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Abstract

Post-translational histone modifications are believed to allow the epigenetic transmission of distinct chromatin states, independently of associated DNA sequences. 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. Utilizing 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 unauthorised 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). Since Suv39/Clr4 can bind the H3K9me2/3 mark that they generate, and 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thought that H3K9 methylation, and heterochromatin, can be maintained by self-propagation, even when the initiator is withdrawn (8, 9).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 non-essential 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 RNAi in a process involving

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heterochromatin nucleation, spreading and maintenance (13-15). The inheritance of heterochromatin on centromere repeat DNA inserted at ectopic locations also requires RNAi and 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). Here we tether a regulatable TetR[off]-Clr4 fusion protein to determine if H3K9me is a persistent histone modification that can be stably copied through mitotic cell divisions and meiosis following 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 (*ago1*, *dcr1*), similar to GBD-Clr4 (18), resulting in reduced RNAPII association, and high H3K9me2 levels and silencing over a broad region (Fig. 1C, fig. S1, and fig. S9C-E). TetR-Clr4* is released within 5 minutes from *tetO* sites by addition of anhydrotetracycline (AHT; Fig. 1B). All strains utilised also express wild-type 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 following TetR-Clr4* release. However, in a time course, H3K9me2 rapidly declines over *4xtetO-ade6+* following AHT addition; >90% is lost within six 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 bp) and *vps1302+* (9,200 bp) exhibit expression levels and rates of H3 turnover that are ~3-fold lower than those of *ade6+* (Fig. 2A-C, and fig. S3). *4xtetO* and *1xtetO* sites were placed within *sib1* and *vps1203*, respectively (Fig. 2D and E). *sib1:4xtetO* and *vps1302:1xtetO* were 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 wild-type 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 to their *own*, or *low-adh1*, promoters (fig. S4). TetR-Clr4* was unable to establish significant levels of 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 likely a general feature of euchromatic loci.

To determine if 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 following 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 non-cycling G2 blocked *cdc25-22* cells (Fig. 3B). TetR-Clr4* was lost from *4xtetO-ade6* within an 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 if mutation of genes for six JmjC domain (Epe1, Jmj1, Jmj2, Jmj4, Lid2, Msc1) (22) or two SWIRM/Amino-oxidase domain proteins (Lsd1 or Lsd2; 23) allowed long-term *4xtetO-ade6+* silencing after tethered TetR-Clr4* release. Wild-type *4xtetO-ade6+ TetR-Clr4** cells form red/*ade6*-repressed colonies on indicator plates lacking AHT, white/*ade6*-expressing colonies appear on +AHT plates due to loss of H3K9me-dependent heterochromatin over *4xtetO-ade6+*. Of the eight tested mutants only *epe1* consistently formed red/pink colonies on +AHT plates, indicating that *4xtetO-ade6+* can remain repressed without bound TetR-Clr4* (Fig. 4A, fig. S5 and fig. 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 colour most likely reflects stochastic events at the *4xtetO-ade6+* locus in *epe1* 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 *epe1* cells is not dependent on the RNAi component Ago1 as *ago1 epe1* cells form red/*ade6*-silent colonies on +AHT plates (fig. S7A) but it does require untethered wild-type 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/division), and a high proportion of descendant cells retain silencing of, and 30-70% of H3K9me2 on, *4xtetO-ade6+* after TetR-Clr4* release by AHT. In contrast, *4xtetO-ade6+* silencing and H3K9me2 are completely lost in wild-type cells (Fig. 4A, and fig. S7B-E). The relative levels of H3K9me2 and H3K9me3 detected on *4xtetO-ade6+* are similar in wild-type and *epe1* cells and surrounding genes are silenced by H3K9me2 in both wild-type and *epe1* . (fig. S9). To determine if H3K9me on *4xtetO-ade6+* in *epe1* cells is maintained through meiosis in the absence of TetR-Clr4*, *epe1 4xtetO-ade6+ tetR-clr4** cells (F0) were crossed to *epe1* cells devoid of both *4xtetO-ade6+* and TetR-clr4* and then F1 *epe1 4xtetO-ade6+* progeny lacking TetR-Clr4* were again crossed to *epe1* cells. A high proportion of resulting F2 *epe1 4xtetO-ade6+* progeny

formed red-pink/*ade6*-repressed colonies and H3K9me2 was retained (Fig. 4B, and fig. S6B). Thus, *epe1* 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 *epe1 4xtetO-ade6+* cells to wild-type *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 *epe1 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 Epe1 is rendered non-functional (Model: fig. S10).

