Heterochromatin formation involves the nucleation and spreading of structural and epigenetic features along the chromatin fiber. Chromatin barriers and associated proteins counteract the spreading of heterochromatin, thereby restricting it to specific regions of the genome. We have performed gene expression studies and chromatin immunoprecipitation on strains in which native centromere sequences have been mutated to study the mechanism by which a tRNAAlanine gene barrier (cen1 tDNA^{Ala}) blocks the spread of pericentromeric heterochromatin at the centromere of chromosome 1 (cen1) in the fission yeast, *Schizosaccharomyces pombe*. Within the centromere, barrier activity is a general property of tDNAs and, unlike previously characterized barriers, requires the association of both transcription factor IIC and RNA Polymerase III. Although the cen1 tDNA^{Ala} gene is actively transcribed, barrier activity is independent of transcriptional orientation. These findings provide experimental evidence for the involvement of a fully assembled RNA polymerase III transcription complex in defining independent structural and functional domains at a euchromatic centromere.

**INTRODUCTION**

Eukaryotic genomes are packaged into two main categories of chromatin that can determine the behavior of the underlying DNA sequence. Euchromatin is typically found in gene rich regions of the genome that are accessible to factors involved in various biological processes including transcription, replication and recombination. In contrast, regions of heterochromatin are generally gene poor, and confer transcriptional repression to inserted reporter genes [1]. Heterochromatin also is required for proper chromosome segregation [2,3]. Hypoacetylation of histones and methylation of histone H3 at lysine 9 are distinguishing marks of heterochromatin in many eukaryotic genomes, including the fission yeast *Schizosaccharomyces pombe* (S. pombe) [4]. In addition to the general categories of heterochromatin and euchromatin, a third type of specialized chromatin exists at centromeres. Centromeric chromatin contains blocks of canonical nucleosomes, methylated on H3 lysine 4, interspersed with blocks of nucleosomes containing the histone H3 variant, cenH3, a protein that provides the structural and functional foundation of all active kinetochores [5].

A key feature of heterochromatin is its ability to spread in cis, causing epigenetic silencing of an otherwise euchromatic gene [6]. The genomic and/or epigenetic features at the confluence of discrete chromatin domains remain poorly understood; however, at some loci a specialized class of DNA element, known as a chromatin barrier [7], plays an active role in demarcating the different chromatin states. Chromatin barriers restrict heterochromatin to specific genomic regions and fall within a broader class of elements called insulators [8]. The range of proteins associated with chromatin barriers is incompletely defined, although increasing evidence suggests that barrier activity correlates with the recruitment of histone acetylase activity and/or the assembly of a transcription complex [9,10,11,12]. Accordingly, many insulators are coincident with the promoters of genes [13,14,15].

We recently defined a novel chromatin barrier element in the fission yeast genome that partitions centromere 1 (cen1) chromatin into structurally distinct domains of pericentromeric heterochromatin and centromeric chromatin [16]. The absence of this barrier results in both propagation of pericentromeric heterochromatin beyond its normal boundary into centromeric chromatin, as well as defects in chromosome segregation during meiosis. Barrier activity is dependent upon an intact transfer RNA alanine gene (cen1 tDNA^{Ala}) that is transcribed from its endogenous, centromeric location [16]. In this study, we further characterize the properties of this novel cen1 tDNA^{Ala} barrier, which differ in several aspects from previously described fission yeast barriers.

**RESULTS**

Barrier activity is a general feature of centromeric tDNAs

tDNAs in the fission yeast genome range in size from 71–102 bp and are characterized by highly conserved internal control elements. To determine whether barrier activity is a general property of tDNAs or if the cen1 tDNA^{Ala} has additional sequence features that convey barrier activity, we engineered strains in which cen1 tDNA^{Ala} (including 40 bp upstream sequence and 25 bp downstream) was replaced with tDNAs that encode two different fission yeast tRNA isotypes. The presence of cen1 tDNA^{Ala} blocks the spread of heterochromatin and permits gene expression from a centromere proximal *ura4* reporter gene (at a moderate level of 27% of wildtype expression).

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ura4 activity), as observed previously ([16]; Fig 1). In the absence of these sequences, however, the spread of pericentromeric heterochromatin results in a significant reduction of ura4 reporter gene expression to only ~6% of wild-type levels. In comparison, replacement of cen1 tDNAAla with non-centromeric tDNAGlu or tDNAIle restored reporter gene expression (35% and 44%, respectively), suggesting that the ability to block the spread of heterochromatin at the centromere is a general property of tDNAs in fission yeast.

