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Report

The Chromatin-Remodeling Factor FACT Contributes to Centromeric Heterochromatin Independently of RNAi

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Summary

Centromeres exert vital cellular functions in mitosis and meiosis. A specialized histone and other chromatin-bound factors nucleate a dynamic protein assembly that is required for the proper segregation of sister chromatids. In several organisms, including the fission yeast, *Schizosaccharomyces pombe*, the RNAi pathway contributes to the formation of silent chromatin in pericentromeric regions. Little is known about how chromatin-remodeling factors contribute to heterochromatic integrity and centromere function. Here we show that the histone chaperone and remodeling complex FACT is required for centromeric-heterochromatin integrity and accurate chromosome segregation. We show that Spt16 and Pob3 are two subunits of the *S. pombe* FACT complex. Surprisingly, yeast strains deleted for *pob3+* are viable and alleviate gene silencing at centromeric repeats and at the silent mating-type locus. Importantly, like heterochromatin and RNAi pathway mutants, Pob3 null strains exhibit lagging chromosomes on anaphase spindles. Whereas the processing of centromeric RNA transcripts into siRNAs is maintained in Pob3 mutants, Swi6-association with the centromere is reduced. Our studies provide the first experimental evidence for a role of the RNA polymerase II cofactor FACT in heterochromatin integrity and in centromere function.

Results and Discussion

FACT Is an Evolutionarily Conserved Nuclear Complex

Centromeres are composed of specialized chromatin in which the histone H3 variant CENP-A underpins the kinetochore and is flanked by heterochromatic regions. This heterochromatin is known to attract cohesin and contribute to centromere function by ensuring physical cohesion between sister chromatids [1]. Significant progress has been made in dissecting the connections between heterochromatin and centromere function. It is known that specific histone modifications [2] and RNAi-related processes [3] contribute to an "epigenetic" mechanism that defines the heterochromatic nature of centromeric DNA.

We wish to further investigate how the correct nucleosomal structure is established and maintained over centromeric repeats. In fission yeast, Pol II is also required for centromere function because it transcribes complementary regions of the centromeric outer repeats [4], but it is not known whether Pol II requires coactivating factors. Evidence already implicates the transcriptional cofactors and chromatin-remodeling complexes RSC (remodeling and spacing complex) and PBAF (polybromo, brahma-related gene 1-associated factor) in centromere-related functions [5, 6]. Pol II transcription is stimulated by the chromatin-remodeling complex FACT (facilitates chromatin transcription) [7]. In order to investigate the possible role of this factor in centromeric chromatin, we first identified the fission yeast FACT complex.

FACT from a variety of organisms contains two core proteins (Spt16 and Pob3/SSRP1). We identified a single set of closely related sequences for these subunits in the *S. pombe* genome (Figure S1 in the Supplemental Data available online). A strain was constructed to express *S. pombe* Pob3 fused to a FLAG-PreScission-HA epitope (FPH-Pob3). Western blots reveal expression of the functional FPH-Pob3 fusion protein. To check whether FPH-Pob3 forms a FACT-like complex with Spt16, we coexpressed green fluorescent protein (GFP)-tagged Spt16 (Figure S2). α-HA antibodies immunoprecipitate Spt16-GFP (Figure 1A). Thus Pob3 and Spt16 associate in vivo.

We next biochemically purified *Sp*FACT from cellular extracts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed two specific bands in the FPH-Pob3 extracts (Figure 1B). Mass-spectrometry analysis identified the two bands as Pob3 and Spt16. To verify whether the two FACT subunits interact, we performed glutathione S-transferase (GST) pulldowns. This demonstrated that Spt16 and Pob3 associate in vitro and that the interaction requires the Spt16-M domain (Figure 1C). Our biochemical data show that *S. pombe* contains a FACT complex similar to other eukaryotes.

To determine whether *Sp*FACT localizes to the nucleus, we imaged functional Spt16- and Pob3-GFP fusions (Figure S2B). Pob3-GFP and Spt16-GFP are nuclear factors (Figure 1D). Together, our biochemical and localization data are consistent with nuclear functions of the *Sp*FACT complex.