Propagation of heterochromatin on *4xtetO-ade6+* in *epe1* mutants requires recognition of TeR-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 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 activitity (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 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 in order 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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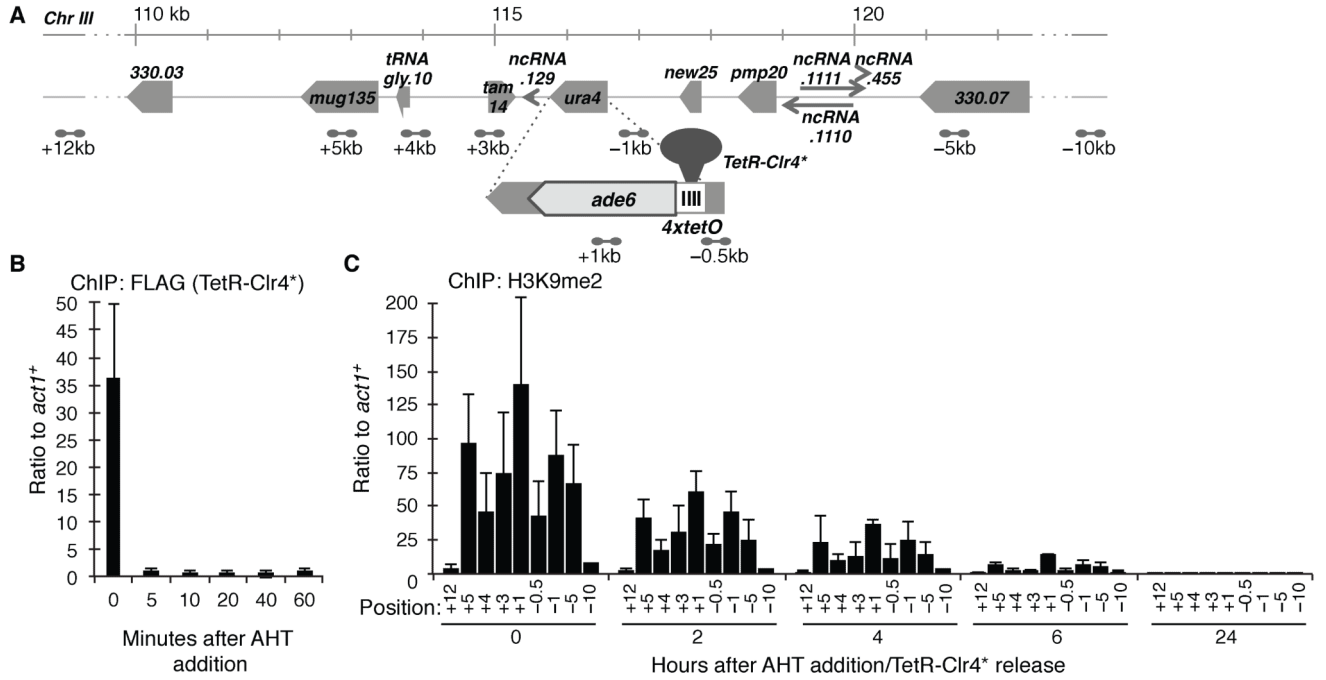
Fig. 1. H3K9 methylation is rapidly lost upon release of tethered TetR-Clr4*