RNA polymerase III and TFIIIC associate with the tDNA barrier

Transcription of tDNAs by RNA polymerase III (Pol III) involves the multi-step assembly of transcription factors into a pre-initiation complex that recruits Pol III [17]. Two highly conserved internal control regions, the A and B boxes, together form a specific binding site for the multisubunit transcription factor IIIC (TFIIIC). Promoter bound TFIIIC provides an interaction platform for the productive assembly of TFIIIB at a TATA box immediately upstream of tDNAs [17,18]. TFIIIB next recruits Pol III and transcription proceeds through a facilitated re-initiation pathway that involves polymerase recapture after transcription termination [19]. Previous work has indicated that cen1 tDNAAla and its surrounding sequences are sensitive to micrococcal nuclease digestion [20] and correspond to DNase I hypersensitive sites [21]. Furthermore, as we have shown previously, cen1 tDNAAla is transcribed and both intact TATA and A box regions are required for robust barrier activity [16]. These findings together suggest that Pol III and its associated transcription complex gain access to the tDNA barrier at the centromere, thus forming a large stable DNA-bound complex.

Figure 1. Barrier activity is a general property of tDNAs. A. (Upper) Representation of S. pombe centromere 1 (cen1). The central core (black) is surrounded by inverted repeats including the inner repeat (imr1; red) and outer repeats (otr1-, light blue, navy and purple). Black vertical lines within imr represent tDNAs in cen1. (Lower) Map of cen1 tDNAAla (green) and surrounding sequence. The ura4 reporter gene (ORF, yellow arrow; surrounding sequence, black line) was inserted into imr1 of cen1. PCR primers are shown as black arrows. B. Illustrations to the left of the graph as above. tDNA alanine (green), isoleucine (gray) and glutamine (light blue) are represented as colored triangles in imr1. Real time RT-PCR analysis of centromeric ura4 transcription was normalized to endogenous ura4 transcription and compared among indicated strains. Error bars represent the standard error of the mean (SEM).

doi:10.1371/journal.pone.0001099.g001
Genome-wide chromatin immunoprecipitation (ChIP on micro-array) previously demonstrated low, but measurable levels of association of both TFIIIC and Pol III with cen1 tDNAs [22]. We confirmed this observation by ChIP analysis over a 1.6 kb region surrounding tDNAAla. These studies revealed that both Sfc6, a TFIIIC subunit, and Rpc130, a Pol III subunit [17], were enriched at cen1 tDNAAla 28- and 3-fold, respectively, over a non-centromeric control locus (Figure 2A).

We next investigated whether components of TFIIIC and Pol III are present at centromeres where barrier activity has been compromised by mutation of either the A box internal control element or the upstream TATA box (Figure 2B, [16]). Using specific primers to distinguish the modified barrier, which also bears the ura4 reporter gene, from the wild-type tDNAAla on the inverted repeat of cen1, we confirmed that both Sfc6 and Rpc130 were present at this locus. However, association of Rpc130 was essentially abolished in strains carrying the mutant barrier (Figure 2B, right panel). Enrichment of Sfc6 was also investigated in these strains. The A box mutation displayed ~49% reduction in Sfc6, whereas the level of enrichment in TATA box mutants was not changed (Figure 2B, middle panel). Importantly, both mutations have reduced levels of ura4 expression (9.4% and 18.5% of wildtype ura4 expression, respectively; Figure 2C left panel), and, therefore, reduced barrier activity. Taken together, these data demonstrate that Sfc6 association is not sufficient to counter the spread of pericentromeric heterochromatin [22] and suggest that robust cen1 tDNAAla barrier activity requires the assembly of a full Pol III transcription complex.

**Barrier activity is independent of tDNA orientation**

Transcriptional interference between Pol III and RNA polymerase II (Pol II) genes has been described in *Saccharomyces cerevisiae*. This tDNA “position effect” has been observed mostly with selected artificial constructions, in which Pol II-transcribed reporter genes were found to be inhibited 2-to 60-fold by a neighboring tDNA [23,24]. However modest tDNA position effects have also been reported to operate at native chromosomal loci, particularly when the Pol II-transcribed gene is less than 1000 bp from a tDNA [25,26,27].