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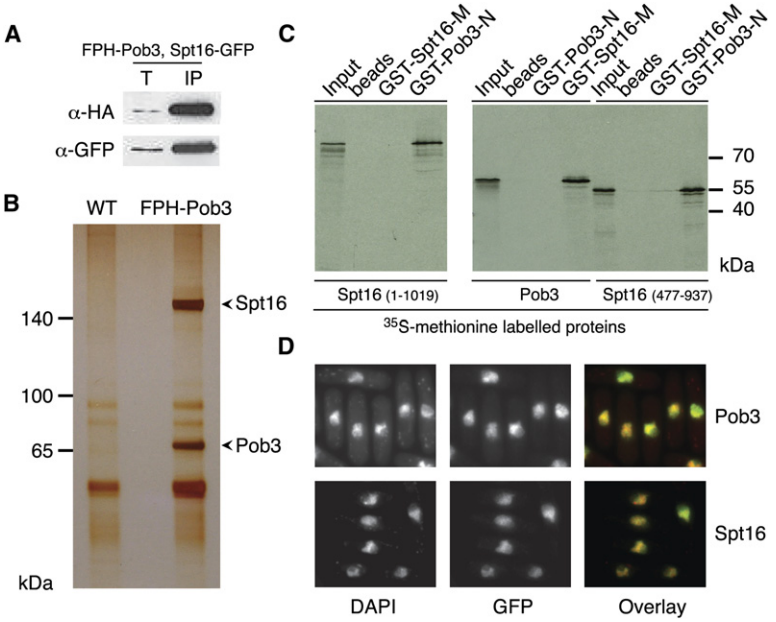


Figure 1. *S. pombe* FACT Is a Heterodimeric, Nuclear-Protein Complex

(A) Coimmunoprecipitation between tagged *S. pombe* Pob3 and Spt16. Extracts prepared from FPH-Pob3 Spt16-GFP strains were incubated with HA-antibody-coupled agarose. Fractions were analyzed by western blotting with either HA polyclonal or GFP monoclonal antibody. Lanes labeled "T" show the equivalent of 10% extract used in the IP.

(B) Biochemical purification of FPH-Pob3 and identification of copurifying proteins. IPs from WT and FPH-*pob3+*-tagged strains were performed with FLAG-epitope affinity purification. Silver staining resolves two specific bands. Peptide sequencing by tandem MS identifies Pob3 and Spt16.

(C) GST pulldowns map necessary interaction domains within *Sp*FACT. Immobilized GST-fusion proteins were incubated with in vitro translated Pob3 and Spt16. GST-Pob3-N (1-448) and GST-Spt16-M (477-937). The input lane contains 10% of the ^{35}S-proteins.

(D) Nuclear localization of Pob3-GFP and Spt16-GFP. The overlay shows the merge between DNA (4',6-diamidino-2-phenylindole [DAPI]) and *Sp*FACT (GFP).

The Small FACT Subunit Pob3 Is Not Essential for Viability in *S. pombe*

In *S. cerevisae*, both subunits of the FACT complex are essential, and mutant alleles with phenotypes in genome stability have been described [8]. As expected, the *S. pombe* ortholog for the large FACT subunit *spt16+* is essential (Figure S3). Surprisingly, a strain bearing the deletion of *pob3+* is viable (Figure 2A, Figures S4 and S5), though it shows temperature sensitivity. No paralog that might account for genetic redundancy can be identified with genomic basic local alignment search tool (BLAST) searches (data not shown). Because the *pob3*Δ strain is viable, we tested its role in distinct chromatin-based events. Cells lacking Pob3 are sensitive to hydroxyurea (HU), camptothecin (CPT), ultraviolet (UV), and (mildly) to 6-azauracil (6AU), suggesting DNA replication, DNA repair, and transcription phenotypes (Figure S6). The sensitivity of *pob3*Δ to these stress agents indicates that *Sp*FACT is involved in multiple chromatin-based cellular functions and participates in genome stability. Yet, its deletion in *S. pombe* is viable.

Deletion of *pob3* Results in Transcriptional-Silencing Defects

In vitro observations have shown that the FACT complex aids Pol II to overcome the nucleosome barrier to transcription [7]. In fission yeast, it is known that Pol II is required to transcribe centromeric, noncoding outer repeats (*otr*) and to form silent heterochromatin [4].

