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## Synthetic heterochromatin bypasses RNAi and centromeric repeats to establish functional centromeres

Alexander Kagansky<sup>1,2,3</sup>, Hernan Diego Folco<sup>2,3</sup>, Ricardo Almeida<sup>3</sup>, Alison L. Pidoux, Abdelhalim Boukaba<sup>3</sup>, Femke Simmer<sup>4</sup>, Takeshi Urano<sup>1</sup>, Georgina L. Hamilton, and Robin C. Allshire<sup>\*</sup>

Wellcome Trust Centre for Cell Biology and Institute of Cell Biology, School of Biological Sciences, The University of Edinburgh, 6.34 Swann Building Edinburgh EH9 3JR Scotland UK.

<sup>1</sup>Department of Biochemistry Shimane University Faculty of Medicine 89-1 Enya-cho, Izumo 693-8501, Japan

### Abstract

In the central domain of fission yeast centromeres the kinetochore is assembled upon CENP-A<sup>Cnp1</sup> nucleosomes. Normally, siRNAs generated from flanking outer repeat transcripts direct histone H3 lysine 9 methyltransferase Clr4 to homologous loci to form heterochromatin. Outer repeats, RNAi and centromeric heterochromatin are required to establish CENP-A<sup>Cnp1</sup> chromatin. We demonstrate that tethering Clr4 via DNA binding sites at euchromatic loci induces heterochromatin assembly, with or without active RNAi. This synthetic heterochromatin completely substitutes for outer repeats on plasmid-based minichromosomes, promoting *de novo* CENP-A<sup>Cnp1</sup> and kinetochore assembly, to allow their mitotic segregation, even with RNAi inactive. Thus, the role of outer repeats in centromere establishment is simply the provision of RNAi substrates to direct heterochromatin formation; H3K9 methylation-dependent heterochromatin is alone sufficient to form functional centromeres.

It is unclear what features define the chromosomal location where histone H3 is replaced by the centromere specific histone H3 variant CENP-A, to allow kinetochore assembly (1). Kinetochores in many organisms are surrounded by heterochromatin (2). In fission yeast (*Schizosaccharomyces pombe*), heterochromatin formed on the outer repeats (*otr*, composed of *dg* and *dh* elements), flanks the central domain chromatin, in which canonical H3 is replaced by CENP-A<sup>Cnp1</sup> that promotes kinetochore assembly (3-7). Outer repeat heterochromatin on minichromosomes is necessary to allow *de novo* establishment of CENP-A<sup>Cnp1</sup> chromatin and kinetochore protein recruitment (8); it also contributes to centromere function by ensuring robust cohesion between sister-centromeres (9, 10). Heterochromatin is formed by the action of RNAi-directed chromatin modification on non-coding outer repeat transcripts to create methyl-H3K9 binding sites for the chromo-domain proteins Swi6, Chp1, Chp2 and Clr4. Thus, RNAi components, histone deacetylases (HDACs), the Clr4 H3K9 methyltransferase and chromo-domain proteins all contribute to heterochromatin integrity (reviewed (11, 12)).

<sup>\*</sup> Corresponding author: robin.allshire@ed.ac.uk Tel: 44-(0)131-650-7117 Fax: 44-(0)845-280-2340.

<sup>1,2</sup>equal contribution

<sup>2</sup>Present Addresses: Dept. Cellular & Mol. Medicine, University of California, San Diego 9500 Gilman Drive, La Jolla, California 92093-0660, USA.

<sup>3</sup>Laboratory of Cancer Epigenetics, Faculty of Medicine, Free University of Brussels, 808 route de Lennik, 1070 Brussels, Belgium.

<sup>4</sup>The Radboud University Nijmegen Medical Centre, Molecular Biology, 6500 HB Nijmegen, The Netherlands.