Figure 2. Barrier activity requires the RNA polymerase III complex. A. Chromatin IP analysis of cen1 for enrichment of Sfc6 (TFIIIC) and Rpc130 (Pol III). The X-axis represents 1.5 kb of cen1 imr including tDNAAla (green) and nearby tDNAAlb (white). Centromere specific primers are listed in Materials and Methods. Error bars represent SEM. B. Real time RT-PCR analysis of centromeric ura4 transcription normalized to endogenous ura4 transcription in strains containing wild-type or mutant barriers (Left). Indicated strains were analyzed by chromatin IP for Sfc6 (Middle, dark gray) and Rpc130 (Right, light gray) enrichment. Error bars represent SEM. doi:10.1371/journal.pone.0001099.g002
Assembly of pericentromeric heterochromatin in fission yeast requires Pol II transcription of the outer repeats [28], followed by processing of the transcripts by the RNAi machinery [1]. Compellingly, siRNAs within 1kb of tDNAAla have been identified [29], although due to both the small size of siRNAs and the sequence redundancy of fission yeast centromeres, their origin cannot be confirmed. These observations suggest the possibility that barrier activity results from a position effect that represses Pol II transcription of repetitive DNA, thus weakening local chromatin structure. Therefore, we hypothesized that the Pol III complex at cen1 likely behaves as a chain terminator (and hence a barrier) to the nucleosome-placement [41,42,43]. Based on the data presented here, we hypothesize that the Pol III complex at cen1 likely behaves as a chain terminator (and hence a barrier) to the nucleosome-placement [41,42,43].

**DISCUSSION**

Transitions between discrete chromatin types have been studied in many organisms, from vertebrates [8,33] to *S. cerevisiae* yeasts: fission yeast tDNA sequences do retain barrier activity in our centromere specific assay (Figure 1). Notably, Pol III transcription complex assembly differs significantly between the budding and fission yeasts: fission yeast tDNAs have an upstream TATA element, centered at position -30 that participates in direct recruitment of TFIIB [18]. Indeed, TATA motifs can be identified at all three tDNAs shown to have barrier activity in this study. Intriguingly, however, a significant difference in the strength of barrier activity was observed between strains containing tDNAAla and tDNAile (p < 0.001) (Fig 1). We suggest that more potent barrier activity requires the association of TFIIC. It is important to note that B box barriers do not associate with Pol III and have been proposed to function by forming chromatin loops and/or by altering the nuclear localization of domains of chromatin, since dispersed sites of TFIIC association coalesce at the nuclear periphery [22].

Here, we demonstrate a fourth type of barrier in fission yeast (Figure 3D), using strains in which native tDNA barrier sequences at cen1 have been altered. Centromeric tDNA barriers require the assembly of a fully functional Pol III promoter complex. This is exemplified most strongly by strains in which the A box internal control element has been mutated (Figure 2). In this case, Sif6 (TFIIC) is recruited, presumably through the unaltered B box sequences; however, it is not sufficient to counter the spread of pericentromeric heterochromatin. Barrier activity is observed only when the Pol III complex is recruited to the centromere. Thus, centromeric tDNA barriers are mechanistically distinct from previously described heterochromatin barriers in fission yeast.

Pol III is a nuclear enzyme that has been specialized to produce small non-translated RNAs in great abundance [38]. While our studies demonstrate that barrier activity is independent of the orientation of tDNA transcription, they do not address whether transcription is required for barrier activity or whether complex formation, in the absence of transcription, is sufficient. Mutations that impair the enzymatic activity of the Pol III complex, but not its assembly, have not been identified, and the development of such mutations represents an important area of future work. The fully assembled Pol III complex is at least 1.3 Mda [39], footprints nearly 150 bp along the chromatin fiber [39,40], preventing nucleosome placement [41,42,43]. Based on the data presented here, we hypothesize that the Pol III complex at cen1 likely behaves as a chain terminator (and hence a barrier) to the nucleosome-depending propagation of pericentromeric heterochromatin.

**Table 1. Barrier activity is independent of tDNA orientation**

| Genotype | tDNAAla | ura4Ala | Normalized ura4Ala expression % | S.E.M.% |
|----------|---------|---------|-------------------------------|--------|
| Cen1:ura4Ala clh4:Leu2 N | F, R | 94.8 | 5.6 |
| Cen1:ura4Ala clh4-4 N | F | 30.1 | 4.7 |
| Cen1:ura4Ala clh4-6 N | F | 29.2 | 3.9 |
| Cen1:ura4Ala clh4-1 N | R, R | 26.4 | 4.0 |
| Cen1:ura4Ala clh4-3 N | F | 31.9 | 5.2 |

*Strains contained the ura4Ala reporter gene at centromere 1 in both wild type and clh4 backgrounds.