We therefore checked whether the loss of Pob3 affects transcriptional silencing within centromeres. Fission-yeast strains with reporter genes inserted at distinct locations within the centromere (Figure 2) allow the assessment of the repressive state of chromatin at these locations [9]. We tested two mutant *pob3*Δ strains with *ura4+* inserted either at the *imrIR*(*Nco*I)::*ura4+* or at *otrIR*(*Sph*I)::*ura4+* repeats. Strains with an active *ura4+* gene grow well in the absence of uracil (−Ura) but are unable to grow on counterselective plates containing 5-fluoro-orotic acid (FOA) [9]. Plating assays show that *pob3*Δ strains grow slower than does a wild-type (WT) strain on FOA medium (Figure 2B). Contrary to FACT's known roles as a transcriptional elongation factor, our results reveal that the loss of Pob3 function allows higher levels of *ura4+* gene expression relative to that of the WT (Figure 2B), with a strong effect at *imr1R* and a weaker one at *otr1R*. This suggests that Pob3 has a novel, repressive role in centromeric transcription. In comparison, mutant strains *clr4*Δ and *tas3*Δ display the complete alleviation of silencing at both loci. Further, *ade6+* reporter assays show that *pob3*Δ loss of silencing is not a *ura4+* gene-specific phenotype (Figure S2). Only a mild effect of *pob3+* deletion is observed when *arg3+* is inserted at the central core region (*cnt1*::*arg3+*; Figure 2B). The silencing assays show that the *pob3*Δ mutation distinctly affects the expression level of centromeric reporter genes, depending on their location. Importantly, these data reveal an unexpected in vivo role for *Sp*FACT in heterochromatin integrity.

We next determined whether *pob3+* deletion affects the silencing of marker genes placed in other transcriptionally silent regions [10, 11]. The results show that *pob3*Δ causes derepression of reporter silencing at the mating-type locus (Figure 2C). In contrast, the *pob3*Δ mutation does not affect the silencing of rDNA and telomeric reporters. Pob3 thus has a role in the formation or maintenance of heterochromatin at the mating locus and at centromeres.

FACT is a general transcription and remodeling factor. Recently, the human Pob3 ortholog, SSRP1, was shown to regulate the expression of a specific subset of genes [12]. The *pob3*Δ loss-of-silencing phenotype could therefore be indirect, altering the expression of specific heterochromatin factors. Thus, we performed gene-expression profiling on *pob3*Δ cells. The results reveal that no such genes were up- or downregulated

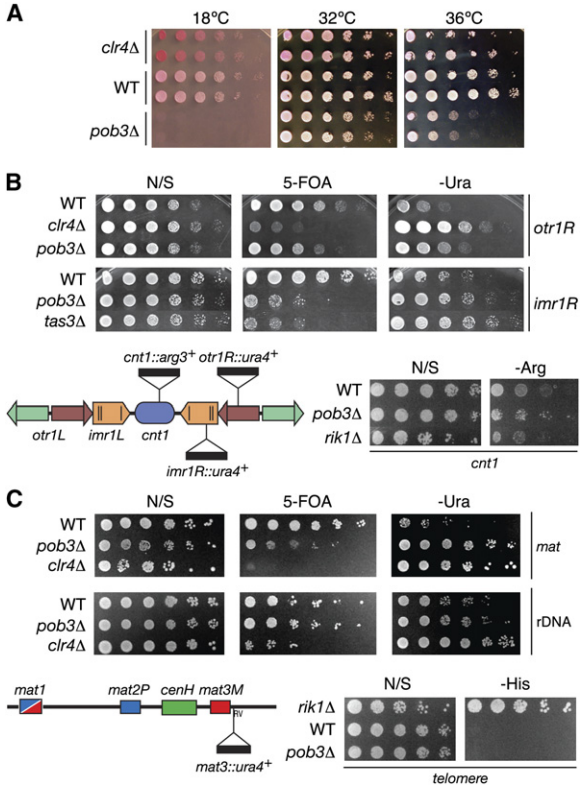


Figure 2. Viability and Heterochromatic Silencing Phenotype of the *S. pombe pob3+* Deletion Strain

(A) Deletion of the small subunit of the chromatin-remodeling complex FACT does not affect *S. pombe* viability.

(B) *pob3+* deletion alleviates heterochromatic silencing at *imr1R* and mildly at *otr1R* centromeric repeats and at the central core (*cnt1*). Mutants in the histone methyl-transferase (*clr4*Δ), in the *rik1+* gene (*rik1*Δ), and in a RITS subunit (*tas3*Δ) serve as positive controls.

