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yFACT Induces Global Accessibility of Nucleosomal DNA Without H2A–H2B Displacement

Hua Xin[1], **Shinya Takahata**[2], **Mary Blanksma**[1], **Laura McCullough**[1], **David J. Stillman**[2], and **Tim Formosa**[1,*]

[1]Department of Biochemistry, 15 N Medical Drive East RM 4100

[2]Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah, 84112

Summary

FACT has been proposed to function by displacing an H2A–H2B dimer to form hexasomes. Results described here with yeast FACT (yFACT) suggest instead that nucleosomes are reorganized to a form with the original composition but a looser, more dynamic structure. First, yFACT enhances hydroxyl radical accessibility and endonuclease digestion *in vitro* at sites throughout the nucleosome, not just in regions contacted by H2A–H2B. Accessibility increases dramatically but the DNA remains partially protected. Second, increased nuclease sensitivity does not require displacement of dimers from the nucleosome. Third, yFACT is required for eviction of nucleosomes from the *GAL1-10* promoter and the adjacent ORF *in vivo*, but most sites do not exhibit the preferential reduction in dimer occupancy expected for hexasome formation. We propose that yFACT promotes a reversible transition between two nucleosomal forms and that this is useful both to overcome the repressive chromatin barrier and to establish and maintain this barrier.

Introduction

DNA becomes relatively inaccessible when it is incorporated into nucleosomes, generally repressing expression of genetic information *in vivo* and inhibiting the activity of enzymes that act on DNA *in vitro* (van Holde, 1988). One strategy for overcoming the barrier posed by nucleosomes is ATP-dependent chromatin remodeling, in which energy from ATP hydrolysis is used to disrupt histone-DNA contacts. This can lead to translocation of histone cores along the DNA, histone eviction, or other outcomes (Cairns, 2007). Specific DNA sequences can become more or less accessible during remodeling as they move between protected nucleosomal sites and the adjacent unrestricted linker regions. Members of the broadly conserved FACT family use a different strategy to make nucleosomal DNA more accessible, as this activity does not involve ATP hydrolysis (Formosa, 2008; Orphanides et al., 1998) or translocation of DNA sequences out of the nucleosome (Rhoades et al., 2004). To distinguish the action of FACT from the process of ATP-dependent remodeling, we have called FACT activity "nucleosome reorganization" (Formosa, 2008). FACT activity is needed both during transcription and DNA replication (Formosa, 2008; Reinberg and Sims, 2006), but its precise roles in these processes remain under investigation.

*Corresponding author; tim@biochem.utah.edu.

Experiments with human FACT suggested that it functions by displacing one H2A–H2B dimer from the nucleosome to form a hexasome (Belotserkovskaya et al., 2003). Observations consistent with this model include binding of the hSpt16 subunit to purified H2A–H2B dimers and of the SSRP1 subunit to $(H3–H4)_2$ tetramers, displacement of about half of the H2A–H2B dimers from immobilized nucleosomes treated with FACT, and formation of some hexasomes during transcription of a nucleosomal template in the presence of FACT (Belotserkovskaya et al., 2003; Orphanides et al., 1999). These results clearly show that FACT destabilizes the association of H2A–H2B dimers with nucleosomes, but they do not show that dimer displacement is necessary for FACT activity. FACT was also unable to promote RNA Pol II progression through nucleosomes treated with crosslinking agents (Orphanides et al., 1999), but this result is consistent with any model in which the arrangement of the subunits of the nucleosome must be altered, not just dimer displacement. Crosslinking should also strongly affect the primary amine-rich N-terminal tails of histones, whose importance in supporting FACT binding has been demonstrated (Stuwe et al., 2008; Vandemark et al., 2008). The requirement for H2A–H2B dimer displacement in FACT activity has been widely accepted but has not been firmly demonstrated.

The FACT homolog from the yeast *Saccharomyces cerevisiae* (yFACT) is an Spt16-Pob3 heterodimer whose function is supported by the HMGB-like DNA-binding protein Nhp6 (Brewster et al., 2001; Formosa et al., 2001). Both human FACT (in which the Pob3 and Nhp6 sequences are fused in the single SSRP1 protein; Orphanides et al., 1999) and yFACT bind nucleosomes (Formosa et al., 2001; Orphanides et al., 1999). Previous studies showed that yFACT binding makes nucleosomal DNA more sensitive to DNase I at specific sites (Rhoades et al., 2004). Most of the DNA remained resistant to digestion, suggesting that yFACT either increases accessibility only in some regions or that it causes changes in the shape of the DNA in a localized way. yFACT also enhanced digestion by some restriction endonucleases, strongly suggesting increased accessibility of their recognition sites (Rhoades et al., 2004). Surprisingly, the sites of enhanced cleavage by both DNase I and by restriction endonucleases included regions expected to be bound by $(H3–H4)_2$ tetramers, not by the H2A–H2B dimers whose removal under the hexasome-formation model would be responsible for the accessibility. Studies with exonuclease III also showed that the pattern of pausing caused when this enzyme encountered a positioned nucleosome was only weakly altered by yFACT binding (Rhoades et al., 2004). Both the initial pause due to $(H3–H4)_2$-DNA contacts at the entry/exit site and secondary pauses that result from the adjacent (H2A–H2B)-DNA contacts were observed during yFACT binding. yFACT therefore alters nucleosome structure, but the altered form does not have the properties expected for a hexasome. It is likely that dimer removal would significantly enhance the exposure of nucleosomal DNA near the entry/exit sites but this cannot be directly tested without stable, homogenous preparations of hexasomes, which have not been reported. Significant questions therefore remain regarding the mechanism of FACT action, particularly concerning the role of H2A–H2B dimer displacement.

In this report, we examine the properties of nucleosomes bound by yFACT to study the mechanism of nucleosome reorganization. Measurements of the rates of digestion by the same set of endonucleases at their cognate sites placed at different locations within positioned nucleosomes indicate that the DNA accessibility induced by yFACT can be surprisingly global. The level of altered accessibility detected depends on the type of probe used; we observe strongly increased accessibility throughout the nucleosome with hydroxyl radicals and with two restriction endonucleases, localized increases with DNase I, and no enhanced accessibility with several other endonucleases. Nucleosomal DNA therefore becomes partially accessible at least briefly at all sites during reorganization, yet it remains in a partially protected environment. We confirm that H2A–H2B dimer loss can be induced by yFACT but the reaction conditions and the source of the histone proteins strongly affect the amount of loss. Importantly, the level of displacement does not predict the degree of nuclease sensitivity induced.

Additionally, sensitivity can exceed dimer loss, so this loss cannot be the proximal cause of the increased accessibility. Finally, we examine the stability of dimers and tetramers during yFACT-dependent transcription *in vivo* and find no evidence of hexasome formation in promoters or in the 5' end of a transcription unit. Some preferential loss of dimers may occur near the 3' end of the transcription unit, consistent with other evidence for an unusual interaction between yFACT and nucleosomes near Pol II termination sites (Duina et al., 2007).

Based on these results, we propose that yFACT establishes an equilibrium between a stable nucleosome and a form in which the same components are more loosely and dynamically associated with one another. The primary purpose of FACT is then to convert nucleosomes between canonical and less stable forms, with dimer loss being an incidental secondary reaction whose probability varies with circumstances. Destabilization of nucleosomes by yFACT is obviously useful for overcoming the inhibitory effects of chromatin, but we also note that the reverse reaction could have a crucial role in forming stable nucleosomes from loose aggregates of components deposited by other chaperones as the chromatin barrier is established and maintained.

Results and Discussion

yFACT enhances *Dra*I sensitivity at many sites throughout a nucleosome

yFACT enhances DNase I digestion at specific sites on nucleosomes reconstituted with recombinant yeast histones and either a sea urchin 5S rDNA nucleosome positioning sequence (5S rDNA NPS) or the Widom 601 NPS (Rhoades et al., 2004; Ruone et al., 2003). yFACT might therefore make nucleosomal DNA more accessible at only these sites, it might alter the shape of the DNA locally to make a better substrate, or it might increase accessibility globally but in a way that only some sites or DNA sequences become good DNase I substrates. Restriction endonucleases should provide a simpler test for accessibility, and the enhanced digestion of 5S rDNA nucleosomes by *Dra*I suggested that a site near the dyad of symmetry that is bound by $(H3–H4)_2$ tetramers becomes much more accessible during yFACT binding (Rhoades et al., 2004). In principle, it should be possible to use a set of restriction endonucleases with different recognition sites to map accessibility at different locations in a single nucleosome and therefore to determine which regions are most affected by yFACT binding. However, different enzymes are also likely to have different substrate accessibility requirements, so any variation in digestion rates observed with different enzymes cannot be interpreted unambiguously.