1. Transcriptional orientation of tDNAAla: N, native (antisense strand); R, reverse (sense strand).

2. Transcriptional orientation of the ura4Ala reporter gene: F, forward (sense strand); R, Reverse (antisense strand).

3. ura4Ala transcript levels were measured as in Figure 1.

4. Standard error of the mean.

DOI: 10.1371/journal.pone.0001099.t001
its association with both TFIIIC and Pol III [22], suggesting that the centromere provides a favorable environment for tDNA barrier activity.

The domain organization at each of the three fission yeast centromeres is similar and involves transitions among three types of chromatin: heterochromatin, euchromatin and centromeric chromatin. Previous genome-wide mapping of covalently modified histones showed an abrupt transition from marks associated with pericentromeric heterochromatin to those associated with centromeric chromatin [29]. Both chromatin types are essential for centromere activity [49], and mis-segregation events are observed in the absence [50,51] or mislocalization [16] of heterochromatin. Compellingly, each chromatin transition correlates with the presence of a pair or cluster of tDNAs [29], including seven different tRNA isotypes [20]. We speculated previously that the neighboring tDNAs perform an essential barrier function at fission yeast centromeres, supported by our observation of chromosome missegregation during meiosis in the absence of tDNAAla [16]. Thus, unlike conventional barriers that ensure appropriate euchromatic gene expression in euchromatin (Figure 3; A–C), cen tDNA barriers may ensure proper centromere assembly and function by protecting the central core domain of centromeric chromatin from the distinct structural changes and epigenetic features associated with pericentromeric heterochromatin.

MATERIALS AND METHODS

Plasmid DNAs
Plasmids SM353 and SM349 [16] contain 1.78 kb of imr1 sequence, including the cen1 tDNAAla and a ura4+ reporter gene at the unique HindIII site. These plasmids were modified by site directed mutagenesis to introduce unique ClaI (–45) and EcoRI (+20) sites flanking tDNAAla, tDNAAs, tDNASe, and reverse tDNAAla were amplified from genomic DNA with primers containing either EcoRI or ClaI restriction sites. The resulting PCR products were purified, digested with EcoRI and ClaI, and ligated with vector that had been digested with EcoRI and ClaI to liberate tDNAAla. Resulting plasmids were purified, sequenced and digested with NcoI and KpnI to generate a 3.5 kb product used for yeast transformation.

Fission yeast strains
The genotypes for S. pombe strains used in this study are listed in Table 2. Media were prepared according to standard procedures [52]. All transformations were performed as in [16]. At least three
PCR was performed in the presence of SYBR Green on a Bio-Rad
preparing oligo dT primed RT-PCR (Invitrogen). Real-time
Yeast were grown in YES to 5
independent transformed strains were established from each

| Table 2. Strains used in this study |
|-----------------------------------|
| 2 | h ade6-210 |
| 3 | h ura4D18 ade6-210 leu1-32 his3D arg3D4 |
| 4 | h ura4D18 ade6-210 leu1-32 his3D arg3D4 |
| 104 | h90 swich arg ura4D18 leu1-32 ade6-210 his3D arg3D4 |
| 139 | h imr1L (Jala HindIII):ura4 ura4D18 ade6-210 leu1-32 his3D arg3D4 |
| 146 | h imr1R (Jala HindIII):ura4 ura4D18 ade6-210 leu1-32 his3D arg3D4 |
| 188 | h imr1L HindIII:ura4 ura4D18 ade6-210 ura4D18 arg3D4 |
| 199 | h imr1L HindIII:ura4 cln4 leu2 ade6-210 ura4D18 arg3D4 |
| 201 | h90 imr1 HindIII:ura4 cln4 leu2 ade6-210 ura4D18 arg3D4 |
| 205 | h imr1L HindIII:ura4 arg3D4 leu1-32 ade6-210 ura4D18 his3D |
| 258 | h imr1L (Aila TATA* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 262 | h imr1R (Aila Abox* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 263 | h imr1R (Aila Abox* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 301 | h7imr1L (Aila TATA* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 302 | h7imr1L (Aila TATA* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 354 | h7imr1R (Aila Abox* HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 504 | h7imr1L (glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 506 | h7imr1L (glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 535 | h7imr1R(glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 699 | h7imr1R(glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 700 | h7imr1R(glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 722 | h7imr1Lala reverse HindIII:ura4 ade6-210 leu1-32 ura4D18 his3D arg3D4 |
| 733 | h7imr1Lala reverse HindIII:ura4 ade6-210 leu1-32 ura4D18 his3D arg3D4 |
| 747 | h7imr1Lala reverse HindIII:ura4 ade6-210 leu1-32 ura4D18 his3D arg3D4 |
| 1024 | h7imr1R(glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |
| 1025 | h7imr1R(glu-glu HindIII):ura4 arg3D4 his3D leu1-32 ura4D18 ade6-210 |