Active RNAi, Clr4 methyltransferase and the Swi6 (HP1) chromo-domain protein are necessary to form heterochromatin on outer repeats and establish CENP-A<sup>Cnp1</sup> chromatin over the adjacent central domain on newly introduced centromeric DNA plasmids (8). To test if artificially tethering Clr4 to a euchromatic locus can promote heterochromatin assembly, and if this alone is sufficient to establish a functional centromere, the DNA binding domain of the *Saccharomyces cerevisiae* Gal4 protein (GBD) was fused to (Fig. 1A): the N-terminus of the gene encoding wild-type Clr4 (*GBD-clr4*), Clr4- $\Delta$ cd lacking the chromo-domain (*GBD-clr4- $\Delta$ cd*) and, Clr4-H410K a catalytically inactive SET-domain mutant (*GBD-clr4- $\Delta$ cd-H410K*). These GBD-to-Clr4 fusions are produced, providing the only source of Clr4 protein (fig. S1A) and were tested in combination with the following reporters: three Gal4 binding sites (*3xgbs*) upstream of *ade6<sup>+</sup>* inserted at the *ura4* (Fig. 1A: *ura4::3xgbs-ade6<sup>+</sup>*) or *arg3* loci, or ten *gbs* sites upstream of *ura4<sup>+</sup>* (fig. S1B, C).

Full *ade6<sup>+</sup>* expression results in white colonies whereas silencing causes pink/red colonies to form. The GBD-Clr4- $\Delta$ cd fusion protein clearly represses the *ura4::3xgbs-ade6<sup>+</sup>* (Fig. 1A) and other reporters (fig. S1B, C) indicating that this silencing is reporter gene and locus independent. It is dependent on Clr4 catalytic activity as no repression occurred with the H410K mutant (Fig. 1A); therefore repression does not result from indirect recruitment of Clr4-associated repressive factors. Although the GBD-Clr4 full-length fusion protein is recruited to the reporter (fig. S2A), it is unable to mediate repression (Fig. 1A). Deletion of the Clr4 chromo-domain affects endogenous heterochromatin and this may release limiting factors for participation in silencing at the tethering site (fig. S2A). Consistent with this, full-length GBD-Clr4 fusion protein can silence the reporter in *dcr1 $\Delta$*  cells (fig. S2B). Analyses of anti-H3K9me2 and Swi6 ChIP demonstrate that in cells expressing GBD-Clr4- $\Delta$ cd protein, H3K9 is dimethylated on, and Swi6 recruited to, a region of approximately 10 kb including and surrounding the *ura4::3xgbs-ade6<sup>+</sup>* reporter (Fig. 1B). Consequently, genes neighbouring the reporter are also repressed in cells expressing GBD-Clr4- $\Delta$ cd (fig. S3). This synthetic heterochromatin is established *de novo*, and maintained, independently of RNAi as the reporter remains largely repressed and assembled in H3K9me2 chromatin in cells lacking Dcr1, Ago1, Tas3, Chp1, or Rdp1 (Fig. 1C; fig. S4). Moreover, no *ade6<sup>+</sup>* or *ura4<sup>+</sup>* reporter gene homologous siRNA were detected (fig. S5). Silencing and H3K9me2 levels are maintained in *dcr1 $\Delta$*  cells (Fig. 1C); thus, although Ago1 associates with *3xgbs-ade6<sup>+</sup>* (fig. S5C), the contribution of it and other RNAi components may only be as bound physical entities. In contrast, silencing is highly dependent on chromatin factors Rik1 (Clr4-associated), Chp2 (a Swi6-related protein), and Sir2 and Clr3 HDACs which must act independently of RNAi (Fig. 1E; fig. S4). Synthetic heterochromatin is essentially insensitive to loss of Swi6; this is consistent the reported role for Swi6 in RNAi-dependent silencing, while Chp2 acts with Clr3 to mediate RNAi-independent transcriptional repression (13). In cells producing only mutant histone H3K9R or H3K9A proteins, *3xgbs-ade6<sup>+</sup>* is expressed, indicating that the Clr4 substrate, H3K9, is critical for silencing by tethered Clr4 activity (fig. S6).

Intact heterochromatin on the outer repeats is necessary for the establishment of CENP-A<sup>Cnp1</sup> chromatin on adjacent central domain DNA (8). Other features of centromeric outer repeats, such as protein binding sites (14) or non-coding RNA (15), could act in combination with heterochromatin to promote CENP-A<sup>Cnp1</sup> incorporation. To determine if heterochromatin alone is sufficient, we substituted the outer repeats on a plasmid with three Gal4-binding sites, or no sites, in close proximity to the central domain, generating the plasmids p*3xgbs-cc2* and p*0xgbs-cc2*, respectively (Fig. 2, fig S7). GBD-Clr4- $\Delta$ cd associates with the plasmid-borne Gal4-sites and H3K9me2 was detected over these and extends into nearby regions suggesting that a 6 kb domain of heterochromatin was formed (Fig. 2A; fig. S7). In support of this, the underlying plasmid-borne marker genes were silenced (fig. S8). Both CENP-A<sup>Cnp1</sup> and CENP-C<sup>Cnp3</sup> associated with the central core of