We therefore produced a series of DNA templates containing variations of the sea urchin 5S rDNA NPS in which the native *Dra*I site was moved to different locations (Fig 1A and Supplemental Materials). Sites were chosen to examine different environments throughout the nucleosome, particularly with respect to whether the DNA is contacted by H2A–H2B dimers or $(H3–H4)_2$ tetramers (Fig 1E). Nucleosomes were reconstituted using these DNA templates and recombinant yeast histones (Luger et al., 1999;Wittmeyer et al., 2003), then the rates of *Dra*I digestion in the presence and absence of yFACT were determined (Fig 1B, 1C, Table 1, and Table S1).

Averaging multiple experiments including all template variants, the rate of *Dra*I digestion was 0.008% per minute per unit of enzyme for nucleosomal DNA, and 3.8%/min/U for free DNA (Table 1). Incorporation into nucleosomes therefore inhibited *Dra*I digestion by about 500-fold. The Nhp6 component of yeast FACT is a DNA-binding protein and it inhibited the rate of digestion of free DNA by *Dra*I by about 13-fold (Table 1). In contrast, Nhp6 *increased* the rate of digestion of nucleosomal DNA about 3-fold. This effect was somewhat higher near entry/exit points (Fig 1C and Table S1, compare *Dra*I-29 and -140 to -78), suggesting that

Nhp6 enhances the normal temporary dissociation of DNA-histone contacts in these regions (Allain et al., 1999;Anderson and Widom, 2000).

Adding Spt16-Pob3 to nucleosomes had no effect (Fig S1), but yFACT (Spt16-Pob3 with Nhp6) caused a dramatic increase in the rate of digestion by *Dra*I at all nucleosomal sites tested (Fig 1C, Table S1), averaging 27-fold faster than digestion of nucleosomes alone (Table 1). Notably, the rate of digestion in the presence of yFACT was consistently lower when nucleosomal DNA was compared with free DNA fragments, with the average being 60% (Table S1). yFACT therefore makes nucleosomal DNA quite sensitive to *Dra*I digestion, but the DNA at each site remains partially protected. The level of enhancement by yFACT varied among the sites, but was significant for every location tested, indicating a global increase in accessibility throughout the nucleosome, without full disruption of the structure (Rhoades et al., 2004 and see below).

Globally enhanced accessibility can also be observed with *Pst*I

To test whether these results reflect unique features of nucleosomes assembled with the 5S rDNA NPS or of the enzyme *Dra*I, we examined a different set of templates that rotationally phase nucleosomes using two repeats of the synthetic TG motif (Simpson and Stafford, 1983) and have recognition sites for the enzyme *Pst*I (Fig 1D; Fan et al., 2003 and Supplemental Materials). The average rate of digestion by *Pst*I for these templates decreased 1100-fold upon incorporation into nucleosomes (Table 1). Nhp6 once again inhibited the rate of digestion of DNA (an average of 5-fold) and enhanced the rate of digestion of nucleosomes (an average of 8-fold), especially near the entry/exit sites (Fig 1F, Table S1). yFACT once again increased the rate of digestion by *Pst*I at all sites, with an average increase of 110-fold relative to nucleosomes alone (Fig 1F, Table 1, and Table S1). This is about 50% of the rate of digestion of free DNA in the presence of yFACT, once again indicating that yFACT induces very high but not complete accessibility of nucleosomal DNA. The global enhancement of endonuclease sensitivity can therefore be observed with two different enzymes and two distinct types of nucleosome positioning sequences.

Accessibility depends on the probe used

The native *Dra*I site at position 78 in the 5S rDNA NPS was mutated to an *Ase*I site, allowing a test of the accessibility of the same site to two different enzymes. yFACT enhanced digestion by *Dra*I 60-fold at position 78 (Fig 1C and Table S1), but *Ase*I digestion at position 78 was not affected or was slightly inhibited by yFACT (Table 1). Similarly, *Pst*I digestion was enhanced 32-fold at position 24, but digestion by *Mlu*I at the same site was unaffected by yFACT (not shown). *Sty*I digestion of nucleosomes assembled with the Widom 601 NPS was also unaffected by yFACT, whereas digestion by *Hha*I was enhanced 26-fold (Rhoades et al., 2004). Digestion by DNase I was enhanced by yFACT at only a subset of possible sites, with the majority of the DNA retaining the relatively protected nucleosomal pattern (Rhoades et al., 2004). If yFACT released nucleosomal DNA to a fully unprotected environment, then all probes should detect the change. These results instead suggest that increased accessibility depends on the size or geometry of the active site of the endonuclease used.

Hydroxyl radicals were therefore used to examine whether structural changes induced by yFACT can be detected with a small molecule probe. Fig 2 shows that the 146 bp 5S rDNA fragment used in these experiments was about equally sensitive to hydroxyl radical damage at all sites (top panel). The DNA-binding protein Nhp6 reproducibly caused a small decrease in hydroxyl radical access at all sites (detected as slightly diminished peak heights in Fig 2, panel 2). Incorporation of this same DNA fragment into nucleosomes resulted in the expected periodic protection of the DNA roughly every 10 nucleotides, as the DNA contacted by the

histones is less accessible to this probe (Fig 2, panel 3). Spt16-Pob3 did not alter the sensitivity patterns for free DNA with or without Nhp6 (not shown) or for nucleosomes (Fig 2, panel 4).

In contrast to the small increase in endonuclease sensitivity induced by Nhp6 alone (Fig 1 and Table S1), hydroxyl radical accessibility was significantly increased throughout the nucleosome by Nhp6 (Fig 2, panel 5). This supports the model that constant cycles of Nhp6 binding, DNA bending, and release in a short time frame cause small, temporary dislocations in histone-DNA contacts. We propose that these gaps can be detected by hydroxyl radicals, but are not large enough or persistent enough to be detected by endonucleases. The periodicity of the hydroxyl radical pattern is dampened but retains the same phase when Nhp6 is added, indicating that the rotational position of the DNA is not altered.

Addition of yFACT caused a small further increase in sensitivity to hydroxyl radicals relative to Nhp6 alone, and diminished periodicity of the accessibility pattern (Fig 2, panel 6). For example, the peak representing the third turn of the DNA helix inside the nucleosome is still distinguishable with Nhp6 alone but not with yFACT (arrows in Fig 2), but the periodic variations near the dyad of symmetry are still observed in the presence of yFACT. The decreased periodicity is due to increased exposure of protected "valley" sites in the nucleosome pattern, not to increased protection of "peak" sites. Overall, the addition of Spt16-Pob3 leads to loss of protection from hydroxyl radical damage throughout the nucleosome.

As described below, most nucleosomes retain their normal composition under these conditions, so the decreased overall protection and the blurring of the periodic pattern of protection suggest the formation of a dynamic structure in which the unique relationship between histones and DNA sequences observed in canonical positioned nucleosomes becomes more variable. One model (see Fig 7 below) is that Nhp6 alone creates small gaps in the histone-DNA contacts but does not alter the position of the contacts. Spt16-Pob3 promotes a more dramatic alteration in structure in which the histone-DNA contacts are not only more transient, they are distributed more dynamically along the DNA. This differs from the hexasome formation model in that the changes occur throughout the nucleosome and do not involve full removal of dimers.

Measuring H2A–H2B dimer displacement

Human FACT removed about half of the H2A–H2B dimers from nucleosomes immobilized on insoluble matrices, suggesting efficient conversion of nucleosomes to hexasomes (Belotserkovskaya et al., 2003). To resolve questions about the disposition of dimers during reorganization by yFACT, we labeled both DNA and H2A and used a native PAGE assay to determine the effect of yFACT binding. Fig 3 shows a typical experiment, in this case with a Cy5-labeled 181 bp 5S rDNA NPS fragment assembled into nucleosomes with recombinant yeast histones including Oregon Green 488-labeled H2A. In the left half of Fig 3A, nucleosomes were incubated for 10 minutes under the conditions indicated, subjected to native PAGE, then the gel was scanned at two wavelengths to detect each dye independently. Each lane is numbered, with "a" indicating the DNA scan and "b" indicating the H2A scan. Lanes 1 and 2 show the migration of nucleosomes alone, and lanes 3 and 4 show the dramatic decrease in migration observed when yFACT is added (Formosa et al., 2001). To reveal changes in the nucleosomes that occurred during yFACT binding, a large excess of unlabeled sheared genomic DNA was added to each sample before electrophoresis in lanes 5–8. This DNA sequesters the Nhp6, causing rapid dissociation of Spt16-Pob3 from the nucleosomes (Rhoades et al., 2004). Lanes 5–8 of Fig 3A show the same samples as in lanes 1–4 after this treatment, revealing full reversal of yFACT binding (compare lanes 7–8 with 3–4).

