this Ub-ligase, Epe1 levels increase within heterochromatin domains resulting in defective centromeric heterochromatin (Braun et al., 2011). It was proposed that Epe1 is degraded specifically within heterochromatin domains, but not at their boundaries, so as to protect heterochromatin from the anti-silencing activity of Epe1. The retention of Epe1 at high levels at heterochromatin boundaries was suggested to prevent heterochromatin from spreading into flanking euchromatic regions (Braun et al., 2011). Epe1 levels may also be regulated by the Cul4-Ddb1(Cdt)² complex at the TetR-Clr4* tethering site. To test this possibility *4xtetO-ade6*⁺ silencing was assessed in cells lacking Cdt2, which is the substrate specifier for the Cul4-Ddb1(Cdt)² ubiquitin ligase (Figure 7.14). *cdt2*Δ cells exhibited alleviation of *4xtetO-ade6*⁺ silencing when TetR-Clr4* is bound, suggesting that the regulation of Epe1 by Cul4-Ddb1(Cdt)² ubiquitin ligase is not restricted to within endogenous centromeric heterochromatin domains. It is likely that Cul4-Ddb1(Cdt)² ubiquitin ligase also regulates the amount of Epe1 recruited to *4xtetO-ade6*⁺ since it regulates the cellular levels of Epe1. ChIP to assess Epe1 enrichment and H3K9me2 levels at the tethering site in *cdt2*Δ cells would confirm if this loss of *4xtetO-ade6*⁺ silencing results from increased Epe1 levels.

DISCUSSION

The results obtained in this chapter show that in the absence of the putative histone demethylase Epe1, heterochromatin can self-propagate through many mitotic and even meiotic cell divisions at *4xtetO-ade6*⁺ in cells lacking TetR-Clr4*. Therefore, the heterochromatin formed possesses bona fide epigenetic properties that allow H3K9 methylation and thus heterochromatin to be propagated in the absence of the initiating signal. This finding demonstrates that H3K9 methylation (and possibly other post-translational modifications on histones) can be transferred faithfully to H3 in newly assembled nucleosomes on daughter strands of DNA through replication.

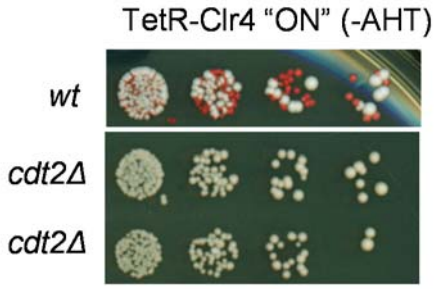

Figure 7.14: alleviation of *4xtetO-ade6*$^{+}$ silencing in cells lacking Cdt2.

Colour assay to assess *4xtetO-ade6*$^{+}$ silencing in wt and *cdt2*Δ in the presence of TetR-Clr4* at the tethering site.

H3K9me must also possess a system of self-reinforcement that acts independently of the DNA sequence and promotes the methylation of new histone incorporated following replication and as a consequence of histone turnover or histones demethylation.

Interestingly, heterochromatin domains carry a regulatory mechanism that is dependent on Epe1, and possibly also the bromodomain protein Bdf2 that prohibits the epigenetic transmission of ectopic H3K9me through cell division. Such a regulatory mechanism plays two important roles.

i) Epe1 protects euchromatic genes from being aberrantly silenced by preventing heterochromatin assembly and spreading over inappropriate places on chromosomes. ii) Epe1 maintains the integrity of heterochromatin domains. Indeed, the loss of Epe1 enhances variegation in silencing at the silent mating type locus and at centromeric repeats (Ayoub et al., 2003; Trewick et al., 2007; Zofall et al., 2012). By preventing heterochromatin assembly at other ectopic loci (outside the main domains) Epe1 may limit the titration of limited core heterochromatin components away from the main heterochromatin domains. It is also possible that the ectopic assembly of heterochromatin in *epe1*Δ mutants might silence genes important for heterochromatin assembly (Wang et al., 2015). Another possibility is that the function of Epe1 in defining heterochromatin/euchromatin boundaries might prevent both heterochromatin spreading into euchromatin regions and euchromatin spreading into heterochromatin domains, thus preserving heterochromatin integrity.

Epe1 is also thought to regulate small domains of facultative heterochromatin that have been detected on *S. pombe* chromosome (Zofall et al., 2012). A category of genes were shown to be silenced by heterochromatin under certain growth conditions and this heterochromatin is disassembled in response to developmental or environmental cues. For example, heterochromatin is formed on meiotic genes such as *mei4*$^{+}$ during vegetative growth (rich medium) but is disassembled upon nitrogen starvation which induces the meiotic program. Because of the epigenetic properties of H3K9me-heterochromatin, this facultative heterochromatin has to be actively disassembled in response to nitrogen starvation in order to activate the meiotic genes. The removal of H3K9me from *mei4*$^{+}$ upon nitrogen starvation is delayed in cells that lack Epe1, thus Epe1 plays a role in the regulation of facultative heterochromatin on specific genes (Zofall et al., 2012).

Epe1 stands out as a major regulator of heterochromatin stability in the genome. Epe1 tightly regulates heterochromatin localisation, spreading and maintenance. However the mechanism by which Epe1 regulates heterochromatin integrity is complex and remains poorly understood. The rapidity of H3K9me loss at *4xtetO-ade6*$^{+}$ upon TetR-Clr4* release and the fact that H3K9me loss from tethering sites requires the putative catalytic JmjC domain of Epe1 strongly points towards Epe1 acting as an H3K9me demethylase (KDM). However, despite numerous attempts Epe1 catalytic activity could not be detected *in vitro* (Tsukada, Fang et al. 2006 and Allshire lab unpublished), suggesting that Epe1 alone cannot demethylate H3K9me and may require other cofactors to gain catalytic activity (Trewick et al., 2007; Tsukada et al., 2006; Zofall and Grewal, 2006). The Epe1 JmjC domain lacks two residues (an aspartic acid replaced by a glutamic acid on position 299 and a histidine replaced by tyrosine in position 370) involved in iron [Fe(II)] binding which is required for the demethylase activity of these proteins (Tsukada et al., 2006). Like Epe1, the human demethylase PHF2 also carries an unusual JmjC domain with a tyrosine in place of a conserved histidine for co-ordinating Fe(II). Remarkably, Phf2 was shown to display H3K9me demethylase activity but only when phosphorylated by Protein Kinase A (PKA) (Baba et al., 2011). The catalytic activity of the PHF8 and KDM3A lysine demethylases is also stimulated by phosphorylation *in vivo* (Cheng et al., 2014; Sun et al., 2015). It has also been proposed that Epe1 might act as a protein hydroxylase rather than a lysine demethylase. Like all JmjC proteins, Epe1 has structural similarity with the JmjC domain protein FIH (factor inhibiting hypoxia inducible factor) that inhibits the transcription factor HIF (hypoxia inducible factor) by hydroxylating the protein. Epe1 could similarly affect the stability of heterochromatin domains by hydroxylating heterochromatin factors (Hewitson et al., 2002; Trewick et al., 2005; Trewick et al., 2007). Epe1 might also remove methyl groups from non-histone proteins such as heterochromatin components required for H3K9me maintenance and spreading.

Interestingly, Epe1 also acts as an anti-silencer at heterochromatin regions independently of its catalytic activity. The overexpression of a version of Epe1 mutated within the JmjC domain results in alleviation of silencing at centromeric repeats (Trewick et al., 2007). The catalytic-independent anti-silencing function of Epe1 might be mediated by the bromodomain protein Bdf2 that is recruited to chromatin via Epe1 but that appears to exert boundary functions independently of Epe1 (Wang et al., 2013). Bdf2 binds histone H4 acetylated on K16 and prevents

Sir2-dependent spreading and maintenance of heterochromatin. The absence of Bdf2 is sufficient to cause aberrant heterochromatin maintenance at *4xtetO-ade6*$^{+}$ although Epe1 is still present in these cells and presumably localised at the tethering site since Bdf2 does not affect Epe1 localisation to chromatin (Wang et al., 2013). It is therefore likely that Epe1-dependent recruitment of Bdf2 prohibits heterochromatin spreading independently of the catalytic activity of Epe1. However, it cannot be ruled out that Bdf2 might also regulate Epe1 catalytic activity. Although we showed that Clr3 HDAC is not necessary for heterochromatin maintenance at the tethering site in *epe1*Δ, cells, it is also possible that Epe1 limits heterochromatin assembly by competing with the Clr3/SHREC complex for the association with Swi6. Both the Clr3 HDAC and Mit1 chromatin remodelling activities of SHREC are important for maintaining heterochromatin integrity (Creamer et al., 2014; Sugiyama et al., 2007).

In conclusion, we have shown that heterochromatin can use a read-write mechanism to both self-propagate through replication and laterally spread along chromatin fibres, independently of the underlying DNA sequence However, *in vivo* this powerful self-reinforcing mechanism is counteracted by a self-regulatory mechanism that is highly dependent on Epe1 which is recruited by the heterochromatin. Epe1 may normally act to limit the spreading of heterochromatin domains into euchromatin and the transmission of inappropriate heterochromatin domains through cell division. Thus, normally the epigenetic inheritance of H3K9me is not sufficient to maintain heterochromatin domains in *S. pombe* that rely on nucleation signals driven by the DNA sequence itself through transcripts that generate siRNA allowing the RNAi machinery to reinforce and stably propagate this heterochromatin.