(**A**) Positions of *4xtetO*, tethered TetR-Clr4* beside *ade6+* at *ura4*, and surrounding *S. pombe* chromosome III genes. Dumbbells indicate primer pairs.
(**B** and **C**) qChIP time course of FLAG-TetR-Clr4* (**B**) and H3K9me2 (**C**) levels on *4xtetO-ade6+* following AHT addition using indicated primers. Data are mean ±SD (*n=3*), *P<0.05* (t-test).
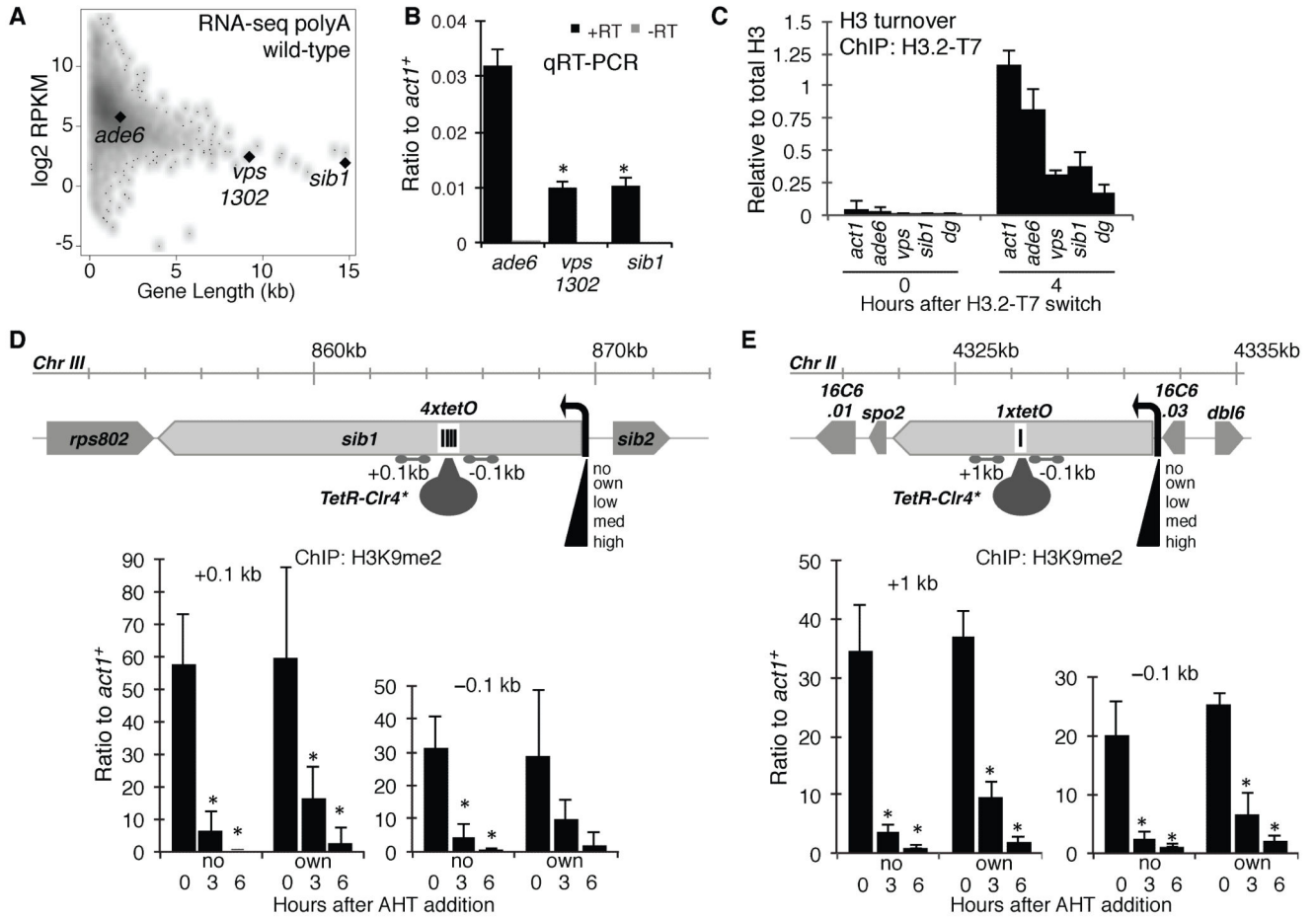
Fig. 2. Tethering TetR-Clr4* at loci with low expression and histone turnover does not stablize H3K9 methylation