Both the nucleosomal DNA and H2A initially comigrated in a relatively tight band (labeled "Nuc" in Fig 3A). Incubation with yFACT in the absence of Mg^{++} ions caused the migration pattern of both DNA and H2A to change, suggesting permanent structural alteration of at least

some nucleosomes (compare Fig 3A lanes 7a and 7b to lanes 5a and 5b). The DNA in the nucleosome band became more diffuse and also smeared significantly to slower-migrating forms (Fig 3A, lane 7a). More extensive changes were observed for H2A, with spreading of the nucleosome band being accompanied by significant retention of signal in the well and the upper portion of the gel (Fig 3A, lane 7b). yFACT binding can therefore induce structural changes in nucleosomes, although this does not result in significant amounts of complete disruption as the amount of free DNA is relatively unchanged (Figs 3A, 3B and Rhoades et al., 2004).

To quantitate these results, each lane was divided into 100 uniform segments and the total signal in each segment was determined (Fig3, right side, and Figs 3B–3D). Figure 3B shows lanes 5 and 7 from panel A, with just the nucleosome region expanded in Fig 3D. Although the peak broadens and the ratio of H2A to DNA decreases somewhat, much of the DNA and H2A comigrate near the original position after yFACT treatment and addition of unlabeled genomic DNA. In contrast, Fig 3C emphasizes the upper half of the gel, revealing the appearance of new species in response to yFACT binding. In the absence of yFACT, tetrasomes (DNA with $(H3–H4)_2$ but no H2A–H2B) were observed in a band centered at segment 42, as confirmed by tests with purified tetrasomes (not shown). The signal for both DNA and H2A increased in this region in samples treated with yFACT, but neither was found in a discrete band. Similarly, both DNA and H2A accumulated in segments 25–40 after yFACT binding. We speculate that these signals may be due to hexasomes, but attempts to confirm this by measuring the ratio of H2A signal to dimer signal gave complex results (Fig S2, S3). We conclude that this region of the gel as well as the nucleosomal band (Fig 3D) contain multiple different forms with 0, 1, or 2 H2A–H2B dimers per $(H3–H4)_2$-DNA complex and the histones occupying different positions along the 181 bp DNA fragment. As described below and in the Supplemental Materials, native PAGE can therefore be used to measure the level of dimer loss induced by yFACT, but other methods will be needed to characterize the complex population of nucleosomal remnants that result.

Significant amounts of H2A were detected in the upper third of the gel but the amount of DNA in this region was minimal in the absence of yFACT treatment, suggesting that this signal represents dimers displaced from nucleosomes. To test this, we mixed purified H2A–H2B dimers with excess unlabeled genomic DNA to mimic the nucleosomal samples, and subjected them to native PAGE in parallel with nucleosomes (Fig 4A). This confirmed that free dimers migrate in this region of these gels. Quantitation shows that about 90% of the free dimers are recovered in segments 1–38 (indicated with a red arrow in Fig 4B) but little DNA signal is found in this region with a nucleosome sample that has not been treated with yFACT. The amount of free dimer in a sample can therefore be determined by summing the total amount of signal in segments 1–38 and subtracting the amount of DNA signal to correct for the small number of nucleosomal remnants. Figure 4C shows an example of this quantitation. Here, each condition was tested in triplicate to estimate the variance among samples, then the amount of DNA and H2A signal in segments 1–38 was determined. The amount of dimer unaccompanied by DNA was determined for each sample (H2A – DNA; for example, 17% - 3.6% = 13% in the absence of yFACT and without Mg^{++}), then the difference between these numbers was calculated to reveal the fraction of dimer displaced from the nucleosomes as a result of yFACT binding (+/−yFACT in Fig 4C; 38% - 13% = 25% in the absence of Mg^{++}). Dimer loss due to yFACT binding is calculated using this method in the remainder of this report. An alternative method based on the ratio of H2A signal to DNA signal remaining in the nucleosome region has also been used and gives qualitatively similar results, but this dimer displacement calculation is favored for reasons discussed in the Supplemental Materials.

Dimer displacement by yFACT is variable

As has been reported with human FACT, these results show that yFACT binding can cause displacement of H2A–H2B dimers from nucleosomes. However, we find that the amount of dimer loss induced by yFACT varies over a wide range depending on the reaction conditions, including conditions that affect restriction enzyme activity. For example, as shown in Fig 3A, yFACT binding was efficient in the absence or presence of Mg^{++} ions, but dimer loss was minimal when Mg^{++} was included (compare Fig 3 lane 7b to lane 8b). Quantitation of multiple samples after incubation at 37° under these buffer conditions shows that about 24% of the dimers are displaced in the absence of Mg^{++} but this drops to 1.5% in the presence of Mg^{++} (Fig 4C). Addition of Mg^{++} also decreased the level of free dimer observed in the absence of yFACT (Fig 4C, 6.6% of the H2A was unaccompanied by DNA in the displaced region with Mg^{++} and 13% without Mg^{++}). Mg^{++} therefore stabilizes nucleosomes against dimer loss, including both spontaneous loss that occurs without yFACT and loss induced by yFACT. Other variables that affect dimer loss (Fig S2, S4–S6) include temperature (dimer loss increases with temperature), the use of acetate as the anion (effects are complex but dimer loss generally increases), and the size of the DNA fragment (smaller DNA fragments generate more dimer loss). In general, dimer loss is therefore influenced by factors expected to change the strength of protein-protein and protein-DNA interactions and the rigidity of DNA. Importantly, yFACT can bind to nucleosomes without inducing significant amounts of dimer loss.

To test the influence of the histone proteins themselves, we assembled nucleosomes with recombinant *X. laevis* histones (Bruno et al., 2004). An additional label was attached to H4 to allow simultaneous detection of $(H3–H4)_2$ tetramers, DNA, and H2A–H2B dimers. The interface between the two H2A–H2B dimers is more extensive with *X. laevis* histones than with yeast histones (White et al., 2001) suggesting that disruption of this interaction should be more difficult and therefore that dimer displacement should decrease. This was true for spontaneous displacement in the absence of yFACT (Fig S5), but dimer loss induced by yFACT was significantly higher with *X. laevis* nucleosomes than with yeast nucleosomes (Fig 4D). About 44% of the H2B signal was displaced under the conditions shown in Fig 4D compared to 16% for a parallel yeast nucleosome (Fig S5). Only 5.4% of the H4 signal was found in the displaced region as a result of yFACT binding under these conditions, supporting the conclusion that $(H3–H4)_2$ tetramers remain stably associated with the DNA even when dimer loss is high (Fig 4D).

Addition of Mg^{++} ions also stabilized the *X. laevis* nucleosomes, leading to 2.4% dimer loss and 4.2% tetramer loss due to yFACT. Other reaction conditions that influenced the stability of yeast nucleosomes also altered the stability of *X. laevis* nucleosomes, with total dimer displacement levels (spontaneous plus yFACT-induced) reaching as high as 70% (not shown). Dimer loss can therefore be too extensive to be accounted for by only hexasome formation and must also be able to proceed to tetrasome formation. The consistent elevation of dimer loss could indicate that *X. laevis* nucleosomes are inherently more prone to losing dimers from the reorganized state induced by yFACT. Alternatively, yFACT might make specific contacts with H2A–H2B proteins to maintain association of the nucleosomal components during reorganization, and *X. laevis* histones might lack some of these binding sites. In this view, yFACT binding always leads to decreased stability of the association of dimers with nucleosomes, but the amount of dimer loss that occurs depends on how firmly yFACT is able to tether the components together. This capacity is influenced by the histones themselves as well as the reaction conditions *in vitro*, and perhaps the action of other factors *in vivo*.

Dimer loss does not account for the level of endonuclease sensitivity

We consider two explanations for the global accessibility of nucleosomal DNA to some endonucleases in the presence of yFACT. First, sensitivity could be the result of dimer loss.