CHAPTER 8: DISCUSSION

For many years it has remained an open question as to whether post-translational modifications are actually true epigenetic marks in the sense that they allow the maintenance of specific chromatin states so that they are transmitted through cell divisions (Bonasio, 2015; Bonasio et al., 2010). Although H3K9 methylation, like other histone marks, is stably associated with the chromatin and possesses mechanisms that allow its spreading independently of the underlying DNA sequence, the maintenance of H3K9me is normally closely associated with DNA sequences such as arrays of repetitive elements, dispersed repeat sequences such as transposons, or transcription factors that bind specific sequences and recruit H3K9 methyltransferases to chromatin, thus promoting the maintenance of H3K9me. In several organisms, the maintenance of H3K9me is coupled to DNA methylation that is thought to epigenetically maintain heterochromatin domains (Cheng, 2014)}. Therefore I have attempted to resolve the question of whether H3K9 methylation can act as a bona fide epigenetic mark independently of the DNA sequence and DNA methylation. H3K9me heterochromatin was assembled ectopically by artificial tethering Clr4 H3K9 methyltransferase activity (TetR-Clr4*) to *tetO* sites inserted at a euchromatic locus and the maintenance of heterochromatin was assessed at that locus following removal of tethered Clr4 activity by dissociation (+AHT) or crossing out. As *S. pombe* completely lacks DNA methylation, the maintenance of H3K9me at an ectopic locus must rely on epigenetic mechanisms only.

Although TetR-Clr4* induces the assembly of a large heterochromatin domain around the tethering site, H3K9me-heterochromatin was found to be rapidly lost from the tethering site upon TetR-Clr4* release even within chromatin regions displaying low levels of histone turnover. The loss of heterochromatin was found to depend on the putative histone H3K9 demethylase Epe1 and the bromodomain protein Bdf2. The Epe1 and Bdf2 proteins must prevent the epigenetic inheritance of H3K9me heterochromatin at ectopic loci and thus limit the inappropriate inheritance of potentially deleterious heterochromatin domains in the genome.

8.1 H3K9me has epigenetic properties

The work described in this thesis demonstrates that the H3K9me2 histone modification can propagate and carry the memory of the heterochromatin state through multiple cell divisions. In wild-type cells H3K9me does not behave as an epigenetic mark since it can not be transmitted through cell divisions. However, the loss of Epe1 function reveals that H3K9me does indeed have true epigenetic properties. Several studies have previously attempted to address this question using similar approaches both in fission yeast and mammalian cells. The "L5" region from the *dg* centromere repeat element has been shown to form ectopic silent heterochromatin when inserted in euchromatin in *S. pombe* (Partridge et al 2002). (Wheeler et al., 2012). Remarkably, following establishment of a heterochromatin domain at the *ura4* locus the subsequent removal of the L5 fragment (using Cre/lox recombination) showed that the H3K9me heterochromatin domain was maintained through multiple mitotic and meiotic divisions in wild-type cells (i.e. *epe1*$^{+}$ cells) (Wheeler et al., 2012). This contrasts sharply with results obtained using TetR-Clr4* to initiate ectopic heterochromatin formation (Audergon et al., 2015; Ragunathan et al., 2015). However, further analyses of heterochromatin induced by the L5 system showed that the maintenance of this heterochromatin at the ectopic locus following deletion of L5 was dependent on the RNAi pathway and therefore it could not be concluded that H3K9 methylated chromatin by itself is capable of self-propagation. Thus, unlike the TetR-Clr4* system which allows H3K9 methylation to persist in the absence of RNAi, the L5 system must induce a local RNAi response so that associated transcripts subsequently attract Clr4 via RNAi.

In mouse cells an elegant study was performed which also tried to address the heritability of heterochromatin by using chemical induced proximity to target HP1α to the Oct4 gene. The heterochromatin domain induced by targeted HP1α was shown to be maintained for several days upon HP1α release from Oct4. This result demonstrated that H3K9me3 could be epigenetically transmitted through numerous cell divisions in the absence of the initial stimulus. However, in ES cells, heterochromatin maintenance at Oct4 was found to be dependent on DNA methylation and cells treated with inhibitors of DNA methylation rapidly lost H3K9me3 upon HP1 release (Hathaway et al., 2012). In mouse embryonic fibroblasts (MEFs) derived from the same mice that underwent Oct4 reactivation by tethering of the VP16 transcription factor at the Oct4 locus, H3K9me3 could be maintained for several

generations upon HP1α release in cells treated with inhibitors of DNA methylation, suggesting a lesser role for DNA methylation in H3K9me3 maintenance in those cells. However, considering that Oct4 is generally silenced and embedded in H3K9me3 chromatin in MEF cells, it is possible that the DNA sequence itself might promote H3K9me3 maintenance at this locus or that remaining DNA methylation in cells treated with inhibitors of DNA methylation facilitate heterochromatin maintenance upon HP1α release. In these two studies, H3K9me maintenance could not be dissociated from RNAi or DNA methylation and therefore the epigenetic properties of H3K9me could not be demonstrated with complete certainty.

The fact that H3K9me can act as an epigenetic mark implies firstly that histone H3 proteins methylated on lysine 9 are faithfully transferred onto the nucleosomes on the two daughter stands of DNA upon replication and secondly, that H3K9me carries a reinforcement mechanism that modifies new histone H3 incorporated throughout the cell cycle and any histone H3 that has been demethylated.

8.1.1. Heterochromatin is restored following replication

To gain access to the DNA, the replication machinery needs to disassemble chromatin in front of the replication fork and then re-assemble chromatin behind following the passage of the replication fork (Burgess and Zhang, 2013). If there was no mechanism to initially distribute modified parental nucleosomes to both daughter DNA strands and then copy the modifications carried by the parental nucleosomes to newly assembled nucleosomes, the modifications and the information it contains would be lost during replication. However, the synchronous cell cycle experiments preformed (Chapter 4) suggests that H3K9 methylated chromatin is restored upon replication even after TetR-Clr4* removal. H3K9me2 is lost at a constant rate through the cell cycle and no accelerated loss was observed during S phase, suggesting that parental histones carrying H3K9me must be transferred to both sister chromatids and that the H3K9 methylation must be copied from histone H3 in parental nucleosomes to neighbouring nascent H3 in newly assembled nucleosomes.

The fact that H3K9 methylation loss remains steady through S phase suggests that the reinforcement mechanism that replenishes H3K9me might be specific to S phase or enhanced during/following S phase, thus allowing newly incorporated histone H3

to be methylated and thus H3K9me levels to be restored after replication despite the presence of Epe1 that promotes H3K9me loss.

Many chromatin modifiers, such as nucleosome remodelers, HDACs and DNMT1 in higher eukaryotes, are associated with the replication fork and these are thought to promote chromatin maturation following replication (Groth et al., 2007). Swi6 in fission yeast and HP1 in higher eukaryotes are known to associate with the CAF1 histone chaperone and Caf1 contacts PCNA and thus may promote the restoration of heterochromatin following replication (Dohke et al., 2008; Murzina et al., 1999). Moreover, the Dos2/Raf2 component of the fission yeast CLRC (Clr4 H3K9 methyltransferase containing complex) interacts with Cdc20, the catalytic subunit of DNA polymerase epsilon, suggesting that the DNA polymerase might recruit CLRC/Clr4 methyltransferase to the replication fork to restore the heterochromatic state (Li et al., 2011).

Instead of the reinforcement mechanism being enhanced in S phase, it is also possible that Epe1 removal of H3K9me might be inhibited during S phase in order to allow heterochromatin to be restored following replication. Interestingly, Epe1 has been shown to be degraded by Cul4-Ddb1^{Cdt2} ubiquitin ligase. This degradation must be limited to S phase since expression of the Cdt2 subunit is restricted to replication in a normal cell cycle (Braun et al., 2011; Liu et al., 2005). Moreover, fission yeast centromeres and the mating type locus replicate early compared to the rest of the genome and this early replication is promoted by the recruitment of replication initiators via Swi6 (Kim et al., 2003; Li et al., 2013). It is possible that this early replication of heterochromatin regions ensures a longer “Epe1-free” period to reinforce the heterochromatin domain following replication.

8.1.2 The H3K9me self-reinforcement mechanism uses a Reader/Writer based recognition of H3K9me by the chromodomains of Swi6 and Clr4

Several histone modifications can be reinforced locally so that they spread along the chromatin fibre independently of the underlying DNA sequence. Many different histone PTMs can be recognized directly by protein binding modules that are directly linked to enzymes responsible for their addition. The ability of a single protein or associated proteins to bind the same mark that it generates can result in reinforcement and cis-spreading of that histone PTM and the factors that it recruits.

In *S. cerevisiae*, Sir2 together with Sir4 promotes H4K16 deacetylation which recruits Sir3 that preferentially binds unacetylated H4K16. Sir3 then recruits additional Sir2-Sir4 dimers which allows heterochromatin spreading along the chromosome. Other examples are the human L3MBTL1 protein which binds H4K20me1 via its 3xMBT domains and directly recruits the H4K20 methyltransferase PR-SET7 to install further H4K20 methylation on neighbouring chromatin (Kalakonda et al., 2008). Such systems are important for the maintenance of a chromatin state since they allow histone PTMs that were removed or lost by histone turnover to be restored on chromatin. In both fission yeast and human cells, the H3K9 methyltransferase SUV39/Clr4 associates with HP1/Swi6 but both these methyltransferases can also bind directly to H3K9me via their N-terminal chromodomains (Bonasio et al., 2010). The chromodomains of Swi6 and Clr4 have been shown to be important for heterochromatin maintenance and spreading away from nucleation sites at domains of constitutive heterochromatin in fission yeast (Bannister et al., 2001; Zhang et al., 2008). In the TetR-Clr4* system utilised here, the Swi6 and endogenous full length Clr4 are essential for the epigenetic inheritance of silent heterochromatin at the tethering site in cells lacking Epe1 function. Thus the read-write reinforcement mechanism based on the recognition of H3K9me by both Clr4 and Swi6 is essential for the epigenetic propagation of heterochromatin.