(C) Gene-silencing phenotype of the *pob3+* deletion in other heterochromatic regions, such as the mating loci (*mat*), ribosomal DNA repeats (*rDNA*), and the telomere.

significantly (Table S1). Importantly, the few genes whose expression is altered are similarly affected in mutants that play a role in heterochromatin integrity, such as *clr1+*, *clr3+*, *clr6+*, and *rpb7+* (Table S2) [13]. For example, a significant fraction of *pob3*Δ upregulated genes are also upregulated in *rpb7-G150D* and in *clr3*Δ (Table S2). Because Clr3, Clr6, and Rpb7 are required for heterochromatin formation at centromeres, it is likely that FACT cooperates with these histone deacetylase (HDAC) enzymes and Pol II in centromere function.

Our data show that the *Sp*FACT complex has a new role in gene silencing at centromeric heterochromatin. Also, the transcriptional phenotype of *pob3*Δ significantly overlaps that of known heterochromatin mutants. Together, our data strongly suggest that Pob3 plays a specific and direct role in the establishment and/or maintenance of heterochromatin.

Pob3 Is Required for Accurate Chromosome Segregation

The observed centromeric-silencing defects in *pob3*Δ cells suggest that centromeric heterochromatin is disrupted. It is well established that mutants affecting heterochromatin integrity at fission-yeast centromeres also exhibit specific defects in mitotic segregation [14, 15]. We therefore conducted three types of test to identify mitotic defects. First, we checked for lagging chromosomes on anaphase spindles. Immunofluorescence staining shows that *pob3*Δ cells display a high incidence of lagging chromosomes (10%) in anaphase (Figure 3A). This represents a more than 200-fold increase over that of the WT (Figure 3B).

Second, we determined whether *pob3+* is required for minichromosome maintenance over several cell divisions. We measured the fidelity of chromosome segregation with two distinct minichromosome-loss assays [14]. In WT cells, the 530 kb linear *Ch16* minichromosome is mitotically stable [16]. Removal of Pob3 function increases the rate of minichromosome loss by more than 20-fold (Figure 3C). Because this phenotype could be due to defective telomere function in linear minichromosomes, we also tested the mitotic stability of the 30 kb circular minichromosome *CM3112* [16]. The loss rate of CM3112 is increased by approximately 30-fold in *pob3*Δ compared to that of the WT (Figure 3C). Thus, the mitotic segregation of both minochromosomes is severely affected in cells lacking *pob3+*.

pob3+ deletion might affect chromosome segregation by altering mitotic spindle function, as is seen in heterochromatin mutants [15]. We thus examined the growth and viability of *pob3*Δ in the presence of the microtubule-destabilizing drug thiabendazole (TBZ) [15]. The plating assays clearly reveal that, compared with the WT, *pob3*Δ cells are TBZ-sensitive (Figure 3D), although to a lesser extent than are *clr4*Δ cells.

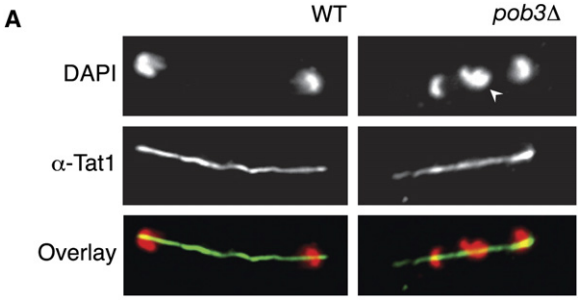
Together, these assays demonstrate that *pob3+* plays a new and important role in accurate chromosome segregation. Pob3's role in centromeric silencing might account for its mitotic functions by contributing to heterochromatin integrity.

Loss of Pob3 Does Not Affect the RNAi Pathway

To investigate the molecular mechanism underlying FACT's novel repressive function, we analyzed the effect of *pob3*Δ on the RNAi-mediated heterochromatin formation at centromeres. In fission yeast, the RNAi pathway directs transcriptional gene silencing to the centromeric outer repeats and is required to assemble intact centromeric heterochromatin [3]. In this pathway, RNA transcripts are generated from *otr* regions and are processed into siRNAs by Dcr1. These siRNAs are incorporated into the ribonucleic acid-induced transcriptional silencing (RITS) effector complex, which is required to establish heterochromatin [3].