For example, the histone cores in hexasomes could be translationally mobile enough to allow all regions of the DNA to escape histone binding at least temporarily. Second, the loose association of components in a reorganized nucleosomal state could be fluid enough to allow the DNA in all regions of the nucleosome to become partially accessible at different times. In the first case, nuclease sensitivity would be limited to those nucleosomes that experienced dimer loss, whereas in the second case nuclease sensitivity and dimer loss would be two independent consequences of the formation of a reorganized nucleosomal state.

To distinguish between these models, we examined dimer loss and endonuclease digestion sensitivity in parallel assays. Two identical samples were prepared, then endonuclease was added to one sample and both were heated to 37°. Aliquots were removed from each reaction at the same time points, then SDS was added to the endonuclease samples for analysis by denaturing PAGE, and excess unlabeled genomic DNA was added to the samples without endonuclease prior to native PAGE to examine dimer loss. Fig 5 shows the results obtained with 181 bp 5S rDNA yeast nucleosomes (*Dra*I-78 substrates in Fig 1). After 32 minutes, 89% of the nucleosomes had been digested by *Dra*I in the sample containing yFACT but only 3% in the sample without it, so yFACT was responsible for digestion of 86% of the nucleosomes. In the parallel sample, yFACT displaced 20% of the dimers to forms unaccompanied by DNA, so at most 40% of the nucleosomes were converted to hexasomes. Stable dimer loss therefore cannot account for the level of endonuclease sensitivity induced by yFACT.

Endonuclease activity requires high concentrations of Mg^{++}, and digestion efficiency is strongly affected by buffer composition and temperature. The range of conditions that could be tested to examine the correlation between dimer loss and endonuclease sensitivity was therefore limited to the size of the DNA fragment and the source of the histones. Nucleosomes formed with a 146 bp 5S rDNA fragment were reproducibly more prone to dimer loss but less nuclease sensitive than those formed with a 181 bp 5S rDNA fragment (Fig S6). Similarly, nucleosomes formed with recombinant *X. laevis* histones displayed much higher levels of dimer loss but lower nuclease sensitivity than yeast nucleosomes formed with the same DNA fragments (Fig S6). Endonuclease sensitivity therefore neither requires nor correlates with dimer loss, making it unlikely that dimer loss is the proximal cause of sensitivity.

yFACT does not induce dimer loss preferentially *in vivo*

To examine whether yFACT causes dimer displacement *in vivo*, we used a chromatin immunoprecipitation (ChIP) assay to simultaneously measure the occupancy of H2B and H3 at sites acted upon by yFACT. Normal induction of transcription from the *GAL1-10* promoter requires yFACT (Biswas et al., 2006), and is accompanied by eviction of nucleosomes from the promoter region (Williams and Tyler, 2007). We therefore asked whether yFACT acts to promote removal of nucleosomes from this promoter. For this experiment, we used a genomic construct in which the *GAL1-10* promoter was fused to the 8 kb *YLR454w* ORF (Mason and Struhl, 2003). Fig 6 (upper left panel) shows that during induction of the *GAL1-10* promoter in a cell with normal yFACT, nucleosome eviction was detected within 10 minutes and continued over the 40 minute time course. Eviction was significantly reduced when yFACT levels were decreased by the *pob3-L78R* mutation (Fig 6, lower left panel, Vandemark et al., 2008). Nucleosome eviction from the *GAL1-10* promoter therefore depends on yFACT activity.

If yFACT functions by displacing H2A–H2B dimers, the ratio of H2B to H3 should decrease at sites acted upon by yFACT. However, no significant decrease was observed at any time during activation of the *GAL1-10* promoter (Fig 6, left panels). Nucleosomes are also evicted from the *HO* promoter during activation in a yFACT-dependent manner (Takahata et al., 2009), and no decrease in the ratio of H2B to H3 was detected at this promoter during its activation (ST, unpublished results). It is possible that dimer displacement by yFACT does occur at these promoters, but the subsequent removal of $(H3–H4)_2$ tetramers occurs too rapidly

to allow detection of the intermediate. Nonetheless, these experiments do not provide support for the idea that FACT activity involves preferential removal of dimers *in vivo*.

We next examined the occupancy of H2B and H3 within the transcribed region of the *GAL1-10:YLR454w* fusion gene. Nucleosomes were gradually displaced from the 5' end of the ORF during the 40 minute time course of induction, but the kinetics of H2B and H3 displacement were identical to one another whether high levels of nucleosome depletion occurred in the presence of normal levels of yFACT or more moderate depletion occurred with lower levels of yFACT (Fig 6 middle panels). In contrast, H2B occupancy did decrease somewhat relative to H3 at the 3' end of the transcription unit after 30 minutes, but only in cells containing normal amounts of yFACT (Fig 6, right panels). A similar result was observed using two independent cultures, so this effect is small but reproducible (not shown). Pol II alone can induce loss of dimers from nucleosomes *in vitro* under high salt conditions, or under lower salt conditions if FACT is present (Belotserkovskaya et al., 2003). The small decrease in H2B relative to H3 at the 3' end of the transcription unit after prolonged high frequency transcription could indicate that yFACT can enhance displacement of dimers by Pol II under some conditions, such as proximity to the termination site.

An H3-L61W mutation causes several defects, including aberrant transcription, and some of these defects can be suppressed by mutations in *SPT16* (Duina et al., 2007). H3-L61W also causes increased retention of yFACT over the 3' ends of transcription units, indicating a link between nucleosome structure, yFACT, and termination of transcription (Duina et al., 2007). Together, these results suggest that displacement of dimers is not an obligate feature of yFACT activity *in vivo*, but that circumstances such as the frequency of Pol II passage or the proximity to a termination site can alter the balance of forces that maintain nucleosome stability.

Possible roles for yFACT activity

These results show that yFACT can promote a significant change in nucleosome structure *in vitro* that at least temporarily exposes all sites tested to digestion by either *Dra*I or *Pst*I using two distinct types of nucleosome positioning sequences. The DNA remains in a partially inaccessible environment, because it remains less sensitive than free DNA is to these same endonucleases under the same conditions, and it remains fully resistant to *Ase*I and *Mlu*I digestion at the same sites where *Dra*I and *Pst*I gain strong access. This change in structure can result in loss of dimers from the yFACT-nucleosome complexes, but most nucleosomes bound by yFACT remain fully intact. Importantly, nuclease sensitivity can be induced without dimer loss, indicating that yFACT causes nucleosomal DNA to become accessible in a structure that retains all of the components of the starting nucleosome.

Fig 7 shows a model for FACT activity that is consistent with these results. Like the widely accepted current model, FACT destabilizes nucleosomes in this model, and this can lead to dimer loss and hexasome production. However, dimer loss here is an indirect consequence of reorganization, not an obligate part of the mechanism. Reorganization occurs in two distinct stages, with Nhp6 causing small dislocations that are significant when hydroxyl radicals are used as the probe, but not when endonucleases are used. Spt16-Pob3 causes a larger alteration in the second stage, with each region of the DNA becoming accessible enough to allow access to some endonucleases. The accessibility is both temporary and incomplete, so steric considerations influence whether a given enzyme can detect this change. Thus, only some endonucleases detect the altered sensitivity, and in the case of DNase I the accessibility is region-specific (Rhoades et al., 2004). This model is also consistent with exonuclease digestion patterns, as the regions near the entry/exit points could remain in a state very similar to the basal nucleosomal state most of the time, but somewhat more prone to disruption by high levels of enzyme, as observed (Rhoades et al., 2004).

We speculate that tethering of the nucleosomal components insures that reorganization *in vivo* typically leads to reestablishment of an intact nucleosome. Mutant yFACT proteins might induce higher than normal levels of dimer loss, leading to the synthetic lethality that was observed when yFACT mutations were combined with mutations in the Hir/Hpc complex that may be needed to repair incomplete nucleosomes (Formosa et al., 2002). It is also possible that dimer displacement is a regulated reaction *in vivo*, as suggested by the role of human FACT in exchanging variant H2A proteins into chromatin (Heo et al., 2008).