Theoretically, Clr4 itself might be considered sufficient to both read and write the H3K9 methylation mark; however, Swi6 is clearly also required for the epigenetic inheritance of H3K9me. Undoubtedly newly deposited H3 needs to be deacetylated and Swi6 is known to be involved in the recruitment of the histones deacetylases Clr6 and Clr3 to ensure heterochromatin integrity (Fischer et al., 2009). However, Swi6 also recruits the putative H3K9 demethylase Epe1 which would prevent heterochromatin maintenance (Trewick et al., 2007; Zofall and Grewal, 2006). In addition, Clr3 was found to be dispensable for the maintenance of H3K9me at the tethering site in the absence of Epe1 (Figure 7.12). A possible explanation is that Swi6 promotes H3K9me maintenance via its direct interaction with Clr4, this might stabilise Clr4 on chromatin by creating a second anchor point for Clr4-Swi6 on chromatin. Swi6 might also physically stabilise H3K9me by enclosing the modified residue in its chromodomain and forming a more compact stable heterochromatin structure that protects H3K9me from demethylation. It would be interesting to assess whether H3K9me has epigenetic properties in plants that lack HP1 proteins or whether in these organisms, H3K9me maintenance relies entirely on DNA and DNA methylation.

8.1.3 HDACs are essential for the epigenetic inheritance of heterochromatin

The histone deacetylase Sir2 plays a role in heterochromatin nucleation, maintenance and spreading at centromeric repeats in fission yeast (Buscaino et al., 2013; Freeman-Cook et al., 2005; Shankaranarayana et al., 2003). How Sir2 promotes heterochromatin assembly and maintenance is unclear although these Sir2 functions require its catalytic activity. Sir2 can deacetylate H3K4ac, H3K9ac, H4K16ac and H3K14ac *in vitro* (Alper et al.). Many studies point towards a key function for transcriptional silencing in maintaining constitutive heterochromatin. Acetylated histones such as H3K9ac, H3K14ac or H4K16ac are associated with transcribed loci (Owen-Hughes and Gkikopoulos, 2012; Smolle and Workman, 2013) and keeping H3K9, H3K14 or H4K16 in their hypoacetylated state is important for the maintenance of repressed heterochromatin domains at fission yeast centromeres (Buscaino et al., 2013; Shankaranarayana et al., 2003; Yamada et al., 2005). It was therefore proposed that Clr3 HDAC, which deacetylates H3K14ac and Sir2 HDAC (deacetylates H3K9ac, H4K16ac and H3K14ac) would act in parallel to RNAi to maintain a hypoacetylated transcriptionally repressed environment essential for heterochromatin maintenance (Buscaino et al., 2013).

With the TetR-Clr4* heterochromatin system, Sir2 is essential for heterochromatin assembly regardless of whether TetR-Clr4* is bound to *4xtetO-ade6*$^{+}$ or not, or whether Epe1 is present or absent. Sir2 is therefore essential for the epigenetic inheritance of TetR-Clr4* initiated heterochromatin. It would be interesting to determine how Sir2 promotes heterochromatin maintenance and whether the deacetylation of a specific histone residue is alone sufficient to promote heterochromatin maintenance. It is possible that Sir2 promotes H3K9 methylation by deacetylating H3K9ac, since removal of the acetyl group is essential for methylation of H3K9. However other deacetylases such as Clr6 are known to participate in the deacetylation of H3K9ac at centromere repeat chromatin (Alper et al.; Nicolas et al., 2007).

Deacetylation of H4K16ac appears to be important for heterochromatin spreading. Indeed, in fission yeast cells expressing histone H4K16R that mimics unacetylated H4K16, heterochromatin can spread beyond its normal boundaries outside of cen1 (Wang et al., 2013). Moreover, in budding yeast, heterochromatin spreading is dependent on Sir2-dependent H4K16 deacetylation (Kueng et al., 2013). H4K16ac

was shown to decrease the compaction of nucleosomes on an array *in vitro* (Shogren-Knaak et al., 2006). This suggests that Sir2 might promote heterochromatin maintenance and spreading by removing H4K16ac and maintaining a compact heterochromatin domain less accessible to the transcription machinery. It would therefore be interesting to test whether cells expressing only H4K16R mutants or lacking Mst1, a known H4K16 acetyltransferase, might bypass the need for Sir2 in both establishing and maintaining TetR-Clr4* mediated heterochromatin.

The epigenetic inheritance of H3K9-methylation dependent heterochromatin relies on Swi6 and Clr4 for its transmission to daughter cells, however, other components such as the histone deacetylase Sir2 also promote H3K9me maintenance by providing a hypoacetylated environment of repressed transcription (Figure 8.1). Thus, any factor that upset the hypoacetylated state by preventing the removal of key acetyl groups from underlying chromatin are likely to prevent heterochromatin stability.

8.2 High transcription levels prevent establishment of H3K9 methylation

The data presented (Chapter 7) clearly shows that strong transcription through the TetR-Clr4* tethering site prevented the establishment of H3K9 at both *sib1* and *vps1302*. It is known that transcription-coupled histone turnover correlates with the levels of gene transcription; thus, it is likely that H3K9me fails to be stably established at either of these tethering sites because methylated H3K9 in resident nucleosomes is turned over when the genes are expressed from the full *adh1* promoter (Svensson et al., 2015). The previous observation that highly transcribed genes can act as boundaries which prevent H3K9me–dependent heterochromatin from spreading from a nucleation site is consistent with the idea that high levels of transcription prevent heterochromatin formation (Wheeler et al., 2009). It remains to be determined if the failure to establish H3K9 methylation at such highly expressed loci is also dependent on Epe1 activity.

In *epe1*Δ cells, compared to wild-type, the levels of gene expression and H4K16ac were observed to be less within the heterochromatin domain around *4xtetO-ade6*$^{+}$ when Clr4 was tethered, however no significant change was observed in H3K9me levels (Fig.7.2 and Fig.7.12). This suggests that Epe1 might promote or stabilise acetylated H4K16 at the tethering site and promote transcriptional activity. Epe1 might prevent heterochromatin assembly on transcribed regions by protecting acetylated

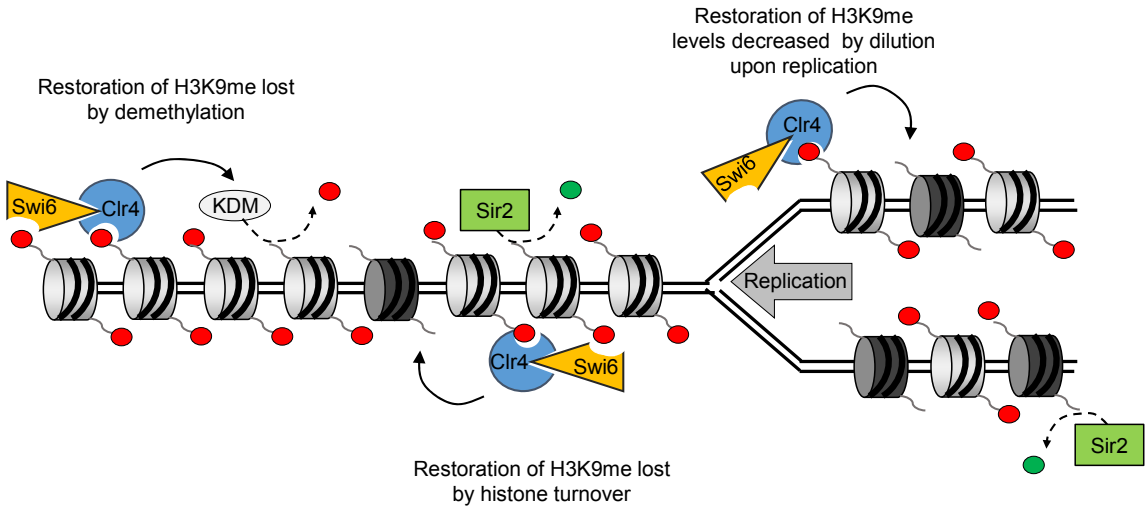

Figure 8.2: Model: H3K9me self-reinforcement mechanism in *S. pombe*

Swi6 and Cl4 are recruited to H3K9me domains via their chromodomain and methylates unmethylated H3K9 in the vicinity thus counteracting H3K9me loss (red dots) resulting from H3K9me demethylation by lysine demethylases (KDMs) or incorporation of new histones H3 on the chromatin during replication or histone turnover. The HDAC Sir2 is required to remove histone acetylation marks such as H3K9ac or H4K16ac (green dots) that prevent H3K9me maintenance.

marks from Clr3 and Sir2-dependent deacetylase activity possibly by recruiting Bdf2 that binds via its two bromodomains to H4K16ac and other acetylated marks and protect acetylated histones from deacetylation (Kim et al., 2014; Wang et al., 2013).