Mutants in the RNAi pathway, such as *dcr1*Δ and *ago1*Δ, are defective in processing noncoding centromeric outer-repeat transcripts to homologous siRNA molecules. As a consequence, unprocessed *otr* transcripts accumulate [3]. In principle, a *pob3*Δ strain could display altered levels of the primary transcript and/or show changes in siRNA accumulation. Any of these phenotypes could explain the centromere-silencing and chromosome-segregation defects.

Northern blots show a clear accumulation of unprocessed *otr* transcripts in *dcr1*Δ cells, but they are not detected in *pob3*Δ or WT cells (Figure 4A). This could



B

	WT	pob3Δ
Total # of cells (late anaphase)	307	347
Lagging chromosomes	1	35
Percentage	<0.05	10.1

C

	WT	pob3Δ
Loss of 530 kb linear minichromosome	0/2199 (<0.05%)	26/2493 (1.04%)
n-fold increase over WT		>21x
Loss of 30 kb circular minichromosome	54/2664 (2.0%)	1299/2240 (58%)
n-fold increase over WT		29x

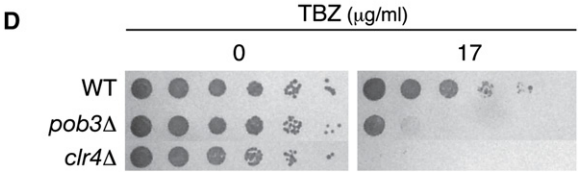


Figure 3. *S. pombe* FACT Subunit Pob3 Is Required for Accurate Chromosome Segregation

(A) *pob3*Δ mutant cells display lagging chromosomes in late anaphase. Cells grown at 25°C were subjected to anti-tubulin (Tat1) immunodetection and DAPI staining. The arrowhead indicates a lagging chromosome in the midzone of the microtubule spindle in *pob3*Δ mutants.

(B) *pob3*Δ deletion increases the frequency of abnormal anaphases. The percentage indicates the fraction of anaphase cells with lagging chromosomes.

(C) Enhanced minichromosome loss in *pob3*Δ mutant versus WT strains.

(D) *pob3*+ deletion strains display a pronounced sensitivity to the tubulin-depolymerizing drug TBZ. *clr4*Δ serves as a positive control.

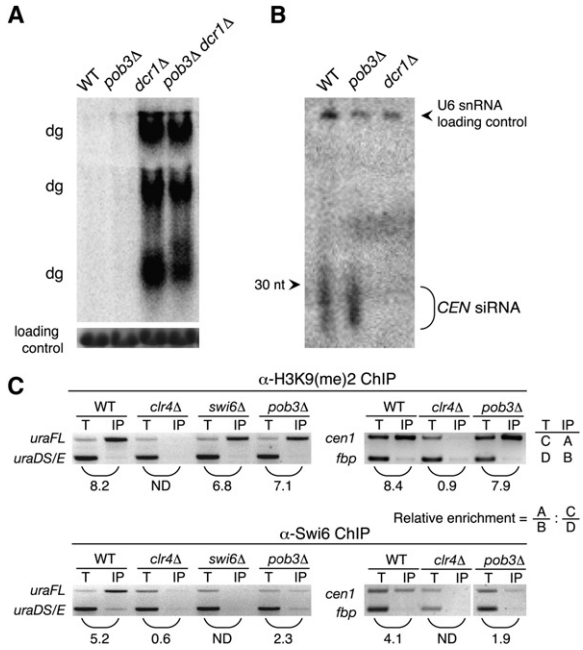


Figure 4. Pob3 Probably Acts Downstream of Dicer and Influences the Deposition of the Heterochromatic Swi6 Protein on Silenced Chromosomal Regions

(A) Northern analysis of noncoding centromeric *dg-dh* transcripts detects no measurable changes in the accumulation of these transcripts in *pob3*Δ mutant strains, in contrast to *dcr1*+ deletion. The *pob3*Δ *dcr1*Δ double mutant does not alter the noncoding *dg* RNA levels. Centromeric transcripts were detected with a probe specific for the *dg-dh* repeat (top). RNA loading controls indicate the total RNA added (ethidium bromide staining, bottom).

(B) Centromeric siRNAs are unaffected by *pob3*+ deletion. A northern blot of small RNAs extracted from WT, *pob3*Δ, and *dcr1*Δ strains was probed with a centromeric (*dg-dh*) probe. U6 snRNA serves as a loading control.