(**A**) Read distribution (log$_2$RPKM) from *S. pombe* polyA RNA-seq relative to gene length. *ade6+, sib1+* and *vps1302+* indicated.

(**B**) qRT-PCR of *ade6+, sib1+* and *vps1302+* RNA levels. Data are mean ±SD (*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 (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. Position of *tetO* sites within *sib1* and *vps1302*. *own* promoters were replaced with *ura4+* (*no*) or swapped to low, medium (*med*), or high *adh1* promoter versions (20). Dumbbells indicate primers. qChIP of H3K9me2 levels, at time points relative to AHT addition, on *sib1:4xtetO* (**D**) and *vps1302:1xtetO* (**E**) with *no* or indicted promoters. Data are mean ±SD (*n=3*), *P<0.05* (t-test). H3K9me2 level within *sib1* carrying its own promoter is decreased with a probability of respectively *P=0.068* and *P=0.051*, 3h and 6h following TetR-Clr4* release.
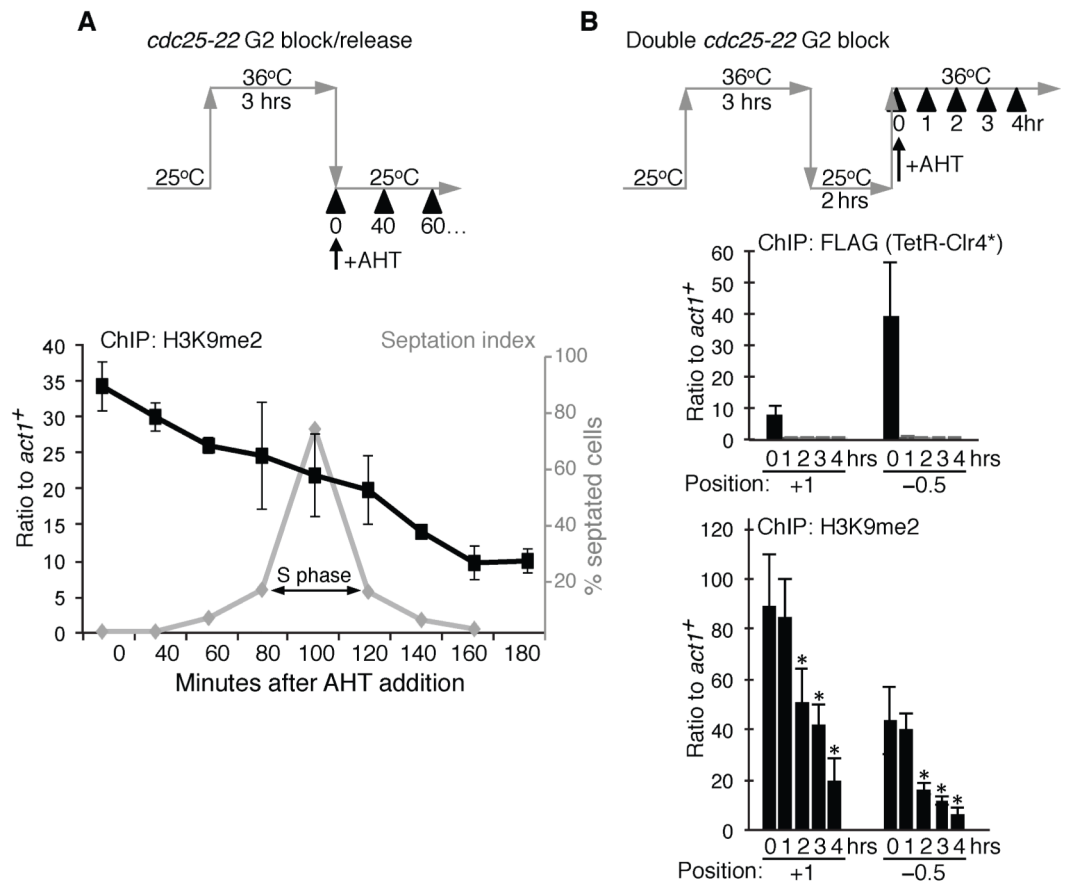
Fig. 3. H3K9 methylation rapidly declines through the cell cycle and in non-cycling cells
Regimes for release of TetR-Clr4* from *4xtetO-ade6*[+] following AHT addition to *cdc25-22*
G2 synchronised cultures (**A**) or double-blocked *cdc25-22* G2 cells (**B**). Synchrony was
assessed by septation index. qChIP time course of H3K9me2 or FLAG-TetR-Clr4* levels on
4xtetO-ade6[+] using indicated primers. Data are mean ±SD (*n=3*).
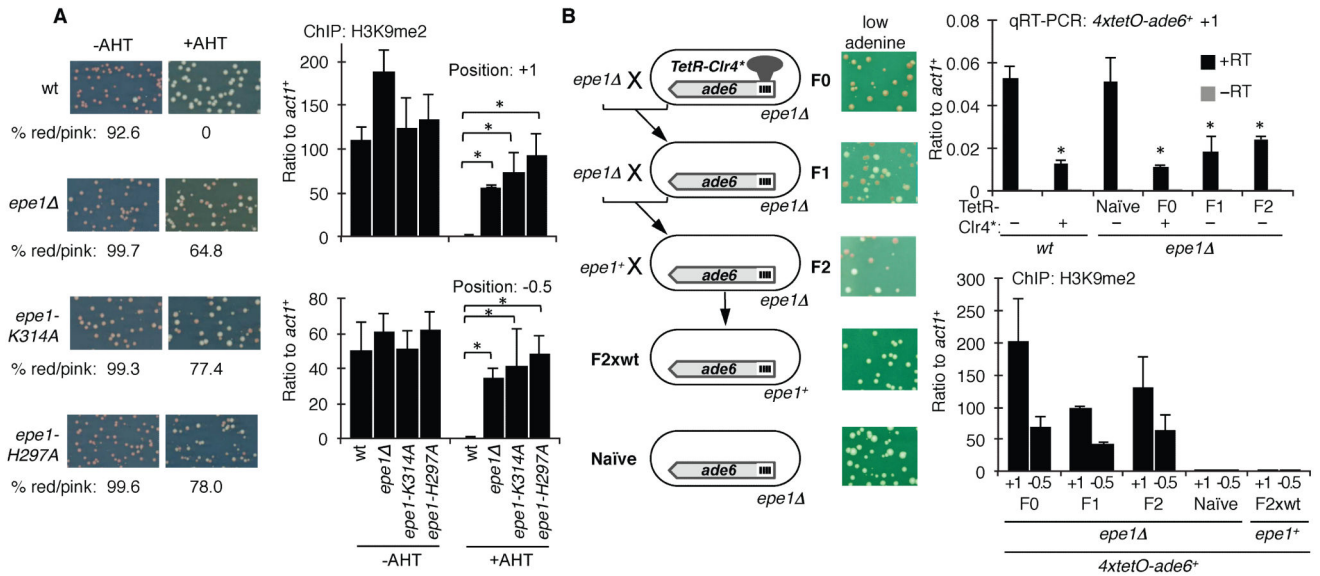
Fig. 4. *epe1* mutants retain heterochromatin without tethered Clr4 methyltransferase through multiple cell divisions and meiosis