8.3 Pro- and anti-silencing factors are finely balanced to control heterochromatin spreading.

Maintaining an equilibrium between factors that promote heterochromatin assembly and those factors that counteract heterochromatin is important to maintain heterochromatin integrity. Overexpression of Epe1 results in alleviation of heterochromatin mediated silencing (Trewick et al., 2007). On the other hand, both the overexpression of Swi6 and the loss of Epe1 or the H3K14 acetyltransferase Mst2 complex allow heterochromatin to spread and heterochromatin assembly at additional loci (Ayoub et al., 2003; Noma et al., 2006){Trewick, 2007 #151; Wang et al., 2015; Zofall et al., 2012). Recently it was shown that cells lacking both Epe1 and the H3K9 HAT Mst2 are extremely slow growing. However, suppressors eventually arise that rescue this slow growth. Detailed analyses suggest that the inappropriate assembly of heterochromatin in the double mutant probably results in the silencing of essential genes, explaining the slow growth phenotype (Wang et al., 2015). Preserving the balance between anti-silencing factors and factors that promote heterochromatin assembly is essential to avoid the unregulated heterochromatinisation of large parts of the genome. The balance of these opposing activities may also limit the epigenetic inheritance of heterochromatin domains if insufficient amounts of repressive heterochromatin factors are available to counteract H3K9me loss by passive histone turnover or by Epe1. Increasing the abundance of various heterochromatin factors such as Clr4, Swi6 or the deacetylases Sir2 and Clr3 might therefore be sufficient to promote the epigenetic inheritance of heterochromatin domains. Indeed the balance between pro- and anti-silencing factors also regulates heterochromatin spreading in *S. cerevisiae* where an increase of Sir3 or a decrease of the H4K16 acetyltransferase Sas2 both lead to heterochromatin spreading at telomeres (Suka et al., 2002).

8.4 Epe1 prevents H3K9me epigenetic maintenance at ectopic heterochromatin loci

8.4.1 Epe1 might act as an H3K9me demethylase that actively removes H3K9me from ectopic heterochromatin loci

The catalytic domain of Epe1 was shown to be important for the removal of H3K9me from *4xtetO-ade6*$^{+}$ following TetR-Clr4* release, suggesting that Epe1 might act to demethylate H3K9me. However, despite several attempts from different laboratories, Epe1 demethylase activity has not been detected *in vitro* (Tsukada et al., 2006{Trewick, 2007 #151; Zofall and Grewal, 2006)}. The over-expression of Epe1 mutated in its putative catalytic domain results in heterochromatin defects at centromeres, suggesting that Epe1 also affects heterochromatin independently of its JmjC domain (Trewick et al., 2007). One explanation is that the additional overexpressed Epe1 titrates Swi6 away from heterochromatin. Consequently, it is difficult to ascribe phenotypes that are specific to the putative catalytic activity of Epe1. It is known that overexpression of the H3K9me3/H3K36me3 demethylase Rph1 from budding yeasts, but not mutant protein, in NIH 3T3 mouse cells reduces the large foci of H3K9 methylation normally observed at pericentromeric region. Therefore as an alternative approach to detect Epe1 demethylase activity we expressed codon-optimised wild-type JmjC domain, or the JmjC domain with a mutated iron binding site, in mouse cells and assessed if Epe1 JmjC domain was able to decrease H3K9me2 or H3K9me3 pericentromeric signals (Klose et al., 2007). Although expression of the FLAG-tagged Epe1 JmjC domain proteins was detected in the nuclei, no decrease of the H3K9me2/me3 signal could be detected by immunofluorescence. These assays indicate that either the expressed form of the JmjC domain of Epe1 does not possess H3K9me2/me3 demethylase activity or is not functional in the form expressed (data not shown; collaboration with Irina Stancheva).

In higher eukaryotes, at least two JmjC domain histone demethylases have been shown to gain demethylase activity upon phosphorylation (Baba et al., 2011; Sun et al., 2015). It is therefore possible that Epe1 catalytic activity needs to be activated by co-factors or signalling pathways. Phospho-proteomic analyses indicate that Epe1 has two residues that are phosphorylated *in vivo*: threonine 770 and serine 932 (Koch et al., 2011; Wilson-Grady et al., 2008). These two phosphorylation sites have been confirmed by mass spectrometry (Tania Auchynnikava, Allshire lab) and are located within C-terminal region of Epe1 that is known to directly interact with Swi6 in yeast two hybrid assays (Trewick et al., 2007). It is possible that phosphorylation of these residues regulates the interaction of Epe1 with Swi6 to promote or hinder Epe1 recruitment to the chromatin. Phosphorylation of these residues might also directly affect Epe1 catalytic activity. In the future it would be instructive to mutate T770 and

S932 to investigate their possible role in regulating Epe1 localisation and activity. Moreover, additional potential phosphorylation sites in Epe1 have been identified that are conserved amongst *Schizosaccharomyces* species, these might also play a role in the regulation of Epe1 activity.

8.4.2 Epe1 might prevent heterochromatin maintenance indirectly by preventing H3K9 methylation

It is possible that Epe1 counteracts heterochromatin maintenance independently of its putative catalytic domain. Epe1 is known to recruit the bromodomain protein Bdf2 to heterochromatin boundaries where Bdf2 was proposed to prevent heterochromatin spreading by protecting H4K16ac from Sir2-dependent deacetylation (Wang et al., 2013). Bdf2 was found to prevent heterochromatin spreading independently of Epe1 when tethered next to a synthetic heterochromatin domain. (Wang et al., 2013). Interestingly, at the *4xtetO-ade6*$^{+}$ locus, loss of Bdf2 was found to promote epigenetic inheritance of H3K9me2 following release of TetR-Clr4* (Chapter7/figure 12). It is therefore possible that Epe1 prevents the epigenetic maintenance of heterochromatin at the *4xtetO-ade6*$^{+}$ locus by recruiting Bdf2 that protects H4K16ac from Sir2-dependent deacetylation and thus prevents heterochromatin spreading.

8.5 Region specific regulation of Epe1

The results obtained in this study indicate that Epe1 prevents the epigenetic inheritance of ectopic heterochromatin through cell division. This raises the question of how Epe1 is specifically targeted to, or activated at ectopic heterochromatin to limit its persistence without affecting endogenous heterochromatin loci. One possibility is that Epe1 is recruited equivalently to all heterochromatin but innate processes such as RNAi and the Atf1/Pcr1 system act to maintain constitutive heterochromatin domains and counteract H3K9me removal by Epe1. However, intermediate levels of H3K9me2 remain detectable on centromere repeats in cells lacking RNAi even though the RNAi pathway is essential for establishing heterochromatin on these repeat elements (Sadaie et al., 2004; Volpe et al., 2002). It is possible that other unknown factors act independently of RNAi to promote the maintenance of this centromeric heterochromatin. Alternatively the fraction of H3K9me that remains on centromere repeats in the absence of RNAi might be maintained by the epigenetic

read-write system despite the presence of Epe1 in the cells. In this scenario, the remaining H3K9 methylation at centromeres would need to be somehow protected from Epe1 activity.

8.5.1 Regulation of Epe1 levels by ubiquitin mediated degradation

There is evidence suggesting that Epe1 and therefore its activity is limited to the boundaries of the main heterochromatin domain by its ubiquitylation mediated degradation (Braun et al., 2011; Trewick et al., 2007). Epe1 is targeted for degradation by the Cul4-Ddb1^{Cdt2} ubiquitin ligase (Braun et al., 2011). The substrate specifier *cdt2*$^{+}$ is expressed in S phase, suggesting that Epe1 degradation is restricted to this phase of the cell cycle (Liu et al., 2005). Epe1 degradation in S phase might provide the opportunity for heterochromatin domains to direct H3K9 methylation of unmethylated histone H3 incorporated during replication and therefore restore full heterochromatin integrity.

Epe1 is recruited to heterochromatin regions via a direct association between its C terminal domain and Swi6 (Trewick et al., 2007). Although Epe1 is generally enriched over regions containing Swi6, the pattern of Epe1 and Swi6 enrichment across heterochromatin domains are different (Zofall and Grewal, 2006). For example, Epe1 is found at higher levels at the boundaries of centromeric heterochromatin than over the body of the centromeric repeats themselves while Swi6 is distributed relatively evenly throughout all heterochromatin domains. Therefore, Epe1 within the body of constitutive heterochromatin domains might be specifically targeted for degradation. This removal of Epe1 would protect heterochromatin over the centromere repeats and the silent mating type locus from Epe1-dependent disassembly. Consistent with this, in cells lacking functional Cul4-Ddb1^{Cdt2} ubiquitin ligase complex, Epe1 levels increase dramatically over centromeric repeats and the mating type locus, but only mildly at heterochromatin boundaries (Braun et al., 2011). The degradation of Epe1 might be differentially regulated on these constitutive heterochromatin compared with ectopic heterochromatin loci. Retention of active Epe1 at ectopic loci such as TetR-Clr4*/*4xtetO-ade6*$^{+}$ would result in the locus specific loss of H3K9-dependent heterochromatin. However, there is no evidence to suggest that Cul4-Ddb1^{Cdt2} is specifically localised at, or displays enhanced activity within, centromere regions or the silent mating type locus. In addition, TetR-Clr4*/*4xtetO-ade6*$^{+}$ silencing was

strongly alleviated in *cdt2*Δ cells suggesting that Epe1 is also targeted for degradation by Cul4-Ddb1^{Cdt2} at ectopic heterochromatin loci.