(C) In *pob3*Δ mutants, Swi6 association is altered at centromeric outer repeats, whereas histone H3K9 me2 levels are maintained. ChIP of H3K9 me2 and Swi6 in WT, *pob3*Δ, and heterochromatin mutants *clr4*Δ and *swi6*Δ detects a significant loss of Swi6 both at the *uraFL* transgene and at the endogenous *cen1* locus (*uraDS/E* and *fbp* serve as euchromatic control regions, respectively). The figure shows a representative example of three independent biological experiments. The relative enrichment of IP/Input is calculated as shown.

also be because of an important role of Pob3 in transcript generation. We therefore tested cells lacking both Dcr1 and Pob3. The results show that these transcripts appear to accumulate to the same extent as observed in *dcr1*Δ single mutants (Figure 4A). Outer-repeat transcripts thus accumulate in a *dcr1*Δ strain independently of Pob3. Consistently, centromere-repeat-homologous siRNAs are detected at similar levels in WT and *pob3*Δ cells, but not in *dcr1*Δ cells (Figure 4B). Taken together, our results indicate that Pob3 function at the centromere does not appear to affect the production or accumulation of both unprocessed transcripts and siRNAs. FACT may thus affect centromeric silencing through changes in the integrity of silent chromatin itself and by acting either downstream of (or parallel to) the RNAi machinery.

The Swi6 Heterochromatin Mark Is Altered in the *pob3*Δ Mutant

To further dissect FACT-mediated heterochromatinization, we analyzed chromatin structure at the centromeric repeats in the *pob3*Δ strain. Other fission-yeast mutants, such as *clr3*Δ and *sir2*Δ, also affect RNAi-directed silent chromatin without affecting the production or abundance of centromeric-repeat siRNAs (unpublished data). In such mutants, the H3K9 me2 levels are reduced, and consequently less Swi6 associates with centromeric repeats [17]. We therefore used chromatin immunoprecipitation (ChIP) assays to determine the levels of H3K9 me2 and Swi6 at centromeric outer repeats. In cells lacking Pob3, the ChIP assays reveal normal levels of histone H3K9 me2 on both the *otrIR::ura4*+ marker gene

and directly on the outer repeats (Figure 4C). Thus, Pob3 is not required to maintain normal levels of H3K9 me2 methylation at centromeres. Because the H3K9 me2 mark is unaffected, we expected the Swi6 protein levels to be maintained in *pob3*Δ. Surprisingly, we find that Swi6 association shows a moderate but reproducible decrease over both the reporter gene and the endogenous centromeric region (Figure 4C). This shows that although centromeric Swi6 association still occurs to some level, its association is disturbed in *pob3*Δ strains. Consistent with this partial effect, we observe that Swi6-GFP remains localized to heterochromatic loci in *pob3*Δ cells, as is seen in several RNAi-pathway mutants (Figure S7) [18]. Thus, although the key histone H3K9 me2 mark is retained on centromeric repeats in cells lacking Pob3, Swi6 association is reduced. *Sp*FACT might thus play a role in assembling or retaining Swi6 on centromeric heterochromatin.

Conclusions

Here we have identified and characterized the *Sp*FACT complex. Surprisingly, we show that deletion of the *Sp*FACT subunit Pob3 is viable. This has allowed us to critically assess Pob3's functions in vivo. Our experiments reveal a conserved biochemical protein assembly that functions in chromatin-based processes. Importantly, we provide the first biological evidence that Pob3 is required for accurate chromosome segregation. We find that this might be because of a novel role of the *Sp*FACT complex in heterochromatin integrity at centromeres. *pob3+* deletion does not affect H3K9 me2 levels, but it leads to decreases in Swi6 association at *otr* repeats. Together, our genetic and biochemical data implicate the chromatin-remodeling complex FACT in forming functional centromeres.

FACT is known to facilitate transcription through chromatin. It has been proposed that FACT can disassemble H2A-H2B from nucleosomes in front of an advancing Pol II enzyme and reassemble H2A-H2B in its wake [19]. At centromeres, mutations in the *Sp*FACT histone chaperone might affect H2A/H2B dimer incorporation and thus change the structural integrity of heterochromatin. Any alterations in the positioning or composition of nucleosomes could interfere with Swi6 association and/or spreading. This could alleviate silencing without noticeably changing H3 K9 methylation, as we observe. Alternatively, FACT might recruit Swi6 directly to *otr* regions. Decreased binding of Swi6 to heterochromatic would be expected to impair sister-chromatid cohesion, resulting in defective chromosome segregation [1].