\[ \text{doi: 10.1371/journal.pone.0001099.t002} \]

Real-Time RT-PCR

Yeast were grown in YES to 5\times10^6 cells/ml at 32°C. cDNA was prepared by oligo dT primed RT-PCR (Invitrogen). Real-time
PCR was performed in the presence of SYBR Green on a Bio-Rad

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iCycler with the following primer pairs for ura4+: 5'-TGGATATTGACCAGAGACGAAGCA-3'; 5026, 5'-AAAAAATGG-TGGCCCTTAGT-3' and act1**: 7453, 5'-AATCCACGCT-TGGAGAGATGA-3'; 7454, 5'-ACGGAGAGGCATACAAAGA-3'. A standard curve of at least four orders of magnitude was generated with genomic DNA isolated from the wild-type strain (ura4+ KY2). Data were analyzed using iCycler iQ Optical System Software. Data were analyzed further only if the PCR efficiency was between 90–110% and the correlation coefficient was between 0.990 and 1.0. ura4+ levels were normalized to act1+ levels and quantified relative to the wild-type strain. At least three biological replicates were performed for each mutant.

CHIP

~2.5\times10^6 cells were grown to mid-log phase at 32°C, then shifted to 18°C for two hours. Cells were pelleted, fixed in 3% paraformaldehyde for 30 min, pelleted and washed twice in ice-cold PBS. Cells were resuspended in 10 ml of 10 mM dimethyladipate, including 0.0025% DMSO and incubated at room temperature for 30 min. Cells were pelleted and washed twice in ice cold PBS. ChIP was performed as previously described [16], 7ul of anti-Sic6 or anti-Rp130 antiserum [17] were used for each ChIP. PCR products were quantified as described for RT-PCR. Enrichment at each query locus was calculated relative to the euchromatin act1+ value and then further normalized for the ratio obtained for the input PCR. Centromeric primers: 5373, 5'-TACCTTGTGTGTTACACACTGCT-3'; 5374, 5'-AACACATGTTTTTTTTTGTTA-3'; 5375, 5'-TCATTGTTGTGACACTGCT-3'; 5376, 5'-TGTTTTGCGATCTTCAAACTCA-3'; 5520, 5'-CACCGATGCCGCTATTGTTT-3'; 5521, 5'-TGCGGTTCA-TCTAAAGCTTGA-3'; 5562, 5'-CGCTACACTCTAAGGTTTTTGCTT-3'; 5563, 5'-GCCGTCAGGAGAATTTTAT-3'; 5582, 5'-CCATGAGGATTCTGTATG-3'; 5585, 5'-GGTTTTGTTTTCTTCCTCCAG-3'; 5564, 5'-GGCAAGAC-TTTTGTGATGAGAAG-3'; 5565, 5'-GGTTTTGTTTTCTTCCTCCAG-3'; ura4/cen primers: 5590, 5'-GCCCTAATTGTTTATTTGGCG-3'; 5591, 5'-CAAAACGATGAAAGATGT-ATATGAG-3'. At least three independent ChIP experiments were performed with each antisera.

ACKNOWLEDGMENTS

We thank Terilyn Gaither for technical assistance; Des. Richard Marra and Ying Huang for reagents; Basyl Wheeler, Dr. Laura Rusche, Dr. Beth Sullivan, and members of the Rusche and Willard laboratories for valuable discussions.

Author Contributions

Conceived and designed the experiments: KS. Performed the experiments: KS CW. Analyzed the data: KS. Contributed reagents/materials/analysis tools: KS. Wrote the paper: KS. Other: Contributed reagents and intellectual support: HW.
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