Interestingly, in cells lacking a functional Cul4-Ddb1^{Cdt2}, ubiquitylated Epe1 is still detected suggesting that other Ubiquitin ligases might also target Epe1 for degradation. The Clr4 containing CLRC complex shares structural similarity to Cul4-Ddb1-Cdt22 ubiquitin ligases as it contains Cul4, Rik1 (a Ddb1 paralog) and Raf1/Dos1 (a Cdt2 paralog) and is also predicted to be a Cul4-Dependent ubiquitin ligase. Because of these similarities between Cul4-Ddb1^{Cdt2} and the CLRC complex, it is possible that Epe1 is also targeted for degradation by this putative CLRC E3 ligase activity (Buscaino et al., 2012).

8.5.2 Regulation of Epe1 recruitment to the chromatin

Swi6-dependent recruitment to the chromatin

Epe1 is recruited to heterochromatin regions via a direct association withSwi6 (Trewick et al., 2007). HP1/Swi6 can have many different functions depending on its interacting proteins. Swi6 and HP1 themselves are subject to many PTMs that affect the binding of interacting proteins. The different isoforms of HP1 have been shown to be acetylated, methylated, ubiquitylated, sumoylated, or formylated (Kwon and Workman, 2011). In *S. pombe*, Ckb1 (Casein Kinase 2) was shown to phosphorylate Swi6 on 5 sites in its N terminal region. Ckb1 also phosphorylates *S. pombe* Chp2, a Swi6 related protein that also contributes to heterochromatin integrity by binding H3K9me via its chromodomain (Shimada et al., 2009). The absence of Ckb1, or mutation of the Ckb1 phosphorylation sites in Swi6, is accompanied by a decrease in the association of the Clr3/SHREC HDAC complex with centromere repeats, increased recruitment of Epe1 and the alleviation of centromere repeat silencing (Braun et al., 2011; Shimada et al., 2009). These observations suggest that Epe1 recruitment to centromere repeats might be limited by phosphorylation of Swi6. A high-throughput GFP-tagged protein localisation database (Orfeome) suggests that Ckb1 may be concentrated near the Spindle Pole Body (SPB). All three centromeres are known to cluster at the SPB in interphase (Funabiki et al., 1993), Ckb1 might specifically phosphorylate Swi6 when associated with the SPB and thereby protect centromeres from Epe1 activity.

Swi6-independent recruitment of Epe1

Low levels of Epe1 remain at heterochromatin boundaries and can be detected at some euchromatic locations in Swi6 deficient cells suggesting that Epe1 might also be recruited independently of Swi6 to these regions (Braun et al., 2011; Zofall and Grewal, 2006). The Swi6 related protein Chp2 also interacts with Epe1 and might play a role in its recruitment to the chromatin (Sadaie et al., 2008). Whether Chp2 or other factors could specially target Epe1 to ectopic heterochromatin such as that created by TetR-Clr4* would need to investigated.

8.6 A role for Epe1 in chromatin reprogramming?

Epe1 might be regulated in response to external stimuli. Epe1 can disassemble heterochromatin domains rapidly and efficiently. It is therefore possible that Epe1 might be activated or inactivated in response to environmental cues in order to rapidly reset the chromatin state. Activation of Epe1 would promote the rapid disassembly of heterochromatin domains and possibly the rapid activation of genes that were silenced by heterochromatin. Such a process might permit cells to adapt to external changes. Epe1 has been shown to regulate H3K9me2 levels at certain meiotic genes and therefore may be important for the rapid activation of those meiotic genes in response to nitrogen starvation by promoting the disassembly of repressive heterochromatin on those genes (Zofall et al., 2012). However it remains to be determined if Epe1 is specifically recruited or activated upon nitrogen starvation.

8.7 A conserved role for histone demethylases in preventing epigenetic inheritance of chromatin domains?

H3K9 methylation dependent heterochromatin can spread *in cis* and self-propagate through multiple cell divisions independently of the underlying DNA sequence. Regulatory mechanisms must operate to prevent H3K9me from spreading and stably invading euchromatic loci where it would silence essential genes. In *S. pombe*, the putative H3K9me demethylase Epe1 prevents the abnormal inheritance of H3K9me through both mitotic and meiotic cell divisions. Several studies suggest that other histone demethylases might also prevent the aberrant transmission of chromatin domains to progeny in higher eukaryotes. In many organisms such as plants, worms and mammals, chromatin reprogramming events occur in gametes and early embryos

during which chromatin marks are passively or actively lost (Feng et al., 2010). In mammals this chromatin reprogramming results in most histone methylation and DNA methylation being erased (Heard and Martienssen, 2014). It has been proposed that like Epe1, histone demethylases erase methylation from chromatin in the gametes and the embryo in order to reset the genome prevent the epigenetic inheritance of aberrant epi-alleles.

In *Arabidopsis*, gametes are differentiated from somatic cells (Kawashima and Berger, 2014). Methylation on histones accumulated in response to external stimuli is removed during meiosis by histone demethylases. Chromatin reprogramming during gametogenesis is known to be required for the erasure of H3K27me3 that silences the *FLOWERING LOCUS C* (FLC) locus following a long period of cold in a process called vernalisation. *FLC* prevents flowering from occurring until plants have undergone vernalisation. Long exposure to the cold during winter time results in H3K27 tri methylation on the FLC gene by Polycomb proteins, resulting in silencing of the gene which allows flowering to occur in response to environmental stimuli. H3K27me3 persists on the FLC gene following winter and is only erased during meiosis by the H3K27 KDM ELF6, ensuring that the requirement for vernalization is reset for flowering each generation (Crevillen et al., 2014). Thus demethylation by ELF6 might prevent the epigenetic transgenerational inheritance of H3K27me3 through meiosis in plants.

The observations described above suggest that although chromatin domains can be epigenetically propagated through cell divisions, it appears that in fission yeast, and also in mammals and plants, the transmission of the epigenetic information carried by histone marks is regulated by mechanisms involving histone demethylases that seem to prevent the transmission of epi-alleles to the progeny. Thus transgenerational maintenance of an epigenetic states appears to be a rare exception rather than a common occurrence.

CONCLUSIONS AND PERSPECTIVES

A common feature of most heterochromatin domains in eukaryotes is the presence of H3 methylated on lysine 9. In fission yeast, we showed that the presence of H3K9me alone is sufficient to form a silent domain of heterochromatin that can self-propagate through mitotic and meiotic cell divisions independently of the underlying DNA

sequence. The epigenetic maintenance of H3K9me heterochromatin relies on the HP1 protein Swi6 and the Clr4 H3K9 KMTase that both bind H3K9me via their chromodomain, recruit factors involved in transcriptional silencing and methylate histone H3 in the vicinity thus allowing heterochromatin domain to spread and to be reinforced through cell divisions. The recognition of H3K9me by HP1 proteins and H3K9 methyltransferase from the Clr4/Suv39 family is conserved in Drosophila and mammals suggesting that H3K9me is likely to act as an epigenetic mark in those organisms. In fission yeast, heterochromatin domains carry a self-regulatory mechanism mediated by Epe1 that prevents inappropriate heterochromatin spreading and epigenetic inheritance of heterochromatin domains. Such a mechanism might be important to avoid the transmission of aberrant epi-alleles to be transmitted to the progeny. Such regulatory mechanisms might be conserved in *Drosophila* and mammals.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S16
References

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EPIGENETICS

Restricted epigenetic inheritance of H3K9 methylation

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Posttranslational histone modifications are believed to allow the epigenetic transmission of distinct chromatin states, independently of associated DNA sequences. Histone H3 lysine 9 (H3K9) methylation is essential for heterochromatin formation; however, a demonstration of its epigenetic heritability is lacking. Fission yeast has a single H3K9 methyltransferase, Clr4, that directs all H3K9 methylation and heterochromatin. Using releasable tethered Clr4 reveals that an active process rapidly erases H3K9 methylation from tethering sites in wild-type cells. However, inactivation of the putative histone demethylase Epe1 allows H3K9 methylation and silent chromatin maintenance at the tethering site through many mitotic divisions, and transgenerationally through meiosis, after release of tethered Clr4. Thus, H3K9 methylation is a heritable epigenetic mark whose transmission is usually countered by its active removal, which prevents the unauthorized inheritance of heterochromatin.

In most eukaryotes, the methylation of nucleosomal histone H3 on lysine 9 (H3K9me) is required for the assembly of constitutive heterochromatin (*1*). H3K9me2/3 is bound by HP1/Swi6 proteins and Suv39/Clr4 H3K9 methyltransferases to form heterochromatic regions (*2*–*6*). Because Suv39 and Clr4 can bind the H3K9me2/3 marks that they generate, and because HP1 proteins may also facilitate recruitment of these methyltransferases (*7*), it is thought that H3K9 methylation and heterochromatin can be maintained by self-propagation, even when the initiator is withdrawn (*8*, *9*). However, in eukaryotic systems that exhibit overtly heritable chromatin states, there is often a tight relationship between DNA methylation, H3K9 methylation, and heterochromatin, confounding analyses of the heritability of H3K9 methylation (*10*, *11*). Fission yeast lacks DNA methylation and a single nonessential methyltransferase, Clr4 (Suv39 ortholog), is responsible for all H3K9me-dependent heterochromatin (*12*). Thus, fission yeast is an ideal system in which to determine whether H3K9me-dependent heterochromatin is truly heritable. Clr4 normally requires sequence-directed targeting to particular chromosomal regions via RNA interference (RNAi) in a process involving