In summary, our results show that the small subunit of the *Sp*FACT complex is required to form normal silent chromatin on the centromeric repeats and for accurate chromosome segregation. Recent studies have shown that both subunits of the human FACT complex biochemically interact with centromeric CENP-A nucleosomes [20, 21]. Although the biological role of this interaction is unclear, our in vivo data now suggest that FACT might use its histone chaperone activity to assemble and maintain the structural integrity of centromeric heterochromatin. Given the high degree of conservation in FACT subunit sequences and in biochemical functions, it is likely that our data point to an important and evolutionarily conserved role for FACT in maintaining centromere integrity.

Experimental Procedures

Strains, Media, Transformation, and Genetic Techniques

Strains are listed in Table S3. Standard genetic techniques were used [22]. Cells were grown in yeast extracts supplemented with adenine (YES) or in synthetic minimal medium [Piperazine-1,4-bis(2-ethanesulfonic acid), $MgSO_4$, glycerol (PMG)]. When required, phloxin B, 6AU, CPT, HU, or TBZ was added. Damage assays [23], minichromosome loss rates [14], silencing assays, comparative plating, and 5-fold serial dilution experiments [9] were performed as described.

Expression Profiling and ChIP Assays

Microarrays were carried out as described [13]. RNA was extracted with a standardized acid phenol protocol. cDNA was generated with *S. pombe*-specific primers and random hexamers and labeled with Cy3 or Cy5. Dye swaps were done for all experiments. Hybridized slides were scanned (Biorad scanner), quantified (ImageQuant 4.2 [Imagene]), and analyzed (Gene Spring [Silicon Genetics]). Similar gene lists were identified with hypergeometric distribution tests (Table S2). Swi6 and H3K9 me2 ChIP assays were performed as described [4]. Bands were quantified with the Eastman Kodak EDAS 290 system and 1D image-analysis software.

Immunofluorescence Microscopy

Cell-growth conditions, TAT1 immunofluorescence, and staining protocol have been described [15]. Images were collected on a Carl Zeiss MicroImaging Axioplan 2 IE fluorescence microscope. Image acquisition was controlled with Metamorph (Universal Imaging).

Northern Blots

RNA was extracted from log-phase cells by acid phenol protocol, and polyethylene glycol (PEG) precipitation to separate high- (HMW) from low-molecular-weight (LMW) RNA followed. Twenty micrograms of HMW RNA and 40 μg of LMW RNA were resolved on 6% formaldehyde gels containing 1% agarose and on 8% urea-denaturating PAGE, respectively. Gels were blotted overnight to a Hybond-XL membrane (GE). DNA probes, complementary to centromeric *dg-dh* repeats, and U6 snRNA were generated with High-Prime labeling (Roche) and T4 polynucleotide kinase (Promega), respectively. HMW and LMW RNA blots were hybridized overnight in a rotating oven at 65°C and 42°C, respectively. Phosphorscreens or films were exposed for between 3 hr and 3 days.

Protein Methods

Immunoprecipitations (IPs) with anti-HA agarose (Sigma) on whole-cell extracts were performed as recommended (Sigma). For GST pulldowns, ^{35}S-Met proteins were expressed by TnT Quick-coupled in vitro transcription and translation (Promega). 20 μl of reaction and 160 μl buffer (1x HEMG, 0.15 M KCl, 1 mM dithiothreitol [DTT], 0.1% NP40) were added to 10–30 μg of immobilized GST fusions, incubated for 1 hr at 4°C, and washed 5×. Gels were exposed on Kodak X-Omat AR. *Sp*FACT was purified with yeast-cell extracts [24]. Lysates were incubated with anti-FLAG M2 agarose (Sigma) for 6 hr at 4°C and washed in ice-cold phosphate buffered saline (PBS), elution was performed with FLAG peptide (Sigma), and SDS-PAGE or mass spectrometry (MS) analysis (Innova Proteomics) followed.

Supplemental Data

Seven figures and three tables are available at http://www.current-biology.com/cgi/content/full/17/14/1219/DC1/.

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