(**A**) wild-type, *epe1Δ*, *epe1-K314A* and *epe1-H297A* cells carrying *4xtetO-ade6+* and expressing TetR-Clr4*, were grown −/+ AHT. Colony colour assay to assess *4xtetO-ade6+* silencing (red-pink colonies; % of total indicated) and H3K9me2 qChIP on *4xtetO-ade6+* with (−AHT) or without (+AHT) tethered TetR-Clr4* Data are mean ±SD (*n=3*), *P<0.05* (t-test).

(**B**) TetR-Clr4* was completely removed from F0 *epe1Δ 4xtetO-ade6+ tetR-Clr4** cells by crossing to *epe1Δ* lacking TetR-Clr4* and *4xtetO-ade6+*. F1 progeny were crossed to *epe1Δ* cells, generating *epe1Δ* F2 progeny. *epe1+* F2xwt progeny were produced by crossing *epe1+* into *epe1Δ 4xtetO-ade6+* F2 cells. Naïve *epe1Δ 4xtetO-ade6+* cells never expressed TetR-Clr4*. Colony colour, qRT-PCR and qChIP assays to assess silencing and transcription of *4xtetO-ade6+*, and H3K9me2 levels on *4xtetO-ade6+* in indicated cell types. Data are mean ±SD (*n=3*). *4xtetO-ade6+* RNA levels are significantly reduced in F0, F1 and F2 compared to wild-type cells without TetR-Clr4*; *P<0.05* (t-test).