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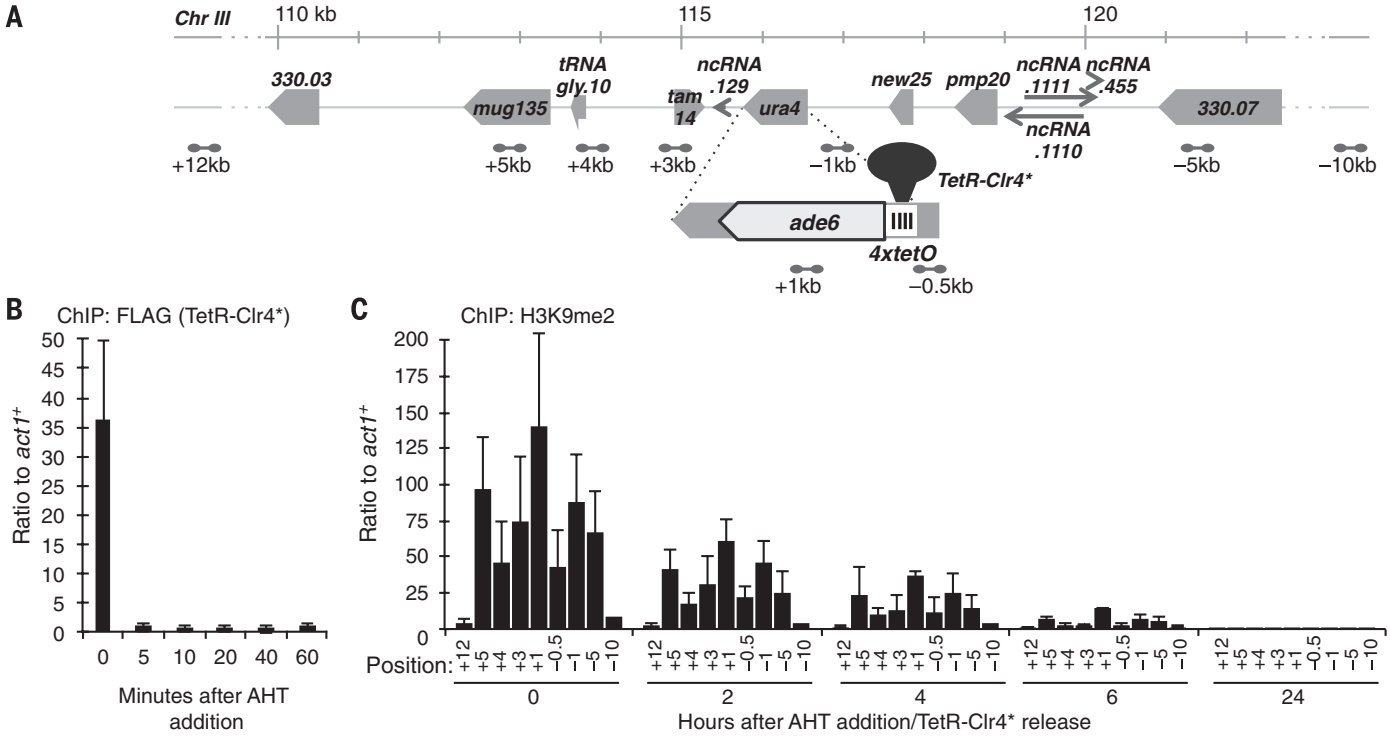

Fig. 1. H3K9 methylation is rapidly lost upon release of tethered TetR-Clr4*. (**A**) Positions of *4xtetO*, tethered TetR-Clr4* beside *ade6*$^+$ at *ura4*, and surrounding *Schizosaccharomyces pombe* chromosome III genes. Dumbbells indicate primer pairs. ncRNA, noncoding RNA. (**B** and **C**) Quantitative chromatin immunoprecipitation (qChIP) time course of FLAG-TetR-Clr4* (B) and H3K9me2 (C) levels on *4xtetO-ade6*$^+$ after AHT addition using the indicated primers. Data are mean ± SD (error bars) (n = 3 experimental replicates). $P < 0.05$ (t test).

heterochromatin nucleation, spreading, and maintenance (*13*–*15*). The inheritance of heterochromatin on centromere repeat DNA inserted at ectopic locations also requires RNAi, but at the mating-type locus heterochromatin is dependent on DNA binding factors in the absence of RNAi (*13*–*17*). However, constitutive tethering of Clr4 to a euchromatic locus via the Gal4 DNA binding domain (GBD) allows the assembly of an extensive domain of H3K9me-heterochromatin, independently of RNAi (*18*). In this study, we tethered a regulatable TetR^{off}-Clr4 fusion protein to determine whether H3K9me is a persistent histone modification that can be stably copied through mitotic cell divisions and meiosis after release or loss of the TetR^{off}-Clr4 initiator.

TetR^{off}-2xFLAG-Clr4-cdd fusion protein (abbreviated TetR-Clr4*), lacking the Clr4 chromodomain, was stably expressed in cells with an *ade6*$^+$ gene downstream of *4xtetO* binding sites at the *ura4* locus (*4xtetO-ade6*$^+$) (Fig. 1A) (*19*). TetR-Clr4* silences *4xtetO-ade6*$^+$ independently of RNAi (*ago1Δ*, *dcr1Δ*), similar to GBD-Clr4 (*18*), resulting in reduced RNA polymerase II association and high H3K9me2 levels and silencing over a broad region (Fig. 1C and figs. S1 and S9, C to E). TetR-Clr4* is released within 5 min from *tetO* sites by addition of anhydrotetracycline (AHT) (Fig. 1B). All strains used in these experiments also express wild-type (WT) Clr4, which can interact via its chromodomain with TetR-Clr4–directed H3K9me and thus potentially use its read-write capabilities to methylate newly incorporated H3 and allow heterochromatin transmission to daughter cells after TetR-Clr4* release. However, in a time course, H3K9me2 rapidly declines over *4xtetO-ade6*$^+$ after AHT addition; >90% is lost within 6 hours (Fig. 1C and fig. S2A). AHT itself does not affect endogenous heterochromatin integrity (fig. S2B). H3 levels do not decline on *4xtetO-ade6*$^+$ over this period (fig. S2A). Swi6^{HP1} is also lost from *4xtetO-ade6*$^+$ when cells are grown with AHT (fig. S1F).

We also tethered TetR-Clr4* within two non-essential genes with long open reading frames, which are less likely to contain unannotated features that might interfere with heterochromatin integrity. Moreover, both *sib1*$^+$ (15,005 base pairs) and *vps1302*$^+$ (9200 base pairs) exhibit expression levels and rates of H3 turnover that are ~three times lower than those of *ade6*$^+$ (Fig. 2, A to C, and fig. S3). *4xtetO* and *1xtetO* sites were placed within *sib1* and *vps1203*, respectively (Fig. 2, D and E). *sib1:4xtetO* and *vps1302:1xtetO* were