The ability to maintain a reversible equilibrium between stable and partially dissociated nucleosome forms is consistent with the wide range of roles proposed for FACT *in vivo* (Formosa, 2008). Producing the unstable form could promote polymerase elongation (Reinberg and Sims, 2006), association of DNA-binding factors with their cognate sites within nucleosomes (Biswas et al., 2006), or eviction of nucleosomes (Fig 6 and Takahata et al., 2009). Promoting the reverse reaction of forming stable nucleosomes from loose aggregates of components could be important during nucleosome deposition or chromatin reestablishment after transcription (Kaplan et al., 2003; Mason and Struhl, 2003; Vandemark et al., 2008). A variety of redundant histone chaperones could all be capable of depositing histones onto DNA, then FACT could promote the transition of these complexes into canonical nucleosomes. The ability to form and resolve reorganized nucleosomes would then be the central feature of FACT activity with an important role in many chromatin-mediated processes.

Experimental Procedures

The *DraI* site in the sea urchin 5S rDNA nucleosome positioning sequence (Simpson and Stafford, 1983) was mutated to an *Ase*I site, then *Dra*I sites were reintroduced as indicated in Fig 1 (Supplemental Materials). *Pst*I variants (Fan et al., 2003) were provided by G. Narlikar (UCSF), or derived from them (Supplemental Materials). DNA fragments were generated by PCR with Cy3 or Cy5 labeled primers and purified from agarose gels. Yeast and *Xenopus laevis* histones were expressed in *E. coli* and assembled into tetramers or octamers and then into nucleosomes as described (Bruno et al., 2004; Luger et al., 1999; Wittmeyer et al., 2003). Mutations (H2A-S113C, H2B-T112C, and H4-T71C for the *X. laevis* proteins, H2A-Q114C for yeast) were introduced by the Quikchange method (Stratagene) to allow labeling of individual denatured histones with maleimide derivatives of Oregon Green 488 or tetramethylrhodamine (Molecular Probes) prior to octamer reconstitution.

For hydroxyl radical mapping, a 161 bp 5S rDNA NPS PCR product (Supplemental Materials) was labeled using polynucleotide kinase and γ-^{32}P-ATP then digested with *Eco*RV. The 146 bp NPS fragment was purified by polyethylene glycol precipitation and then used to assemble nucleosomes. Hydroxyl radicals were generated essentially as described (Hampel and Burke, 2001) and analyzed as described in the Supplemental Materials.

The rate of restriction digestion was determined as described (Rhoades et al., 2004), except SDS-PAGE was used instead of urea-PAGE, and DNA fragments were detected by Cy3 or Cy5 fluorescence (Typhoon, GE). Enzyme concentrations were adjusted to obtain linear initial rates for different conditions using the recommended buffers (New England Biolabs). Four time points at 8–10 minute intervals were tested with 100 fmol of DNA or nucleosome in a 10 µl volume. yFACT reactions contained 10 µM Nhp6 and either 0.2 µM Spt16-Pob3 (yeast nucleosomes) or 0.5 µM Spt16-Pob3 (*X. laevis* nucleosomes). Nhp6 was purified from *E. coli* as described (Ruone et al., 2003), and Spt16-Pob3 with a histidine tag on the Pob3 was purified from yeast cells overexpressing both proteins using nickel affinity and size exclusion chromatography (Biswas et al., 2005). All reactions contained final concentrations of 17 mM HEPES (pH 7.6), 2 mM Tris·Cl (pH 7.5), 0.8 mM Na_3EDTA, 0.11 mM 2-mercaptoethanol, 11 mM NaCl, 1.1% glycerol, and 12% sucrose. Where noted, additional components were

included for NaCl-HSA (100 mM NaCl, 0.9 mg/ml human serum albumin; Sigma) or acetate conditions (50 mM KOAc, 20 mM Tris·OAc (pH 7.9), 1 mM dithiothreitol), with or without 10 mM $MgCl_2$ or magnesium acetate as indicated. For experiments with parallel restriction digestions, the recommended buffer supplied with the enzyme was added to the base buffer components. Native PAGE was performed as described (Ruone et al., 2003), then gels were scanned at multiple wavelengths to detect individual labels (Typhoon, GE) and quantitated with ImageQuant software (Molecular Dynamics). yFACT-nucleosome complexes were disrupted by adding 32 μg per 10 μl sample of salmon testes genomic DNA (Sigma) sheared by sonication to about 1–3 kbp.

ChIP methods are described in the Supplemental Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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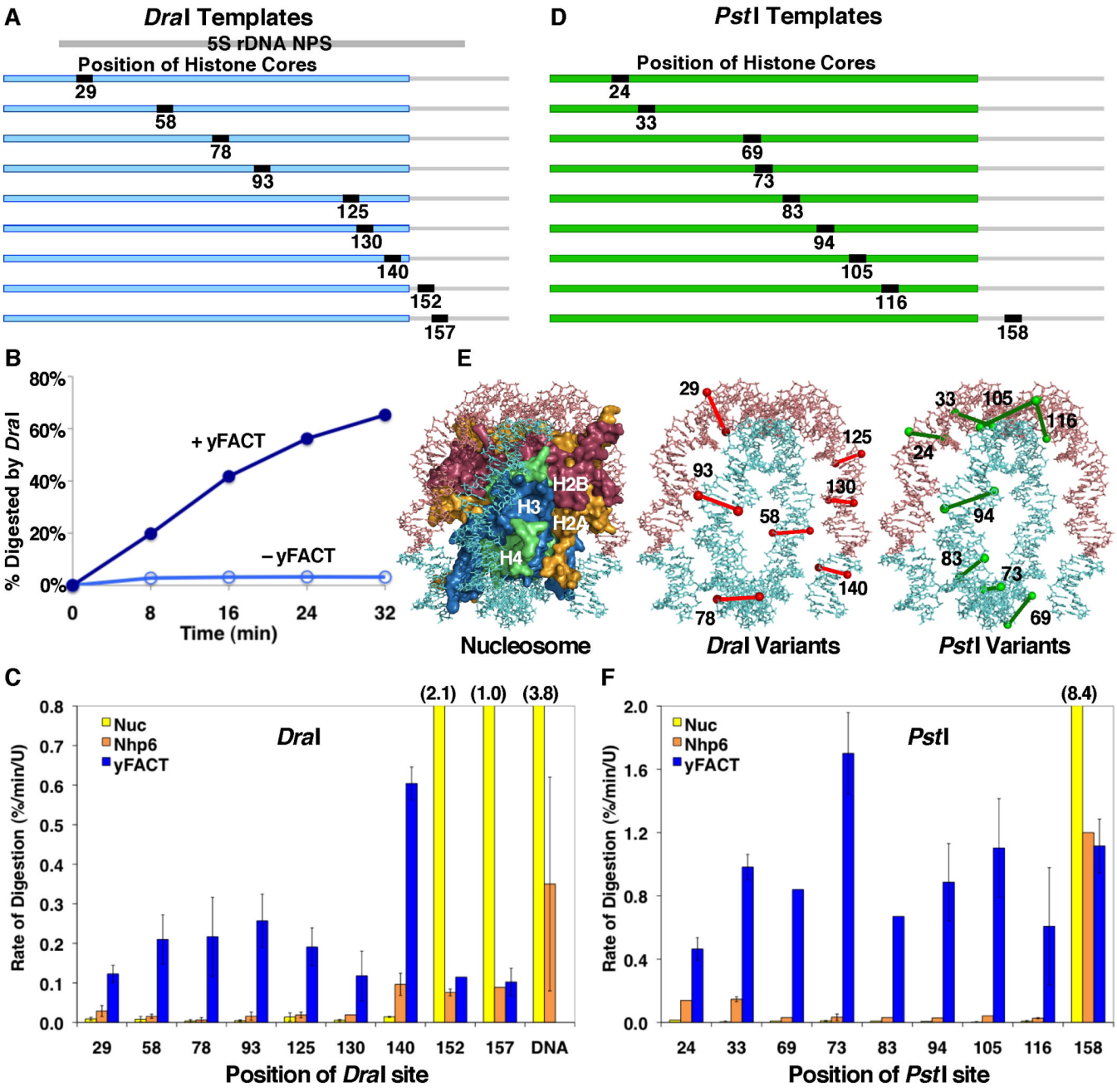

Fig 1. yFACT induces sensitivity to two restriction endonucleases at all nucleosomal sites tested

A) Nucleosomes were assembled with yeast histones and 181 bp DNA fragments with variants of the sea urchin 5S rDNA NPS in which the native *Dra*I site was moved from position 78 to the sites indicated (Supplemental Materials). The NPS identified in animal histones (Simpson and Stafford, 1983) is shown in the grey bar, and the observed location of yeast histone cores on these DNA fragments is shown in blue. B) Results of a typical *Dra*I digestion with a 181 bp *Dra*I-78 nucleosome with or without yFACT. C) *Dra*I digestion rates were calculated for nucleosomes alone, with Nhp6 added, or with yFACT added (Nhp6 and Spt16-Pob3). Averages and standard deviations from at least three measurements with each variant template are shown (Table S1). Results for *Dra*I-152, *Dra*I-157, and free DNA are given above the chart and in

Table 1 or Table S1. D) As in panel A, except templates with a recognition site for *Pst*I are shown with the observed position of histone cores in green. E) The approximate locations of the recognition sites for *Dra*I and *Pst*I are mapped onto the structure of the yeast nucleosome (White et al., 2001) assuming alignment of the left ends of the linear DNAs in each case. DNA regions contacted by H2A–H2B are colored red, and those contacted by H3–H4 are colored blue. An intact nucleosome is shown on the left, with just the DNA shown for the variant template sets. Red (*Dra*I) or green (*Pst*I) spheres indicate the phosphodiester linkage digested. F) As in panel C, except the rates of *Pst*I digestion are shown for the templates in panel D.