the p3x*gbs*-cc2 plasmid, but only in cells expressing GBD-Clr4- $\Delta$ cd (Fig. 2B), and CENP-A<sup>Cnp1</sup> was also detected at the extremities of the 8.5 kb central domain, but not on other regions of the plasmid (fig. S9). Thus, the synthetic heterochromatin domain formed by Clr4 methyltransferase bound to these Gal4-sites is sufficient to promote the assembly of CENP-A<sup>Cnp1</sup> across, and recruit other kinetochore proteins to, the central domain.

Only plasmids carrying Gal4-sites formed white/sectorized colonies when combined with GBD-Clr4- $\Delta$ cd (Fig. 2C; see SOM), indicating that a functional centromere, capable of mitotic segregation, had formed. Similar results were obtained with Gal4-sites on the opposite side of cc2 (pcc2-3x*gbs*) (fig. S9). Both 3x*gbs* plasmids exhibit ~2 fold greater mitotic stability than pH'-cc2 which contains native outer repeat heterochromatin on a similarly sized functional minichromosome (fig. S9). This indicates that the function of natural outer repeat DNA at a plasmid-borne centromere can be substituted by artificially recruiting Clr4 to DNA. Using this sensitive plasmid-based assay, we conclude that centromeric outer repeats have no hidden unknown features, and apart from its role in directing heterochromatin assembly, the primary outer repeat DNA sequence is dispensable with respect to establishing centromere-kinetochore function.

Centromeric outer repeats may just provide a dsRNA substrate for RNAi to direct H3K9 methylation and centromeric heterochromatin formation. To test if synthetic heterochromatin is completely RNAi independent, p3x*gbs*-cc2 was transformed into wild-type, *dcr1* $\Delta$  and *rik1* $\Delta$  cells expressing GBD-Clr4- $\Delta$ cd. GBD-Clr4- $\Delta$ cd was bound to the 3x*gbs* in all strains (Fig. 3A) whereas H3K9me2 was detected over this region in wild-type and *dcr1* $\Delta$  but not *rik1* $\Delta$  cells. This confirms that the establishment of H3K9me2 modified chromatin by tethered Clr4 occurs independently of RNAi, but depends on Rik1. In support of this, silencing with full-length GBD-Clr4 only occurred upon deletion of Dcr1 (fig. S2B). Anti-CENP-A<sup>Cnp1</sup> and CENP-C<sup>Cnp3</sup> ChIP indicated that recruitment of GBD-Clr4- $\Delta$ cd to the plasmids' Gal4-sites allowed the establishment of CENP-A<sup>Cnp1</sup> chromatin on, and recruitment of CENP-C<sup>Cnp3</sup> to, the central core of p3x*gbs*-cc2 in wild-type and *dcr1* $\Delta$  cells, but not *rik1* $\Delta$  cells (Fig. 3B). The minichromosome with the Gal4-sites exhibited mitotic stability when combined with GBD-Clr4- $\Delta$ cd in *dcr1* $\Delta$  cells (Fig. 3C; fig. S9). We conclude that the requirement for outer repeats and RNAi in the *de novo* establishment of functional heterochromatin to promote CENP-A<sup>Cnp1</sup> and kinetochore assembly, and form mitotically active centromeres, on plasmid-based minichromosomes can be fully substituted by Clr4-tethered synthetic heterochromatin formed adjacent to a central domain.

Three Gal4-binding sites, which directly recruit Clr4 methyltransferase activity, can replace the normal requirement for at least 2.1 kb of centromeric outer repeat DNA adjacent to a central domain on naïve plasmids to form active centromeres (3, 7, 8). This synthetic heterochromatin is therefore functional in that it generates sufficient sister-centromere cohesion and promotes assembly of CENP-A<sup>Cnp1</sup> in place of histone H3 to provide a foundation for kinetochore formation. This indicates that no other contribution of the outer repeats is required in terms of their primary DNA sequence in this plasmid-based establishment assay. RNAi components were previously shown in similar assays to be necessary for CENP-A<sup>Cnp1</sup> chromatin establishment (8). By artificially recruiting Clr4 activity we have bypassed RNAi and rule it out as being directly involved in promoting CENP-A<sup>Cnp1</sup> deposition. Thus the non-coding outer repeat transcripts themselves, and the resulting siRNA, are not required to form a mitotically functional minichromosomal centromere. It remains to be determined how heterochromatin promotes CENP-A<sup>Cnp1</sup> incorporation. Recent analyses suggest that the acetylated state of histones is important and HDAC inhibitors rescue CENP-A<sup>Cnp1</sup> chromatin assembly defects (4, 16). Synthetic heterochromatin placed close to a central domain may provide a favourable chromatin environment to attract key remodelling and modifying factors. Ultimately, similar

manipulations as described here may improve the efficiency of human artificial chromosome formation.