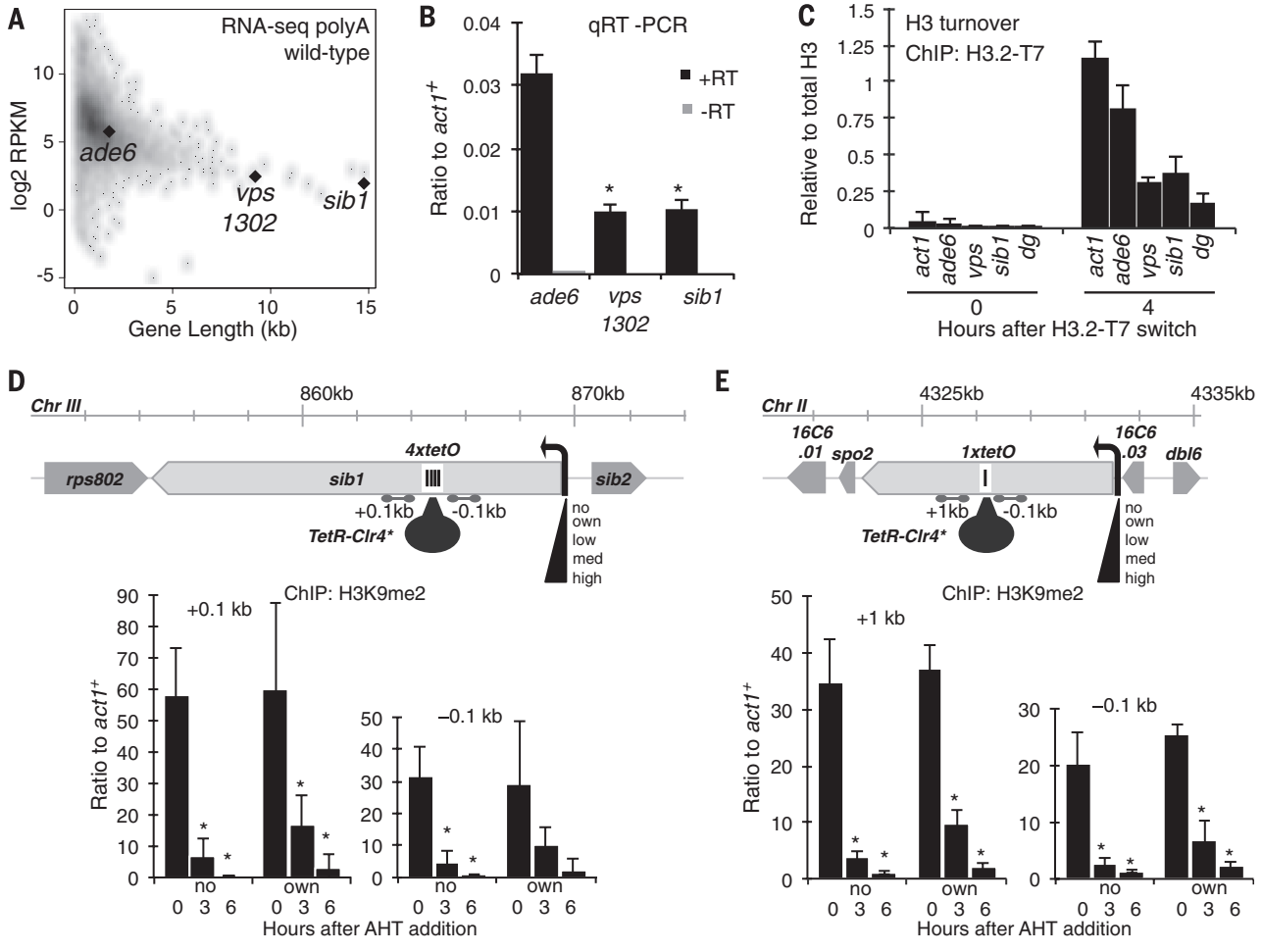

Fig. 2. Tethering TetR-Clr4* at loci with low expression and histone turnover does not stabilize H3K9 methylation. (**A**) Read distribution (log_2RPKM, where RPKM is reads per kilobase per million) from *S. pombe* polyA RNA sequencing (RNA-seq) relative to gene length. *ade6*$^+$, *sib1*$^+$, and *vps1302*$^+$ are indicated. (**B**) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of *ade6*$^+$, *sib1*$^+$, and *vps1302*$^+$ RNA levels. Data are mean ± SD (error bars) (n = 3). *P < 0.005 (t test). (**C**) Recombination-induced tag exchange monitoring incorporation of new H3-T7 on *act1*$^+$, *ade6*$^+$, *sib1*$^+$, *vps1302*$^+$, and cen-*dg* repeats. Data are mean ± SD (error bars) (n = 3). H3 turnover on *sib1*$^+$ and *vps1302*$^+$ was significantly lower than on *act1*$^+$ and *ade6*$^+$. P < 0.05 (t test). (**D** and **E**) *sib1*$^+$ and *vps1302*$^+$ lose H3K9me2 after TetR-Clr4* release. The position of *tetO* sites within *sib1* and *vps1302* is shown. *own* promoters were replaced with *ura4*$^+$ (*no*) or swapped to low-, medium (*med*)–, or high-*adh1* promoter versions (*20*). Dumbbells indicate primer pairs. qChIP of H3K9me2 levels, at time points relative to AHT addition, on *sib1:4xtetO* (D) and *vps1302:1xtetO* (E) with *no* or indicted promoters is shown. Data are mean ± SD (error bars) (n = 3); P < 0.05 (t test). The H3K9me2 level within *sib1* carrying its own promoter is decreased with a probability of, respectively, P = 0.068 and 0.051, 3 and 6 hours after TetR-Clr4* release.

also placed under the control of low, medium (*med*), and high versions of the constitutive *adh1* promoter (*20*). We also generated *sib1:4xtetO* and *vps1302:1xtetO* without promoters (*no*). All strains expressed WT Clr4 and TetR-Clr4*. Both *sib1*$^+$ and *vps1302*$^+$ were expressed at low levels when their promoters were removed and at much higher levels from *med-adh1* or *high-adh1* compared with their *own* or *low-adh1* promoters (fig. S4). TetR-Clr4* was unable to establish much H3K9me2 when tethered to *sib1:4xtetO* or *vps1302:1xtetO* expressed from *hi-adh1* and relatively low levels when expressed from *med-adh1*, but substantial H3K9me2 occurred when either gene had *no*, its *own*, or the *low-adh1* promoter (fig. S4). However, as with *4xtetO-ade6*$^+$, rapid loss of H3K9me2 followed TetR-Clr4* release from even *no* and *own* promoter constructs; again, >90% was lost within 6 hours (Fig. 2, D and E). Although high levels of transcription across tethering sites prevents the establishment of H3K9me by TetR-Clr4*, neither low promoter strength nor low H3 turnover renders H3K9me more persistent upon methyltransferase release. Thus, the inability to maintain H3K9 methylation upon removal of the initiating tethered Clr4 methyltransferase is probably a general feature of euchromatic loci.

To determine whether the loss of H3K9 methylation from the tethering site is coupled to replication or passage through the cell cycle, we released TetR-Clr4* from *4xtetO-ade6*$^+$ in *cdc25-22* synchronized cultures (Fig. 3A). H3K9me2 levels on *4xtetO-ade6*$^+$ dropped by 70% within one cell cycle after the addition of AHT to these synchronized cultures, and no accelerated H3K9me2 loss was evident during S phase, which is coincident with septation (*21*). We also released TetR-Clr4* from *4xtetO-ade6*$^+$ in noncycling G_2 blocked *cdc25-22* cells (Fig. 3B). TetR-Clr4* was lost from *4xtetO-ade6* within 1 hour, and H3K9me declined to less than 25% of initial levels within 4 hours. Thus, after release of the initiating methyltransferase, rather than being passively diluted through chromatin replication, H3K9 methylation must be removed by an active process.

Known and putative histone demethylases might act to remove H3K9me and thus disassemble heterochromatin from TetR-Clr4* tethering sites. We therefore tested whether mutation of genes for six JmjC domain (Epe1, Jmj1, Jmj2, Jmj4, Lid2, and Msc1) (*22*) or two SWIRM/amino-oxidase domain proteins (Lsd1 and Lsd2) (*23*) allowed long-term *4xtetO-ade6*$^+$ silencing after tethered TetR-Clr4* release. WT *4xtetO-ade6*$^+$ *TetR-Clr4** cells form red/*ade6*-repressed colonies on indicator plates lacking AHT, whereas white/*ade6*-expressing colonies appear on +AHT plates due to loss of H3K9me-dependent heterochromatin over *4xtetO-ade6*$^+$. Of the eight tested mutants, only *epe1Δ* consistently formed red-pink colonies on +AHT plates, indicating that *4xtetO-ade6*$^+$ can remain repressed without bound TetR-Clr4* (Fig. 4A and figs. S5 and S6). Catalytically inactivating mutations in the Fe(II) or 2-oxyglutarate binding sites of the Epe1 putative demethylase (*epe1-H297A* and *epe1-K314A*) had a similar phenotype (Fig. 4A, fig. S6, and table S3). The variable silencing and colony color most likely reflects stochastic events at the *4xtetO-ade6*$^+$ locus in *epe1Δ* cells in which H3K9me domains are known to expand and additional heterochromatin islands also appear, potentially titrating and redistributing heterochromatin proteins between various loci in individual cells (*24*–*27*). Maintenance of the silenced state in *epe1Δ* cells is not dependent on the RNAi component Ago1, as *ago1Δepe1Δ* cells form red/*ade6*-silent colonies on +AHT plates (fig. S7A), but it does require untethered WT Clr4 with an intact Clr4 chromodomain and Swi6 (fig. S8). This reliance on untethered, intact Clr4 and Swi6 is consistent with a simple read-write propagation mechanism (fig. S10).

Silencing of *4xtetO-ade6*$^+$ can be propagated through multiple cell divisions in *epe1* mutants (lost in 4% of cells per division), and a high proportion of descendant cells retain silencing of, and 30 to 70% of H3K9me2 on, *4xtetO-ade6*$^+$ after TetR-Clr4* release by AHT. In contrast, *4xtetO-ade6*$^+$ silencing and H3K9me2 are completely lost in WT cells (Fig. 4A and fig. S7, B to E). The relative levels of H3K9me2 and H3K9me3 detected on *4xtetO-ade6*$^+$ are similar in WT and *epe1Δ* cells, and surrounding genes are silenced by H3K9me2 in both WT and *epe1Δ*. (fig. S9). To determine whether H3K9me on *4xtetO-ade6*$^+$ in *epe1Δ* cells is maintained through meiosis in the absence of TetR-Clr4*, *epe1Δ 4xtetO-ade6*$^+$ *tetR-clr4** cells (F_0) were crossed to *epe1Δ* cells devoid of both *4xtetO-ade6*$^+$ and TetR-Clr4*, and then F_1 *epe1Δ 4xtetO-ade6*$^+$ progeny lacking TetR-Clr4* were again crossed to *epe1Δ* cells. A high proportion of resulting F_2 *epe1Δ 4xtetO-ade6*$^+$ progeny formed red-pink/*ade6*-repressed colonies, and H3K9me2 was retained (Fig. 4B and fig. S6B). Thus, *epe1Δ* allows silencing and H3K9me to persist through multiple mitotic divisions and meiosis, in the complete absence of the tethered TetR-Clr4* that initiated H3K9me-dependent heterochromatin on *4xtetO-ade6*$^+$. Crossing of red F2 *epe1Δ 4xtetO-ade6*$^+$ cells to WT *epe1*$^+$ cells resulted in loss of silencing (white colonies only) and H3K9me2 from the *4xtetO-ade6*$^+$ locus. Thus, provision of *epe1*$^+$ results in removal of persistent H3K9me and loss of silencing (Fig. 4B). Genetically identical naïve *epe1Δ 4xtetO-ade6*$^+$ cells that were never exposed to the TetR-Clr4* initiator formed only white/*ade6*-expressing colonies, and H3K9me2 was absent (Fig. 4B). We conclude that the transient tethering of TetR-Clr4* adjacent to *4xtetO-ade6*$^+$ allows establishment of H3K9me-dependent heterochromatin, which can be propagated epigenetically through mitotic cell divisions and meiosis using endogenous read-write copying mechanisms, provided that Epe1 is rendered nonfunctional (for a model, see fig. S10).