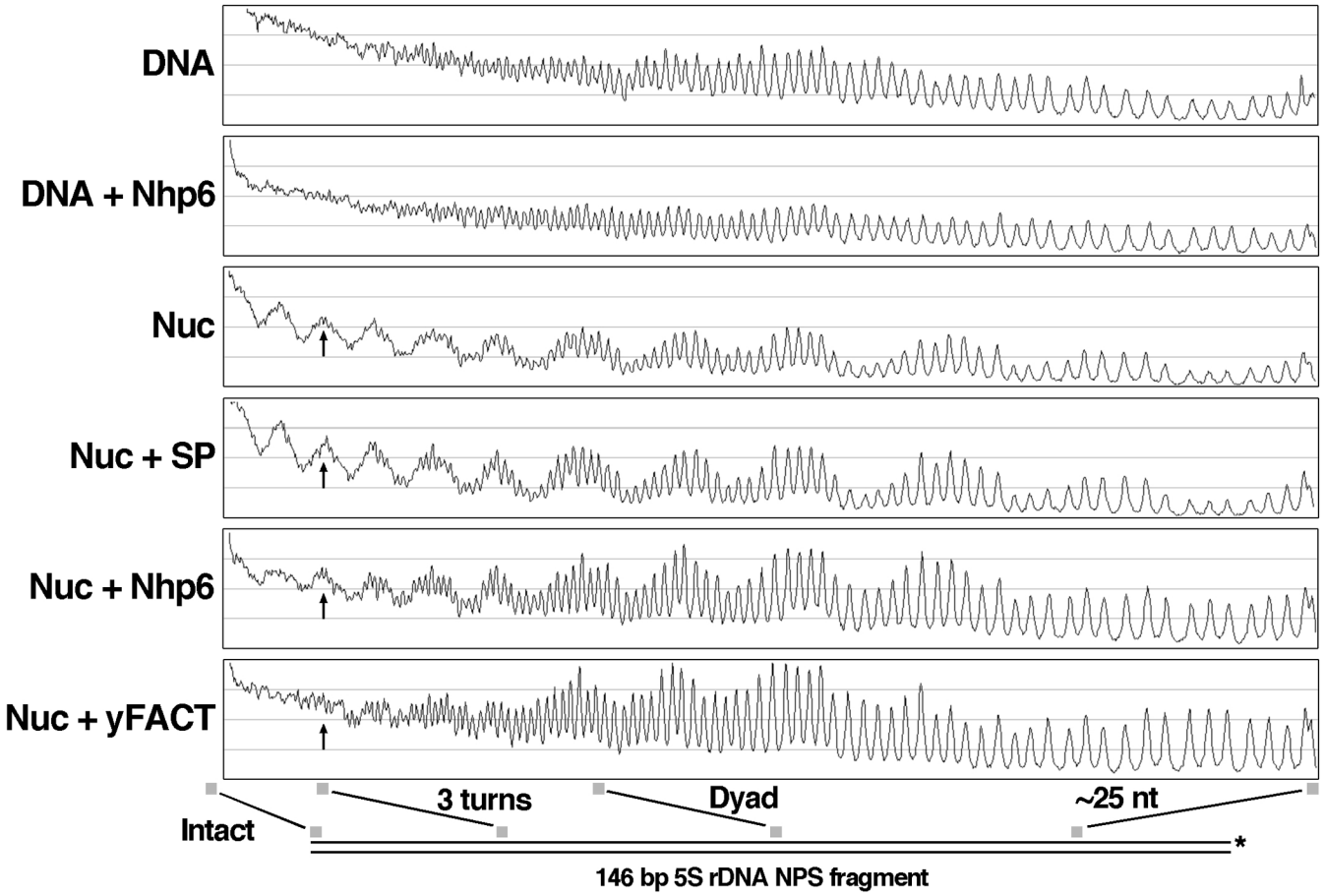

Fig 2. Global accessibility induced by Nhp6 or yFACT is detected by hydroxyl radicals

A 146 bp 5S rDNA fragment (Supplemental Materials) labeled with ^{32}P at the 5' end of the bottom strand (*) was used for hydroxyl radical analysis (Experimental Procedures). The trace here shows the region below the intact 146 nt band. Each scan is from the same gel and is presented on the same scale, so the height of individual peaks represents the relative yield of that product under identical conditions. Grey boxes on the cartoon indicate the undigested 146 nt band, the region 30 bp from the left end of the DNA fragment (with arrows on the nucleosomal traces), the center of the DNA fragment/nucleosomal dyad, and the last product resolved on the gel (~25 nt).

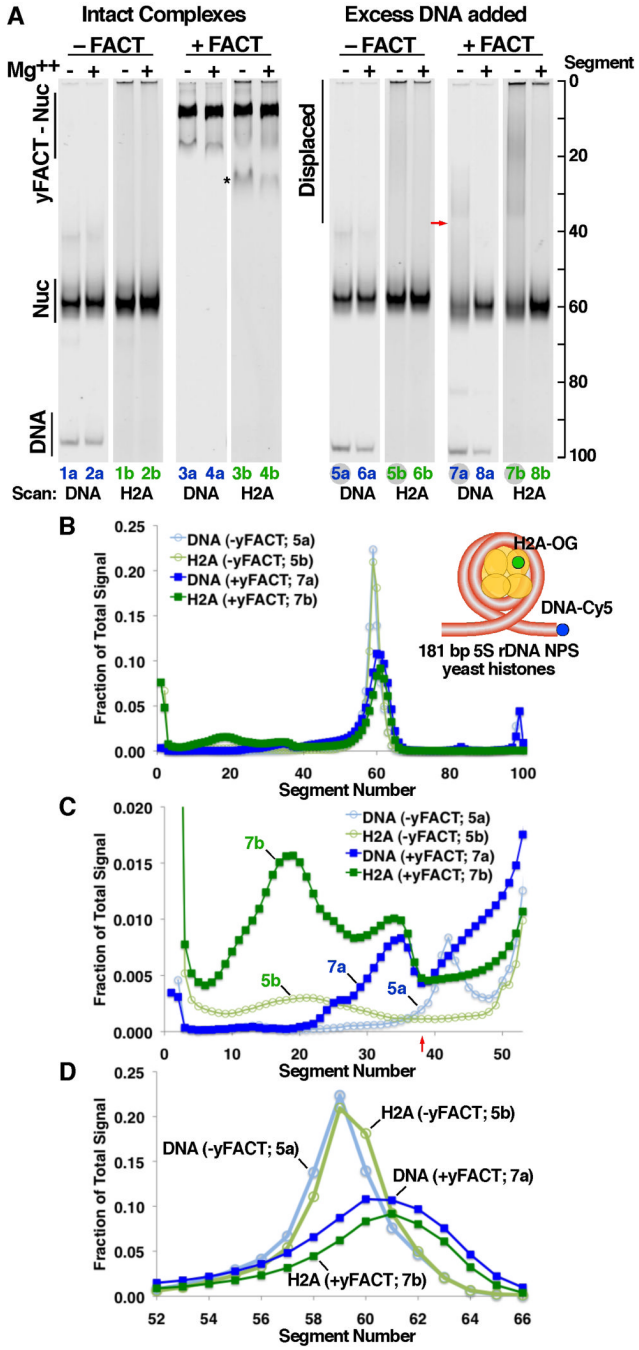

Fig 3. Native PAGE reveals changes in migration induced by yFACT

A) Nucleosomes (181 bp 5S rDNA, yeast histones) were incubated for 10 minutes at 30° under NaCl-HSA conditions with or without 10 mM $MgCl_2$ (Experimental Procedures) and with or without yFACT. Aliquots were then subjected to native PAGE either without (lanes 1–4) or with (lanes 5–8) addition of excess unlabeled genomic DNA. The single gel with 8 lanes was scanned to detect DNA (Cy5, labeled "a" in blue) and H2A (Oregon Green 488, labeled "b" in green), then the panels were reassembled to simplify comparison. The regions containing free DNA (DNA), nucleosomes (Nuc), and intact yFACT-nucleosome complexes (yFACT-Nuc) are indicated. The asterisk indicates the position of Spt16-Pob3 complexes with H2A–H2B dimers, and the red arrow indicates segment 38, the last one included in calculation of the

material in the displaced region (Displaced). The segment numbering scale for quantitation is shown on the right. B–D) The region from the well to the free DNA in lanes 5 and 7 was quantitated in 100 segments, then the signals were normalized by subtracting the background level and dividing by the total amount of signal for lane 5. Panels B–D show the same data adjusted emphasize different features.