## Supplementary Material

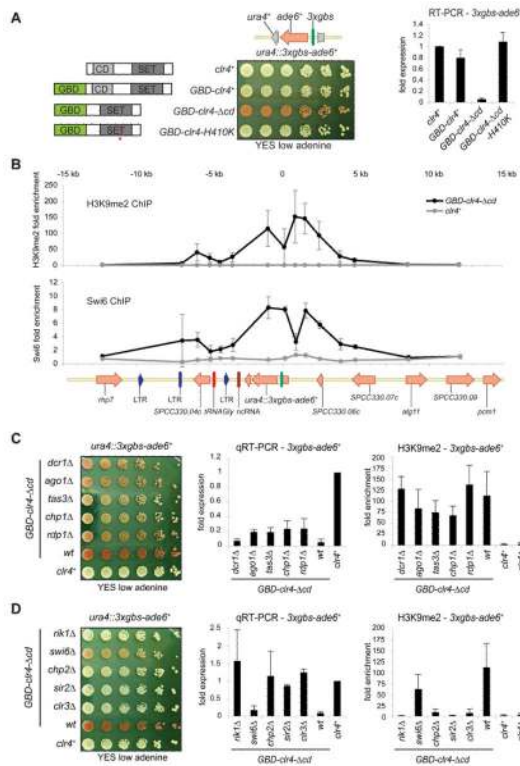
Refer to Web version on PubMed Central for supplementary material.

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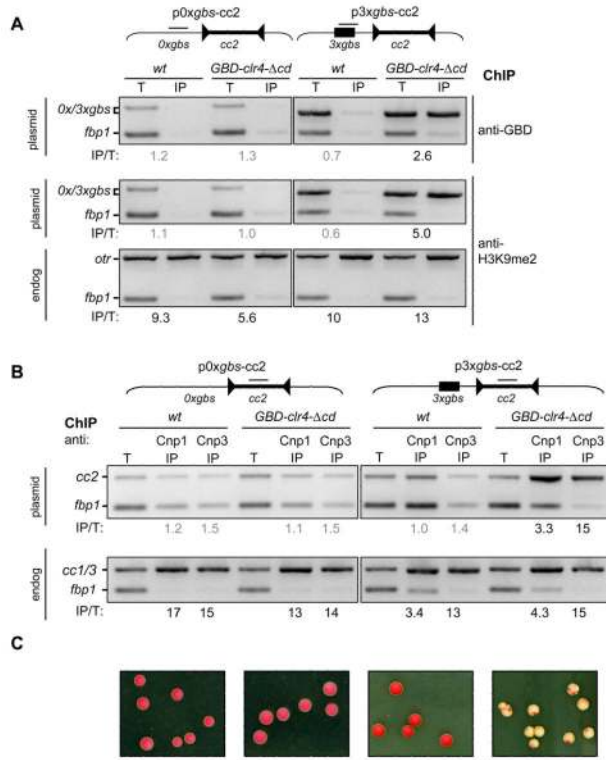
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**Fig. 1.**