Propagation of heterochromatin on *4xtetO-ade6*$^+$ in *epe1* mutants requires recognition of TetR-Clr4*–mediated H3K9me by the chromodomain of Clr4 and also Swi6 (fig. S8). Epe1 associates with Swi6^{HP1} and clearly opposes heterochromatin formation (*24*–*28*). Indeed, Epe1 associates with TetR-Clr4*–mediated heterochromatin (fig. S7C). Although

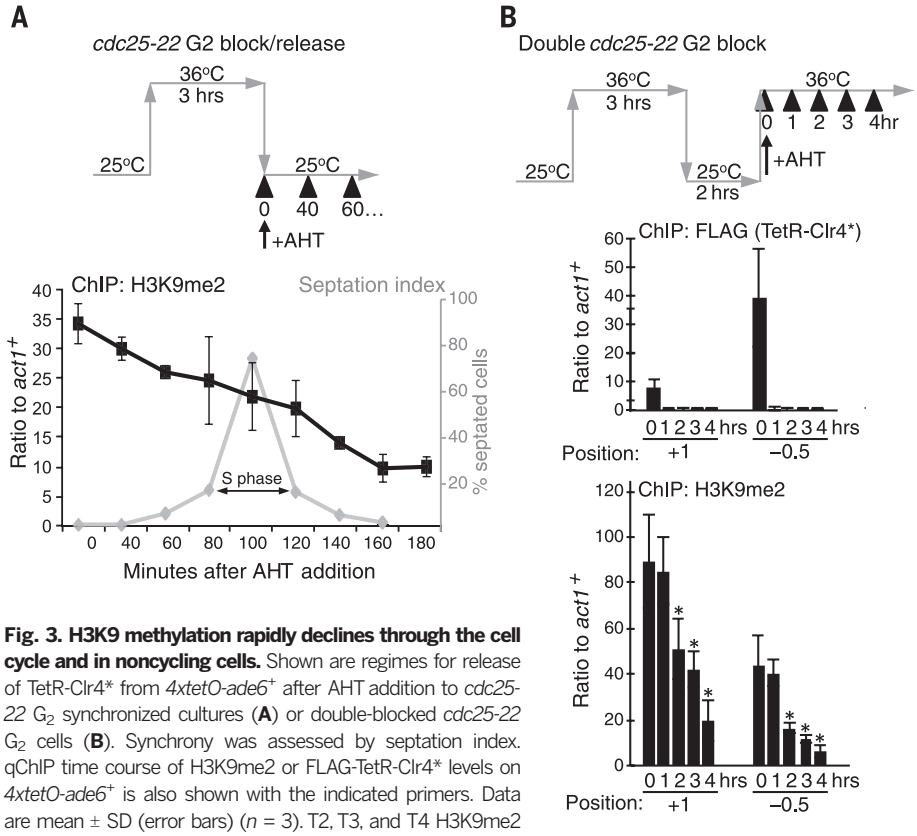

Fig. 3. H3K9 methylation rapidly declines through the cell cycle and in noncycling cells. Shown are regimes for release of TetR-Clr4* from *4xtetO-ade6*$^+$ after AHT addition to *cdc25-22* G_2 synchronized cultures (**A**) or double-blocked *cdc25-22* G_2 cells (**B**). Synchrony was assessed by septation index. qChIP time course of H3K9me2 or FLAG-TetR-Clr4* levels on *4xtetO-ade6*$^+$ is also shown with the indicated primers. Data are mean ± SD (error bars) (n = 3). T2, T3, and T4 H3K9me2 levels are significantly less than T0; *P < 0.05 (t test).

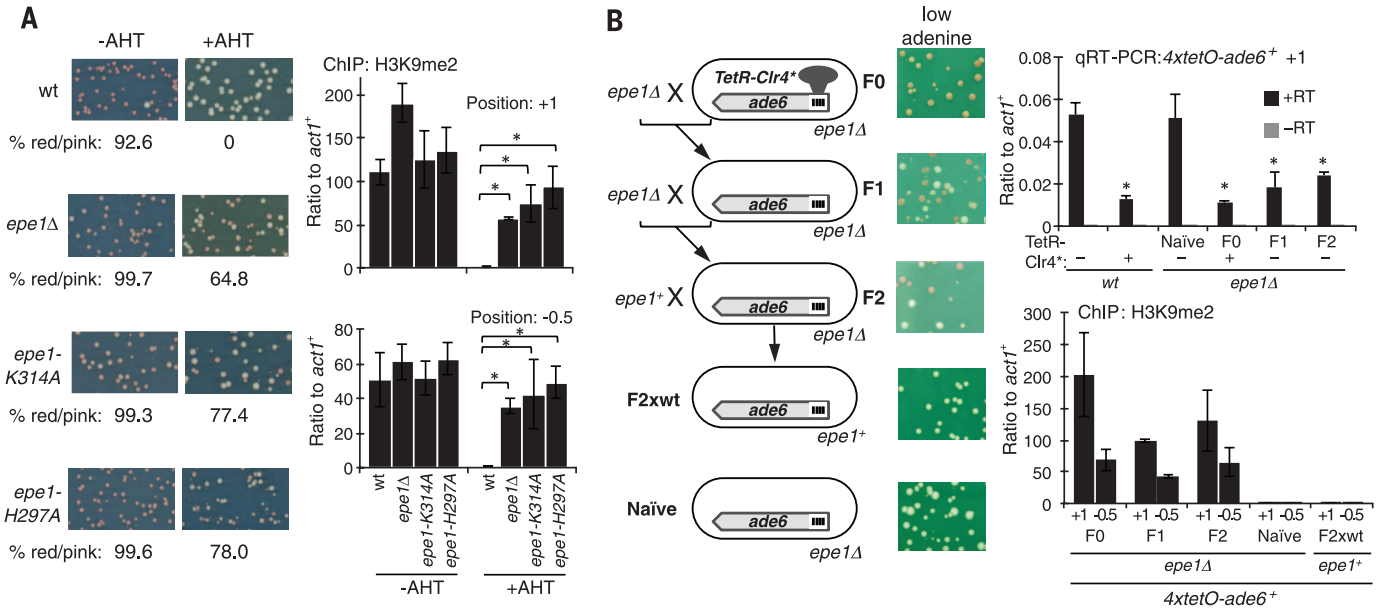

Fig. 4. *epe1* mutants retain heterochromatin without tethered Clr4 methyltransferase through multiple cell divisions and meiosis. (**A**) WT, *epe1Δ*, *epe1-K314A*, and *epe1-H297A* cells carrying *4xtetO-ade6*$^+$ and expressing TetR-Clr4* were grown with or without AHT. Colony color assay to assess *4xtetO-ade6*$^+$ silencing (red-pink colonies; percent of total indicated) and H3K9me2 qChIP on *4xtetO-ade6*$^+$ with (-AHT) or without (+AHT) tethered TetR-Clr4* are shown. Data are mean ± SD (error bars) ($n = 3$). $P < 0.05$ (*t* test). (**B**) TetR-Clr4* was completely removed from F_0 *epe1Δ 4xtetO-ade6*$^+$ *tetR-Clr4** cells by crossing to *epe1Δ* lacking TetR-Clr4* and *4xtetO-ade6*$^+$. F_1 progeny were crossed to *epe1Δ* cells, generating *epe1Δ* F_2 progeny. *epe1*$^+$ F_2×WT progeny were produced by crossing *epe1*$^+$ into *epe1Δ 4xtetO-ade6*$^+$ F_2 cells. Naïve *epe1Δ 4xtetO-ade6*$^+$ cells never expressed TetR-Clr4*. Colony color, qRT-PCR, and qChIP assays were performed to assess silencing and transcription of *4xtetO-ade6*$^+$, and H3K9me2 levels on *4xtetO-ade6*$^+$ in indicated cell types are shown. Data are mean ± SD (error bars) ($n = 3$). *4xtetO-ade6*$^+$ RNA levels are significantly reduced in F_0, F_1, and F_2 compared with WT cells without TetR-Clr4*. $P < 0.05$ (*t* test).

Epe1 contains a JmjC domain, its Fe(II) binding site is unusual, and histone demethylase activity has not been detected (*22*). However, the human PHF2 JmjC domain bears a similar anomaly but phosphorylation activates its latent H3K9 demethylase activity (*29*). The analyses presented here are consistent with Epe1 normally acting as an H3K9 demethylase that removes H3K9 methylation from ectopic sites of heterochromatin formation. Moreover, additional heterochromatin islands and domain expansion in *epe1* mutants are best explained by the loss of an H3K9 demethylase that prevents excessive H3K9me-dependent heterochromatin formation. Epe1-dependent removal of H3K9me ensures regulation of centromeric heterochromatin and makes the RNAi pathway essential for the systematic replenishment of H3K9me every cell cycle (*30*, *31*). Epe1 itself may be regulated in response to environmental cues to retain or eliminate H3K9 methylation at specific locations (*26*). Indeed, Epe1 levels are regulated, and this may aid the persistence of centromeric H3K9me-dependent heterochromatin (*28*). Thus, opposing H3K9 methyltransferase and demethylase activities must be finely tuned to allow controlled heterochromatin formation and prevent its inappropriate mitotic and transgenerational inheritance. It seems counterintuitive for heterochromatin to carry a means of self-destruction; however, such an inbuilt safety mechanism averts the inappropriate, and potentially deleterious, silencing of genes by removing repressive heterochromatin and preventing its propagation.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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Restricted epigenetic inheritance of H3K9 methylation
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