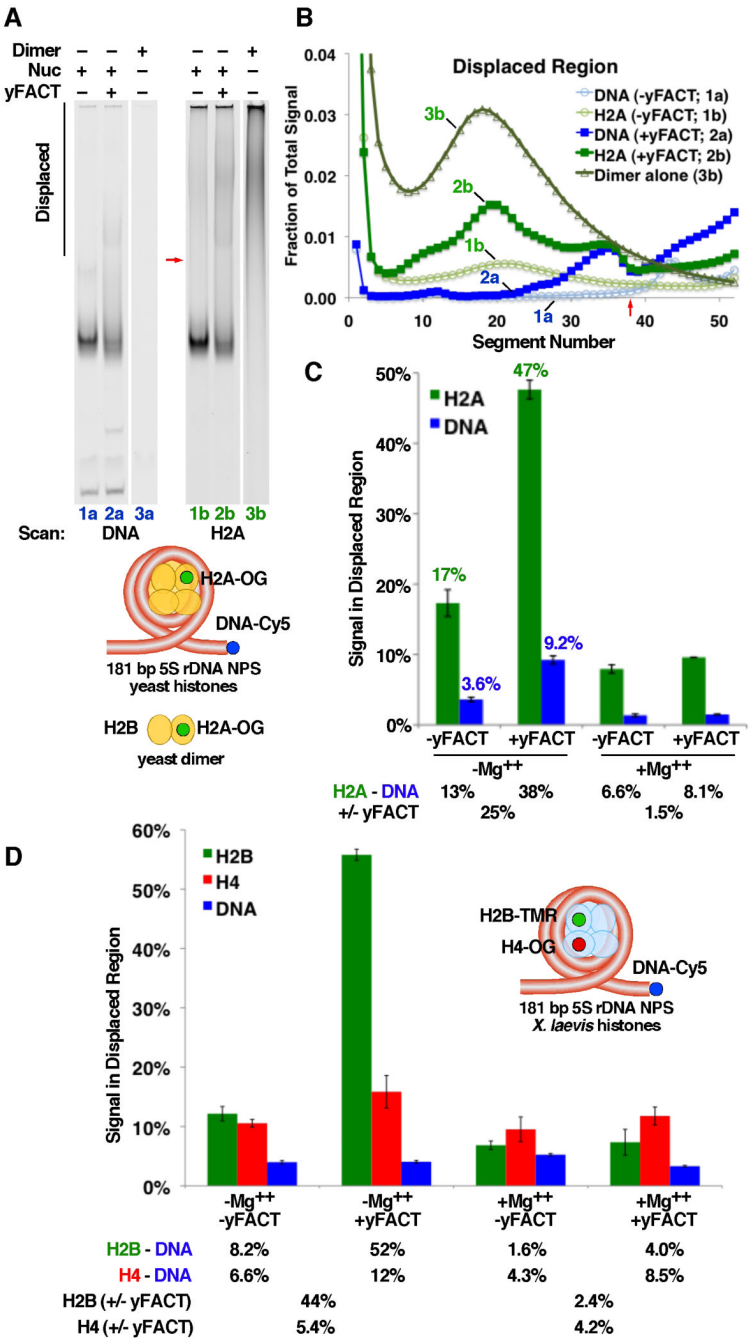

Fig 4. Examination of free dimers and quantitation of dimer displacement

A) A nucleosome (181 bp 5S rDNA, yeast histones) with (lane 1) or without (lane 2) yFACT, and a similar amount of purified yeast H2A–H2B dimer (lane 3) were incubated under NaCl-HSA conditions without $MgCl_2$ (Experimental Procedures) at 30° for 10 minutes. Unlabeled genomic DNA was added to all samples prior to native PAGE analysis as in Fig 3. Quantitation of segments 1–52 is shown in panel B. Curves are labeled with the lanes scanned, and segment 38 is indicated with a red arrow. C) Three repeats of the nucleosome samples in panel A were analyzed, except $MgCl_2$ was added as indicated and the incubation was at 37°. The amount of signal in segments 1–38 was determined with the average and standard deviation plotted. The difference between the H2A and DNA values was calculated (shown in the row labeled "H2A

– DNA"), then the difference between these values for the samples with and without yFACT was determined (shown in the "+/− yFACT" row) to give the amount of dimer displaced as a result of yFACT binding. D) An experiment similar to the one in panel C was performed, except the nucleosome was assembled with *X. laevis* histones, H4 was labeled in addition to the H2B and DNA, and samples were incubated at 30°. Samples were then analyzed as in panel C.

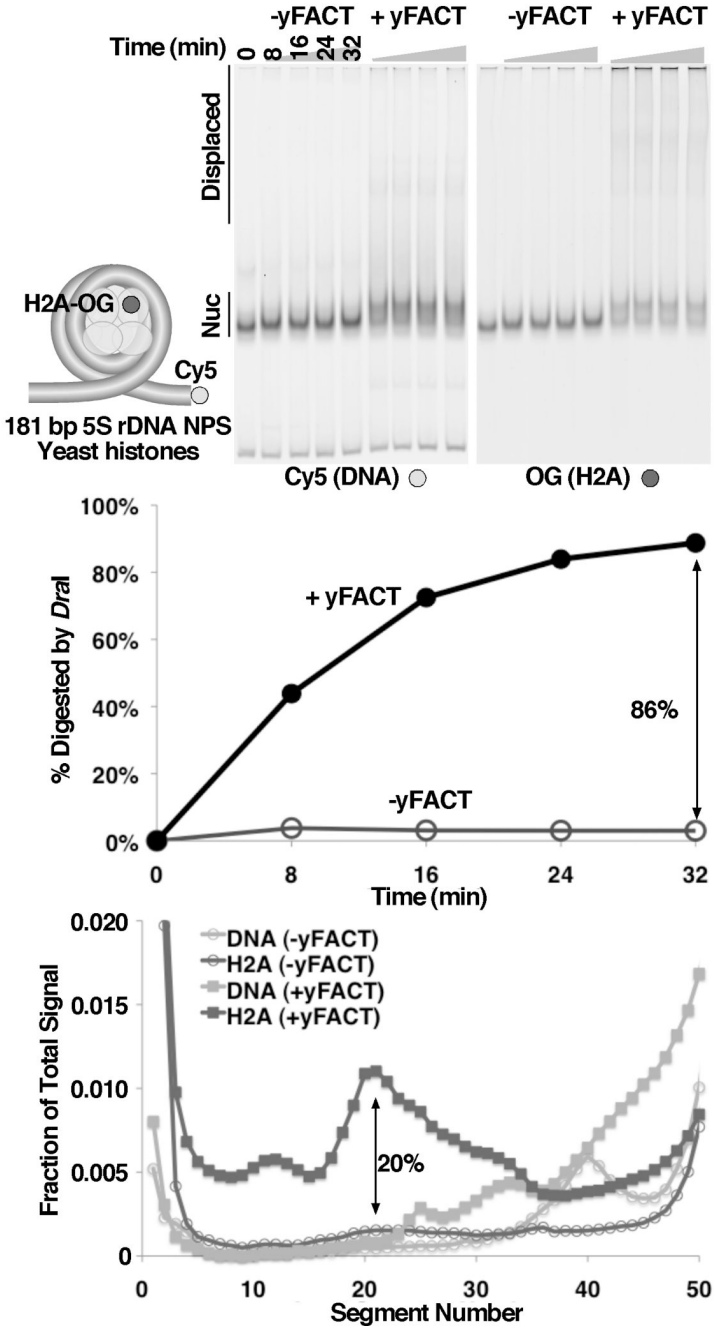

Fig 5. Dimer loss is lower than the level of endonuclease sensitivity

Nucleosomes were assembled with yeast histones and the 181 bp 5S rDNA *Dra*I-78 fragment and digested with *Dra*I with or without yFACT, or incubated under the same conditions without *Dra*I for analysis by native PAGE after adding excess unlabeled genomic DNA. The top panel shows the dimer and DNA distributions for each set of samples after native PAGE, the middle panel shows the accumulation of digestion products with time, and the bottom panel shows analysis of the "displaced" region of the native gel for the 32 minute time points, as in Fig 3. The total dimer loss due to yFACT binding as calculated in Fig 4 was 20%.