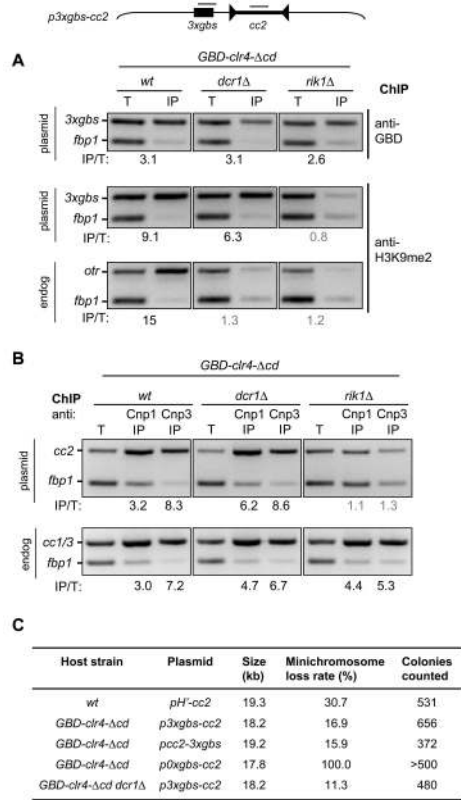
Tethered Clr4 silences transcription and forms a 10 kb domain of heterochromatin and requires HDACs, Rik1 and Chp2, but not RNAi. **(A)** Left: Clr4 fusion proteins used. Gal4 DNA binding, chromo, and SET domains, and H410K (asterisk) mutation, are indicated. Centre: Plating assay on low adenine of cells expressing the indicated Clr4 proteins and containing the *3xgbs-ade6<sup>+</sup>* reporter inserted at *ura4<sup>+</sup>*. *ade6<sup>+</sup>* expressing cells form white colonies; *ade<sup>-</sup>* and *ade6<sup>+</sup>* repressed cells form red colonies. Right: qRT-PCR showing *3xgbs-ade6<sup>+</sup>* transcript levels (error bar is SD, n=3). **(B)** ChIP analysis with  $\alpha$ -H3K9me2 or  $\alpha$ -Swi6 antibodies of 25 kb region surrounding *ura4::3xgbs-ade6<sup>+</sup>* in presence of Clr4 or GBD-clr4- $\Delta$ cd. Genomic features are depicted below.

**(C)** Plating assay on low adenine of cells with indicated genes deleted expressing Clr4 or GBD-clr4- $\Delta$ cd with the *ura4::3xgbs-ade6<sup>+</sup>* reporter (left). qRT-PCR for *3xgbs-ade6<sup>+</sup>* transcript levels (middle) and qChIP for H3K9me2 levels on *ura4::3xgbs-ade6<sup>+</sup>* in the indicated strains (Error bar: SD, n=3). **(D)** As above with indicated mutants.





**Fig. 2.** Tethered Clr4 promotes CENP-A<sup>Cnp1</sup> deposition, kinetochore assembly and centromere activity. **(A)** ChIP with anti-GBD (top) and anti-H3K9me2 (middle and bottom) of *wt* and *GBD-clr4-Δcd* cells transformed with *p0xgbs-cc2* or *p3xgbs-cc2* as indicated. Enrichment values (IP/T) were calculated from the intensity of the *0xgbs* or *3xgbs* band normalized to the control *fbp1* locus. Grey values indicate no enrichment. H3K9me2 levels on endogenous centromeric *otr* repeats (bottom). **(B)** ChIP with anti-Cnp1 and anti-Cnp3 of *wt* and *GBD-clr4-Δcd* cells transformed with *p0xgbs-cc2* or *p3xgbs-cc2* as indicated. Enrichment values (IP/T) were calculated from the intensity of the plasmid *cc2* band (top) or the endogenous *cc1/3* central cores (middle), normalized to the control *fbp1* locus (see SOM). **(C)** Colonies from strains indicated in C. transformed with *p0xgbs-cc2* or *p3xgbs-cc2* grown on low adenine. Plasmid mitotic stability is determined by colony colour: white/red sectored indicates stable; uniform red indicates complete loss (see SOM).

**Fig. 3.**

Synthetic heterochromatin allows establishment of functional centromeres in cells lacking RNAi. **(A)** ChIP with anti-GBD (top) and anti-H3K9me2 (middle and bottom) of *wt*, *dcr1Δ* and *rik1Δ* cells expressing *GBD-clr4-Δcd* cells transformed with p3xgbs-cc2. Enrichment values (IP/T) calculated as described in SOM. Grey values indicate no enrichment. H3K9me2 levels on endogenous centromeric *otr* repeats (bottom). **(B)** ChIP with anti-Cnp1 and anti-Cnp3 on identical cells as in A. Levels on endogenous centromeric *cc1/3* sequences are shown (bottom). **(C)** Loss rate of indicated plasmid based minichromosomes in indicated host strains expressing Clr4 or GBD-clr4-Δcd. pH-cc2 containing natural outer repeat heterochromatin is included for comparison (3). pcc2-3xgbs has the 3xgbs to the right of cc2 (see fig S9).