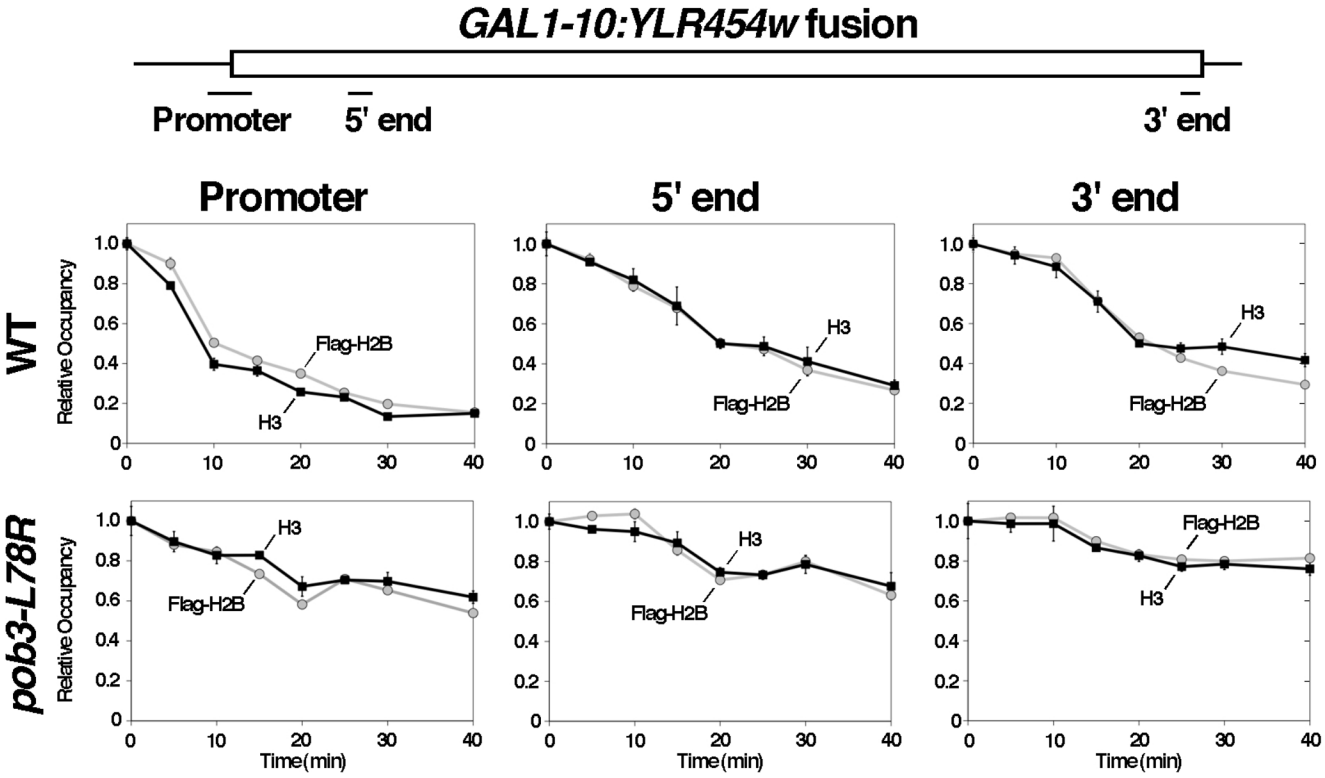

Fig 6. Histone occupancy at yFACT targets *in vivo*

W303 strains were constructed with the *GAL1-10* promoter integrated adjacent to the 7887 bp *YLR454w* ORF (Mason and Struhl, 2003), both copies of the genes encoding H2A and H2B deleted, and a plasmid expressing H2A and an N-terminal fusion of the FLAG epitope to H2B (generously provided by MA Osley; Supplemental Methods). Versions with the *POB3* (WT; DY13736) or *pob3-L78R* alleles (DY13744) were grown at 25° in raffinose medium, then the promoter was induced by the addition of 2% galactose. Samples were collected at times after induction as indicated, then H2B and H3 occupancy was measured by ChIP and quantitative PCR using primers for the promoter (−192 to +168 relative to the ORF), the 5' end (+945 to +1147), and the 3' end (+7701 to +7850) (Experimental Procedures). PCR was performed in triplicate with each primer set, with the standard deviation shown (some error bars are obscured by the data symbol).

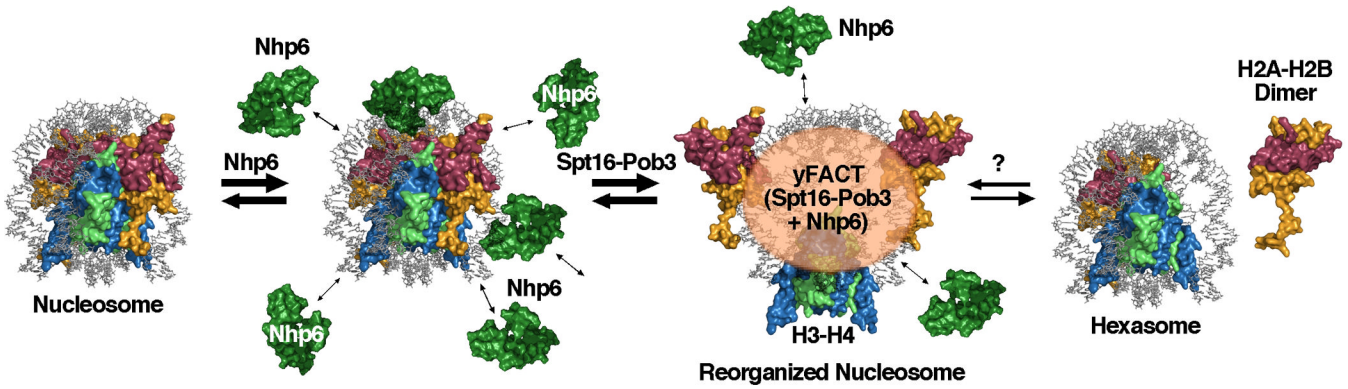

Fig 7. A model for FACT activity

See text for details. Reinsertion of dimers from a free pool may be mediated by yFACT or by other factors.

Table 1

Restriction Digestion Rates

	DNA	DNA + Nhp6 or yFACT	Nuc	Nuc + Nhp6	Nuc + yFACT
*Dra*I rate (%/min/U)	3.8	0.35	0.008	0.027	0.22
Std Dev	+/− 1.4	+/− 0.27	+/− 0.006	+/− 0.031	+/− 0.14
(n)	(18)	(52)	(28)	(21)	(35)
Normalized Rate	470	44	1.0	3.4	27
*Pst*I rate (%/min/U)	8.9	1.7	0.008	0.067	0.87
Std Dev	+/− 4.9	+/− 1.5	+/− 0.005	+/− 0.051	+/− 0.43
(n)	(7)	(23)	(15)	(13)	(32)
Normalized Rate	1100	210	1.0	8.2	110
*Ase*I rate (%/min/U)		2.3	0.009		.005
Std Dev			0.002		.003
(n)		(1)	(3)		(3)
Normalized Rate		270	1.0		0.6

Digestion rates were determined from initial slopes as in Fig 1B from n independent measurements. The values presented here are cumulative for all variant templates used under the conditions noted; the values for individual templates are presented in Table S1 and plotted in Fig 1. Normalized rates were obtained by dividing each rate by the rate observed with nucleosomes alone.

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